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STUDY OF THE TOXICOLOGICAL INTERACTIONS BETWEEN AMPHETAMINE DESIGNER DRUGS IN THE CONTEXT OF POLYDRUG ABUSE

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Aos meus incansáveis pais que me deram asas para voar.

À Avó Ester, à Ângela, à Filipa e à Lara.

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3. Dias da Silva D, Silva E, Carmo H (2010). What combination effects can we expect from amphetamine designer drugs? *Toxicology Letters*, 196:S148-S148 doi:10.1016/j.toxlet.2010.03.512.
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Author's declaration

The author states to have afforded a major contribution to the conception, design, and technical execution of the work, acquisition of data, analysis and interpretation of the results and manuscript preparation of the published or under publication articles included in this thesis.

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RESUMO

O padrão de consumo da 3,4-metilenodioximetanfetamina (MDMA, *ecstasy*) está intimamente associado à administração concomitante de uma panóplia de outras substâncias. Além de várias drogas que são intencionalmente ingeridas com o intuito de alcançar efeitos específicos, outras substâncias são por vezes administradas como parte de esquemas terapêuticos, ou inadvertidamente consumidas como contaminantes das pastilhas de *ecstasy*. Não obstante o facto da presença simultânea destas substâncias ter um potencial efeito negativo na toxicidade da MDMA, o efeito pernicioso da administração de misturas tem recebido relativamente pouca atenção por parte da comunidade científica, já que a maioria dos estudos disponíveis está confinada à análise toxicológica das substâncias individualmente.

Deste modo, o nosso grupo propôs-se averiguar se a combinação de substâncias anfetamínicas seria capaz de produzir efeitos tóxicos significativos, distintos daqueles observados para as substâncias individualmente. Uma vez que estas substâncias apresentam mecanismos farmacológicos e metabólicos sobreponíveis, existe um claro potencial para a ocorrência de interações.

Os perfis de citotoxicidade da MDMA e de três outras anfetaminas, a metanfetamina (METH), a 4-metiltioanfetamina (4-MTA), e a *d*-anfetamina (*d*-AMP), frequentemente presentes nas pastilhas de *ecstasy*, como contaminantes, ou em substituição da MDMA, ou ainda intencionalmente ingeridas para aumentar os efeitos desejados desta droga, foram analisados utilizando o ensaio de redução do brometo de 3-(4,5-dimetil-tiazol-2-il)-2,5-difeniltetrazólio (MTT) numa linha celular de hepatocarcinoma humano (as células HepG2). Em seguida, os efeitos de três combinações diferentes foram previstos a partir dos perfis citotóxicos individuais de todos os componentes da mistura, aplicando dois conceitos matemáticos amplamente utilizados em farmacologia, o modelo de *adição de concentração* (CA) e o de *ação independente* (IA). A verificação experimental da toxicidade originada pela mistura confirmou as expectativas da CA, revelando um evidente efeito aditivo. De particular interesse, foi a demonstração de efeitos de mistura relevantes mesmo quando cada anfetamina estava presente em níveis que eram individualmente inócuos, enfatizando os riscos associados ao abuso de múltiplas drogas.

Outros fatores inerentes ao consumo da MDMA, nomeadamente a hipertermia induzida pela droga, a bioativação metabólica produzindo espécies reactivas, a alteração na liberação de catecolaminas e respectivo metabolismo, o comprometimento da função

mitocondrial, a apoptose, e as respostas imunes, parecem estar implicados na hepatotoxicidade induzida por esta substância. Por conseguinte, a nossa investigação pretendeu confirmar a aplicabilidade da CA em cenários experimentais mais realistas que procuram mimetizar o fígado *in vivo* de forma mais fidedigna. Especificamente, nós pretendemos (i) avaliar os efeitos de mistura em hepatócitos primários, (ii) perceber a influência da hipertermia nesta avaliação, e (iii) investigar o impacto do efeito de mistura da MDMA com os seus três principais metabolitos humanos.

Quando a influência do aumento da temperatura na aplicabilidade dos dois modelos de previsão foi investigada, confirmou- se que a CA é o modelo mais adequado para prever os efeitos da mistura de anfetaminas também em condições de hipertermia (40,5 ºC), em células HepG2. Surpreendentemente, os nossos resultados demonstraram que, para esta temperatura, incrementos muito pequenos nas concentrações de cada substância ou da mistura promoviam aumentos muito acentuados na resposta citotóxica, oscilando rapidamente entre efeitos quase indetectáveis a efeitos altamente deletérios. Estas observações reforçam a hipótese de uma provável redução da 'janela de segurança' destas drogas, quando combinadas, *in vivo* e, consequentemente, de um aumento dos riscos a que os consumidores de *ecstasy* estão expostos nos cenários de consumo. Os mecanismos subjacentes à toxicidade exigem uma investigação mais aprofundada, no entanto, os nossos dados indicam que a perturbação da homeostase antioxidante (através do aumento da produção de espécies reactivas de oxigénio e de azoto e depleção de glutationa) com insuficiência mitocondrial e consequente perturbação do estado energético celular parecem desempenhar um papel crítico na citotoxicidade observada.

Os efeitos de mistura de anfetaminas também foram analisados em hepatócitos primários de rato, um modelo que é metabolicamente mais representativo dos hepatócitos *in vivo*. A uma temperatura de incubação fisiológica (37 °C), os resultados obtidos em hepatócitos primários e células HepG2 foram muito semelhantes. No entanto, a uma temperatura de incubação superior (40,5 °C), a toxicidade dos composto individualmente e da mistura aumentam significativamente nas células primárias. A aceleração da taxa metabólica esperada a temperaturas de incubação mais elevadas pode explicar, pelo menos parcialmente, o aumento da susceptibilidade dos hepatócitos primários à citotoxicidade das drogas e da sua mistura. A previsão dos efeitos citotóxicos pela CA mostrou-se novamente precisa em células primárias para ambas as temperaturas de incubação.

Estudos suplementares foram realizados aplicando o mesmo modelo experimental para avaliar os efeitos de uma mistura equipotente (uma mistura em que cada um dos seus

componentes está presente numa concentração que induz um efeito citotóxico semelhante) de MDMA e dos seus três principais metabolitos humanos, a 3,4-metilenodioxianfetamina (MDA), a *N*-metil-*α*-metildopamina (*N*-Me-*α*-MeDA) e a *α*-metildopamina (*α*-Meda), com o objetivo de simular a mistura produzida *in vivo* após bioactivação metabólica da MDMA. Também neste caso, foram observados efeitos aditivos significativos, em conformidade com as previsões pela CA, mesmo quando as substâncias estavam presentes em concentrações baixas, não citotóxicas, salientando a contribuição do metabolismo da MDMA para citotoxicidade nas células hepáticas.

Finalmente, investigámos os mecanismos subjacentes à morte celular induzida pelas anfetaminas testadas. Após 24 h de exposição às quatro anfetaminas, individualmente ou em mistura, a concentrações equieffectivas (*i.e.* concentrações que induziram 25% de morte celular, conforme avaliado pelo ensaio de redução do MTT), a mortalidade das HepG2 ocorreu preferencialmente por apoptose, quando testada a uma temperatura de incubação fisiológica (37 °C). Esta letalidade foi exacerbada com o aumento do período de incubação para 48 h. Quando a temperatura de incubação foi aumentada para 40,5 °C, a necrose tornou-se o tipo de morte celular privilegiado, para ambos os períodos de incubação (24 h e 48 h). Em consonância com estes resultados, os níveis de ATP intracelular sofreram uma redução acentuada quando as células foram incubadas com as substâncias individuais e em mistura a 40,5 °C, efeito esse que não foi observado a 37 °C. Uma investigação mais profunda dos mecanismos apoptóticos intervenientes mostraram um declínio nos níveis de mRNA dos genes *BCL-2* e *BCL-XL*, com um simultâneo aumento de *BAX*, *BIM*, *BAD* (apenas para MDMA), *PUMA* e *BID*. A elevação dos níveis proteicos de Bax, Bim (fracções EL, S e L), Bid clivado, Puma e Bak foram, posteriormente, confirmados. De acordo com a informação presentemente disponível na literatura, os marcadores Puma, Bim e Bak nunca antes tinham sido associados com a toxicidade induzida por anfetaminas. Resultados adicionais mostraram o envolvimento da ativação da caspase-3/-7, mas não de alterações no potencial de membrana mitocondrial ($\Delta\psi m$), na apoptose induzida por estas anfetaminas, isoladamente e em mistura, nas condições experimentais testadas. A proteólise da poli (ADP-ribose) polimerase (PARP), após 24 h de exposição aos compostos referidos, confirmou a lesão celular. De um modo geral, para todos os parâmetros avaliados, não foram observadas diferenças significativas entre as anfetaminas individualmente e a sua mistura, suportando a hipótese de que os efeitos da combinação de anfetaminas são aditivos e que os seus componentes agem de uma forma semelhante para promover a morte da célula hepática.

Concluindo, os resultados apresentados na presente dissertação permitiram-nos concluir que os derivados anfetamínicos atuam aditivamente para produzir efeitos de mistura expressivos, mesmo quando as substâncias estão presentes em quantidades que, individualmente, não apresentam efeito citotóxico relevante. Esta citotoxicidade foi particularmente significativa em hipertermia. Os nossos resultados evidenciaram ainda que os efeitos de misturas de anfetaminas podem ser previstos pelo modelo da CA. O modelo de IA, por sua vez, mostrou-se ineficaz na antecipação da resposta deste tipo de combinações, subestimando a toxicidade inerente à administração concomitante de derivados anfetamínicos.

ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA, *ecstasy*) is often consumed in a polydrug abuse pattern, where the co-occurrence of other substances is common. In addition to several drugs that are intentionally taken to achieve certain effects, other substances are ingested as part of therapeutic schemes, or inadvertently consumed as contaminants in *ecstasy* tablets. Notwithstanding the great potential of this polydrug abuse to negatively impact on MDMA-elicited toxicity, joint detrimental effects have received rather little attention from the scientific community with most available investigations heavily relying on single-drug studies.

We became very interested in evaluating whether amphetamines in combination were able to yield significant effects, distinct from those observed for the individual drugs. We had anticipated that potential interactions between amphetamines could occur, since these drugs have overlapping mechanistic, metabolic and detoxifying pathways.

The cytotoxic profiles of MDMA and three other amphetamines, methamphetamine (METH), 4-methyltioamphetamine (4-MTA), and *d*-amphetamine (*d*-AMP), widely found in *ecstasy* pills in addition to, or instead of MDMA, or intentionally co-ingested to enhance the desired MDMA effects, were recorded using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay in a human liver carcinoma cell line (HepG2 cells). Then, the combination effects of three different mixtures were predicted on the basis of the individual concentration-response curves of all the components, by using two mathematical concepts broadly employed in pharmacology, the *concentration addition* (CA) and the *independent action* (IA) models. Experimental mixture testing confirmed CA expectations, revealing a clear additive effect. Of particular interest, we demonstrated that there were substantial mixture effects even when each amphetamine was present at levels that were individually ineffective, thus emphasizing the risks linked to polydrug abuse.

Other detrimental factors, such as hyperthermia, bioactivation into reactive species, the altered release and metabolism of neurotransmitters, impairment of mitochondrial function, apoptosis, and immune responses are thought to play a decisive role in amphetamine-induced hepatotoxicity. Accordingly, our research further aimed at confirming the applicability of CA in experimental scenarios that more closely mimic the liver *in vivo*. Specifically, we sought (i) to evaluate mixture effects in primary hepatocytes, (ii) to assess the

influence of increased temperature on these evaluations and (iii) to investigate the significance of the three major human MDMA metabolites in the mixture-related toxicity.

When the influence of temperature on the applicability of the two predictive models was investigated, CA was confirmed to be the most appropriate model in predicting amphetamine mixture effects also in hyperthermic conditions (40.5 ºC), in HepG2 cells. Remarkably, our data evidenced that, at this temperature, very small variations in the concentrations of each drug or the mixture prompted sharp increases in cytotoxic responses, which quickly shifted from roughly undetectable to highly detrimental effects. These observations emphasize the potential reduction in the 'safety window' of these drugs *in vivo* and the consequent increased risks that *ecstasy* abusers are exposed to. The mechanisms underlying the observed toxicity require exhaustive clarification, but our data indicate that antioxidative homeostasis disruption (increased production of reactive species and glutathione depletion) with consequent mitochondrial impairment and disturbance of the energetic cellular status seems to play a critical role in the observed cytotoxic effects.

Amphetamine mixtures were also analysed in primary rat hepatocytes, a model that is metabolically more representative of the hepatocytes *in vivo*. At a physiological incubation temperature (37 °C), the results obtained in primary hepatocytes and HepG2 cells were very consistent. Nevertheless, at a higher incubation temperature (40.5 °C), individual and mixture toxicities were significantly higher in primary cells. We hypothesized that the expected acceleration of the metabolic rate at higher incubation temperatures could explain the increased susceptibility of the primary hepatocytes. Additionally, the accuracy of the CA prediction was also demonstrated in primary cells at both incubation temperature settings.

Further studies were undertaken by applying the same experimental design to assess the effects of an equipotent mixture (a mixture containing the individual drugs at concentrations eliciting similar cytotoxic effects) of MDMA and its three main human metabolites 3,4-methylenedioxyamphetamine (MDA), *N*-methyl-*α*-methyldopamine (*N*-Me-*α*-MeDA) and *α*-methyldopamine (*α*-MeDA), aiming to simulate the mixture produced *in vivo* after MDMA administration. Also in this case, significant additive effects conforming to CA predictions were observed, even when the substances were present at low, non-cytotoxic concentrations, emphasizing hazard implications of MDMA metabolism to liver cells.

Ultimately, we searched for the mechanisms underlying the observed amphetamine-induced cell death. For that purpose, the substances were tested at equieffective concentrations (*i.e.* at concentrations producing 25% of cell death, as measured by the MTT assay). After 24 h of exposure to the individual and combined amphetamines at a

physiological incubation temperature (37 ^{o}C), HepG2 mortality occurred preferentially by apoptosis. Lethality was exacerbated with the increase of the incubation period to 48 h. When the incubation temperature was raised to 40.5 ^{o}C, necrosis became the privileged type of cell death, for both incubation times (24 h and 48 h). Consistent with these findings, ATP intracellular levels suffered a marked depletion at 40.5 ^{o}C, an effect which was not observed at 37 ^{o}C. Thorough investigations of the triggered apoptotic mechanisms showed a decline in *BCL-2* and *BCL-$_{XL}$* mRNA levels, with concurrent upregulation of *BAX*, *BIM*, *BAD* (only for MDMA), *PUMA* and *BID* genes. Elevation of Bax, cleaved Bid, Puma, Bak and Bim (EL, S and L isoforms) proteins were further confirmed. To the best of our knowledge, Puma, Bim and Bak had never been linked with the toxicity induced by amphetamines. Additional results showed that time-dependent caspase-3/-7 activation, but not mitochondrial membrane potential ($\Delta\psi_m$) disruption, may also mediate amphetamine-induced apoptosis, at least under our experimental conditions. The cell dismantling was confirmed by poly (ADP-ribose) polymerase (PARP) proteolysis, after 24 h exposures. Overall, for all the parameters evaluated, no relevant differences were detected between individual amphetamines and the mixture (all tested at equipotent concentrations), strengthening the hypothesis that amphetamine combination effects are additive and the entire mixture components act in a similar way to promote hepatic cell death.

In conclusion, the results presented in this thesis demonstrated for the first time that the amphetamine derivatives act additively to produce substantial mixture effects, even when the substances are present in amounts that individually do not elicit detrimental responses. This cytotoxicity was particularly significant in hyperthermia. Additionally, our results showed that the effects of mixtures of amphetamines can be predicted with accuracy by the CA model. The model of IA, in turn, was ineffective in anticipating mixture effects of such combinations, vastly underestimating the inherent toxicity of concomitant administration amphetamine derivatives.

LIST OF ABBREVIATIONS

4-MTA	4-Methyltioamphetamine
5-HT	Serotonin
ALDH	Aldehyde dehydrogenase
ATP	Adenosine 5'-triphosphate
CA	Concentration addition
COMT	Catechol-*O*-methyltransferase
CYP450	Cytochrome P450
Cyt *c*	Cytochrome *c*
DA	Dopamine
d-AMP	*d*-Amphetamine
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DXM	Dextromethorphan
ES	Effect summation
EtOH	Ethanol
GABA	Gamma-aminobutyric acid
GHB	Gamma-hydroxybutyrate
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HMA	4-Hydroxy-3-methoxyamphetamine
IA	Independent action
LSD	Lysergic acid diehtylamide
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MDA	3,4-Methylenedioxyamphetamine
MDEA	*N*-Ethyl-3,4-methylenedioxyamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
METH	Methamphetamine
MPT	Mitochondrial permeability transition
NA	Noradrenaline
NEC	No effect concentration
NF-κB	Nuclear factor kappa B
N-Me-α-MeDA	*N*-Methyl-α-methyldopamine
NOEC	No observed effect concentration
$ONOO^{\cdot-}$	Peroxynitrite
PARP	Poly (ADP-ribose) polymerase
Pgp	P-Glycoprotein
PMA	*p*-Metoxyamphetamine
Redox	Reduction-oxidation
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SERT	Serotonin transporter
SSRI	Selective serotonin reuptake inhibitor
T4	Thyroxine
tBid	Truncated Bid
TH	Tryptophan hydroxylase
THC	Δ^9-Tetrahydrocannabinol
TNF-α	Tumor necrosis factor alpha
UCP	Uncoupling protein
UGT	UDP-Glucuronosyltransferase

α-MeDA	α-Methyldopamine
Δψm	Mitochondrial membrane potential

PART I – GENERAL INTRODUCTION

The current dissertation is divided into three parts. The first part is an introduction in which the information available in the scientific literature will be presented. This section will focus on the toxicocokinetics, toxicodynamics and toxicological properties of MDMA, as well as on the influence of various aspects on the occurrence of adverse reactions to this drug, with emphasis on the potential for interactions with other substances, particularly with other amphetamines, including methamphetamine (METH), *d*-amphetamine (*d*-Amp) and 4-methylthioamphetamine (4-MTA). Then, a brief description of the methodology currently employed for the evaluation of mixture effects will be presented. In the second part, the experimental work is presented in the form of the five publications that were originated. These include the specific aims of each study, the detailed description of the experimental protocols, the attained results, the statistical analysis of the data, and the discussion for each study. The third part consists of the general and integrated discussion of the results obtained in the five different studies. The final conclusions of this dissertation and the references consulted are also presented in the fourth and fifth sections, respectively.

1. 3,4-Methylenedioxymethamphetamine (MDMA)

In the late 1980s, the recreational use of synthetic drugs like amphetamines became quite popular, attracting the attention of the scientific community, the media, and the society in general, and in particular of the young individuals that are the 'victims' of this phenomenon. Among this type of drugs, also known as *designer drugs*, one of the most notorious is 3,4-methylenedioxymethamphetamine (MDMA), commonly known as *ecstasy*. MDMA abuse is associated with all-night dance sessions occurring during the weekend, specifically in the techno music scenes designated as *raves*. The popularity of the drug can be explained by the range of effects that its users experience after consumption, which include euphoria, increased energy, increased sexual desire, and facilitation of social interaction (Baylen and Rosenberg 2006).

Because the number of severe and/or fatal acute intoxications is relatively low when the vast number of consumers is considered, one of the most worrisome particularities associated with the consumption of this drug is the widespread misconception that it

represents little danger for the user. However, *designer drugs* comprise genuine hazards and severe and even fatal adverse reactions can arise as a direct consequence of the drug's action, or associated with the adoption of other risk behaviours, such as driving under the influence of stupefacients, promiscuous and unprotected sexual intercourse (linked with sexually-transmitted diseases), among others (Becona Iglesias et al. 2011; Dunn et al. 2010; Hittner and Schachne 2012). These concerns are further reinforced by the ease with which recreational drugs, in particular amphetamines, are acquired and even synthesized (Carmo 2007).

One additional critical problem associated with *ecstasy* intake is the enormous unpredictability and variability of the possible detrimental effects of this drug, often independent of the amount of drug ingested or the circumstances in which it is taken (Carvalho et al. 2010). This suggests a strong variation among individual responses to the drug and the existence of predisposing factors for the expression of *ecstasy* toxicity. Also, among the plethora of factors that may impact the toxic effects elicited by *ecstasy* misuse is the simultaneous intake of other substances (Carvalho et al. 2012).

The exposure of *ecstasy* abusers to other substances relates not only to the intentional licit and/or illicit polydrug abuse patterns (aiming at modulating the effects of MDMA), which are conventionally associated with this drug, but also to the intake of pharmaceuticals during treatment regimens and to the presence of several other substances (stimulants or not) that appear as contaminants in *ecstasy* tablets (Carvalho et al. 2012; Mohamed et al. 2011). Contrasting with pharmaceuticals that are comprehensively studied in clinical trials, particularly in what concerns the characterization of their pharmacokinetics, amphetamine derivatives appearing on the illicit drug market are consumed without any sort of risk assessment. In fact, depending on the illegal source of these so-called 'party drugs' the content and purity can vary enormously. The analysis of seized tablets disclosed a large disparity in the amount of active substance per tablet and also revealed a large number of other substances present along with MDMA in *ecstasy* pills, including excipients (sodium bicarbonate, talc, sugars), and other drugs (stimulant or not), such as caffeine, lysergic acid diethylamide (LSD), paracetamol and other amphetamines (Carmo 2007; Carvalho et al. 2012; Mohamed et al. 2011). The awareness of the influence of these factors on the toxicity of MDMA is crucial for the establishment of measures to prevent risk and to undertake appropriate therapeutic strategies for the treatment of intoxications.

1.1. Historical perspective: from the clinic to the *dance scene*

MDMA was originally synthesized by Merck in 1912, and later patented in Germany in 1914. The objective of its synthesis was the development of an anorectic drug for use as an appetite suppressant (Hegadoren et al. 1999a), or as a precursor agent for new therapeutically active compounds (Green et al. 2003). MDMA was never used with such purposes but the psychiatric community advocated its use as a therapeutic adjunct to enhance the ability of introspection and induce feelings of trust and empathy between patients and psychotherapists (Green et al. 2003). This property has led to the classification of MDMA as belonging to a new pharmacological class, the *entactogens* (from the Greek *contact with the intimate*) (Nichols et al. 1986). The first report of MDMA psychostimulant activity belongs to Alexander Shulgin, who was, consequently, considered the 'father' of the drug (Shulgin and Nichols 1978).

MDMA emerged as a drug of abuse in the late 1970s. Despite the controversy surrounding the therapeutic potential of MDMA, its use was prohibited due to its widespread use and high potential for abuse (Shulgin 1986). Albeit the restrictions on its usage and marketing, since the mid 1980s MDMA achieved impressive popularity as a drug of abuse worldwide, particularly among the youth. Its stimulant effects, associated with increased alertness, induced sense of well-being, feelings of trust and empathy, decreased inhibitions and moderate hallucinogenic effects, together with a false perception of safety, are responsible for its popularity (Green et al. 2003).

1.2. MDMA misuse

1.2.1. Epidemiology, consumption venues and patterns

Several studies on the consumption patterns of *ecstasy* have been carried out. The 2011 Annual Report on the State of Drugs Problem in Europe, reported that amphetamines, including *ecstasy*, are among the most commonly used illicit drugs in Europe, being the second most abused illicit substance (after cannabis), in many countries (EMCDDA 2011). About 11 million Europeans have tried *ecstasy* and about 2.5 million have used the drug during 2010 (EMCDDA 2011). MDMA abuse is more frequent among young adults, with males reporting levels of use much higher than females. Lifetime prevalence of MDMA use

among the 15–34 age group ranges from 0.6 % to 12.7 %, with most countries recording estimations between 2.1 % and 5.8 % (EMCDDA 2011). The epidemiology and pattern of *ecstasy* abuse have been the object of a number of reviews (Baghianimoghadam et al. 2009; Becona Iglesias et al. 2011; Breen et al. 2006; Chen et al. 2011; Degenhardt et al. 2009; EMCDDA 2011; Kinner et al. 2012; Krul et al. 2012; Licht et al. 2012; Mendes and Lomba 2008; Ramtekkar et al. 2011; Scholey et al. 2004; Shamshiri Milani et al. 2011; Soar et al. 2006; van Ours 2005; Wilkins et al. 2011). A detailed and updated analysis of these data on MDMA consumption patterns is presented in Table 1.

MDMA is usually consumed at *rave* events or at parties and techno clubs that typically take place in overcrowded spaces with high ambient temperatures, loud repetitive electronic music and light shows (Klein and Kramer 2004). At these venues, MDMA consumers take the drug along with other psychoactive substances in a polydrug abuse pattern (Breen et al. 2006; Bruggisser et al. 2010; Chu et al. 2012; Falcon et al. 2010; Kinner et al. 2012). Among the substances most often co-consumed with MDMA are tobacco, cannabis and ethanol (EtOH) (Lora-Tamayo et al. 2004; Scholey et al. 2004). Another common consumption pattern involves the use of anxiolytics and sedatives (benzodiazepines and opiates) together with stimulants, such as cocaine and, in many cases other amphetamines (Williamson et al. 1997; Wu et al. 2009).

It is believed that many of these factors might impact the toxicity of MDMA, as in fact, most fatal intoxications reported in the literature are associated with MDMA abuse in these settings (CDC 2010; Cole and Sumnall 2003; Vanden Eede et al. 2012).

1.2.2. *Ecstasy* trading: presentation forms and routes of administration

Commonly, MDMA is marketed for oral use as tablets of diverse colours, shapes and sizes, decorated with a wide assortment of logos (Green et al. 2003).

Table 1. Patterns of recreational consumption of MDMA.

Year	Location	Population	n	Age (years)	Individuals consuming MDMA	Number of pills	Reference
2011	On a cruise ship on the Baltic Sea	Swedish clubbers attending a 40h electronic music dance event	401 (when boarding the ship, all passengers were required to pass by police officers and drug detecting dogs)	18-45	16.2 % have tried 0.3% uses a couple of times/month 0.5% in the last 48h 1.8 % when clubbing 20.0% were polydrug users: the most commonly used drugs were amphetamines, followed by *ecstasy*/MDMA, cannabis, and cocaine		(Kinner et al. 2012)
2006-2011	Victoria, Australia	Drivers tested for presence of drugs	853		17% used MDMA alone and 3.9% combined with METH and Δ(9)-tetrahydrocannabinol (that were the most common drugs with 77% and 42% prevalence, respectively)		(Chu et al. 2012)
2010	Los Angeles County, USA	Emergency department (ED) visits, all linked to a New Year's Eve event attended by approximately 45,000 persons	30	16-34	18 patients visited EDs for MDMA-related illness within 12 h of the rave. 9 were female. In addition to using MDMA, 13 also had used alcohol or other drugs, including marijuana and prescription medications. For the 6 patients (3 of whom were aged <21 years) with available serum alcohol levels, the mean blood alcohol concentration was 0.31 g/dL (range: 0.19 g/dL-0.33 g/dL)	1-6 tablets (MDMA and caffeine in nearly equal proportions, and a minor amount of N-methylphthalimide)	(CDC 2010)
2010	Galicia (Santiago de Compostela, Lugo, A Coruña, Ferrol, Pontevedra, Ourense and Vigo)	Young people (49.7% males)	1214	15-25	33.4% have tried MDMA 22.3% in the last year		(Becona Iglesias et al. 2011)
2010	Tehran (Iran)	Iranian high school female students	2350		2.3% (polydrug use linked with *ecstasy* misuse $p < 0.001$)		(Shamshiri Milani et al. 2011)
2010	Ibiza (Spain)	Mothers after giving birth (a 3-cm-long proximal segment of maternal hair corresponding to the last trimester of pregnancy was analyzed)	107		0.9 %		(Friguls et al. 2012)
2010	Web-based survey (country of residence: 84% UK, 4% Ireland, 8% Other in Europe, 5% North America, 2% Australia)	Mephedrone users (individuals had taken mephedrone on at least one occasion in the past)	1506	10-73	87% have tried 11% typically taken with mephedrone		(Carhart-Harris et al. 2011)

Table 1. Continued.

Year	Location	Population	n	Age (years)	Individuals consuming MDMA	Number of pills	Reference
2009	France	Student population seen during the visit at the University medical facility	808	20 (mean age)	4% (at least once)		(Morvan et al. 2009)
2007-2009	Murcia, Spain	Pregnant women at 12th week gestation who decided voluntarily to interrupt their pregnancy	142		1.4% were positive for MDMA 5.6% cases of polydrug abuse		(Falcon et al. 2010)
1997-2009	Swiss	Exposure/abuse cases with psychostimulants reported to the Swiss Toxicological Information Centre	433	11-62	Approximately 56.7% of MDMA exposures with a known medical outcome exhibited a polydrug abuse pattern.		(Bruggisser et al. 2010)
2008	Europe	Night club attenders	1341	16-35	28.7% (at least once in a lifetime)		(Bellis et al. 2008)
2008	UK	Drug abusers	80	20-22	86.3% (at least once in a lifetime)	1-6	(Cole et al. 2008)
2008	Portugal	MDMA abusers	223	13-23	53.8% occasional consumption (0-1times/month); 6.7% polydrug abuse. 34.5% regular consumption (> than once a month); 35% polydrug abuse. 11.7% excessive consumption (> than once a week); 58.3% polydrug abuse.		(Mendes and Lomba 2008)
2008	USA	High school students (9^{th}–12^{th} grade)	13917		4.9-7.4% (in the preceding month of the study).		(Pisetsky et al. 2008)
2008	New Zealand	General population	1902 (year 2006) 5475 (year 1998)	15-45	1.5% in 1998 3.9% in 2006		(Wilkins and Sweetsur 2008)
2007	Switzerland	Rave party attenders	302	16-46	40.4% (at least once in a lifetime) 14.7% (1 to 3 times on the previous month of the study)		(Chinet et al. 2007)
2007	Germany	Disqualified drivers	247	18-29	14.6% had only consumed MDMA 27% polydrug ingestion of designer drugs		(Dresen et al. 2007)
2007	UK	Drug users	40	18-45	85% at least once in a lifetime	1-4	(Goudie et al. 2007)
2007	New York (USA)	Gay and bisexual men	450	18-67	74.7%		(Halkitis et al. 2007)
2007	USA	MDMA users	48	18-35	97.9% (in the previous year of the study)		(Medina and Shear 2007)

Table 1. Continued.

Year	Location	Population	n	Age (years)	Individuals consuming MDMA	Number of pills	Reference
2007	Taiwan	MDMA users	200	14-19	62.5% used 20 times 29.5% polyconsumers		(Yen et al. 2007)
2006	Taipei (Taiwan)	Teenagers	2126	12-18	2-14.5%(at least once in a lifetime)		(Chou et al. 2006)
2006	Australia	MDMA users	216	19-39	28.2% combine MDMA with pharmaceuticals	2	(Copeland et al. 2006)
2006	China	Drug users			5.37%		(Fang et al. 2006)
2006	New York (USA)	Gay and bisexual man (GHB consumers)	131	18-40+	56.5%		(Halkitis et al. 2007)
2006	Colorado (USA)	Young people in polydrug abuse treatment	782	10-32	34% (at least once in a lifetime)		(Hopfer et al. 2006)
2006	New York (USA)	Women attending night clubs	1104	18-49	44% (at least once in a lifetime)		(Parsons et al. 2006)
2006	Italy	Rave party attenders	2015	20-40	17-27.1% (at least once in a lifetime) 9.5-16.2% (in the last year) 4.4%-10% (in the last month)		(Pavarin 2006)
2006	USA	University students	1206		9% (at least once in a lifetime)		(Wish et al. 2006)
2006	Texas (USA)	Low income users	696	18-31	15.2% at least once in a lifetime; of these, 53% in the last year; 20% in the last 20 days)	0,5-4	(Wu et al. 2006b)
2006	New York (USA)	Women attending to night clubs	1104	18-49	44% (at least once in a lifetime)		(Parsons et al. 2006)
2006	USA	Designer drug users	19084	16-23	13% (at least once in a lifetime) 6% (in the last year)		(Wu et al. 2006a)
2005	Montreal (Canada)	Rave party attenders	186	17-47	50% (at least once in a lifetime; 92.3% of these uses MDMA in combination with other drugs)	1	(Barrett et al. 2005)
2005	New York (USA)	Gay and bisexual young man	569	17-28	47.9% (at least once in a lifetime) 24.3% (chronic use; of these. 82.8% used at least once and 13.3% used more than 5 times in the last month)		(Clatts et al. 2005)
2005	USA	Gay and bisexual hispanic man	172		14% (in the last 6 months)		(Fernandez et al. 2005)
2005	New York (USA)	6^{th}, 7^{th} and 8^{th} grade students	23780		2.3% (at least once in a lifetime)		(Goldsamt et al. 2005)
2005	New York (USA)	Drug users	715	17-64	23.4% (at least once in a lifetime) 11.9% (in the last 6 months)		(Ompad et al. 2005)

Table 1. Continued.

Year	Location	Population	n	Age (years)	Individuals consuming MDMA	Number of pills	Reference
2004	USA	Gay and bisexual men	150	31-50	14% (in the last 3 months)		(Crosby and Diclemente 2004)
2004	Ibiza (Spain)	People arrested for drug possession	70		77% (had only MDMA in urine) 20% (MDMA + another designer		(Lora-Tamayo et al. 2004)
2004		Internet users	763	21-35	37% (at least once in a lifetime) 39% beginners (1-9 times) 48% moderate use (10-99 times) 13% heavy use (more than 100 times)	Beginners: 1-2 pills 3% moderate users: > 4 pills 14% heavy users: >4 pills	(Scholey et al. 2004)
2004	Denver, Colorado (USA)	Homeless	186	16-25	25% (in the last 9 months)		(Van Leeuwen et al. 2004)
2003	Australia	Women	9512	22-27	15% (at least once in a lifetime)		(Turner et al. 2003)
2003	Chicago (USA)	Adult population	627	18-40	49% (at least once in a lifetime)		(Fendrich et al. 2003)
2002	Montreal (Canada)	Rave party attenders	210	16-32	65.2% (at least once in a lifetime)		(Gross et al. 2002)
2001/2002	USA	Asian American adolescents and young adults	996 adolescents 1108 adults	12-25	3.7 % (Ages 12–17) 12.8% (Ages 18–25)		(Wu et al. 2011)
2001/2002	USA	Adult participants in the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC)	43093		1.6% reported lifetime *ecstasy* use (37% *ecstasy*-polydrug users; 29% *ecstasy*-marijuana-cocaine-moderate amphetamines users; 34% *ecstasy*-marijuana-low prescription-type drugs users)		(Wu et al. 2009)
2001	UK and Italy	Young people	768	14-28	15% (less than 20 times) 15.5% (more than 20 times)		(Parrott et al. 2001)
2001	Edinburgh	Drug users	122	16-47	80% *ecstasy* or amphetamine (of these 35% take MDMA on a weekly basis)		(Riley et al. 2001)
2001	UK	Drug users	1151	18-29	96% (at least once in a lifetime) In the last year, 12% has used 2-3 times/week; and 22% has used once a week	55% consumed 2 or less 25% consumed 4 or more	(Winstock et al. 2001a)
1999	South England	Drug and alcohol users	100	16-21	22% (in the last 90 days) 38% (at least once in a lifetime)		(Boys et al. 1999)
1999	Australia	MDMA users	329	15-46	89% (at least once a month)	1	(Topp et al. 1999)
1997/1999	USA	University students	14000	21-24	2.8% in 1997 and 4.7% in 1999		(Strote et al. 2002)

Table 1. Continued.

Year	Location	Population	n	Age (years)	Individuals consuming MDMA	Number of pills	Reference
1998	Italy	MDMA users	150	19-28	50% polydrug abusers	1 or less	(Schifano et al. 1998)
1997	West Australia	Rave attenders	83	13-48	76% (at least once in a lifetime) 41% (polydrug abuse) 31.3% (on the last rave party)		(Lenton et al. 1997) (Boys et al. 1997)
1997	UK	Drug users	158	14-59	52% used during last year 70% (on last month)	2	(Williamson et al. 1997)
1996	UK	High School students	7722	15-16	7.3% of girls (at least once in a lifetime) 9.2% of boys (at least once in a lifetime)		(Miller and Plant 1996)
1996	UK	University students (second year)	3075	18-65	13% (at least once in a lifetime) 5.2% (more than once or twice) 2.7% (at least once a week)		(Webb et al. 1996)
1995	Glasgow (UK)	Rave party attenders	135	14-44	95% (at least once in raves)		(Forsyth 1995)
1992	Sydney (Australia)	MDMA users	100	16-48	68% (> 3 times) 33% use drugs once a month or per trimester) 18% use in special occasions	9% less than 1 71% used 1 13% used 2 7% used more than 2	(Solowij et al. 1992)
1986/1990	USA	University students	742 (in 1986) 1264 (in 1990)		15.5% (at least one time in 1986) 24.3% (at least one time in 1990)		(Cuomo et al. 1994)
1987	USA	University students	369		39% at least once last year		(Peroutka 1987)

The most common names by which it is recognized in the market of illicit drugs are: *Ecstasy*, *XTC*, *E*, *X*, *Adam*, *Lover's Speed* and *love drug*. *Ecstasy* is now considerably cheaper than it was in the 1990s, when it originally became widely available. While there are some reports of tablets being sold for as little as EUR 1, most countries report mean retail prices in the range of EUR 4 to EUR 9 per tablet (http:// www.emcdda.europa.eu/publications/annual-report/2011).

Different administration routes are experienced to modulate the attained effects (Mechan et al. 2006; Pontes 2009; Solowij et al. 1992). Although MDMA oral administration is the most common (as pills, capsules or powder dissolved in water), nasal (snorting by inhalation of powder obtained by crushing tablets) (Gahlinger 2004), rectal (suppositories, known as *shafting* or *shelving*) and intravenous administrations (unusual) have also been described (Ramsey et al. 2001; Solowij et al. 1992).

1.2.3. *Ecstasy* tablets – purity concerns

Since MDMA is a drug clandestinely synthesized, dose and purity of the pills vary considerably, as can be seen in Table 2. Generally, the tablets contain between 80 mg and 150 mg of MDMA, but higher dosages have also been found (up to 250 mg per tablet) (Green et al. 2003; Holden and Jackson 1996). The average MDMA content of *ecstasy* tablets tested in Europe in 2009 was between 3 and 108 mg, but the availability of high-dose *ecstasy* tablets containing higher amounts of MDMA was reported by several countries in 2011 on the Annual Report on the State of Drugs Problem (http:// www.emcdda.europa.eu/publications/annual-report/2011). Since *ecstasy* is a term often used to describe other chemically and pharmacologically related compounds, MDMA consumers may, inadvertently or not, ingest substances other than MDMA, which are marketed under the same designation. Additionally, other substances may be present in the tablets, including psychoactive analogues of amphetamines (present in a high percentage of *ecstasy* tablets), caffeine, ephedrine/pseudoephedrine, dextromethorphan (DXM), ketamine, acetaminophen, and atropine salicylates (Baggott et al. 2000; Carvalho et al. 2012; Mohamed et al. 2011; Parrott 2004a). These drugs are included in *ecstasy* tablets with the aim of alleviating the unpleasant ‘come-down’ effects of MDMA, such as anhedonia, dysphoria, and depression or to extend the duration of the ‘high’ (Mohamed et al. 2011).

Table 2. Impurity profile and MDMA content of pills sold as *ecstasy*.

Study	Year	Local	n	Origin	MDMA dose (mg/tablet)	With MDMA	Without MDMA	Other substances in *ecstasy* tablets with MDMA
(Morefield et al. 2011)	2011	Adelaide, Australia.	28 different batches	Participants donated *ecstasy* pills for chemical analysis	0-124	46.4% MDMA only 17.9% MDMA + MDEA 14.3% MDMA + METH		MDEA, ketamine, caffeine, MDA, pseudoephedrine
(Macias and Furton 2011)	2011	Florida, USA	3 different batches (#1, #2 and #3)	Three batches of seized *ecstasy* were provided by the Florida Highway Patrol Contraband Interdiction Program Division		25%, 17% and 8% MDMA for batch #1, #2 and #3, respectively		Batch #1 also presented piperonal, MDP-2-POH, and MDP-2-P; Batch #2 also presented isosafrole, piperonal, and MDP-2-POH; Batch #3 presented 10% caffeine and also METH, isosafrole, MDP-2-POH, MDP-2-P, MDEA
(Wood et al. 2011)	2011	London, UK	101	*Ecstasy* tablets seized from individuals attending nightclubs	58.7±22.9, with a range of 20-131 mg per tablet.	The majority (96.0%) of tablets contained less than 100 mg MDMA per tablet. There appeared to be a bimodal distribution of MDMA content at approximately 20-40 mg per tablet and 60-80 mg per tablet.		
(Brunt et al. 2012)	2000-2010	Netherlands	5,786	Drug users that utilized voluntarily and anonymously the drug testing facilities of DIMS	2.0–218.0	69.9% MDMA only 2.5% MDMA+MDEA 2% MDMA+mCPP 1.2% MDMA+PMMA 1.1% MDMA+AMP 0.7% MDMA+MDA <0.1% MDMA+2-CB	12.2% mCPP 1.8% MDA 1.6% BZP 1.5% AMP 1.5% mephedrone 1.3% 2 C-B 0.6% MDEA	Metoclopramide, BZP, mephedrone, caffeine, p-fluoroamphetamine
(Shetab Boushehri et al. 2009)	2009	Tehran, Iran	13	The most abundant *ecstasy* type pills	23.9-124.5mg			
(Mitrevski et al. 2011)	2006-2009	Macedonia and Australia	24	Nine tablets were seized in Macedonia (2006–2007) and provided by Macedonian Police, and 15 tablets were seized in Australia (2009) and provided by Australian Federal Police				Macedonian samples were rich in *N*-formyl-MDMA and *N*-acetyl-MDMA and poor in 3,4-MD-propane and 3,4-MDAcPh as compared to Australian samples. 2-(Dimethylamino)-2-methyl-3-(3,4-methylenedioxyphenyl)-propanenitrile has been detected only in samples from Macedonia. Presence of 3,4-methylenedioxyphenyl derivative and apiperonyl methyl ether have been detected only in samples from Australia.

AMP, amphetamine. BZP, benzylpiperazine. 2-CB, 4-bromo-2,5-dimethoxyphenethylamine. mCPP, meta-chlorophenylpiperazine. DIMS, Drug Information and Monitoring System. DOB, 2,5,-dimethoxy-4-bromoamphetamine. DXM, dextromethorphan. N-formyl-MDMA, N-formyl-3,4-methylenedioxymethamphetamine. LSD, lysergic acid diethylamide. MBDB, *N*-methyl-1,3-benzodioxolylbutanamine. MDA, 3,4-methylenedioxyamphetamine. 3,4-MDAcPh, 3,4-methylenedioxyacetophenone. MDB, 3,4-methylenedioxy-N-methylbenzylamine. MDEA, 3,4-methylenedioxy-*N*-ethylamphetamine. 3,4-MD propane, 3,4-methylenedioxyphenylpropane. MDP, 3,4-methylenedioxyphenyl-2-propanol. MDP-2-P, 3,4-methylenedioxyphenyl-2-propanone. MDP-2-POH, 1-(3,4-methylenedioxyphenyl)-2-propanol. METH, methamphetamine. MTA, 4-methylthioamphetamine. PCP, phencyclidine. PMA, *para*-methoxyamphetamine. PMMA, p-methoxymethamphetamine.

Table 2. Continued.

Study	Year	Local	n	Origin	MDMA dose, mg/tablet	With MDMA	Without MDMA	Other substances in *ecstasy* tablets with MDMA
(Vogels et al. 2009)	1993-2008	Netherlands	33,006	*Ecstasy* tablets that were handed in by numerous individual (potential) substance users		The number of tablets containing only MDMA was lowest around 1997, reaching the highest levels in 2000 and 2004. From 1998 to 2008, the number of high-dose tablets (> or = 106 mg MDMA) gradually increased.	Many tablets contained other substances in addition to or instead of MDMA (e.g. MDA, MDEA, MBDB, AMP and caffeine).	After 2004, the purity of ecstasy tablets decreased again, caused mainly by a growing proportion of tablets containing meta-chlorophenylpiperazine (mCPP).
(Cheng et al. 2006)	2006	Hong Kong	89	Seizure	43 (average)			Ketamine, caffeine, salicylates, acetaminophen, chlorpheniramine, metronidazole, by-products of synthesis.
(Tanner-Smith 2006)	2006	USA	1214	Submitted anonymously		39% only MDMA	46%	MDA, DXM, caffeine, pseudoephedrine, 4-MTA, low concentrations of ketamine, DOB, heroin, PCP, PMA.
(Teng et al. 2006)	2002-2005	Taiwan	181	Seizure	16-193	66-71% only MDMA (the content of MDMA in MDMA only tablets varied from 89 to 133 mg/tablet)		There was a decreasing trend in MDMA content in these tablets over time. Other components commonly found besides MDMA included caffeine (18%), METH (7%), MDEA (7%) and AMP (4%). MDA, ketamine, ephedrine, diazepam, chlorzoxazone and nicotinamide were also detected. During the study period, the number of other drugs found as well as the combinations of different drugs detected in these tablets increased.
(Mitrevski and Zdravkovski 2005)	2000-2004	Skopje, Macedonia	24,229	Seized by Macedonian police		4880 tablets or 20% tablets contained MDMA as active ingredient	19,287 tablets or 79% contained d-AMP as active ingredient	METH, MDEA, MDA and MBDB
(Drugs 2004)	2004	New Zealand	8604	Seizure	30.2-172.4	Most of it		Ketamine, cocaine

AMP, amphetamine. BZP, benzylpiperazine. 2-CB, 4-bromo-2,5-dimethoxyphenethylamine. mCPP, meta-chlorophenylpiperazine. DIMS, Drug Information and Monitoring System. DOB, 2,5,-dimethoxy-4-bromoamphetamine. DXM, dextromethorphan. N-formyl-MDMA, N-formyl-3,4-methylenedioxymethamphetamine. LSD, lysergic acid diethylamide. MBDB, *N*-methyl-1,3-benzodioxolylbutanamine. MDA, 3,4-methylenedioxyamphetamine. 3,4-MDAcPh, 3,4-methylenedioxyacetophenone. MDB, 3,4-methylenedioxy-N-methylbenzylamine. MDEA, 3,4-methylenedioxy-*N*-ethylamphetamine. 3,4-MD propane, 3,4-methylenedioxyphenylpropane. MDP, 3,4-methylenedioxyphenyl-2-propanol. MDP-2-P, 3,4-methylenedioxyphenyl-2-propanone. MDP-2-POH, 1-(3,4-methylenedioxyphenyl)-2-propanol. METH, methamphetamine. MTA, 4-methylthioamphetamine. PCP, phencyclidine. PMA, *para*-methoxyamphetamine. PMMA, p-methoxymethamphetamine.

Table 2. Continued.

Study	Year	Local	n	Origin	MDMA dose, mg/tablet	With MDMA	Without MDMA	Other substances in *ecstasy* tablets with MDMA
(Cheng et al. 2003)	2000-2001	Hong Kong	212	Seizure		MDMA tablets have made up 98 and 71% of the total *ecstasy* tablets	4-MTA, MDA and AMP	Ketamine (present in 12% of tablets in 2000 and 42% in 2001). MDP-2-P, MDP, MDB, piperonal and N-formyl-MDMA were the most common impurities detected in MDMA tablets seized
(Cole et al. 2002)	2002	UK	80	Seizure	Ranged from 20 to 109 mg and the mean was in the 60-69 mg range	100%		MDEA
(Gimeno et al. 2002)	2002	France	10	Seizure		33.7±3.1%		Benzaldehyde, 1,3-benzodioxole, 3,4-methylenedioxytoluene, AMP, METH, 1,2-dimethyl-3-phenyl-aziridine, safrole, 3,4-(Methylenedioxy)-phenylpropane, piperonal, *p*-Methoxymethamphetamine, MDA, *N*-Methyl-1-[1,2-dimethoxy-4-(2-aminopropyl)]benzene, among others
(Spruit 2001)	2001	Netherlands	10,000	Seizure		48-60% only MDMA 7-13% MDMA+X	2-7% MDA ou MDEA	MDA, MDEA, AMP
(Baggott et al. 2000)	2000	USA	107	Submitted for analysis of tablets		63% MDMA, MDA or MDEA	29%	21% contain DXM; Caffeine, ephedrine, pseudoephedrine, salicylates
(Sherlock et al. 1999)	1999	UK	25	Amnesty	19-140	64% MDEA or MDMA	24%	Ketamine, caffeine, 4-MTA, AMP paracetamol
(Schifano et al. 1998)	1998	Italy	20,000	Seizure	100-150	85-90%	MDA, MDEA, MBDB	
(Giroud et al. 1997)	1997	Switzerland						MDA, MDEA, MBDB, 2-CB, caffeine, ephedrine, polyols, LSD, testosterone, chloroquine, vasodilators
(Milroy et al. 1996)	1996	UK	13		0,21-12	77% MDEA, MDMA, MDA, or mixtures		
(Renfroe 1986)	1986	USA	101			58 % only MDMA 26% MDMA+X	16%	MDA

AMP, amphetamine. BZP, benzylpiperazine. 2-CB, 4-bromo-2,5-dimethoxyphenethylamine. mCPP, meta-chlorophenylpiperazine. DIMS, Drug Information and Monitoring System. DOB, 2,5,-dimethoxy-4-bromoamphetamine. DXM, dextromethorphan. N-formyl-MDMA, N-formyl-3,4-methylenedioxymethamphetamine. LSD, lysergic acid diethylamide. MBDB, *N*-methyl-1,3-benzodioxolylbutanamine. MDA, 3,4-methylenedioxyamphetamine. 3,4-MDAcPh, 3,4-methylenedioxyacetophenone. MDB, 3,4-methylenedioxy-N-methylbenzylamine. MDEA, 3,4-methylenedioxy-*N*-ethylamphetamine. 3,4-MD propane, 3,4-methylenedioxyphenylpropane. MDP, 3,4-methylenedioxyphenyl-2-propanol. MDP-2-P, 3,4-methylenedioxyphenyl-2-propanone. MDP-2-POH, 1-(3,4-methylenedioxyphenyl)-2-propanol. METH, methamphetamine. MTA, 4-methylthioamphetamine. PCP, phencyclidine. PMA, *para*-methoxyamphetamine. PMMA, p-methoxymethamphetamine.

1.3. Pharmacokinetics

1.3.1. Absorption, distribution and excretion

The half-life of MDMA varies between 6 and 12 h and excretion is mainly hepatic and renal in the first 24 h after intake (delaTorre et al. 2004a; delaTorre et al. 2004b; Fallon et al. 1999; Hegadoren et al. 1999; Mas et al. 1999; Ramcharan et al. 1998). Despite being extensively metabolised by the liver, a significant fraction of the drug is generally excreted without biotransformation (delaTorre et al. 2004a). Since amphetamine derivatives are compounds with alkaline characteristics (pKa~ 9.9), and with relatively low molecular weight, these drugs readily cross cell membranes and lipidic layers, reaching the tissues and biological fluids with pH more acidic than blood, including saliva and sweat (delaTorre et al. 2004a). MDMA may be additionally detected in vitreous humor, hair and nails (delaTorre et al. 2004a; Samyn et al. 2002). Due to its inherent properties (e.g. significant volume of distribution), MDMA is liable to *post-mortem* redistribution (De Letter et al. 2010). *Post-mortem* studies revealed that MDMA accumulates in many tissues and organs where it reaches concentrations much higher than in the plasma: for example, it may be up to 18 times higher in the liver (De Letter et al. 2006; deLetter et al. 2004) and 30 times higher in the brain (Garcia-Repetto et al. 2003). Furthermore, literature suggests that the dose of MDMA ingested and the plasma concentrations achieved in fatal poisoning are not necessarily superior to those found in survival cases (Milroy 2011).

MDMA pharmacokinetics also appear to be stereoselective (Fallon et al. 1999; Fitzgerald et al. 1990; Lanz et al. 1997; Lin et al. 1997; Meyer et al. 2002a; Moore et al. 1996; Pizarro et al. 2002; Pizarro et al. 2004; Ramcharan et al. 1998)

1.3.2. Metabolism

The metabolism of MDMA in humans is well documented (Carvalho et al. 2012; delaTorre et al. 2004a; delaTorre et al. 2004b; Kraemer and Maurer 2002). Due to the presence of the methylenedioxy ring, MDMA undergoes extensive metabolism, greater than other amphetamine-type compounds (delaTorre et al. 2004a). About 80% of MDMA is metabolized in the liver (delaTorre et al. 2004a). Foltz and Lim (1988; 1989) characterized the metabolism of MDMA and identified the four major metabolic pathways for MDMA in humans and rats,

namely, (i) *N*-demethylation, (ii) *O*-dealkylation (demethylenation) (iii) deamination and (iv) conjugation by methylation, glucuronidation or sulfonation. Figure 1 is a schematic representation of the metabolic pathways of MDMA.

The metabolism of MDMA occurs via two main pathways: (i) methylenedioxy ring opening (the major metabolic pathway), followed by methylation of the resulting hydroxyl groups and/or conjugation with glucuronic acid or with the sulfonate anion (delaTorre et al. 2004a; delaTorre et al. 2000a; Lim and Foltz 1989; Maurer et al. 2000) and (ii) the dealkylation of the amine group with formation of 3,4-methylenedioxyamphetamine (MDA) that retains biological activity (Johnson et al. 1988). The subsequent oxidation and deamination of the side chain leads to the formation of phenylketones that are oxidized to benzoic acid derivatives (Maurer 1996), which are then conjugated with glycine and excreted as hippuric acid derivatives (hippurates) (delaTorre et al. 2004b).

The opening of the methylenedioxy ring of MDMA and MDA originates two catechol metabolites, *N*-methyl-α-methyldopamine (*N*-Me-α-MeDA) and α-methyldopamine (α-MeDA), respectively. Both are subsequently methylated by the enzyme catechol-*o*-methyltransferase (COMT). These metabolites are mostly present in plasma and urine as conjugates with glucuronide or with sulfonate anions (delaTorre et al. 2000a; delaTorre et al. 2000b; Kraemer and Maurer 2002; Maurer 1996). The presence of the catechol group confers high reactivity to these metabolites, which easily oxidize, giving the corresponding *o*-quinone (Cho et al. 1999). The oxidation of quinones may also cause the formation of aminochromes (Bindoli et al. 1992), whose subsequent oxidation leads to the production of polymers of melanin type (Zhang and Dryhurst 1994). The cycles of reduction-oxidation (redox) associated with this metabolic conversion result in the formation of reactive oxygen (ROS) and nitrogen (RNS) species, which similarly to what occurs with quinones, might attack relevant intracellular nucleophilic groups, such as cysteine, reduced glutathione (GSH) and protein sulfhydryl groups, resulting in significant modification of macromolecules, including proteins, lipids and deoxyribonucleic acid (DNA) (Bindoli et al. 1992; Bolton et al. 2000).

These quinones combine with one molecule of GSH to form adducts, which can further combine with a new molecule of GSH in a process that is also accompanied by the formation of ROS and RNS (Hiramatsu et al. 1990; Monks et al. 2004). The conjugation of catechols with glutathione has also been demonstrated, with consequent formation of toxic adducts (*e.g. 5*-(glutathion-*S*-yl)-α-MeDA) (Bai et al. 1999; Easton et al. 2003; Jones et al. 2005; Miller et al. 1997).

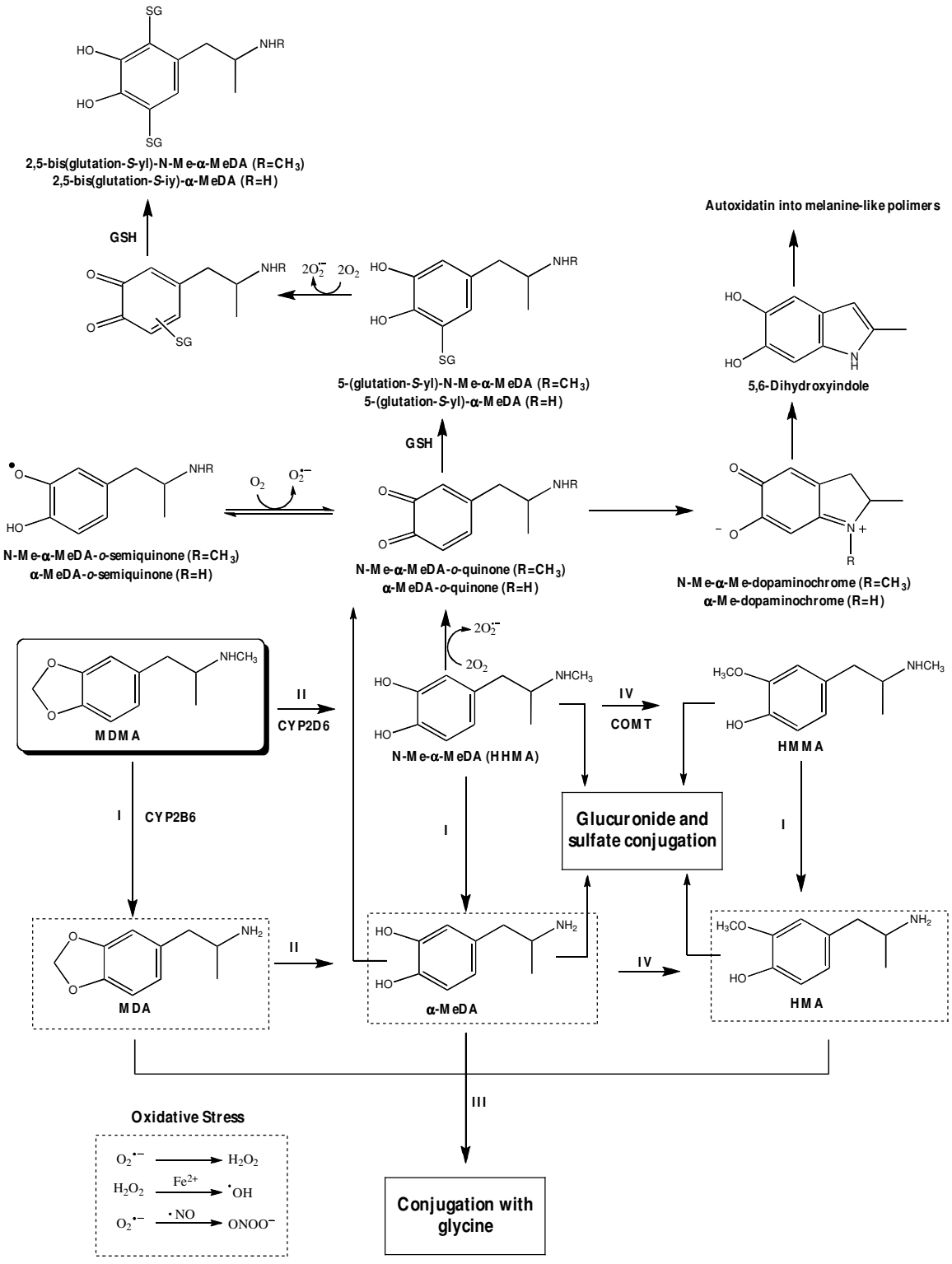

Figure 1. MDMA metabolic pathways. I, *N*-demethylation; II, demethylenation; III, oxidative deamination; IV, *o*-methylation; MDMA, 3,4-methylenedioxymethamphetamine; MDA, 3,4-methylenedioxyamphetamine; N-Me-*α*-MeDA or HHMA, N-methyl-*α*-methyldopamine or 3,4-dihydroxymethamphetamine; *α*-MeDA, *α*-methyldopamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; HMA, 4-hydroxy-3-methoxyamphetamine. Adapted from Carvalho (2012).

The demethylenation of MDMA displays a two-step Michaelis-Menten kinetics, which comprises a high affinity component and another one of low affinity (Kreth et al. 2000). The isoform 2D6 of cytochrome P450 (CYP450) catalyzes the low affinity component, while the high affinity component is mostly catalyzed by CYP1A2 and to a lesser extent by CYP2B6 and CYP3A4 (Kreth et al. 2000; Maurer et al. 2000; Tucker et al. 1994). The contribution of CYP2D6 to MDMA demethylenation was assessed *in vitro* and corresponds to approximately 50% in human liver and in microsomes expressing CYP2D6 (Ramamoorthy et al. 2002; Tucker et al. 1994), and to approximately 30% when assessed *in vivo* in humans (Segura et al. 2005).

The velocity of the *N*-demethylation is approximately one order of magnitude lower than the demethylenation and is characterized by monophasic kinetics. In humans, it is catalyzed by the enzyme CYP2B6 with contribution of CYP2D6, CYP1A2 and CYP3A4,and in the rat by CYP2D1 and CYP1A2 (Kreth et al. 2000; Maurer et al. 2000).

Since MDMA is predominantly metabolized by the CYP2D6 isoform of the CYP450 enzyme complex (Ingelman-Sundberg et al. 1999), it is vulnerable to pharmacokinetic/toxicokinetic interactions with other substances (pharmaceuticals, drugs of abuse, food, etc.) that share or interfere with this metabolic pathway (Oesterheld et al. 2004).

MDMA presents linear kinetics at low doses (delaTorre et al. 2004a; Mas et al. 1999) but non-linear pharmacokinetics at higher doses (similar to those commonly used for recreation) (delaTorre et al. 2004a; delaTorre et al. 2004b). This might be explained by the potential saturation of the metabolic pathways and by the interaction of some metabolites with the metabolic enzymes (delaTorre et al. 2004a). At higher concentrations, MDMA itself has been shown to be a CYP2D6 inhibitor through mechanisms that include a competitive interaction and/or the formation of a complex between the enzyme and the metabolites (Delaforge et al. 1999; delaTorre et al. 2000a; Heydari et al. 2004; Wu et al. 1997). This inhibition may occur within one hour after ingestion of MDMA, and the basal enzyme activity may only be restored after a period of at least 10 days (Yang et al. 2006). For this reason, repeated MDMA administration has the ability to transform the metabolizing phenotype of its users from an extensive metabolizer (expressing a fully functional enzyme) phenotype into a poor metabolizer (expressing non-functional or low levels of the enzymes) phenotype, regardless of the genotype of the individual.

1.4. Pharmacology

As mentioned previously, MDMA causes an euphoric state, including an increased sense of energy, emotional openness, increased capacity for introspection, empathy, intense perception of sounds and colours, reduced negative thoughts and reduced inhibitions (Davison and Parrott 1997; delaTorre et al. 2004a; Hegadoren et al. 1999; Liechti and Vollenweider 2000a; Morgan 2000; Parrott and Stuart 1997). In addition to the desired effects, MDMA prompts acute sympathomimetic reactions, *e.g*., increased heart rate and blood pressure, and transient anxiety (Greer and Strassman 1985; Grinspoon and Bakalar 1986). The mechanisms involved in the onset of these effects will be briefly discussed.

1.4.1. Effects on release, transport and neurotransmitter receptors of monoamines

MDMA induces the release of sympathetic neurotransmitters from the vesicles in nerve terminals and in the adrenal medulla, specifically serotonin (5-HT) and the catecholamines noradrenaline (NA), adrenaline, and dopamine (DA). These neurotransmitters activate their respective receptors, resulting in the exacerbation of sympathetic stimulation (Carvalho et al. 2012).

The ability of MDMA to induce 5-HT release is greater than that recorded for the release of DA and NA in cells expressing the respective transporters (Verrico et al. 2005). MDMA selectivity for affecting 5-HT release was demonstrated both *in vivo* (Mechan et al. 2002; Sabol and Seiden 1998; Yamamoto et al. 1995) and *in vitro* (Berger et al. 1992; Crespi et al. 1997; Nichols et al. 1982). The putative mechanisms for MDMA action at the serotonergic neuronal terminal are displayed in Figure 2. The increased release of 5-HT activates pre- and post-synaptic receptors, and triggers a sequence of intracellular pathways (*e.g*., via adenylate cyclase), resulting in the subsequent molecular and neurochemical effects (Callaway et al. 1990; Green et al. 2003; Morley et al. 2005; Simantov 2004).

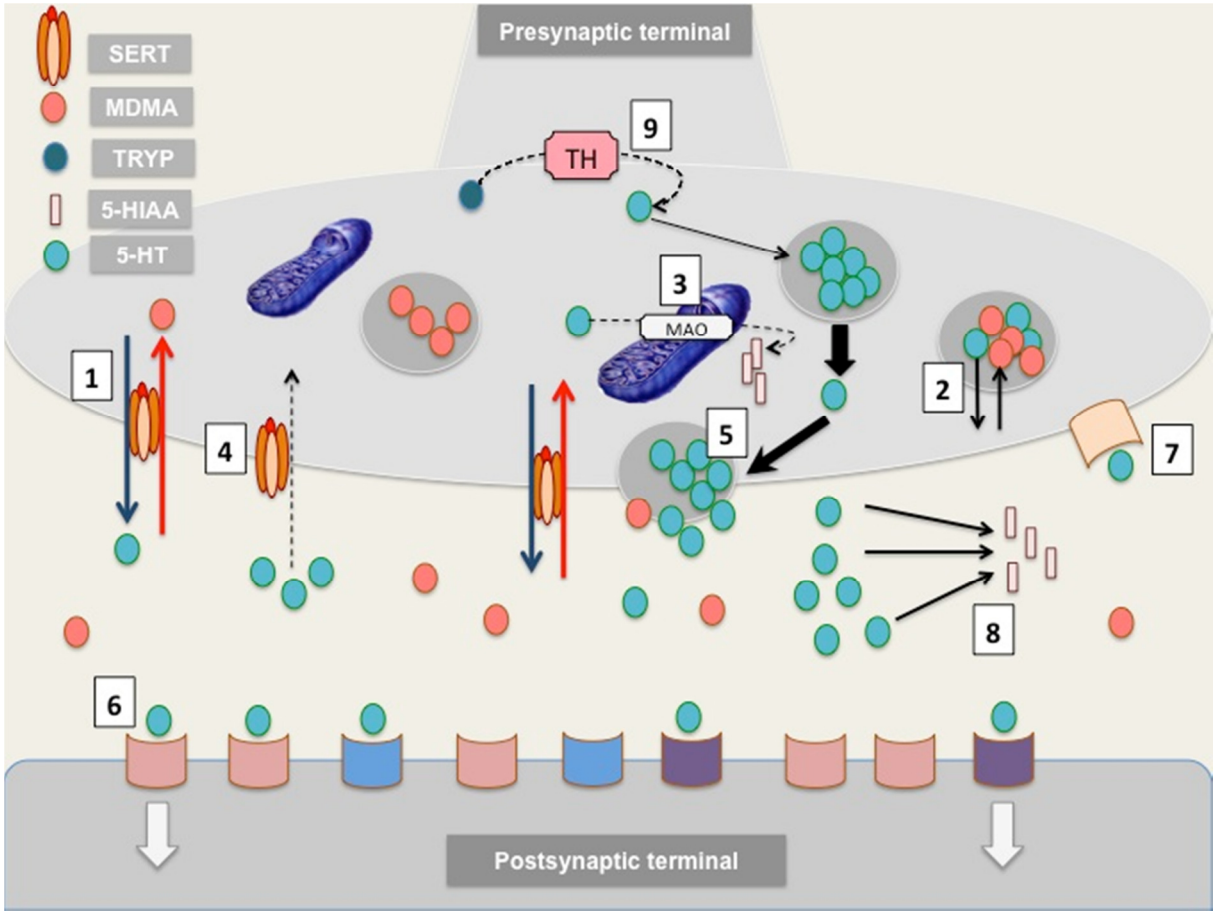

Figure 2. Pharmacological mechanisms of action of MDMA at the synapse of the serotonergic neuronal terminal. MDMA binding to the presynaptic serotonin transporter (SERT) inhibits neuronal reuptake of serotonin (5-HT) (4) and increases 5-HT release by exchange with MDMA (1), in a process designated by diffusion-exchange (Rudnick and Wall 1992; Simantov 2004). As a consequence of MDMA transport into the neuron, the carrier is available on the internal surface of the membrane to bind to 5-HT and transport it into the synaptic cleft (Rudnick and Wall 1992). Along with the effect at the neuronal reuptake level, MDMA also exerts its action on vesicular uptake of 5-HT. At low concentrations, MDMA inhibits vesicular uptake of 5-HT (Gudelsky and Nash 1996; Nichols et al. 1982). At higher concentrations, MDMA penetrates into the synaptic vesicles by a passive diffusion process (Green et al. 2003). Once inside these vesicles, and since MDMA chemically behaves as a weak base, pH increases promoting the efflux of 5-HT contained therein (2), as 5-HT is maintained inside the vesicles through a proton gradient that is regulated by an ATP-dependent proton pump (Rudnick and Wall 1992). The increase of 5-HT levels is also due to the inhibition of the metabolic degradation of this neurotransmitter by mitochondrial monoamine oxidase (MAO) (3), and to the stimulation of 5-HT release into the synaptic cleft (5). The released 5-HT accumulates in the synaptic cleft where it stimulates 5-HT postsynaptic receptors (Simantov 2004) (6). Nevertheless, mechanisms that prevent disproportionate increase of 5-HT levels in the synaptic cleft operate by self-regulating the release of 5-HT by presynaptic receptors (7), and by increasing formation of 5-HT metabolites and products of autoxidation in the synaptic cleft (8). MDMA also promotes the depletion of 5-HT reserves by causing inhibition of the biosynthesis of 5-HT by tryptophan hydroxylase (TH) (9). In addition to the indirect action of MDMA in stimulating the release of 5-HT, MDMA itself has affinity for 5-HT receptors and may activate them directly. Discontinuous arrows indicate inhibition and larger arrows indicate induction. Adapted from Carmo (2007).

The increase in DA release prompted by MDMA can result from: (i) the reversion of the transporter (Crespi et al. 1997) and/or (ii) the stimulation of DA synthesis by MDMA agonism of the 5-HT receptors (Bankson and Yamamoto 2004; Nash et al. 1990; Sprague et al. 1998; Yamamoto et al. 1995). Bruxism, tremors, hyperlocomotion, clonus and other long-term neurotoxic outcomes are among the dopaminergic effects elicited by MDMA (Ball et al. 2003; Bubar et al. 2004; Easton et al. 2003; O'Shea et al. 2001; Risbrough et al. 2006; Saadat et al. 2006).

MDMA also acts on central and peripheral adrenergic system, triggering effects such as an increase in systolic and diastolic blood pressure (Green et al. 2003), peripheral vasoconstriction, pupil dilation, decreased salivation, decreased secretion of pancreatic enzymes and insulin, urinary retention, decreased gastric motility, piloerection (Vander et al. 2001), and hyperactivity (Bexis and Docherty 2006; Selken and Nichols 2007).

Due to the MDMA stimulating action on the release of 5-HT, repeated exposure to the drug depletes the reserves of these neurotransmitters, provoking depression in MDMA abusers a few days after consumption (mainly due to the MDMA action on the 5-HT system) (Gahlinger 2004). Repeated administration also diminishes the biological effects (acute tolerance by MDMA action on NA/adrenaline and DA systems) (delaTorre et al. 2004a).

Since MDMA exerts serotonergic, dopaminergic and adrenergic actions it has the potential to undergo toxicodynamic interactions with compounds having the same or antagonistic mechanisms of action (Oesterheld et al. 2004). This potential interaction is discussed with further detail in section 2 of the introduction.

1.4.2. Tryptophan hydroxylase (TH) and monoamine oxidase (MAO) inhibition

The effects of MDMA on the neurotransmitter monoamines are also a consequence of the drug inhibitory effect on two important enzymes, tryptophan hydroxylase (TH) and monoamine oxidase (MAO).

MDMA, at high doses, prevents the metabolic inactivation of the catecholamines by competitively inhibiting MAO (preferentially MAO-A, which has higher affinity for 5-HT) (Fig. 2). At low MDMA doses (below inhibitory effects), MAO is still involved in the metabolism of DA and 5-HT, leading to the formation of DA reactive metabolites and

hydrogen peroxide (H_2O_2) (Alves et al. 2007; Bolton et al. 2000; Carmo 2007; Carvalho et al. 2012).

MDMA and/or its metabolites also inhibit the enzyme TH (Fig. 2), which is the rate-limiting enzyme in 5-HT synthesis (Green et al. 2003).

1.4.3. Neuroendocrine effect

The administration of MDMA leads to increased serum levels of corticosterone, prolactin, renin, aldosterone, thyroxine (T4) (Green et al. 2003; Sprague et al. 2003), cortisol, prolactin (Grob et al. 1996; Harris et al. 2002; Mas et al. 1999) adrenocorticotrophic hormone (ACTH or corticotrophin), antidiuretic hormone (or vasopressin) (delaTorre et al. 2004b; Grob et al. 1996; Henry et al. 1998), gonadotropins follicle-stimulant hormone (FSH), luteinizing hormone (LH), testosterone (Dickerson et al. 2008), and oxytocin (Forsling et al. 2002; Wolff et al. 2006). The effect of MDMA on the release of oxytocin may play a critical role in MDMA pro-social effects and form the basis of reinforcement properties of this drug (Thompson et al. 2007). Some of the detrimental actions of the drug can be attributed to this neuroendocrine disturbance, as is the case of the potentially lethal hyponatremic effect of the drug associated with the increases in vasopressin (Green et al. 2003).

1.4.4. Thermoregulation disturbance

In humans, the hyperthermic effect of MDMA is well documented and increased body temperature is considered to be a potentially lethal consequence of MDMA intoxications (Henry et al. 1992), often associated with other complications elicited by the drug, such as rhabdomyolysis, acute renal failure, disseminated intravascular coagulation (DIC), multiple organ failure, and acidosis (Henry et al. 1992; Kalant 2001; Kendrick et al. 1977). Body temperatures greater than 41.7 ºC (Chadwick et al. 1991; Coore 1996; Hall and Henry 2006; Henry et al. 1992; Milroy et al. 1996; Screaton et al. 1992; Vanden Eede et al. 2012) and as high as 43.9 ºC (Milroy et al. 1996) have been reported in human MDMA intoxications. A rise in body temperature above 42 ° C is considered lethal although survival cases have been recorded (Logan et al. 1993). To aggravate this scenario, it is unlikely that a single

pharmaceutical will be entirely successful in reversing the hyperthermic effect (dantrolene has some efficiency), so careful body cooling using cold baths or ice packs remain the main clinical approach (Carvalho et al. 2012).

The induction of hyperthermia is highly variable because it is dependent on environmental settings (such as ambient temperature) (Broening et al. 1995; Carvalho et al. 2002a; Malberg and Seiden 1998), administered dose, and individual characteristics. The increased muscle activity associated with intense dancing, dehydration and high temperature of the venues that are often crowded aggravate the body temperature rise prompted by MDMA (delaTorre et al. 2004b). Supporting the importance of environmental factors is the fact that, when MDMA was administered in controlled clinical trials, the sharp increase in body temperature was not observed (temperature increments did not exceed 0.5 °C) (delaTorre et al. 2000b; Grob et al. 1996; Mas et al. 1999; Vollenweider et al. 1998). There are cases in which (i) MDMA-elicited hyperthermia is not observed, (ii) only one tablet triggered a fatal hyperthermic response (Gahlinger 2004) or (iii) the individual survived after checking in the emergency department with a body temperature of 42.9 ° C after ingestion of three tablets of MDMA (Mallick and Bodenham 1997; Pontes 2009).

There is considerable evidence suggesting that 5-HT does not play a direct role in the acute hyperthermic response of MDMA (Beveridge et al. 2004; Carvalho et al. 2012; Colado et al. 2004; Docherty and Green 2010; Green et al. 2004; Mechan et al. 2002; Saadat et al. 2005) and the effect appears more related to the increased release of DA (Mechan et al. 2002). Nevertheless, 5-HT appears to modulate DA release (Alex and Pehek 2007) and also seems to influence the hyperthermic response, due to its effect on heat dissipation mechanisms (Saadat et al. 2005).

Additionally, the hyperthermia resulting from the consumption of MDMA may be due to its action on both the central thermoregulatory centre, and on peripheral changes in blood flow and brown fat thermogenesis (Carvalho et al. 2012; Docherty and Green 2010; Pedersen and Blessing 2001; Pontes 2009). Several studies revealed a complex interaction between the hypothalamic–pituitary–thyroid (HPT) axis, the sympathetic nervous system, and the activity of uncoupling proteins (UCP) (Carvalho et al. 2012; Sprague et al. 2003). By activating anterior hypothalamus neurons, MDMA induces NA release and slightly increases T4 serum levels (Pontes 2009). NA, in turn, promotes vasoconstriction through the activation of vascular α_1-adrenoreceptors, impairing heat dissipation, and together with thyroid hormones modulates thermogenesis by regulating the expression of UCP and/or synthesis of adrenergic receptors (Carvalho et al. 2012; Mills et al. 2004; Rusyniak and Sprague 2005). Through

activation of α_1- and β_3-adrenergic receptors, UCPs incorporate in mitochondria, dissociating the mitochondrial proton gradient from adenosine 5'-triphosphate (ATP) synthesis (mitochondrial uncoupling) and releasing the free energy as heat, in skeletal muscle and brown fat (Carvalho et al. 2012; Mills et al. 2004; Rusyniak and Sprague 2005; Sprague et al. 2007).

1.4.5. MDMA addiction, tolerance and withdrawal

The addictive character of MDMA is presently a controversial matter, with a few studies providing evidence of MDMA abuse and dependence potential (Cottler et al. 2001; Schuster et al. 1998; Topp et al. 1999; Yen et al. 2007). In debate is the fact that criteria for abuse and dependence in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (DSM-IV) are the same for all substances, with no regards to the pharmacology of the drug, which ultimately impacts its use pattern (Cottler et al. 2001).

Symptoms of tolerance have been frequently reported, as the increasing frequency of MDMA use leads to a decrease of the emphatogenic effects desired by the abusers, which normally causes a gradual disinterest in the drug. However, in several cases, some of these individuals may increase the intake to overcome the tolerance effects and develop dependency (Jansen 1999).

There are reports of individuals presenting withdrawal symptoms, including fatigue, loss of appetite, mood swings, anxiety, sleep disturbances, feelings of depression, and trouble concentrating (Cottler et al. 2001; Jansen 1999). The *National Institute on Drug Abuse* (NIDA) reported that withdrawal symptoms affect almost 60% of MDMA users (http://www.drugabuse.gov/pdf/rrmdma.pdf).

1.5. Toxicity of MDMA

MDMA became a popular drug of abuse due to its intense stimulating effects and because it was believed that toxic effects would be negligible (Pontes 2009). However, as its popularity increased, so did the number of fatalities and severe adverse events related to its use (Carvalho et al. 2012; De Letter et al. 2006; Pontes 2009). Some aspects of the pathogenesis

associated with MDMA-elicited toxicity remain unclear, such as the variability in the delay between drug exposure and the onset of the injury. Also, MDMA-induced toxicity is not always related to the dose intake and/or plasma concentrations (Milroy 2011), indicating the existence of alternative mechanisms for the occurrence of toxic effects (Carvalho et al. 2012; Pontes 2009). For example, there is one report of a fatal intoxication occurring with the ingestion of a single tablet (MDMA plasma concentration detected was 0.424 mg/L) (Chadwick et al. 1991), while in two other serious poisonings the individual survived after ingesting 30 pills (Shannon 2000), or 18 pills (plasma concentration detected was 4.05 mg/L) (Roberts and Wright 1993). Also, a virtually asymptomatic case was recorded after the ingestion of 42 tablets (resulting in a plasma concentration of 7.72 mg/L) (Henry et al. 1992). As presented in Table 3 and Table 4, the variability in plasma concentrations found in fatal and non-fatal poisonings is overwhelming.

1.5.1. Lethal intoxications

Deaths in which *ecstasy* is considered the causal agent are not very frequent, when compared with the high frequency of use (Carvalho et al. 2012; Pontes 2009). As an example, in the UK, in 1996, the mortality rate of people aged between 15 and 24 years old showed an index of 2-53 deaths per 100,000 *ecstasy* users (Greene et al. 2003). Recent data from the 2011 European report suggest that deaths related to *ecstasy* are rare in most countries of the European Union (EU), particularly if *ecstasy* is the only drug involved (EMCDDA 2011). However, these deaths raise great concern, as they mostly occur sudden and unexpectedly in socially integrated young people (Carvalho et al. 2012; Pilgrim et al. 2009).

Of concern are not only the directly-induced lethal intoxications, but also some deaths that are indirectly associated with MDMA consumption (*e.g.* depression that arises from consumption of MDMA leading to suicide, accidents due to risky behaviours while under the influence of *ecstasy*, among others) (Brookhuis et al. 2004; Kalant 2001; Lora-Tamayo et al. 1997; Morland 2000; Verschraagen et al. 2007). Extensive reviews of *ecstasy*-related deaths are available in the literature (Carvalho et al. 2012; De Letter et al. 2006; Milroy 2011; Patel et al. 2004; Pontes 2009; Schifano 2004; Schifano et al. 2010; Schifano et al. 2003). Table 5 summarizes some of these reported data.

Table 3. Blood and tissue concentrations of MDMA determined at autopsy after fatal intoxication. Adapted from Carvalho (2012).

Blood	Liver[b]	Kidney[b]	Brain[b]	Bile[b]	Lung[b]	Heart[b]	Stomach	
mg/L (µM)	µg/g	µg/g	µg/g	mg/L	µg/g	µg/g	µg/g	Reference
3.9 (20.2)	34							(Fernando et al. 2012)
	22.26	801.14					835.97	(Dordevic and Tomasevic 2007)
2.8[a] (14.5)	20.2		13.7					(Rohrig and Prouty 1992)
0.58[a] (2.99)	1.8							
0.18 (0.96)	13.23	9.82	12.79	27.34	10.7			(Fineschi and Masti 1996a)
2.9 (14.9)	6.4			73				(Moore et al. 1996)
3.10 (16.0)	26.20	13.0	15.6	14.2	13.0	14.0		(deLetter et al. 2002)
3.18 (16.5)	4.86			1.41				(Garcia-Repetto et al. 2003)
0.28 (1.45)	5.13		8.42	1.23	2.64			
0.17[§] (0.88)	0.18	0.05	0.14		1.46			
1.13 (5.8)	6.66	4.06	2.25	25.42	10.9	1.73		(Dams et al. 2003)
7.2* (37.3)	29.7		29.1		36.6			(Sticht et al. 2003)
0.271 (1.4)	4.87	1.44	0.69	22.07	3.62	0.38		(deLetter et al. 2004)
13.51 (70)	103.5	111.9		86.95	101.2	140.1		

* mg/kg
§ Determined before death
[a] Peripheral blood levels (femoral)
[b] Mean concentration values if different tissue portions were analyzed

Table 4. Blood concentrations of MDMA found at non-fatal and fatal intoxications. Adapted from Carvalho (2012).

		MDMA blood concentration (µg/L)		Study	Reference
Dose	**Number of pills**		***post-mortem***		
			87	Fatal intoxication	(Diffley et al. 2012)
			9300	Fatal intoxication	(Fernando et al. 2012)
	3		1500	Fatal intoxication	(Vanden Eede et al. 2012)
		1200		Fatal intoxication	(Sano et al. 2009)
	1.5	<500		Fatal intoxication	(Sauvageau 2008)
			2900	Fatal intoxication	(Moore et al. 1996)
			3100[a]	Fatal intoxication	(deLetter et al. 2002)
		1090	3180	Fatal intoxication	(Garcia-Repetto et al. 2003)
		40	280	Fatal intoxication	
		170		Fatal intoxication	
			1130[a]	Fatal intoxication	(Dams et al. 2003)
			7200[a] **	Fatal intoxication	(Sticht et al. 2003)
			271[b]	Fatal intoxication	(deLetter et al. 2004)
			13510[a]	Fatal intoxication	
	4	230		Non-fatal intoxication	(Greene et al. 2003)
	5	350		Non-fatal intoxication	
	2	250		Non-fatal intoxication	
	2	130		Non-fatal intoxication	
	1	< 100		Non-fatal intoxication	
100-150		[§]7000		Non-fatal intoxication	(Brown and Osterloh 1987)
	50	[§]R (-) 44000 S-(+) 42000		Non-fatal intoxication	(Ramcharan et al. 1998)
	42	7720		Non-fatal intoxication	(Randall 1992)
	18	4050		Non-fatal intoxication	(Roberts and Wright 1993)
			1100	Fatal intoxication	(Dowling et al. 1987)
150	1,5		1000	Fatal intoxication	
	1		424	Fatal intoxication	(Chadwick et al. 1991)
			2000	Fatal intoxication	(Suarez and Riemersma 1988)
		2400		Fatal intoxication	(Greene et al. 2003)
		930		Fatal intoxication	
			2800[a]	Fatal intoxication	(Rohrig and Prouty 1992)
			580[a]	Fatal intoxication	
			180	Fatal intoxication	(Fineschi and Masti 1996a)

C_{max}
[§] Approximately 4 h after ingestion
[†] n = 2 (individual values)
[‡] n = 2 (mean ± SD)
** mg/kg
[a] Peripheral blood levels (femoral)
[b] Subclavian blood values

As can be depicted in Table 5, Milroy (2011) reviewed seventy-seven deaths where MDMA was detected in body fluids/organs. In 13 of these cases, death was due to the toxic effects of MDMA alone, in 22 cases due to polydrug use, and in 24 cases death was attributed to trauma. The main outcome of his study was that no specific concentration can be considered lethal, as fatal MDMA concentrations overlap with non-fatal concentrations (Milroy 2011). As noted before, it can be clearly concluded from the data presented in Table 5 that, in most cases of severe intoxication or death, MDMA is consumed in combination with alcohol and/or other drugs, such as cocaine, cannabinoids and other amphetamines (Morland 2000; Verschraagen et al. 2007), which may also aggravate behavioural changes, increasing the risk of accident and also increasing the direct toxicity exerted by each drug.

1.5.2. MDMA acute and long-term toxicity

The acute adverse effects that occur after ingestion of MDMA in humans include increased blood pressure, increased heart rate, palpitations (delaTorre et al. 2000b; Downing 1986; Grob et al. 1996; Harris et al. 2002; Lester et al. 2000; Liechti et al. 2001; Liechti and Vollenweider 2000a; Liechti and Vollenweider 2000b; Mas et al. 1999; Vollenweider et al. 1998), nausea, vomiting, hyponatremia (delaTorre et al. 2004b; Henry et al. 1998; Holden and Jackson 1996; Holmes et al. 1999; Kessel 1994; Matthai et al. 1996; Maxwell et al. 1993; Milroy et al. 1996; Satchell and Connaughton 1994), mydriasis, chills, sweating, tremor, trismus (jaw clenching), bruxism (teeth grinding) (Redfearn et al. 1998), sudden sensation of cold or heat, nystagmus, restlessness, irritability, and insomnia (Cole and Sumnall 2003; Green et al. 2003; Peroutka et al. 1988). It can also induce panic attacks, delirium and brief psychotic episodes that disappear quickly when the MDMA action ends (delaTorre et al. 2004b). In addition to these immediate toxic effects, acute administration of MDMA can also lead to toxic effects of long duration that might persist for up to seven days after drug ingestion. These include fatigue, irritability, anxiety, depressed mood, insomnia, dizziness, and muscle tension (Cole and Sumnall 2003; delaTorre et al. 2004b).

Table 5. Deaths related to *ecstasy*.

Reference	Year	Local	Cases	Sex/age (years)	Cause of death/autopsy findings	*Postmortem* toxicological analysis
(Diffley et al. 2012)	2012	Oakland, USA.	n=1	M/15	Catecholaminergic polymorphic ventricular tachycardia	87 ng/mL MDMA in blood, approximately 12 hrs after ingestion; Marijuana
(Fernando et al. 2012)	2012	Adelaide, Australia	n=1	F/15	MDMA toxicity (edema, lungs congestion, neuronal hypoxic-ischemic changes in hippocampus), the manner suicide	Blood (mg/L): 9.3 MDMA, 0.28 MDA and 25 caffeine; Liver (mg/kg): 34 MDMA, 0.9 MDA and 25 caffeine; Stomach (mg/kg): 530 MDMA and 550 caffeine; Urine (mg/L): 2.4 MDMA, 0.2 MDA and 8.4 caffeine.
(Vanden Eede et al. 2012)	2012	Hague, Netherlands	n=1	M/19	Massive rhabdomyolysis (hyperkalemia unresponsive to treatment)	T=39.5°C at the moment of death; massive rhabdomyolysis and evidence of myocardial damage Serum potassium = 9.4 mmol/L. MDMA = 1.5 mg/L. No other amphetamines or other drugs were found.
(Sano et al. 2009)	2009	Maebashi, Japan	n=1	F/39	Multi-organ failure involving the brain, heart, lung, kidney, and liver	Small foci of myocyte necrosis with a surrounding macrophage inflammatory response, foci of fibrosis, and calcification accompanied by myocyte necrosis. Centrilobular and mid-zonal hepatic necrosis with fatty degeneration and inflammation. Myoglobinuria. Degeneration of neurons throughout the whole brain and haemorrhagic foci in the pons and medulla. Serious bronchopneumonia.
(Sauvageau 2008)	2008	Montreal, Canada	n=1	F/13	Anaphylactic reaction to ecstasy	Massive brain edema with anoxic/ischemic encephalopathy; lings congested; Laryngeal edema; increased neutrophils (16.2×10^9/L [reference range 1.4 to 6.5×10^9/L])
(Pilgrim et al. 2012)	2002-2008	Victoria, Australia	n=4	M/31	Mixed drug toxicity *Serotonin toxicity* Hepatitis C Pulmonary edema Contraction band necrosis left ventricular myocardium	Cavity blood: MDMA 1.7 mg/L; MDA 0.1 mg/L; Amphetamine 0.06 mg/L; Moclobemide 0.3 mg/L; THC 6 ng/mL
				M/24	Amphetamine toxicity *Serotonin toxicity* Pulmonary edema Cerebral edema	Femoral blood: MDMA 2.6 mg/L; MDA 0.1 mg/L; Methamphetamine 0.1 mg/L; Moclobemide 0.7 mg/L
				M/24	Mixed drug toxicity and coronary artery disease *Serotonin toxicity* Coronary artery atherosclerosis Pulmonary edema	Femoral blood: MDMA 1 mg/L; MDA 0.04 mg/L; Methamphetamine 0.2 mg/L; Amphetamine 0.02 mg/L; Moclobemide 15 mg/L
				M/45	Mixed drug toxicity *Serotonin toxicity* Coronary artery atherosclerosis Pulmonary edema	Femoral blood: MDMA 2.0 mg/L; MDA 0.07 mg/L; Methamphetamine 0.12 mg/L; Amphetamine 0.4 mg/L; Moclobemide 8.7 mg/L
(Lin et al. 2009)	2001-2008	Taiwan	n=59	66.1% M/ 14-46	Acute intoxication (n= 40); mechanical injury (n= 19, including 3 hanging and 2 drowning); The manners of death were accidental (n= 44); homicidal (n= 6); suicidal (n= 7); and undetermined (n= 2)	Ketamine was found in 28 of these cases; 0.12-40.41 microg/mL MDMA *postmortem* whole blood; MDA (0.05-1.81 microg/mL) was found in 30 cases

DIC, disseminated intravascular coagulation. DXM, dextromethorphan. EtOH, ethanol. F, female. GHB, γ-Hydroxybutyric acid. M, male. MBDB, N-methyl-1,3-benzodioxolylbutanamine. MDA, 3,4-methylenedioxyamphetamine. MDEA, N-ethyl-3,4-methylenedioxyamphetamine. MDMA, 3,4-methylenedioxy-N-methamphetamine. PMA, p-metoxyamphetamine. THC, Δ^9tetrahydrocannabinol.

Table 5. Continued.

Reference	Year	Local	Cases	Sex/age (years)	Cause of death/autopsy findings	*Postmortem* toxicological analysis
(Milroy 2011)	1992-2008	Sheffield, UK	n=73		MDMA intoxication (n=17): water intoxication, myocardial necrosis, hyperpyrexia, etc; Polydrug use (n=30): MDMA was the main drug in 28 cases. The other principal drugs in these cases were MDEA (n=1), MBDB (n=1), cannabinoids (n=16), ethanol (n=13), heroin (n=11), benzodiazepines (n=9), amphetamine (n=8), antidepressants (n=6), methadone (n=5), cocaine (n=5), GHB (n=2). Trauma (n=28) Homicide (n=8), Vehicular collision (n=10) Fall from height (n=6) Drowning (n=4) Ischaemic heart disease (n=1).	Blood MDMA concentration at autopsy: 0.478–53.9 mg/L in MDMA deaths (n=13); 0.04–41.5 mg/L MDMA, 0.187 mg/l MDEA and 0.44 mg/l MBDB in mixed drug deaths (n = 22); and 0.035–4.81 mg/L in traumatic deaths (n = 24)
(Kaye et al. 2009)	2000-2005	Australia	n=82	83% M/ 17-58	Drug toxicity (n=61): MDMA-only (n=17) or combined drug toxicity (n=44)	Blood: 0.03–93.0 mg/L MDMA and 0.01–1.0 mg/L MDA; In 87% cases there were other drugs present (especially methamphetamine/ amphetamine, morphine, alcohol, codeine and benzodiazepines)
(Gill et al. 2002)	2002	New York (USA)	n=22	17-41	Acute intoxication (n=13); Mechanical injury (n=7); Combination of previous illness with acute intoxication (n=2)	MDMA (n=22) Opiates and cocaine (n=7)
(Fineschi et al. 1999)	1999	Italy	n=2	M/19	Hyperpyrexia ,DIC	MDMA (7.15 mg/L serum; 31 mg/L urine) MDA (0.25 mg/L serum; 0.85 mg/L urine)
				M/20	Hyperpyrexia	MDMA (0.18 mg/L serum; 263.13 mg/L urine); MDA (0. 12 mg/L serum; 5.25 mg/L urine); MDEA (1.59 mg/L serum; 183.73 mg/L urine)
(O'Connor et al. 1999)	1999	New Zeland	n=1	F/27	Cerebral edema	
(Walubo and Seger 1999)	1999	USA	n=1	M/53	Suicide: Hyperpyrexia (40.4ºC), Rhabdomyolysis, DIC	MDMA (3.05 mg/L serum) amphetamines in urine
(Byard et al. 1998)	1998	South Australia	n=2	F/22	Hyperpyrexia (42.5ºC), DIC, Hyperkalaemia, Rhabdomyolysis	Serum: 1.32 mg/L PMA and 0.3 mg/L MDMA; high levels of MTA and THC
				F/26	Cyanosis, Hyperpyrexia (46.1ºC), Irregular heartbeat, Pulmonary edema	Serum: 2.2 mg/L PMA; 0.82 mg/L MDMA; 0.09 mg/L MTA
(Henry and Hill 1998)	1998	UK	n=1	M/32	Heart Failure	Serum: 4.56 mg/L MDMA; 0.36 mg/L MDA; 0.24 mg/mL EtOH
(Mueller and Korey 1998)	1998	USA	n=1	F/20	Hyperpyrexia	MDMA in serum

DIC, disseminated intravascular coagulation. DXM, dextromethorphan. EtOH, ethanol. F, female. GHB, γ-Hydroxybutyric acid. M, male. MBDB, N-methyl-1,3-benzodioxolylbutanamine. MDA, 3,4-methylenedioxyamphetamine. MDEA, N-ethyl-3,4-methylenedioxyamphetamine. MDMA, 3,4-methylenedioxy-N-methamphetamine. PMA, p-metoxyamphetamine. THC, Δ^9-tetrahydrocannabinol.

Table 5. Continued.

Reference	Year	Local	Cases	Sex/age (years)	Cause of death/autopsy findings	*Postmortem* toxicological analysis
(Lora-Tamayo et al. 1997)	1997	Spain	n=10	M/23	Hypovolemic shock	Serum: 0.23 mg/L MDMA; 0.77 mg/L MDEA; 0.05 mg/L MDA; 0.62 mg/L amphetamine
				M/17	Fall from height	Serum: 0.23 mg/L MDMA; 0.10 mg/L amphetamine; 0.04 mg/L MDA; 0.35 mg/L MTA
				M/32	Hit by car	Serum: 0.27 mg/L MDMA; 2.47 mg/mL EtOH
				M/39	Sudden death at nightclub; Ischemic heart disease; Occlusion of the right coronary artery and the circumflex artery; stroke in the left ventricle	Serum: 0.60 mg/L MDMA; 0.22 mg/L MDEA; 0.12 mg/L MDA; 0.22 mg/L amphetamine; 0.54 mg/mL EtOH
				M/21	Car accident	Serum: 0.17 mg/L MDMA; 1.07 mg/L MDEA; 0.18 mg/L MDA; 0.10 mg/L amphetamine; 0.71 mg/mL EtOH
				M/26	Car accident	Serum: 0.03 mg/L MDMA; 2.44 mg/L MDEA; 0.15 mg/L MDA; 0.05 mg/L benzoylecgonine; 0.88 mg/mL EtOH
				M/29	Adverse reaction to drugs	Serum: 4.07 mg/L MDMA; 0.49 mg/L MDA; 0.38 mg/L morphine; 0.1 mg/L alprazolam; 0.92 mg/mL EtOH
				M/30	Acute pulmonary edema	Serum: 0.98 mg/L MDMA; 0.06 mg/L amphetamine (in serum); 0.15 mg/L morphine; 0.38 mg/mL EtOH
				M/27	Acute pulmonary edema Pulmonary hemorrhage	Serum: 8 mg/L MDMA; 0.18 mg/L amphetamine; 1.2 mg/L MDA; 0.04 mg/L cocaine; 0.13 mg/L benzoylecgonine; 0.9 mg/mL EtOH
				M/19	Adverse reaction to drugs	Serum: 0.49 mg/L MDMA; 4.32 mg/L MDEA; 0.29 mg/L MDA; 0.20 mg/L amphetamine; 1.36 mg/L dipyrone
(Parr et al. 1997)	1997	Australia	n=1	F/15	Hyponatraemia Cerebral edema	MDMA (0.05 mg/L in serum and 430 ng/mL in urine)
(Coore 1996)	1996	Ireland	n=1	F/18	Hyperpyrexia (43ºC) Kidney injury; DIC Rhabdomyolysis	MDA (0.246 mg/L in serum) MDMA and MDA in urine
(Cox and Williams 1996)	1996	UK	n=1	M/22	Hyperpyrexia; DIC	Serum: 0.43 mg/L MDMA; 0.30 mg/L MDEA; 0.25 mg/L MDA
(Crifasi and Long 1996)	1996	USA	n=1	M/29	Accident	MDMA (2.14 mg/L serum; 118.8 mg/L urine); MDA (< 0.25 mg/L serum; 3.86 mg/L urine)
(Dar and McBrien 1996)	1996	USA	n=1	M/17	Hyperpyrexia; Rhabdomyolysis; DIC	
(Ellis et al. 1996)	1996	UK	n=4	F/21	Hyperpyrexia (41ºC) High blood pressure Tachycardia; Acute Renal failure; DIC; Transplant; Sepsis	MDMA (0.11 mg/L serum ; 0.04 mg/L urine)
				F/18	Progressive jaundice Liver failure (regular consumption of *ecstasy*)	
				F/36	Jaundice after consuming 1 tablet of *ecstasy*; Transplant; Sepsis	
				F/22	*Ecstasy* use for 6 months Jaundice; Transplant; Sepsis	

DIC, disseminated intravascular coagulation. DXM, dextromethorphan. EtOH, ethanol. F, female. GHB, γ-Hydroxybutyric acid. M, male. MBDB, N-methyl-1,3-benzodioxolylbutanamine. MDA, 3,4-methylenedioxyamphetamine. MDEA, N-ethyl-3,4-methylenedioxyamphetamine. MDMA, 3,4-methylenedioxy-N-methamphetamine. PMA, p-metoxyamphetamine. THC, Δ^9-tetrahydrocannabinol.

Table 5. Continued.

Reference	Year	Local	Cases	Sex/age (years)	Cause of death/autopsy findings	*Postmortem* toxicological analysis
(Fineschi and Masti 1996)	1996	Italy	n = 1	M/20	Hyperpyrexia DIC	MDMA (0.18 mg/L in serum) MDEA in serum
(Milroy et al. 1996)	1996	UK	n=7	M/21	Collapse in the rave hyperpyrexia (44ºC) Heat failure	MDMA (4.2 mg/L) and amphetamine (1.4 mg/L) in serum
				M/20	Collapse in club High blood pressure Hyponatraemia Water intoxication	MDMA (0.04 mg/L in serum)
				M/24	Death at the disco Unknown cause	MDEA (0.187 mg/L) and amphetamine (0.453 mg/L) in serum
				M/21	Death in bed after party Unknown cause	MDMA (2.1 mg/L). MDEA (3.5 mg/L); MDA (8.5 mg/L); and amphetamine (0.256 mg/L) in serum
				M/20	Found unconscious in bed; rigidity; hyperpyrexia (39,5ºC) Cerebral hypoxia	MDMA (0.09 mg/L) and MDA (0.13 mg/L) in serum
				M/25	Sudden death in the street	Traces of MDMA and MDA in urine
				M/23	Progressive jaundice and liver failure; excessive consumption of *ecstasy*	
(Moore et al. 1996)	1996	USA	n=1	M/20	Acute intoxication with MDMA, cocaine and heroin	MDMA (2.8 mg/L), benzoylecgonine (0.97 mg/L), and morphine (0.11 mg/L) in serum MDMA and MDA in urine
(Squier et al. 1995)	1995	UK	n=1	M/30	Seizures Hyperpyrexia Cerebral necrosis	MDMA. Heroine. Amphetamine and EtOH in serum.
(Forrest et al. 1994)	1994	UK	n=1	M/21	Asphyxia Seizures	MDA, MDEA and MDMA in serum
(Hooft and van de Voorde 1994)	1994	Belgium	n=1	M/26	Road accident in an attempt to car surfing	MDMA (0.63 mg/L) and EtOH (1.23 g/L) in serum; Amphetamines in urine
(Watson et al. 1993)	1993	UK	n=1	M/16	Hyperpyrexia Rhabdomyolysis, DIC	
(Campkin and Davies 1992)	1992	UK	n=1	M/18	Hypotension Tachycardia Hyperpyrexia (42ºC) Rhabdomyolysis, DIC	MDMA (1.26 mg/L in serum)
(Rohrig and Prouty 1992)	1992	USA	n=2	M/35	Unknown cause	MDMA (2.8 mg/L in serum)
				F	Suicide Depression?	MDMA (0.58 mg/L) and diazepam in serum
(Screaton et al. 1992)	1992		n=1	M/19	Hyperpyrexia Rhabdomyolysis, DIC	
(Henry 1992)	1992	UK	n=6	M/18	Hyperpyrexia Cardiovascular failure	
				M/17	Hyperpyrexia; DIC Seizures	
				M/20	Hyperpyrexia Rhabdomyolysis; DIC	
				M/18	Hyperpyrexia Rhabdomyolysis; DIC	
				M/21	Car accident	
				M/23	Car accident	
(Chadwick et al. 1991)	1991	UK	n=1	F/16	Hyperpyrexia; DIC	MDMA (0.424 mg/L in serum)
(Suarez and Riemersma 1988)	1988	USA	n=1	M/34	Heart Failure	MDMA (2 mg/L in serum)

DIC, disseminated intravascular coagulation. DXM, dextromethorphan. EtOH, ethanol. F, female. GHB, γ-Hydroxybutyric acid. M, male. MBDB, N-methyl-1,3-benzodioxolylbutanamine. MDA, 3,4-methylenedioxyamphetamine. MDEA, N-ethyl-3,4-methylenedioxyamphetamine. MDMA, 3,4-methylenedioxy-N-methamphetamine. PMA, p-metoxyamphetamine. THC, Δ9-tetrahydrocannabinol.

Hyperthermia can lead to serious and often fatal complications, which include rhabdomyolysis (Cunningham 1997; Fineschi et al. 1999; Greene et al. 2003; Henry et al. 1992; Kendrick et al. 1977; Screaton et al. 1992), DIC (with consequent bleeding and widespread tissue necrosis) and acute renal failure (Carvalho et al. 2002b; Chadwick et al. 1991; Cunningham 1997; Green et al. 2003; Holt and Moore 2001; Ishigami et al. 2003; Liechti et al. 2005; Terada et al. 1988). Other severe symptoms of intoxication with MDMA include hyperkalemia (Ravina et al. 2004), hyponatremia (Traub et al. 2002), tachycardia, coagulopathy, thrombocytopenia, leucocytosis, late acidosis, hypoglycaemia, pulmonary congestion, oedema and liver toxicity (cases of fulminant hepatitis and hepatic necrosis) (Chadwick et al. 1991; Dowling et al. 1987; Henry et al. 1992; Kendrick et al. 1977; McCann et al. 1996; Screaton et al. 1992; Terada et al. 1988; Traub et al. 2002). Other potentially fatal neurological effects include subarachnoid haemorrhage, intracranial haemorrhage, cerebral infarction and cerebral thrombosis. These complications may result from hypertension, cerebral angiitis or dehydration associated with the ingestion of MDMA (McCann et al. 1996; Milroy et al. 1996; Rutty and Milroy 1997). The necrosis of the liver and heart tissue was also observed in *post-mortem* studies (Milroy et al. 1996; Rutty and Milroy 1997).

The most worrisome long-term toxic effect of MDMA is neurotoxicity (Boot et al. 2000; Capela et al. 2009; Dafters et al. 1999; Gerra et al. 2000; Gerra et al. 1998; Gouzoulis-Mayfrank and Daumann 2006b; Green et al. 2003; McCann et al. 1999a; McCann et al. 2000; McCann et al. 1999b; McCann et al. 1994; McCann et al. 2005; Price et al. 1989; Reneman et al. 2001; Reneman et al. 2002; Ricaurte et al. 1990; Ricaurte et al. 2000; Semple et al. 1999), an effect that appears to be reversible after a long period of abstinence, although some damages seem to persist (Gouzoulis-Mayfrank and Daumann 2006b). Chronic use of MDMA has also been associated with persistent sleeping problems (Allen et al. 1993), various psychopathological problems, including eating disorders (Schifano et al. 1998) and cardiac (Brody et al. 1998; Screaton et al. 1992) and renal impairment (Carvalho et al. 2002b; Cunningham 1997; Holt and Moore 2001; Ishigami et al. 2003; Liechti et al. 2005).

Toxicity is therefore diffuse and, among the target organs of MDMA toxicity, the liver is of special concern because it not only suffers from the detrimental actions of MDMA itself, but also from the metabolic bioactivation processes that render this organ particularly vulnerable to the toxicity of MDMA and many other amphetamines.

1.5.3. Liver toxicity

MDMA is among the most common causes of drug-induced liver failure, accounting for up to 20% of all cases in young patients (Andreu et al. 1998; Carvalho et al. 2012; Carvalho et al. 2010). This drug was even described as the second most common cause of liver injury (after EtOH) in young people admitted to intensive care units (Andreu et al. 1998; Carvalho et al. 2012; Carvalho et al. 2010; Jones and Simpson 1999), but it is believed that several cases of MDMA-elicited liver failure are not correlated to the drug intake. In fact, the highly variable interval between exposure and the onset of the symptoms (from a few days to 2 or 3 weeks) hamper the recognition of the aetiological agent involved (Brncic et al. 2006; Carvalho et al. 2012; Carvalho et al. 2010; Henry 1992; Nunez et al. 2002; Shulgin and Nichols 1978).

Notwithstanding, multiple cases of hepatotoxicity mediated by MDMA have been described (Andreu et al. 1998; Brncic et al. 2006; Coore 1996; de Man et al. 1993; Dykhuizen et al. 1995; Fidler et al. 1996; Henry et al. 1992; Khakoo et al. 1995; Roques et al. 1998) and were subject of several reviews (Aknine 2004; Antolino-Lobo et al. 2011b; Carvalho et al. 2010; Jones and Simpson 1999). Toxic liver effects are characterized by distinct patterns, ranging from asymptomatic hepatic injury (only denoted by the altered liver function tests), mild benign forms (Dykhuizen et al. 1995; Ellis et al. 1996), serious liver dysfunction with loss of liver parenchyma, as a result of extensive and/or focal hepatic necrosis (Henry et al. 1992; Milroy et al. 1996; Sano et al. 2009) and, in worst cases, to fulminant liver failure (Brauer et al. 1997; Caballero et al. 2002; Dykhuizen et al. 1995; Ellis et al. 1996; Fineschi et al. 1999; Garbino et al. 2001; Henry et al. 1992; Khakoo et al. 1995; Liechti et al. 2005; Milroy 1999; Milroy et al. 1996; Varela-Rey et al. 1999) that can be fatal or require liver transplantation (Brauer et al. 1997; Caballero et al. 2002; Carvalho et al. 2012; Carvalho et al. 2010; Ellis et al. 1996; Lange-Brock et al. 2002). After exposure to MDMA, the liver architecture can show dramatic modifications mainly due to necrosis accompanied by an acute inflammatory reaction, particularly in the cases accompanied by hyperpyrexia (Carvalho et al. 2012; Carvalho et al. 2010; Ellis et al. 1996; Fidler et al. 1996). Microvesicular fatty changes may be present, with the portal tracts being expanded by oedema and inflammatory infiltrates (Andreu et al. 1998; Milroy et al. 1996).

This multiplicity of MDMA-induced hepatotoxic manifestations suggests different mechanisms of pathogenesis (Andreu et al. 1998; Brauer et al. 1997; Carvalho et al. 2012; Carvalho et al. 2010; Henry et al. 1992; Jones and Simpson 1999; Milroy et al. 1996; Schwab et al. 1999). Further complicating this scenario, is the fact that the severity of the symptoms

and injuries previously mentioned do not link with the chronicity of exposure nor to the dose of drug consumed (Andreu et al. 1998; Dykhuizen et al. 1995; Garbino et al. 2001). In some cases, the damage occurs after the ingestion of a small amount of drug and cases of fulminant acute hepatitis have been described after the ingestion of a single tablet of MDMA (Dykhuizen et al. 1995; Ellis et al. 1996; Garbino et al. 2001; Henry et al. 1992). In other cases, liver injury appears as a consequence of chronic MDMA use (de Man et al. 1993; Fidler et al. 1996). Repeated or/and prolonged exposures appear to increase the extent of liver damage, suggesting the possible involvement of immune mechanisms (Carvalho et al. 2012; Carvalho et al. 2010; Jones and Simpson 1999). Also, chronic use of MDMA can lead to the development of progressive liver fibrosis (Carvalho et al. 2012; Carvalho et al. 2010; Khakoo et al. 1995; Varela-Rey et al. 1999).

For all the presented reasons, MDMA hepatocellular toxicity is characterized by its random occurrence, with unpredictable severity (Brncic et al. 2006; Carvalho et al. 2012; Carvalho et al. 2010). A brief overview of the mechanisms involved is presented below, with some of particular interest to the experimental work herein presented being discussed with further detail in the discussion section of the thesis.

1.5.3.1. Putative mechanisms underlying MDMA-elicited hepatotoxicity

The mechanisms through which the intoxications occur are complex and have not been completely elucidated but it is acknowledge the high probably of a multifactorial origin being at play. Carvalho *et al.* (2012; 2010) extensively reviewed the multiple pathways that have been proposed to explain the liver damage induced by *ecstasy*. Among others, these effects have been attributed to: (i) idiosyncratic reactions (Henry et al. 1992), (ii) the action of MDMA and/or its metabolites either by a direct toxic action, or indirectly through the release of endogenous catecholamines, (iii) induction of oxidative stress, (iv) hyperthermia (Andreu et al. 1998), (v) changes in liver blood flow, (vi) apoptosis, (vii) hypersensitivity/ inflammation mechanisms (Andreu et al. 1998), (viii) circulatory collapse and hypoxic damage (Mustafa et al. 1985), and also (ix) to the impact of other substances ingested with MDMA (Milroy et al. 1996; Parrott 2004a), *etc.*

1.5.3.1.1. Oxidative stress

The generation of ROS and/or RNS and the subsequent oxidative/nitrosative stress is inherent to several mechanisms underlying MDMA-induced hepatocellular toxicity (Beitia et al. 1999; Beitia et al. 2000; Carvalho et al. 2002a; Cerretani et al. 2011; Custodio et al. 2010; Darvesh et al. 2005; Gow et al. 2004; Johnson et al. 2002; Moon et al. 2008; Ninkovic et al. 2004; Pacher et al. 2007; Zheng and Laverty 1998), such as hyperthermia, oxidative metabolism of MDMA and released catecholamines, mitochondrial impairment, and immune responses. With the ability to disrupt cellular homeostasis and produce cellular death, oxidative stress biomarkers were considered of particular interest and included in the experimental tasks of this thesis.

1.5.3.1.2. Altered release of biogenic catecholamines and oxidative metabolism

The altered release of biogenic catecholamines and its consequent oxidative metabolism plays a role in MDMA hepatotoxicity. The activation of α-adrenergic receptors located in the hepatocytes by endogenous catecholamines, which are released due to MDMA action, can result in decreased levels of GSH (James et al. 1983; Sies and Graf 1985), increased mitochondrial respiration and increased concentration of intracellular free calcium (Pontes 2009; Taylor et al. 1983). Liver injury can also result from the oxidative metabolism of these catecholamines, mostly metabolized by MAO and COMT (Carvalho et al. 2012; Hoffman and Lefkowitz 1996; Lefkowitz et al. 1996; Pontes 2009). On the other hand, when the metabolic pathways become saturated or inhibited by MDMA (Leonardi and Azmitia 1994), catecholamines can undergo auto-oxidation with formation of highly reactive species, including reactive metabolites, ROS and RNS, with the ability to trigger intracellular oxidative stress (Bindoli et al. 1992; Bindoli et al. 1989; Carvalho et al. 2012; Pontes 2009). The oxidation of the catecholamines (Bindoli et al. 1992; Bindoli et al. 1989) begins with the oxidation of the catechol group into a semiquinone form, which rapidly oxidizes into a quinone (Figure 3). The reaction of the oxidation products of the catecholamines with cysteine and GSH results in the formation of adducts with high reactivity and potentially cytotoxic (Bindoli et al. 1992). Once the cell is depleted from endogenous defence mechanisms, including enzymatic and non-enzymatic antioxidants, it can enter into a death

process either by necrosis or apoptosis, depending mostly on its energetic status (Carvalho et al. 2012; Pontes 2009).

1.5.3.1.3. Mitochondrial disruption

Mitochondria are preferential sites for ROS/RNS production, and, consequently, vital targets of oxidative/nitrosative stress. It is argued that MDMA and/or its reactive metabolites (especially the quinones) disrupt mitochondrial functionality (Burrows et al. 2000; Carvalho et al. 2004b; Fisher et al. 2007; Miller et al. 1997; Montiel-Duarte et al. 2004; Moon et al. 2008; Nakagawa et al. 2009) by directly inhibiting the activities of mitochondrial complexes I, II, IV, and V (Alves et al. 2009; Moon et al. 2008) or by inactivating various mitochondrial proteins, as already demonstrated with cytochrome *c* (cyt *c*) (Fisher et al. 2007), which is likely to contribute to mitochondrial dysfunction (Carvalho et al. 2012; Pontes 2009). It is possible that concurrent production of peroxynitrite (ONOO·-) after MDMA exposure leads to the oxidative modifications of some mitochondrial complex proteins, resulting in their inhibition (Cardoso et al. 1999; Murray et al. 2003). Interruption of the mitochondrial electron transport chain would lead to increased ROS leakage (Amacher 2005; Begriche et al. 2006; Lin and Beal 2006; Moon et al. 2008), which in turn, may also cause mitochondrial dysfunction (Brown and Yamamoto 2003). Mitochondrial dysfunction was also evaluated in the experimental work of this thesis.

1.5.3.1.4. Hyperthermia

In vitro studies have shown that the elevation of incubation temperature, aiming at simulating the MDMA-induced hyperthermia, enhances the hepatotoxic effects of MDMA, particularly those closely related to oxidative stress (Carvalho et al. 2001; Carvalho et al. 2002a; Pontes et al. 2010; Pontes et al. 2008a; Pontes et al. 2008b; Pourahmad et al. 2010; Santos-Marques et al. 2006). In several of the reported human intoxications where liver damage occurred the intoxicated individuals presented increased body temperature (Table 5). For any mechanistic evaluation of MDMA-induced toxicity the influence of hyperthermia has to be necessarily addressed.

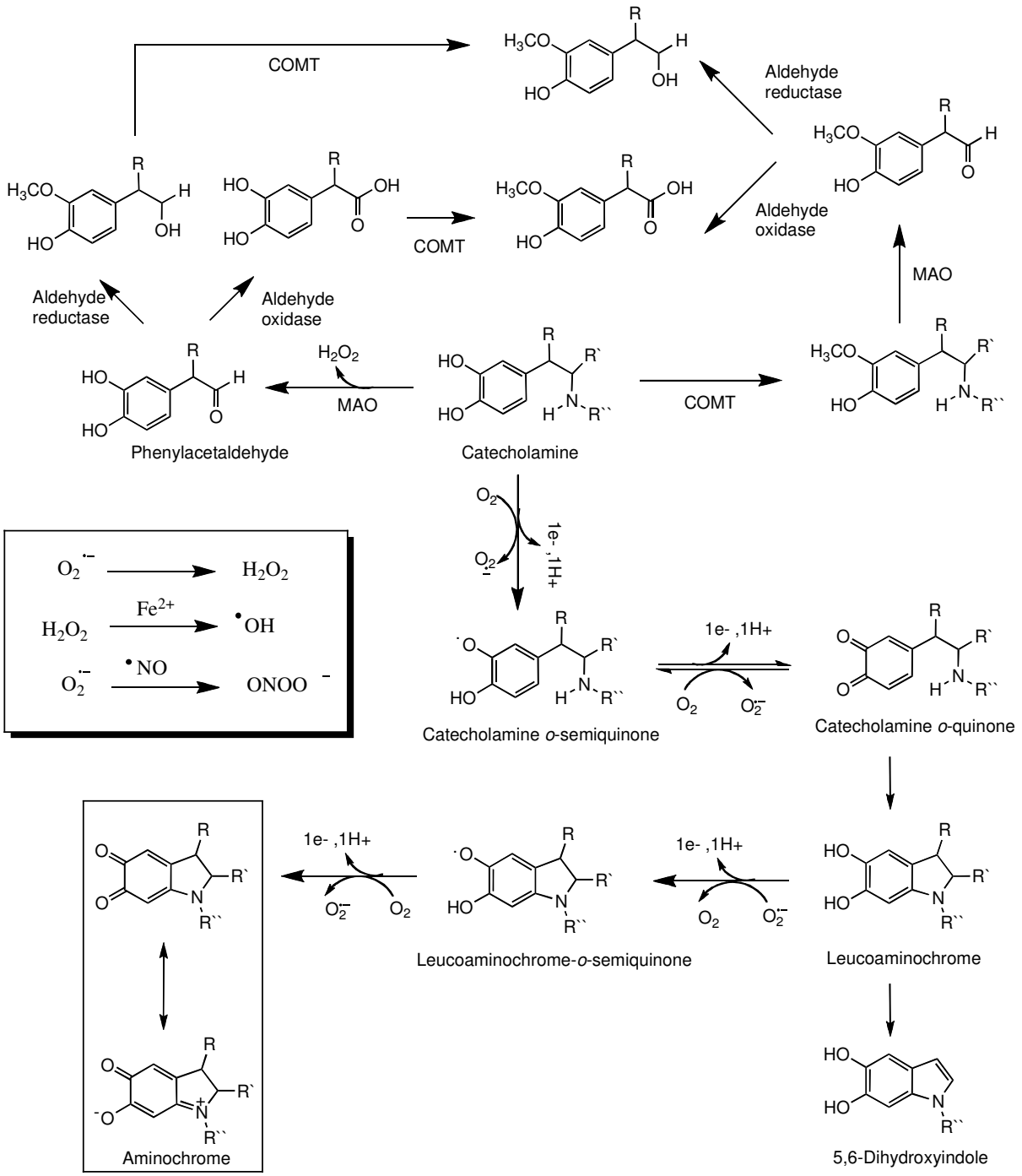

Figure 3. Metabolism of catecholamines and their autoxidation process. Catecholamines-o-quinones are unstable and by deprotonation of the amino group, can endure a nucleophilic attack at the nitrogen, resulting in the formation of leucoaminochrome (1,4-intramolecular cyclization). The leucoaminochrome is oxidized forming the respective aminochrome. The speed of this metabolic process depends on the substituent groups of catecholamines being, therefore, variable for different catecholamines (dopamine <α-methyldopamine <noradrenaline <N-methyl-α-methyldopamine <adrenaline) (Chavdarian et al. 1978). The increase of catecholamine-o-quinones lifetime subsequently increases the likelihood for exerting intracellular oxidative damage. Aminochromes are chemically unstable and may undergo intramolecular rearrangements (with formation of dihydroxyindoles which polymerize yielding dark, insoluble melanin-like pigments), oxidations and reductions, regenerating leucoaminochromes. The reduction of aminochromes may occur in the presence of antioxidants, such as glutathione, yielding highly reactive intermediate semiquinones (Bindoli et al. 1992). The reactivity of quinones and aminochromes formed during these oxidation processes are directly related to their ability to enter in redox cycles with consequent formation of ROS and RNS. Adapted from Carmo (2007).

1.5.3.1.5. Metabolic bioactivation of MDMA

The metabolic pathways of MDMA that were previously described provide many possibilities for inducing cellular damage. Several *in vitro* studies have demonstrated the toxicity of MDMA metabolites and have been extensively reviewed (Carvalho et al. 2012; Green et al. 2003; Pontes 2009). These *in vitro* and *in vivo* studies showed that metabolic bioactivation plays an important role in the MDMA toxic expression not only in the liver (Carvalho et al. 2004a; Carvalho et al. 2004b), but also in neurons (Capela et al. 2006; Gollamudi et al. 1989; Mueller et al. 2009; Patel et al. 1991), kidney (Carvalho et al. 2002b) and heart (Carvalho et al. 2004c).

1.5.3.1.6. Immune responses and inflammation

Another pathway that has been suggested to play a role in the onset of MDMA-induced liver injury is linked to adverse immune responses, since activated Kupffer cells (KC) and infiltrated inflammatory cells (lymphocytes, neutrophils, and eosinophils) have been found in the liver after fatal MDMA intoxications (Andreu et al. 1998; Antolino-Lobo et al. 2011b; Ellis et al. 1996; Khakoo et al. 1995; Schwab et al. 1999). Additionally, MDMA has been shown to induce a transient immune dysfunction (declining the number of T helper lymphocytes CD4 +, increasing the number of natural killer cells (NK), and activating nuclear factor kappa B (NF-κB), a transcription factor involved in the activation of response genes to inflammatory stimuli (Chen and Shi 2002; Montiel-Duarte et al. 2004; Pacifici et al. 2000; Pacifici et al. 2001a; Pacifici et al. 1999; Pacifici et al. 2002; Tiangco et al. 2005). Oxidative stress is also believed to play a distinct role in the development of such mechanisms.

1.5.3.1.7. Genetic polymorphisms of MDMA abusers

Polymorphisms of genes coding for proteins involved in the pharmacokinetics and pharmacodynamics of MDMA may help explain the fact that some individuals can consume MDMA regularly without apparent damage, while others die or experience severe toxic

reactions with sporadic or low-dose consumptions (Carmo 2007; Carvalho et al. 2012; Carvalho et al. 2010; Pontes 2009; Wolff et al. 1995).

It has already been described that genetic polymorphisms affect the toxicity profile of MDMA due to alterations on expression and/or activity of CYP450 (CYP1A2, CYP2B6, CYP2D6, CYP3A4) (Ingelman-Sundberg 2004; Parkinson et al. 2004), COMT (Mattay et al. 2003; McLeod et al. 1994; Weinshilboum et al. 1999), UDP-glucuronosyltransferase (UGT) (Felton and Malfatti 2006; Miners et al. 2002), sulfotransferase (SULT) (Nagata and Yamazoe 2000), 5-HT receptor (Masellis et al. 1995), DA receptor (Cichon et al. 2000; Cravchik and Gejman 1999; Li et al. 2004; Persico et al. 1996; Steen et al. 1997), serotonin transporter (SERT) (Lesch and Gutknecht 2005; Reneman et al. 2006; Roiser et al. 2005; Roiser et al. 2006), among others (Carmo 2007; Carvalho et al. 2012; Carvalho et al. 2010; Pontes 2009).

1.2.1.1.1. Induction of apoptosis

As mentioned before, cellular death mechanisms are mostly decided by the energetic status of the cell. Despite the main clinically significant toxic features of amphetamines in general, including hyperthermia and hepatic failure, correlate with histopathological demonstration of liver necrosis (Brauer et al. 1997; Garbino et al. 2001; Ibranyi and Schonleber 2003; Kamijo et al. 2002; Marinkovic et al. 2011; Sano et al. 2009), there are plenty of data in the literature supporting that also apoptotic pathways are involved in MDMA-induced liver toxicity (Cerretani et al. 2011; Montiel-Duarte et al. 2004; Montiel-Duarte et al. 2002; Upreti et al. 2011). The apoptotic pathways of cellular death were also included in the experimental work of the thesis when mechanistic evaluations were performed.

1.2.1.1.1. Co-exposure with other substances

As can be easily concluded from what has been presented so far, the type and severity of MDMA-induced hepatotoxicity is influenced by assorted factors. These features, when combined with the heterogeneity of substances included in *ecstasy* tablets or the drugs that are inadvertently or intentionally co-ingested by abusers (to extend and exacerbate the stimulating

effect or to alleviate the undesired ones) increase the risk for developing toxicity and complicate the determination of the circumstances involved in MDMA intoxications (Carvalho et al. 2012; Cole and Sumnall 2003; Gowing et al. 2001; Mohamed et al. 2011). Given the importance of this subject for the work herein presented, the detrimental consequences originated by the co-exposure of MDMA and other substances will be extensively debated in section 2 of the introduction.

1.2.1.2.Factors affecting liver toxicity induced by MDMA

1.2.1.2.1. Dose ingested

The ingested dose of MDMA impacts on its biological/toxicological effects, particularly due to the phenomenon of MDMA self-induced metabolic inhibition. Pro-social effects, propensity to addiction, 'hangover' effects, disorientation, hallucinations and other side effects are known to increase in a dose-depend fashion (Chesher 1990; Ho et al. 2001; Plessinger 1998; Solowij et al. 1992). But as already noted, it is not only the amount of MDMA ingested on each single occasion that accounts for the toxicity of the drug. The frequency of administration was shown to highly impact the hyperthermic effect, hyperkinetic responses, long-lasting serotonergic deficits, tachyphylaxis, psychological disorders, and tolerance induced by the drug (Dafters 1995; Green et al. 2004; Mechan et al. 2006; Schifano et al. 1998; Solowij et al. 1992).

1.2.1.2.2. Ambient settings

Despite the existence of some sporadic episodes providing compelling evidence of a direct toxic action of MDMA regardless of the circumstances of its use (Bedford-Russell et al. 1992; Chang et al. 2005; Eifinger et al. 2008; Hall et al. 1996), environmental conditions of misuse venues greatly affect MDMA-induced toxicity (Bellis et al. 2002; Parrott 2004b). In fact, MDMA abuse in hot, crowded environments, with loud repetitive electronic music and prolonged physical exertion, seems to exacerbate the toxicity of the drug mainly because high temperature increases MDMA toxicity (Capela et al. 2007; Capela et al. 2006; Carvalho et al. 2002a; Fineschi et al. 1999; Malberg and Seiden 1998; O'Shea et al. 2006). Several *in vivo*

studies provide evidence that the effects of MDMA on body temperature are dependent on ambient temperature (Banks et al. 2007; Carvalho et al. 2002a; Gordon et al. 1991; Green et al. 2005; Malberg and Seiden 1998; Sanchez et al. 2004; Von Huben et al. 2007). When administered to rats at high ambient temperatures, MDMA causes hyperthermia by increasing heat production in brown adipose tissue and reducing heat loss due to peripheral vasoconstriction. Conversely, when administered to rats in a cold environment, MDMA causes hypothermia by inhibiting brown adipose tissue thermogenesis and due to the tail artery vasoconstriction caused by the cold (Rusyniak et al. 2008). Furthermore, high ambient temperatures increased the number of MDMA self-administrations by rats, appearing to affect both the social and reinforcing effects of MDMA (Chesher 1990; Cornish et al. 2003).

Loud noise has been associated with an increase in MDMA-induced neurotoxicity (Walubo and Seger 1999). Noise can also exacerbate myocardium damage, rhabdomyolysis, by causing ultra-structural changes on mitochondria of cardiac muscle (Gesi et al. 2002a; Gesi et al. 2002b). Stress causes an increase in MDMA brain concentrations (Johnson et al. 2004) and increases psychostimulant self-administration (Piazza and Le Moal 1996). Aggregation potentiates the acute effects of MDMA (Fantegrossi et al. 2003; Parrott 2004b), and increases addictive behaviours (Meyer et al. 2002b) raising concern due to the frequently overcrowded consumption settings. Physical activity also contributes to the worsening of MDMA acute toxicity (Parrott 2004b), probably by increasing heat production and, thus, contributing to hyperthermia (Miller and O'Callaghan 1995). Also, the excessive water intake can lead to hyponatremia resulting in cerebral oedema (Cole and Sumnall 2003; Finch et al. 1996; Wilkins 1996).

1.2.1.2.3. Abuser features

Several factors inherent to *ecstasy* misusers determine the risks associated with drug consumption. Among the features of consumers that may impact MDMA toxicity are age (Broening et al. 1994; Broening et al. 1995; Marchesini et al. 1988; Morley-Fletcher et al. 2004; Parkinson et al. 2004; Schmucker 2005; Sotaniemi et al. 1997; Wiley et al. 2008; Wynne et al. 1989), ethnicity (Clatts et al. 2005; Ingelman-Sundberg et al. 1999; Ompad et al. 2005; Parkinson et al. 2004; Relling et al. 1992; Wu et al. 2006a), gender (Kawas et al. 1997; Liechti et al. 2001; McCann et al. 1994; Parkinson et al. 2004; Relling et al. 1992; Reneman

et al. 2001; Sherlock et al. 1999; Swaab and Fliers 1985; Vistisen et al. 1991; Wyeth et al. 2009), sexual orientation (Degenhardt 2005; Parsons et al. 2006), and physiological and pathophysiological individual conditions (*e.g.* pregnancy, hypo/hyperthyroidism, liver and renal deficiencies, infectious and inflammatory pathologies, heart pathology, *diabetes mellitus* and obesity), as these characteristics determine individual pharmacokinetics/pharmacodynamics, therefore, impacting the pharmacological/toxicological profile of the drug, and also influences consumption patterns.

2. MDMA multiple-drug consumption patterns – Interaction with other substances

Ecstasy misusers repeatedly abuse several drugs simultaneously, combining legal and illegal substances to modulate the expected effects of MDMA (Carvalho et al. 2012; Drugs 2004; Pontes 2009; Schifano et al. 1998). Studies on the progression of drug use revealed that the recreational use of MDMA is usually preceded by legal drugs (EtOH and nicotine), followed by cannabis, and later by other illicit drugs, including other amphetamines (Pedersen and Skrondal 1999; Pontes 2009). A recent study observed that polydrug abuse is more frequent with the initiation of 'heavier' drugs, including MDMA, when compared to alcohol, tobacco or cannabis (Olthuis et al. 2013). Also, a direct correlation seems to exist between the frequency of *ecstasy* usage and the propensity of individuals to consume other illicit drugs, such as cocaine, amphetamines, LSD, hallucinogenic psilocybin mushrooms, ketamine, gamma-hydroxybutyrate (GHB) (Ho et al. 2001; Mendes and Lomba 2008; Mohamed et al. 2011; Scholey et al. 2004). Frequently, the abusers notice a decrease in the drug acute effects after continued use (pharmacodynamic tolerance), increasing the propensity for the use of other psychoactive substances or drug cocktails aiming at accomplishing the desired pleasant experiences (Merrill 1996; Parrott 2001; Peroutka et al. 1988).

This polydrug abuse pattern is one the most serious confounding factors when evaluating MDMA toxicity, as it hampers the correlation of toxicological events with one specific drug (Cole and Sumnall 2003; Gouzoulis-Mayfrank and Daumann 2006a). In fact, unique and unpredictable reactions can be produced from the joint action of MDMA and other substances in the body, greatly aggravating the associated health risks (Carvalho et al. 2012). Nevertheless, the understanding of the toxicity and the investigation of potential neurobehavioral, pharmacodynamic and/or pharmacokinetic drug–drug interactions between

MDMA and other recreational or therapeutic agents has received rather little attention, and most of the information on the topic is inferred from clinical cases (human reports from emergency room visits) and from preclinical *in vitro* studies (Carvalho et al. 2012; Mohamed et al. 2011).

Behavioural and neurobiological interactions are the most frequently reported by the abusers. They usually deliberately engage in polydrug abuse patterns aiming at heightening social and psychological experiences, or at mitigating aversive physiological effects (Mohamed et al. 2011).

Pharmacokinetic interactions may arise when a particular compound interferes with absorption, distribution, metabolism and/or excretion of the drug. For instance, P-glycoprotein (Pgp) is an ubiquitous ATP-dependent efflux transporter that eliminates a wide variety of xenobiotics from the cells (Ketabi-Kiyanvash et al. 2003). Pgp inhibitors (*e.g.* ritonavir or paroxetine) are able to improve the bioavailability of MDMA and other amphetaminic analogues (*e.g.* 3,4-methylenedioxy-N-ethylamphetamine or MDE, *p*-methoxyamphetamine or PMA), by reducing their renal elimination and by increasing their levels in the brain (through a decrease in the blood-brain barrier clearance) leading to severe adverse effects (Ketabi-Kiyanvash et al. 2003; Pontes 2009). Also, drugs that are metabolized by, or disturb the CYP450 complex (by inducing or inhibiting the isoforms involved in MDMA metabolism) are prone to interact with MDMA pharmacokinetics. For instance, an increase in MDMA plasma concentrations due to reduced hepatic metabolism is likely to occur. Therefore, also potential toxicodynamic interactions are expected when a competitive inhibitor of CYP2D6 is co-ingested with MDMA (Mohamed et al. 2011; Oesterheld et al. 2004). Examples of these drugs include fluoxetine, paroxetine, cocaine (Mohamed et al. 2011; Ramamoorthy et al. 2002), other amphetamine analogues (Mohamed et al. 2011; Wu et al. 1997), and haloperidol metabolites (Mohamed et al. 2011; Shin et al. 2001). The simultaneous ingestion of drugs that additionally inhibit other CYP450 isoforms (*e.g.*, CYP3A4) will further induce an even more marked effect on MDMA concentrations. Also, the self-inhibition of CYP2D6 by MDMA at high concentrations might disproportionately increase the levels of the co-administered drugs, triggering toxicity phenomena (delaTorre et al. 2000a). Of particular concern is the fact that several substrates of CYP2D6 display a narrow safety window (*e.g.* some tricyclic antidepressants, antiarrhythmics, β-blockers, antipsychotics and tramadol). Thus, notorious adverse reactions with MDMA can worryingly be anticipated (Carvalho et al. 2012).

Pharmacodynamic interactions occur when other substances with similar or opposite mechanisms of action are present (Carvalho et al. 2012). It is well known that MDMA promotes serotonergic, dopaminergic and noradrenergic actions (Lyles and Cadet 2003). The most disturbing effect that may arise from the simultaneous ingestion of pro-serotonergic drugs and MDMA is the serotonergic syndrome, whose effects include sweating, fever, tremors, hyperreflexia, agitation, and myoclonus and that can be fatal (Sternbach 1991). Cocaine, theophylline, caffeine LSD, and other amphetamines are drugs able to aggravate these MDMA effects (Carvalho et al. 2012; Greene et al. 2008; Oesterheld et al. 2004). But also some prescribed pro-serotonergic pharmaceuticals (*e.g.*, anticholinergics, antidepressants, tramadol, lithium, salicylates, DXM, etc.), compounds inadvertently ingested in the diet, contaminants of the tablets of MDMA (*e.g.*, *d*-AMP, MDA, METH) (Teter and Guthrie 2001), drugs used intentionally to enhance the effects of MDMA (*e.g.*, cocaine, DXM) (Ramamoorthy et al. 2002) or to decrease the undesirable ones (*e.g.*, EtOH, benzodiazepines, sildenafil, 5-hydroxytryptophan) (Breslau 2002; Copeland et al. 2006; Winstock et al. 2001a) may render individual more susceptible to MDMA pharmacodynamic complications (Carvalho et al. 2012; Oesterheld et al. 2004).

Some drugs can simultaneously act as sympathomimetic/ serotonergic/ dopaminergic agents and as CYP2D6 inhibitors, thus having the potential to cause pharmacodynamic and pharmacokinetic interactions with unpredictable consequences (Carvalho et al. 2012; Oesterheld et al. 2004).

2.1. Therapeutic drugs

As noted above, a popular practice among *ecstasy* abusers is to consume medications or dietary supplements to strengthen the 'drug high' or counteract some of the undesired 'come down' effects of MDMA, such as depression and insomnia. Pilgrim *et al.* (2011) reviewed the MDMA-related fatalities occurred in Victoria, between 2002 and 2008 and found that 41% cases involved the concomitant use of MDMA with pharmaceuticals. There are abundant data in the literature concerning MDMA interactions with therapeutic drugs regimens, that have been reviewed elsewhere (Carvalho et al. 2012; Copeland et al. 2006; Mohamed et al. 2011). The most common drugs intentionally used with MDMA are benzodiazepines and sildenafil,

followed by MAO inhibitors (MAOI) and selective serotonin reuptake inhibitors (SSRI) (Carvalho et al. 2012; Copeland et al. 2006).

Benzodiazepines enhance the effect of the neurotransmitter gamma-aminobutyric acid (GABA), resulting in central nervous system inhibition properties. Due to their sedative, anxiolytic, anticonvulsant, and muscle relaxant effects, these drugs are a first-line strategy employed to control the sympathomimetic effects of amphetamines (Derlet et al. 1990; Nisijima et al. 2003). Notwithstanding, chlordiazepoxide potentiates amphetamine-induced hyperactivity (Carvalho et al. 2012; Kelly et al. 2009). Starcevic and Sicaja (2007) proposed that dual intoxication with amphetamine and benzodiazepine enhances the toxic effects on cardiac tissue and coronary arteries, resulting in larger myocardial injury. Enhanced CYP3A inhibition was also noted in rat liver microsomes co-incubated with MDMA and clozapine (Antolino-Lobo et al. 2011a).

Ecstasy abusers combine MDMA and sildenafil (a combination known as '*sextasy*') to enhance the sexual experience (Breslau 2002). There are data demonstrating that sildenafil produces a long-lasting neuroprotective effect against MDMA-induced 5-HT deficits, apparently due to the increased expression of manganese superoxide dismutase (MnSOD) and a subsequent reduced susceptibility to the oxidative stress caused by MDMA (Puerta et al. 2012).

Antidepressants are used by *ecstasy* abusers to enhance and prolong the drug 'high', to counteract the 'come down' stage, and for sleeping aid (Copeland et al. 2006). The antidepressants most commonly used in combination with *ecstasy* are SSRIs (*e.g.,* citalopram, fluoxetine, paroxetine, sertraline) and MAOIs (*e.g.,* phenelzine, moclobemide) (Carvalho et al. 2012), both promoting an increase of the neurotransmitters in the synaptic cleft.

SSRIs can rise MDMA plasma levels increasing the potential for toxicodynamic acute reactions (Carvalho et al. 2012), but can also promote beneficial effects regarding the long-term cellular damage, by decreasing the formation of MDMA toxic metabolites (Rietjens et al. 2012) through CYP2D6 inhibition such as is the case of fluoxetine (Ramamoorthy et al. 2002). Also, clinical effects (*e.g.* blood pressure, heart rate, body temperature) and positive subjective effects can be reduced, possibly due to a pharmacodynamic interaction at the SERT (Rietjens et al. 2012). This reduction in MDMA subjective effects may in turn lead users to consume higher doses of MDMA, to achieve the desired effects, thus exposing themselves to potentially lethal toxic risks (Farre et al. 2007).

The MAOIs most frequently involved in lethal MDMA poisonings are those with greater selectivity for MAO-A, such as moclobemide (Vuori et al. 2003) and clorgyline

(Carvalho et al. 2012). MAOIs are likely to potentiate pharmacodynamic interactions with MDMA in humans (delaTorre et al. 2004a; Kaskey 1992; Smilkstein et al. 1987) and the most worrisome effect is the induction of the 5-HT syndrome (Pilgrim et al. 2010; Pilgrim et al. 2011; Pilgrim et al. 2012; Scorza et al. 1999; Vuori et al. 2003). Selective MAO-B inhibitors (*e.g.*, selegiline, L-deprenyl), on the contrary, appear to confer some protection against MDMA-induced neurotoxicity by several mechanisms (Carvalho et al. 2012; Pontes 2009) including: (i) decreasing the depletion of 5-HT in the brain (Sprague and Nichols 1995), (ii) decreasing the formation of reactive species resulting from the MAO-B metabolism of the released catecholamines (Hrometz et al. 2004), and consequently, (iii) decreasing of lipid peroxidation (Sprague and Nichols 1995), and of (iv) mitochondrial oxidative damage (Alves et al. 2007).

The most serious cases of serotonergic syndrome seem to occur when the drugs block both 5-HT reuptake and MAO metabolic degradation pathways, greatly increasing the 5-HT levels in the synaptic cleft.

2.2. Interaction with food

Several constituents of the diet may affect the CYP450 isoenzymes (Ioannides 1999; Parkinson et al. 2004), potentially raising the risk for pharmacokinetic interactions with MDMA. The induction of these enzymes may be especially problematic due to the expected increase in metabolic bioactivation. Also the unpredictable effect of food xenobiotics present in the diet (*e.g.* mycotoxins, dioxins, products generated during food processing and food additives, such as growth promoting hormones and pesticides) on the activity of the CYP450 and, therefore, on MDMA pharmacokinetics should not be neglected (Ioannides 1999; Pontes 2009).

2.3. Contaminants and adulterants of *ecstasy* pills

Most studies on the content and purity profile of pills sold as *ecstasy* so far conducted (Cheng et al. 2003; Parrott 2004a; Sherlock et al. 1999; Stoové et al. 2006; Tanner-Smith 2006) lead to the same conclusions (Carvalho et al. 2012; Pontes 2009): (i) not all the tablets contain

MDMA and some have a completely different drug (Baggott et al. 2000; Cheng et al. 2003; Giroud et al. 1997; Sherlock et al. 1999; Tanner-Smith 2006); (ii) the MDMA content of *ecstasy* tablets differs greatly even within the same batch (Giroud et al. 1997; Sherlock et al. 1999; Tanner-Smith 2006), (iii) the majority of the pills present inert substances/excipients in their composition (Carvalho et al. 2012; Giroud et al. 1997), and (iv) many pills contain other stimulants in addition to MDMA (*e.g.*, 4-MTA, ketamine, caffeine, ephedrine, LSD, among many others) (Baggott et al. 2000 633; Cheng et al. 2003; Drugs 2004; Giroud et al. 1997; Parrott 2004a; Tanner-Smith 2006). Several relevant studies provide compelling evidence that extremely harmful and unpredictable reactions on consumers do occur due to such combinations (De Letter et al. 2006; Schifano et al. 2003).

2.3.1. Products derived from chemical synthesis

While in certain batches there is no evidence of the presence of compounds derived from the synthesis process (Parrott 2004a 126; Pontes 2009; Schifano 2000), reagents, intermediates and by-products of synthesis have already been found in seized tablets (Cheng et al. 2006). The presence of these impurities is worrisome from a toxicological perspective as they may also have inherent severe toxicity (Table 6).

Table 6. Potential toxicity derived from precursors, intermediates, and by-products of MDMA chemical synthesis. Adapted from Pontes *et al.* (2009).

Compound	Putative effect
Starting materials	
MDA	MDMA addictive toxicodynamic and toxicokinetic effects (Crean et al. 2006)
Saphrol and analogues	Potential carcinogenicity in humans by forming adducts with DNA (Phillips 1994)
Tetrahydrofuran (THF)	Hepatocellular dysfunction at high doses (Moody 1991) Inhibition of some CYP450 isoenzymes (mainly CYP2E1) (Moody 1991)
Methanol	Metabolic acidosis and inhibition of cell respiration due to accumulation of formic acid that may decrease the half-life of MDMA by increasing urinary excretion (Lanigan 2001)
N-methylformamide	Hepatoxicity (Clagett-Carr et al. 1988)
Pyridine (Cheng et al. 2006)	Induction of CYP2E1 Hepatotoxicity and nephrotoxicity
Intermediates of synthesis	
1,3-bis-(*3,4*-methylenedioxyphenyl)-*2*-propanamine *N-formyl-1,3-bis(3,4*-methylenedioxyphenyl)-*prop-2*-ylamine	Inhibition of monoamine transporters with similar intensity to MDMA (Pifl et al. 2005)

2.3.2. Other substances

Apart from synthesis several other substances have been found as contaminants of *ecstasy* pills. These include caffeine, DXM, amphetamine derivatives, among others.

Caffeine exposure results not only from contamination of the pills (Brunt et al. 2012; Klingler et al. 2005; Morefield et al. 2011; Vogels et al. 2009), but also from the ingestion of caffeine-rich drinks including, tea, coffee, coke, and non-alcoholic energy beverages that are consumed to reduce drowsiness and fatigue (Carvalho et al. 2012). While caffeine is considered safe at low doses (Daly and Fredholm 1998) it can exacerbate the acute MDMA toxicity and has been shown to increase mortality after acute administration of MDMA (Carvalho et al. 2012; Derlet et al. 1992; McNamara et al. 2006; Vanattou-Saifoudine et al. 2012b). The involved mechanisms and associated effects were reviewed by Vanattou-Saifoudine *et al.* (2012b) and included: (i) potentiation of serotonergic toxicity (Camarasa et al. 2006; McNamara et al. 2006; Sprague et al. 1998), (ii) increased MDMA-induced release of DA (Carvalho et al. 2012; Okada et al. 1997; Vanattou-Saifoudine et al. 2011) and enhanced intracellular response mediated by D1 receptor signalling pathway (Vanattou-Saifoudine et al. 2012a), (iii) increased body temperature (McNamara et al. 2006; Vanattou-Saifoudine et al. 2010a; Vanattou-Saifoudine et al. 2010b), (iv) increased MDMA-induced locomotor activity (Camarasa et al. 2006), (v) anorectic effects (Camarasa et al. 2006; McNamara et al. 2006) and (vi) profound and persistent tachycardia, (McNamara et al. 2007; Vanattou-Saifoudine et al. 2010b; Vanattou-Saifoudine et al. 2012b). Notwithstanding, recent studies suggest that chronic caffeine consumption at low doses exerts anti-inflammatory effects and prevents MDMA-induced neuroinflammation, as it completely prevented MDMA-induced glial activation without inducing physiological or behavioural alterations (Ruiz-Medina et al. 2012). Caffeine can also interfere with MDMA pharmacokinetics, as shown *in vitro* by the enhanced permeation through intestinal Caco-2 cells (Kuwayama et al. 2007), and *in vivo* with increases in the area under the plasma concentration-time curve (AUC) of MDMA in rats (Kuwayama et al. 2007).

DXM is an antitussive cough suppressant that has been found in the constitution of *ecstasy* tablets at doses considerably higher than the usual therapeutic dose of this drug (Pontes 2009). It has been responsible for severe and even fatal intoxications (http://metro.co.uk/2011/07/07/a-level-student-kate-mclaughlin-killed-by-mix-of-ecstasy-and-cough-medicine-70133/) since it potentiates the serotonergic effects of MDMA (Baggott et al.

2000; Kamei et al. 1992; Urbain et al. 2008) and competes for the CYP2D6 metabolic pathways (Delaforge et al. 1999).

Paracetamol has been found in *ecstasy* tablets but not at hazardous doses (generally less than 200 mg per tablet) (Sherlock et al. 1999). The CYP450-catalysed metabolism of paracetamol results in formation hepatotoxic metabolites that can deplete GSH (similar to MDMA) and exacerbate liver injury (Plaisance 2000; Pontes 2009).

Some pharmacologically inert excipients can also be intentionally included in the pills. For example, sodium bicarbonate causes an alkalinisation of the excretion fluids (*e.g.,* urine, sweat) leading to a delay in MDMA clearance (Carvalho et al. 2012) and prolonging its effects.

Among the drugs of abuse that most often appear in *ecstasy* pills, the least frequent seem to be cocaine, heroin and other opioids, since the inclusion of these ingredients in the tablets is not an economical benefit to manufacturers (Pontes 2009; Sherlock et al. 1999).

On the contrary, the presence of amphetamine analogues is very common in *ecstasy* pills in addition to MDMA (Barrett et al. 2005; Carvalho et al. 2012). Some may arise in *ecstasy* tablets as precursor of the MDMA synthesis, but in most cases these drugs are intentionally included in the composition of the tablets to enhance the effects of the drug (Kalasinsky et al. 2004). The consequences of such interactions will be further discussed in section 2.4.11 with greater detail.

2.4. Intentional co-ingestion of other recreational drugs

As already mentioned, *ecstasy ab*users frequently ingest other recreational drugs of abuse along with MDMA (Drugs 2004; Schifano et al. 1998). Scholey *et al.* (2004) reported that the abuse practices of MDMA misusers include LSD (60%), psilocybin (56%), and opiates (23%). But other studies disclosed the tendency for the use of other illicit drugs, such as ketamine (9%), GHB (7%), and psilocybin-containing ‘magic mushrooms’ (4%) (Ho et al. 2001; Mohamed et al. 2011). Compelling evidence on polydrug abuse reported by *ecstasy* misusers are compiled in Table 1.

Among the drugs that are legally consumed, tobacco and EtOH are the most frequently reported by MDMA abusers (Carvalho et al. 2012).

2.4.1. Tobacco

Ecstasy consumers have a high prevalence of smoking. Roughly 90% of American adolescents using MDMA consume tobacco (Martins et al. 2008; Mohamed et al. 2011), as do approximately 64% of drug-using university students in Canada (Barrett et al. 2006).

Pharmacokinetic interactions between some of the constituents of tobacco and MDMA are expected due to the potent CYP450 (mainly, CYP1A2 and CYP2E1) and UGT induction by the polycyclic aromatic hydrocarbons (*e.g.*, benzo [a] pyrene) and other pyrolysis products (Benowitz 2008; Carvalho et al. 2012; Parkinson et al. 2004). Also, nicotine *per se* induces CYP2E1 *in vivo* and inhibits CYP2A6 *in vitro* (Benowitz 2008).

Pharmacodinamically, it was hypothesised that nicotine-mediated DA release may facilitate MDMA reinforcement response (Mohamed et al. 2011). Also due to the high affinity of MDMA for receptors containing β_2 subunits (such as the nicotinic receptors) (Picciotto et al. 1998) MDMA can modulate nicotine addiction (Chipana et al. 2008; Pontes 2009). Nicotine can relieve the negative effects of MDMA (Parrott 2005) and MDMA can, in turn, reduce the irritability and anxiety associated with the decrease of nicotine levels during cigarette abstinence (Parrott 1999).

2.4.2. Ethanol (EtOH)

One of the substances consumed more frequently with MDMA is EtOH (Barrett et al. 2006; Barrett et al. 2005; Breen et al. 2006; Carvalho et al. 2012; Lora-Tamayo et al. 2004; Mohamed et al. 2011; Riley et al. 2001; Winstock et al. 2001a), since it is legal and easily accessible in most countries (Lora-Tamayo et al. 2004; Scholey et al. 2004). Mohamed *et al.* (2009) comprehensively reviewed MDMA/EtOH interactions in humans and rodents.

Relevant pharmacodynamic interactions may arise from the EtOH ability to counteract MDMA effects due to its GABAergic actions (Carvalho et al. 2012; delaTorre et al. 2004a). On the other hand, both drugs can produce serotonergic and dopaminergic effects, provoking detrimental effects at various levels including, muscular, neurologic (Izco et al. 2007), endocrine, immune (Pacifici et al. 2001b), cardiovascular, hepatic (Pontes et al. 2008a; Pontes et al. 2008b), and renal (Carvalho et al. 2012).

Pharmacokinetically, EtOH affects several CYP450 isoenzymes (Busby et al. 1999) and it can increase MDMA blood levels (Hernandez-Lopez et al. 2002; Mohamed et al. 2011; Oesterheld et al. 2004; Ramaekers and Kuypers 2006) probably due to the inhibition of MDMA metabolism (Ben Hamida et al. 2007). However, controversial *in vitro* studies also reported that EtOH increased the metabolism of MDMA to 4-hydroxy-3-methoxymethamphetamine (HMA) and MDA in a temperature-dependent fashion, in a pathway mediated, at least partially, by CYP3A and CYP2E1 (Pontes et al. 2010).

2.4.3. Δ^9-tetrahydrocannabinol (THC; cannabis)

The vast majority of *ecstasy* consumers use cannabis derivatives more or less regularly, before starting the consumption of MDMA and continue to consume these products simultaneously with MDMA (Carvalho et al. 2012; Parrott et al. 2007; Pontes 2009; Reid et al. 2007; Rodgers 2000; Scholey et al. 2004; Turner et al. 2003). The combination of these drugs has already been linked to a fatal outcome (Diffley et al. 2012).

The actions of MDMA and Δ^9-tetrahydrocannabinol (THC), the main psychoactive component of cannabis, converge to a common mechanism that modulates the efflux of DA (Robledo et al. 2007). Nevertheless, due to their antioxidant, anti-inflammatory and anti-excitatory properties (Gouzoulis-Mayfrank and Daumann 2006a; Morley et al. 2004b; Pacifici et al. 2007) THC seems to protect the deleterious effects of oxidation due to the excess of DA (Chipana et al. 2008). A study conducted in rats showed that THC prevented the hyperthermia and decreased the 5-HT depletion induced by prolonged consumption of MDMA (Morley et al. 2004b). However, the use of larger doses is associated with several psychobiological problems that can disappear after a period of abstinence of both drugs (Milani et al. 2005). Several detrimental effects have been observed in cannabis and MDMA users including, psychological problems (depression, psychosis, demotivation, increased impulsivity, anxiety), cognitive impairment (attention, memory, learning, verbal fluency, processing speed and manual acuity deficits) and other neuropsychobiologic disorders (sleep disorders, sexual dysfunction, reduced immunocompetence, somatic complaints, obsessive-compulsive patterns and psychotic behaviour) (Dafters et al. 2004; Gouzoulis-Mayfrank and Daumann 2006a; Pacifici et al. 2007; Parrott 2006; Young et al. 2005).

Additionally, since THC is metabolized by CYP3A4 and CYP2C9, pharmacokinetic interactions between MDMA and cannabis may arise (Carvalho et al. 2012; Matsunaga et al. 2000). An extensive review on MDMA/THC interactions was conducted by Mohamed *et al.* (2011).

2.4.4. Cocaine

The combination between cocaine and MDMA (the so-called '*bumping up*' or '*cloud mind*') is often adopted with the intent of intensifying the desired psychostimulant effects (Daza-Losada et al. 2008).

Although the affinity and action of cocaine and MDMA for the different monoamine transporters is not comparable either in humans or in rodents (Han and Gu 2006), both drugs share pharmacodynamic mechanisms (cocaine also elevates the synaptic levels of DA, 5-HT and NA) (Daza-Losada et al. 2008), so pharmacodynamic interactions may be expected (Carvalho et al. 2012). Both substances induce a rise in body temperature that is dependent on the environment temperature (Ansah et al. 1996; Gonzalez 1993). Concurrent administration of MDMA/cocaine produces locomotor activation and enhances dopaminergic responses in rats to a greater extent than administration of either drug alone (Panos and Baker 2010). A recent study demonstrated that MDMA increases cocaine reward in male adolescents (Starosciak et al. 2012). Repeated administration of MDMA to rats increases the cocaine-induced release of DA, suggesting that MDMA can alter the vulnerability to develop cocaine dependence and abuse (Morgan et al. 1997).

Cocaine was demonstrated to inhibit *in vitro* the metabolism of MDMA (Ramamoorthy et al. 2002; Shen et al. 2007). Thus, substantial increases on the concentrations of MDMA may be attained when the drugs are co-ingested.

2.4.5. Lysergic acid diethylamide (LSD)

LSD is one of the most potent hallucinogens, widely used all over the world (Golub et al. 2001; Schiff 2006). Its combination with MDMA is called *candyflipping* or *XL* on the streets, and has reached high prevalence in the last decade (Carvalho et al. 2012; Pontes 2009; Wish

et al. 2006), as supported by a few reports (Licht et al. 2012; Miller and Gold 1994; Millman and Beeder 1994).

The pharmacodynamic interactions between these drugs seem to result from the increased levels of synaptic 5-HT, which activates the receptors involved in the stimulating properties of LSD and MDMA (Schechter 1998). Because the acute toxic effects of LSD include cardiovascular symptoms (tachycardia, hypertension), renal failure and rhabdomyolysis (Berrens et al. 2010; Schiff 2006), the toxic response of MDMA might be aggravated when the drugs are co-ingested.

As LSD metabolism appears to involve CYP2D6 (Yu 2008), it can be expected that the drug interferes with MDMA pharmacokinetics by altering the blood levels of the drug.

2.4.6. γ-Hydroxybutyric acid (GHB, liquid *ecstasy*)

GHB is an endogenous inhibitory neurotransmitter (Bessman and Fishbein 1963) often sold as a recreational drug in form of powder, which dissolves in water (Freese et al. 2002). Its unpleasant taste is often masked with alcohol, which increases the potent hypnotic/sedative effects of GHB (Gahlinger 2004). GHB carries the 'street' names *liquid ecstasy* or *liquid X*, as it produces MDMA-like effects including, euphoria, reduced anxiety, enhanced social interactions, disinhibition (Doyon 2001), progressing to amnesia (Gahlinger 2004), deep sedation, coma and even death (Drasbek et al. 2006). It is often used as a 'rape drug'.

Neurochemically, GHB and MDMA have counteracting mechanisms since GHB inhibits DA release and increases the availability of 5-hydroxytryptophan (the precursor of 5-HT), thus potentiating presynaptic 5-HT turnover (Mohamed et al. 2011). So, the concurrent administration of GHB has allegedly the ability of attenuating dysphoric or unpleasant 'comedown' after-effects of MDMA (Carvalho et al. 2012; Uys and Niesink 2005). Van Nieuwenhuijzen *et al.* (2010) showed that combined MDMA and GHB were responsible for MDMA-like deficits in memory and social behavior and that the combination causes long-term neuro-adaptation in the hypothalamic oxytocin system, probably related to the long-term social interaction deficits caused by both drugs (van Nieuwenhuijzen et al. 2010).

2.4.7. Ketamine

Ketamine is a derivative of 1-(1-phenylcyclohexyl)piperidine (PCP, phencyclidine) anaesthetic used in human and veterinary medicine, with analgesic, hallucinogenic and dissociative properties – characterised by a sense of detachment from the physical body and the external world (White et al. 1982; Wolff and Winstock 2006). Recreationally, ketamine is often snorted (*known as K, Super K, Special K, Vitamin K, Green, Mean Green, kat-kit, Keets, super acid, cat valiums and Jet*) but is also marketed to be smoked or ingested (as a liquid, powder or capsules) (Pontes 2009; Wolff et al. 1995). Ketamine has been identified in *ecstasy* tablets known as '*strawberry milkshake*' and '*white lightning*' in dosages ranging from 185 to 197 mg and the effects elicited by the drug (immobility, hallucinations, among others) often surprises consumers that expect empathy and euphoria (Pontes 2009; Winstock et al. 2001b; Wolff et al. 1995).

Ketamine has the ability to inhibit the reuptake of NA, DA and 5-HT in a dose-dependent manner, thus having a sympathomimetic action (Nishimura et al. 1998), that may account for the pharmacodynamic interactions with MDMA. In fact, the risk of over-stimulation of the heart is a potentially fatal consequence of combining ketamine with amphetamines, and the symptoms associated with ketamine overdose include, hyperactivity, anxiety, chest pain, palpitations, tachycardia, hyperthermia and, in more severe cases, rhabdomyolysis (Gill and Stajic 2000; Jansen 1993; Wolff and Winstock 2006). Any of these effects can add to those caused by MDMA, increasing toxicity (Pontes 2009).

Besides pharmacodynamic interactions ketamine also has the potential to cause pharmacokinetic interactions, since most of the drug is metabolized by CYP2B6 (Carvalho et al. 2012; Hijazi and Boulieu 2002; Yanagihara et al. 2001). On the other hand, ketamine inhibited *in vitro* the uptake and permeation of MDMA through the epithelial cells of the ileum (Kuwayama et al. 2007), showing the potential to reduce MDMA absorption at the intestinal level (Carvalho et al. 2012).

2.4.8. Ephedrine

Ephedrine is found in *Ephedra vulgaris natural* (*Ma-Huang* or *Herbal ecstasy*). It causes the release of NO and, to a lesser extent, the release of DA and 5-HT (Rothman et al. 2001).

Ephedrine promotes thermogenic, sympathomimetic, and pro-dopaminergic effects and it is therefore likely to interact with MDMA and increase its addictive and toxic potential (Carvalho et al. 2012; Chen et al. 2004; Pontes 2009; Soni et al. 2004; Yates et al. 2000). The metabolism of ephedrine may be responsible for a pharmacokinetic interaction with MDMA (Soni et al. 2004), since both drugs compete for the same metabolic pathways (Carvalho et al. 2012).

2.4.9. Opiates and derivatives

Heroin and MDMA are often combined to favour the stimulant effects of MDMA and ease the 'come down' associated with *ecstasy* intake (Gervin et al. 2001; Gervin et al. 1998), but this combination was already linked to fatal outcomes (Gerevich et al. 2000).

It is possible that the serotonergic effects of MDMA may be enhanced by heroin (Oesterheld et al. 2004) elevating the risk for the 5-HT syndrome to occur. Compan *et al.* (2003) showed that the opioid antagonist naloxone suppressed the hyperlocomotor effects of MDMA in mice. Also modifications in the opioid system may negatively influence the abuse potential of MDMA (Bilsky et al. 1991; Daza-Losada et al. 2008; Mohamed et al. 2011; Reid et al. 1996).

2.4.10. Psilocybin-containing mushrooms ('*magic mushrooms*')

Some mushrooms have hallucinogenic properties due to its psychoactive indole alkaloids content (colloquially, *shrooms* or *magic mushrooms*). The effects of psilocybin mushrooms come from psilocybin that is converted by hydrolysis to its active metabolite, psilocin, which is responsible for the psychedelic effects (Passie et al. 2002).

Psilocybin/psilocin has sympathomimetic serotonergic and dopaminergic effects (binding with high affinity to presynaptic 5-HT_{2A} receptors and, to a lesser extent, to 5-HT_{1A} receptors) (Passie et al. 2002). This sympathomimetic action also leads to (Passie et al. 2002; Pontes 2009) DA D_2 receptors activation. Therefore, psilocybin/psilocin have great potential for pharmacodynamically interacting with MDMA (Carvalho et al. 2012).

2.4.11. Amphetaminic stimulants

In addition to the amphetamines that are present in *ecstasy* pills, the interaction of MDMA with other amphetamines also occurs when these are intentionally co-ingested to modulate the effects of MDMA. The combined ingestion of MDMA and other amphetaminic analogues (MDA, *d*-AMP, 3,4-methylenedioxy-N-ethylamphetamine or MDEA and PMA) has already been linked to the onset of fatal poisonings, usually accompanied by hyperthermia and DIC. De Letter and collaborators (2001) described eight severe intoxications, one with a fatal outcome, involving the highly toxic 4-MTA. This drug is frequently found contaminating or substituting MDMA in clandestine *ecstasy* pills, along with other amphetamines. Also PMA, METH and *d*-AMP were identified in toxic/lethal amounts at *postmortem* analysis of six fatalities caused by the intake of tablets marketed as *ecstasy* (Byard et al. 1998). Since these drugs share biokinetic and pharmacologic pathways, the potential for interaction is very high.

The study of the possible interactions between MDMA and three other amphetamines in this polydrug abuse context, and the mechanisms involved, is the major purpose of the experimental work of the current dissertation. For this reason, in the following sections, the main pharmacokinetic and pharmacodynamic properties of our test drugs, METH, *d*-AMP and 4-MTA will be discussed in greater detail.

2.4.11.1. Amphetamine (AMP) and methamphetamine (METH)

d-AMP, the dextrorotatory stereoisomer of the prototype of this group of drugs, and METH (*speed, ice, crystal, crank*), the *N*-methylated analogue of AMP, are abused worldwide due to their euphoric and powerful stimulant properties. Epidemiological evidence reveals that METH abuse rates are greater than those of *d*-AMP (SAMHSA 2009) possibly due to the presence of the methyl group that makes METH more lipophilic (therefore more potent). Also, because its synthesis is cheaper and easier, this drug is more readily available compared to *d*-AMP. Recreational METH abuse allegedly involves larger doses and routes of administration that produce a more rapid onset of effects (users often begin with intranasal or oral use and progress to intravenous use and, occasionally smoking) (Simon et al. 2002). Both drugs are approved in several countries to treat similar medical conditions, being *d*-AMP one of the most frequently prescribed medications for the treatment of attention deficit disorder,

and narcolepsy. Despite METH being usually perceived to have a greater abuse potential, findings from behavioral studies indicate that, at equivalent doses, both drugs produced similar discriminative stimulus effects in rats (Kuhn et al. 1974), and are self-administered by rhesus monkeys and rats at similar rates (Balster and Schuster 1973; Yokel and Pickens 1973). Kirkpatrick *et al.* (2012) compared intranasal METH and *d*-AMP self-administration and concluded that both produced a similar dose-related profile of effects in humans, which supports their equivalence for abuse potential.

2.4.11.1.1. Pharmacokinetics

Generally, amphetamines have high oral bioavailability, high volume of distribution, low plasma protein binding (usually less than 20%) and both renal and hepatic elimination (Carvalho et al. 2012). Amphetamine is usually consumed orally as the S-(+)-enantiomer (*d*-AMP) while METH can be used orally, intravenously, snorted, vapor inhaled, or smoked as S-(+)-METH hydrochloride salt. Following administration, peak METH concentrations occur in 1.5-3.5 hours (depending on the route of administration) and the mean elimination half-life is 10 hours (Mendelson et al. 2006). The bioavailability varies between 67-90% (smoked > snorted > oral) (Cook et al. 1993). Amphetamine blood concentration peaks at 4 hours after ingestion. The distribution of AMP and METH is similar and is purportedly independent of the administration route (Carvalho et al. 2012). Volkow and co-workers (2010) proved that after intravenous administration the highest uptake occurred in lungs (22%), liver (23%) and in brain (10%). Of forensic relevance, the drugs have been shown to accumulate in saliva, hair, and nails. The main metabolic reaction of METH is the *N*-demethylation into AMP (Lin et al. 1997). The metabolic pathways described for METH and AMP are presented in Figure 4. These drugs are metabolized to a lower extent than MDMA, and the urinary levels of the unchanged drugs are accordingly much higher (approximately up to 50% eliminated without biotransformation) (de la Torre et al. 2004). There is wide variability in the elimination half-life of amphetamines, since it is, to a great extent, dependent on urine pH (de la Torre et al. 2004). About 15% of METH is eliminated in the urine as 4-hydroxymetamphetamine and 10% as AMP (Cook et al. 1993).

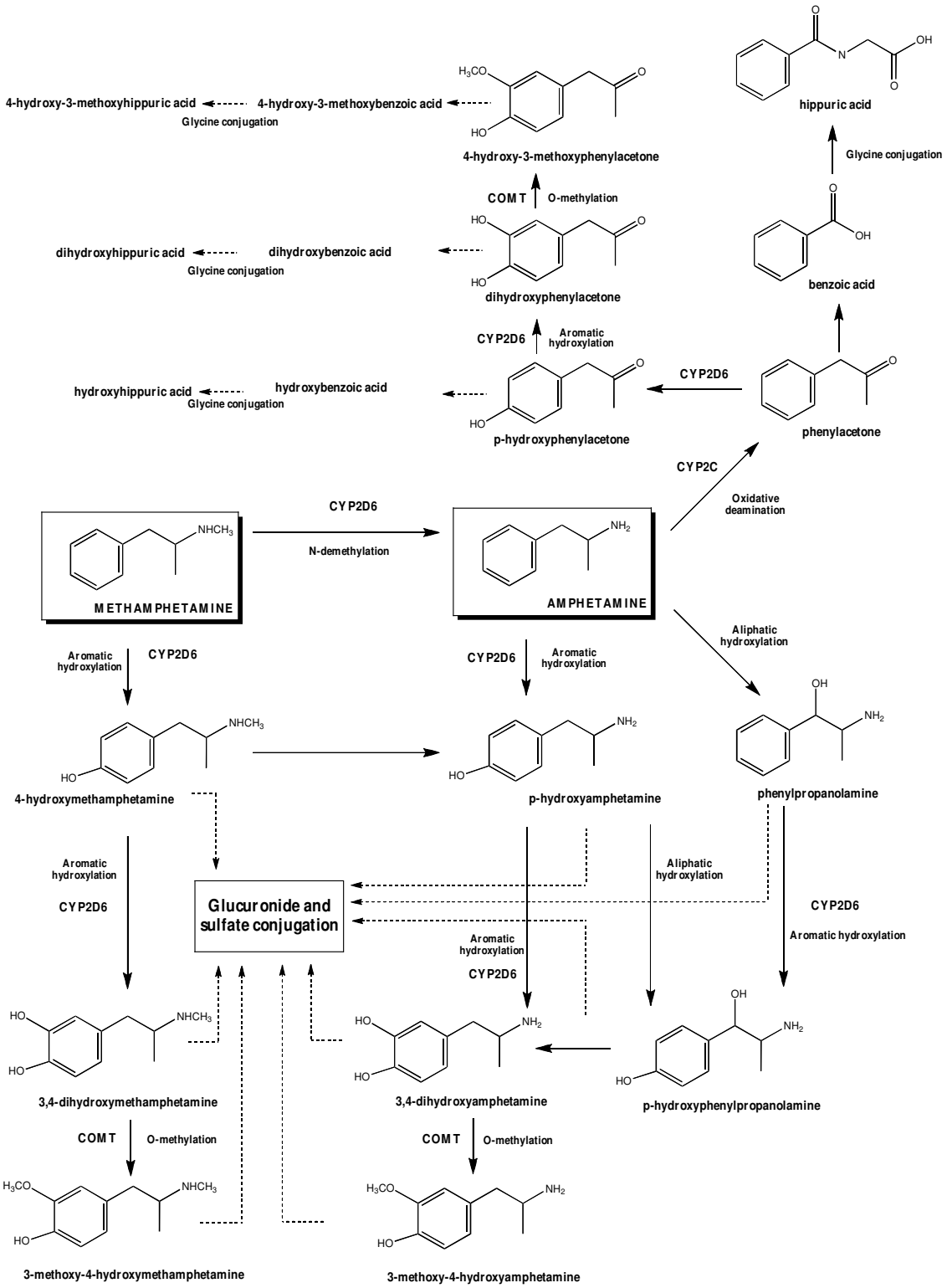

Figure 4. Metabolic pathways of amphetamine and methamphetamine. METH is (i) N-demethylated to AMP and (ii) 4-hydroxylated to 4-hydroxymethamphetamine by CYP2D6. AMP is metabolized mainly through (i) N-deamination and oxidation into the corresponding benzoic acid derivatives that are further conjugated with glycine and excreted as the corresponding hippuric acids and (ii) 4-hydroxylation of the aromatic ring, generating 4-hydroxyamphetamine, followed by conjugation of the phenol group with sulfate or glucuronic acid. ß-oxidation of the side chain leads to the formation of norephedrine that is oxidized in the aromatic ring into hydroxynorephedrine. Amphetamine N-deamination seems to be catalyzed by CYP450 isoenzymes of the CYP2C subfamily, whereas CYP2D6 is involved in the hydroxylation of the aromatic ring. Adapted from Carvalho *et al.* (2012).

2.4.11.1.2. Pharmacology

Similar to MDMA and the other ring-substituted amphetamine derivatives, there is plenty of evidence, both experimental and clinical, that METH and AMP act by increasing the release of the monoamine neurotransmitters (DA, NA and 5-HT) from their respective axon terminals either by (i) redistributing catecholamines from the synaptic vesicles to the cytosol and by (ii) reversing the transport of the neurotransmitter through plasma membrane transporters. However, the affinity of these drugs for different monoamine transporters differ. Some amphetamines, such as MDMA and 4-MTA, preferentially inhibit the reuptake and stimulate the release of 5-HT, while others, such as METH and *d*-Amp, preferentially inhibit the reuptake and stimulate the release of DA. There is also evidence that METH and *d*-Amp can increase the cytosolic levels of monoamines by inhibiting the activity of MAO, as well as by increasing the activity and expression of the DA-synthesizing enzyme, tyrosine hydroxylase (Sulzer et al. 2005). S-(**+**)-METH and S-(**+**)-*d*-Amp stereoisomers are generally associated with more potent physiological effects.

1.2.1.1.1. Toxicity

The addition of a methyl group in METH has a major influence on the neurotoxicity of the drug. By virtue of its higher lyphofily, METH allegedly crosses the blood-brain barrier more readily. Also, the duration of its effect is longer.

METH and *d*-Amp use has been related to several clinical, neurological, and psychiatric problems. The main toxic effects occur at the nervous system level (primarily affecting the dopaminergic system). The initial feeling of physically and mentally enhanced powers following METH/AMP use can quickly deteriorate with high doses or chronic use, resulting in emotional lability, confusion, paranoia, hallucinations, delirium, anxiety, mood disorders, behavioural disinhibition, agitation, psychosis, deterioration in memory and learning capacity, psychomotor slowdown, and problems in processing information (Schep et al. 2010). Some of these psychiatric symptoms are similar to those of schizophrenia. It is proposed that these neurotoxic effects are a result of i) chronic methamphetamine use, ii) a severe acute selective decrease in the various dopaminergic markers in various cerebral structures, and iii) major changes in cerebral blood flow (Schep et al. 2010). Cerebrovascular accidents due to hemorrhage or vasospasm, cerebral edema, and cerebral vasculitis are very

common features of METH/AMP intoxications. Also, cardiovascular effetcs are a major reason of concern, and a frequent cause of death and they include chest pain, palpitations, myocardial infarctions, hypertension, necrosis, and ischemia. Significant hypotension with bradycardia and metabolic acidosis were seen in massive amphetamine overdoses (Schep et al. 2010). Dyspnea and breath impairment are frequently associated with amphetamines intake. Necrotizing angiitis with arterial aneurysms were observed in the kidney, liver, and pancreas, in METH drug abusers. Amphetamine-induced seizures seem to occur as isolated events or associated with hyperthermia, coma, muscle hyperactivity, metabolic acidosis, secondary rhabdomyolysis, renal failure, or shock .

1.2.1.2.4-Methylthioamphetamine (4-MTA)

4-Methylthioamphetamine (4-MTA), also known as *p*-methylthioamphetamine (*p*-MTA), 1-(4-methylthiophenyl)-2-aminopropane, and *α*-methyl-4-methylthiophenylethylamine was firstly synthesized in 1963 as a potential anorectic agent. In 1992, David Nichols and colleagues (Purdue University) synthesized this molecule again, this time aiming at obtaining new molecules pharmacologically active in the treatment of depression. During the late 1990s, 4-MTA emerged on the streets as a drug of abuse. Tablets containing 4-MTA were first seized in Europe in 1997, with the street name *flatliners* (nickname adopted by virtue of the flat half-scored white pills), *5-S, MK* and *Golden Eagle* (Groombridge 1998; Poortman and Lock 1999). They were usually marketed as *ecstasy*. 4-MTA rapidly became a major cause of concern due to the several deaths linked to its consumption and to its high risk of severe side effects. Tablets sold on the illicit market revealed a 4-MTA content ranging between 100-140 mg/tablet (Elliott 2000; Poortman and Lock 1999).

1.2.1.2.1. Pharmacokinetics

In 2000, Elliott (2000) analyzed *perimortem* and *postmortem* blood specimens in a fatal case involving 4-MTA. The results indicated the presence of two possible metabolites of 4-MTA, presumably a sulfoxide and a hydroxyl derivative. The results also suggested that there is little difference in the concentration of 4-MTA in *perimortem* and *postmortem* femoral blood,

as can be observed in Table 7. This could indicate that 4-MTA may not undergo significant *postmortem* redistribution and may have a relatively low volume of distribution (Elliott 2000).

Later, Elliott (2001) reported, for the first time, data regarding the possible pharmacokinetics of 4-MTA in humans. The author studied three intoxication cases implicating 4-MTA, in which the patients survived. In all three cases other amphetamines, such as *d*-AMP, MDMA and pseudoephedrine, were present (Elliott 2001). In all three cases there was a relatively low concentration of MDMA and/or amphetamine in the plasma, compared to 4-MTA (table 7). Presumed metabolites were also detected, with compounds identified as being 4-MTA sulphoxide and hydroxy-MTA. In one patient the 4-MTA concentration was determined in a series of plasma samples (table 7) and this allowed the estimation of a presumptive half-life of approximately 7 h (Elliott 2001).

De Letter *et al* (2001) reported a case study involving one fatal and seven survived cases of intoxication with 4-MTA (table 7). In this study they observed that, similar to what happens with other amphetamines in which the urinary excretion is pH-dependent, the individuals with the lowest urinary pH showed the highest urinary 4-MTA levels (De Letter et al. 2001). Decaestecker and colleagues (2001) performed a detailed distribution study by measuring 4-MTA levels in various tissues and fluids samples after autopsy in the fatal outcome case (Decaestecker et al. 2001). The drug suffered *postmortem* diffusion from the liver (30.8 mg/kg) and lungs (16.6 mg/kg) to the cardiac atriums (7.9 mg/L) via the vascular pathway. The lungs showed levels that were almost 3 times higher than those in the peripheral blood. Also, 4-MTA levels in liver and bile (36.4 mg/L) were 6-7 times higher than the peripheral blood concentration (5.23 mg/L collected from the femoralis vein) (Decaestecker et al. 2001). Given the significant 4-MTA levels attained in the liver and the bile, it seems likely that 4-MTA is eliminated by excretion via the bile. On the contrary, 4-MTA cardiac and skeletal muscle levels were similar to peripheral blood concentrations, denoting that the drug does not tend to concentrate in these tissues. It was also suggested a certain accumulation of 4-MTA in red blood cells. As the brain is the most worrisome target organ, 4-MTA levels in the various lobes of the brain and the brainstem (ranging between 31.7 mg/kg and 36.5 mg/kg) were high and in line with the aforementioned toxicity expectations.

Carmo *et al.* (2002) investigated the metabolic profile of 4-MTA after acute administration to Charles-River CD1 mice. Their results showed that 4-MTA was partially metabolized with the parent compound and its respective metabolites being excreted in urine.

Table 7. Concentrations of 4-MTA found at non-fatal and fatal intoxications.

	4-MTA urine concentration		4-MTA blood concentration		4-MTA liver concentration	Study	Reference
Number of pills		***Post-mortem***		***Post-mortem***	***Post-mortem***		
			0.760 µg/L (admission) 0.280 µg/L (after 7 h; also detected 40 µg/L MDMA and 250 µg/L *d*-AMP) 0.144 µg/L (after 14 h) 0.154 µg/L (after 17 h; also detected 20 µg/L MDMA and 110 µg/L *d*-AMP) 0.131 µg/L (after 16.5 h; also detected 20 µg/L MDMA and 50 µg/L *d*-AMP) 0.189 µg/L (also detected 60 µg/L MDMA and 20 µg/L *d*-AMP)			Human non fatal intoxication‡	(Elliott 2001)
						Human non fatal intoxication‡	(Elliott 2001)
						Human non fatal intoxication‡	(Elliott 2001)
7-8		87.2 mg/L	4.3 mg/L	4.6 mg/L †		Human fatal intoxication	(Elliott 2000)
≥ 6		100 mg/L (also 1.2 mg/L MDMA)		8.38 mg/L	30 mg/L	Human fatal intoxication&	(De Letter et al. 2001)
2	10 mg/L		0.63 mg/L			Human non fatal intoxication&	(De Letter et al. 2001)
1	4 mg/L		1.08 mg/L			Human non fatal intoxication&	(De Letter et al. 2001)
1	8 mg/L		2.08 mg/L			Human non fatal intoxication&	(De Letter et al. 2001)
1 or 1.5	4 mg/L		1.93 mg/L			Human non fatal intoxication&	(De Letter et al. 2001)
3, 5 or 6	40 mg/L		1.26 mg/L			Human non fatal intoxication&	(De Letter et al. 2001)
≥ 2	32 mg/L		0.43 mg/L			Human non fatal intoxication&	(De Letter et al. 2001)
						Human non fatal intoxication&	(De Letter et al. 2001)
				1,500 mg/L §			(Poortman and Lock 1999)

‡ At the time of specimen collection the patient was in a serious clinical condition requiring ventilation and sedation after suffering convulsions.
& Positive for tetrahydrocannabinoic acid. The 4-MTA pills, weighing 345 mg, contained 97 mg 4-MTA.
† Blood specimens from the femoral vein.
§ Levels of *d*-AMP in the blood sample were 1,500 mg/L.

In addition to β-hydroxylated isomers and *4*-methylthiobenzoic acid metabolites, they observed the presence of a third hydroxylated metabolite resulting from either aromatic hydroxylation or hydroxylation on the methylthio group. These metabolic pathways are similar to those of *d*-AMP and many of its derivatives (Caldwell 1980; Foster et al. 1993; Staack and Maurer 2005). In another study using primary hepatocytes of different animal species (monkey, dog, rabbit, rat, and mouse) and three male human donors (Carmo et al. 2004), they observed that 4-MTA was not extensively metabolised by the hepatocytes obtained from all the species examined. The main metabolite was identified as *4*-methylthiobenzoic acid which, for the first time was described as a human metabolite. Similarly to what was observed in the *in vivo* study (Carmo et al. 2002), two β-hydroxylated isomers (4-methylthiophenylpropanolamine and 4-methylthiocathine) metabolites were observed. However, the authors concluded that those compounds and another 4-MTA sulfoxide metabolite could be formed spontaneously without the interference of hepatic metabolism.

After investigating the urine samples (collected 8–15 h after reported ingestion) of five individuals taking 1-5 pills of 4-MTA, Ewald *et al.* (2005) identified four metabolites and postulated the metabolic routes presented in Figure 5. The metabolites: ring hydroxy 4-MTA, β-hydroxy 4-MTA and *4*-methylthiobenzoic acid had already been identified by Carmo *et al.* (Carmo et al. 2002; Carmo et al. 2004). The two isomers of the ß-hydroxy metabolite that were detected by Carmo *et al.* (2004) in hepatocytes, could not be detected, maybe due to low urine concentrations. In addition, in this study 4-MTA sulfoxide was also identified as a probable artifact. As the peak of the deamino-hydroxy metabolite was more abundant in urine after hydrolysis with glucuronidase and arylsulfatase, it was concluded that it is partly excreted as glucuronide and/or sulfate. The presented study revealed that 4-MTA was poorly metabolized by humans. Therefore, the drug screening in case of intoxication should focus on the parent compound.

Carmo *et al.* (2007) tested in an *in vitro* model whether CYP2D6 as well as CYP3A4 could influence the susceptibility to the toxicity of 4-MTA. Their results showed that the expression of wild type CYP2D6*1 greatly enhanced the susceptibility to the cytotoxic effects of 4-MTA compared to the cells devoid of CYP-dependent enzymatic activity. Toxicity in cells expressing CYP2D6*1 was also higher compared to the cells expressing the low activity alleles CYP2D6*2 and CYP2D6*9. In contrast to CYP2D6, the CYP3A4 isoenzyme did not enhance 4-MTA toxicity.

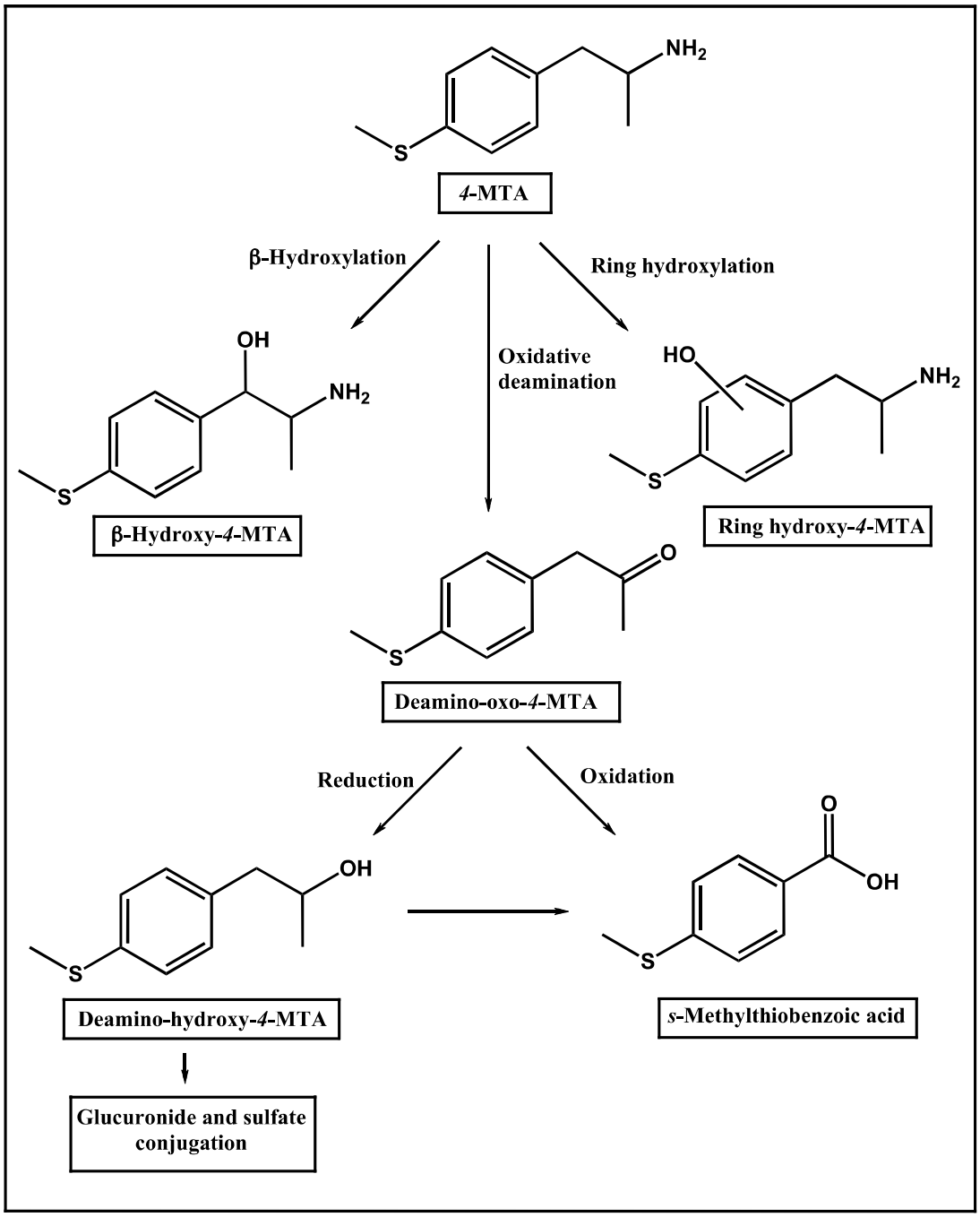

Figure 5. Proposed pathways by Ewald and co-workers (2005) for the phase I metabolism of 4-MTA in humans. 4-MTA suffers oxidative deamination to the corresponding deamino-oxo metabolite (1-[4-(methylthio)-phenyl]propan-2-one), followed by reduction to the corresponding alcohol deamino-hydroxy 4-MTA (1-[4-(methylthio)phenyl]propan-2-ol) or degradation of the side chain to 4-methylthiobenzoic acid; ring hydroxylation to a phenolic structure ((2-aminopropyl)-(methylthio)phenol) and β–hydroxylation of the side chain to 4-methylthionorephedrine or ß-hydroxy 4-MTA (2-amino-1-[4-(methylthio)phenyl]-propan-*1*-ol). The deamino-hydroxy metabolite is partly excreted as glucuronide and/or sulfate.

These data indicate that metabolism may play a role in the toxic effects of the drug. However, since the drug is metabolised *in vivo* to a relatively low extent it may not significantly modify the toxicity of the drug.

1.2.1.1.1. Pharmacology

There is no systematic data available on the neuropsychological effects of 4-MTA in man, however limited animal data suggests effects similar to the entactogen class of compounds such as MDMA (Kavanagh et al. 1999). 4-MTA is indeed a potent, selective, serotonin releaser and uptake inhibitor, apparently devoid of serotonin neurotoxic effects (Huang et al. 1992; Scorza et al. 1999). In rats, 4-MTA caused a dose-dependent serotonin release but had no long-term serotonin depleting effects in frontal cortex, hippocampus and striatum at behaviorally relevant doses (Huang et al. 1992). With regard to the inhibition of the neuronal serotonin uptake 4-MTA was about six times more potent than MDMA (Huang et al. 1992; Murphy et al. 2002). The pro-serotoninergic activity of the drug was responsible for a dose-dependent increased secretion of several hormones including, oxytocin, corticosterone and renin, in rats (Li et al. 1996).

4-MTA has also a high MAO-A inhibitory capacity, being a more potent inhibitor than the other amphetamine derivatives (Scorza et al. 1999). This property was deemed responsible for the cytotoxicity elicited by 4-MTA in RCHT cells derived from adult rat hypothalamus due to the consequent increase in the intracellular concentration of dopamine that is further autoxidised into the respective aminochrome with simultaneous production of reactive oxygen species (Hurtado-Guzman et al. 2002). Additionally, MAO-A inhibition might be responsible for the much higher and sustained effect on the release of serotonin, which is observed with administration of 4-MTA (Scorza et al. 1999). As this is not an immediate effect it may also explain why there is a delay in producing the maximum stimulating effect desired by drug abusers. There is a likely possibility that potentially deleterious drug-drug interactions with other MAO inhibitors may occur through the potentiation of the serotonin syndrome. The drug also proved to be relatively inert as a catecholaminergic agent (the capacity of inhibition of the uptake of DA and NE by 4-MTA is two and five times, respectively, lower than that of MDMA) (Huang et al. 1992).

Carmo *et al.* (2003) suggested that the 4-MTA-induced hyperthermia might be highly influenced by the catecholaminergic and serotonergic receptor activation and the MAO activity as, in mice, the blockade of the 5-HT receptors with methysergide and the MAO inhibition with pargyline resulted in the potentiation of the 4-MTA-induced hyperthermic effect. Also, the blockade of the α_1-adrenergic receptors with prazosin completely reverted the 4-MTA-induced hyperthermia while with the β-adrenergic receptor blocker propranolol this reversal was not complete (Carmo et al. 2003).

1.2.1.1.2. Toxicity

Users have noted that 4-MTA produces a greater 'high' and has a delayed reaction compared to other *ecstasy* pills. The slow onset of the effects of the drug (up to 2 h) may encourage abusers to administer further doses assuming that the first dose was inadequate, which can result in accidental overdose and possible death (Elliott 2001). In fact, 4-MTA was involved in several fatal and non-fatal intoxication cases in the United Kingdom, Netherlands, France and Belgium (De Letter et al. 2001; Decaestecker et al. 2001; Elliott 2000; Elliott 2001; EMCDDA 1999; Poortman and Lock 1999). Most of the 4-MTA fatal and non-fatal intoxications involved additional drugs such as MDMA, caffeine, cannabis, other amphetamines, methadone and alcohol (de Boer et al. 1999; De Letter et al. 2001; Elliott 2001; EMCDDA 1999; Poortman and Lock 1999). Based on the data obtained from those intoxications, Elliott (2001) suggested that moderate toxicity may be associated with plasma 4-MTA concentrations of between 0.20 and 0.60 mg/L, severe toxicity with concentrations above 0.60mg/L, and death with concentrations above 1.50mg/L

It is suspected that the toxicity of 4-MTA is related to the serotonin syndrome. The side-effects of 4-MTA are mainly related to the sympathicomimetic overstimulation and include tachycardia, tremors, palpitations, diaphoresis, paraesthesias, trismus and bruxism, agitation and insomnia (De Letter et al. 2001). Fatal cardiac arrhythmia and respiratory failure are considered probable causes of death (De Letter et al. 2001; EMCDDA 1999). The intoxication reports point to a stronger and prolonged effect than that caused by MDMA, and 4-MTA also appears associated with serious unpleasant effects, including nausea, nystagmus, hyperthermia, glaucoma, thirst, tremors, confusion, memory loss, seizures, respiratory depression and increased heart rate (De Letter et al. 2001; Elliott 2000; EMCDDA 1999). De

Boer *et al.* (1999) reported a patient who suffered from amnesia during a 2-week period following the ingestion of a single pill.

Custodio *et al.* (2010) studied the involvement of mitochondria in 4-MTA-induced hepatotoxicity and observed that 4-MTA (from 0.025 up to 0.1mM) was more potent than MDMA (from 0.2 up to 0.5mM) in decreasing the sensitivity of rat liver mitochondria to mitochondrial permeability transition. However, higher concentrations of 4-MTA (from 0.5 up to 2mM) were highly toxic to mitochondria. 4-MTA simultaneously increased H_2O_2 production in a MAO-dependent way, and uncoupled and inhibited mitochondrial respiration. In contrast, MDMA had only limited or no effects on these mitochondrial parameters (Custodio et al. 2010).

1.2.1.2.Combination effects between amphetamine derivatives

All amphetamines with phenylisopropylamine structure are inhibitors of CYP2D6 (Lin et al. 1997), their potency being dependent on the presence of substituent functional groups (Pontes 2009; Wu et al. 1997). The simultaneous ingestion of MDMA and other amphetamines will probably lead to increased MDMA plasma concentrations, thereby, increasing the risk of acute toxicity (Hashimoto et al. 1993). Also, the intestinal absorption of compounds with amine moieties, such as the amphetamines, are regulated by a common transport system. Therefore, amphetamine-type stimulants present in *ecstasy* tablets, such as AMP, METH, MDEA and MDA may inhibit the uptake of MDMA via ileum epithelial cells, delaying its intestinal absorption (Kuwayama et al. 2007; Pontes 2009).

Pharmacodynamic interactions may arise as a result of combining MDMA with other amphetaminic congeners, as these drugs are also neurotoxic affecting the dopaminergic and serotonergic pathways through mechanisms that are similar to those of MDMA (Gouzoulis-Mayfrank and Daumann 2006a; Seiden and Sabol 1996). Thus, the co-consumption of MDMA and other amphetaminic stimulants can increase serotonergic neurotoxicity and also the toxic effects on the dopaminergic system induced by MDMA (Gouzoulis-Mayfrank and Daumann 2006a). For instance, MDMA/PMA combination induces functional changes in 5-HT neurotransmission (Mohamed et al. 2011). MDA, by itself, increases blood pressure, induces bradycardia and locomotor activity, and is more potent than MDMA in activating α_{1D}, α_{2A} and α_{1A} receptors (Bexis and Docherty 2006; Pontes 2009). Additionally, it is also

capable of inducing hyperthermia (Crean et al. 2006; Pontes 2009). *d*-AMP also induces an hyperthermic response that is dependent on ambient temperature (Hamida et al. 2008). In spite of some differences in the effects on the thermoregulatory system of MDMA and *d*-AMP or its derivatives (Jaehne et al. 2005), an increased risk for hyperthermia is expected when these drugs are co-ingested.

Also, the reinforcing, physiological, and subjective effects of MDMA in humans are similar to those of *d*-AMP (Tancer and Johanson 2003). MDMA pretreatment increases the locomotor stimulant effect of *d*-AMP in rats (Callaway and Geyer 1992). Garcia-Ratés *et al.* demonstrated that METH and MDMA have affinity for, and can interact with, nicotinic acetylcholine receptors, inducing their up-regulation, especially when higher doses are used. Such effects may have a role in METH- and MDMA-induced neurotoxicity, cholinergic neurotransmission, and in processes related with addiction and dependence (Garcia-Rates et al. 2007). Also, if the relative proportion of dopaminergic amphetamines in the pills is higher, the addictive potential of these pills will probably be increased (Carvalho et al. 2012; Garcia-Rates et al. 2007). In rats, pre-exposure to MDMA delays the initial acquisition of amphetamine self-administration and encourages amphetamine-seeking behaviour along with MDMA-induced hyperactivity (Morley et al. 2004a). Yet, pre-treatment with MDMA diminishes self-administration of METH (Clemens et al. 2006). MDMA itself does not have a high potential to disturb the dopaminergic system, but the increase in DA produced by the other congeners leads to feelings of self-confidence, energy, sexual stimulation and euphoria and is also responsible for the addictive potential (Pontes 2009). It was also demonstrated, in rats, that the MDMA/METH combination may produce adverse neurochemical and behavioural effects (euphoric and pro-social effects) (Clemens et al. 2007b) and marked monoamine depletion (Clemens et al. 2004) that are greater than those observed with similar doses of each drug administered separately. However, these differences seem to disappear with a chronic consumption pattern (Clemens et al. 2007a). Other authors reported that, in humans, regular use of combined MDMA/METH cause severe long-term cognitive, behavioural, and neurological changes (Brecht and von Mayrhauser 2002; Reneman et al. 2002). This enhanced neurotoxicity may be caused by oxidative stress mechanisms that arise after exhaustion of the endogenous free-radical scavenging processes (Hanson et al. 2004).

Recently, new amphetamine derivatives that are not controlled in many countries and are easily available, mainly through the Internet, have raised concern. These so-called '*legal highs*' are mainly constituted of cathinone derivatives and include mephedrone, methylone, methedrone, and buthylone, among others. Because they share similar structures and

mechanisms of action with the classical amphetamines, there are increasing numbers of case reports involving intoxications also with these drugs (Carvalho et al. 2012).

3. Evaluation of mixture effects in terms of additivity, antagonisms or synergisms – comparison of the accuracy of prediction models

As outlined above, the threshold levels of amphetamines capable of causing severe detrimental health effects in humans are controversial and widely discussed (Milroy 2011). Unfortunately, low-dose and, often, first time exposures to MDMA are recurrently associated with severe and life-threatening accidental intoxications. The incongruence between concentrations of drug found in the serum and/or tissues of intoxicated abusers and the toxic effects elicited by these drugs, are one of the main arguments in support of the hypothesis that other factors might be at play to justify the risk posed by amphetamines to humans.

Reported misuse settings show that abusers ingest multiple substances concurrently (Barrett et al. 2006; Mohamed et al. 2011; Wu et al. 2006a). Along with the intentional intake of several types of drugs (alcohol, tobacco, cannabis, other amphetamines, etc.), inadvertent consumption of multiple substances often occurs, for instance, due to contaminants frequently present in *ecstasy* tablets (Morefield et al. 2011; Pavlaki et al. 2011) or as a part of prescribed therapies. It is, therefore, conceivable that a number of these drugs may act together to produce effects of different magnitude to those induced by the individual drugs alone.

In fact, polydrug abuse is one of the most relevant, yet poorly understood factors in evaluating MDMA toxicity and has been pointed out as one of the putative justifications for the unpredictability of its effects. It is possible that the combination with other chemicals will exacerbate the severity and/or widen the range of the toxic effects of the drug, resulting in serious and potentially lethal intoxications (De Letter et al. 2006; Verschraagen et al. 2007).

Evaluating the effects of mixtures of MDMA and other substances is paramount in order to elucidate the realistic risks associated with *ecstasy* abuse. Notwithstanding, it has long been highlighted that there is still a lack of information regarding the toxicity and lethality of drugs in co-administration, as most efforts have been put into studying isolated, single substances. In recent years, some attempts to improve the understanding of mixture effects have been made (Clemens et al. 2005; Clemens et al. 2007b; Clemens et al. 2004; Dumont et al. 2009; Pontes et al. 2008b; van Nieuwenhuijzen et al. 2010). However, much of the published literature on the subject shows that the theoretical basis for mixture analysis is

poorly understood in the field (Pontes et al. 2008b; Schechter 1998). For this reason, many of the studies tend to be ill-conceived, and often lead to erroneous conclusions (Kortenkamp and Altenburger 1998). Some authors have fuelled the belief that synergisms between drugs need to be evoked to prove enhanced toxic risks associated with mixture exposures (Pontes et al. 2008b; Robledo et al. 2007; Schechter 1998). Although the effective demonstration of synergisms will unequivocally always heighten concerns about health risks, the possible implications of apparently less spectacular additive combination effects have not received adequate attention (Charles et al. 2002). In our opinion, the persistent seek for synergisms in an attempt to justify deleterious consequences is inadequate and unhelpful, and the lack of synergisms may lead many to assume that it indicates 'no risk', as 'mere' additive combinations are not relevant to the observed toxicity.

3.1. Defining additivity

From a realistic point of view, the direct testing of all the hypothetical combinations of drugs is unfeasible. Thus, predictive approaches that permit the mixture assessment are exceptionally important. Hence, in order to correctly assess combination effects of chemicals, it is crucial to formulate a hypothesis about the expected effect of the mixture. This hypothesis ('*null-hypothesis*') provides the necessary basis for evaluating mixture effects in terms of synergy or antagonism. An appropriate reference point for such analyses is the expectation that joint effects are the result of purely additive interactions of all mixture components (Berenbaum 1989; Kortenkamp 2007).

It is often understand that the effects of a mixture of drugs may be smaller or greater than the sum of the individual effects of all mixture components. However, from this premise it is often assumed that expectations of joint effects are obtainable by straightforwardly calculating the arithmetic sum of all individual effects. As recognized by Kortenkamp (2007), the fallacy of this assumption is evident: 'when we consider, for example, 10 agents, each of which provokes 15% of a certain response. The anticipation that the resulting joint effect should be 10 x 15 % = 150% turns out to be biologically impossible if the maximally inducible effect can only be 100%'. This misconception is based on the predictive model of *effect summation* (ES) and has been highlighted in several works of experts in the field of mixture toxicology (Berenbaum 1981; Kortenkamp and Altenburger 1998). In the ES model

the effects of the individual compounds are added up to predict the global effect of the mixture. Unfortunately, this approach is not valid with chemicals that produce sigmoidal concentration–response curves (as it is the case of the majority of chemicals) and is only applicable to linear concentration–response curves. The ES concept is all too often mistakenly used, leading to very erroneous conclusions. Such an example is a publication by Arnold *et al* (1996) in *Science*. The authors claimed to see strong synergistic effects with binary mixtures of weak estrogens. However, there were severe issues with their work, including the fact that they used the ES model to predict their additive effects. These strong mixture effects could not be repeated by other researchers, and when it was shown that the paper was incredibly flawed, it had to be retracted from the journal.

Consequently, reliable approaches are required to provide consistent computations of mixture effects, and those should be able to consider saturation effects at high effect levels, this way avoiding inconsistent expectations of supramaximal joint effects, such as in the example described above.

3.2. Models for calculating additivity expectations

The assessment of the combination effects in terms of synergy, antagonism and additivity, relies on the accurate prediction of the expected effects of a mixture. There is much debate on how additive effects should be calculated. All too often, mixture effects have been assessed without explicit reference to additive expectations, or even these expectations were inaccurately set (Arnold et al. 1997; Soto et al. 1994).

Despite a number of reviews on the topic have developed few methods to assess combination effects (Berenbaum 1985; Berenbaum 1989; Greco et al. 1995), the main adversity encountered when evaluating mixture effects based on individual responses of mixture components, relies on validating the most accurate model from the vast assortment of models seeking to precisely estimate these joint effects.

Thus, how can the expected effect of a mixture be defined?

3.2.1. Concentration addition (CA)

The first and most frequently acknowledged model to predict additive effects has been put forward by Loewe and Muischnek (1926) based on the work of Fraser (1872). This approach, known as concentration addition (CA) or *dose addition*, rests on the assumption that the components of a mixture act in a similar way, *i.e.* produce the same toxic effect in the same target organ via the same mechanism of action. So, if two agents share the same shape and slope for their concentration-response curves and differ only in potency, then it can be said that they act as a dilution of each other if combined together. Consequently, each compound in the mixture can be replaced by an equi-effective concentration of another, without diminishing the overall mixture effect. By implication, this means that every mixture component contributes to the overall combination effect in proportion to its concentration, even below zero effect levels. This model has been further developed and described by Berenbaum (1985; 1989) and have been widely accepted by a number of scientists within the field.

CA can be mathematically expressed by:

$$\sum_{i=1}^{n} = \frac{c_i}{EC_i} = 1 \qquad \textit{(equation 1)}$$

Where n is the number of mixture components, EC_i is the concentration of the mixture component i that provokes an effect E of known magnitude when applied singly, and ci is the concentration of the respective component in the mixture. Every quotient (c_i/EC_i), which has been termed *toxic unit* (Sprague 1970), gives the concentration of a compound in the mixture scaled for its relative toxicity. If the sum of each fraction equals 1, then CA holds true and the joint effect can then be called additive. The mixture components act as dilutions of the same compound.

Equation 1 can be applied to calculate a mixture concentration that produces a defined effect, when the EC_i of the individual mixture components and their relative abundance in the mixture are known. The concentration ci of agent i in the mixture is related to the total mixture concentration by:

$$c_i = p_i \times EC_{mix} \qquad \textit{(equation 2)}$$

Where p_i is the fraction of chemical i in the total mixture concentration EC_{mix} that is required to produce effect E. Substitution of ci in equation 2 gives:

$$\sum_{i=1}^{n} = \frac{(p_i \times EC_{mix})}{EC_i} \qquad \textit{(equation 3)}$$

And rearranging yields:

$$\sum_{i=1}^{n} = \frac{(p_i \times EC_{mix})}{EC_i} \qquad \textit{(equation 3)}$$

Equation 4 allows the calculation of the concentration EC_{mix} of a mixture that provokes an effect E of defined magnitude, provided we have knowledge of the mixture composition and of the concentration of each mixture component that, on its own, produces effect E. By repeating this calculation for a large range of different effects, it is possible to construct a concentration-response relationship that describes the expected additive effects of the mixture. The effect concentrations ECi of the single agents are calculated using the best-fit regression model inverse formulae of the concentration–response models of the single mixture components.

It is assumed that chemicals with the same molecular target site have similar slopes (Berenbaum 1989; Gennings et al. 2005; Greco et al. 1995). The importance of the assumptions of CA concerning both the degree of similarity of site of action and the similarity of slope has been debated since the introduction of the model (Berenbaum 1989; Greco et al. 1995; McCarty and Borgert 2006).

3.2.2. Independent Action (IA)

It is unanimous that CA is a suitable and valid concept for the prediction of mixture effects of similarly acting agents (Altenburger et al. 2000), whether it is applicable to mixtures of agents that show differing modes of action is the topic of a long-standing controversy (Berenbaum 1985) and is vehemently refuted by some experts (Pöch 1993).

The concept of independen action (IA) or *Bliss independence* was first developed by Bliss (1939) and has also been referred to as *joint independent action* (Plackett and Hewlett 1948), *response addition* and *effect multiplication* (Colby 1967). This concept, that has been successfully applied to the assessment of mixtures when the components produce the same toxic effect but via dissimilar mechanisms of action (Backhaus et al. 2000a; Backhaus et al. 2004a), is based on the idea of probabilistic independence (Greco et al. 1995). IA rests on the assumption that the effects of the individual mixture constituents are independent from one another, so each component of a mixture acts in a way that does not interfere with the others, but all contribute to the overall effect. Therefore, the relative effect of a chemical remains unchanged in the presence of another compound (Faust et al. 2001). For example, substance A acts in the presence of substance B as it would in the absence of substance B. This implies that the action of substance A is independent and not influenced by substance B.

This concept assumes that mixture effects are the result of interactions of individual mixture constituents with different targets of a biological system. Thus, chemicals that are present below 'zero effect' levels are not expected to contribute to the total mixture effect.

The model of IA allows calculating the predicted effects EC_{mix} of a mixture of known composition by using:

$$\mathrm{EC_{mix}} = 1 - \prod_{i=1}^{n} [1 - E(\mathrm{c_i})] \qquad \textit{(equation 5)}$$

Where, *E(ci)* is the effect *E* produced by compound *i* at concentration *c*, when applied singly. IA is a probabilistic model, *i.e.*, *E(ci)* is a fraction of a maximal possible effect that cannot exceed 1.

Thus, when applying this model to measures a toxic effects, $E_{tox}(ci)$, a maximal effect, *Emax*, must be defined as a reference point. The effects of test drugs are expressed relative to that effect maximal (*e.g.* 100% cell killing), and this can be represented by:

$$E(\mathrm{c_i}) = \frac{E_{\mathrm{tox}}(\mathrm{c_i})}{E_{\mathrm{max}}} \qquad \textit{(equation 6)}$$

If the concentration-response relationships of all mixture constituents *i* are described by an appropriate regression model *Mi*, the effect $E_{tox}(ci)$ can be estimated from the mean

effect *Mi* (ci) predicted by the regression model, and this modifies equation 6 in the following manner:

$$E_{\text{tox}}(c_i) = M_i(c_i) \quad \textit{(equation 7)} \qquad \text{and} \qquad E(c_i) = \frac{M_i(c_i)}{E_{\max}} \quad \textit{(equation 8)}$$

The overall fractional effects are given by substituting this back into equation 5 and gives:

$$EC_{\text{mix}} = 1 - \prod_{i=1}^{n}\left[1 - \frac{M_i(c_i)}{E_{\max}}\right] \qquad \textit{(equation 9)}$$

By multiplying the fractional effects (equation 9) by E_{max} we can compare the predicted effects with the CA predictions:

$$EC_{\text{mix}} = E_{\max}\left[1 - \prod_{i=1}^{n}\left[1 - \frac{M_i(c_i)}{E_{\max}}\right]\right] \qquad \textit{(equation 10)}$$

There are practical limitations on the accurate calculation of IA, as the effect *E(ci)* elicited by each individual mixture component at the concentration *ci* that it is present in the mixture (equation 5) must be known. This becomes more difficult with decreasing agent concentrations and decreasing single agent effects.

3.2.3. Discriminating between both models

In recent years, several attempts have been made to discriminate methodically between predictions derived from the two concepts of CA and IA (Altenburger et al. 2000 ; Backhaus et al. 2000a; Marques et al. 2012).

It is generally accepted that CA would produce valid predictions for the joint effects of drugs that interfere with one and the same target. Conversely, the concept of IA should prove to be unreliable in anticipating the joint actions of these agents, as it assumes that the different components of a mixture cause a common integral biological effect (*e.g.* cell death) through

interaction with different molecular target sites. Thus, based on the understanding of the assay system utilised and the concentration-response relationships obtained, it should be fairly straightforward to define the more adequate prediction model.

Notwithstanding, it often happens that CA and IA generate very identical expectations of additive combination effects, which cannot be distinguished from one another. Examples of this problem can be found in a number of reports. Payne and collaborators used these approaches to predict and assess the effect of mixtures of up to four environmental estrogens. They found that both models produced almost undistinguishable additivity predictions, when applied to mixtures of oestrogen receptor agonists (similarly acting agents) (Payne et al. 2000) and to combinations of mitogenic agents which were expected to have diverse modes of action (Payne et al. 2001). Takakura and co-workers (2012) investigated the cytotoxicity induced by two cocktails composed of two and five pesticides previously identified in the French diet, on human cell lines, using the neutral red uptake assay. Two fixed proportions of pesticides in the cocktails were used: an equimolar proportion and an exposure proportion based on consumption data. The two models of CA and IA gave almost identical concentration–response curves in all cases. Villa *et al.* (2012) tested on the bacterium *Vibrio fisheri* the responses to complex equitoxic mixtures of a high number of individual components (up to 84 individual chemicals in the mixtures) with different chemical structures and toxicological modes of action (narcotics, polar narcotics, herbicides, insecticides, fungicides). It is remarkable that neither CA or IA could be rejected in the case of 4 mixtures involving narcotics and polar narcotics alone, as well as in combination with pesticides, since the difference between the curves calculated with both models was negligible, even in the case of high number of chemicals. For pesticides alone and narcotics and pesticides mixture, the two predictions gave different results, but the difference was negligible. Also, Backhaus and colleagues (2004b) validated CA and IA to predict toxicities of phenylurea multicomponent mixtures, as the both concepts yielded identical mixture calculations. Scelfo *et al.* (2012) studied the effects of binary mixtures with both similar and dissimilarly-acting compounds on neuronal networks cultured on multielectrode array chips and verified that CA and IA were comparable in predicting additive mixture effects. Other examples for overlapping predictions are widely reported in the literature (Backhaus et al. 2004b; Cedergreen et al. 2007a; Huang et al. 2011; Ohlsson et al. 2010; Petersen and Tollefsen 2012).

Although the difference between the two predictive models is regularly slight or, as mentioned above, inexistent, CA often consistently predicts a higher toxicity effect than IA

(Altenburger et al. 2000 ; Backhaus et al. 2000a; Faust et al. 2003). According to Drescher and Boedeker (1995), the discrimination between predictions derived from *CA* and *IA* relies on the number of mixture components, their concentration ratio in the mixture, the slopes of their concentration-response functions and the regression model used to describe the relationships between concentrations and effects of single agents. It has been shown that, by increasing the number of individual mixture components, the separation between the two predictions will occur (Drescher and Boedeker 1995).

The idea that all mixture components should display parallel concentration-response curves to formulate a prediction based on CA was first discussed by Bliss (Bliss 1939). This however was dropped as a requisite following revisions made by Hewlett and Plackett (1959). The predictions based upon IA and CA have been shown to cope well with response curves of varying slopes and maximal effects (Altenburger et al. 2000 ; Berenbaum 1985; Rajapakse et al. 2002; Silva et al. 2002).

The slope of a concentration–response curve can only be defined either as the ratio between two EC-values (*e.g.* the EC_{50}/EC_{05} ratio) or in relation to a specific concentration–response model. Despite their mutually exclusive conceptual positions, it is accepted that *CA* and *IA* will compute identical toxicities if the concentration–response curves of all mixture components can be described by a Weibull-model with a common slope parameter of 1 (on a *ln*-transformed concentration axis) (Backhaus et al. 2004b; Brosche and Backhaus 2010). In this situation, an EC_{50}/EC_{05} ratio corresponds to 13.5. If the ratio for the concentration–response curves of the mixture components is lower (*i.e.* the steepness is higher), CA predicts a greater effect (*i.e.* a lower EC_{50}) for the mixture than IA and *vice versa* (Brosche and Backhaus 2010). Typically, EC_{50}/EC_{05} ratios seem to be substantially smaller than the critical threshold of 13.5 (Faust 2004; Smit et al. 2001) and consequently, CA frequently predicts a higher toxicity than IA in most situations, a pattern which seems to be confirmed by empirical evidence (Kortenkamp et al. 2009).

3.2.4. Applicability of CA and IA to studies with similarly and dissimilarly acting agents

The existence of both CA and IA as models for mixture risk assessment has brought about a great deal of discussion surrounding their appropriate applications. In the last decades, the reliability of both concepts has been widely evaluated.

From a theoretical point of view, it has often been argued that *CA* is a precise reference model for calculation of joint effects when compounds are known to have similar target sites. Arrhenius *et al.* (2004) evaluated mixtures of structurally related and similar-acting phenylurea herbicides on two types of marine microalgal communities. Faust *et al.* (2001) performed analyses with mixtures containing 18 s-triazine herbicidals at two different concentration ratios in freshwater algae. Also Junghans *et al*. (2003b) examined mixture toxicity of herbicides to aquatic systems and found that the mixture effects conformed more accurately to CA predictions. Parallel outcomes were obtained in several studies performed with mixtures of similar-acting components, in assorted fields (Cedergreen et al. 2007b; Hass et al. 2007; Svendsen et al. 2010). Tammer (2007) evaluated and predicted the combined effects of mycotoxin mixtures upon the functional activity of immune cells and observed that the experimental design using the concept of *CA* was suitable for predicting the combined effects of their tested mixtures. Backhaus *et al.* (2000b), proved that the mixture toxicity of quinolones is best predicted by CA. Also in the anticancer research context, much debate has centred on a single, appropriate model to predict combined drug effects (Berenbaum 1989; Greco et al. 1995). A combination of two taxanes, paclitaxel and cisplatin, has shown close support for CA (Levasseur et al. 1997), especially for predicting joint effects of similarly acting agents. Orton and her group (2012) demonstrated a good agreement between the effects of a mixture of eight 'pure' androgen receptor antagonists and the responses predicted by *CA*. A multi-component mixture composed of 16 similarly and specifically acting chemicals, anticipated to have a common mode of action via weak acid respiratory uncoupling of oxidative phosphorylation, demonstrated highly accurate mixture predictions based upon *CA* (Altenburger et al. 2000), and this was further supported by the work of Silva and colleagues (Silva et al. 2002) with endocrine disrupting agents. Xing *et al.* (2012) showed that the CA approach was an effective method for the calculation of toxicity of binary system mixtures of chlorophenols, commonly used as pesticides, herbicides, and disinfectants, to *Daphnia magna*. Finally, recent findings demonstrate that CA predicts well the toxicities of simple binary as well as a more complex 6-component mixture of crude naphthenic acids to fish liver cells (Tollefsen et al. 2012). All together these investigations show the potential of using the CA concept to obtain accurate predictions of joint mixture effects, especially in the case of similar-acting compounds.

CA has also been demonstrated as well suited in to predicting mixture toxicity of unclearly or differently acting substances/chemicals (Backhaus et al. 2004b; Evans et al. 2012; Zhang et al. 2010; Zhou et al. 2010) and generally it is the more conservative of the two

models (Altenburger et al. 1996; Faust 2004). This is very important when doing cumulative risk assessment. It has been reasoned that CA should be used for the assessment of every mixture which risk is being evaluated, independently of the mode of action of the single agents. It is argued that, even if the chemicals act dissimilarly and IA is more appropriately, CA would be more conservative predicting mixture effects for a lower concentration range and, therefore, more protective of the exposed population. If not much about the chemicals in the mixture is known is preferable to be safe than sorry. This point of view is being considered very seriously in the regulatory world.

However, the results for some mixture studies of chemicals with similar overall mode of action have also been equivocal (Loureiro et al. 2010; Marques et al. 2012). The reasoning for these discrepancies may rely on the ambiguity of the interpretation of the term 'similar action'. From a mechanistic point of view, 'similar action' has been defined to apply to agents with an identical binding site (Pöch 1993). 'Similar action' was also taken to mean that drugs can trigger the same toxicological response (Berenbaum 1989). Considering endpoints such as cell death, then this could include an extensive range of drugs. *CA* has, in fact, been suggested to be the 'general solution' to compute expected effects for any combination of agents without the need to refer to their mechanisms of action (Berenbaum 1985). Furthermore, the designations of site and mode of action are equivocal, as they are concentration dependent, with a small minority of drugs having one site or mode of action at low concentrations and others at higher concentrations (McCarty and Borgert 2006). Additionally, sites and modes of action are still undetermined for a multitude of drugs (Borgert 2004a; McCarty and Borgert 2006).

Conversely, experimental evidence from studies with algae (Backhaus et al. 2004a; Faust et al. 2003) and bacteria (Backhaus et al. 2000a), has shown that chemicals with specific and dissimilar modes of action are better described using *IA*. Work using the marine bacterium, *V. fischeri*, has shown that *IA* has excellent predictive power when combining fourteen chemicals of well-known differing mechanisms of action in bacteria (Backhaus et al. 2000a). The alternative concept of CA resulted in an overestimation of the mixture toxicity. This has further been supported by algal toxicity of sixteen dissimilarly acting biocides, where IA proved to be superior and more accurate in predicting combined toxic effects (Faust et al. 2003). Notwithstanding, theoretically, the applicability of IA is seriously questioned by those who argue that the combination of chemicals can never act independently due to the elevated level of integration of a living system (Gessner 1988).

Some authors reported controversial predictive trends, regarding the mechanism of action. Barata and collaborators (2006) showed that each model's predictive abilities changed across endpoints. They observed that *IA* concept was able to predict accurately the toxicities of binary combinations of dissimilarly acting chemicals (metals and pyrethroid insecticides) when measuring mortality of *Daphnia magna*, whereas the *CA* concept did so in three pairings for feeding behavior response, irrespective of the chemical mode of action. Cedergreen and co-authors (2008) compared the two models, *CA* and *IA* to determine which best described data for 158 binary mixtures of chemicals which acted in different sites of action (primarily pesticides and pharmaceuticals), when tested in seven different test systems. The analyses showed that approximately 29 of the mixtures were adequately predicted only by IA, 16 were adequately predicted only by CA, and both models could predict the outcome of another 34 combinations. Approximately half of the experiments (78) could not be described adequately by either of the two models. This level of predictability is rather low, considering that, in this situation (evaluation of joint toxicity of chemicals with a different molecular target site) *IA* allegedly should provide the better fit (Cedergreen et al. 2008). A comment on the state of the art of mixture toxicology for endocrine disrupters (an area where a large amount of mixture work has been carried out) offered an identical position, considering *CA* to be the best reference model even for differently acting chemicals, for a range of mammalian whole organism end points (Boekelheide 2007).

Summarizing, the *CA* concept has some theoretical underpinnings and some experimental data supporting its use for mixtures of chemicals with the same site of action. In contrast, *IA* is not as well grounded in theory, nor is it unambiguously supported by data. Despite *CA* being usually considered a somewhat conservative default approach for the predictive assessment of mixture toxicities in general (Berenbaum 1985), the toxicity of antibiotics to microbial communities might be an exception from this pattern, as the vast differences in sensitivity of the involved species seems to lead to extraordinary flat concentration–response curves (Brosche and Backhaus 2010). This is also supported by mixture studies with bacterial communities, in which *IA* systematically predicted higher mixture toxicities of antibiotics (Christensen et al. 2006). The results of many studies encourage the view that mixture toxicity outcomes on an organism level are way too complex to be predicted solely on the basis of the *a priori* knowledge of components mode of action. Secondary mechanisms of action, uptake kinetics, transportation, metabolism, compartmentalization, and excretion of the drugs are implicated and have possibly great impact on the joint effects (Borgert 2004a; Borgert et al. 2004; Cedergreen et al. 2008;

McCarty and Borgert 2006). Nevertheless, they are not considered by the predicting models. Large deviations from either of the reference models can, however, be used to form hypotheses in terms of types of interactions (Cedergreen et al. 2008). More complex, mechanistic approaches, which take into consideration toxicokinetics and toxicodynamics of the drugs would be more accurate. However, to date, there are no pragmatic and well-implemented alternatives. Taking the limited available information of most individual chemicals, the models CA and IA are, at the moment, the best option.

3.3. Relevance of synergisms and antagonisms

One of the main challenges in toxicology has been to develop approaches to address whether combinations of drugs will interact adversely, by either enhancing toxicity or diminishing efficacy. Plackett and Hewlett (1952) proposed that chemicals may basically behave in two ways: they can have a joint action (zero-interaction) or they can interact. In the first case they may act through the Bliss independence criterion, based on the *IA* concept, or through the Loewe additivity model, based on the *CA* concept, as aforesaid. Both are ‘‘non-interaction’’ models assuming that, in combination, drug effects are simply additive, and are neither infra- nor supra-additive. In the second case, the effects of the interaction may be antagonistic or synergistic.

Antagonism is largely defined as a type of ‘interaction’ in which compounds produce lower toxicity when combined than would be expected by their actions separately. Accordingly, greater amounts of drugs are needed to yield exactly the same effect when the substances are present together than would be predicted from their individual effects. Synergism is the converse, *i.e.*, higher effect produced, or less drug required, than predicted.

The concept ‘predicted’ is of utmost importance in these designations, as the classification demands a comparison of the observed effect with the effect predicted for the mixture. As the interactions (synergism and antagonism) cannot be directly tested, they are deduced from experimental results that deviate from a model of non-interaction.

One of the main motivations for mixture evaluation is to address the concern that hazard expectations for single drugs might grossly undervalue toxicity due to the potential for synergism between mixture components. Indeed, the anticipation of synergism has been used to raise fears about toxicity of a range of distinct mixtures. Concerns apart, a synergistic

interaction can also be particularly convenient when it confers therapeutic advantages. For instance, in an effort to seek for therapeutically beneficial synergistic combinations, pharmaceutical and pesticide industries have spent great scientific, professional and financial resources. Unfortunately, these efforts have been deeply unsuccessful, as synergisms have not been broadly identified. Contrary to synergisms, pharmacological addition and toxicological antagonism are among the more common means of conferring therapeutic advantage (Berenbaum 1988). An argument supporting that synergisms are probably rare in the environment, is the fact that interactions have been mathematically shown to be more likely to occur in the mid-range of the concentration-response curve than at high or low extremes (Berenbaum 1989). But despite pharmaceuticals being used in biologically active concentrations (contrary to environmental contaminants often present at concentrations below the observable effect range), pharmacologists and toxicologists have to date, found only a few biologically significant synergistic interactions.

3.4.Characterising individual and joint toxicities

Human populations are inexorably exposed to a virtually infinite number of complex mixtures, albeit the human health risk exposure is currently assessed on the basis of toxicity data on individual agents. The ability to employ the previous concepts to compute the overall joint effects depends on the knowledge of the precise response profile of single components and on the exact composition of the tested mixture.

3.4.1. Selection of the assay system

For the prediction of accurate and thorough concentration-response relationship at a given mixture ratio, information about the concentration-response curves of all single drugs is required (Backhaus et al. 2000a; Berenbaum 1989).

Recognisably, to ensure greater precision of these predictions, high reproducibility in the assay of choice is demanded. This has to be able to produce large amounts of highly reliable data for every component of the mixture, in a relatively short period of time.

In vivo assays are, obviously, regarded as the '*gold standards*' in the appraisal of drugs toxicity, as these systems incorporate all drug pharmacokinetic aspects including absorption, metabolism, distribution and excretion. For this reason, *in vivo* confirmation of the *in vitro* actions is generally required before any considerations about an agent's effects. Nevertheless, animal testing for toxicity identification is a costly, time-consuming and labour intensive approach, and is not pragmatic for hazard identification of large numbers of chemicals. Moreover, most governments have increasingly restrictive policies on the use of animal testing, so it is minimised when possible. Thus, alternative methods that are rapid, sensitive, reproducible, have higher throughput ability and are predictive of *in vivo* effects are needed (Coecke et al. 2007; Lilienblum et al. 2008).

3.4.2. Composition of the mixture

The careful selection of each component's concentration in the mixture avoids disproportionate contribution of a single agent to the overall mixture effect. The so-called *fixed ratio design* (Altenburger et al. 2000 ; Backhaus et al. 2000a) has been developed to address this issue. In it, each component of the mixture is mixed at a fixed mixture ratio such as concentrations that produce *x%* of the maximum effect. An approach such as this is particularly well suited for analyzing multiple mixtures by employing the models of *CA* and *IA,* as it ensures that all individual components contribute equally to the overall mixture effect and no single agent overpowers the effect of the others. In other words, it ensures that the more potent components are present at lower concentrations in the mixture than less potent agents. It is also appropriate for proof of principle and to identify which model best describe the mixture effects being studied. Conversely, when studying more realistic mixtures, chemicals are seldom combined at equieffective concentrations. The frequently used alternative approach of varying the concentration of one agent while keeping the others fixed leads to complications in the computation of expected responses, because mixture ratios change continuously. As will be discussed later, this approach is well suited to characterize potential interactions between chemicals and identify additive, synergistic or antagonistic effects. As described in Chapter I, we have deviated from the *fixed mixture ratio design* when studying the potential interaction of amphetamines that could be present as contaminants in *ecstasy* pills with MDMA.

3.4.3. Modelling individual and mixture toxicities: nonlinear regression analysis

All single agents and mixtures should be extensively tested using a wide range of several different concentrations, each with a variable set of replicates run in parallel, depending on the variability of the assay. The most suitable concentration-response relationship for each drug is often selected from a number of non-linear regression models using a best-fit approach, as described by Scholze and colleagues (2001). Here, a number of different models are fitted to the data and the one that best describes the observed effects is selected (best-fit criterion). Some of the regression models used for describing sigmoidal concentration-response curves of single and combined agents are listed in Table 8.

Table 8. Regression models used to calculate the concentration–response curves.

Regression model (acronym)	Function (f)[a]
Logit	$f(x)= \theta_{min} + (\theta_{max} - \theta_{min})/(1+\exp(-\theta_1-\theta_2*\log(x)))$
Generalized Logit I (GLI)	$f(x)= \theta_{max} *1/(1+\exp(-\theta_1- \theta_2*\log10(x)))^{\wedge} \theta_3$
Generalized Logit II (GLII)	$f(x)= \theta_{max} * (1-1/(1+\exp(\theta_1+ \theta_2*\log10(x)))^{\wedge} \theta_3)$
Gompertz	$f(x)= \theta_{max} * (1-\exp(-\exp(\theta_{min} + \theta_{max} *(x))))$
Probit	$f(x) = \theta_{max} *$ PAREA$(\theta_1+ \theta_2*(x))$
Langmuir	$f(x)= 1/(\theta_1+ \theta_2* x)^{\wedge}(- \theta_3-1)$
Logit with Box-Cox transformation	$f(x)=$ tetmax $* 1/(1+\exp(-$tet1$- \theta_2*(x ^{\wedge} \theta_3-1)/ \theta_3))$
Gompertz with Box-Cox transformation	$f(x)=$ tetmax $* (1-\exp(-\exp($tet1$+ \theta_2*(x ^{\wedge} \theta_3-1)/ \theta_3)))$
Hill	$f(x)= \theta_{min} + (\theta_{max} - \theta_{min})/((1+ x /EC_{50}) \exp(-\theta_2)$
Weibull	$f(x)=\exp(-\exp(\theta_1+\theta_2\log_{10} x))$

[a] **Where θ_{min} and θ_{max} are the minimal and maximal observed effects, respectively; x is the concentration of the test agent; θ_1 is the parameter for location; θ_2 is the slope parameter; θ_3 describes the degree of asymmetry around the EC_{50} and PAREA is the area under the normal probability distribution curve from infinity to x.**

Once dose-response relationships are obtained for each of the single agents and suitable fits established, the parameters from the fitted concentration-response curves of all single components are used to model the responses of well-defined mixtures using the two concepts for mixture analysis as described before, *IA* and *CA*.

3.5. Low-Dose Mixture Effects

The relevance of mixture effects at extremely low levels of individual components may be controversial. From a theoretically perspective, it is clear that the significance of 'low-effect'

concentrations is different, depending on the concept employed. Under the assumption of *CA*, there is no threshold concentration other than zero itself as any concentration, no matter how low it is, of any mixture constituent is expected to contribute to the overall mixture effect in an additive fashion. In contrast, *IA* admits that only those drugs that are present at effective concentrations, *i.e.*, that cause individual effects greater than zero, will contribute to the whole joint effect. By other words, *IA* would predict a mixture effect of zero when all mixture constituents are present below the threshold concentration, whereas *CA* could predict a supra-threshold response. Hence, the predicted joint effect of components at low effect concentrations will vary, depending on the value of the estimated 'zero effect' concentrations. Clearly, an exact estimation of low effect concentrations, particularly that 'zero effect' levels for *IA*, are required to carry out consistent mixture assessments when drugs are combined at low or very low amounts.

3.5.1. Calculating low effect levels

Recurrently 'zero effect' level, or *no effect concentration* (NEC), is defined as the highest tested concentration that produces no effect. In toxicity tests performed on animals, using a limited and well-defined number of test individuals, NEC is the concentration that would not lead to the observed end point (*e.g.* death) in any of the individuals tested (Villa et al. 2012). This definition is not feasible for tests with microorganisms (algae, bacteria), where a functional parameter of the population (growth, luminescence, etc.) is measured. Therefore, a NEC may be defined as the concentration that would not produce a response different from the control (Villa et al. 2012).

It is well established that it is impossible to ascertain NECs with confidence, because genuine 'zero effects' cannot be distinguished from very small, albeit unnoticeable responses. The statistical approach most commonly used to deal with this uncertainty is hypothesis testing in which treatment responses are compared with negative controls to test the *null hypothesis* that they are drawn from the same population. If the *null hypothesis* is rejected, a multiple comparison test (*e.g.* Dunnett, Newman-Keuls, or Tukey test) is used to generate a *no observed effect concentration* (NOEC). Thus, a NOEC value is derived from experimental data by applying statistical hypothesis testing procedures to distinguish between the highest

tested concentration at and below which the effect is not significantly different from untreated controls (Van der Hoeven 1997).

NOEC is a quite controversial concept and its use as the basis for estimating 'zero effects' has been severely criticised (Chapman 1996). It has been shown that what is estimated as a NOEC depends to a large degree on the biological variability of the test system and the number and spacing of test concentrations (Moore 1997). Often, effects below 10% of a maximal possible response cannot be distinguished reliably from the effects seen in untreated controls (Moore 1997). NOECs tend to increase the fewer concentrations tested and the larger the spacing between each concentration (Moore 1997). Thus, one of the major criticisms on the use of NOECs is the fact that this can lead to false negative results, as NOECs are highly dependent on the quality of the data being analysed. There is a high risk of overlooking real low dose effects, if the data is poorly distributed (Kortenkamp and Altenburger 1998). Furthermore, most of the information in the concentration-response curves (*e.g.* the slope and confidence intervals) is lost and cannot be used, for example, to estimate risks of different severity. In summary, NOECs define a range of concentrations where low effects can neither be quantified nor ruled out with certainty.

The so-called benchmark concentrations (EC_x) are increasingly viewed as an alternative to NOEC determinations (Chapman 1996; Faust et al. 2001; Van der Hoeven 1997). These are estimated by interpolation from the best-fitting regression model equation of the individual observed effects (Moore 1997). Unlike NOECs, which are derived from comparisons of only two concentrations ('treated' *versus* 'controls'), the benchmark concentration procedure utilizes the information contained in the entire concentration-response curve. Moreover, one can confidently interpolate effects to untested concentrations.

3.5.2. Combining substances at very low concentrations

Risk assessment authorities provide threshold doses or concentrations of regulatory concern such as *acceptable daily intakes* (ADI) or *predicted no effect concentrations* (PNECs) for individual chemicals that are based on reference points (*no observed adverse effect levels*, NOAELs, NOECs, or benchmark doses). Exposures below these levels are usually considered safe. But is there adequate protection also against joint exposures, if each component is present below its individual threshold doses or concentrations?

The data reported in the literature provide evidence that low levels of individual drugs, far below concentrations traditionally assumed as NOECs, are suitable to contribute to the toxicity of the mixture, particularly those comprising a higher number of individual components. Silva *et al.* (2002) found significant mixture effects, when eight xenoestrogens were mixed together at levels well below their NOECs and EC_{01}. Villa and collaborators (Villa et al. 2012) described that an effect of 50% had been obtained by testing a mixture composed of 84 chemicals, in which concentrations of individual chemicals were orders of magnitude lower than the traditional NOEC. Actually, for about 90% of the individual substances the concentration was below EC_{01}. The results of these and other investigations (Faust et al. 2001; Junghans et al. 2003a) are corroborated by the theoretical assumptions of the CA model, as the concentrations of the chemicals, however low they are, are accounted for the overall mixture effect.

Notwithstanding, Backhaus *et al* (2011) have shown clear mixture effects when five distinct pharmaceuticals and personal care products were present at their individual NOECs (an effect accurately predicted by *IA*) highlighting the fact that, even with respect to mixtures of chemically and functionally dissimilar compounds, possible mixture effects from low-effect concentrations of individual compounds must be taken into consideration.

In summary, albeit the traditional approaches may be suitable to signpost a level at which a drug is considered more or less ‘safe’, evidence supports that this may not be a real ‘zero effect’ concentration, if the drugs are combined in complex mixtures.

PART II – OBJECTIVES OF THE THESIS

From the information previously presented in the introduction section it can be concluded that the effects caused by the ingestion of an *ecstasy* pill may be influenced by a plethora of factors that converge in a given individual at a given moment, thus complicating the evaluation of the mechanisms associated with the toxic effects of the drug.

Of particular concern are the interactions that may arise between MDMA and other drugs, intentionally or accidentally co-consumed, which can be potentially lethal. Polydrug abuse is, undoubtedly, one of the most relevant confounding factors in determining the toxicological potential of MDMA and several studies support the evidence that polydrug abuse is of serious consequence for the development of toxicity upon exposure to MDMA (Cole and Sumnall 2003; Schifano 2004; Schifano et al. 1998).

From a clinical pharmacokinetic perspective, amphetamine-type drugs share many of their pharmacological and toxicological effects, as well as the same metabolic bioactivation pathways. It is, therefore, reasonable to anticipate that the co-occurrence of these stimulants could result in toxicities that differ from those observed when they are present individually. Whilst the risk of interaction between *ecstasy* and other drugs, including amphetamine-like stimulants, have been clearly pointed out, a lack of systematic information regarding the toxicity and lethality of drugs in simultaneous administration in the context of *ecstasy* abuse has been noticed.

This project aimed at increasing the understanding of the toxicological interactions between MDMA and other amphetamine-type drugs. More specifically, the purposes of this work were:

1. To assess whether the joint cytotoxic effects of mixtures of amphetamine-like substances could be accurately predicted based on the concentration-response data of the individual components, using the models of CA and IA.

2. To classify the mixtures as additive, synergistic or antagonistic by comparing experimental observations with the additivity expectations according to both models.

3. To determine if there were significant combination effects, when the individual components were present in the mixture at low, 'realistic' concentrations, even when these did not produce measurable effects when tested individually.

4. To improve the understanding of the mechanisms of interaction between the components of the mixture, especially if deviations from additivity were observed. Due to their recognized influence on the toxicity of amphetaminic drugs, the contributions of hyperthermia and metabolic bioactivation were specially considered.

PART III – ORIGINAL RESEARCH

This section presents a detailed description of the experimental protocols, the results and their statistical treatment, as well as the discussion of the data within each experimental work, in the form of the publications that were originated or the manuscripts that were submitted for publication in peer-reviewed international journals listed in the Journal Citation Reports of the ISI Web of Knowledge. Five different studies are presented:

Chapter I. The risky cocktail: what combination effects can we expect between *ecstasy* and other amphetamines? Reprinted from *Archives of toxicology* **87**(1)**:** 111-122 (doi: 10.1007/s00204-012-0929-9). Copyright (2012) with kind permission of Springer-Verlag.

Chapter II. Combination effects of amphetamines under hyperthermia – The role played by oxidative stress. *S*ubmitted to *Journal of applied toxicology*.

Chapter III. Cytotoxic effects of amphetamine mixtures in primary hepatocytes are severely aggravated under hyperthermic conditions. *S*ubmitted to *Toxicology in vitro*.

Chapter IV. Mixtures of MDMA and its major human metabolites induce significant toxicity to liver cells when combined at low, ineffective concentrations. *Journal of applied toxicology*. In press. Copyright (2013) with kind permission of Wiley-Blackwell.

Chapter V. A mechanistic insight into the hepatocellular death induced by amphetamines, individually and in combination – The involvement of necrosis and apoptosis. *S*ubmitted to *Archives of toxicology.*

Chapter I. The risky cocktail: what combination effects can we expect between *ecstasy* and other amphetamines?

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TOXICOKINETICS AND METABOLISM

The risky cocktail: what combination effects can we expect between *ecstasy* and other amphetamines?

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Abstract The recreational and illicit use of amphetaminic designer compounds, specially 3,4-methylenedioxymethamphetamine (MDMA; *Ecstasy*), is of concern worldwide. Such psychostimulating drugs are frequently present as complex mixtures in 'rave' pills, making concomitant polysubstance use a common trend. However, the understanding of possible combination effects with these substances is still scarce. The present study was aimed at predicting the cytotoxic effects of mixtures of four amphetaminic derivatives: MDMA, methamphetamine, 4-methylthioamphetamine and d-amphetamine in a human hepatoma cell line. Concentration–response curves for all single-mixture components were recorded by the MTT assay. Data obtained for individual agents were then used to compute the additivity expectations for mixtures of definite composition, using the pharmacological models of *concentration addition* (CA) and *independent action*. By comparing the predicted calculations with the experimentally observed effects, we concluded that CA accurately predicts the combination of amphetamines, which act together to generate additive effects over a large range of concentrations. Notably, we observed substantial mixture effects even when each drug was present at low concentrations, which individually produced unnoticeable effects. Nonetheless, for all tested mixtures, a small deviation from additivity was observed towards higher concentrations, particularly at high effect levels. A possible metabolic interaction, which could explain such deviation, was investigated, and it was observed that at higher mixture concentrations increased MDMA metabolism could be contributing to divergences from additivity. In conclusion, the present work clearly demonstrates that potentially harmful interactions among amphetaminic drugs are expected when these drugs are taken concomitantly.

Keywords 3,4-methylenedioxymethamphetamine (*ecstasy*, MDMA) · Amphetamine-related toxicity · Hepatocytes · Combination effects · *Concentration addition* (CA) · *Independent action* (IA)

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Introduction

Amphetamine designer drugs are widely abused addictive psychostimulants. 3,4-methylenedioxymethamphetamine (MDMA), commonly known as *ecstasy*, is the most popular analogue and its use has increased in all social settings, all over the world. As a result, *ecstasy* has often been associated with toxic episodes, including fulminant hyperthermia, disseminated intravascular coagulation, rhabdomyolysis and multi-organ failure (Walubo and Seger 1999).

Reported drug abuse scenarios show that it is common practice among misusers to consume multiple substances

concomitantly (Barrett et al. 2006; Wu et al. 2006; Mohamed et al. 2011). In addition to the deliberate intake of different types of drugs by the users, inadvertent consumption of multiple substances often occurs, as large number of other chemicals are regularly found in *ecstasy* party pills (Pavlic et al. 2010; Morefield et al. 2011), including lysergic acid diethylamide (LSD), dextroamphetamine (d-AMP), methamphetamine (METH), ketamine, mephedrone, cocaine and even the highly toxic 4-methylthioamphetamine (4-MTA), which has been linked to several fatalities (Elliott 2000; De Letter et al. 2001). In fact, polydrug abuse is one of the most pertinent confounding factors in predicting MDMA toxicity, since the combination with other chemicals can exacerbate the severity or widen the range of the toxic effects of this drug, resulting in potentially lethal intoxications (De Letter et al. 2006; Verschraagen et al. 2007). Nevertheless, when evaluating MDMA toxicity, most studies focus on MDMA alone rather than in combination with other substances. Moreover, the few combination studies reported so far between MDMA and other psychoactive drugs have been conducted without reference to the expected joint effects (Clemens et al. 2005; Pontes et al. 2008). Consequently, whilst the risk of interaction between MDMA and other stimulants has been widely acknowledged, there is still a lack of information regarding the toxicity and lethality of drugs in co-administration. To define the way in which amphetamine-like substances interact may represent an important improvement for understanding their toxicity mechanisms.

Over the last decades, several studies on mixture toxicology have compared two well-established models for the calculation of expected additive mixture effects (Drescher and Boedeker 1995; Payne et al. 2000; Rajapakse et al. 2001; Silva et al. 2002; Pavlaki et al. 2011): *concentration addition* (CA), first defined by Loewe and Muchnik (1926), and *independent action* (IA) as described by Bliss (1939). The concept of CA is based on the assumption that the mixture constituents have similar modes of action, which means that any component can be replaced partially or totally with another without changing the overall mixture effect. This means that each individual component contributes to the global joint effect by acting in proportion to its concentration, even below concentrations producing no effect. This model has been used to assess combination effects of agents with a common site of action (Backhaus et al. 2000b; Silva et al. 2011a). Experimental evidence from some studies showed that combination effects of drugs with dissimilar mechanisms of action are better described using the alternative approach, that is, IA, which considers each agent interacting at differing sites of action (Backhaus et al. 2000a). The fractional response of one individual component is supposed to be independent from those induced by other components, presuming that mixture components present at zero effect concentrations will not contribute to the overall effect.

The two models can produce very distinct expectations and, to our knowledge, have never been applied to amphetamine-like compounds. For this reason, the main aim of this study was to compare the applicability of CA and IA models in predicting the joint toxic effects of amphetaminic drugs in immortalized hepatoma Hep G2 cells, based on comprehensive information on the individual drugs. In addition to MDMA, the amphetamines d-AMP, METH and 4-MTA were selected for this study, due to their widespread presence in *ecstasy* pills. As amphetamine derivatives are often found as low-level contaminants in street drugs offered as *ecstasy* (Becker et al. 2003), we were also interested in investigating the potential for significant joint effects to occur, even when these individual components were combined at low concentrations, representative of real exposure scenarios. In order to address these questions, three specifically designed mixtures of the same four amphetamines, but combined at three different ratios, were tested. With these, we were able to compare the applicability of the described prediction models and evaluate amphetamine interactions in relevant exposure situations. Being able to accurately predict and study combination effects of amphetamines will improve the understanding of potential chemical interactions when simultaneous consumption occurs, as well as provide some potential insight into the reasons behind random occurrence of extreme toxicity and even fatalities after consumption of *ecstasy* pills, when this per se are not associated with high rates of mortality.

Materials and methods

Chemicals

All reagents were of analytical grade or of the highest grade available. Minimum Essential Medium Alpha (MEM Alpha) with GlutMAX, foetal bovine serum (FBS), 0.05 % trypsin/1 mM EDTA, antibiotic (5,000 U/ml penicillin, 5,000 µg/ml streptomycin), fungizone (250 µg/ml amphotericin B), human transferrin (4 mg/ml) and Hanks balanced salt solution (HBSS) without Ca or Mg were purchased from Invitrogen Corporations (Paisley, UK). 4-MTA (HCl salt) was synthesized at REQUIMTE/Toxicology Laboratory, Biological Sciences Department of Faculty of Pharmacy, University of Porto. MDMA (HCl salt) was extracted and purified from high purity MDMA tablets that were provided by the Portuguese Criminal Police Department. The obtained salts were purified and fully characterized by nuclear magnetic resonance (NMR)

and mass spectrometry (MS) methodologies. d-AMP sulphate was generously provided by Dr Frederico Pereira (IBILI, Faculty of Medicine, University of Coimbra, Portugal), and 4-hydroxy-3-methoxymethamphetamine (HMMA, 3-*O*-Me-N-Me-α-MeDA) and 4-hydroxy-3-methoxyamphetamine (HMA, 3-*O*-Me-α-MeDA) were synthesized at the Chemistry Department, Faculty of Science and Technology, University Nova de Lisboa, Portugal, following a previously described procedure (Capela et al. 2006). 1 cc (30 mg) OASIS MCX SPE extraction cartridges were purchased from Waters (Lisbon, Portugal). Trifluoroacetic anhydride (TFAA), 4-hydroxy-3-methoxybenzylamine hydrochloride, Type HP-2β-glucuronidase from *Helix pomatia*, 4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT), Triton X-100, 3,4-methylenedioxyamphetamine (MDA) and (+)-METH hydrochloride (98 % purity) were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Sodium acetate, *n*-hexane, ethyl acetate, ammonium hydroxide, methanol, dimethyl sulfoxide (DMSO), ethanol and all other chemicals were purchased from Merck (VWR, Leicestershire, UK).

All amphetamines were used as supplied and stock solutions made up in deionized sterile water. Stock solutions were at least 20 times more concentrated than the highest concentration tested, in order to prevent media dilution. Subsequent dilutions were freshly prepared before each experiment. All solutions were stored at −20 °C.

Hep G2 routine cell culture

As the liver is known to be one of the main targets for amphetaminic toxicity in humans (Carvalho et al. 2010), the immortalized human hepatoma cell line Hep G2 was chosen for the cytotoxicity studies. These cells have been widely used to assess the chemical liver toxicity (Chen and Cederbaum 1997; Tang et al. 2012) and were, therefore, considered an appropriate model for the work described here.

Hep G2 cells were kindly provided by Dr Maryam Modarai from UCL School of Pharmacy, London, UK. Cells were routinely cultured in 75 cm^2 flasks in MEM alpha medium supplemented with 10 % heat-inactivated FBS, 1 % antibiotic, 1 % fungizone and 6 μg/ml transferrin (complete culture medium) and maintained in a humidified atmosphere of 5 % CO_2 at 37 °C. The medium was changed every 4 days. When cells reached 80 % confluence, cells were detached by trypsinization and subcultured over a maximum of 10 passages. Hep G2 cells were routinely tested for mycoplasma contamination.

MTT reduction assay

To produce reliable additivity expectations, concentration–response relations of the individual mixture constituents had to be accurately recorded, which required the use of a reproducible and robust system, enabling high throughput, with minimum variability. To meet those requirements, the cytotoxic effects of the amphetamine-like drugs were determined using the MTT reduction assay, which measures succinate dehydrogenase activity, an indicator of metabolically active mitochondria and, therefore, an indicator of cell viability. A previously described protocol (Silva et al. 2011b) was adopted and optimized to a 96-well plate format (Falcon; BD Biosciences, Oxford, UK).

Briefly, Hep G2 cells were seeded onto the central 60 wells of 96-well plates, at a density of 100,000 cells per well, in a volume of 200 μl of complete culture medium, to obtain confluent monolayers within 2 days. Peripheral wells on the plate were filled with sterile water. On the day of the experiment, the media was gently aspirated and the cells exposed to MDMA, METH, 4-MTA, d-AMP or mixture solutions in fresh cell culture medium, for 48 h. Each individual plate also included six replicates of negative controls (i.e. no test agents) and six replicates of positive controls (culture media containing 1 %Triton X-100).

At the selected time point, the culture medium was aspirated and frozen at −80 °C for future metabolic profile analysis. The attached cells were rinsed with 200 μl HBSS, followed by the addition of fresh culture medium containing 0.25 mg/l MTT and incubation at 37 °C in a humidified 5 % CO_2 atmosphere for 30 min. The formed intracellular formazan crystals were then dissolved in 100 μl 100 % DMSO and absorbance measured at 570 nm, using a multi-well plate reader (Labsystems Multiskan, Basingstoke, UK). To reduce inter-experimental variability, data were normalized on a plate-by-plate basis and scaled between 0 % (negative controls) and 100 % effect (positive controls). Results were graphically presented as percentage of cell death versus concentration (mM).

All individual compounds were tested in nine independent experiments, run on up to two plates per experiment, with each plate containing eight increasing concentrations of the test chemical in triplicates.

Mixture testing

In this work, three mixtures containing the same four selected amphetamines but combined in different ratios were tested. In mixture A, all chemicals were combined at their EC_{50}, such that they were present at concentrations that produced the same effect, that is, individual compounds were combined at equipotent concentrations. For this, a master solution of mixture A was prepared containing the individual components at the concentrations presented in Table 1 (corresponding to their individual EC_{50}) and a range of concentrations for testing was subsequently prepared by

Table 1 Parameters for the test amphetaminic agents in the MTT reduction assay

	Estimated parameters for the best-fit regression model of each individual agent					EC_{50} (mM)	EC_{01} (mM)	Relative proportion (%)	
	Regression model	$\theta 1$	$\theta 2$	$\theta 3$	θmax			Mixture A	Mixture B
MDMA	Logit	−1.616E+00	4.644E+00	–	1.019E+02	2.228E+00	2.283E−01	20.94	20.93
METH	Logit	−3.328E+00	4.618E+00	–	1.129E+02	5.256E+00	5.316E−01	49.39	48.75
4-MTA	GL-1	−3.622E+01	4.338E+02	7.471E − 03	9.837E+01	7.407E−01	4.598E−02	6.96	4.22
d-AMP	GL-1	−4.182E+01	7.635E+01	5.516E − 02	9.944E+01	2.416E+00	2.847E−01	22.71	26.10
Mixture A	Logit	−2.360E+00	4.477E+00	–	1.167E+02	2.90E+00	2.924E−01	100	–
Mixture C	Logit	−1.652E+00	4.610E+00	–	1.131E+02	2.03E+00	2.161E−01	–	–

employing the *fixed mixture ratio design*, as described by Altenburger et al. (2000) and Backhaus et al. (2000a). Briefly, the master stock was serially diluted maintaining the ratio between each constituent unchanged. Serial dilutions covered a wide range of concentrations; so that a complete concentration–response relationship could be recorded. Mixture B was prepared in a similar way, but this time, the four-mixture components were combined at their individual EC_{01} (individual concentrations presented in Table 1). A serial dilution of this mixture covering a wide range of concentrations was also prepared using the *fixed mixture ratio design*.

The final mixture, mixture C, was prepared by fixing the concentration of MDMA at 0.5 mM and modifying the remaining components over a wide range of concentrations. In order to achieve this, a 2× concentrated master stock solution containing METH, 4-MTA and d-AMP in equal proportions was prepared following the *fixed mixture ratio design* and then serially diluted in a large range of concentrations, as described above. This ensures the ratio between the three components is kept constant. An equal volume of 1 mM MDMA was then added to each three-component mixture concentration previously prepared, in order to obtain a final concentration of MDMA of 0.5 mM in all tested concentrations of mixture C.

In other words, for all concentrations of the four-component mixture tested, the concentration of MDMA remained 0.5 mM, whereas the concentrations of the remaining three amphetamines increased throughout the tested range, which means that the ratio between the four amphetamines differs for each mixture concentration tested.

Calculation of predicted mixture effects

Based on the complete concentration–response curves of the single agents, the overall effect of each mixture with defined composition was predicted applying both CA and IA models, as described in Payne et al. (2000).

Determination of MDMA and metabolites (MDA, HMA, HMMA), METH, 4-MTA and d-AMP by GC/MS

MDMA, METH, 4-MTA and d-AMP, as well as the MDMA metabolites MDA, HMA and HMMA, were quantified in the extracellular media and in the cellular content after cell cleavage by adding 200 µl water with overnight incubation at 0 °C. For METH, 4-MTA and d-AMP, a qualitative analysis was carried out, where the chromatograms of individual and mixture samples were compared to the chromatograms of controls and the presence of other molecules (including metabolites) was not detected. For this reason, further quantitative analysis was not performed for these substances. The GC/MS determination was carried out as previously described (da Silva et al. 2010; Pontes et al. 2010).

Quantitative GC/MS analysis was performed with a Varian CP-3800 gas chromatograph (USA) equipped with a VARIAN Saturn 4000 Ion Trap (IT) mass selective detector (USA) and a Saturn GC/IT-MS workstation software version 6.8. The capillary column VF-5 ms (30 m × 0.25 mm × 0.25 m) was from VARIAN. The gas chromatography was conducted with high purity helium C-60 (Gasin, Portugal) at a constant flow of 1 ml/min with a 1:30 split ratio. A CombiPAL automatic autosampler (Varian, Palo Alto, CA) equipped with a 10-µl liquid syringe was used for all analysis. 2 µl of sample was injected into the system, in splitless mode. The injection port was at 220 °C. An initial column temperature of 100 °C was held for 1 min, followed by a ramp of 15 °C/min to 300 °C, with a 10 min post-run hold. The injection port temperature was maintained at 250 °C. Total chromatographic separation was achieved in 9 min. The IT detector was set as follows: the transfer line, manifold, and trap temperatures were 280, 50 and 180 °C, respectively. All mass spectra were acquired in the electron impact (EI) mode. To avoid solvent overloading, ionization was maintained off during

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the first 4 min. The mass range was 50–600 m/z, with a scan rate of 6 scan/s. The emission current was 50 A, and the electron multiplier was set in relative mode to autotune the procedure. The maximum ionization time was 25,000 s, with an ionization storage level of 35 m/z.

The data analysis was performed in full scan mode, and the chromatograms were reprocessed by selecting the characteristic ions for each molecule. The selected ions were as follows: IS m/z = 232 and m/z = 345; MDMA and MDA m/z = 135 and m/z = 162; HMMA, m/z = 154 and m/z = 260; HMA m/z = 140 and m/z = 260; METH m/z = 118, m/z = 154 and m/z = 246; 4-MTA m/z = 137, m/z = 164 and m/z = 277; d-AMP m/z = 118, m/z = 140 and m/z = 232. Standard curves were plotted for each compound. Linearity, precision, accuracy and recovery were all within the accepted values for these parameters (da Silva et al. 2010).

Regression modelling and statistical analysis

Nonlinear regression analysis of all 4 amphetamines, individually or in mixture, was carried out using a best-fit approach as described by Scholze et al. (2001). The cytotoxicity data obtained with the MTT reduction assay (% cell death) were fitted to appropriate dosimetric models (Gompertz, Logit, Probit, Weibull, Langmuir, General Logit I and II) by using the specialized software program NLREG—Nonlinear Regression, version 5.4 (Phillip H. Sherrod, USA). All of the nonlinear regression models describe sigmoidal concentration–response relationships. A suitable best-fit model was selected based on a statistical goodness-of-fit principle, after independently fitting each equation to the same data set (Table 1), and the results presented including the 95 % confidence intervals (CI).

MTT data from mixture B are presented as mean ± 95 % CI and are from five independent experiments. Normality of the data distribution was assessed by three tests (KS normality test, D'Agostino and Pearson omnibus normality test and Shapiro–Wilk normality test), and statistical comparison between groups was estimated using the nonparametric method of Kruskal–Wallis [one-way analysis of variance (ANOVA) on ranks] followed by Dunn's post hoc test.

Data from GC–MS analysis are from at least three independent experiments, run in triplicate and are expressed as mean ± SEM (standard error of the mean). Normality of the data distribution was assessed by three tests (KS normality test, D'Agostino and Pearson omnibus normality test and Shapiro–Wilk normality test) and differences analysed by the Student's unpaired *t* test. *p* values lower than 0.05 were considered statistically significant.

All statistical calculations were performed using GraphPad Prism software, version 5.01 (GraphPad Software, San Diego California, USA).

Results

Concentration–response relationship of individual mixture agents

One of the main aims of this work was to investigate potential interactions between four amphetaminic drugs and evaluate whether these interactions could be accurately predicted using the mathematical CA and IA models. In order to produce the data required for calculating predictions of mixture effects, extensive concentration–response analyses of all the individual mixture components had to be carried out. Moreover, this toxicity information for the single drugs must be of reliable and reproducible significance to ensure consistent predictions of combination effects (Rajapakse et al. 2002).

In the MTT assay, all tested single agents yielded reproducible effects in a concentration-dependent fashion, resulting in decreased cell viability with the rising of chemical concentration (increased percentage of cell death). The data were produced on several occasions, using independently prepared serial dilutions of all chemicals. There was always good agreement between experiments. The cytotoxicity curves for each of the tested drugs, including the upper and lower 95 % CI, are displayed in Fig. 1. A summary of the best-fit regression models and the concentrations which individually produce 1 and 50 % of the maximal effect (EC_{01} and EC_{50}, respectively) for each drug are presented in Table 1. All drugs produced complete curves of percentage of cell death versus drug concentration. Individual concentration–response curves for the tested chemicals were relatively similar, as were their maximal effects.

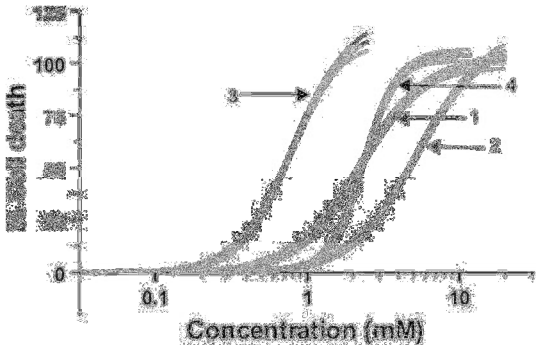

Fig. 1 Regression models for the cytotoxicity effects of all four-mixture components in Hep G2 cells. The *grey solid lines* represent the regression models for 4-methylthioamphetamine (4-MTA), d-amphetamine (d-AMP), 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine (METH) obtained in the MTT assay, following 48 h incubations. The *dashed grey lines* are the *upper* and *lower* 95 % CI of the best estimate of mean responses. The *labels* are as follows: *1* MDMA, *2* METH, *3* 4-MTA and *4* d-AMP. Data were from a minimum of nine independent experiments run in triplicate

Differences were observed essentially in the EC_{50} values and the slopes. MDMA with an EC_{50} of 2.23 mM shared similar potency with d-AMP (EC_{50} 2.42 mM). Comparatively, 4-MTA showed considerably higher potency (EC_{50} 0.74 mM), whilst METH was the least potent chemical tested (EC_{50} 5.26 mM). These differences between EC_{50}s of the test chemicals were deemed statistically significant, as there was no overlap between the corresponding 95 % CI of the concentration–response curves (Fig. 1).

In order to obtain an in-depth understanding of the potential interactions between the tested amphetamines, three different mixtures combining MDMA, METH, 4-MTA and d-AMP at varying ratios were tested.

Effects of a mixture prepared at a combination ratio proportional to the potency of each individual component (mixture A)

As described previously, in mixture A, all chemicals were combined at their EC_{50}, such that they were present at equieffective concentrations (2.23 mM MDMA, 5.26 mM METH, 0.74 mM 4-MTA and 2.42 mM d-AMP) (Table 1), ensuring that each drug contributed equally to the overall mixture effect and avoiding the disproportionate contribution of any one single agent. This mixture was designed with the main aim of assessing the validity of the two competing prediction models. Based on the concentration–response relationships of the individual chemicals, the concepts of IA and CA were used to predict the additive joint effects of the four drugs. As seen in Fig. 2, the slope for each prediction model was similar, both ranging the same order of magnitude from minimal to maximal mortality. However, the curve according to CA was shifted to lower concentrations, assuming stronger mixture effects than IA. The combined effects were then tested experimentally (Fig. 2). The obtained data revealed low variability and led to a complete concentration–effect curve. As shown in Fig. 2, additive expectations according to CA agreed well with the experimental observations, especially in the low effect range. At the higher effect range, a slight deviation of the curve was observed towards higher concentrations.

In contrast, the IA prediction clearly underestimated the mixture effects. Comparing the relative concentrations for the EC_{50}, the median effect concentrations were 2.9 and 2.51 mM for the mixture and CA, respectively, whereas for IA this value was much higher (4.68 mM).

Combination effects at low, ineffective concentrations (mixture B)

In mixture B, chemicals were mixed in a similar manner to mixture A, but this time, in proportion to their EC_{01}, to test possible joint effects when individual components are present at statistically ineffective concentrations. As shown in Fig. 3, when each component of the mixture was individually tested at the concentrations of 0.21 mM MDMA, 0.5 mM METH, 0.047 mM 4-MTA and 0.29 mM d-AMP, they produced very low effects, which could not be statistically differentiated from negative controls. Nonetheless, when mixed at those ineffective concentrations, they were able to act together to produce significant additive responses, which were accurately predicted by CA (Fig. 3). As shown, 1.058 mM of mixture B was responsible for 13.98 ± 2.34 % of cell killing. CA predicted effect was slightly lower but not significantly different (11.57 %).

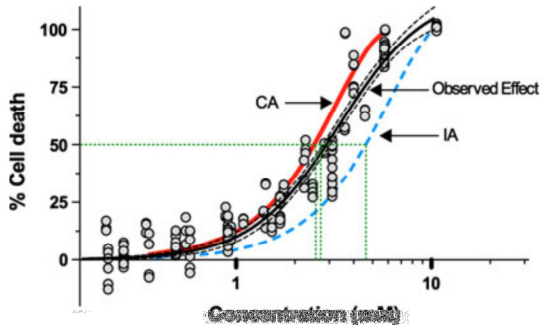

Fig. 2 Predicted and observed effects of a mixture of the four tested amphetamine-designer drugs in the MTT assay—mixture A. Individual data points are represented by *grey circles*, and the best-fit regression model is shown by the *black line*, labelled 'observed effect'. *Black dashed lines* represent the *upper* and *lower* 95 % CI for the regression fit. The *dashed blue line* shows the predicted combined effects derived from *independent action* (IA). The *solid red line* shows the prediction according to *concentration addition* (CA). The *green dotted lines* show the EC_{50} for each response curve (for values see Table 1). Experimental data derive from five independent experiments run in triplicate (color figure online)

Combination effects of a mixture representative of a 'realistic' exposure scenario (mixture C)

Finally, mixture C was conceived with the aim of confirming the CA additivity expectations in a more realistic exposure scenario associated with the consumption of *ecstasy* pills containing MDMA as a main constituent and the remaining amphetamines as contaminants.

It is often reported that amphetamine-related compounds can appear as contaminants in MDMA pills or even as substitutes of the supposed main component (Milroy 1999; Tanner-Smith 2006). To recreate these eventual situations, mixture C was prepared by fixing the MDMA concentration at 0.5 mM and gradually increasing the levels of the remaining components over a wide range of concentrations, while ensuring the ratio between them remained unaltered.

Although CA accurately predicted mixture effects in the lower concentration range (up to concentrations producing about 40 % effect), a deviation from additive expectations

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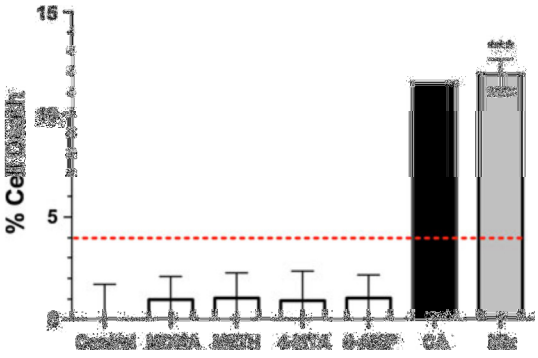

Fig. 3 Individual effects of MDMA, METH, 4-MTA and d-AMP at the concentrations present in 1.058 mM of mixture B (1.058 mM is the concentration of the mixture when all compounds are mixed at their EC_{01}). CA: *Concentration addition* prediction. MIX: observed effect of 1.058 mM of mixture B. The concentrations of the four-mixture components in 1.058 mM of the mixture B are 0.21 mM MDMA, 0.5 mM METH, 0.047 mM 4-MTA and 0.29 mM d-AMP. Data are from five independent experiments run in triplicate. The *dashed red line* corresponds to the sum of the individual effects of all mixture components. *Error bars* represent the 95 % CI. Statistical comparisons were made using the Kruskal–Wallis test followed by the Dunn's multiple comparison post hoc test. *** show statistically significant differences between the mixture and all other treatments. (***$p < 0.001$)

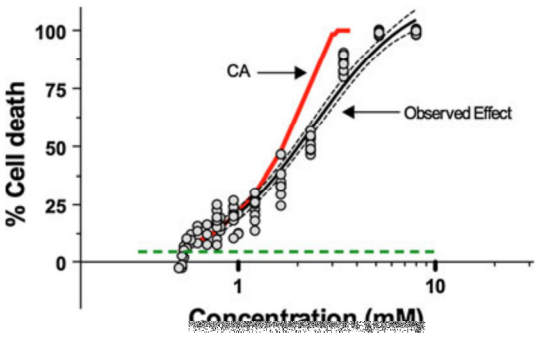

Fig. 4 Predicted and observed effects of mixture C in the MTT assay. Individual data points (*grey circles*) are from four independent experiments run in six replicates. Best-fit regression model is illustrated by the *black line*, labelled 'observed effect'. *Black dashed lines* represent the *upper* and *lower* 95 % CI for the regression fit. The *solid red line* (CA) shows the prediction based on *concentration addition*. The *horizontal green dashed line* represents the effect of 0.5 mM of MDMA when tested alone (color figure online)

was seen at higher concentrations (Fig. 4). The observed deviation was indicative of a weak antagonism, as concentrations higher than expected were necessary to produce the same effects, experimentally.

Evaluation of the metabolism of MDMA in the presence of other amphetamines (impact of mixtures)

As mentioned earlier, by evaluating the effects of mixtures A and C, it became clear that the effect concentrations predicted by CA agreed well with those experimentally observed throughout most of the effect range, except at higher effect levels, where a small deviation from additivity was seen. This deviation was particularly obvious in mixture C, for effects above 40 % cell death.

It is widely reported that the amphetamines tested in this study share common mechanisms of metabolism and detoxification and, consequently, can compete with each other in these processes (de la Torre et al. 2004). For that reason, it is conceivable that a different metabolic profile of the mixture components could occur in a combination setting. However, such interactions would not be accounted for when calculating mixture expectations according to CA, as the concept assumes the compounds do not interact in a pharmacological manner. This could explain the deviations found between the predicted and experimentally mixture effects and the weak antagonistic effects observed.

We tested this hypothesis by investigating potential unexpected changes in the metabolic profile of the individual compounds, when these were present in the mixture, by GC/MS analysis. For that, parent compounds and corresponding MDMA metabolites were quantified in the extracellular media and in Hep G2 cells, after incubation with two selected concentrations of both mixture A and C: For mixture A, a concentration of 1.75 mM (A1; composed of 0.383 mM MDMA, 0. 820 mM METH, 0.131 mM 4-MTA and 0. 424 mM d-AMP) was chosen, as it induced an effect that fell within the range accurately predicted by CA (effect level approximately 24.99 %). A second tested concentration of mixture A was 4.0 mM (A2; constituted by 0.871 mM MDMA, 1.87 mM METH, 0.298 mM 4-MTA and 0.965 mM d-AMP), as this was shown to produce effects that deviated from additivity, as observed in Fig. 5.

The tested concentrations of mixture C were selected on the same basis as for mixture A. Therefore, a concentration of 1.64 mM (C1, containing 0.5 mM MDMA plus 0.381 mM of each one of the other components METH, 4-MTA and d-AMP), which yielded an effect well predicted by CA, and a concentration of 2.33 mM (C2, 0.5 mM MDMA and 0.6104 mM of each one of the remaining amphetamines), which produced an effect that deviated from that predicted by CA, were chosen. For all mixture concentrations tested for metabolic profiling (A1, A2, C1 and C2), the corresponding concentrations of the individual components were also evaluated.

Under our experimental model, intracellular levels of parent compounds and MDMA metabolites were below the quantification limit (3.5 ng/ml) of the analytical method for all the tested samples. Analysis of the extracellular media showed no significant alterations of METH, 4-MTA and d-AMP individual profiles for either mixture tested (data not shown). Also, no biotransformation of MDMA into HMA or HMMA was detected in either media.

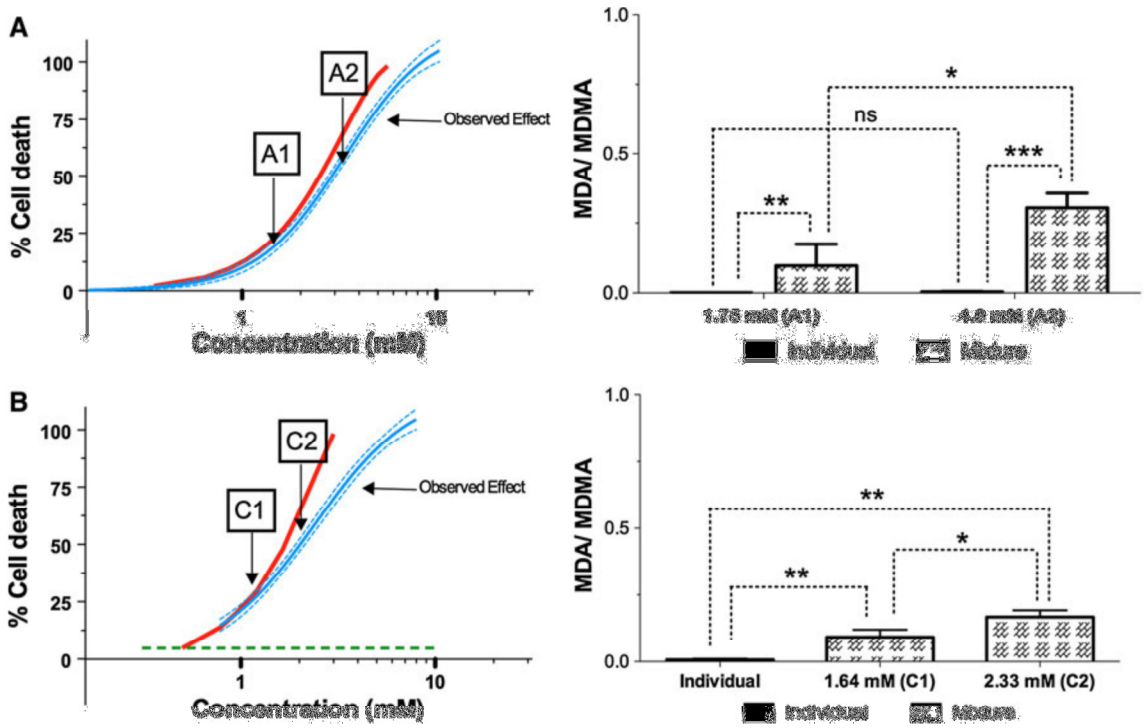

Fig. 5 Metabolism of MDMA alone and in combination as tested by GC–MS. **a.** A1 is the concentration of mixture A tested (1.75 mM) where the effect coincides with the CA prediction. A1 is composed of 0.383 mM of MDMA, 0. 820 mM of METH, 0.131 mM of 4-MTA and 0.424 mM of d-AMP; A2 is a concentration of mixture A (4.0 mM) that deviates from CA expectations and is constituted by 0.871 mM of MDMA, 1.87 mM of METH, 0.298 mM of 4-MTA and 0.965 mM of d-AMP. **b.** Concentration C1 of mixture C induces an effect that falls within the range estimated by CA and corresponds to 1.64 mM. It contains 0.5 mM of MDMA and 0.3815 mM of each one of the components METH, 4-MTA and d-AMP, while C2 corresponds to a concentration of mixture C (2.33 mM) that fails to meet the prediction by CA and is constituted by 0.5 mM MDMA and 0.6104 mM of each one of the remaining amphetamines. *Solid red lines* in the *graph plots* represent predictions by CA, while the *solid* and *dashed blue lines* are the experimental effects with the correspondent 95 % CI, respectively. Data are mean ± SEM and were obtained from three independent experiments run in duplicate. *Dotted lines*, in the *bar graphs*, represent comparisons between groups of two. Differences between groups were analysed by Student's unpaired *t* test. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ (color figure online)

Conversely, as depicted in Fig. 5, MDA was detected in the extracellular samples, an indicator of MDMA metabolism. The analysis of mixture A showed an increase in the metabolic rate of MDMA when the chemical was in the presence of other amphetamines. Moreover, the metabolism of this amphetamine increased even further in the concentrations where a deviation from CA was observed (A2). When MDMA was tested alone, there were no significant differences in its metabolic profile between the concentration present in A1 (0.383 mM) and A2 (0.871 mM). Here, the MDA/MDMA ratios were 0.0034 and 0.007, respectively. However, when combined, the metabolism of the same concentrations of MDMA was significantly higher in A2 (MDA/MDMA ratio 0.3045) than in A1 (MDA/MDMA ratio 0.0968).

A similar observation was made with mixture C (Fig. 5). In this case, the concentration of MDMA in both C1 and C2 remained constant at 0.5 mM. The MDA/MDMA ratio of this concentration tested alone was 0.0052. When in combination, the metabolism of MDMA increased with an increase in the concentrations of the remaining three amphetamines, which was clearly seen by the differences in MDA/MDMA ratios. For concentration C1, the MDA/MDMA ratio was 0.0917, whereas for C2, this was 0.1668 ($*p < 0.05$).

Discussion

The use of CA and IA models to predict additive combination effects requires an exhaustive characterization of the concentration–effect relationships of individual mixture components, in terms of shape, position (along the concentration axis) and maximal effect (Drescher and Boedeker 1995).

The MTT assay proved to be an effective method to meet these requirements, allowing the performance of high-throughput experiments with relative small variability. It produced reproducible results and complete curves that span a wide concentration–effect range.

In line with earlier reports (Carmo et al. 2004; Cloonan et al. 2010; Custodio et al. 2010), in this work, 4-MTA revealed to be a powerful cytotoxic agent (EC_{50} 0.75 mM) yielding more potent responses than any other tested amphetamine, including MDMA (EC_{50} 2.19 mM). In contrast, METH presented the least cytotoxic profile (EC_{50} 4.69 mM) corroborating previous studies, which identified it as a less effective drug than MDMA and d-AMP (EC_{50} 2.42 nM) in inducing in vitro cell death (Stumm et al. 1999b; Jimenez et al. 2004).

Considering the comparisons between computed and experimentally observed effects, the CA model proved to be a valuable tool for the assessment of additive joint effects of mixtures of amphetamines in this in vitro system. The overlap between the predicted data and the 95 % CI of the best-fit regression model showed good conformity, particularly at low effect levels. The work presented here demonstrates, for the first time, the excellent prediction power of CA when applied to combinations of amphetamines, proving that the four tested chemicals act in an additive fashion to produce the overall mixture effect. The concept of IA, on the other hand, is undoubtedly inappropriate for the assessment of the joint effects of these compounds in the MTT assay, suggesting that there is a possible similarity in the way in which these agents lead to Hep G2 cell death. So, assuming that all of our mixture components operate in a similar manner, we can expect the same mixture effect being produced by replacing one constituent totally, or in part, by other, at an equieffective concentration. For that reason, each individual component is thought to contribute to the overall mixture effect by acting proportionally to its concentration, even at concentrations that individually yield undetectable effects.

As shown, when each component of the mixture was individually tested at its EC_{01}, very low effects were produced, which could not be statistically differentiated from untreated controls. Others also confirmed these noncytotoxic concentrations in the immortalized human choriocarcinoma JAR cells at the same time point (Hayat et al. 2006). Nevertheless, when mixed at these concentrations, the four substances were able to act together to produce very significant effects. In fact, the effect of 1.058 mM of mixture B does not correspond to 4 % of cell killing, as it could be mistakenly believed by the simple sum of the component effects, but to 13.98 ± 2.34 %. This is very close to the value estimated by the CA prediction, once again demonstrating the applicability of the model.

Accordingly, we do not need to invoke synergistic combinations to prove that low levels of amphetamines present in illicitly consumed 'rave pills' can produce adverse effects, as significant mixture effects already occur in an additive fashion. Understanding this concept is crucial in the evaluation of mixture interactions, as studies frequently rely heavily on the search for synergisms to justify observed joint effects. A consequence of this approach is that often conclusions of synergisms are made, even in the absence of appropriate additive expectations.

As mentioned earlier, besides MDMA, *ecstasy* pills often contain amphetamine-like products of uncontrolled and clandestine synthetic processes. Several previous publications highlighted the fact that many of the MDMA pills available in illicit markets contain a number of other substances, sometimes cheaper and easily obtained, like METH (Camilleri and Caldicott 2005), d-AMP (Sherlock et al. 1999; Teng et al. 2006), 4-MTA (Tanner-Smith 2006; Teng et al. 2006) and other related derivatives. In order to assess whether the prognostic value of CA expectations fitted to more realistic scenarios, we studied mixture C, where the influence of varying concentrations of a three-component mixture (4-MTA, METH and d-AMP) was combined with a constant concentration of MDMA. In a similar way to the observations made with mixtures A and B, our results demonstrated a good agreement with CA especially at low concentrations and joint effects were slightly lower than additivity for higher effect levels (above 40 %) indicating weak antagonisms.

For the model of CA to be applicable, it relies on the assumption that all mixture components share the same toxicity mechanism and do not interact with, potentiate or antagonize each other. For this reason, this model does not take into account potential pharmacokinetic interactions between chemicals, such as the induction or inhibition of metabolic pathways. However, we know that amphetamine-related drugs have a close structural and functional relationship and use the same pharmacological and detoxification pathways (de la Torre et al. 2004). Therefore, it is plausible that all four amphetamines will compete and consequently interact with the metabolism and detoxification of each other in an unexpected manner. Ultimately, this could result in the deviations from additivity here reported.

The effects caused by the consumption of amphetamines can be conditioned by a plethora of factors that converge in a certain individual, on a certain moment. The mechanisms involved in liver damage induced by amphetamines are complex and still not completely understood. A variety of hypotheses have been proposed including the increased efflux of neurotransmitters, the oxidation of biogenic amines, mitochondrial impairment and apoptosis, and a direct effect of amphetamines and/or reactive metabolites (Carvalho et al. 2012). In addition, genetic polymorphism of metabolizing enzymes (particularly CYP2D6), polydrug abuse, and environmental features accompanying illicit amphetamine use may increase the risk for liver complications (Carvalho et al. 2012). Hyperthermia is thought to greatly contribute to liver toxicity. However, in some cases,

liver damage appears unrelated to hyperpyrexia (Milroy et al. 1996; Jones and Simpson 1999).

A well-known mechanism of toxicity in humans implies hepatic MDMA bioactivation into reactive species (de la Torre et al. 2004). The metabolism of MDMA is mainly regulated by cytochrome P450 (CYP450) enzymes and catechol-*O*-methyltransferase (COMT) in the liver. *N*-demethylation to MDA is a reaction mainly catalysed by CYP2B6. Both MDMA and MDA are then *O*-demethylated by CYP2D6, and to a lesser extent by CYP1A2, CYP2B6 and CYP3A4, to 3,4-dihydroxymethamphetamine (HHMA, *N*-methyl-α-methyldopamine, *N*-Me-α-MeDA) and 3,4-dihydroxyamphetamine (HHA, α-methyldopamine, α-MeDA), respectively. These catechol intermediates can undergo oxidation to the corresponding highly redox active ortho-quinones, which can enter in redox cycling, originate semiquinone radicals and lead to the generation of ROS or RNS, which are highly toxic for the cell (de la Torre et al. 2004; Shenouda et al. 2009; Barbosa et al. 2012). In light of this, and because all chemicals tested herein share the same biotransformation pathways, we hypothesized that they would promote the saturation of specific enzymes involved in oxidative metabolism and, therefore, reduce the formation of reactive species and so, cytotoxicity. However, instead, we observed a statistically significant increase in the formation of MDA ($^{**}p < 0.01$), an indication of increased metabolism. A possible explanation for this might be linked to the fact that in the mixture setting, the MDMA fraction bound to serum proteins or retained in the lipid bilayer membrane might decrease, as it is displaced from the binding sites by the remaining mixture components. This would increase the levels of free MDMA available for metabolism. Then, a preferential overexpression of CYP2B6, promoting *N*-demethylation with MDA formation, in detriment of *O*-demethylation, which would produce highly toxic reactive species, may play a role. As MDA has been shown to have slightly lower toxic effects than the parent compound MDMA in human proximal tubular cells (Carvalho et al. 2002), an increase in this metabolic product would justify the weak antagonisms observed. Nevertheless, the precise molecular interactions between amphetaminic drugs are still not fully understood, and it is possible that additional factors are involved in the deviations observed, requiring further biological and molecular investigation.

The tested concentrations used in the present study are in the range of concentrations used in several mechanistic in vitro studies (Simantov and Tauber 1997; Stumm et al. 1999a; Carvalho et al. 2004a, b; Capela et al. 2006). They are higher than concentrations commonly found in human abusers. However, it should be noted that high interindividual variations of blood levels in cases of severe and even fatal intoxications have been observed. For MDMA, for example, blood concentrations can be as high as 13.5 mg/l (approximately 70 μM) (De Letter et al. 2004, 2006). In such cases, the autopsy findings have shown that the tissue levels of the drug in the liver can be up to 18 times higher than blood concentrations (De Letter et al. 2006) and 30 times higher in the brain (Garcia-Repetto et al. 2003). Amphetamines in general have low protein binding (usually under 20 %), which confers high bioavailability to these drugs and favours their easy diffusion from the plasma to the extravascular compartment (de la Torre et al. 2004). Moreover, these concentrations found at autopsy are probably lower than the peak concentrations that are expected to occur after drug intake, especially in the cases where the victims are submitted to emergency-care treatments to control the intoxications.

One final point to consider is the fact that in the present study, we have explored combination effects in an in vitro setting, using a hepatocarcinoma cell line model. The Hep G2 cell line, in spite of retaining metabolic capacity and being able to respond to metabolic inducers, as well as bioactivate chemical substances by cytochrome P450 isoforms (Doostdar et al. 1993; Darroudi et al. 1996; Ripp et al. 2003; Knasmuller et al. 2004; Donato et al. 2008), has weaker metabolic activity than primary hepatocytes or normal liver tissue (Donato et al. 2008). For this reason, an important task for the future is to investigate whether our findings hold true for biological effects at higher levels of complexity, such as in primary hepatocyte cultures or in an in vivo scenario, where other factors, such as metabolic competency, immune-mediated responses and polymorphisms, can be taken into account. However, to the best of our knowledge, CA and IA models are yet to be applied to the study of mixtures of amphetamines in vivo.

In conclusion, our results emphasize the limitations of the traditional focus on single agents, as they would completely overlook these potentially hazardous events and would lead to significant underestimations of toxicity. Given this, assessing combination effects of amphetamines is of utmost importance from a toxicological point of view, as the majority of *ecstasy* users, consciously or not, take a wide variety of distinct drugs on the same night out. Understanding the impact of other drugs in *ecstasy* pills might provide valuable information to dissect the causes behind reported sudden and random lethal intoxications and aid diagnostics and treatment of nonfatal cases.

We strongly believe that a better understanding of the joint effects of these uncontrolled illicit drugs might have a considerable influence on public health by raising the awareness of potential severe toxicity and, consequently, encouraging behavioural changes in consumers worldwide.

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Conflict of interest The authors declare that there are no conflicts of interest.

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Chapter II. Combination effects of amphetamines under hyperthermia – The role played by oxidative stress.

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Combination effects of amphetamines under hyperthermia - The role played by oxidative stress

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Abstract

We evaluated the impact of hyperthermia on the cytotoxicity of combinations of *3,4*-methylenedioxymethamphetamine (MDMA, *ecstasy*) and three other amphetamines, often co-ingested with MDMA. With this purpose, HepG2 cells were exposed to MDMA, *d*-amphetamine, methamphetamine and *4*-methylthioamphetamine, individually or combined, in hyperthermia (40.5 ºC). The obtained data were compared with information from normothermia (37.0 ºC). Mixture additivity expectations were calculated by mathematical models of *independent action* and *concentration addition* (CA). To delineate the mechanism(s) underlying the elicited effects, a range of stress endpoints were evaluated, including quantification of reactive species (ROS/RNS), lipid peroxidation, GSH and GSSG, ATP, and changes on mitochondrial membrane potential ($\Delta\psi m$).

Our data show that, in hyperthermia, the joint effects of the amphetamines were additive and accurately predicted by CA. Interestingly, at 40.5 ºC, even slight increases in the concentrations of each drug/mixture promoted significant rises in cytotoxic effects, which quickly shifted from roughly undetectable to maximal mortality. Additionally, the observed increase of RNS/ROS production, decrease of GSH, and ATP depletion, accompanied by mitochondrial impairment were greatly exacerbated under hyperthermia Importantly, when equieffective cytotoxic concentrations of the mixture and individual amphetamines were compared for all tested stress endpoints, it was observed that the effects of the mixture did not deviate from those elicited by individual drug treatments, suggesting that these chemicals have a similar mode of action, which is not altered in combination.

Concluding, our data indicate that mixtures of amphetamines produce deleterious effects, even when the individual drugs are combined at very low concentrations. These effects are strongly exacerbated in hyperthermia, emphasizing the potential increased risks of *ecstasy* intake, especially when hyperthermia occurs simultaneously as polydrug abuse.

Keywords

Amphetamines; *Concentration addition* (CA); Hepatocytes; Hyperthermia; Mixtures; Oxidative Stress.

Introduction

In spite of the increasing evidence of severe and even fatal toxicity, *3,4*-methylenedioxymethamphetamine (MDMA, *ecstasy*) abuse is still considered by many of its users to be relatively 'safe', since the reported number of fatalities or severe morbidity events related with this drug do not appear to be proportional to the high frequency of misuse. Nevertheless, the consumption of this drug often results in unexpected and random severe toxicity, for which there is still no clear explanation (deLetter et al. 2004; Greene et al. 2003; Henry 1992; O'Donohoe et al. 1998; Schifano 2004). In fact, the concentrations of MDMA found in human abusers following symptomatic and/or fatal intoxications vary enormously (deLetter et al. 2004; Sticht et al. 2003) and, consequently, threshold toxic concentrations or potentially lethal concentrations are currently unknown for this drug. Also, there is apparently no correlation between the amount or the frequency of MDMA intake and the severity of toxic symptoms. It is remarkably striking that, in some cases, MDMA-related deaths occur for first time users or after consumption of only a single tablet of *ecstasy*, while in other cases mild intoxication symptoms and full recovery occurred after the ingestion of up to a few dozens of tablets on a single occasion (Coore 1996b; deLetter et al. 2004; Henry et al. 1992b; Milroy et al. 1996; O'Donohoe et al. 1998; Ramcharan et al. 1998; Schifano 2004).

Contributing to this unpredictable acute toxicity of MDMA is undoubtedly the frequently reported concomitant abuse of other substances. It is widely acknowledged that a number of recreational drugs, including other amphetamines, are often taken together with MDMA, either accidentally or deliberately (Baggott et al. 2000; Camilleri and Caldicott 2005; Dams et al. 2003; Greene et al. 2008). *Ecstasy* pills often contain more than one type of these stimulants including methylenedioxyamphetamine (MDA), *d*-amphetamine (*d*-AMP), methylenedioxyethylamphetamine (MDEA), methamphetamine (METH), among others (Camilleri and Caldicott 2005; Carvalho et al. 2012; Sherlock et al. 1999; Tanner-Smith 2006; Teng et al. 2006). Consequently, this simultaneous use of a wide variety of drugs can have a significant impact on the toxicity of MDMA, as combination and interactions with other chemicals can potentially increase the severity or broaden the range of its toxic effects (deLetter et al. 2006; Verschraagen et al. 2007) either through toxicokinetic (*e.g.* metabolic impairment) or toxicodynamic interactions (*e.g.* increased sympathomimetic and serotonergic effects) (Oesterheld et al. 2004). This issue has been recently addressed in a recent publication from our group that unequivocally demonstrated that amphetamine-like compounds that are known to be simultaneously abused *in vivo* (MDMA, *d*-AMP, METH and 4-methylthioamphetamine, 4-MTA), were able to interact *in vitro* in an additive fashion, producing considerable cytotoxic mixture effects, even when combined at individually ineffective concentrations (Dias da Silva et al. 2012).

Also, the environmental conditions in the recreational venues where MDMA intake regularly takes place have been implicated in its health-threatening risks (Bellis et al. 2002). Effectively, it has been argued that MDMA toxicity may be strongly potentiated by the overcrowded and overheated environments as they exacerbate one of the most toxic effects of the drug, the impairment of thermoregulation. The thermogenic actions of MDMA, combined with the mentioned environmental conditions, as well as the excessive dancing and inadequate fluid intake have been shown to induce dramatic increases in body temperature, with reported values as high as 43.3 ^{o}C (Armenian et al. 2012; Coore 1996b; Green et al. 2003; Henry et al. 1992b). This MDMA-induced hyperthermia has been associated with the most serious detrimental health effects of the drug. Even though fatalities elicited by MDMA-induced overheating are not very frequent high body temperature has been described as one of the most aggressive physiological consequences of amphetamines in acute poisonings and has been recurrently pointed out as a cause for life-threatening complications, such as acute liver failure, rhabdomyolysis, disseminated intravascular coagulation, acidosis and multiple organ failure (Armenian et al. 2012; Henry et al. 1992a; Kalant 2001; Kendrick et al. 1977; Patel et al. 2005; Screaton et al. 1992).

One of the main target organs for MDMA toxicity that is especially sensitive to the hyperthermic effect of the drug is the liver. MDMA has been deemed responsible for several cases of acute liver failure in young people (Andreu et al. 1998). The hepatotoxic action of MDMA stems from multiple interacting mechanisms that become exacerbated at high body temperatures. These mechanisms include the increased efflux of neurotransmitters, the oxidation of biogenic amines, formation of highly reactive metabolites, mitochondrial impairment, and apoptosis (Carvalho et al. 2012; Carvalho et al. 2010). Several studies have shown that an increase in body temperature is not only directly related with hepatotoxic damage *per se*, but also further exacerbates the amphetamines-related liver toxicity (Carvalho et al. 1997a; Carvalho et al. 2001; Carvalho et al. 2002a; Pontes et al. 2008b; Skibba et al. 1991). Both in *in vivo* and *in vitro* study models, hyperthermic

conditions were shown to potentiate the hepatotoxic effects, namely those related with oxidative stress, including depletion of GSH levels, lipid peroxidation and loss of cell viability (Carvalho et al. 2012). As noted above, the severity of the symptoms and the hepatic lesions do not seem to correlate either with the duration of use or with the amount of drug consumed (Andreu et al. 1998; Dykhuizen et al. 1995; Garbino et al. 2001). In some cases, liver damage appeared only after regular use for weeks or months, but two reported cases of fulminant hepatitis (Coore 1996a; Ellis et al. 1996) and two cases of acute hepatitis (Dykhuizen et al. 1995; Henry et al. 1992a) have been reported after ingestion of a single tablet of *ecstasy*. The pathogenesis of MDMA-induced liver injury remains, therefore, poorly understood.

A major challenge in elucidating such mechanisms is to account for complex factors like polydrug abuse and hyperthermia in the study models. As mentioned above, we have previously tested the combined effects of commonly abused amphetamines in an *in vitro* model using human hepatoma HepG2 cells. We demonstrated that, even when each components was present at very low concentrations, mixtures of these drugs had significant hepatotoxic effect (Dias da Silva et al. 2012).We have now extended our previous research by applying this study model to hyperthermic conditions, which better reflect the physiological environment of the liver in an acute intoxication scenario, due to the thermogenic action of the drugs and the environmental circumstances of the abuse. We presently report the investigation of the cytotoxic effects elicited by the tested amphetamines, when these drugs were incubated individually and in combination at a temperature of 40.5 ºC. Our observations were compared with our previous data, obtained under normothermia (*i.e.* 37 ºC) to highlight potential changes in cytotoxicity associated with an increase in body temperature. Furthermore, in a similar way to our previous study (Dias da Silva et al. 2012), we tested the applicability of two mathematical models, *concentration addition* (CA) and *independent action* (IA), with the aim of investigating whether good agreement was still observed with the CA expectations, under the new experimental conditions. Finally, in order to delineate the mechanisms underlying the observed cytotoxicity, we further evaluated the involvement of several stress indicators in the promoted effects, namely the increased formation of reactive oxygen (ROS) and nitrogen (RNS) species, the contents of reduced (GSH) and oxidised (GSSG) glutathione, the loss of mitochondrial membrane potential ($\Delta\psi m$), lipid peroxidation markers, and the ATP intracellular levels.

Our results indicated a disturbance in the cell redox status and antioxidant defences, with consequent mitochondrial injury and energetic deficit that seem to play an important role in the observed individual and mixture effects, under hyperthermic settings. Of particular note, was the increase in the steepness of the cytotoxicity curves displayed by each one of these drugs individually, as well as in combination, at hyperthermic incubations, emphasizing the potential reduction of the 'safety window' of these drugs and the consequent increased risks for *ecstasy* abusers in the high temperature misuse scene.

Materials and Methods

Chemicals

MDMA (HCl salt) was extracted and purified from high purity MDMA tablets that were provided by the Portuguese Criminal Police Department and 4-MTA (HCl salt) was synthesized at REQUI*M*TE/ Department of Biological Sciences, Laboratory of Toxicology, Faculty of Pharmacy, University of Porto. The obtained MDMA and 4-MTA salts were pure and fully characterized by nuclear magnetic resonance (NMR) and mass spectrometry (MS) methodologies. *d*-AMP (sulfate salt) was purchased from Tocris Bioscience (Bristol, UK) and (+)-METH hydrochloride was purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA).

All chemicals were used as supplied. Stock solutions were prepared in ultra-purified sterile water and were at least 20 times more concentrated than the highest concentration tested to prevent media dilution. All stock solutions were stored at –20 °C and subsequent dilutions were freshly prepared in cell culture medium before each experiment. A range of test concentrations were prepared by successive dilutions employing the *fixed mixture ratio design,* as described by Altenburger (2000) and Backhaus (2000a). Briefly, the master stock solution was serially diluted without changing the ratio between each constituent. These serial dilutions covered a broad range of concentrations, so that a complete concentration–response relationship could be recorded allowing robust computation of the data.

Cell culture routine

HepG2 cells were routinely maintained in 75 cm^2 canted-neck tissue culture flasks (Helena Biosciences; Gateshead, UK) in Minimum Essential Medium Alpha (MEM Alpha) with GlutMAX^{TM} supplemented with 10% foetal bovine serum (FBS), 1 % antibiotic (5000 U/ml penicillin, 5000 µg/ml streptomycin), 0.5% fungizone (250 µg/ml amphotericin B) and 0.0015% human transferrin (4 mg/ml) in a humidified incubator, at 37 °C, with 5% CO_2. Cells were subcultured at approximately

70% confluence over a maximum of 10 passages and regularly tested for *Mycoplasma* contamination.

All reagents used in routine cell culture, including 0.05% trypsin/1 mM EDTA and Hanks balanced salt solution (HBSS) without Ca and Mg were obtained from GIBCO, Invitrogen Corporations (Paisley, UK), unless stated otherwise.

MTT reduction assay

The protocol described previously by Dias da Silva *et al*. (2012) was used. Briefly, HepG2 cells were seeded onto the central 60 wells of 96-well microtiter plates (Falcon; BD Biosciences, Oxford, UK), at a density of 8 x10^4 cells per well. Peripheral wells on the plate were filled with sterile water to prevent evaporation and concentration of test solutions. Forty eight hours after seeding, the media was gently aspirated and the cells were incubated with the test drugs MDMA, METH, 4-MTA, *d*-AMP individually, and with the tested mixture dilutions prepared in fresh culture medium, in a humidified air atmosphere containing 5% CO_2, at 40.5 °C. The concentrations were selected to cover the whole effect range, from undetectable effects (when compared to negative controls) to 100% mortality. Each individual plate included six replicates of negative controls (*i.e.*, no test agents) and six replicates of positive controls (full media with 1% Triton X-100). After incubation for 48 h, at 40.5 °C, the medium was removed and the attached cells rinsed with 200 µl HBSS (pH 7.4), followed by the addition of fresh cell culture medium containing 0.25 mg/l MTT (Sigma-Aldrich, Co.; St. Louis, USA). Plates were incubated at 37 °C, for 30 min. Finally, the cell culture medium was aspirated and the formed intracellular formazan crystals dissolved in 100% DMSO (Fisher Scientific Ltd; Loughborough, UK). The spectrophotometric analysis was run at 570 nm using a multi-well plate reader (Labsystems Multiskan, Basingstoke, UK). To reduce inter-experimental variability, data were normalized plate-by-plate as described elsewhere (Rajapakse et al. 2004), with all data scaled between 0 (negative controls) and 1 (positive controls). Data were obtained from at least five independent experiments, with each test plate containing six replicates of eight increasing concentrations of the tested individual drugs and mixtures.

Regression modelling

The normalised MTT data were fitted to the dosimetric logit model, that was chosen based on a statistical goodness-of-fit principle (Scholze et al. 2001): $Y= \theta_{min} + (\theta_{max} - \theta_{min})/(1+\exp(-\theta_1-\theta_2*\log(x)))$, where θ_{min} and θ_{max} are the minimal and maximal observed effects, respectively; x is the concentration of test agent; θ_1 is the parameter for location; and θ_2 is the slope parameter.

Mixture design and prediction of combination effects

The mixture ratio was chosen such that all chemicals were present at concentrations eliciting 50% of their individual maximal effect (EC_{50}) as determined in the MTT assay. This ensured that all mixture components were present at equipotent concentrations, thus preventing disproportionate contributions from any single component. The overall effect of the designed mixture was predicted using the concepts of *concentration addition* and *independent action* (IA), first described by Loewe and Muchnik (1926) and Bliss (1939), respectively. These concepts are based on general assumptions regarding the mechanisms of action of the mixture constituents and their details have been discussed elsewhere (Kortenkamp 2007). Briefly, the concept of CA is based on the assumption that the mixture constituents have similar modes of action, which means that any component can be replaced partially or totally with another without changing the overall mixture effect (Backhaus et al. 2000b; Silva et al. 2011). This means that each individual component contributes to the global joint effect by acting in proportion to its concentration, even at concentrations producing no effect. The IA alternative approach better describes combination effects of drugs with dissimilar mechanisms of action with each agent interacting at differing sites of action (Backhaus et al. 2000a). In this case, the fractional response of one individual component is supposed to be independent from those induced by other components, presuming that mixture components present at zero effect concentrations will not contribute to the overall effect. The two models can produce very distinct expectations and, to our knowledge, have only been applied to amphetamine-like compounds in our former study conducted at normothermic (37 ºC) incubations (Dias da Silva et al. 2012). The mathematical derivations of prediction models (CA and IA) were described by Payne *et al.* (2000).

Drugs challenge for mechanistic studies

Based on the individual cytotoxicity data obtained in the MTT viability assay, four concentrations were chosen for each individual amphetamine and also for the mixture. The concentrations selected were 0.3 mM, 1 mM, 1.5 mM and 1.8 mM for MDMA; 0.8 mM, 1.8 mM; 2.3 mM and 3 mM for METH; 0.3 mM, 0.5 mM 0.7 mM and 0.9 mM for 4-MTA; 0.8 mM, 1.7 mM, 1.9 mM and 2.2 mM for *d*-AMP; and, 0.7 mM, 1.5 mM, 2.1 mM and 3 mM for the mixture.

Table 1. Concentration of each individual amphetamine at the tested mixture concentrations.

	Tested mixture concentration	0.7 mM	1.5 mM	2.1 mM	3.0 mM
Individual concentration present in the mixture (mM)	MDMA	0.186	0.399	0.559	0.798
	METH	0.270	0.578	0.810	1.156
	4-MTA	0.069	0.149	0.208	0.298
	d-AMP	0.174	0.374	0.523	0.747

These concentrations were selected to cover a wide cytotoxic effect range. Although they are generally higher than those found in human abusers, which are usually in the low micromolar range (Garcia-Repetto et al. 2003), they can still be considered physiologically relevant since blood concentrations found in victims of MDMA-related fatal intoxications can be as high as 13.5 mg/L (approximately 70 µM) (deLetter et al. 2004; deLetter et al. 2006). In such cases the autopsy findings have shown that the tissue levels of the drug can be much higher (Garcia-Repetto et al. 2003; Sticht et al. 2003), up to 18 times higher than blood concentrations in the liver (deLetter et al. 2006) and 30 times higher in the brain (Garcia-Repetto et al. 2003). Moreover, these concentrations found at autopsy are probably lower than the peak concentrations that are expected to occur after drug intake, especially in the cases where the victims are submitted to emergency-care treatments to control the intoxications.

Table 1 presents the individual concentrations of each amphetamine at different tested mixture concentrations. Incubations were conducted both under normothermic (37 ºC) and hyperthermic (40.5 ºC) conditions. The following biochemical endpoints were determined.

ROS and RNS

The intracellular ROS and RNS production was monitored by means of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a lipophilic sensitive probe that readily penetrates the cells producing 2',7'-dichlorodihydrofluorescein (DCFH) after hydrolysis, that further reacts with intracellular ROS and RNS, including the hydroxyl radical and hydrogen peroxide, generating green fluorescent 2',7'-dichlorofluorescein (DCF), which is polar and trapped within the cells (Rao et al. 1992; Smith and Weidemann 1993).

For this determination, 8 x10^4 cells per well were seeded onto 96-well black plates (Greiner CELLSTAR® from Sigma) and allowed to attach for 24 h. On the day of the experiment, the cells were rinsed twice with HBSS and pre-incubated with 10 µM DCFH-DA (Sigma) for 30 min, at 37 °C. DCFH-DA was stored in an opaque airtight container at –20 °C. As DCFH-DA is a non water-soluble powder, it was initially prepared as a 4 mM stock solution in DMSO (Merck) and made up to the final concentration in fresh culture media (ensuring that the final concentration of DMSO did not exceed 0.5%) immediately before each experiment. The cells were then rinsed twice with HBSS and incubated with 200 µl/well of each test concentration of MDMA, METH, 4-MTA, *d*-AMP and mixture dilutions prepared in fresh medium at either 37 ºC or 40.5 ºC. A blank (no cells) and a negative control (no test drugs) were also included in the plate. After a 24 h treatment period, fluorescence was recorded at 37 ºC, on a fluorescence microplate reader (FLUOstar Optima, BMG Labtech GmbH) set to 485 nm excitation and 530 nm emission. The data obtained for the individual drug and mixture treatments were normalized to negative controls on a plate-by-plate basis and presented as fold increase over control conditions at 37 ºC or 40.5 ºC. This normalization is required to minimize the variability between plates and between independent experiments and has been extensively described elsewhere (Rajapakse et al., 2004).

GSH and GSSG

The cellular GSH and GSSG levels were determined by the DTNB–GSSG reductase recycling assay, as described before (Anderson 1985), with some adaptations. Briefly, 2 x10^6 cells were seeded onto 10 cm^2 petri dishes and allowed to attach for 24 h. On the day of the experiment, the media was gently aspirated and the cells were incubated with each test concentration of MDMA, METH, 4-MTA, *d*-AMP, and mixture dilutions, prepared in fresh medium, at either 37 ºC or 40.5 ºC. Twenty-four hours after cell treatment, the cells were rinsed twice with HBSS and scrapped/precipitated with 5% perchloric acid ($HClO_4$, w/v). After centrifugation for 5 min at 16,000g (at 4 °C), the supernatants were collected and kept frozen at –80 °C until further quantification, and the pellet was used for protein quantification. The thawed acidic supernatant was neutralized with an equal volume of 0.76 M potassium bicarbonate ($KHCO_3$) and centrifuged for 5 min at 16,000g (at 4 °C). Total glutathione (tGSH) was determined by transferring, in

triplicate, 100 µl of the neutralized supernatants, standards, or blank (5% $HClO_4$, w/v) to a 96-well plate, followed by the addition of 65 µl of freshly prepared reagent containing 0.24 mM NADPH and 1.3 mM 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) in phosphate buffer (71.5 mM Na_2HPO_4, 71.5 mM NaH_2PO_4 and 0.63 mM EDTA, pH 7.5). The plates were then incubated for 15 min, at 30 °C, in a microplate reader (SPECTRAmax 340PC ROM v3.13, Molecular Devices, US), prior to the addition of 40 µl per well of a freshly prepared 10 IU/ml glutathione reductase solution (Sigma) in phosphate buffer. The stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB) was followed every 10 sec for 3 min at 415 nm (at 30 °C), and compared to a standard curve performed for all readings. For the determination of GSSG, 10 µl of 2-vinylpyridine were added to 200 µl aliquots of the acidic supernatants and mixed continuously for 1 h (at 0 °C) for derivatization of the sulfhydryl groups (SH). GSSG was then measured as described for total glutathione. The molar reduced glutathione (redGSH) content was calculated by subtracting the GSSG from the total glutathione values (redGSH = tGSH −2x GSSG). The results were normalized to the total protein amount, and the final results were expressed as nmol GSH or GSSG.mg-^{1} protein.

Protein determination

Protein was quantified by the Bradford assay (Bradford 1976). The absorbance measurement was taken at 595 nm using a multi-well plate reader (Labsystems Multiskan, Basingstoke, UK) and was compared with a bovine serum albumin (BSA, Sigma) standard curve to assess protein amount.

ATP content

Cellular ATP levels can be rapidly detected through a bioluminescent luciferase-based assay. Luciferase catalyses the formation of light from the reaction of ATP and luciferin. For this determination, 2 x10^6 cells were seeded onto 10 cm^2 petri dishes to obtain confluent monolayers 24 h later. On the day of the experiment, the media was gently aspirated and the cells were incubated with each test concentration of MDMA, METH, 4-MTA, *d*-AMP and mixture dilutions prepared in fresh medium at either 37 ºC or 40.5 ºC. After 24 h, the cells were rinsed twice with HBSS and scrapped/precipitated with 5% $HClO_4$ (w/v). After centrifugation for 5 min at 16,000g (at 4 °C), the supernatants were collected and kept frozen at –80 °C until quantification, and the pellet was used for protein quantification. The thawed acidic supernatant was neutralized with an equal volume of 0.76 M $KHCO_3$ and centrifuged for 5 min at 16,000g (at 4 °C). The ATP contents were then measured in triplicate in 96-well white plates (Greiner CELLSTAR® from Sigma), using a luciferin/luciferase bioluminescence ATP determination kit (Invitrogen) and following the manufacturer instructions. The emitted light intensity was determined by using a luminescence microplate reader (FLUOstar Optima, BMG Labtech GmbH) and compared to a standard curve performed within each experiment. The results were normalized to the total protein amount, and the final results were expressed as nmol ATP.mg-^{1} protein.

Lipid peroxidation

The extent of lipid peroxidation, which can be related with the formation of malondialdehyde (MDA) after the hydrolysis of polyunsaturated fatty acids, was measured by the thiobarbituric acid reactive substances (TBARS) assay (Buege and Aust 1978), with some variations from the original protocol. Briefly, 1 x10^6 cells were seeded onto 6-well plates and allowed to attach for 24 h. On the day of the experiment, the media was gently aspirated and the cells were incubated with each test concentration of MDMA, METH, 4-MTA, *d*-AMP and mixture dilutions prepared in fresh cell culture medium at either 37 ºC or 40.5 ºC. Following a 24 h incubation period, the cells were scrapped with 250 μl HBSS and precipitated with 500 µl of 10% trichloroacetic acid (TCA, w/v). The mixture was incubated on ice for 30 min before centrifugation at 16,000 g, for 10 min (at 4 ^{o}C). Aliquots of 500 µl of the supernatants were added to an equal volume of 1% thiobarbituric acid solution (TBA, w/v in distilled water). The mixture was heated for 10 min at 80-95 ^{o}C and allowed to cool down to room temperature. The absorbance readings were performed at 535 nm using a multi-well plate reader (Labsystems Multiskan, Basingstoke, UK). TBARS were expressed as MDA equivalents per milligram of protein after calculation with the extinction coefficient of 1.56 x10^5 M^{-1} cm^{-1} (Buege and Aust 1978).

Mitochondrial integrity

Assessment of mitochondrial integrity was performed by measuring the tetramethylrhodamine ethyl ester perchlorate (TMRE) inclusion. TMRE is a cell permeable fluorescent dye that specifically stains live mitochondria, and accumulates in proportion to mitochondrial membrane potential ($\Delta\psi_m$) (Scaduto and Grotyohann 1999). For this determination, 8 x10^4 HepG2 cells were seeded onto 96-well black plates (Greiner CELLSTAR® from Sigma). After 24 h, the media was gently aspirated and the cells were incubated with each test concentration of MDMA, METH, *4*-MTA, *d*-AMP, and mixture dilutions prepared in fresh

medium at either 37 ºC or 40.5 ºC. At the end of the 24 h incubation period, the cells were rinsed twice with HBSS and incubated at 37 °C with 100 ul of 2 µM TMRE (Sigma), for 30 min. As TMRE is a non water-soluble powder, a 2 mM stock solution was initially prepared in DMSO (Merck) and stored in the dark. The final 2 µM concentration was prepared in fresh culture media, shortly before usage (the final concentration of DMSO did not exceed 0.5%). Afterwards, the media was gently aspirated and replaced by 0.2% BSA in HBSS. Fluorescence was measured at 37 °C on a fluorescence microplate reader (FLUOstar Optima, BMG Labtech GmbH) set to 544 nm excitation and 590 nm emission. The data obtained for the individual drug and mixture treatments were normalized on a plate-by-plate basis to the values of the respective controls and calculated as the percentage of control conditions at 37 ºC or 40.5 ºC. This normalization is required to minimize the variability among the individual plate readings and between independent experiments (Rajapakse et al., 2004).

Statistical analysis

The cytotoxicity data from the MTT reduction assay were obtained from at least five independent experiments and fitted to the logit regression. The results were presented including the 95 % confidence intervals (CI) of the mean values. For the biochemical assays, results are presented as mean ± standard error of the mean (SEM) from at least four independent experiments. Normality of the data distribution was assessed by the Kolmogorov–Smirnov normality test. Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test. The control values (raw data) obtained for hyperthermic and normothermic incubations in the determination of ROS and RNS and mitochondrial integrity assays were compared by the Student's unpaired t-test for identification of potential effects on these endpoints of the increase in temperature alone. Significance was accepted at $p < 0.05$. All statistical calculations were performed using GraphPad Prism software, version 5.01 (GraphPad Software, San Diego, CA).

Results

The cytotoxicity of amphetamines is significantly exacerbated under hyperthermia

A comprehensive concentration–response analysis of all single drugs was carried out under hyperthermic conditions by incubating the cells at 40.5 ºC with the test drugs over a wide concentration range. In the MTT assay, all the tested drugs yielded reproducible concentration-dependent cytotoxic effects. The data were produced on five different occasions, using independently prepared serial dilutions of all chemicals and an excellent agreement between experiments was consistently noted. The mortality curves for each of the tested amphetamines, including the upper and lower 95% confidence intervals (CI), are displayed in figure 1. A summary of the parameters of the best-fit regressions is provided in table 2.

For all the tested drugs, complete lethality curves were obtained that significantly differed in their slope and EC_{50} values, as shown in figure 1. MDMA (EC_{50} 1.77 mM) and *d*-AMP (EC_{50} 1.66 mM) showed comparable potencies. A considerably lower median effect concentration was obtained for 4-MTA (EC_{50} 0.66 mM) that showed the highest potency, whilst METH was the least potent drug tested (EC_{50} 2.97 mM).

For the sake of comparison, our previously reported data obtained under similar experimental conditions, but with the incubations performed under normothermia (*i.e.* 37 ºC) (Dias da Silva et al. 2012) were also included in figure 1. The EC_{50} values obtained under hyperthermic circumstances were lower than those obtained at a physiological incubation temperature (Dias da Silva et al. 2012): MDMA EC_{50} 2.23 mM at 37 ºC *versus* 1.77 mM at 40.5 ºC; METH EC_{50} 5.26 mM at 37 ºC *versus* 2.97 mM at 40.5 ºC; 4-MTA EC_{50} 0.74 mM at 37 ºC *versus* 0.66 mM at 40.5 ºC; and d-AMP EC_{50} 2.42 mM at 37 ºC *versus* 1.66 mM at 40.5 ºC, thus indicating an increase in toxicity at the higher incubation temperature that was observed for all tested amphetamines with a remarkable difference noted for METH.

By comparing the slopes of the regression models at hyperthermic incubations with those achieved at normothermic incubations, it became clear that the curves obtained at 40.5 °C were significantly steeper than those at 37 °C, and that very small increases in the concentration of each test drug provoked much greater increases in cell mortality than what could be observed at 37 ºC (figure 1). These results indicate that increasing the incubation temperature to values that mimic the target-organ temperatures expected to occur *in vivo,* in an intoxication scenario (Armenian et al. 2012; Henry et al. 1992b) has a strong impact on the cytotoxic profile of the test drugs, with very similar concentrations inducing effects that can quickly shift from roughly undetectable to high mortality levels.

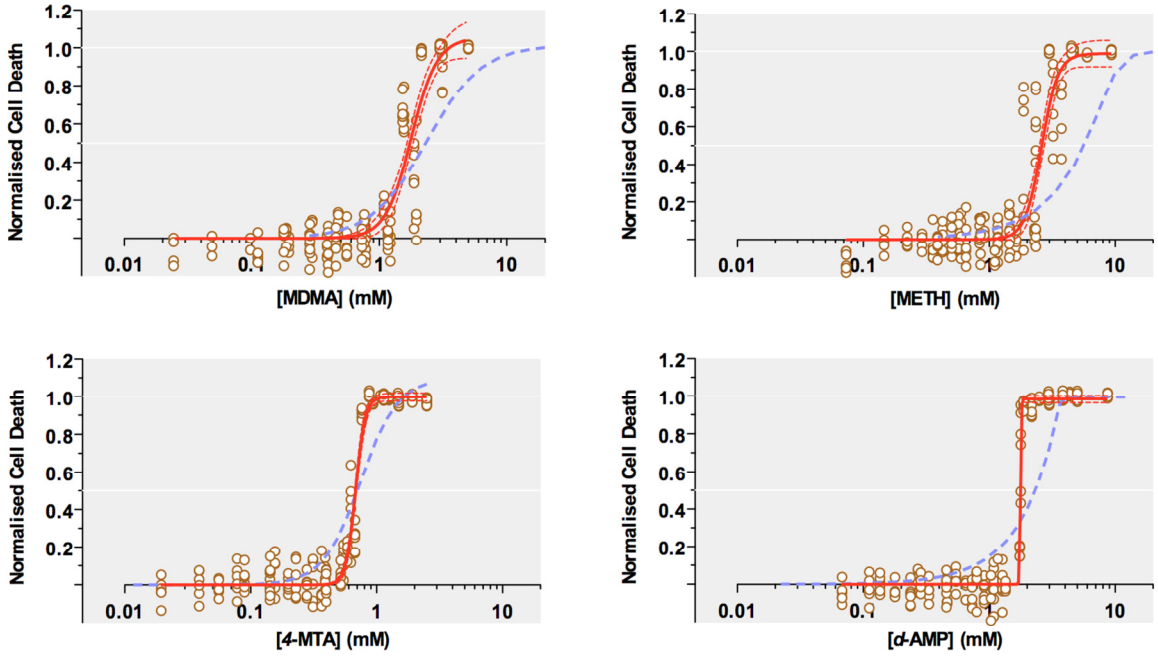

Figure 1. Concentration–response curves obtained with the MTT reduction cytotoxicity assay for all four individual amphetamine mixture components, in HepG2 cells after 48 h incubations at 40.5 °C. The open circles represent data from five independent experiments for MDMA, METH, 4-MTA and *d*-AMP, following 48 h incubations. The red solid lines represent the regression model (logit) and the dashed red lines represent the corresponding 95% CI. The dashed blue lines represent the previously reported data obtained under similar experimental conditions when incubations were performed at normothermic (37 ºC) conditions (Dias da Silva *et al.* 2012).

Table 2. Logit parameters for the test amphetaminic agents in the MTT reduction assay, at 40.5 °C.

Drug	Estimated parameters for the best-fit regression model of each individual agent		EC_{50} (mM)	Relative proportion in mixture (%)
	$^{0}1$	$^{0}2$		
MDMA	-4.42E+00	1.78E+01	1.77E+00	20.94
METH	-9.74E+00	2.38E+01	2.97E+00	49.39
4-MTA	6.12E+00	3.39E+01	6.60E-01	6.96
d-AMP	-5.20E+00	2.37E+01	1.66E+00	22.71
Mixture	-9.84E+00	2.94E+01	2.06E+00	100

Amphetamines interact with each other to produce significant additive effects at hyperthermic incubations

Based on the individual information of the single agents, the mathematical models CA and IA were applied to predict the additive mixture effects of the four amphetamines for the complete concentration–response range. The computed data yielded well-discriminated predictions, enabling a good validation of the most suitable model in predicting the joint effects of amphetamines, under hyperthermic conditions (figure 2). We then tested the mixture experimentally. As formerly described in the mixture design used in this study, the 4 drugs were combined in proportion to their EC_{50}, such that they were present in the mixture at equieffective concentrations. The composition of the mixture analysed in the experimental studies is provided in table 2.

As shown in figure 2, the slope for each prediction model was similar. However, the curve according to CA was shifted to lower concentrations, assuming greater combination effects than with the IA model.

When the mixture was experimentally tested, the obtained data revealed low variability and the observed responses were well estimated by CA, up to concentrations producing 50% mortality.

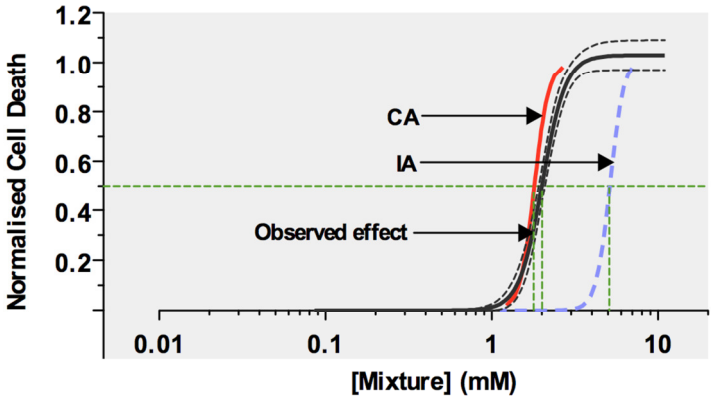

Figure 2. Predicted and observed effects obtained with the MTT reduction cytotoxicity assay for an equipotent mixture of MDMA, METH, 4-MTA and *d*-AMP in HepG2 cells, after 48 h incubations at 40.5 °C. Best-fit of observed mixture effects (black solid line) is from six independent experiments, with dashed black lines indicating the 95% CI. Predicted effects were calculated using the models of *concentration addition* (CA; solid red line), and *independent action* (IA; dashed blue line). Dotted green lines indicate the EC_{50} of predicted and experimentally observed concentration–response curves: observed mixture effect - EC_{50} 2.06 mM; CA - EC_{50} 1.91 mM; and IA - EC_{50} 5.14 mM.

The EC_{50} value of the experimentally tested mixture was 2.06 mM and the estimated value according to CA was 1.91 mM, proving good prediction power. On the other hand, IA predicted a much higher EC_{50} value of 5.14 mM, clearly underestimating the mixture effects and proving inappropriateness to predict the joint effects of amphetamines under our experimental conditions. At concentrations higher than the EC_{50}, the curve obtained with the experimental data deviated from CA, with a minimal shift to the right, suggesting a potential slight antagonism.

Amphetamines, individually and in combination, generate reactive species in a concentration- and temperature-dependent manner

Following environmental stress, such as that elicited by cytotoxic agents or overheating, some cellular redox modifications may happen through the production of peroxides and free radicals. Nonetheless, the cells contain complex antioxidant defense mechanisms to counteract the risk of oxidative injury. When the generation of reactive species (RNS/ROS) exceeds the capability of the cell machinery to scavenge and detoxify the reactive intermediates, oxidative stress arises with the damage of vital cellular structures, such as nucleic acids, proteins, and lipids (Powers and Lennon 1999). The existing literature suggests that oxidative burst is implicated in the cytotoxicity elicited by amphetamine derivatives (Barbosa et al. 2012; Carvalho et al. 2002a; Cerretani et al. 2011). So, in order to elucidate the mechanisms underlying the observed hepatotoxicity in the MTT assay, we examined the involvement of oxidative/nitrosative stress in the effects of the tested chemicals. As shown in figure 3, the DCFH assay revealed that 24 h after normothermic incubations, all 4 drug treatments significantly increased the formation of reactive species in a concentration-depend manner, being 4-MTA the most effective. These effects were significantly potentiated when the drugs and the mixtures were incubated at 40.5 °C (figure 3). Significant differences in the ROS and RNS intracellular levels were also observed ($p<0.05$) between control incubations at 37 ºC (39939 ± 8096 fluorescence units) and at 40.5 ºC (43768 ± 13045 fluorescence units), showing that an increase in temperature had an impact on ROS and RNS formation *per se*.

With the aim of directly comparing the individual potencies of each drug at 40.5 ºC, as well as the effects of the individual chemicals with those of the mixture, we focused on the effects elicited by the individual EC_{50} under hyperthermia, *i.e.*, those concentrations that elicited 50% cell death in the MTT assay. These concentrations were: 1.8 mM for MDMA, 3.0 mM for METH, 1.7 mM *d*-AMP, 0.7 mM 4-MTA and 2.1 mM for the mixture. The obtained data show that, at these equieffective cytotoxic concentrations, MDMA and METH induced a similar effect in ROS/RNS increase over controls (2.25 ± 0.08 and 2.50 ± 0.09, respectively), while *d*-AMP and 4-MTA proved to be much more potent, inducing 3.72 ± 0.11 and 3.86 ± 0.08 fold increases, respectively. The mixture exhibited an intermediate response yielding a 3.01 ± 0.08 fold increase over control values.

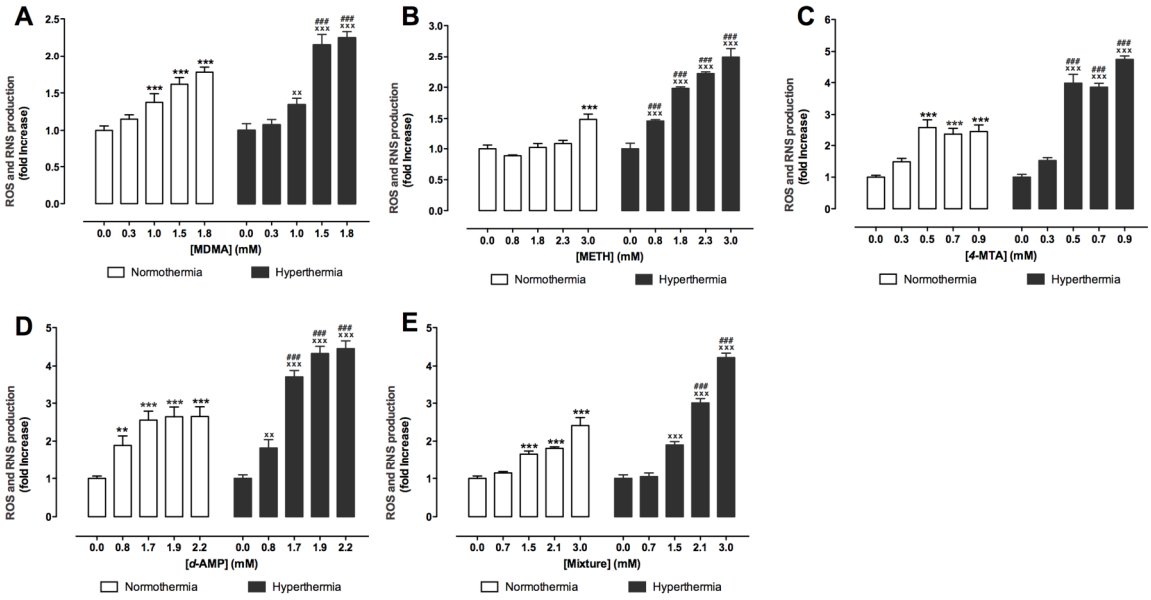

Figure 3. Effect of MDMA (A), METH (B), 4-MTA (C), *d*-AMP (D) and mixture (E) on reactive species (ROS and RNS) production in HepG2 cells, after 24 h incubations at 37 ◦C (white bars) or 40.5 ◦C (black bars). Results represent the mean ± SEM of the fluorescence emitted when 2',7'-dichlorodihydrofluorescein (DCFH) reacts with ROS and RNS and were normalized to the control (fluorescence of non-treated cells was set to 1). Data are from at least 4 independent experiments, run in triplicate. Statistical comparisons were made by one-way ANOVA followed by the Tukey's multiple comparison *post hoc* test. ***p*<0.01, *** *p*<0.001, compared with control at 37 ◦C; xx *p*<0.01, xxx *p*<0.001, compared with control at 40.5 ◦C; ###*p*<0.001, compared with the same concentration at 37 ◦C.

Amphetamines, individually and in combination, induce a concentration-dependent decrease in reduced and total intracellular glutathione, an effect that is exacerbated at hyperthermic incubations

The presence of GSH in the cytosol and aqueous compartments of mitochondria is essential to counteract the detrimental effects of free radicals in the cell. This tripeptide has several antioxidant functions, including the scavenging of a variety of radicals and the reduction of a diversity of cellular antioxidants. Acting as a co-substrate of glutathione peroxidase (GPx) it contributes to the elimination of H_2O_2 and other peroxides. By donating a pair of hydrogen ions, GSH is oxidized into GSSG and the regeneration into GSH is catalyzed by glutathione reductase (GR) using NADPH as the reducing substrate (Meister 1995).

As depicted in figure 4, the results from our study on GSH and GSSG intracellular levels indicate that there were no significant alterations in the intracellular levels of glutathione disulfide between the control incubations at 37 ºC (6.78 ± 0.36 nmol GSSG/mg protein) and at 40.5 ºC (5.84 ± 0.40 nmol GSSG/mg protein). Also, no significant differences were observed in GSSG levels for any of the drug incubations at both incubation temperatures. These observations were expected, as GSSG in the liver is maintained at very low levels to keep the intracellular ratio of GSH/GSSG high and minimize GSSG toxicity (Kretzschmar et al. 1992).

After the disulfide production, the cells excrete GSSG to the extracellular space (Leier et al. 1996).

A significant decline in reduced GSH, and consequently, in the total glutathione levels (tGSH), was observed in a concentration-dependent fashion, when HepG2 cells were incubated with the test drugs at 37 ^{o}C (figure 4A). In fact, significant decreases from control values (97.9 ± 7.5 nmol tGSH/mg protein) were observed for all drugs and for the mixture. These effects were significantly exacerbated at 40.5 ºC incubations, as can be seen in figure 4 (B). For all test drugs and the mixture, tGSH levels were significantly lower than those obtained with the same concentrations at 37 ºC incubations, including the control incubations (78.7 ± 4.1 nmol tGSH/mg protein, p<0.05 *versus* control at 37 ºC).

At EC_{50} in the MTT assay (40.5 ºC), METH induced the smallest decrease in tGSH levels to 37.4 ± 1.8 nmol tGSH/mg protein. The drugs MDMA and 4-MTA displayed very similar potencies, with MDMA inducing a decrease in tGSH levels to 10.2 ± 0.9 nmol tGSH/mg protein and 4-MTA down to 11.5 ± 1.6 nmol tGSH/mg protein. Both *d*-AMP and the mixture exhibited an intermediate effect by inducing a decrease down to 13.6 ± 1.1 nmol tGSH/mg protein and 14.1 ± 3.5 nmol tGSH/mg protein, respectively.

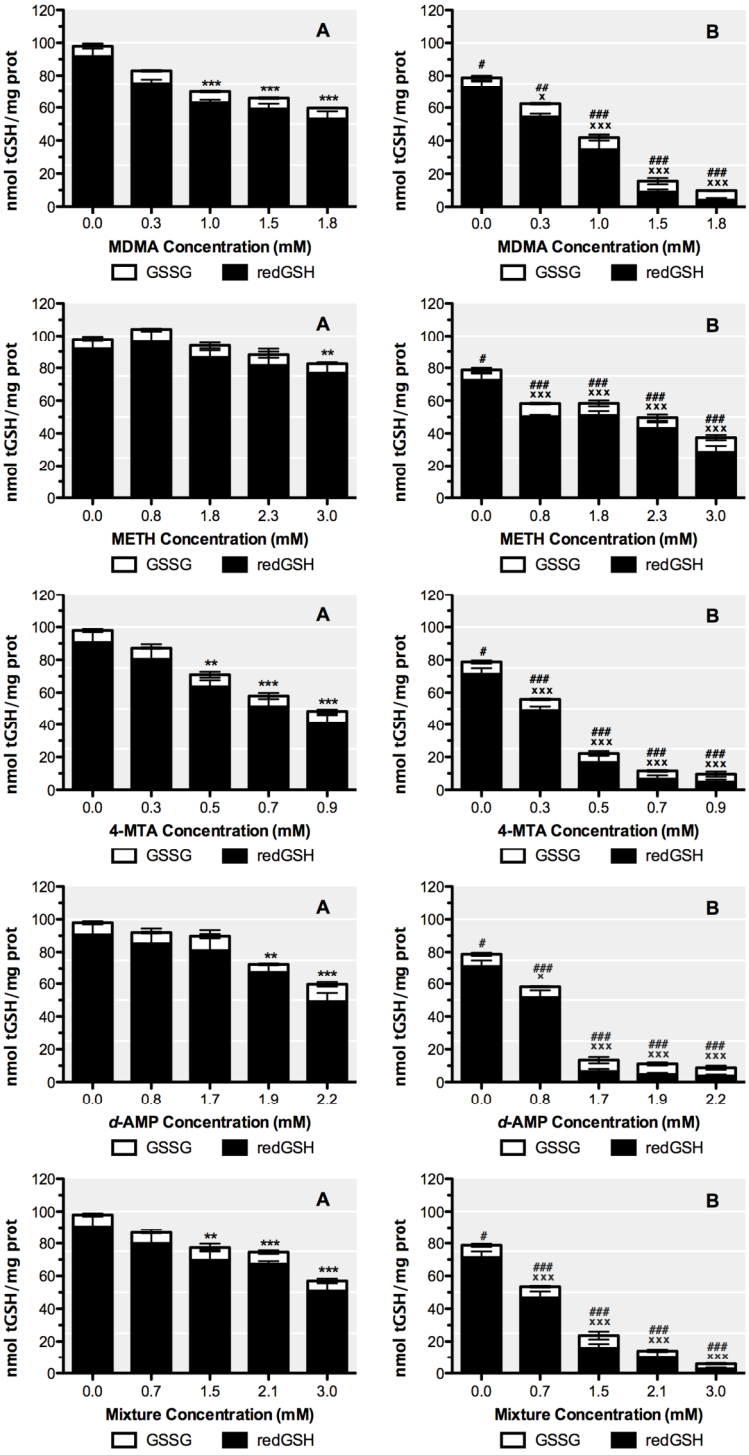

Figure 4. Effect of MDMA, METH, 4-MTA, *d*-AMP, and mixture on the total glutathione (tGSH), reduced glutathione (redGSH) and glutathione disulfide (GSSG) contents in HepG2 cells, after 24 h incubations at 37 ◦C (A) or 40.5 ◦C (B). Results represent the mean ± SEM and are from at least five independent experiments, run at least in triplicate. Statistical comparisons were made by one-way ANOVA followed by the Tukey's multiple comparison *post hoc* test. **p*<0.05, ***p*<0.01, *** *p*<0.001, compared with control at 37 ◦C; x *p*<0.05, xx *p*<0.01, xxx *p*<0.001, compared with control at 40.5 ◦C; ###*p*<0.001, compared with the same concentration at 37 ºC.

Amphetamines, individually and in combination, altered the intracellular energetic status, particularly at hyperthermic incubations

ATP, the key intermediate for energy exchange, is considered the 'primary energy currency' in living cells (Knowles 1980). ATP serves as a means for storing energy and its break down, with consequent energy release, is engaged in a variety of cellular activities, including cellular energetics, metabolic regulation and signalling. Since all cells require ATP to remain alive and carry out their specific function and, because ATP is transiently depressed by many forms of cellular stress, its levels reflect the functional integrity of viable cells.

The observed changes in intracellular ATP levels are presented in figure 5.

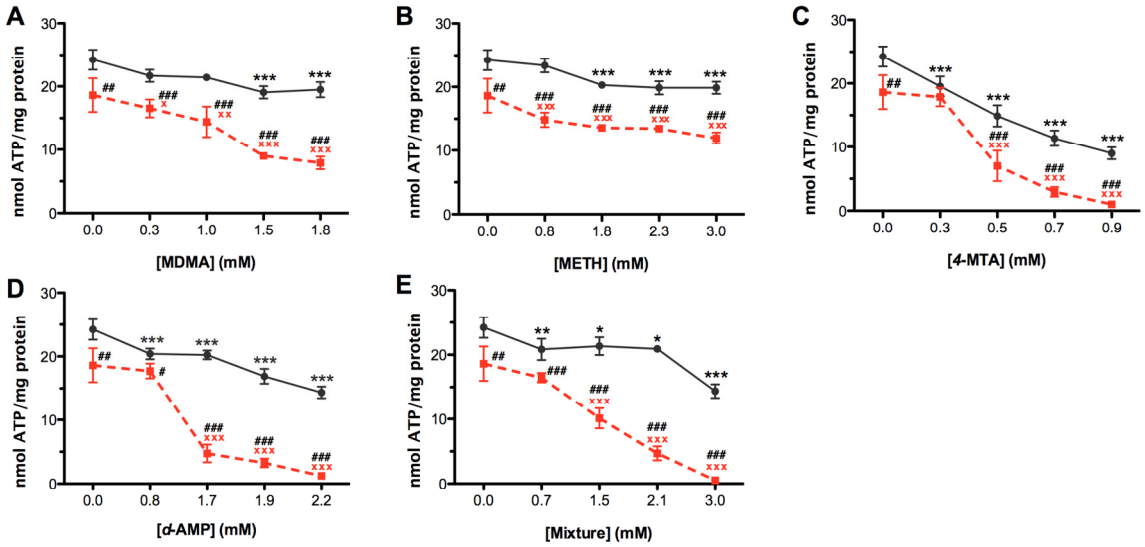

Figure 5. Effect of MDMA (A), METH (B), 4-MTA (C), *d*-AMP (D) and mixture (E) on ATP intracellular content in HepG2 cells, after 24 h incubations at 37 ◦C (dark solid line) or 40.5 ◦C (dashed red line). Results represent the mean ± SEM and are from at least four independent experiments, run in triplicate. Statistical comparisons were made by one-way ANOVA followed by the Tukey's multiple comparison *post hoc* test. **p*<0.05, ***p*<0.01, ****p*<0.001, compared with control at 37 ◦C; x *p*<0.05, xx *p*<0.01, xxx *p*<0.001, compared with control at 40.5 ◦C; #*p*<0.05, ##*p*<0.01, ###*p*<0.001, compared with the same concentration in normothermia.

Control values were 24.23 ± 0.30 nmol ATP/mg protein and 18.62 ± 0.50 nmol ATP/mg protein at 37 ºC and 40.5 ºC incubations, respectively (p<0.01 *versus* control at 37 ºC). At 37 ºC, significant decreases in ATP intracellular levels were observed for 4-MTA, *d*-AMP and mixture incubations with all tested concentrations. Smaller, but still significant ATP decreases could also be observed for MDMA and METH incubations.

At median cytotoxic effect concentrations (EC_{50} values in the MTT assay) tested at 40.5 ºC, 4-MTA significantly decreased the ATP cellular levels (3.0 ± 0.8 nmol ATP/ mg protein) and was the most potent drug, while higher values were observed for MDMA and METH (7.9 ± 1.0 nmol ATP/ mg protein and 12.0 ± 0.9 nmol ATP/ mg protein, respectively). Intermediate values were obtained for *d*-AMP (4.8 ± 0.7 nmol ATP/ mg protein) and mixture (4.7 ± 1.1 nmol ATP/ mg protein).

Amphetamines did not induce lipid peroxidation in HepG2 cells, neither at normothermic nor hyperthermic incubations

Due to their high reactivity, free radicals can interact with the lipids of cell membranes, resulting in their oxidative degradation, a process known as lipid peroxidation, which can result in destruction of the cell membrane. The end-products of lipid peroxidation, such as malonildialdehyde (MDA), are also injurious and can react with intracellular targets including DNA, forming adducts (Marnett 1999).

After 24 h of incubations at 37 ºC and at 40.5 ºC no detectable signs of modification in the formation of MDA (data not shown), a general indicator of the decomposition of polyunsaturated fatty acids, were observed for any of the tested concentrations of the amphetamines and the mixture as determined by the TBARS assay.

Mitochondrial function of HepG2 cells is severely affected by amphetamines incubation, individually and in combination, at 40.5 ºC

$\Delta\psi_m$ is crucial for maintaining the physiological function of the mitochondrial respiratory chain, a process responsible for ATP generation. A significant loss of $\Delta\psi_m$ impairs oxidative phosphorylation, depleting cells of energy and inducing cell death. Therefore, the determination of $\Delta\psi_m$ by the TMRE mitochondrial inclusion assay provides important information about the mitochondrial function and the physiological status of the cell.

When incubated at 37 ºC, the cells suffered $\Delta\psi_m$ dissipation only at the highest concentration tested (down to 89.2 ± 2.3% of control for 1.8 mM MDMA, 36.8 ± 5.5% of control for 0.9 mM 4-MTA, 69.8 ± 2.7% of control for 2.2 mM *d*-AMP, and 48.9 ± 8.5% of control for 3 mM mixture) (figure 6).

Mitochondrial damage was significantly aggravated under hyperthermic conditions, as significant differences were observed in response to lower concentrations of the tested drugs.

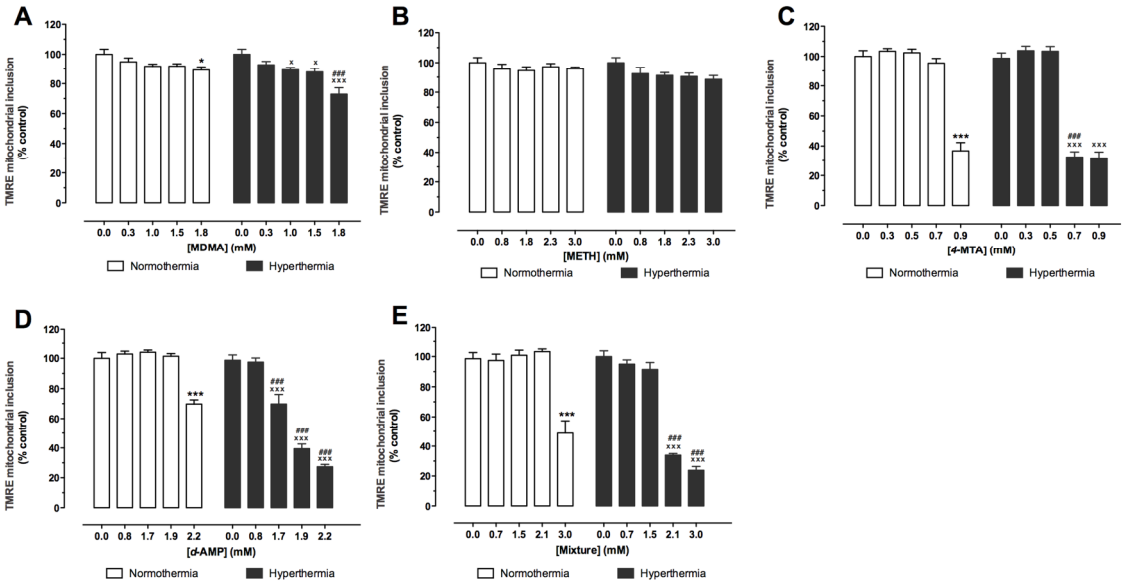

Figure 6. Effect of MDMA (A), METH (B), 4-MTA (C), *d*-AMP (D) and mixture (E) on the amount of tetramethylrhodamine ethyl ester (TMRE) incorporated within the mitochondria as a measure of mitochondrial membrane integrity, after 24 h incubations at 37 ◦C (white bars) or 40.5 ◦C (black bars). Data are expressed in percentage of control at 37 ◦C or 40.5 ◦C (fluorescence of non-treated cells set to 100%) and represented as the mean ± SEM. Results were obtained from four independent experiments run in six replicates. Statistical comparisons were made by one-way ANOVA followed by the Tukey's multiple comparison *post hoc* test. **p*<0.05, *** *p*<0.001, compared with control at 37 ◦C; x *p*<0.05, xxx *p*<0.001, compared with control at 40.5 ◦C; ###*p*<0.001, compared with the same concentration in normothermia.

At 40.5 ºC and for the highest concentrations tested, the following values were observed: 72.9 ± 4.3% of control for 1.8 mM MDMA; 31.8 ± 4.1% of control for 0.9 mM 4-MTA; 27.4 ± 1.6% of control for 2.2 mM *d*-AMP; and 23.8 ± 2.6% of control for 3 mM mixture). No significant differences were identified (p>0.05) between control incubations at 37 ºC (45875 ± 11516 fluorescence units) and at 40.5 ºC (47364 ± 14120 fluorescence units) highlighting that an increase in temperature in the absence of amphetamine treatment had no impact on mitochondrial function.

For the 50% effective concentrations in the MTT assay, the results show that MDMA and *d*-AMP were the least effective in generating significant mitochondrial disruption (with decreases in TMRE inclusion down to 72.9 ± 4.3% of control and 69.8 ± 6.3% of control, respectively). Notwithstanding, 4-MTA and the four-component mixture were able to produce greater effects (down to 32.6 ± 3.5% of control and 34.0 ± 1.1% of control, respectively). METH, by itself, did not have a significant effect on $\Delta\psi_m$, since no changes in TMRE inclusion in relation to control values were detected at 37 ºC or 40.5 ºC incubations for any of the tested concentrations of the drug.

Discussion

Remarkable interindividual differences in the toxicity of amphetamine-like drugs, including MDMA, have been reported (Carmo et al. 2004; Carmo et al. 2005; deLetter et al. 2004; Henry et al. 1992a; O'Donohoe et al. 1998). However, it is still unclear why, under similar circumstances, the consumption of this drug can either induce no noticeable clinical symptoms or result in life-threatening and lethal complications, even when the drug is taken in relatively small amounts (1 or 2 tablets on a single occasion) (Coore 1996b; Mueller and Korey 1998). Among the most probable contributing factors that have been put forward to explain these random toxicity events are the concomitant intake of other drugs and the hot, crowded, dance clubs conditions which can enhance the thermogenic actions of amphetamines (Green et al. 2004). Understanding the influence of drug combinations and environmental factors on *ecstasy* toxicity, as well as clarifying the mechanisms involved in their toxicological effects is, therefore, crucial to predict and consequently prevent random toxicity events.

Nevertheless, only a scarce number of studies has so far addressed the investigation of the putative interactions between amphetamines in mixture (Clemens et al. 2007; Clemens et al. 2005; Clemens et al. 2004). Also, these studies are often impaired by the lack of adequate study models that can accurately reflect the human situation and bring together factors, such as combination effects and hyperthermia. In recently published work, Dias da Silva and colleagues (2012) were able to demonstrate that in an *in vitro* model using immortalized human hepatoma HepG2 cells, four amphetamines that are frequently co-ingested in *ecstasy* pills (4-MTA, *d*-AMP and METH and MDMA) act together to produce significant cytotoxic mixture effects, even when combined at very low, ineffective concentrations (Dias da Silva et al. 2012). This work clearly demonstrated that potentially harmful interactions among amphetamines can be expected when these drugs are taken concomitantly.

In the present study, we took this approach one step further and showed, for the first time, that the same four amphetamines act together to produce significant mixture effects, and that these effects are further exacerbated when the temperature is raised from physiological (37°C) to hyperthermic (40.5 °C) levels. An example of these observations can be provided by using the concentration of the four-component mixture that prompts 50% of effect (EC_{50} 2.9 mM). At this concentration (37°C), all four individual components were present in the mixture at concentrations that individually yielded effects indistinguishable from controls (below 6% of maximal responses). Yet, when combined, they were able to induce 50% of cell mortality, indicating a significant additive mixture effect. Strikingly, when this same concentration (2.9 mM) of the same mixture was tested in the same *in vitro* system, but at a temperature of 40.5 ºC, it induced 97.7% mortality, substantiating an astonishing exacerbation of toxicity. Thus, this mixture effect that was significant at 37 ºC was greatly exacerbated under hyperthermic settings.

In terms of our ability to predict mixture toxicity in hyperthermia, of note was the excellent discrimination achieved between the expectations according to *CA* and *IA* at 40.5 ºC, which allowed an accurate determination of the model that better described the experimental outcomes. Similarly to what was observed at 37 °C, the model of CA provided accurate estimations of amphetamine combination effects at 40.5 °C, proving that the tested chemicals acted together in an additive fashion (Dias da Silva et al. 2012). Since the CA model assumes that all our mixture components operate in a similar manner and that each individual drug contributes to the overall mixture effect in proportion to its concentration, we can expect that the same overall effect will be observed if one constituent were totally or partially replaced by an equieffective concentration of another drug. As a consequence, significant mixture effects can occur even when the drugs are mixed at individually ineffective concentrations. It is, therefore, plausible that low levels of amphetamines present in *ecstasy* pills can produce adverse effects by virtue of its additive mixture effects.

In spite of the excellent agreement between CA and experimental data, a very slight deviation from additivity was detected at higher effect concentrations, indicating a weak antagonism. This was also previously observed with incubations at 37 ºC and was attributed to a possible metabolic interaction since at higher mixture concentrations an increase in the metabolic conversion into MDA, a metabolite that retains pharmacological action but is less cytotoxic than MDMA (Carvalho et al. 2002b; Johnson et al. 1988), was observed (Dias da Silva et al. 2012). Yet, this hypothesis awaits experimental confirmation, since additional factors may be involved such as the availability of the drugs at the binding sites, or unspecific serum protein binding that may be altered due to the combination of the mixture components.

The inappropriateness of the IA model for the assessment of the joint effects of these drugs in the MTT assay was shown by the model's significant underestimation of the experimentally observed mixture toxicity. This further suggests that there is, in fact, a similarity in the way in which these agents lead to HepG2 cell death. Accordingly, an overall analysis of our data on the oxidative stress, energetic status, and mitochondrial dysfunction of hepatocytes exposed to the drugs either individually or in mixture showed that, for all evaluated parameters, the mixture exhibited a similar response profile when compared with the individual drug treatments.

A remarkable feature of the cytotoxicity curves obtained at 40.5 ºC was the steepness of the concentration–response relationships for all drugs, meaning that, in the linear part of the curve, very small increases in the concentrations of the test chemicals elicited substantial increases in cytotoxicity and, consequently, the differences between concentrations inducing no noticeable effects and those leading to significant toxicity were extremely small. This was not observed when the incubation temperature was set at 37 °C, suggesting that this was a temperature-related event. Consequently, it could be hypothesized that, in an *in vivo* situation, the 'safety window' of these drugs would become narrower and the associated hazards increased under hyperthermia.

Our data also agrees well with previous studies that have provided experimental support for the influence of hyperthermia in the severity of the cytotoxic effects that MDMA produces in the target organs, namely in the liver (Carvalho et al. 2001;

Carvalho et al. 2002a; Pontes et al. 2010; Pontes et al. 2008a). It has been postulated that hyperthermia *per se* induces a pro-oxidant state (Flanagan et al. 1998; Skibba et al. 1991) that may aggravate the oxidant effects of amphetamines (Carvalho et al. 2001; Carvalho et al. 2002a; Pontes et al. 2010; Pontes et al. 2008a) and that amphetamines, directly or through the production of reactive metabolites, are able to disturb the pro-oxidant/antioxidant cellular status, in favour of the first, in several systems (Barbosa et al. 2012; Carvalho et al. 2004; Cerretani et al. 2011). Our data support such mechanistic interactions, given that our results revealed a concentration-dependent generation of ROS/RNS and a significant decrease in GSH intracellular levels, when HepG2 cells were exposed to the test amphetamines both individually and in combination. Hepatotoxicity provoked by hyperthermia has been previously linked to lipid peroxidative damage as a consequence of oxidative stress (Skibba et al. 1991; Skibba et al. 1990) and was observed in freshly isolated rat hepatocytes incubated with amphetamines (Carvalho et al. 1997a; Carvalho et al. 2001), but we did not observe this end-effect of oxidative stress. In addition, we did not observe an intracellular change in GSSG levels since, once formed, it is extruded to the extracellular medium (Leier et al. 1996). It cannot be, therefore, excluded that the observed decrease in tGSH and reduced GSH levels result at least partially from the loss due to the high reactivity of the amphetamine metabolites that can conjugate with GSH (Bindoli et al. 1992; Carvalho et al. 1997b; Hiramatsu et al. 1990) and not only from oxidation into GSSG.

Overall, all the tested stress endpoints were greatly intensified when the incubation temperature was increased. Accordingly, the effect of hyperthermia was particularly notorious in the dramatic depletion of intracellular stores of ATP that was observed for the amphetamines individually, as well as in combination. The consequent mitochondrial dysfunction was also confirmed by the loss in $\Delta\psi_m$, at 40.5 ^oC. The similarities between the magnitude of effects elicited by the amphetamines alone and combined suggest a similarity in the mechanism of action of the individual drugs, which is maintained in a mixture setting. In other words, as the concentrations tested were equieffective in terms of cytotoxicity and for this endpoint additivity was confirmed, a similar equieffectiveness for the remaining endpoints, suggests the drugs still act in a similar way and the mixture remains additive. On the other hand, it is important to highlight that the individual concentrations of the drugs in the mixture are much lower than those required to produce the same effect when tested individually. As an example, the 1.5 mM concentration of the mixture for which significant effects were noted in the majority of the tested endpoints (both at normothermia and hyperthermia), contains 0.399 mM MDMA, 0.578 mM METH, 0.149 mM 4-MTA, and 0.374 d-AMP. At these individual concentrations, no effect would be noted. These data demonstrates that the simultaneous drug abuse under hyperthermia can induce toxic effects that are much higher than those that could be anticipated based on the individual actions of the drugs.

Concluding, our study brings experimental support to the concerns raised by the amphetamine polydrug abuse especially when body temperature is increased, either due to the thermogenic action of the drug or due to the influence of high environmental temperatures, or both simultaneously. Taking into account the potential impact of each (hyperthermia and joint effects) individually, severely increased toxicity might be anticipated in the corresponding misuse scene, but the combination of both has the potential to dramatically aggravate the severity of the intoxications. For accurate *in vivo* extrapolations further investigation is required but an alarming picture of the potential risks of *ecstasy* abuse is already exposed.

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Conflict of Interest statement

No conflicts declared.

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Chapter III. Cytotoxic effects of amphetamine mixtures in primary hepatocytes are severely aggravated under hyperthermic conditions.

*S*ubmitted to *Toxicology in vitro*.

Cytotoxic effects of amphetamine mixtures in primary hepatocytes are severely aggravated under hyperthermic conditions

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Abstract

Amphetamine consumers are often, deliberately or not, polydrug abusers. Predicting combination effects based on concentration–response analysis of individual components has revealed to be a valid strategy for accurate toxicological assessment of mixtures. We previously reported that the deleterious joint effects of *3,4*-methylenedioxymethamphetamine (MDMA, *ecstasy*) and three other often co-ingested amphetamines (methamphetamine, *4*-methylthyoamphetamine and *d*-amphetamine) could be predicted by *concentration addition (CA)* model in HepG2 cells. We sought to further evaluate the relevance of these findings by extending these studies to a primary cell model that more closely mimics *in vivo* situation.

Detailed cytotoxic information of the four individual amphetamines on primary rat hepatocytes was recorded by the MTT assay, under physiological (37 ^{o}C) and hyperthermic (40.5 ^{o}C) conditions. The latter simulates the rise in body temperature induced by amphetamines intake. Mixture expectations were calculated using the CA and *independent action (IA)* models.

Combined effects were accurately predicted by CA, while IA underestimated the observed toxicity. Incubation at 40.5 ºC further aggravated the cytotoxic effects. Our findings provide evidence of the increased risks associated with the abuse of amphetamine mixtures, especially under hyperthermia, and emphasize the need to increase the awareness of misinformed users who believe these drugs are safe.

Keywords

Amphetamine-related toxicity; Primary hepatocytes; Polydrug abuse; *Concentration addition* (CA); *Independent action* (IA); Hyperthermia.

Introduction

Reports of acute intoxications related with amphetamine abuse have been increasing in recent years (De Letter *et al.*, 2004; De Letter *et al.*, 2001; Lin *et al.*, 2011; Papaseit *et al.*, 2012; Sano *et al.*, 2009; Schifano, 2004; Vanden Eede *et al.*, 2012). In view of the random nature of the toxic effects of *3,4*-methylenedioxymethamphetamine (MDMA, *ecstasy*) in *ecstasy* abusers (Milroy, 2011) and the large discrepancy between the blood concentrations found in intoxicated individuals and the magnitude of the induced toxicity (Coore, 1996; Milroy *et al.*, 1996; Ramcharan *et al.*, 1998), possible health risks based solely on exposure to the drug are difficult to explain. Consequently, it is plausible that other factors must be at play.

One of the aspects that have been highlighted as a potential justification to these inconsistencies is the polydrug intake frequently associated with misuse contexts (Carvalho *et al.*, 2012; Carvalho *et al.*, 2010; Mohamed *et al.*, 2011a). Often, uninformed abusers are encouraged to ingest several drugs in combinations with the aim of maximizing the desired effects or ameliorating the unpleasant ones. Also, inadvertent ingestion of therapeutic drugs and contaminants or adulterants present in *ecstasy* party pills is frequent (Schifano, 2004). Since MDMA consumers are exposed to a plethora of other drugs, including other amphetamines (Camilleri *et al.*, 2005; Sherlock *et al.*, 1999; Tanner-Smith, 2006; Teng *et al.*, 2006), which are often involved in fatal intoxications (Carvalho *et al.*, 2012; Carvalho *et al.*, 2010; Mohamed *et al.*, 2011a), the toxicological impact of combined effects of these drugs needs to be considered.

Despite putative interactions being of particular concern and, requiring examination, particularly because they can be lethal, *in vivo* investigation of the multitude of conceivable mixtures is often hampered by ethical, economical, or practical aspects. Recurrently, risk assessment strategies rely on quantitatively estimations of joint effects of substances that are based on the knowledge of individual mixture constituents. In effect, two reference concepts broadly employed for predicting mixture outcomes can be distinguished: the *Loewe additivity* or *concentration addition* (CA), originally introduced by Loewe and Muschnik (1926); and *independent action* (IA), also known as *response addition*, or *Bliss independence,* developed by Bliss (1939).

The first model, *CA*, is based on the principle that the components of a certain mixture have a common site/mechanism of action (Dias da Silva *et al.*, 2013; Silva *et al.*, 2002). It is based on the assumption that, if all mixture components act in a similar way, then the same mixture effect can be obtained by replacing one chemical, entirely or in part, by a portion, with the same effectiveness, of another. Conversely, the competing approach, *IA*, relies on the assumption that the compounds of a given mixture act independently, by acting in different molecular targets and having different modes of action (Backhaus *et al.*, 2000).

In order to evaluate the mixture effects of amphetamines that are often taken concomitantly by *ecstasy* abusers, we have previously studied, in the immortalised hepatocarcinoma HepG2 cell line (Dias da Silva *et al.*, 2013), the cytotoxic effect of MDMA in combination with three other amphetamines, commonly found in *ecstasy* tablets: methamphetamine (METH), *4*-methylthyoamphetamine (4-MTA) and *d*-amphetamine (*d*-Amp) (Camilleri *et al.*, 2005; Sherlock *et al.*, 1999; Tanner-Smith, 2006; Teng *et al.*, 2006). In this *in vitro* system, we showed that CA provided accurate expectations of the mixture effects of amphetamines and revealed that these drugs acted together in an additive fashion to produce significant effects, even when combined at very low levels (Dias da Silva *et al.*, 2013).

Although it is recognizable that an accurate investigation of drug interactions based on the assessment of mixture effects would ideally rely on the integrated use of *in vivo* and *in vitro* systems, the extensive concentration–response descriptions that are required for these studies are currently only feasible at reasonable cost with *in vitro* assays, that provide high through-put testing with minimum biologic variability and maximum reproducibility.

To further investigate the relevance of these findings and overcome some of the limitations of previously used human hepatoma HepG2 cell model, we have now extended our previous investigations to an *in vitro* model that more closely mimics the *in vivo* situation, the rat primary hepatocyte, and compared these two cellular models.

Amphetamines intake is associated with an increase in body temperature (Greene *et al.*, 2003) due to the drugs' thermogenic actions, which are further aggravated by the crowded, hot environments where drug abuse often takes place (Henry *et al.*, 1992a). This hyperthermia has been associated with some of the toxic events linked to *ecstasy* use, including acute liver failure, rhabdomyolysis, and disseminated intravascular coagulation (Armenian *et al.*, 2012; Henry *et al.*, 1992b; Kalant, 2001; Kendrick *et al.*, 1977; Patel *et al.*, 2005; Screaton *et al.*, 1992). Consequently, we set out to also evaluate the impact of raised temperature on the toxicity of our test amphetamines by incubating the primary hepatocytes with the drugs both alone and in combination at a temperature of 40.5 ºC. This aimed at mimicking the hyperthermic state of the

liver, which can occur in a real intoxication scenario.

Materials and Methods

Test drugs

4-Methylthioamphetamine hydrochloride (racemic mixture) was kindly gifted by Doctor David Nichols (Purdue University, West Lafayette, IN). *3,4*-Methylenodioxymethamphetamine hydrochloride was extracted and purified from high purity MDMA tablets provided by the Portuguese Criminal Police Department at REQUI*M*TE/ Department of Biological Sciences, Laboratory of Toxicology, Faculty of Pharmacy, University of Porto. The obtained salt was pure and fully characterized by nuclear magnetic resonance (NMR) and mass spectrometry (MS) methodologies. *d*-Amphetamine (*d*-AMP) sulfate) was purchased from Tocris Bioscience (Bristol, UK) and *(+)*-METH hydrochloride (98% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used were of analytical grade and were employed as supplied. Master stock solutions of all amphetamines and mixtures were prepared in ultra-pure sterile water and were at least 20 times more concentrated than the highest concentration tested, to prevent media dilution. All stock solutions were preserved at –20 °C and subsequent dilutions were freshly prepared in cell culture media before each experiment.

Primary culture of cryopreserved rat hepatocytes

High quality plateable cryopreserved male Wistar rat hepatocytes were purchased from Invitrogen Corporations (Paisley, UK). The vials were thawed in a water bath at 37 ^{o}C, until ice disappeared and the hepatocyte suspension was gently transferred into 45 ml of thawing/plating medium containing Williams' medium E (1x) without L-glutamine and phenol red, supplemented with 5% fetal bovine serum (FBS), 10 µM dexamethasone solution in DMSO, 100 U/mL penicillin-streptomycin, 4 mg/L bovine insulin, 1% GlutaMAX™ 100x and 15 mM HEPES pH 7.4. After centrifuging cells at room temperature at 55 x g for 3 min, the supernatant was carefully aspirated and the pellet gently resuspended in approximately 5 ml of the thawing/plating medium. The viability of the isolated hepatocytes was estimated by the trypan blue exclusion test and was always greater than 80%. Subsequently, a suspension containing 0.5 x 10^6 viable cells/ml was prepared in thawing/plating medium and seeded onto the central 60 wells of collagen-coated clear 96-well culture plates at a density of 8 x 10^4 viable cells/cm^2. The peripheral wells were filled with sterile water to prevent media evaporation. The cells were incubated at 37 ^{o}C in a humidified atmosphere containing 5% CO_2/95% air, for cell adhesion. After 4-6 h the medium was replaced and the cells incubated overnight at 37 ^{o}C in the maintenance medium containing Williams' medium E (1x) without L-glutamine, phenol red or FBS, and supplemented with 10 µM dexamethasone solution in DMSO, 50 U/mL penicillin-streptomycin, 1% ITS+ (insulin, transferrin, selenium complex, BSA, linoleic acid), 1% GlutaMAX™ 100x and 15 mM HEPES pH 7.4pH 7.4. The experiments were carried out 24 h later.

The MTT reduction viability assay

The mitochondrial reductases of viable cells reduce the *3*-(*4,5*-dimethylthiazol-*2*-yl)-*2,5*-diphenyl tetrazolium bromide (MTT) dye to purple formazan crystals that are spectrophotometrically quantified to produce a measure of cell viability (Mosmann, 1983). The protocol described by Dias da Silva *et al.* (2013) was followed with some modifications. Briefly, 24 h after cell seeding, the maintenance media was gently aspirated and the cells were incubated for 48 h, at 37 ^{o}C or 40.5 ^{o}C with the selected concentrations of MDMA, METH, 4-MTA, *d*-AMP or mixture, prepared in fresh cell maintenance medium. Each individual plate included six replicates of negative controls (*i.e.*, no test agents) and three replicates of positive controls (full media with 1%Triton X-100). To obtain complete concentration–response, a broad range of concentrations was tested for each individual drug and for the mixture, at least in triplicate, in a minimum of three independent experiments. After 48 h, the incubation medium was removed and the attached cells rinsed with 200 µL HBSS (pH 7.4), followed by the addition of fresh maintenance medium containing 0.5 mg/L MTT. The cells were incubated at 37 °C in a humidified environment, with 5% CO_2/95% air atmosphere, for 3 h. Finally, the cell culture medium was removed and the formed insoluble formazan crystals dissolved in 100% DMSO. The spectrophotometric analysis was run at 570 nm using a multi-well plate reader (Labsystems Multiskan, Basingstoke, UK). To reduce inter-experimental variability, data were normalized on a plate-by-plate basis and scaled between 0% (negative controls) and 100% effect (positive controls). Results from at least three independent experiments were graphically presented as the percentage of cell death *versus* concentration (mM).

Regression modelling

Scatter plots of normalized cellular mortality values (effect) *versus* log concentration were constructed

and analysed through the best-fit approach (Scholze *et al.*, 2001). For each individual data set, the model that best fitted the experimental values was selected from a range of nine different two or three-parametric nonlinear regression models (including the commonly used probit, Gompertz, and Hill models). This selection was accomplished on the basis of the sum of absolute errors and an analysis of residues, performed on the specialised software program NLREG – Nonlinear Regression, version 5.4 (Phillip H. Sherrod, USA). In the present study, the generalised logit function was employed, as described by Dias da Silva *et al.* (2012).

Table 1. Parameters derived from nonlinear fits of single agent concentration–response data to the asymmetric logit function, in the MTT reduction assay, at normothermic conditions, 37 ºC (Figure 1). These parameters were used to compute the predicted mixture effect curves shown in Figure 4.

Compound	Tested concentration range (mM)	Estimated parameters for the best-fit regression model			Fraction in the mixture
		θ1[1]	θ2[2]	θmax[3]	
MDMA	6.64E-02 – 1.7E+01	-5.18E+00	1.28E+01	1.04E+02	2.82E-01
METH	6.64E-02 – 1.7E+01	-1.06E+01	1.75E+01	1.03E+02	4.52E-01
4-MTA	9.76E-03 – 2.5E+00	6.23E+00	2.06E+01	1.07E+02	5.52E-02
d-Amp	3.91E-02 – 1.0E+01	-4.36E+00	1.58E+01	1.02E+02	2.11E-01
Mixture	4.29E-02 – 1.1E+01	-3.64E+00	1.35E+01	1.00E+02	1.00E+00

[1]Location parameter of the Logit function. [2]Slope parameter. [3]Maximal effect, expressed as corrected % cell death

Table 2. Parameters derived from nonlinear fits of single agent concentration–response data to the asymmetric logit function, in the MTT reduction assay, at hyperthermic conditions, 40.5 ºC (Figure 2). These parameters were used to compute the predicted mixture effect curves shown in Figure 4.

Compound	Tested concentration range (mM)	Estimated parameters for the best-fit regression model			Fraction in the mixture
		θ1[1]	θ2[2]	θmax[3]	
MDMA	9.07E-02 – 10E+01	1.36E+00	7.67E+00	9.98E+01	2.10E-01
METH	9.07E-02 – 10E+01	-1.37E+00	8.64E+00	9.69E+01	4.62E-01
4-MTA	1.17E-02 – 1.5E+00	1.10E+01	1.96E+01	9.62E+01	8.72E-02
d-Amp	7.25E-02 – 8E+00	2.07E+00	1.69E+01	9.58E+01	2.41E-01
Mixture	6.35E-02 – 7E+00	6.24E+00	3.70E+01	9.61E+01	1.00E+00

[1]Location parameter of the Logit function. [2]Slope parameter. [3]Maximal effect, expressed as corrected % cell death

Table 3. Comparison between median effect concentrations (EC_{50}[a]) obtained in HepG2 cells and primary rat hepatocytes, using the MTT reduction assay, at hyperthermic (40.5 ºC) and normothermic (37 ºC) conditions.

Compound	Primary rat hepatocytes		HepG2 cells	
	37 ºC	40.5 ºC	37 ºC (Dias da Silva *et al.*, 2013)	40.5 ºC (Dias da Silva *et al.*, 2012)
MDMA	2.50E+00	6.66E-01	2.23E+00	1.77E+00
METH	4.01E+00	1.47E+00	5.26E+00	2.97E+00
4-MTA	4.90E-01	2.76E-01	7.40E-01	6.60E-01
d-Amp	1.87E+00	7.63E-01	2.41E+00	1.66E+00
Mixture	1.87E+00	6.81E-01	2.90E+00	2.06E+00

[a] *I.e.*, concentration yielding 50% of the maximal effect in the MTT assay

A summary of the logit parameters for the best-fit regression model of each individual agent are presented for normothermic (Table 1) and for hyperthermic (Table 2) incubations.

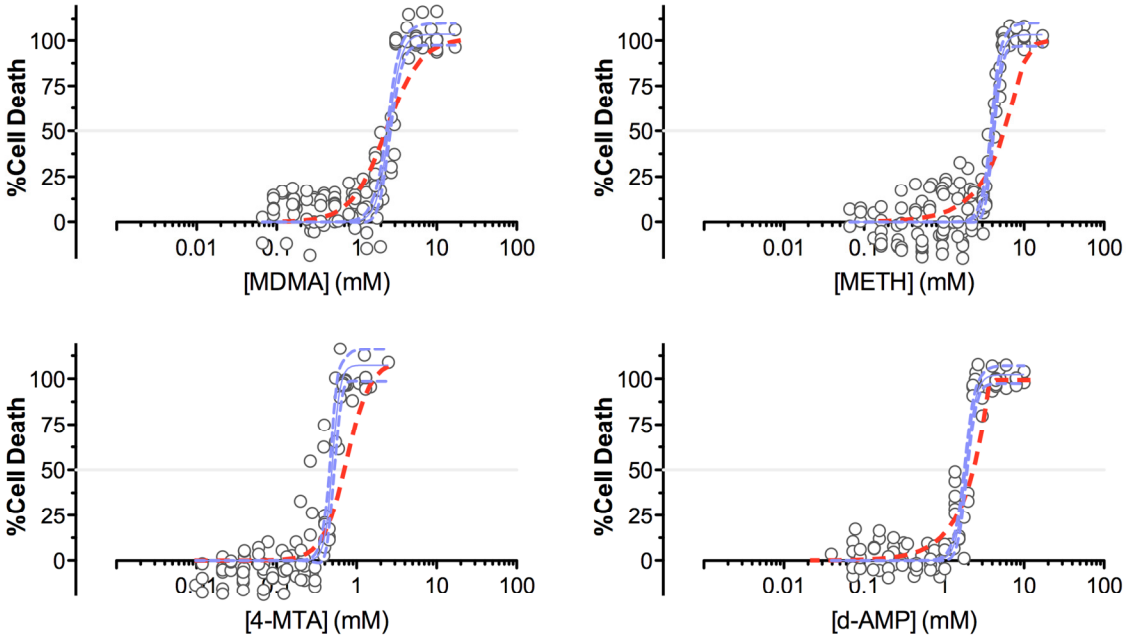

Figure 1. Mortality induced by MDMA, METH, 4-MTA, and *d*-Amp 48 h after incubation with primary rat hepatocytes, at 37 ºC. Cellular mortality data as evaluated by the MTT reduction assay following 48 h incubations of primary rat hepatocytes to MDMA, METH, 4-MTA, and *d*-Amp. Data were fitted to the logit function represented by the solid blue line. Dashed blue lines show the 95% confidence interval belt of the fit. Data were normalized to negative (untreated) and positive (1%Triton X-100) controls. The results were obtained from four independent experiments where a range of different concentrations of each drug was tested in series of three to six replicates. The dashed red line represents the cytotoxicity curves of the substances towards the human hepatoma HepG2 cell line under the same experimental setting (Dias da Silva *et al.*, 2013).

Mixture design

All chemicals were combined in proportion to the concentration producing 50% of the maximal effect in the MTT assay (EC_{50}), calculated by interpolation from the best-fit regression model for each tested agent. The estimated median effect concentrations for the best-fit regression model of each individual agent are presented in Table 3, for hyperthermic and normothermic incubations.By using this design, it was ensured that all mixture components were present at equipotent concentrations in the mixture, so that no single drug contributed excessively and disproportionately to the overall mixture effect. For this purpose, a master solution of the mixture containing all the individual components in proportion to the EC_{50} was prepared. A range of test concentrations was obtained through serial dilutions by employing the *fixed mixture ratio design* (Altenburger *et al.*, 2000), so that the ratio between each constituent was kept constant. This mixture design was selected with the aim of exploring the predictability of combination effects in primary rat hepatocytes, under our experimental conditions. In other words, the mixture tested here is intended as a proof of concept. If the observed effects meet the expectations, the applicability of the concept will be possibly extended to other potential mixture ratios, as observed formerly (Dias da Silva *et al.*, 2013).

Predicting combination effects

After exhaustive characterization of the concentration–response curves of the single agents, the effects of the mixture were predicted assuming additive joint responses. The expected effects were calculated using the CA and IA approaches, as reported by Payne *et al.* (Payne *et al.*, 2000) and used as a reference for the assessment of combination effects in terms of synergisms (if the observed effects are greater than additive predictions), additivity (if the experimental mixture outcomes equal the prediction), and antagonism (if the experimental joint effects fall short of additivity).

Statistical analysis

Nonlinear regression analysis was carried out using the best-fit approach and results were presented as the best estimate of the mean effects.

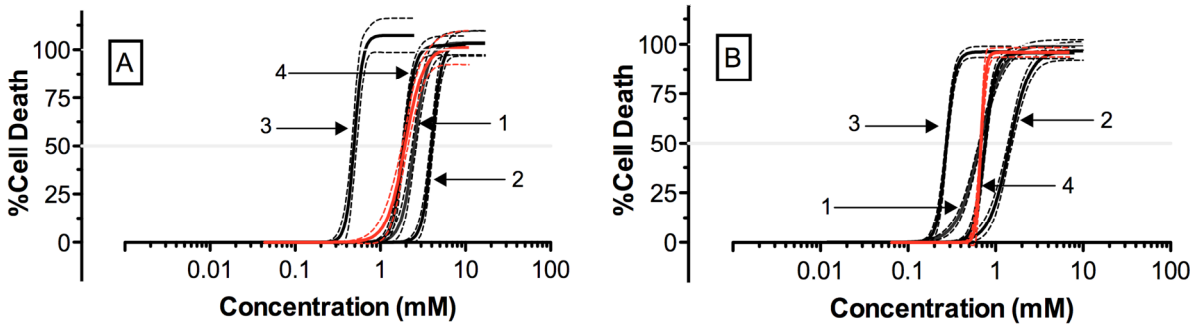

Figure 2. Regression models for the mortality of all four amphetamines, individually and combined 48 h after incubation with primary rat hepatocytes, at 37 °C (A) and at 40.5 °C (B). The black solid and dashed lines are the mean and 95% confidence interval of the mean responses of the individual drugs, respectively. The numbering of the drugs is as follows: 1: MDMA, 2: METH, 3: 4-MTA, 4: *d*-Amp. The red solid and dashed lines are the mean and 95% confidence interval of the mean responses of the mixture, respectively.

Statistical uncertainties were expressed as the 95% confidence intervals (CI) and differences between the best fits were deemed statistically significant when their corresponding CI belts did not overlap. Additionally, comparisons between HepG2 cells and primary hepatocytes curve parameters for maximal effects, slope and location (θ_{max}, θ_1 and θ_2, respectively) were performed using the extra sum-of-squares *F* test. *p* values lower than 0.05 were considered statistically significant. Nonlinear curve fitting and all statistical calculations were performed using GraphPad Prism software, version 5.01 (GraphPad Software, San Diego California, USA).

Results

4-MTA and METH were the most and the least potent amphetamines, respectively, eliciting cytotoxicity in primary rat hepatocytes, at a physiological incubation temperature (37 °C).

In order to accurately estimate the effects of a mixture with well-defined composition, detailed and reliable information on the individual responses of each of the constituents is required. Therefore, a comprehensive range of concentrations was tested for all test substances to describe the complete response range (from 0 to 100% effect), for the MTT cytotoxicity assay.

Figure 1 shows the cytotoxicity curves obtained for the single substances, at a physiological incubation temperature (37 °C).

Table 1 provides the individual curve parameters for all the tested drugs, including slope, and maximal effects at the same temperature. Median effect concentrations (EC_{50}) are displayed in Table 3. The regression lines of all substances were similar in shape and slope, but exhibited different individual potencies (Fig. 2). For example, *d*-AMP potency (EC_{50} 1.87 mM) was approximately 4 times lower than that of the most potent tested amphetamine, 4-MTA (EC_{50} 0.49 mM), and over 2 times higher than that of METH (EC_{50} 4.01 mM), the least cytotoxic of the four tested amphetamines. MDMA exhibited an EC_{50} of 2.50 mM.

Primary rat hepatocytes are more sensitive to the detrimental effects of MDMA, METH, 4-MTA, *d*-AMP, when incubated under hyperthermic conditions (40.5 °C) then under physiological circumstances (37 °C)

Figure 3 depicts the scattergrams and nonlinear regression models (blue solid lines), including the 95% confidence intervals for the mean effects (dashed blue lines), when primary hepatocytes were incubated with the four tested amphetamines, under hyperthermic conditions (incubation temperature of 40.5 °C).

As previously noted with the incubations performed at 37 °C, under this new temperature setting, the MTT assay proved to be a rapid and sensitive method, yielding reproducible results with minimum variability, for all the tested drugs (Fig. 3). Table 2 provides the individual curve parameters, including slope and maximal effects. The median effect concentrations (EC_{50}) are shown in Table 3. The comparison between the calculated median effects (Fig. 2) shows that METH is the least potent cytotoxic amphetamine tested (EC_{50} 1.47 mM) while 4-MTA is the most potent (EC_{50} 0.28 mM). MDMA and *d*-Amp showed intermediate median effect concentrations (EC_{50} 0.67 mM and 0.76 mM, respectively).

Amphetamines act in an additive fashion in primary rat hepatocytes

Assessments of combination effects in terms of synergisms, antagonisms, or additivity critically

depend on the determination of the expected effect of a given mixture. After thoroughly characterising the concentration–effect relationships of the individual agents in terms of shape, slope, and maximal effects, the CA and IA mathematical models were applied for quantitatively modelling the expected effects of an equimolar mixture of MDMA, METH, 4-MTA, and *d*-Amp. As depicted in Fig. 4, the two additive assumptions, CA and IA, produced very distinct expectations. This good discrimination between both models allowed the correct evaluation of which of the concepts best described the amphetamines mixture effects. The CA model estimated stronger effects, predicting EC_{50} values of 1.98 mM and 0.74 mM for normothermic and hyperthermic incubations, respectively, while the median effect concentrations estimated by IA were 7.14 mM for normothermic (Fig. 4A) and 2.28 mM for hyperthermic incubations (Fig. 4B).

The experimental mixture testing, confirmed that the CA model was the best suited for predicting mixture effects at both incubation temperatures, as this model was able to prediction accurately the responses over the entire range of effect levels. Experimentally obtained EC_{50} values provided in Table 3 (1.87 mM for normothermia and 0.68 mM for hyperthermia) agreed with those calculated using the CA approach. Conversely, the *independent action* concept clearly underestimated the mixture toxicity.

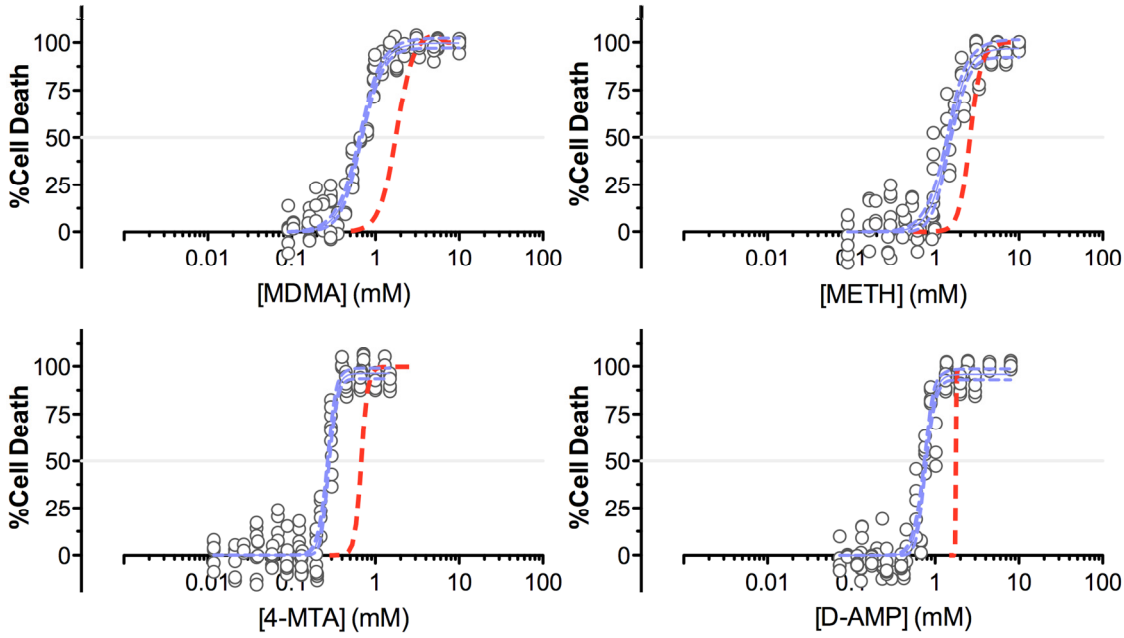

Figure 3. Mortality induced by MDMA, METH, 4-MTA, and *d*-Amp 48 h after incubation with primary rat hepatocytes, at 40.5 ºC. Data obtained with the MTT reduction assay were from three independent experiments where several different concentrations of MDMA, METH, 4-MTA, and *d*-Amp were tested in series of three to six replicates. Mean effects (lethality) were fitted to the logit function represented by the solid blue line. Dashed blue lines show the 95% confidence interval belt of the fit. Data were normalized to negative (untreated) and positive (1%Triton X-100) controls. The dashed red line represents the cytotoxicity curves of the substances towards the human hepatoma HepG2 cell line, under the same experimental setting (Dias da Silva *et al.*, 2012).

Discussion

Remarkable inter-individual differences in the susceptibility to the toxic effects of MDMA and other amphetamines have been noted on several occasions (De Letter *et al.*, 2004; Henry *et al.*, 1992a; O'Donohoe *et al.*, 1998). The plasma or target-organ concentrations of the drugs often fail to explain the random, severe and sometimes lethal intoxications that often occur after *ecstasy* use (Greene *et al.*, 2003). Polydrug abuse, either due to unintentional ingestion of impure *ecstasy* tablets, or deliberately aimed at increasing the desired effects of the drugs is thought to be, at least partially, responsible for these unexpected intoxication events (Schifano, 2004).

Assessing the risk associated with the ingestion of drug mixtures is a challenge often hampered by the immense number of possible combinations and their permanent change in quality and quantity within the organism, due to metabolism and transport mechanisms, among others (Borgert, 2004).

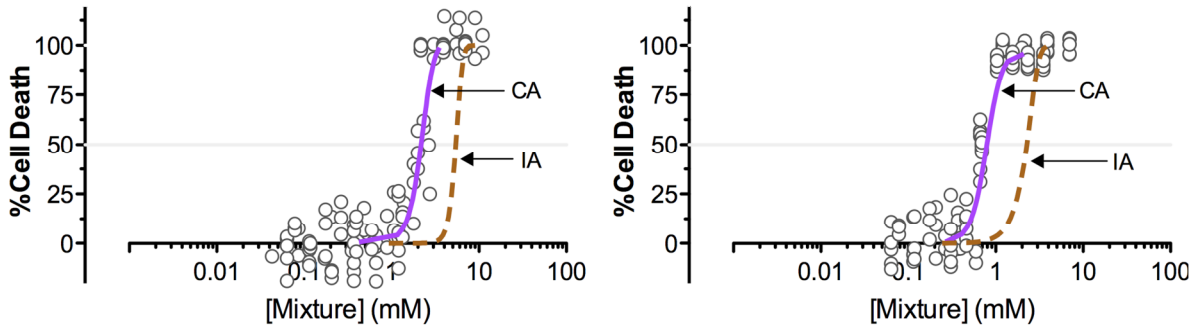

Figure 4. Predicted and observed cytotoxic effects of a mixture of MDMA, METH, 4-MTA, and *d*-Amp, obtained with the MTT assay after 48h incubations of primary rat hepatocytes, at either 37 ºC (A) or 40.5 ºC (B). The individual components were mixed in proportion to their EC_{50} values calculated from the best-fit regression model for each individually tested drug (Table 3). On the basis of the single drug concentration–response relationships (Fig. 1 and 2, for the physiological and hyperthermic settings, respectively), additive combination effects were predicted using the models of *concentration addition*, given by the solid line labelled CA, and *independent action*, as represented by the dashed line, labelled IA. Data were from at least three independent experiments where different concentrations were tested in six replicates.

We have previously tested the combination effects of four different amphetamines that are known to be simultaneously abused, using the human hepatoma HepG2 cell line. In the mentioned study, we found significant additive effects, even at very low levels of the individual substances, which can potentially explain the severe effects that can arise when these are ingested concomitantly (Dias da Silva *et al.*, 2013). In the present study we extended these previous investigations to an *in vitro* model that is known to be more representative of the *in vivo* situation, cultured primary hepatocytes (Donato *et al.*, 2008; Hengstler *et al.*, 2000a; Hengstler *et al.*, 2000b).

Due to the thermogenic action of the drugs combined with high ambient temperatures, the body temperature of intoxicated individuals is often increased. This phenomenon has also been suggested as potentially linked to the unexpected effects of amphetamines (Carvalho *et al.*, 2012). Consequently, we also investigated the impact of raising the incubation temperature to levels that mimic the hyperthermic state induced by these drugs on their joint cytotoxic effects.

With the work presented here, we have shown that the tested MDMA, 4-MTA, *d*-AMP and METH amphetamines have joint effects in primary rat hepatocytes. These effects were observed for both incubations temperatures, but the overall cytotoxicity was severely increased when the drugs were incubated at 40.5 ºC, both individually and in combination. Our data, therefore, supports that hyperthermia can greatly aggravate the harmful effects attributed to polydrug abuse. These results agree well with those previously obtained with the immortalized HepG2 cells (Dias da Silva *et al.*, 2012; Dias da Silva *et al.*, 2013).

In spite of the acknowledged importance in assessing the effects of mixtures in this field, research has focused predominantly on the activity of single drugs and the few mixture studies conducted so far have suffered from inadequate theoretical and conceptual foundations, that have led some authors to claim synergisms even when no references to the expected mixture outcomes were provided (McNamara *et al.*, 2006; Mohamed *et al.*, 2011b; Pontes *et al.*, 2008; Schechter, 1998; Young *et al.*, 2005). We have now demonstrated that in primary hepatocytes these four amphetamines interact in an additive manner and therefore, based on CA postulations, significant toxicity is expected to occur when the amphetamines are mixed at individual ineffective concentrations, as shown before with the HepG2 cells (Dias da Silva *et al.*, 2013). The *in vivo* relevance of such interactions can be clearly demonstrated by several reports of human intoxications involving MDMA and other amphetamines (Dams *et al.*, 2003; Felgate *et al.*, 1998; Fineschi *et al.*, 1996; Johansen *et al.*, 2003).

From a pharmacokinetic perspective, the four tested amphetamines share pharmacological and toxicological mechanisms and also the same metabolic bioactivation routes. Therefore, we anticipated that the CA model would more accurately predict the mixture effects than the IA model. In effect, despite only few studies employing both concepts in a comparative manner for mixture toxicity prediction be available, there is a clear understanding that CA concept is most appropriately used for mixtures constituted by similar agents while the contrary occurs with IA (Kortenkamp, 2007). Our data corroborated these assumptions, given that the CA model provided the best estimates, with EC_{50} values that were very close to those experimentally observed both at 37

ºC and 40.5 ºC. This suggests that equal overall effects will be promoted in case one drug is replaced by an equieffective concentration of another one. Should this prove to be true, amphetaminic agents, when present in mixtures, may act together additively even when each component is present at concentrations that individually produce no noticeable effects. In this case, it may not be necessary to invoke synergisms to elucidate how low, ostensibly irrelevant, levels of drug present in illicit formulations may produce significant effects as mixtures.

Conversely, marked deviations between IA predictions and observations were identified. For both mixtures (37 ^{o}C and 40.5 ^{o}C), this model vastly underestimated the mixture cytotoxic effects proving to be an inappropriate concept to model combination effects of these substances.

By comparing these results in primary rat hepatocytes with those produced in HepG2 cells under the same experimental settings (data published previously by our group (Dias da Silva *et al.*, 2013)), we observed no significant differences (p >0.05), for all the treatments, at 37 ^{o}C. In spite of the similarity in terms of shape and position, the corresponding curves obtained from experiments in HepG2 cells (Dias da Silva *et al.*, 2013) are somewhat shallower (lower slopes) (Fig. 1), which, in some cases, can be translated into median effects slightly different from those seen with primary cells (Table 3). Notwithstanding this good agreement between the effects in primary and immortalised cells at physiological temperature, the cytotoxic effects were significantly aggravated in the primary hepatocytes compared to the HepG2 cells at 40.5 ºC. Effectively, a significantly lower cytotoxic effect was observed for all the treatments in the HepG2 cells (p <0.0001) as shown by the remarkable rightwards shift of the lethality curves (Figure 3) and by the significantly higher calculated EC_{50} values (Table 3), suggesting that, under hyperthermic conditions, the primary hepatocytes may be more susceptible than HepG2 to the toxicity of the tested amphetamines, both individually and mixed. These differences may be explained by the changes in drug metabolism that are expected to occur under a hyperthermic state and to which the primary hepatocytes would naturally display a higher susceptibility compared to the HepG2 cells. In fact, it is recognized that improved metabolic efficiency occurs when the rat liver is subjected to heat stress (40 ^{o}C and 42 ^{o}C) (Li *et al.*, 2012). The role of the hepatic bioactivation of amphetamines in toxicity has been extensively studied and it is known to greatly contribute to the *in vivo* toxicity of these drugs in rodent experimental models as well as and in humans (de la Torre *et al.*, 2004; Easton *et al.*, 2003; Gollamudi *et al.*, 1989; Jones *et al.*, 2005). The oxidative metabolites that are produced in the liver are highly reactive and generate ROS and RNS, inducing an oxidative stress that is extremely damaging for the cell (Antolino-Lobo *et al.*, 2011; Barbosa *et al.*, 2012; Carvalho *et al.*, 2012; Carvalho *et al.*, 2010; de la Torre *et al.*, 2004). Admitting that primary cells are metabolically more competent than cell lines, they might be susceptible to higher toxicity when exposed to substances that are metabolised into more harmful derivatives than the parent compound. Whether an increased production of these metabolites and their deleterious effects occurs in these primary hepatocytes *in vitro* system needs further experimental confirmation.

Human hepatoma cell lines, such as the HepG2 cells, are highly differentiated, exhibit numerous genotypic qualities of the liver cells from which they are derived and retain many of the biochemical synthesis pathways (Sassa *et al.*, 1987). Nonetheless, in general, continuous cell lines (tumour or artificially immortalized) express a limited range of phase I enzymes and UDP-glucuronosyl transferases found in hepatic tissue and, in spite of preserving some metabolic functions, the enzyme activity may show variability with increasing number of cell passages (Donato *et al.*, 2008; Gerets *et al.*, 2012; Westerink *et al.*, 2007; Xu *et al.*, 2004), becoming less representative of the *in vivo* situation. The accumulation of successive replicative-related variations also introduces variability in the original cellular background. Using primary hepatocytes substantially overcomes these limitations. In spite of the additional costs, limited life span, and the complexity of the cell isolation and culture techniques (Madan *et al.*, 2003) the use of primary hepatocytes is clearly advantageous over the immortalized cell lines when metabolism is thought to contribute to the toxic effects, such as in the amphetamines case. The use of more complex systems, including *in vivo* experimental models will undoubtedly bring additional confidence in what concerns the extrapolation from rat to human hepatocytes and from the *in vitro* to the *in vivo* situation.

In conclusion, the additive effects of amphetamines that are often ingested simultaneously raise concern about the detrimental effects that are expected to occur even if the drugs are mixed at low concentrations that would individually produce no effect. Also, a dramatic increase in toxicity can occur if body temperature increases. These data provides experimental evidence supporting that the severity of the intoxications is not reflected by the blood and tissue concentrations of these drugs and mixture effects must be taken into account. Accurate risk assessment of these drugs of abuse is strengthened by these powerful prediction tools. Knowledge on the nature of such interactions is of utmost

importance for increasing the awareness of the risks associated with simultaneous amphetamine abuse.

Abbreviations

d-Amp, *d*-Amphetamine; CA, *Concentration addition*; CI, Confidence intervals; EC_{50}, Concentration producing 50% of the maximal effect in the MTT assay; FBS, Fetal bovine serum; IA, *Independent action*; MDMA, *3,4*-Methylenedioxymethamphetamine, *ecstasy;* METH, Methamphetamine; 4-MTA, 4-Methylthyoamphetamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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Conflict of Interest statement

There are no conflicts or competing interests to declare.

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Chapter IV. Mixtures of MDMA and its major human metabolites induce significant toxicity to liver cells when combined at low, ineffective concentrations.

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Mixtures of MDMA and its major human metabolites induce significant toxicity to liver cells when combined at low, ineffective concentrations

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Abstract

Hepatic injury following *3,4*-methylenedioxymethamphetamine (MDMA; *Ecstasy*) intoxications is highly unpredictable and does not seem to correlate with either dosage or frequency of use. The mechanisms involved include the metabolic bioactivation of the drug and the hyperthermic state of the liver triggered by its thermogenic action and exacerbated by the environmental circumstances of the abuse at hot and crowded venues. We therefore became interested in understanding the interaction between *ecstasy* and its metabolites generated *in vivo* as misusers are always exposed to mixtures of parent drug and metabolites.

With this purpose, HEPG2 cells were incubated with MDMA and its main human metabolites *3,4*-methylenedioxyamphetamine (MDA), *α*-methyldopamine (*α*-MeDA), and *N*-methyl-*α*-methyldopamine (*N*-Me-*α*-MeDA), individually and in mixture, at normal (37 ^{o}C) and hyperthermic (40.5 ^{o}C) conditions. After 48h, viability was assessed by the MTT assay. The extensive concentration–response analysis performed with single drugs was used to predict joint effects by using the well-founded models of *concentration addition* (CA) and *independent action*.

Experimental testing revealed that mixture effects conformed to CA, for both temperature settings. Additionally, substantial combination effects were attained even when each substance was present at low concentrations that individually produced unnoticeable effects. Hyperthermic incubations dramatically increased the toxicity of the tested drug and metabolites, both individually and in mixture. These outcomes suggest that the MDMA metabolism has hazard implications to liver cells even when metabolites are found in low concentrations, as they contribute additively to the overall toxicological effect.

Keywords

3,4-methylenedioxymethamphetamine (MDMA; *Ecstasy*); Metabolites; Mixture effects; Hyperthermia; *Concentration addition* (CA); *Independent Action* (IA).

Introduction

The recreational use of the ring-substituted amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA, *ecstasy*) has been recognized as one of the most significant trends in drug abuse over the past decades. Acting as a potent releaser and/or reuptake inhibitor of presynaptic serotonin, dopamine, and noradrenaline (Capela et al., 2009; de la Torre et al., 2004a; de la Torre et al., 2004b; Green et al., 2003) it induces intense feelings of euphoria, friendliness, comfort, intimacy, pleasure, empathy, and hyperactivity (Parrott, 2001). This psychoactive drug is commonly misrepresented among its young users as being safe. However, the number of *ecstasy*-related deaths and emergency department visits for overdose and unexpected reactions seem to have peaked in the last decade (CDC, 2010; Sano et al., 2009). Severe acute toxic effects associated with MDMA abuse are, in part, related to its pharmacological actions and include multiorganic failure, severe hyperthermia, tachycardia, hypertension, myocardial ischemia, psychosis, cerebral haemorrhage, and the serotonin syndrome (increased muscle rigidity, hyperreflexia, and hyperthermia), which can ultimately lead to death (Brauer et al., 1997; de la Torre et al., 2004b; Greene et al., 2003; Henry et al., 1992; Jones and Simpson, 1999; Schwab et al., 1999). Among these, an increase in reported cases of acute and chronic hepatic injury secondary to *ecstasy* intake has been noticed (Andreu et al., 1998; De Carlis et al., 2001; Ellis et al., 1996; Sano et al., 2009). These effects do not rely on dosage and may occur with the administration of a single dose or only after repeated use (Coore, 1996; Dykhuizen et al., 1995; Ellis et al., 1996; Henry et al., 1992). The produced hepatic lesions range from benign forms similar to viral hepatitis (Carvalho et al., 2010; Dykhuizen et al., 1995; Ellis et al., 1996) to extensive necrosis causing severe acute hepatic failure that require hepatic transplantation (Carvalho et al., 2010; Ellis et al., 1996; Henry et al., 1992; Milroy et al., 1996). The mechanisms that have been proposed to explain these hepatotoxic effects induced by MDMA are diverse and include the increased release of endogenous catecholamines, metabolic bioactivation, oxidative stress, liver blood flow changes, immunological mechanisms and hyperthermia.

MDMA interferes with thermoregulation and the hyperthermia induced by ingestion of the drug can be greatly exacerbated by the hyperactivity and physical exertion of the user, as well as the crowded overheated settings of nightclubs or dance parties, where its consumption often occurs (Henry et al., 1992). These factors combined can induce significant increases in body temperature, with temperatures as high as 43 ºC having been reported (Green et al., 2003). Hyperthermia is, consequently, considered one of the potentially lethal consequences of MDMA acute intoxications and has been implicated in MDMA-induced hepatotoxicity through the induction of deleterious oxidative stress (Jones and Simpson, 1999). The hepatocellular damage observed in MDMA intoxication cases is, in several aspects, similar to that produced by hyperthermic injury to isolated hepatocytes (Santos-Marques et al., 2006). Moreover, incubation of freshly isolated hepatocytes with MDMA under hyperthermic conditions has been shown to further increase the cellular mortality and aggravate toxicity, particularly oxidative stress biomarkers, including decreased GSH levels and lipid peroxidation (Carvalho et al., 2001).

There is also increasing evidence that the acute effects of MDMA intoxications do not correlate entirely with MDMA plasma concentrations (Carvalho et al., 2012; Greene et al., 2003), suggesting the involvement of the metabolic bioactivation of the drug (Fig. 1).

N-dealkylation of MDMA leads to the formation of the corresponding primary amine *3,4*-methylenedioxyamphetamine (MDA or *love*) which retains pharmacological action (Johnson et al., 1988) and has also been extensively used as a recreational drug of abuse. MDMA and MDA hepatic oxidative metabolism consists mainly of demethylenation into the highly reactive catechol metabolites *N*-methyl-*α*-methyldopamine (*N*-Me-*α*-MeDA) and *α*-methyldopamine (*α*-MeDA) that are *o*-methylated by catechol-*o*-methyltransferease and/or conjugated with glucoronide or sulphate prior to excretion (de la Torre et al., 2004a; de la Torre et al., 2000; Lim and Foltz, 1989; Maurer et al., 2000). Both *N*-Me-*α*-MeDA and *α*-MeDA can be further oxidized into the corresponding ortho-quinones. These highly redox-active molecules can enter a redox cycling with their semiquinone radicals, leading to the generation of reactive species of oxygen (ROS) and nitrogen (RNS) (Bolton et al., 2000; Remiao et al., 2004). Parallel side-chain degradation pathway leads to the formation of oxidatively deaminated metabolites (Lim and Foltz, 1988) that are excreted as their benzoic derivatives (hippuric acids) (Maurer, 1996; Maurer et al., 2000).

The aforementioned oxidative metabolites have been implicated in the mechanisms underlying the toxic effects of MDMA (Capela et al., 2006; Carvalho et al., 2002; Carvalho et al., 2004a; Carvalho et al., 2004b; Carvalho et al., 2004c; Easton et al., 2003; Escobedo et al., 2005; Forsling et al., 2002; Gollamudi et al., 1989; Hartung et al., 2002; Jones et al., 2005).

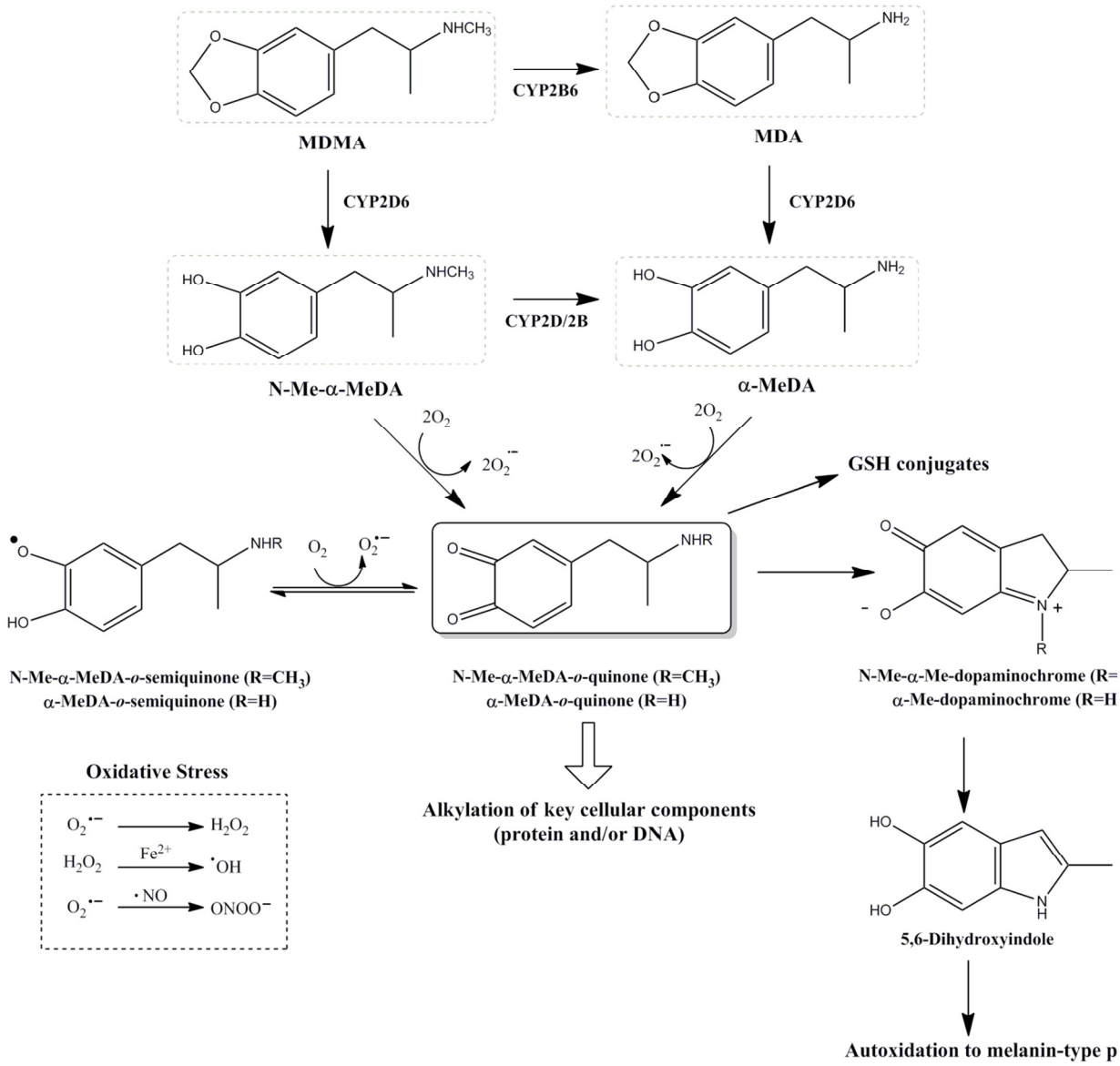

Figure 1. Proposed mechanisms for oxidative *3,4*-methylenedioxymethamphetamine (MDMA) metabolism into hepatotoxic metabolites. MDMA and MDA are *o*-demethylenated, predominantly by cytochrome P450 2D6 (CYP2D6) in humans, to *N*-methyl-*a*-methyldopamine (*N*-Me-*a*-MeDA) and *α*-methyldopamine (*α*-MeDA). *N*-Me-*α*-MeDA can be subsequently *N*-demethylated to give *α*-MeDA through CYP2D and 2B isoforms. Both reactive catechols are readily oxidized to the corresponding ortho-quinones, highly redox-active molecules that can enter a redox cycling with their semiquinone radicals leading to the injury of cellular components through the generation of pro-oxidant reactive species (ROS and RNS), conjugate with glutathione (GSH), or further oxidize into aminochromes through cyclization and polymerize leading to melanin type polymers. Adapted from (Carmo et al., 2006).

In vitro studies using freshly isolated rat hepatocytes have shown that the toxicity of the metabolites was much higher than MDMA under the same experimental settings (Carvalho et al., 2004a; Carvalho et al., 2004b). This metabolic bioactivation of MDMA is particularly significant in the liver, where MDMA is extensively metabolized, with approximately 80% of the ingested dose being excreted after hepatic metabolism (de la Torre et al., 2004b). In spite of the abundance of *in vitro* evidence, the *in vivo* relevance of the metabolic bioactivation of MDMA in the development of hepatotoxicity is still a matter of debate. Extrapolation of these *in vitro* findings to the *in vivo* situation has often been limited by the relatively high concentrations of the metabolites that are tested *in vitro*. However, a few *in vivo* studies have already provided evidence on the role of MDMA oxidative metabolism on neurotoxicity (Easton et al., 2003; Gollamudi et al., 1989; Jones et al., 2005).

In the liver, due to the potential MDMA bioactivation, it is possible that the metabolites contribute to the overall toxicity of the drug by acting together with MDMA. However, to our knowledge, this hypothesis has not been addressed yet. With the present investigation we aimed at evaluating the contribution of the metabolites to the

hepatotoxicity of MDMA by studying their effects when mixed with the parent compound.

To better understand the putative interactions between MDMA and its metabolites generated *in vivo* we used a previously developed study model that compares the applicability of two well-established mathematical models for the calculation of expected additive mixture effects (Pavlaki et al., 2011; Payne et al., 2000; Rajapakse et al., 2001; Silva et al., 2002): *concentration addition* (CA) and *independent action* (IA). *Concentration* addition is usually applied to mixtures of substances that exert their effects through similar modes of action, while *independent* action is usually suitable to predict additive responses of combinations of drugs with diverse modes of action. It is in relation to the additivity expectations given by these models that combination effects are evaluated in terms of synergisms (when the experimentally observed effects are greater than the additivity expectations) or antagonisms (when the experimentally observed effects fall short of the additivity expectations) (Kortenkamp, 2007). These models also enable investigating the potential for significant joint effects to occur, even when the components are mixed at low physiologically relevant concentrations. We have previously successfully applied these models for predicting, in immortalized hepatoma HEPG2 cells, the joint toxic effects of amphetaminic drugs often found as low-level contaminants in street drugs offered as *ecstasy* (Dias da Silva et al., 2012). Given the influence of hyperthermia on the outcome of the hepatotoxic effects, the interaction between MDMA and metabolites was also tested in this system under hyperthermic incubation conditions.

Materials and Methods

Test drugs

MDMA (HCl salt) was extracted and purified from high purity MDMA tablets provided by the Portuguese Criminal Police Department, at REQUI*M*TE/ Toxicology Laboratory, Biological Sciences Department of the Faculty of Pharmacy, University of Porto. MDA (HCl salt) was synthesized in the Organic Chemistry Department of the Faculty of Pharmacy, University of Porto. The obtained salts were purified and fully characterized by nuclear magnetic resonance (NMR) and mass spectrometry (MS) methodologies. Synthesis of *N*-Me-α-Me-DA (HBr salt) and α-MeDA (HBr salt) was prepared following the procedure of Borgman *et al.* (1974), at REQUI*M*TE/CQFB, Chemistry Department, Faculty of Science and Technology, Universidade Nova de Lisboa, as described by Capela *et al.* (2006).

Master stock solutions of all amphetamines and the mixture were prepared in deionised sterile water and were at least 20 times more concentrated than the highest concentration tested. Subsequent serial dilutions were freshly prepared before each experiment. All solutions were stored at –20 ^{o}C, protected from light.

HepG2 routine cell culture

The human hepatocarcinoma HepG2 cell line was routinely maintained as previously described (Dias da Silva et al., 2012). Briefly, cells were cultured in 75 cm^2 flasks in minimum essential medium alpha (MEM alpha) supplemented with 10% heat-inactivated FBS, 1% penicillin (5,000 units/ml)/streptomycin (5,000 µg/ml) mixture, 1% fungizone and 6 µg/ml transferrin (complete culture medium) and incubated in a humidified atmosphere of 5% CO_2 at 37 ^{o}C. All the cell culture reagents were purchased from Invitrogen Corporations (Paisley, UK). The medium was changed every 3 days. When cells reached 70% confluence, cells were detached by trypsinization and subcultured over a maximum of 10 passages. HepG2 cells were routinely tested for Mycoplasma contamination.

For the MTT assay, 80,000 cells were seeded onto the central 60 wells of 96-well plates in order to obtain confluent monolayers within two days. Peripheral wells on the plate were filled with sterile water to avoid evaporation of the treatment solutions. On the day of the experiment, the seeding media was gently aspirated and the cells exposed to eight different concentrations per plate (six replicates each) of MDMA, MDA, *N*-Me-α-Me-DA, α-MeDA, and mixture solution, prepared in fresh culture medium, for 48 h, at 37 ^{o}C or 40.5 ^{o}C. Each individual plate also included six replicates of negative controls (*i.e.*, no test agents) and six replicates of positive controls (full media with 1%Triton X-100). A broad range of concentrations was tested for each individual chemical and mixture, in order to obtain complete and well-described concentration-response curves.

The MTT reduction assay

The *3*-(*4,5*-dimethylthiazol-*2*-yl)-*2,5*-diphenyl tetrazolium bromide (MTT) is reduced to purple formazan by mitochondrial reductases of living cells, providing a measure of the cell viability. The protocol described by Mosmann (1983) was followed with some modifications (Dias da Silva et al., 2012).

Table 1. Parameters derived from nonlinear fits (logit model) of single agent concentration–response data, in the MTT reduction assay, at normothermic conditions, 37 °C (Figure 2). These parameters were used to compute the predicted mixture effect curves shown in Figure 3.

Compound	Tested concentration range (mM)	Estimated parameters for the best-fit regression model			EC_{50} (mM) [4]	EC_{01} (mM) [5]	Fraction in the mixture
		θ_1 [1]	θ_2 [2]	θ_{max} [3]			
MDMA	6.6E-03 – 12	-7.97E-01	2.44E+00	1.17E+02	1.62E+00	2.40 E-02	6.53E-02
MDA	9.22E-04 – 10	-1.45E+00	1.97E+00	1.74E+02	1.88E+00	1.31E-02	3.56E-02
α-MeDA	2.32E-03 – 5	4.03E+00	1.59E+01	9.05E+01	7.51E-01	2.91E-01	7.90E-01
N-Me-*α*-MeDA	2.36E-03 – 5	1.58E+00	4.17E+00	7.05E+01	5.83E-01	4.02E-02	1.09E-01

[1]Location parameter of the Logit function. [2]Slope parameter. [3]Maximal effect, expressed as % cell death. [4]Median effect concentration, *i.e.*, concentration yielding 50% of the maximal effect produced by the drug in question. [5]Concentration yielding 1% of the maximal effect in the MTT assay.

Table 2. Parameters derived from nonlinear fits (logit model) of single agent concentration–response data, in the MTT reduction assay, at hyperthermic conditions, 40.5 °C (Figure 2). These parameters were used to compute the predicted mixture effect curves shown in Figure 3.

Compound	Tested concentration range (mM)	Estimated parameters for the best-fit regression model			EC_{50} (mM) [4]	EC_{01} (mM) [5]	Fraction in the mixture
		θ_1 [1]	θ_2 [2]	θ_{max} [3]			
MDMA	3.46E-03 – 12	-9.04E-01	4.89E+00	1.12E+02	1.39E+00	1.67E-01	3.51E-01
MDA	8.65E-04 – 10	-7.68E-01	4.13E+00	1.10E+02	1.39E+00	1.12E-01	2.36E-01
α-MeDA	1.17E-03 – 5	-2.08E-01	4.03E+00	1.52E+02	6.83E-01	6.41E-02	1.35E-01
N-Me-*α*-MeDA	1.183E-03 – 5	1.94E+00	7.32E+00	8.99E+01	5.75E-01	1.32E-01	2.78E-01

[1]Location parameter of the Logit function. [2]Slope parameter. [3]Maximal effect, expressed as % cell death. [4]Median effect concentration, *i.e.*, concentration yielding 50% of the maximal effect produced by the drug in question. [5]Concentration yielding 1% of the maximal effect in the MTT assay.

Briefly, at the selected time point, the culture medium was aspirated and the attached cells were rinsed with 200 µl HBSS, followed by the addition of fresh culture medium containing 0.25 mg/l MTT. After a 30 min incubation at 37 °C in a humidified, 5% CO_2 atmosphere, the formed intracellular crystals of formazan were dissolved in 100 µl 100% DMSO. The absorbance was measured at 570 nm, using a multi-well plate reader (Labsystems Multiskan, Basingstoke, UK).

To reduce inter-experimental variability, data from each test plate were normalized and scaled between 0% (negative controls) and 100% of effect (positive controls). Single agent data were obtained from at least six independent experiments run in six replicates, and mixture samples were run in six replicates on at least five independent occasions. Results were graphically presented as percentage of cell death *versus* concentration (mM).

Regression modelling

Curves of normalized mortality values (effect) *versus* concentration (mM) were constructed and analyzed through the best-fit approach (Scholze et al., 2001). The final data analysis was carried out with the best-fit model, selected from a number of nonlinear regressions. In the present study, the logit function was employed:

$$Y = \theta_{min} + (\theta_{max} - \theta_{min})/(1+\exp(-\theta_1-\theta_2*\log(x)))$$

where θ_{min} and θ_{max} are the minimal and maximal observed effects, respectively; x is the concentration of test agent; θ_1 is the parameter for location; and θ_2 is the slope parameter.

Mixture design

All chemicals were combined in proportion to their EC_{01} (concentration producing 1% of the maximal inducible toxic effect in the MTT assay). Using these criteria, all mixture components were present at equipotent concentrations, ensuring that no single drug contributed disproportionately to the overall mixture effect. For this purpose, a master solution of mixture was prepared containing the individual components in proportion to the EC_{01} and a range of concentrations for testing were subsequently prepared by employing the *fixed mixture ratio design* (Altenburger et al., 2000). The ratio between each constituent was maintained. EC_{01} values were determined by interpolation from the best-fit regression model for each tested agent. A summary of the logit parameters and estimated 1% effect concentration for the best-fit regression model of each individual agent are presented for normothermia (Table 1) and for hyperthermia (Table 2).

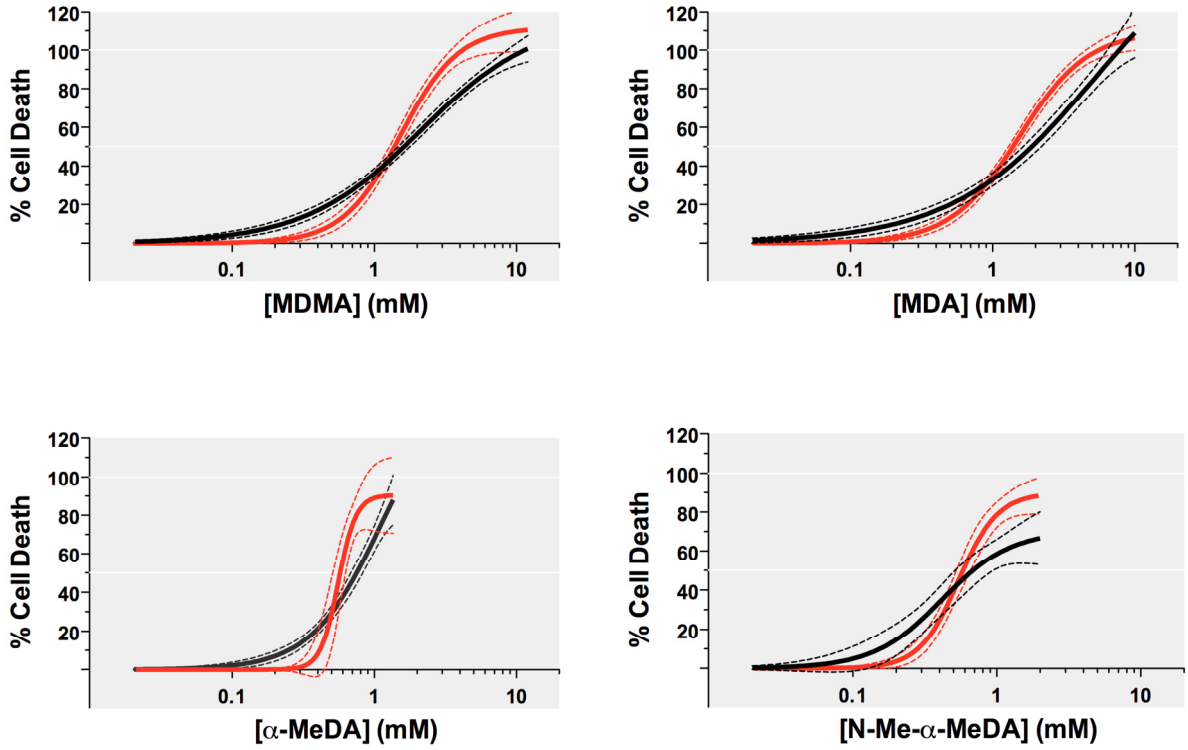

Figure 2. Regression models for the mortality induced by MDMA and the 3 tested metabolites MDA, *N*-Me-*α*-Me-DA, and *α*-MeDA, at normal and hyperthermic conditions, in HepG2 cells. Cellular mortality data as evaluated by the MTT reduction assay obtained following 48 h exposures of HepG2 cells to MDMA, MDA, *α*-MeDA, and *N*-Me-*α*-MeDA. Mean effects (lethality) were fitted to the logit function (best fit: black and red solid lines for 37 °C and 40.5 °C incubations, respectively). Dashed lines show the 95% confidence belt of the fit (mean responses). Responses were normalized to negative (untreated) and positive (1% Triton X-100) controls. Data were obtained from at least six independent experiments where fifty-eight different concentrations were tested in series of six replicates. Comparisons between normothermic and hyperthermic curves of each substance were made using the extra sum-of-squares *F* test. In all cases, the differences were considered statistically significant (curve *p* values <0.0001).

Computing combination effects

After exhaustive concentration–response analysis of all single agents, the effects of the mixture (with all chemicals combined in proportion to their EC_{01} values; Table 1 and Table 2) were predicted assuming additive joint responses. The expected effects were calculated using the CA and IA approaches. The mathematical derivation of the concepts was published by Payne *et al.* (2000).

Statistical analysis

Nonlinear regression analysis was carried out using a best-fit approach as described by Scholze and colleagues (2001) and the results are presented as the best estimate of the mean effects. Statistical uncertainties are expressed as 95% confidence intervals (CI) and differences between the best fits were deemed statistically significant when their corresponding 95% confidence belts did not overlap. Additionally, comparisons between curves (θ_{max}, θ_1 and θ_2) were also performed using the extra sum-of-squares *F* test. *p* values lower than 0.05 were considered statistically significant. Normality of the data distribution was assessed by three tests (KS normality test, D'Agostino and Pearson omnibus normality test and Shapiro–Wilk normality test) and statistical comparison between groups was estimated using the nonparametric method of Kruskal–Wallis [one-way analysis of variance (ANOVA) on ranks] followed by Dunn's *post hoc* test. Nonlinear curve fitting and all statistical calculations were performed using GraphPad Prism software, version 5.01 (GraphPad Software, San Diego California, USA).

Results

N-Me-*α*-MeDA and *α*-MeDA are more potent cytotoxic metabolites than the respective parent compounds MDMA and MDA

The cytotoxic effects induced by MDMA, MDA, *α*-MeDA, and *N*-Me-*α*-MeDA to the HepG2 cells after 48 h incubations at 37 °C and 40.5 °C are presented in Fig. 2, and the curve parameters are

listed in Table 1 and Table 2, respectively. Under these experimental settings all tested substances induced a concentration-dependent cytotoxic effect, as evaluated by the MTT reduction assay. The metabolite MDA produced an effect that was very similar to that observed with MDMA. Both substances showed very reproducible effects in all independent experiments and the resulting curves were highly consistent in terms of slope, maximal effect, and median effect concentrations (MDMA EC_{50} 1.62 mM and 1.39 mM, MDA EC_{50} 1.88 mM and 1.39 mM, at 37 ^{o}C and 40.5 ^{o}C, respectively). In contrast, the catechol metabolites *N*-Me-α-Me-DA and α-MeDA produced effects greater than MDA and MDMA, both at 37 ºC and 40.5 ºC (curve *p* values <0.0001). In line with earlier observations (Carvalho et al., 2004c), α-MeDA was less potent (*i.e.*, showed a higher median effect concentration) than *N*-Me-α-MeDA, both at 40.5 ºC (α-MeDA EC_{50} 0.68 mM and *N*-Me-α-MeDA EC_{50} 0.57 mM) and 37 ºC (α-MeDA EC_{50} 0.75 mM and *N*-Me-α-MeDA EC_{50} 0.58 mM) in inducing cell death.

For all the tested substances, incubations at 40.5 ºC produced steeper curves with significantly higher slopes (*p* values <0.0001, when curves were compared for θ_2) (Table 1 and Table 2) indicating a higher mortality variation within a smaller concentration range, under hyperthermic conditions.

Of note, the incubations with *N*-Me-α-Me-DA and α-MeDA failed to produce a maximal lethal effect, *i.e.* maximal responses were always below those of the positive control. α-MeDA yielded a maximal effect of 90.53% at 37 ºC, while *N*-Me-α-MeDA only induced up to 70.46% and 89.9% maximal responses under normothermic and hyperthermic conditions, respectively. This is due to the turbidity resulting from the incubation with high concentrations of the catechol metabolites and has been previously observed *in vitro* (Carmo et al., 2006; Carvalho et al., 2004c). At the higher concentration range, these metabolites produce ortho-quinones that are further oxidized in a process that involves an irreversible 1,4- intramolecular cyclization, resulting in the formation of aminochromes (orange-colored pigments) and related compounds, such as 5,6-dihydroxyindoles, which eventually leads to the appearance of dark-brown or black insoluble polymers of the melanin type (Figure 1) (Bindoli et al., 1992; Bindoli et al., 1989).

In the MTT assay, the formation of these pigments is accounted as formazans, which interfere with the optical density readings and, for this reason, higher metabolite concentrations were not considered.

CA is the most accurate model to predict combination effects between MDMA and its metabolites at normothermic and hyperthermic incubations

Based on the single agent concentration–response relationships, shown in Fig. 2, we calculated the predictions for an equipotent mixture on the basis of the EC_{01} of individual components, so that none of the constituents contributed disproportionately to the overall mixture effect. The predictions were made assuming additive combination effects according to the CA and IA models. At both incubation temperatures, the two models produced well-differentiated additivity expectations that allowed the clear identification of the model that better correlated with the experimental observations (Fig. 3). The experimentally observed mixture effects agreed perfectly with the CA estimations, as the CA prediction was able to model responses over the entire range of effect levels and the predicted CA curve overlapped with the 95% confidence intervals of the best-fit regression model for the mixture (Fig. 3). Conversely, IA led to a clear underestimation of the observed joint effects. Accordingly, at 37 ^{o}C, the EC_{50} were 0.72 mM for the experimentally tested mixture and 0.79 mM for the CA prediction curve, while a much higher EC_{50} value of 1.93 mM was observed for the IA prediction curve (Fig. 3A). Likewise, for the hyperthermic incubations (40.5 ^{o}C), the experimental median effect concentration was 0.92 mM for the mixture, 0.92 mM for the CA prediction, and 1.59 mM for the prediction derived from IA (Fig. 3B).

Maximal observed lethal effects also agreed well with the predicted values according to the CA model, both at normothermic and hyperthermic incubations. However, as denoted above, the maximal responses for the α-MeDA and *N*-Me-α-MeDA metabolites were below those for the positive controls, so that CA predictions were limited in their full effect range.

Significant mixture effects are observed even when the drugs are combined at individually ineffective concentrations

The results displayed in Fig. 4, convincingly demonstrate that MDMA and its metabolites act together to produce significant additive mixture effects when each chemical was present at its individual EC_{01} value (Table 1 and 2). These effects were accurately predicted by the CA model.

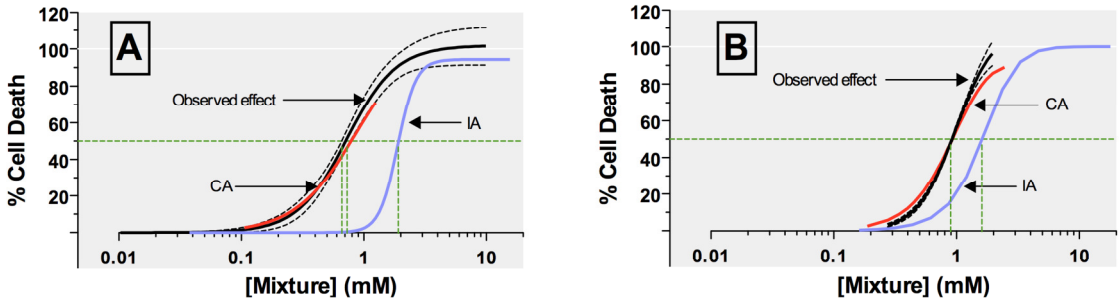

Figure 3. Predicted and observed effects of a mixture of MDMA and the 3 tested metabolites MDA, *N*-Me-α-Me-DA, and α-MeDA, in the MTT assay in normothermic (A) and hyperthermic (B) conditions. On the basis of the single agent concentration–response relationships (Fig. 2), additive combination effects were predicted using the models of *concentration addition* (CA), given by the solid red line; and *independent action* (IA), as represented by the solid blue line. The individual components were mixed in proportion to their EC_{01} values calculated from the data obtained with the single incubations (Fig. 2). The best-fit regression model (logit) for the experimental data obtained following 48 h of incubation with the mixture is shown by the solid black line, labelled 'observed effect'. Black dashed lines represent the upper and lower 95 % CI for the regression fit. The green dotted lines show the EC_{50} for each response curve (A: 0.72 mM observed effect, 0.79 mM CA and 1.93 mM IA at 37°C; B: 0.918 mM observed effect, 0.920 mM CA and 1.59 mM mM IA at 40.5°C). Data are from five independent experiments with sixty-four different concentrations tested in six replicates.

When tested individually at 37 ºC, 0.024 mM MDMA, 0.013 mM MDA, 0.29 mM α-MeDA, and 0.04 mM *N*-Me-α-MeDA produced 1% cell death each (such an effect is within the negative control variation range). When mixed together (0.368 mM mixture total concentration) the observed cell death effect was significantly different from untreated controls, reaching a mean level of 19.85 ± 1.07 % cell killing, that was well estimated by the CA model (19.6%, Fig. 4A).

Similarly, at 40.5 ºC, 0.167 mM MDMA, 0.112 mM MDA, 0.064 mM α-MeDA, and 0.132 mM *N*-Me-α-MeDA incubations produced an individual 1% cell death effect while the corresponding mixture (0.476 mM total concentration) induced a significantly higher lethal effect of 17.33 ± 1.55 % cell death, that was also previously well estimated by the CA model (17% estimated cell death, Fig. 4B).

This demonstrates that there were substantial joint effects even when the test agents were combined at concentrations that individually yielded responses indistinguishable from those of untreated controls.

Discussion

Intoxications following MDMA abuse are worrisome in terms of acute and/or long lasting effects and are frequent causes of emergency department admissions (Carvalho et al., 2012).

The toxicological actions of this drug are due to complex and interacting mechanisms resulting in remarkable interindividual differences in toxicity (Henry et al., 1992; O'Donohoe et al., 1998), which adds to the complexity and the severity of the intoxications and renders their treatment unspecific and mostly empirical. Among the putative aspects that may contribute for the described toxicity is MDMA metabolism. In humans, MDMA is principally cleared by hepatic metabolism, which results in production of redox-active metabolites (Antolino-Lobo et al., 2011; Carvalho et al., 2012; Carvalho et al., 2010; de la Torre and Farre, 2004). These metabolites formed *in vivo* may act jointly with the parent compound to produce an overall effect that can be different than that elicited by MDMA alone. We, therefore, investigated the combined effects of MDMA and its three major human hepatic metabolites (MDA, *N*-Me-α-MeDA and α-MeDA) in a human hepatoma cell line and found that together, the parent drug and its main metabolites significantly increase liver toxicity in relation to the parent compound alone, by acting in an additive way. We have further tested whether hyperthermia could aggravate these combination effects. Hyperthermia is a common feature of MDMA-induced intoxications, as it has been linked to the majority of the acute toxicity cases reported, including fatalities (Armenian et al., 2012; Coore, 1996; Dar and McBrien, 1996; Garcia-Repetto et al., 2003). Also, the hot, crowded, dance club conditions where MDMA abuse often takes place are expected to enhance the thermogenic actions of the amphetamines, with well-known clinical implications (Green et al., 2004). Our data show that the effects of MDMA and its main metabolites are severely aggravated when the cells are incubated at a temperature of 40.5 ºC. Even more remarkable was the observation that, when MDMA and its main metabolites were combined at individually low ineffective concentrations, significant cytotoxicity occurred, independently of the incubation temperature. These results clearly support the putative contribution of metabolic bioactivation to the toxicity of MDMA.

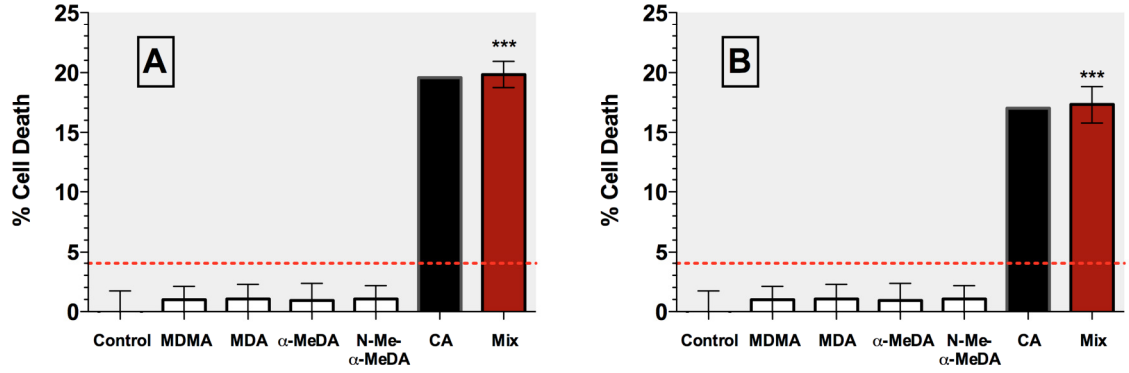

Figure 4. Individual and mixture effects of MDMA and the metabolites MDA, *α*-MeDA and *N*-Me-*α*-MeDA mixed at their EC_{01} effect level, in normothermic (A) and hyperthermic (B) conditions. CA: *Concentration addition* prediction. MIX: experimentally observed effect of 0.368 mM of mixture incubated at 37 ºC (A) and 0.476 mM of mixture incubated at 40.5 ºC (B). The individual concentrations of the four-mixture components in 0.368 mM of the mixture (A) were 0.024 mM MDMA, 0.013 mM MDA, 0.29 mM *α*-MeDA and 0.04 mM *N*-Me-*α*-MeDA. The individual concentrations of the four-mixture components in 0.476 mM of the mixture (B) were 0.167 mM MDMA, 0.112 mM MDA, 0.064 mM *α*-MeDA and 0.132 mM *N*-Me-*α*-MeDA. The dashed red line corresponds to the sum of the individual effects of all mixture components (4%). Data (mean results) were from five independent experiments run in six replicates. Error bars represent the 95% CI. Statistical comparisons were made using the Kruskal–Wallis test followed by the Dunn's multiple comparison *post hoc* test. *** Show statistically significant differences between the mixture and all other treatments. (***p<0.001).

The cytotoxicity of the oxidative metabolites has been previously recognized in several *in vitro* studies that have shown that the metabolic bioactivation of the drug has an important role in its neuronal (Gollamudi et al., 1989; Patel et al., 1991), hepatic (Carvalho et al., 2004a; Carvalho et al., 2004b), renal (Carvalho et al., 2002), and cardiac (Carvalho et al., 2004c) toxicities. These studies have demonstrated that the cytotoxic effects of the metabolites, especially those of the metabolites that retained the catechol moiety were greatly increased relative to the effects of MDMA. For example, the oxidative MDMA metabolite *N*-Me-*α*-MeDA proved to be more than 100-fold more cytotoxic than the parent compound MDMA (Carmo et al., 2006). The toxicity of *N*-Me-*α*-MeDA and *α*-MeDA involves the reactivity of the catechol moiety of these molecules that can be easily oxidized into the corresponding *o*-quinones (figure 1). These quinones directly react with cellular nucleophiles, such as sulfhydryl groups, including free cysteine, GSH and cysteinyl residues on proteins, rendering the cells more susceptible to the effects of the reactive compounds generated by these metabolic reactions. Further redox cycling occurs generating reactive species (ROS and RNS) and causing oxidative stress through the formation of oxidized cellular macromolecules, including lipids, proteins, and DNA (Bindoli et al., 1992; Bolton et al., 2000). *N*-Me-*α*-MeDA also depletes intracellular GSH (Carvalho et al., 2004b; Carvalho et al., 2004c), and the *o*-quinones can conjugate with GSH to form a redox-active glutathionyl adduct that is subsequently oxidized to a quinone thioether, which, after the reductive addition of a second molecule of GSH, yields a bis-glutathionyl conjugate (Hiramatsu et al., 1990; Monks et al., 2004). Further oxidation of the *o*-quinones produces aminochromes, a late stage event of catecholamine oxidation (Bindoli et al., 1989). *α*-Methyldopaminochrome and *N*-methyl-*α*-methyldopaminochrome are, in turn, oxidized and lead to melanin type polymers with a dark brown/black colour (Zhang and Dryhurst, 1994). Accordingly, a dark brown/black turbidity occurred in the cell culture media of the cells incubated with *N*-Me-*α*-MeDA and *α*-MeDA, in our study. In fact, due to the formation of such coloured compounds that significantly interfered with the absorbance readings of the MTT assay, we could not obtain complete concentration-response curves after the individual incubations with *N*-Me-*α*-MeDA and *α*-MeDA, since this was specially notorious at high concentrations of the metabolites. For this reason, it was not possible to test these highest concentrations in our system. The intracellular formation of such polymers is believed to occur only when the antioxidant defence system of the cell is significantly compromised. Additionally, *N*-Me-*α*-MeDA, *o*-quinones, and the aminochromes can inhibit the activity of important enzymes involved in the cellular antioxidant defence, namely, glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione *S*-transferase (GST) (Carvalho et al., 2004b; Remiao et al., 1999; Remiao et al., 2002). *N*-Me-*α*-MeDA also depletes ATP (Carvalho et al., 2004b), which combined to the disturbance in the thiol homeostasis, may contribute to mitochondrial dysfunction and disruption of cellular energetics.

As can be seen in figure 2, our results confirm the greater cytotoxicity of the catechol metabolites when compared to MDMA, with lower EC_{50} values and a left-shift in the concentration-response plots. On the other hand, the *N*-demethylated metabolite MDA produced very similar cytotoxic effects compared to the parent drug, with similar EC_{50} and slope values obtained for both substances. Other studies have also shown that MDA had comparable or even slightly lower cytotoxic effects than those elicited by MDMA (Carvalho et al., 2002).

In spite of all the evidence supporting the cytotoxicity of MDMA metabolites, the confirmation of the *in vivo* relevance of these *in vitro* findings has been hindered due to a lack of adequate *in vivo* models that accurately distinguish the toxic effects caused by the parent drug from those of the toxic metabolites. Also, confounding factors including redundant metabolic pathways, interspecies differences in the extent of metabolite formation, and the relative amounts of the formed metabolites preclude these *in vivo* investigations. With this study model we were able to bring together two important features of MDMA toxicity: metabolic bioactivation and hyperthermia, and were able to demonstrate for the first time that significant additive joint effects occur between MDMA and its metabolites under circumstances that are highly representative of the human situation in an acute intoxication scenario.

With the present data, we were able to calculate quantitative estimations of expected mixture effects that were accurately predicted by the CA model. An excellent discrimination achieved between the expectations according to CA and IA models allowed an accurate determination of the model that better described the experimental outcomes. The model of CA provided accurate estimations of combination effects proving that the tested chemicals acted together in an additive fashion. Since the CA model assumes that all our mixture components operate in a similar manner and that each individual drug contributes to the overall mixture effect in proportion to its concentration, we can expect that the same overall effect will be observed if one constituent were totally or partially replaced by an equieffective concentration of another. As a consequence, significant mixture effects can occur even when the individual test drugs are mixed at individually ineffective concentrations. This was clearly demonstrated for the mixture of MDMA and the metabolites, since when these were mixed at their individual EC_{01} concentrations (that failed to produce measurable effects relative to untreated controls) significant cytotoxicity was observed. It is, therefore, plausible that even when low levels of metabolites are produced in the liver adverse effects can occur by virtue of these additive effects. The model of IA, on the other hand, proved to be inappropriate for the assessment of the joint effects of MDMA and its major metabolites. This model that describes combination effects of drugs with dissimilar mechanisms of action and/or interacting at differing sites of action (Backhaus et al., 2000; Faust et al., 2003), severely underestimated the experimentally observed toxicity of the mixture. This suggests that there is a similarity in the way in which MDMA and its major metabolites lead to HepG2 cell death. However, the mechanistic interactions that explain these observed mixture effects await further investigation.

Of note is the fact that our data agrees well with a previous study that recently demonstrated that, in HepG2 cells, four amphetamines that are frequently co-ingested in *ecstasy* pills (*4*-methylthioamphetamine, *d*-amphetamine, methamphetamine, and MDMA) acted together producing additive cytotoxic mixture effects that could be accurately predicted by the CA model (Dias da Silva et al., 2012) whereas the IA model failed to produce accurate estimations of the cytotoxic joint effects of that mixture.

The finding of significant additive cytotoxic effects when MDMA and metabolites were combined at low, physiologically relevant concentrations is of special relevance. The reported concentrations of MDMA found in the blood and tissues at autopsy after fatal intoxications vary enormously and range from 1.0 to 70 µM (Carvalho et al., 2012). In the tissues, much higher concentrations of the drug are achieved. For example, in the liver, the drug levels can be up to 18 times higher than blood concentrations (Garcia-Repetto et al., 2003). Very limited information is available on metabolite levels in human specimens. However, data from pharmacokinetic studies obtained with volunteers after the ingestion of relatively small doses of MDMA, revealed that the maximal *N*-Me-α-MeDA plasma concentrations are similar to those attained by MDMA (Segura et al., 2001).

A limitation to our prediction analysis was the production of an incomplete cytotoxicity concentration-response curve for *N*-Me-α-MeDA (both at normothermia and hyperthermia) and α-MeDA (at normothermia), since maximal effects could not be recorded due to the absorbance interference of the pigments produced by the metabolites incubations. Due to the mathematical features of CA, this concept cannot be applied to effect levels that exceed the maximal effect of the chemical with the shortest (most incomplete) concentration-response curve (Silva et al 2007). However this had no significant impact on the accuracy of the estimation achieved by this model and our ability to compare it with experimental outcomes in the work reported here.

An important point to consider is the possibility that MDMA could be metabolized into the tested

mixture components by the HepG2 cells, with consequent changes in the contributions of individual agents to the overall mixture effect, as these cells retain a certain metabolic activity regarding the main cytochrome P450 isoenzymes involved in the hepatic metabolism of these metabolites (CYP2D6 for the catechols and CYP2B6 for MDA) (Donato et al., 2008). However, we did not observe any deviation from additivity, indicating that such putative metabolic interactions are probably not contributing to the observed effects.

Conclusions

Overall, we have shown, for the first time, that MDMA and its three major hepatic metabolites act in an additive manner producing joint effects that were accurately predicted by the *concentration addition* model. As such, when mixed at individually ineffective concentrations, significant cytotoxicity is produced. This was clearly demonstrated both at normothermic and hyperthermic incubations experimentally suggesting that, at physiologically relevant conditions, the metabolic bioactivation greatly contributes to the liver toxicity of the drug in intoxicated individuals. This is also a strong reminder that when concentrations of MDMA are evaluated in plasma of intoxicated individuals, these largely underestimate the overall exposure of cells to the toxic mixture of MDMA and its metabolites. Whether such additive combination effects between the parent drug and metabolites also occur in more complex experimental models deserves further investigation.

Abbreviations

CA, Concentration addition; CI, Confidence interval; EC_{01}, Concentration producing 1% of the maximally inducible effect in the MTT assay; EC_{50}, Concentration producing 50% of the maximally inducible effect in the MTT assay; IA, Independent Action; MDA, *3,4*-Methylenedioxyamphetamine; *Love*; MDMA, *3,4*-methylenedioxymethamphetamine; *Ecstasy*; α-MeDA, α-Methyldopamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; N-Me-α-MeDA, N-methyl-α-methyldopamine; RNS, Reactive species of nitrogen; ROS, Reactive species of oxygen.

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Chapter V. A mechanistic insight into the hepatocellular death induced by amphetamines, individually and in combination – The involvement of necrosis and apoptosis.

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An insight into the hepatocellular death induced by amphetamines, individually and in combination – The involvement of necrosis and apoptosis

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Abstract

The liver is a vulnerable target for amphetamine toxicity, but the hepatotoxicity mechanisms involved remain poorly understood. The purpose of the current research was to characterize the mode of death elicited by four amphetamines and to evaluate whether their combination triggered the same mechanisms in immortalised human HepG2 cells.

The obtained data revealed a time- and temperature-dependent mortality of HepG2 cells exposed to *3,4*-methylenedioxymethamphetamine (MDMA, *ecstasy;* 1.3 mM), methamphetamine (3 mM), *4*-methylthioamphetamine (0.5 mM) and *d*-amphetamine (1.7 mM), alone or combined (1.6 mM mixture). At physiological temperature (37 ^{o}C) 24 h-exposures caused HepG2 death preferentially by apoptosis, while a rise to 40.5 ^{o}C favoured necrosis. ATP levels remained unaltered when the drugs where tested at normothermia, but incubation at 40.5 ^{o}C provoked marked ATP depletion for all treatments. Further investigations on the apoptotic mechanisms triggered by the drugs (alone or combined) showed a decline in *BCL-2* and *BCL-$_{XL}$* mRNA levels, with concurrent upregulation of *BAX*, *BIM*, *PUMA* and *BID* genes. Elevation of Bax, cleaved Bid, Puma, Bak and Bim protein levels was also seen. To the best of our knowledge, Puma, Bim and Bak have never been linked with the toxicity induced by amphetamines. Time-dependent caspase-3/-7 activation, but not mitochondrial membrane potential ($\Delta\psi_m$) disruption also mediated amphetamine-induced apoptosis. The cell dismantling was confirmed by PARP proteolysis.

Overall, for all evaluated parameters, no relevant differences were detected between individual amphetamines and the mixture (all tested at equieffective cytotoxic concentrations), suggesting that the mode of action of the amphetamines in combination does not deviate from the mode of action of the drugs individually, when eliciting HepG2 cell death.

Keywords

3,4-Methylenedioxymethamphetamine (*ecstasy*, MDMA); Amphetamine-related toxicity; Hepatocytes; Combination effects; Apoptosis; Necrosis.

Introduction

It has been widely recognised that *3,4*-methylenedioxymethamphetamine (MDMA, *Ecstasy*,) is hepatotoxic to humans, in spite of this toxicity being random and associated with variable clinical outcomes, ranging from asymptomatic elevation of hepatic enzymes (ALT, AST, alkaline phosphatase) to lethal liver failure (Andreu et al. 1998; Dykhuizen et al. 1995; Ellis et al. 1996; Henry et al. 1992; Khakoo et al. 1995). The underlying mechanisms are not yet fully understood, but a plethora of factors have been proposed to justify these effects, including hyperthermia, drug-drug interactions from polydrug abuse, altered efflux of neurotransmitters, oxidation of biogenic amines, mitochondrial function impairment, direct effect of reactive metabolites, immune responses and activation of apoptotic pathways (Antolino-Lobo et al. 2011; Carvalho et al. 2012; Carvalho et al. 2010).

Results from previous studies from our group have supported the fact that hepatotoxicity can be exacerbated by the combination of MDMA with other amphetamines, which are either intentionally ingested or present in *ecstasy* pills as contaminants, even for combinations at concentrations that individually produce no detectable effects (Dias da Silva et al. 2013). In addition, we have shown that temperatures higher than those physiologically normal, such as those reached *in vivo* after amphetamine intake, further enhance the hepatotoxicity of amphetamines, as well as of their mixtures (Dias da Silva et al. 2012).

The hepatotoxicity of amphetamines has been associated with an increase in apoptosis and the mechanisms involved in this effect include a decrease in the levels of anti-apoptotic genes and related proteins, such as Bcl-2 and Bcl-$_{XL}$ (De Miglio et al. 2000; Jayanthi et al. 2001; Montiel-Duarte et al. 2002; Upreti et al. 2011), an overexpression of pro-apoptotic factors, like Bax and Bad (De Miglio et al. 2000; Genc et al. 2003; Jayanthi et al. 2001), Bid (Cerretani et al. 2011; Jayanthi et al. 2001) and p53 (De Miglio et al. 2000), the release of cytochrome *c* (cyt *c*) (Beauvais et al. 2011; Montiel-Duarte et al. 2002), SMAC/DIABLO (Cerretani et al. 2011) and apoptosis inducing factor (AIF) from the mitochondria, the activation of caspase-3 (Montiel-Duarte et al. 2002), the proteolysis of poly(ADP-ribose)polymerase (PARP) (Montiel-Duarte et al. 2002), the condensation of nuclei chromatin (Montiel-Duarte et al. 2002) and the accumulation of oligonucleosomal fragments in cytoplasm (Montiel-Duarte et al. 2002). Upreti *et al.* (2011) also demonstrated that MDMA activates c-Jun N-terminal protein kinase and p38 kinase. These protein kinases phosphorylate the anti-apoptotic Bcl-2 protein, so, indirectly, their activation may promote apoptosis in MDMA-exposed tissues.

Conversely, numerous clinical hepatotoxic descriptions of amphetamines correlate with morphological evidence of acute necrosis, rather than apoptosis, particularly in cases of intoxicated patients, who died or required liver transplantations (Brauer et al. 1997; Garbino et al. 2001; Ibranyi and Schonleber 2003; Kamijo et al. 2002).

It is clear from the aforementioned studies that the relative contribution of apoptosis and/or necrosis to amphetamine-induced liver injury remains controversial, as evidence for both types of cell death has been reported in hepatocytes. In addition to the uncertainties surrounding the precise mechanisms of cytotoxic action of individual amphetamines is the question of whether the mode of action of these drugs remains unaltered when they are combined. Does the simple combination of these drugs impact on the mode of cell death? Furthermore, taking into account the substantial increase in body temperature that often occurs with amphetamine abuse, would the mechanism of cell death, for both the individual and the mixed amphetamines, also be affected in a hyperthermic situation?

In order to address these questions and provide new insights into the putative mechanisms underlying the cytotoxicity linked to amphetamine intoxications, the present study attempted to delineate the mechanisms of cell death under normothermic (37 ^{o}C) and hyperthermic (40.5 ^{o}C) conditions caused by MDMA, methamphetamine (METH), *4*-methylthioamphetamine (4-MTA), *d*-amphetamine (*d*-AMP), and their mixture in HepG2 cells. For that, we evaluated the mode of HepG2 cell death (apoptosis or necrosis) and the cellular energetic status. Additionally, the relative mRNA expressions of *BCL-2*, *BCL-$_{XL}$*, *BAX*, *BIM*, *BAD*, *PUMA* and *BID*, as well as levels of PARP/cleaved PARP, Bak, Puma, Bim (EL, S and L isoforms), Bid/cleaved Bid and Bax proteins were assessed at 37 ^{o}C (the temperature condition showing higher apoptosis rates). Ultimately, and since the tested drugs induced differential expression of anti- and proapoptotic BCL-2 family members, we tested mitochondrial permeability impairment.

Overall, a better understanding of the cellular and molecular mechanisms involved in amphetamine toxicity should help to generate more adequate therapeutic approaches to lessen the long-term consequences of amphetamine use.

Materials and Methods

Test Drugs

All chemicals used were of analytical grade. *d*-AMP (sulphate salt) was purchased from Tocris Bioscience (Bristol, UK) and (+)-METH

hydrochloride (98% purity) was purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). MDMA (HCl salt) was extracted and purified from high purity MDMA tablets provided by the Portuguese Criminal Police Department, at REQUI*M*TE/ Toxicology Laboratory, Biological Sciences Department of Faculty of Pharmacy, University of Porto. The obtained salts were purified and fully characterized by nuclear magnetic resonance (NMR) and mass spectrometry (MS) methodologies. 4-MTA hydrochloride (racemic mixture) was generously provided by Dr David Nichols (Purdue University, West Lafayette, IN).

All chemicals were used as supplied. Stock solutions were prepared in ultra purified water and kept at –20 °C. Dilutions were freshly prepared before each experiment and were at least 20 times more concentrated than the highest concentration tested, so that media dilution was avoided.

Table 1. Tested drug concentrations and composition of the tested mixture. Adapted from Dias da Silva *et al*. (2013).

Compound	MDMA	METH	4-MTA	*d*-AMP	Mixture
EC_{50} (mM)[a]	2.23	5.260	0.741	2.42	2.90
Fraction in the mixture (%)	20.90	49.40	6.96	22.70	100
EC_{25} (mM)[b]	1.3	3.0	0.5	1.7	1.6[c]

[a]The EC50 were used to design the mixture, and to determine the ratio between the individual components of the equipotent mixture.
[b] Correspond to the concentrations tested in this study.
[c] 1.6 mM of mixture is constituted by 334 µM MDMA, 790 µM METH, 113 µM 4-MTA and 363 µM *d*-AMP.

HepG2 cells culture

HepG2 cells were kindly provided by Dr Maryam Modarai from UCL School of Pharmacy, London, UK. HepG2 cells were routinely maintained in 75 cm^2 canted-neck tissue culture flasks (Helena Biosciences; Gateshead, UK) in Minimum Essential Medium Alpha (MEM Alpha) with GlutMAX supplemented with 10% fetal bovine serum (FBS), 1% antibiotic (5,000 U/ml penicillin, 5,000 µg/ml streptomycin), 0.5% fungizone (250 µg/ml amphotericin B) and 0.0015% human transferrin (4 mg/ml) in a humidified incubator, at 37 °C, with 5% CO_2. Cells were sub-cultured at approximately 70% confluence over a maximum of 10 passages and regularly tested negative for *Mycoplasma*. All the reagents used in routine cell culture including 0.05% trypsin/1 mM EDTA and Hanks balanced salt solution (HBSS) without Ca and Mg were obtained from GIBCO, Invitrogen Corporations (Paisley, UK), unless stated otherwise.

Mixture design

The tested mixture was planned as described before (Dias da Silva et al. 2013). The composition of the mixture was based on parameters derived from previous experimental studies, using the tetrazolium-based (MTT) viability assay (Dias da Silva et al. 2013) and is shown in Table 1. The ratio between the four amphetamines was based on the concentrations eliciting 50% of the maximal mortality in the MTT assay (EC_{50}), after 48h incubations at 37 °C. This mixture design ensures that all mixture components are present at equipotent concentrations, thus preventing non-proportional individual contributions.

Drug treatments

In a previous study, we evaluated the cytotoxic effects of the mixture shown in Table 1 using the MTT assay and demonstrated that all four amphetaminic mixture components acted in an additive way to induce the overall mixture effect. Also, as the toxicity of the mixture could be accurately predicted by the mathematical model of *concentration addition*, it was suggested that all tested drugs acted via a similar mechanism of action (Dias da Silva et al. 2013). In the work presented here, it was important to select test concentrations (of individual amphetamines and mixture) that produced an equal level of cell death, *i.e.* were equipotents. In doing so, we ensured an easy and direct comparison between individual drugs and their mixture in the outcomes evaluated. Based on the additivity interactions shown previously, it is expected that all chemicals will have the same mechanism of action and therefore the equipotency of all tested drugs, as well as the mixture should be maintained in all studied endpoints. A deviation from those will suggest a difference in mechanism of action, rather than a difference in potency.

Consequently, the individual drugs and mixture were tested at concentrations that induced 25% of the maximum effect observed in the MTT reduction assay (EC_{25}), *i.e.* 1.3 mM MDMA, 3 mM METH, 0.5 mM 4-MTA, 1.7 mM *d*-AMP and 1.6 mM mixture (Dias da Silva et al. 2013) (Table 1). This effect level was selected as it corresponds to some degree of toxicity by moderately decreasing cell metabolic competence/viability, allowing the assessment of mechanisms of action and avoiding high levels of mortality.

Neutral red uptake for cell viability assessment

The neutral red (NR) viability assay, originally used by De Renzis and Schechtman (1973), is based on the ability of viable cells to incorporate and subsequently retain the supravital dye neutral red (*3*-amino-*m*-dimethylamino-*2*-methylphenazine hydrochloride; Sigma-Aldrich). This weak cationic compound readily penetrates the cellular membrane and accumulates in the lysosomes (lower pH) by binding the matrix through its anionic sites. Modifications of cell surface or delicate lysosomal membrane determine lysosomal fragility that become permanent, with consequent decreased in the dye uptake (Borenfreund and Puerner 1985).
$8x10^4$ cells were seeded per well on the 96-well plates and allowed to attach. After 24h, the culture medium was replaced by treatment solutions (six replicates), freshly prepared in cell culture medium. Each plate also included a negative control. After the incubation periods (6h, 12h, 24h and 48h), at 37 ^oC (physiological temperature) or 40.5 ^oC (hyperthermic conditions), the culture medium was removed and the plate was rinsed with HBSS. 100 µl medium containing neutral red (50 µg/ml) was added to each well. After 2 h of incubation at 37 ^oC, the medium was removed and the plate was rinsed twice with HBSS. Then, 200µl lysis solution, containing 1% glacial acetic acid, 50% ethanol and 49% H_2O, was added to each well, and shaken for 15 minutes in the dark. The absorbance (Abs) of the coloured solution was measured with a multi-well plate reader (Labsystems Multiskan, Multisoft Primary EIA V.1.3-0, Basingstoke, UK) at 540 nm, and is directly correlated to the number of cells with an intact membrane and unimpaired lysosomal activity (Borenfreund and Puerner 1985).

ImageStream multispectral imaging flow cytometry

Flow cytometry is a technique with great sensitivity and accuracy that allows discrimination between cellular populations based on parameters such as cell size, complexity, and fluorescence (usually derived from antibodies labelling specific cellular structures). In order to distinguish between live, necrotic, and early and late apoptotic cells, the combination propidium iodide (PI)/ fluorescein labelled Annexin V (AnV-FITC) was used. Annexin V (AnV) is a Ca^{2+}-dependent protein with high affinity for phosphatidylserine (PS), a phospholipid component that is usually held on the inner layer of cell membrane by the enzyme flippase. When cells undergo apoptosis, PS is no longer constrained to the cytosolic side of the membrane, becoming exposed to the outer leaflet, where proapoptotic agents (highly conjugated with Ca^{2+}) readily and irreversibly bind to PS (Vermes et al. 1995). In contrast, PI enters the cell and stains the nucleus, after the membrane becomes permeable. Late apoptotic and necrotic cells (nonviable) promptly stain with PI (Vermes et al. 2000; Vermes et al. 1995). In late apoptotic cells, this is a consequence of the increased permeability instigated by nuclear pores, permitting the entrance of large molecules to the nucleus, such cationic nucleotide-binding dyes (Faleiro and Lazebnik 2000). In necrotic cells, mitochondrial disruption leads to the impairment of nuclear membrane integrity (Kroemer et al. 1998). After entering the cells, PI passes through the disrupted nuclear membrane and intercalates between the double-stranded DNA, causing red fluorescence in the nucleus (Vermes et al. 2000; Vermes et al. 1995). Cells with intact plasma membranes (viable and early apoptotic cells) exclude PI.

To characterize the type of cell death elicited by amphetamines, isolated or in mixture, on HepG2 cells, and evaluate the influence of temperature and time of exposure, $1x10^6$ cells were seeded onto 10 cm^2 petri dishes and left to attach for 24 h. Then, the cells were exposed to the test solutions and incubated at 37 ^oC or at 40.5 ^oC, for 24 h or 48h. At the end of the treatment periods, the cells were rinsed twice with HBSS and trypsinized for 5 min with 300 µl 0.05% Trypsin-EDTA, at 37 °C. The cell culture media was kept and processed since adherent cells undergoing apoptosis may detach from the culture surface. Cells were washed in cold HBSS at 500 x g for 5 min (pre-cooled centrifuge, at 4 ^oC) and the supernatant was discarded. The cell pellet was resuspended in approximately 100 µl of 1x annexin-binding buffer (BD Pharmigena. Oxford, UK). Cell counting was carried out using a Neubauer chamber and the density of the cell suspension determined. Final cell density was then adjusted to ~$1x10^7$ cells per ml. The samples were stained with 5 µl of AnV-FITC and 5 µl PI (BD Pharmigena. Oxford, UK). After gently vortexing, cells were incubated for 15 min, at room temperature (RT, 25°C), in the dark. All the cell preparation procedures were carried out with care to maintain cellular integrity. The samples were placed on ice and the analysis was performed within 1 h on the ImageStream multispectral imaging flow cytometer (Amnis Corporation, Seattle, WA). The system provides both photometric and morphometric classifications, as it enables visualization of individual cells passing through the flow chamber, offering additional information regarding morphology and spatial distribution of each cell. At least 5-10 x 10^4 images were collected for each sample and images were divided into four sub-groups: live cells (unstained), cells at early apoptotic state (stained with AnV-FITC), cells at late apoptotic state (stained by both AnV-FITC and PI) and necrotic cells (stained with PI). Debris, including cell dublets and clumps, was

eliminated from the data as the machine gates around only single cells for analysis. A non-treated sample was divided into 3 tubes corresponding to negative control (AnV−/PI−), AnV-positive control (AnV+/PI−) and PI-positive control (AnV−/PI+) groups, which were used for compensation. Acquired results were processed using the ImageStream Data Analysis and Exploration Software (IDEAS 5.0).

ATP content

Cellular ATP levels can be rapidly detected through a bioluminescent luciferase-based assay, since luciferase catalyses the formation of light from the reaction of ATP and luciferin. Briefly, $1x10^6$ cells were seeded onto 10 cm^2 petri dishes to obtain confluent monolayers 24 h later. On the day of the experiment, the media was gently aspirated and the cells were incubated for 24 h with MDMA, METH, 4-MTA, *d*-AMP and mixture dilutions prepared in fresh medium, at 37 ºC or at 40.5 ºC. Then, the cells were rinsed twice with HBSS and scrapped/ precipitated with 5% perchloric acid ($HClO_4$, w/v). After centrifugation for 5 min at 16,000 x g (4 °C), the supernatants were kept frozen at –80 °C until further quantification. The pellet was used for protein quantification. The thawed acidic supernatant was neutralized with an optimised equal volume of 0.76 M potassium bicarbonate ($KHCO_3$) and centrifuged for 5 min at 16,000 x g (4 °C). The ATP contents were measured in triplicate in 96-well white plates (Greiner CELLSTAR® from Sigma), using an ATP determination kit (Invitrogen Corporations) following the manufacturer instructions. ATP levels are linearly related to the emitted light intensity that was determined by using a luminescence microplate reader (FLUOstar Optima, BMG Labtech GmbH) and compared to a standard curve performed with each experiment. The results were normalized to the total protein amount, and the final results were expressed as nmol ATP.mg^{-1} protein.

Protein determination

Protein was quantified through the Bradford assay (Bradford 1976). The absorbance measurements were taken at 595 nm using a multi-well plate reader (Labsystems Multiskan, Basingstoke, UK) and were compared with a bovine serum albumin (BSA, Sigma) standard curve to assess protein amount.

Cell treatment for RNA isolation

HepG2 cells were seeded at a density of $1x10^6$ cells per 10 cm^2 petri dish and grown for 24 h. The cell culture media was then replaced by selected dilutions of MDMA, METH, 4-MTA, *d*-AMP and mixture. Non-treated cells were used as controls. Following the dosing period (1 h, 4 h, 8 h, 16 h, 24 h and 48 h at 37 ºC), the cells were harvested by the addition of 300 µl 0.05% trypsin-EDTA and incubated at 37 °C for 5 min. The cell suspension was centrifuged at 500 x g for 5 min and the supernatant was removed. The pellet was immediately frozen in liquid nitrogen and stored at −80 °C until further use for RNA isolation.

Isolation and quantification of total RNA

Total RNA was isolated from cell suspensions using the Nucleospin RNA II kit (Macherey-Nagel, Abgene, Epson, UK) following the manufacturer instructions and the obtained RNA pellets were solubilised in RNAse/DNAse free water to prevent traces of genomic contamination. The concentration and purity of the final preparations were determined after measuring the absorbances at 230 nm, 260 nm and 280 nm, by calculating the 260/230 and 260/280 absorbance ratios, respectively (NanoDrop 1000-v3.7 spectrophotometer, Thermo Fisher Scientific).

Reverse transcription

Approximately 2.5 µg of total RNA was reverse transcribed into cDNA using the following protocol: 7 µl 5x RT buffer (Promega, Southampton, UK), 4 µl 10 mM dNTP mix (Agilent Technologies), 1 µl RNAse inhibitor (Promega), 1 µl hexamer random primers (Invitrogen), 2.5 µg RNA and 15 µl of RNAse/DNAse free water (Sigma-Aldrich) were mixed and heated at 62 °C for 10 min. The samples were then cooled on ice for 2 minutes before adding 2 µl M-MLV Reverse Transcriptase (Promega). The reaction was then allowed to proceed for another 90 min at 42 °C. The reverse transcribed products were stored at −80 °C until further use.

Primer design and quantitative real-time PCR (qPCR)

Primers for qPCR were designed using Beacon designer 5.1 software (Premier Biosoft International, Palo Alto USA), and purchased as high quality, purified OliGold Primers from Eurogentec Ltd (Hampshire, UK). The sequences of the primers used are presented in Table 2.

cDNA was used in qPCR reactions performed on the iCycler iQ qPCR detection system with the iCycler v3.1. Software (Bio-Rad Laboratories, Hertfordshire, UK).

Table 2. Oligonucleotide primer sequences for real-time PCR.

Gene	Genebank accession number	Primer	Sequence	Conc. (nM)	Prod. length (bp)
β-ACTIN	X00351	Forward	**5′-TCAGCAAGCAGGAGTATG-3′**	300	97
		Reverse	**5′-GTCAAGAAAGGGTGTAACG-3′**		
GAPD	NM_002046	Forward	**5′-TCTCTGCTCCTCCTGTTC-3′**	900	120
		Reverse	**5′-GCCCAATACGACCAAATCC-3′**		
HPRT1	M31642	Forward	**5'-CCTTGGTCAGGCAGTATAATCC-3′**	100	135
		Reverse	**5′-GGGCATATCCTACAACAAACTTG-3′**		
BCL-2	EU287875	Forward	**5′-CCTGGTGGACAACATC-3'**	100	160
		Reverse	**5′- GAGCAGAGTCTTCAGAG-3'**		
BCL-xL	BT007208	Forward	**5′- TGAACAGGTAGTGAATGAAC-3'**	100	105
		Reverse	**5′-TCCTTGTCTACGCTTTCC-3'**		
BAX	BC014175	Forward	**5′- CCTCCTCTCCTACTTTGG-3'**	150	103
		Reverse	**5′-GCCTCAGCCCATCTTC-3'**		
BIM	O43521	Forward	**5′- GCCACTACCACCACTTG-3'**	150	84
		Reverse	**5′-GCAGCGAACCGAATACC-3'**		
BAD	BT006678	Forward	**5′-GATGAGTGACGAGTTTGTG-3'**	150	133
		Reverse	**5′-GCCCAAGTTCCGATCC-3'**		
PUMA	AF354654	Forward	**5′-AGGCGATTGCGATTGG-3'**	150	107
		Reverse	**5′-CCCTGCTCTGGTTTG-3'**		
BID	AY005151	Forward	**5′-GTGAACCAGGAGTGAGTC-3'**	200	112
		Reverse	**5′-AAGCCAAACACCAGTAGC-3'**		

For a total volume of 20 µl per reaction, 0.8 µl cDNA and 10 µl 1x iQ SYBR Green Supermix (Bio-Rad) were mixed with 9.2 µl of an adequate dilution of forward and reverse primers mixture (1 µM stock) in a 96-well low profile PCR plate (Bio-rad). Optimised primer concentrations are listed in Table 2. Melting curves were generated for every primer pair following every qPCR reaction, to ensure that a single amplification product was obtained. Amplification was optimised for each gene with regard to temperature, and primer and template concentration (data not shown). PCR conditions were considered optimized when PCR efficiency ranged between 98 and 100%. Relative quantification was performed using the comparative threshold cycle method. *GAPDH*, *HPRT1* and *β-ACTIN* were tested as internal controls (reference genes). As neither gene's expression changed with the treatments, *HPRT1* was used to normalize gene expression throughout.

Data analysis for qPCR

Expression data were analysed in terms of relative quantification. *HPRT1* was used as reference gene. Data were normalized and relatively compared to the control group according to the ΔΔCt model with the following formulas (Livak and Schmittgen 2001):

$$\Delta Ct = Ct_{\text{(target gene)}} - Ct_{\text{(reference gene)}}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{(treatment group)}} - \text{mean } \Delta Ct_{\text{(control group)}}$$

The expression ratio of the treatments compared to the controls is expected as $2^{-\Delta\Delta Ct}$. Results were displayed as fold increase over controls standardized such that values obtained in controls were set to 1.

Immunoblotting

HepG2 cells were seeded at a density of $1x10^6$ cells per 10 cm^2 petri dish and cultured for 24 h. Then, cells were incubated with the test drugs for 24 h (PARP/cleaved PARP detection) or 12 h (all other proteins), at 37 ^oC. After the incubation period, HepG2 cells were lysed by incubating for 20 min with a freshly prepared ice-cold Laemmli sample buffer, containing: 62 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulphate (SDS), 10% glycerol, 5% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1:100 Protease Inhibitor Cocktail (Sigma) (Laemmli 1970). Cells were harvested from the petri dishes with a rubber scraper and transferred to pre-chilled tubes. Lysates were boiled at 95 ^oC for 10 minutes and pulsed spun. Equal amounts of whole-cell lysates (25-40 µg of total protein) were loaded onto 9-12% polyacrylamide gels and submitted to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).At the end of each

run, proteins were electrophoretically transferred from the gels onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) at 40 V for 1 h, at RT, using a transfer buffer containing 25 mM Tris, 190 mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol. After blotting, non-specific binding was blocked by soaking the membranes in 20mM Tris-base, 0.1 M NaCl, 0.1% (v/v) Tween-20, pH 8 (TBS-T buffer) containing 5% non-fat dry milk and incubated for 1 h. Afterwards, the membranes were washed three times for 5 min, with 15 ml TBS-T buffer. They were then incubated overnight at 4 °C with rabbit polyclonal anti-Puma antibody (Cell Signaling Technology, Inc.; 1:1000 in 5% w/v BSA/TBS-T); rabbit monoclonal anti-Bak antibody (Cell Signaling Technology, Inc.; 1:1000 in 5% w/v BSA/TBS-T); and rabbit monoclonal anti-Bax antibody (Cell Signaling Technology, Inc.; 1:1000 in 5% w/v BSA/TBS-T). Rabbit polyclonal anti-Bid antibody (Cell Signaling Technology, Inc.; 1:1000 in 5% w/v nonfat dry milk/TBS-T) was employed for detection of both full length (22 kDa) and cleaved large fragment (15 kDa) of human Bid; whereas levels of total Bim (EL, L and S isoforms) protein were analysed with rabbit monoclonal anti-Bim antibody (1:1000 in 5% w/v BSA /TBS-T). Rabbit polyclonal anti-PARP antibody was used for detection of endogenous levels of full length PARP (116 kDa), as well as the large fragment (89 kDa) of PARP resulting from caspase cleavage (Cell Signaling Technology, Inc.; 1:1000 in 5% w/v nonfat dry milk/TBS-T). Thereafter, the membranes were washed 3 times for 5 min with TBS-T buffer. After washing, the membranes were incubated with secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signaling Technology, Inc.), diluted 1:3000 in 5% w/v non-fat dry milk /TBS-T, for 1 h at RT. Finally, the membranes were washed again following the procedure described above. Detection of bound antibodies was carried out using the enhanced chemiluminescence (ECL) system, consisting of the incubation of the membranes for 1 min with the ECL buffer (Trisma-HCL 100 mM, pH 8.5) supplemented with 250 mM luminol (Avocado Research Chemicals, Lancashire, UK), 90 mM cumaric acid (Sigma) and hydrogen peroxide (H_2O_2, Sigma). The luminescent signal was captured by ChemiDoc XRS System (Bio-Rad). To ensure that the exact same amount of protein was evenly loaded into the SDS-acrylamide gels, the membranes were stripped of the antibody and re-probed with anti-β-Actin antibody (Cell Signaling Technology, Inc.; 1:1000 in 5% w/v BSA/TBS-T).

Stripping and re-probing of membranes

Membranes were incubated at 50 °C with stripping buffer (0.5 M Tris-HCl (pH 6.8), 20% SDS, 0.8% β-mercaptoethanol) for 45 min with gentle agitation. The blots were then rinsed with TBS-T, re-blocked and re-probed with anti-β-Actin antibody as described above.

Mitochondrial integrity by TMRE inclusion

Assessment of mitochondrial integrity was evaluated by measuring the tetramethylrhodamine ethyl ester (TMRE) inclusion. TMRE is a cell permeable fluorescent dye that specifically stains viable mitochondria, being accumulated in proportion to mitochondrial membrane potential ($\Delta\psi_m$) (Scaduto and Grotyohann 1999). Briefly, 8 x 10^4 HepG2 cells were seeded onto 96-well black plates (Greiner CELLSTAR® from Sigma). After 24 h, the media was gently aspirated and the cells were exposed to MDMA, METH, 4-MTA, *d*-AMP, as well as mixture solutions prepared in fresh medium at 37 ºC or at 40.5 ºC. At the end of the 24 h treatment period, cells were rinsed twice with HBSS and incubated at 37 °C with 100 µl of 2 µM TMRE (Sigma), for 30 min. As TMRE is a non water-soluble powder, a 2 mM stock solution was initially prepared in DMSO (Merck) and stored in the dark. The final 2 µM concentration was prepared in fresh culture media, shortly before usage (the final concentration of DMSO did not exceed 0.5%). Afterwards, the media was gently aspirated and replaced by 0.2% BSA in HBSS. Fluorescence was measured at 37 °C on a fluorescence microplate reader (FLUOstar Optima, BMG Labtech GmbH) set to 544 nm excitation and 590 nm emission wavelengths. The data obtained for the individual drug and mixture treatments were normalized on a plate-by-plate basis to the values of the respective controls and calculated as the percentage of control conditions at 37 ºC or 40.5 ºC. This normalization is required to minimize the variability among the individual plate readings and has been extensively described elsewhere (Rajapakse et al. 2004).

Measurement of caspase-3/7 activity

Caspase-3/7 activation assay was performed using a Caspase-Glo 3/7 assay kit according to the manufacturer's instructions (Promega, Southampton, UK). Briefly, HepG2 cells were seeded onto Greiner Bio-One CELLSTAR® 96-well white plates (Fisher, UK) at a density of 10^4 cells/well. After 24 h, the cells were exposed to the 4 tested amphetamines, individually or in mixture at 37 ºC. Each plate also included a blank (medium alone), a negative control (untreated cells) and a positive control (15 µM Mitomycin C, MMC, derived from *Streptomyces caespitosus*, Sigma-Aldrich).

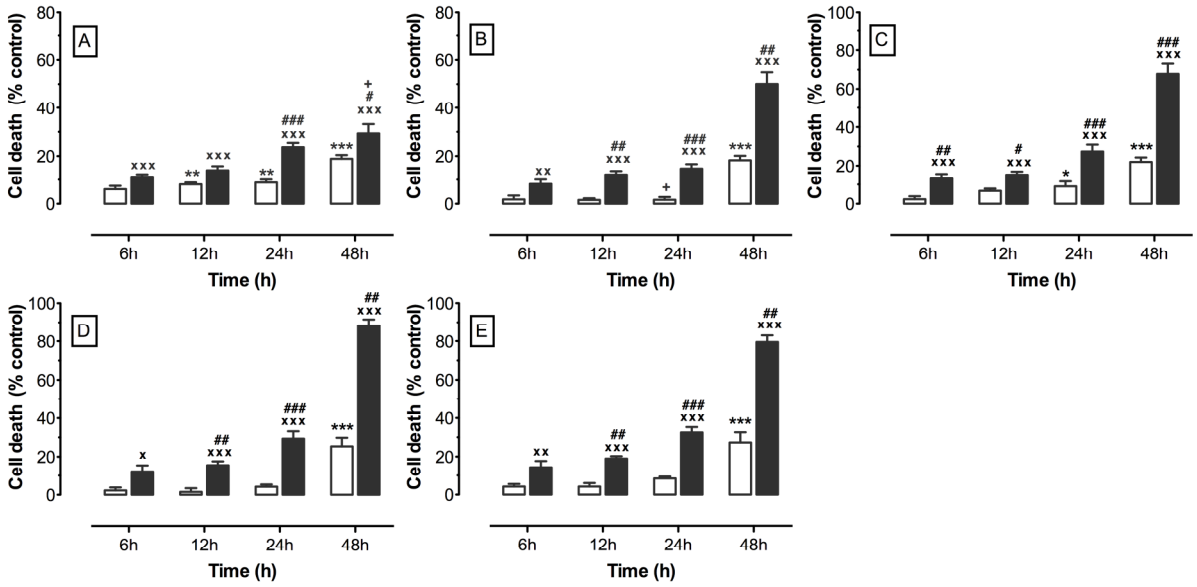

Figure 1. Time-course of HepG2 cell death, as indicated by NR lysosomal inclusion, after exposure to MDMA (A), METH (B), 4-MTA (C) and *d*-AMP (D), individually or in mixture (E), at 37 °C (white bars) or 40.5 °C (black bars). The compounds were tested at concentrations that induce 25% of the maximum effect observed in the MTT reduction assay (EC_{25}), *i.e.* 1.3 mM MDMA, 3 mM METH, 0.5 mM 4-MTA, 1.7 mM *d*-AMP and 1.6 mM mixture (Dias da Silva et al. 2013). Results presented as mean ± SEM are from five independent experiments, run in six replicates. Statistical comparisons were made by Kruskal–Wallis test followed by the Dunn's *post hoc* test. *p<0.05, **p<0.01 ***p<0.001 show statistically significant differences *versus* controls at 37 ◦C. ×p<0.05, ××p<0.01 and ×××p<0.001, indicate significant differences *versus* controls at 40.5 ◦C. #p<0.05, ##p<0.01 and ###p<0.001 show statistically significant differences *versus* 37 °C. +p<0.05 indicates differences between individual treatments and the mixture at same incubation time and temperature.

All the treatments were run at least in triplicate. Following the dosing period (1 h, 4 h, 8 h, 16 h, 24 h, 36 h and 48 h), Caspase-Glo 3/7 reagent was added to each well. The plate was then incubated in the dark at RT, for 1 h and the luminescence was measured with a multiwell plate-reading luminometer (FLUOstar Optima, BMG Labtech GmbH). Luminescence is proportional to the amount of caspase activity present.

Sulforhodamine B Assay

As the different treatments and time points may influence the cellular density per well in the Caspase 3/7 assay, the Sulforhodamine B (SRB, Sigma-Aldrich) assay was run in parallel for each treatment and used for normalization of the cell number. Briefly, after the testing periods, the assay was finished by placing a clear 96-well plate on ice, for 1 min. After gently removing the experimental media, the cells were fixed with 200 µl of 10% trichloroacetic acid, for 20 min and rinsed 5 times with tap water. After air-drying, 50 µl 4% SRB was added to each well and incubated for 10 min. The cells were then washed with 1% acetic acid until the solution ran clear, to remove unbound dye. After air-drying in the dark, the bound dye was solubilised with 100 µl of 10 mM Tris solution (pH 10.5). Absorbances were measured at 510 nm, directly in the plate, using a multi-well plate reader (Labsystems Multiskan, Basingstoke, UK). The data obtained was correlated with the cell number of each well by means of a calibration curve previously plotted.

Statistical analysis

All assays were performed in at least four independent experiments (with exception of the cytometry assay performed in two) and SD values were always < 10%. Data achieved were expressed as mean ± SEM (standard error of the mean). All statistical calculations were performed using the GraphPad Prism version 5.0a for Mac OS X (GraphPad Software, San Diego California, USA). Normality of the data distribution was assessed by three tests (Kolmogorov–Smirnov normality test, D'Agostino and Pearson omnibus normality test and Shapiro–Wilk normality test). The control values (raw data) obtained for hyperthermic and normothermic incubations in the determination of the mitochondrial integrity were compared by the Student's unpaired *t*-test in order to investigate potential effects associated with the increase of temperature alone. All the other statistical comparisons were estimated by the nonparametric method of Kruskal–Wallis [one-way analysis of variance (ANOVA) on ranks] followed by the

Dunn's multiple comparison test. *p* values lower than 0.05 were considered statistically significant.

Results

Viability assessment by NR lysosomal uptake

In order to corroborate the previously published cytotoxicity of MDMA, METH, 4-MTA, d-Amp and mixture in HepG2 cells (Dias da Silva et al. 2013) and confirm that the concentrations selected were in fact equieffective and corresponded to 25% toxicity effects, we evaluated the viability of HepG2 cells in response to the amphetamines and their mixture in the NR lysosomal uptake assay. The data presented in Fig. 1 (white bars) indicate a time-dependent increase in cytotoxicity, when the HepG2 cells were exposed at 37 oC to the 4 tested amphetamines, alone or combined. At 37 ºC, the observed cytotoxicity in the NR assay only reached significance ($p<0.001$) when the cells were incubated for 48 h, with the exception of MDMA and 4-MTA. Conversely, when the incubation temperature was raised to 40.5 oC, all the drugs (alone or combined) induced effects significantly different to controls ($p<0.001$), for all exposure periods.

Overall, we observed that, at 48 h, under normothermia (the temperature used to determine the equieffective cytotoxic concentrations in the MTT assay) the cytotoxicty determined by the NR assay showed effect levels that were similar to those obtained in the MTT, confirming that, at the selected concentrations, the cytotoxic effect of the individual amphetamines and mixture is similar. Accordingly, no substantial disparities were observed when comparing responses elicited by individual amphetamines with those obtained from the mixture. The noted exceptions were for MDMA (at 40.5 ºC after 48 h, $p<0.05$) and METH (at 37 ºC after 24 h, $p<0.05$).

Differential contributions of necrosis and apoptosis to HepG2 cell death elicited by amphetamines

To investigate which mechanisms mediate the cytotoxic effects of the four amphetamines and their combination we analysed, by flow cytometry, the preponderance of apoptosis and necrosis to the observed hepatocyte death. HepG2 cells had been exposed to individual and combined drugs at different time points (24 h and 48 h) and different temperatures (37 ^{o}C and 40.5 ^{o}C). After incubation, the cells were stained with AnV-FITC and PI and evaluated using the ImageStream multispectral flow cytometer (Amnis Corporation, Seattle, WA). On the ImageStream, each cell was concomitantly imaged in dark field (488 nm laser side-scatter), green fluorescence (500–550 nm, annexin V channel), orange fluorescence (550–600 nm, PI channel), and bright field (660–720 nm). Based on the total intensities of AnV-FITC and PI staining as well as morphological features, the cells were grouped into live (AnV−/PI−), early apoptotic (AnV+/PI−), late apoptotic (AnV+/PI+) and necrotic (AnV−/PI+) populations (Fig. 2A).

Of note, is the fact that positive ANV/PI double staining is not always specific to cells in late stages of apoptosis and may also be present in cells undergoing necrosis, so they cannot be distinguished based solely on fluorescence descriptions. A unique aspect of the ImageStream flow cytometry system is that it allows visual examination of the images taken, allowing the differentiation between advanced apoptotic and necrotic populations, based on their specific morphology. Cells in late apoptosis exhibit shrunken, irregular shape and condensed (pyknosis), fragmented nuclei (karyorrhexis).

Necrotic cells are larger, with large, unfragmented nuclei that uniformly stain with PI. Additionally, apoptotic cells exhibit lower bright field intensity and greater granularity compared to necrotic cells (Fig. 2A).

Another exclusive quality of data obtained with the ImageStream system is that each data point on the scatter graph can be directly related to the image of the contributing cell (Fig. 2B). After categorizing live, early apoptotic, late apoptotic and necrotic populations using fluorescence intensities, the identities of cell populations were confirmed using this 'virtual cell sorting' function (George et al. 2004) of the IDEAS data analysis software that analyses the data from the ImageStream, by inspecting the bright field, fluorescence, and dark field (laser side-scatter) imagery obtained from each cell. These population gates were adjusted based on the morphology of cells, permitting better resolution of necrotic from late apoptotic cells.

Figure 3 depicts the relative amounts (% of cell population) of live, early apoptotic, late apoptotic and necrotic HepG2 cells at the time of the staining. The vast majority of untreated cells (negative controls) cultivated at 37 ^{o}C were viable after 24 h exposures (75.5 ± 0.6%). Usually, the mortality in the controls is not as high (up to 15%). The reasoning for the relatively elevated amount of cells undergoing cell death processes might be related with the basal levels within the cell line population, the after-staining waiting time before measurement, or the damage to the plasma membrane caused during the preparation procedures, which could have occurred, despite all the care taken during technique execution.

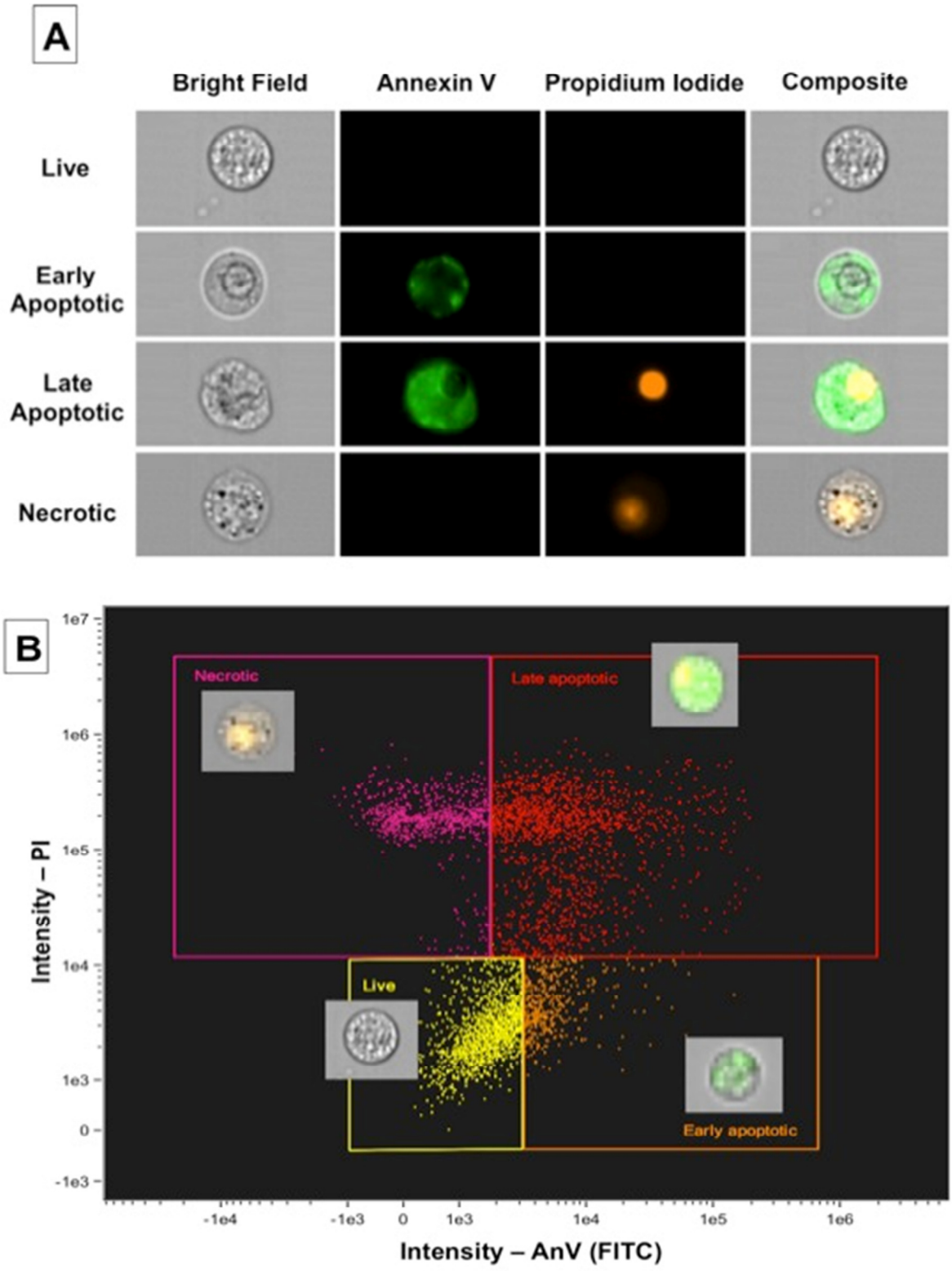

Figure 2. Imaging flow cytometry-based cellular classification according to fluorescent staining and morphological examination of HepG2 cells using ImageStream multispectral flow cytometer (Amnis Corporation, Seattle, WA). A. Morphological examination of HepG2 cells classified into live (AnV−/PI−), early apoptotic (AnV+/PI−), late apoptotic (AnV+/PI+) and necrotic (AnV−/PI+) cells based on staining profile. **B.** Representative analysis of gated cells to show procedure for classification. PI = Propidium iodide; AnV = Annexin V; Composite image = an overlay of AnV, PI, and bright field.

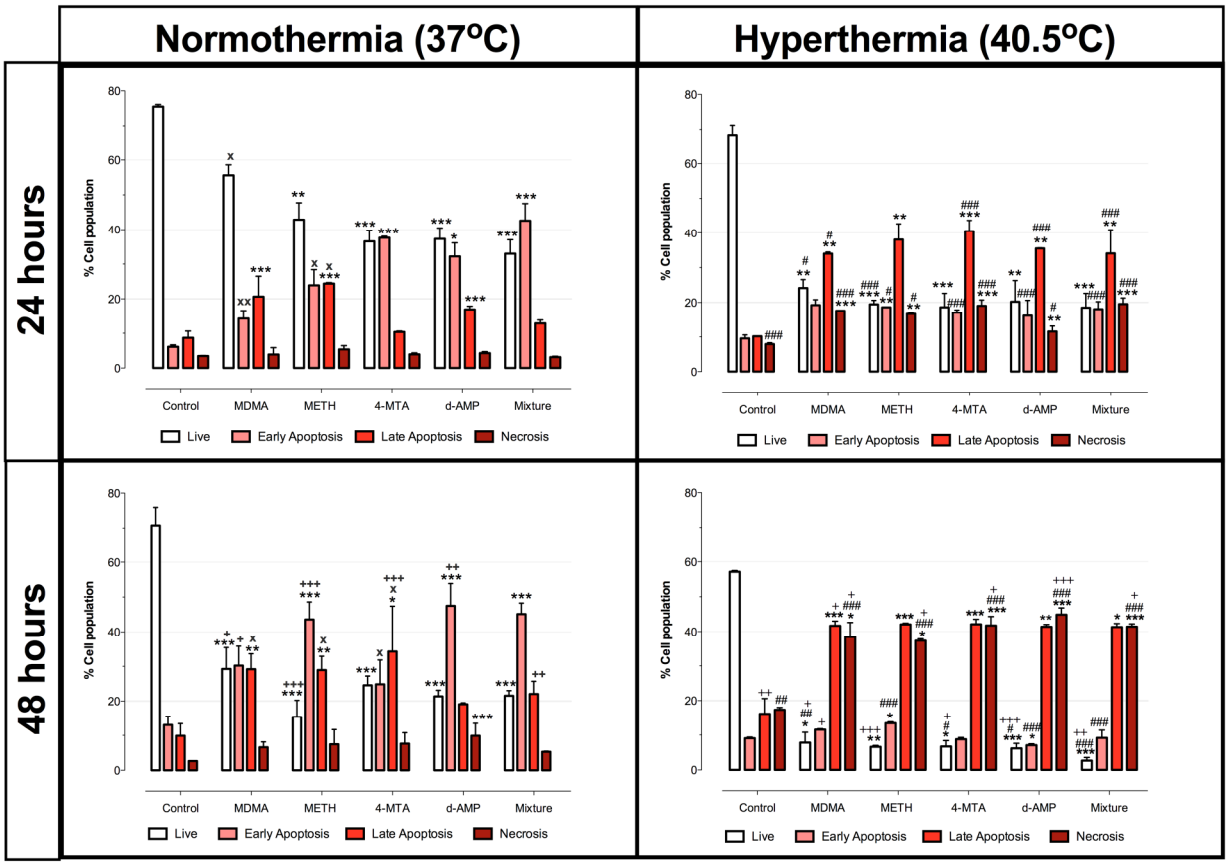

Figure 3. Effect of both temperature (37 ºC or 40.5 ºC) and incubation time (24h or 48h) on AnV/PI staining of HepG2 cells after exposure to MDMA, METH, 4-MTA and *d*-AMP, individually or in mixture. The treatments were tested at concentrations that induced 25% of the maximum cytotoxic effect observed in the MTT reduction assay (EC_{25}), *i.e.* 1.3 mM MDMA, 3 mM METH, 0.5 mM 4-MTA, 1.7 mM *d*-AMP and 1.6 mM mixture (Dias da Silva et al. 2013). HepG2 population was separated into live (AnV−/PI−), early apoptotic (AnV+/PI−), late apoptotic (AnV+/PI+), and necrotic cells (AnV−/PI+), according to cellular staining with AnV-FITC and PI and cell morphology. Results are mean ± SEM from two independent experiments, run in triplicate. Statistical comparisons were made by using Kruskal–Wallis test followed by the Dunn's *post hoc* test. *p<0.05, **p<0.01, ***p<0.001 show statistically significant differences *versus* controls at the same incubation time and temperature. ×p<0.05, ××p<0.01 represent statistical comparisons between individual drugs and the mixture at the same incubation time and temperature. #p<0.05, ###p<0.01 and ###p<0.001 show statistical comparison *versus* the 37 ºC incubation temperature. ++p<0.01 and +++p<0.001, represent statistically significant differences *versus* the 24h incubation time.

The viable fraction in controls slightly decreased with the increase of the temperature to 40.5 ºC (68.4 ± 2.8% at 24 h) and time of incubation to 48 h (70.75 ± 5.15% at 37 ºC), reaching the highest drop when cells were incubated at 40.5 ºC for 48 h (57.15 ± 0.35%). The number of living cells suffered a substantial decline when HepG2 cells were treated with all the amphetamines, alone or combined (p<0.05 *versus* control). Analysis of cell populations revealed that the distribution of the cells between the population sets (live, early apoptotic, late apoptotic, and necrotic cells) varies with temperature and incubation time. An overall evaluation indicated that, at 37 ºC, with the increase of incubation time from 24 h to 48 h, there is a decrease in cell viability that is mostly counterbalanced by an increase in cells in early and/or late stages of apoptosis; while at 40.5 ºC the decrease in cell viability predominantly gives place to a slight rise in the late apoptosis population. A more obvious tendency exists regarding the temperature increment from 37 ºC to 40.5 ºC as, along with the decrease of cell viability, there is a noteworthy expansion in the number of cells undergoing necrosis, what is clearly observed for all treatments (p<0.05), for both 24 h and 48 h incubations.

In general, no significant differences were detected when HepG2 cells were exposed to amphetamines individually or in mixture. Notwithstanding, some exceptions occurred at 37 ºC, with early apoptosis more evident in the mixture relative to the MDMA and METH individual incubations after 24h, and late apoptosis less evident in the mixture relative to the individual MDMA, METH, and 4-MTA individual treatments at 48h incubations. This indicates that, although these drugs lead to the same cytotoxic outcome, the underlying mechanisms may slightly differ.

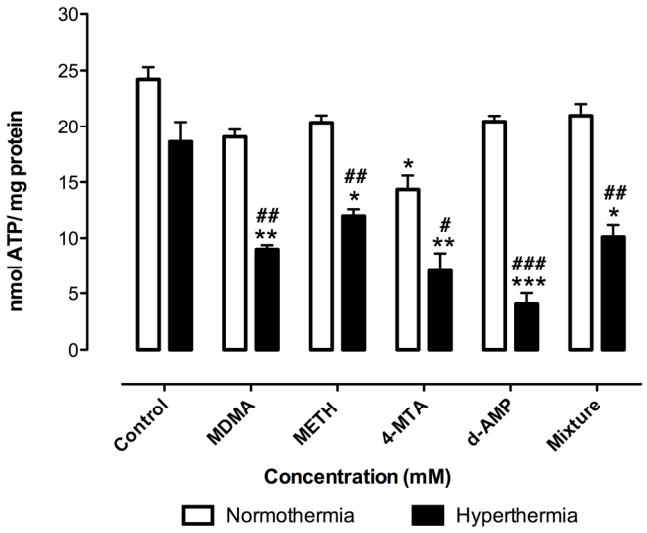

Figure 4. Effect of MDMA, METH, 4-MTA, *d*-AMP and the mixture on ATP intracellular storages in HepG2 cells, at 37 ◦C (white bars) or 40.5 ◦C (black bars), after 24 h exposure. HepG2 cells were exposed to amphetamines, for 24h, at the following equieffective concentrations ($EC_{25\ MTT\ Assay}$, after 48h at 37 ºC): 1.3 mM MDMA, 3 mM METH, 0.5 mM 4-MTA, 1.7 mM *d*-AMP and 1.6 mM mixture (Dias da Silva et al. 2013). Results from at least four independent experiments are presented as mean ± SEM. Statistical comparisons were made by using Kruskal–Wallis test followed by the Dunn's *post hoc* test. **p*<0.05, ***p*<0.01, ****p*<0.001 represent statistical differences *versus* controls within the same incubation temperature (37 ºC or 40.5 ºC). #*p*<0.05, ###*p*<0.01 and ###*p*<0.001 show statistical comparison between different incubation temperatures.

Intracellular energetic levels

Research evidence indicates that the cell death fate, either by apoptosis or necrosis, is a condition determined by intracellular ATP levels, as apoptosis is an extremely controlled phenomena involving a number of energy-dependent processes, such as enzymatic hydrolysis (*e.g.* caspase activation), chromatin condensation, contraction and membrane blebbing (Eguchi et al. 1997; Zamaraeva et al. 2005).

Results displayed in Fig. 4 suggest there were no significant differences in cellular ATP between controls and drug treatments, when HepG2 cells were exposed at normothermia (37 ºC). Nonetheless, the increase in the temperature of incubation to 40.5 ºC prompted significant alterations in energy storages for all amphetaminic exposures (p<0.05). No statistically significant differences were observed between mixture and the drugs alone under the same temperature conditions.

Expression profiles of BCL-2 family genes

Our results revealed that apoptosis plays a highly significant role in the mechanism of cell death provoked by the amphetamines in HepG2 cells, particularly at a physiological temperature (37 ºC). With the aim of exploring the specific pathways responsible for initiating apoptosis in the presence of the test substances, early intracellular events linked to apoptotic phenomena, such as the regulation of pro and antiapoptotic factors, were studied under the same experimental settings.

BCL-2 is a family of interrelated proteins that regulate mitochondrial or intrinsic pathway of apoptosis via its proapoptotic and prosurvival (antiapoptotic) members. BCL-2 factors are also involved in the enhancement of the extrinsic death receptor pathway. So far, approximately 20 BCL-2 family members have been identified and are classically divided into three subfamilies: Bcl-2 pro-survival subfamily (including Bcl-2 and Bcl-x_L), Bax-like pro-apoptotic subfamily (comprises Bax and Bak), and BH3-only pro-apoptotic subfamily. These proteins are crucial controllers of the permeabilization of the outer membrane of the mitochondria, which promotes the release of cyt *c* and other apoptotic factors to the cytosol, activating the downstream effector caspase-3/-7 cascade.

As this is considered an irreversible point in the programmed cell death, these proteins play a pivotal role in deciding whether a cell lives or dies. Pro- and antiapoptotic BCL-2 members may be regulated by mechanisms other than post-translation alterations. So, to avoid harmful actions in viable cells, constitutively active proapoptotic regulators are transcriptionally silent, but in response to selected death stimuli transcription is initiated. To ascertain to what extent proapoptotic and antiapoptotic BCL-2 family regulators may account for triggering apoptosis in HepG2 cells, after exposure to amphetamines, the expression of some apoptosis-regulating genes was investigated by qPCR. BCL-2 family genes were found to be differentially expressed in HepG2 cells after incubation with equieffective cytotoxic concentrations of individual and combined amphetamines at 37 ºC, particularly after 8 h exposures (Fig. 5). In fact, this was the earliest incubation period, which exhibited statistically significant differences, despite several time points (1 h, 4 h, 8 h, 16 h, 24 h and 48 h) having been tested (data not shown). As shown in Fig. 5, a remarkable down-regulation of the antiapoptotic *BCL-2* and *BCL-$_{XL}$* genes was observed for all the treatments (p<0.001).

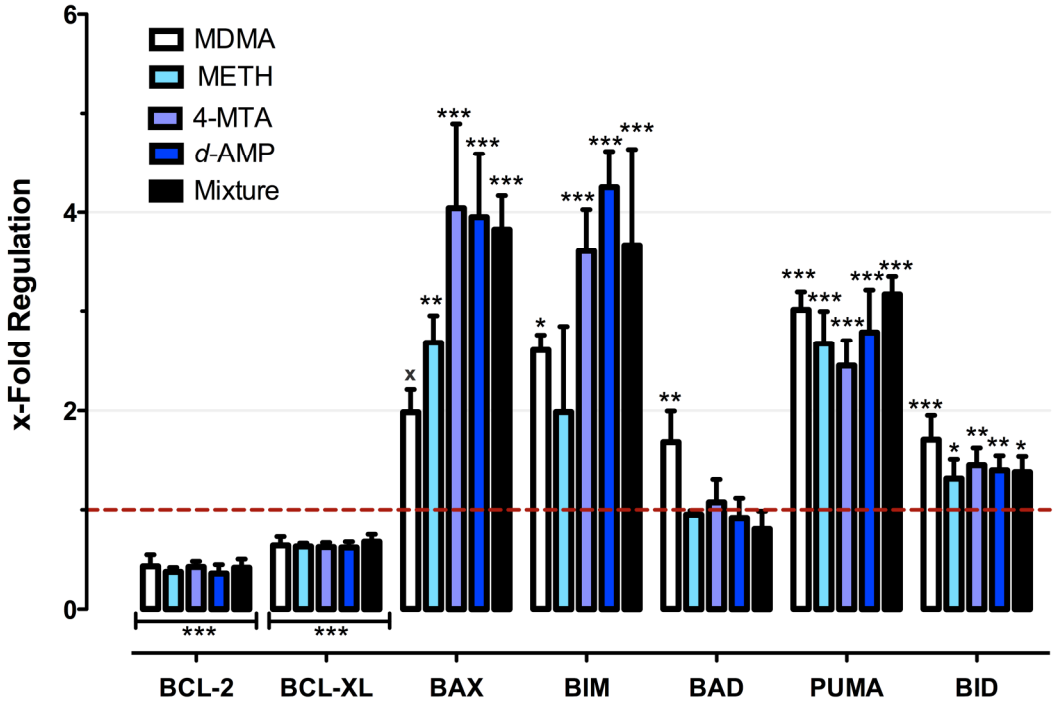

Figure 5. Gene expression of proapoptotic and antiapoptotic regulators involved in HepG2 cell death, elicited by individual and combined amphetamines. qPCR analysis of *BCL-2*, *BCL-$_{XL}$*, *BAX*, *BIM*, *BAD*, *PUMA* and *BID* mRNA levels was performed after incubation of HepG2 cells at 37 ºC, for 8 h with 1.3 mM MDMA, 3 mM METH, 0.5 mM 4-MTA, 1.7 mM D-AMP and 1.6 mM of mixture (concentrations inducing 25% of the maximum effect observed in the MTT reduction assay, EC_{25}) (Dias da Silva et al. 2013). Expression of each gene was normalized to *HPRT1* (reference gene) and compared to the control group, according to the $2^{-\Delta\Delta Ct}$ model. Control values were set to 1 (dashed red line), so values higher than 1 are indicative of up-regulation and values lower than 1 reveal down-regulation. Results are at least from four independent experiments and are given as mean ± SEM. Statistical comparisons were made using Kruskal–Wallis test followed by the Dunn's *post hoc* test. *p<0.05, **p<0.01, ***p<0.001 show statistically significant differences *versus* controls. ×p<0.05 indicates significant differences between treatments with individual drugs and the mixture.

Accordingly, a significant increase in the expression rates of the genes coding for proapoptotic factors, *BAX*, *BIM*, *PUMA* and *BID* was also found, with slight variations among the different drugs (Fig. 5). No alteration in the expression pattern of *BAD* was detected, with the exception of HepG2 cells exposed to MDMA (p<0.05). Individual and mixture results revealed great concordance, as no significant differences were observed; with exception of *BAX* in MDMA-treated cells (p<0.05).

Protein expression analysis

In order to confirm at the protein level the increased expressions observed at the gene level, the amount of proapoptotic BCL-2 family markers were investigated, after 12 h treatments by immunoblotting. Results showed an increase in the levels of proapoptotic Puma, Bim, Bid and Bax proteins in HepG2 cells, when these were treated with the test amphetamines. Similarly, an increase in Bak levels in relation to those present in untreated controls, was also observed for all treatments (Fig. 6). Nuclear PARP, a protein involved in DNA repair, is one of the main cleavage targets of caspase-3 and caspase-7 *in vivo* (Fig. 5). PARP inactivation has been proposed to prevent depletion of ATP, which is thought to be required for the latest events of apoptosis. Our data suggest that, at 24 h, there are signs of PARP cleavage, as given by the reduction in the intensity of the PARP (116 kDa) band and the presence of an 89 kDa fragment (corresponding to the cleaved protein), particularly evident in MDMA and *d*-AMP cases (Fig. 6).

Mitochondrial integrity assessed by TMRE inclusion

The mitochondria has been shown to play a major role in apoptosis by mediating and propagating death signals originated from the inside (intrinsic apoptotic pathway) or outside (extrinsic apoptotic pathway) of the cell.

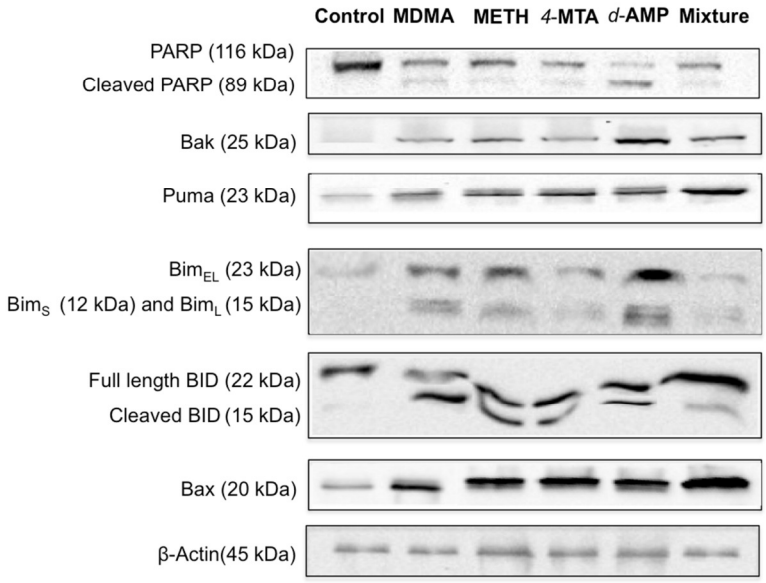

Figure 6. Immunoblotting analysis of the effect of MDMA, METH, 4-MTA, *d*-AMP and their mixture on the expression of proapoptotic markers. Western blotting of poly (ADP-ribose) polymerase (PARP)/cleaved PARP, Bak, Puma, Bim (EL, S and L isoforms), Bid/cleaved Bid and Bax protein expressions performed after incubation of HepG2 cells at 37 ºC, for 24 h (PARP/cleaved PARP) or 12 h (all other proteins), with 1.3 mM MDMA, 3 mM METH, 0.5 mM 4-MTA, 1.7 mM *d*-AMP and 1.6 mM mixture (concentrations inducing 25% of the maximum effect observed in the MTT reduction assay, EC_{25}) (Dias da Silva et al. 2013). β-Actin was used as an internal loading control.

Most apoptotic events involve the dissipation of the mitochondrial inner transmembrane potential ($\Delta\psi_m$), which is crucial for maintaining the physiological function of the mitochondrial respiratory chain. A significant loss in $\Delta\psi_m$ impairs oxidative phosphorylation, which depletes the cells of energy and promotes the release of apoptotic factors, with subsequent death. Therefore, the $\Delta\psi_m$ can provide important information about the mitochondrial function and the physiological status of the cell.

Our data on the accumulation of TMRE inside HepG2 cells exposed to test amphetamines for 24 h revealed only slight, insignificant (p>0.05) declines in $\Delta\psi_m$, when incubations were carried out at 37 ºC (Fig. 7). However, considerable deviations from controls were obtained in hyperthermic conditions (p<0.01), as observed by the decline in TMRE mitochondrial inclusion, indicating significant mitochondrial impairment.

No significant differences were identified (p>0.05) between control incubations at 37 ºC (40702 ± 12264 fluorescence units) and at 40.5 ºC (44255 ± 10562 fluorescence units), showing that the increase in temperature alone had no impact on $\Delta\psi_m$. Moreover, at 40.5 ºC, mixture results presented relevant dissimilarities from those obtained with MDMA, METH and 4-MTA (p<0.05). At the other tested conditions, no significant differences were observed between individual and combined drugs.

Measurement of caspase-3/-7 activity

As noted before, programmed cell death can be triggered by the activation of cell surface death receptors (extrinsic apoptosis pathways) or in response to signals instigated from inside the cell (intrinsic apoptosis pathways). Nonetheless, the signals of such diverse sources seem to ultimately converge to activate common downstream mechanisms, specifically the apoptosis executioner caspase cascade. Among effector caspases that are proteolytically activated are the caspase-3 and -7, two cysteine-aspartic acid proteases that, following activation, cleave a specific set of protein substrates, including procaspases themselves and PARP, resulting in the spreading of apoptotic death stimulus and eventually in the execution of cell suicide. The purpose of the current determination was to define the contribution of caspase-3/7 activity to the extent of observed cell death and identify the onset and peak of this molecular event for each treatment, in order to identify possible differences between the mechanisms underlying the cytotoxicity of individual and combined amphetamines.

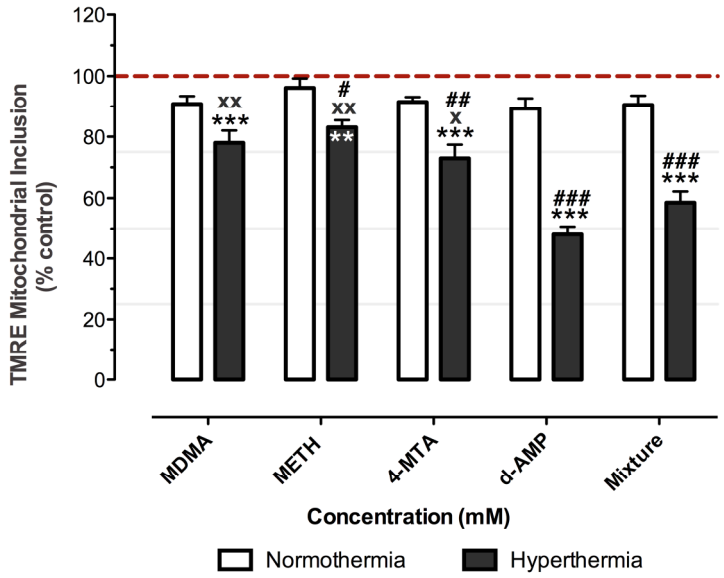

Figure 7. Effect of MDMA, METH, 4-MTA, *d*-AMP and mixture on mitochondrial membrane potential in HepG2 cells, at 37 ◦C (white bars) or 40.5 ◦C (black bars). HepG2 cells were exposed to amphetamines, for 24 h, at the following equieffective concentrations ($EC_{25\ MTT\ Assay}$): 1.3 mM MDMA, 3 mM METH, 0.5 mM 4-MTA, 1.7 mM *d*-AMP and 1.6 mM mixture (Dias da Silva et al. 2013). Shown are the mean ± SEM from four independent experiments, with six replicates each, expressed as percentage of controls (fluorescence of non-treated cells) for 37 ºC or 40.5 ºC incubations. The red dashed line indicates the control values, set to 100%. Statistical comparisons were made using the Kruskal–Wallis test followed by Dunn's *post hoc* test. **p*<0.05, ***p*<0.01, ****p*<0.001 show statistically significant differences *versus* controls, at the same incubation temperature. ×*p*<0.05, ××*p*<0.01 indicate differences between the individual treatments and the mixture, at the same incubation temperature. #*p*<0.05, ###*p*<0.01 and ###*p*<0.001 show statistical differences *versus* 37 ºC incubations.

As observed in Fig. 8, MDMA and METH induced a short, but significant increase in enzyme activity from 8-16 h of incubation (maximal effects of 1.34 ± 0.14 fold increase and 1.36 ± 0.21 fold increase, respectively), which remained more or less constant after 16 h of exposure (p<0.001). Earlier and more pronounced effects were observed when HepG2 cells were exposed to 4-MTA and to *d*-AMP, as significant effects were seen as early as 1h (4-MTA) and 4h (*d*-AMP) of incubation. A peak in the effects of these drugs was achieved at 16 h (5.08 ± 0.36 fold increase) and 24 h (3.91 ± 0.69 fold increase), for 4-MTA and *d*-AMP, respectively.

There were no statistically significant differences between the mixture and all other treatments, for any of the incubation periods. In fact, mixture effects were clearly seen at 8 h of treatment (p<0.05) and seem to lie between all individual responses, being more pronounced at 16 h (2.16 ± 0.40 fold increase).

Discussion

Years of clinical and experimental evidence have demonstrated hepatotoxicity after amphetamine exposure in human abusers and laboratory animals (Andreu et al. 1998; Beitia et al. 1999; Brncic et al. 2006; Khakoo et al. 1995; Montiel-Duarte et al. 2002). Accordingly, our *in vitro* findings in the human hepatoma HepG2 cell line indicate that all tested amphetamines and their combination promote time-(6 h – 48 h) and temperature-(37 ºC and 40.5 ºC) dependent mortality, when evaluated by the NR reuptake assay. Under normothermic conditions, the NR assay demonstrated similar sensitivity to the MTT reduction assay, which was used in previous studies to assess cytotoxicity at 48h (Dias da Silva et al. 2013).

Several lines of investigation suggest that either necrosis or apoptosis mediate the reported amphetamine-related toxicity. Previous reports have shown that amphetamine designer drugs can induce apoptosis in human serotonergic JAR cells (Simantov and Tauber 1997), rat cortical neurons (Cunha-Oliveira et al. 2006; Stumm et al. 1999; Warren et al. 2007), immortalized mesencephalic neurons (Cadet et al. 1997; Kanthasamy et al. 2006), rat cerebellar granule cells (Jimenez et al. 2004), striatal glutamic acid decarboxylase (GAD)-containing neurons (Jayanthi et al. 2004), and in mice striatal dopaminergic neurons (Krasnova et al. 2005; Zhu et al. 2005), among others. Capela *et al.* (2012) have additionally demonstrated MDMA-induced programmed neuronal death, involving both the mitochondria machinery and the extrinsic cell death pathway in primary cultures of hippocampus obtained from rat embryos.

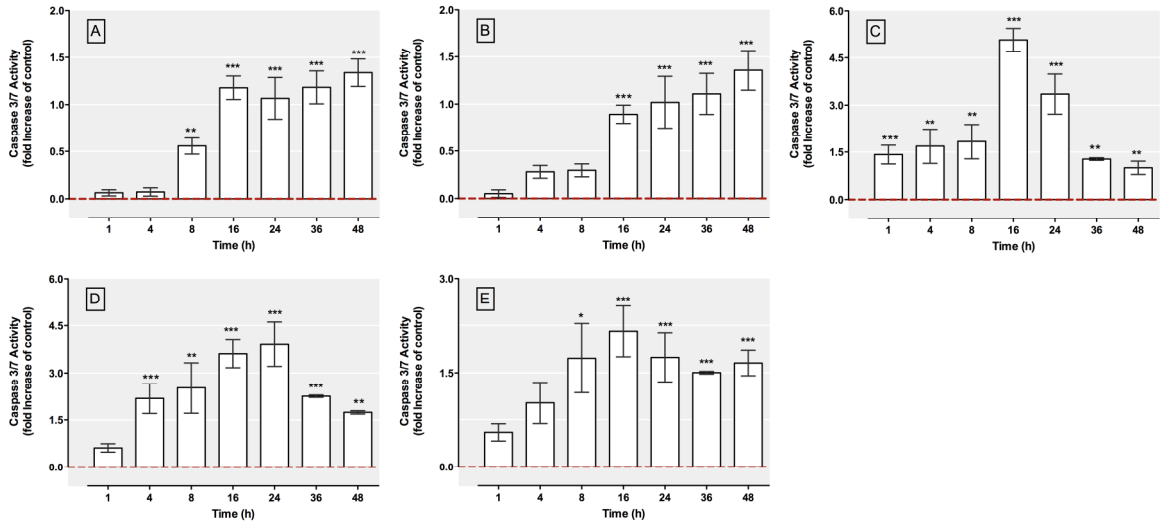

Figure 8. Time-course of caspase-3/-7 activation in HepG2 cells exposed to MDMA (A), METH (B), 4-MTA (C), *d*-AMP (D), individually and in combination (E). The compounds were tested at concentrations that induce 25% of the maximum effect observed in the MTT reduction assay (EC_{25}), *i.e.* 1.3 mM MDMA, 3 mM METH, 0.5 mM 4-MTA, 1.7 mM *d*-AMP and 1.6 mM mixture (Dias da Silva et al. 2013). Caspase-3/-7 activation was measured using a caspase-Glo 3/7 assay kit (Promega) after 1h, 4h, 8h, 16h, 24h, 36h or 48h of incubation, at 37 °C. Results are from five independent experiments performed in six replicates and are presented as mean ± SEM. Control values are set to 0 (dashed red line). Statistical comparisons were made using the Kruskal–Wallis test followed by Dunn's *post hoc* test. **p*<0.05, ***p*<0.01, ****p*<0.001 show statistically significant differences *versus* controls.

Albeit the neuronal system being the most widely studied, apoptosis has also been demonstrated in cultured hepatic stellate cells (Montiel-Duarte et al. 2004; Montiel-Duarte et al. 2002) and in freshly isolated rat hepatocytes exposed to amphetamines (Montiel-Duarte et al. 2002). Conversely, *post-mortem* reports of intoxicated abusers describe cases of human livers exhibiting clear necrotic effects, from foci of individual necrotic cells to massive hepatic necrosis (Milroy et al. 1996), which seems to indicate that apoptosis is not the only mechanism of cell death attributed to amphetamines. In addition to liver necrosis, which is accompanied by fatty degeneration and inflammation, Sano *et al.* (2009) have also observed small foci of myocyte necrosis providing further evidence of the extension of this cell death process to other organ systems.

A classical definition of necrosis, based on morphological criteria, presumes an unregulated phenomenon involving damage of membrane integrity, cellular disruption and swelling of cytoplasmic organelles; whereas apoptosis is characterized by organised plasma membrane blebbing, cell shrinkage and typical modifications of nuclear morphology, such as chromatin condensation and fragmentation.

With the current investigation, we expected to provide new insights into the putative mechanisms underlying the reported cellular death observed here and in previous studies (Dias da Silva et al. 2012; Dias da Silva et al. 2013), under physiological and hyperthermic settings. Additionally, we expected to provide new understandings of the way amphetamines work when in combination. Effectively, a few studies aimed at establishing the type of interaction between these four agents have already been carried out (Dias da Silva et al. 2012; Dias da Silva et al. 2013), and support the conviction that amphetamines act in a similar way to prompt the overall mixture cytotoxicity. However, the mechanisms underlying such additive interactions are yet to be fully understood.

We, therefore, evaluated the rate of necrosis *vs.* apoptosis in HepG2 cells following incubation with these drugs, under different time and temperature conditions. Our findings indicate that in the absence of heat stress (37 °C), HepG2 cells preferentially exhibited death with apoptotic characteristics. The extent of hepatocellular apoptosis is dependent on the duration of exposure to the tested drugs, being more pronounced after 48 h incubations. Apoptosis is a highly effective cell-suicide program, which entails the orchestrated interplay of a range of promoter and suppressor agents belonging to the BCL-2 family. The results presented herein clearly shown the significant downregulation of *BCL-2* and *BCL-$_{XL}$* gene expressions in response to amphetamine treatment. Other authors have already reported a differential expression of these antiapoptotic markers in the rat liver after exposure to MDMA (Cerretani et al. 2011; Montiel-Duarte et al. 2002; Upreti et al. 2011) and *d*-AMP (De Miglio et al. 2000), but this correlation was never reported

for hepatotoxicity provoked by METH and 4-MTA, nor for amphetamine combinations.

The proteins encoded by these antiapoptotic genes have the role of preventing the activation of a proteolytic caspase cascade that condemns cells to death, through direct interactions with the proapoptotic members (Cory et al. 2003; Garcia-Saez 2012; Gross et al. 1999). Indeed, it had become evident that it is the imbalance between the opposing BCL-2 family members, in favor of proapoptotic regulators, rather than the proteins themselves, that controls the release of downstream effector caspases.

In accordance with results obtained in other cellular systems for METH (Genc et al. 2003; Pereira et al. 2006; Shao et al. 2012) and *d*-AMP (De Miglio et al. 2000; Krasnova et al. 2005), anti-survival Bax was found to be differentially expressed in HepG2 cells after amphetamine treatment. Notwithstanding, as far as we know, this is the first study reporting Bax overexpression induced by MDMA and 4-MTA. Also, this is the first time that an association between Bak overexpression and apoptosis have been reported for our test drugs. It is conceivable that in our cellular model, upon a death stimulus, Bax, which is present in the cell in the form of cytosolic monomers, translocates to the mitochondria. Once there, Bax and Bak, the latter being constitutively bound to the outer membrane, suffer conformational changes, integrate into the membrane and oligomerize (Nechushtan et al. 2001). The formed oligomers are alleged to provoke the permeabilization of the outer mitochondrial membrane, permitting the release of several crucial pro-death factors from the mitochondrial intermembrane space into the cytoplasm, which in turn activates the effector caspase-3 and -7 that execute apoptosis (Susin et al. 1999; Verhagen and Vaux 2002).

Under normal cell conditions, the BH3-only regulators (*e.g.*, Bid, Bim and Puma) have no activity or exist only at residual levels. Upon stimuli, like the one induced by amphetamines, they are transcriptionally upregulated (similarly to what was observed with Bim, Bad, Puma and Bid) or activated by post-translational modifications, such as dephosphorylation, subcellular translocation, or proteolysis (as with Bim and Bid). To the best of our knowledge, Puma and Bim have never been linked with the toxicity induced by amphetamines. Increased Bad has been associated with apoptosis induced by METH in mouse neocortex (Jayanthi et al. 2001) and rat striatum (Beauvais et al. 2011);and upregulation of Bid has been reported in MDMA-rat liver exposed to MDMA (Cerretani et al. 2011) and in mouse brain exposed to METH (Deng et al. 2007; Jayanthi et al. 2001) but not in hepatocytes. When activated, these proteins govern apoptosis either directly activating Bax/Bak or neutralizing the role of prosurvival members, such as Bcl-2, Bcl-x_L.

In the particular case of Bid, it was observed that its expression was increased at both transcript and protein levels. This p22 protein is generally confined to the cytosol of the cell in an inactive form. When activated, caspase-8 mediates the cleavage of Bid into one major p15 and two minor p13 and p11 fragments, and leads to the translocation of the p15 truncated form (tBid) to the mitochondrial outer membrane (Li et al. 1998; Luo et al. 1998; Yin et al. 1999). The translocation of tBid is associated with cyt *c* efflux from the mitochondria, due to Bax/Bak oligomerization. Since the apical caspase-8 is activated by cell surface death receptors, such as Fas and tumor necrosis factor (TNF), it is plausible that extrinsic signalling pathways mediate, at least in part, apoptotic responses triggered by amphetamines in HepG2 hepatocytes. Supporting this possibility are the results presented by Capela and co-workers (2012) that demonstrated programmed cell death of primary cultures of hippocampal neurons exposed to amphetamine derivatives, an effect involving receptor stimulation, caspase-8 activation, significant increase in AIF and release of cyt *c* to the cytoplasm.

Our results seem to indicate that apoptosis induced by amphetamines in HepG2 occurred primarily through extrinsic and/or mitochondrial pathways. This was further confirmed by the observed activation of caspases-3 and-7 and cleavage of cellular components, like PARP, in the presence of the test substances. We did not detect any disruption of mitochondrial membrane potential when HepG2 cells were exposed to amphetamines, at 37 ^{o}C. Notwithstanding, the importance of mitochondrial dysfunction in hepatocyte programmed death is unclear. Despite the loss of $\Delta\psi_m$ being allegedly an early apoptotic event, there is emerging evidence advocating that this may not be the case. Depending on the cell system under investigation and the apoptotic stimuli, the loss of $\Delta\psi_m$ may not be required for cyt *c* release (Ly et al. 2003). These inconsistencies may also be attributed to the distinct abilities of fluorochromes to accurately discriminate changes in $\Delta\psi_m$.

From our results, it was also clear that the increase in temperature (from 37 ^{o}C to 40.5 ^{o}C) rendered cells more vulnerable to necrosis, for both time conditions (24 h and 48 h). This suggests that is there is a change in the mode of cell death with the increase in temperature. At physiological temperature, amphetamines led preferentially to apoptosis, but under hyperthermia, cellular death was mostly due to necrosis and not apoptosis. This observation indicates that a 3.5 ^{o}C increment was sufficiently detrimental to provoke premature death of HepG2 cells by necrosis, even in controls,

advocating that hyperthermia *per se* is deleterious enough to trigger necrotic pathways. The vulnerability of the cells exposed at hyperthermia was further exacerbated with the exposure to amphetamines, as necrosis was greatly enlarged in the treatment groups in comparison to controls. Necrotic effects were additionally enhanced with the widening of drug exposure period (from 24 h to 48 h).

Intracellular ATP levels corroborate these results. Although energy contents slightly decrease in HepG2 cells treated with amphetamines at 37 ^{o}C, the ATP decline only presented statistically substantial differences (p<0.05) when cells where exposed at 40.5 ^{o}C. Actually, as observed in this study, when temperature was set to physiological conditions (37 ^{o}C), cells exposed to amphetamines preferentially died by apoptosis. This programmed process is highly regulated and requires energy for a number of ATP dependent stages (Richter et al. 1996; Zamaraeva et al. 2005), such as caspase activation (Li et al. 1997), chromatin condensation (Kass et al. 1996), and blebbing (Barros et al. 2003). On the other hand, it has been proven that the exhaustion of ATP cellular storages redirects the type of death from apoptosis to necrosis (Leist et al. 1997; Nicotera et al. 1998). This is in agreement with our results, considering that hyperthermia (40.5 ^{o}C) *per se* was capable of reducing cellular ATP and that this effect was further exacerbated in the presence of amphetamines.

Apoptosis is a physiologically occurring programmed and targeted way of cell death that normally provides benefits to the organism. In contrast, despite investigation suggesting that necrosis occurrence and progression might be tightly controlled (Golstein and Kroemer 2007; Proskuryakov et al. 2003), it almost always implies negative consequences. Contrary to apoptosis, in which apoptotic bodies containing cytosol, condensed nuclear material, and organelles are ultimately engulfed by phagocytes without causing any damage; cells undergoing necrosis suffer early plasma membrane rupture, loss of selective permeability, dilatation of cytoplasmic organelles, particularly mitochondria, and uncontrolled leakage of cellular contents into the extracellular space. This results in injury of neighbouring cells and severe inflammatory reaction in the corresponding tissue, which could become chronic and cause irreparable damage. Given the fact that the higher temperature used in our experiment (40.5 ^{o}C) coincides with the body temperatures that recreational abusers may attain after *ecstasy* consumption, our results support the hypothesis that amphetamine-induced thermodysregulation, which is also exacerbated by the hot and overcrowded venues were consumption often occurs, is one of the key causes of necrosis-mediated acute liver failure.

Overall, no major differences were observed between the independently tested amphetamines and their mixture for any of the investigated endpoints. This supports the assumption that these compounds, tested at equal effect concentrations, behave similarly and do not interact with each other in any unexpected way. In other words, this supports previous observations which showed that, when combined, these amphetamines act in an additive manner (Dias da Silva et al. 2013).

In conclusion, the cell death mechanism (apoptosis or necrosis) induced by amphetamines, alone and in mixture, depends on both temperature and duration of exposure. It is possible that under less hostile temperature settings, psychostimulant amphetamines promote programmed hepatocyte death involving both the mitochondrial machinery, (through the synchronized interaction between BCL-2 members), and the extrinsic cell death, possibly by activating caspase-8. Although upstream mechanisms were not totally clarified, our results evidenced that apoptosis common pathways were eventually activated, after HepG2 cells exposure to amphetamines. Further approaches aimed at elucidating the involvement of the mitochondrial and, particularly, extrinsic receptor pathway should be considered in the future. We stress the importance of these results for an improved knowledge of the molecular mechanisms involved in amphetamines toxicity, which should aid in implementing novel therapeutic approaches with a view to avoid or attenuate the acute and/or chronic consequences of *ecstasy* misuse.

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Conflict of Interest statement

The authors declare that they have no conflict of interest.

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PART IV – GLOBAL DISCUSSION

1. Evaluating the mixture effects of MDMA and other amphetamine designer drugs

MDMA abuse is due to the euphoric state induced by the drug, that includes an increased sense of energy, emotional openness, increased capacity for introspection, empathy, reduced negative thoughts and reduced inhibitions (Davison and Parrott 1997; delaTorre et al. 2004a; Hegadoren et al. 1999; Liechti and Vollenweider 2000a; Morgan 2000; Parrott and Stuart 1997; Peroutka et al. 1988). The perception of sounds and colours can also seem more intense (Davison and Parrott 1997). To obtain such effects, MDMA is rarely ingested alone (Carvalho et al. 2012; Mohamed et al. 2011). There is a wide variety of drugs that are used for such purposes. Mixtures of several amphetamine-like drugs are among the most frequently used drug combinations (Carvalho et al. 2012; Mohamed et al. 2011). Therefore, considering the high incidence of the simultaneous administration of different amphetamine-designer drugs (Barrett et al. 2005; Carvalho et al. 2012; Dams et al. 2003; Johansen et al. 2003; Kunsdorf-Wnuk et al. 2005; Lin et al. 2007; Mohamed et al. 2011) and the severity of the intoxications that have been associated with this polydrug abuse pattern (Dams et al. 2003; Felgate et al. 1998; Fineschi and Masti 1996; Johansen et al. 2003; Kunsdorf-Wnuk et al. 2005; Lin et al. 2007), the present work aimed at understanding the nature of the interactions between these drugs to help predict and explain their toxic effects. So far, mixture effects had been mostly neglected since the vast majority of the studies on the toxicity of this type of drugs addresses individual effects, underestimating the real risk associated with multidrug 'cocktails'.

MDMA toxicity is diffuse and produces a wide variety of adverse health effects. Long-term toxicity is particularly worrisome, due to the neurotoxic effects that can irreversibly damage the brain of its young users. On the other hand, acute intoxications can be lethal and virtually all organs are potentially affected by the detrimental actions of this drug.

The liver is a remarkably vulnerable target for MDMA toxicity because the drug is extensively metabolised in this organ generating very reactive metabolites that are accompanied by the formation of deleterious ROS and NOS (Figure 1). Several studies have contributed to the characterization of the toxicity of MDMA metabolites (Barbosa et al. 2012; Barenys et al. 2012; Capela et al. 2006; Carvalho et al. 2002b; Carvalho et al. 2004a; Carvalho et al. 2004b; Carvalho et al. 2004c; Ferreira et al. 2013; Gollamudi et al. 1989; Patel et al. 1991), demonstrating that metabolic bioactivation plays an important role in the

expression of MDMA toxicity. As a consequence, clinical cases of hepatotoxicity secondary to *ecstasy* abuse have been frequently and increasingly reported. Among the most commonly noted symptoms are jaundice, hepatomegaly, centrilobular necrosis, hepatitis, and fibrosis (Aknine 2004; Brncic et al. 2006; Dykhuizen et al. 1995; Fidler et al. 1996; Garbino et al. 2001).

Although to different extents, other amphetamines are also metabolised in the liver and share many of the bioactivation pathways that have been well characterized for MDMA (Caldwell et al. 1972; delaTorre et al. 2000a; Ewald et al. 2005; Kraemer and Maurer 2002; Kuwayama et al. 2012; Shima et al. 2006). Therefore, we were particularly interested in studying the impact of amphetamines mixtures in liver toxicity.

Evaluating joint toxicity is a project with immense scope. At any given instant, misusers are exposed to a multitude of unique mixtures, resulting both from intentional and unpremeditated (*e.g.*, food, medicines) drug exposures. Also, the number and concentrations of individual mixture constituents are permanently fluctuating within the organism, due to, among others, pharmacokinetic phenomena (Borgert 2004a; Faust et al. 2003). The number of potential drug combinations is virtually immeasurable and makes the *in vivo* investigations of these combined effects challenging, considering both cost and ethical restrictions. To overcome these limitations, assessment of mixture effects often relies on high throughput *in vitro* assays that estimate joint effects based on the detailed knowledge of the toxic responses of the individual constituents. The choice of adequate study models and toxicity endpoints are, thus, crucial for the success of these investigations. We chose two complementary *in vitro* models that have been widely used to study liver toxicity: a human immortalized hepatoma cell line (HepG2) and primary rat hepatocytes. Human hepatoma cell lines, such as HepG2, are highly differentiated, exhibit numerous genotypic qualities of the liver cells from which they are derived and retain many of the morphological features and the metabolic pathways (Sassa et al. 1987). Nonetheless, in general, continuous hepatic cell lines (tumour or artificially immortalized) express a limited range of the phase I enzymes and UGT that are found in hepatic tissue. Even when the metabolic functions are preserved, the enzyme activity may show variability with increasing number of cell replications during cell culture, becoming less representative of the in *in vivo* situation (Donato et al. 2008; Gerets et al. 2012; Westerink and Schoonen 2007; Xu et al. 2004). In fact, because cell lines undergo several passages, they may accumulate variations introducing significant transformations relative to the original background. Primary rat hepatocytes on the contrary, are metabolically more competent, but are derived from a rodent species (Donato et al. 2008; Gerets et al. 2012;

Westerink and Schoonen 2007; Xu et al. 2004). Several studies proved a good correlation between *in vitro* results in primary rat, mouse and human hepatocytes and those obtained *in vivo* (Akrawi et al. 1993; Carmo 2007; deSousa et al. 1995; Kelly et al. 1992; Li et al. 1997), supporting that primary hepatocytes are an excellent tool in the assessment of the toxicity and the metabolism of drugs. This holds true also in the case of the cryopreserved cells (Bronley-DeLancey et al. 2006; Brown et al. 2006; Carmo 2007; Carmo et al. 2004; Carmo et al. 2005; deSousa et al. 1999; Guillouzo et al. 1999; Hewitt et al. 2000; Koebe et al. 1999). Nevertheless, Carmo *et al.* (2004) examined the cytotoxicity and the metabolic profile of 4-MTA in primary hepatocytes isolated from three male human donors and Sprague-Dawley rats, and observed that human hepatocytes are more metabolically competent (as shown by the increased 4-methylthiobenzoic acid formation) (Figure 5). Accordingly, comparative studies of metabolism performed with *d*-AMP and other related compounds have shown moderate interspecies differences (Caldwell 1980).

When we started our investigations, all the studies conducted with amphetamines and their associations, including those claiming synergisms (Diffley et al. 2012; McNamara et al. 2006; Mohamed et al. 2011; Pontes et al. 2008b; Robledo et al. 2007; Schechter 1998; Song et al. 2010; Vanattou-Saifoudine et al. 2012a; Vanattou-Saifoudine et al. 2011; Young et al. 2005), were designed with neither reference to the expected additivity joint effects, nor to the conceptual principles, such as the CA and IA models, that are the foundation of mixture toxicology. This evidenced some knowledge gaps in the field, related to the application of fundamental concepts and approaches essential to accurately study mixtures of amphetamines. Thus, toxicity predictions with theoretical modelling methods represented, at that time, an open issue of critical urgency.

1.1. Model appropriateness in predicting amphetamine joint effects

The first part of our work with mixtures of amphetamines focused on the issue of validating joint assessment concepts. The first challenge was to construct a mixture where the combination effect predictions derived from *CA* and *IA* were sufficiently different to permit sound identification of the most accurate model in predicting mixture effects. To avoid the disproportionate contribution of a single drug to the overall mixture effect, it was imperative to choose concentration ratios that reflected the individual potency of each mixture

component. With this purpose the first mixture to be experimentally tested included MDMA, METH, 4-MTA and *d*-AMP at concentrations corresponding to their individual EC_{50} values, *i.e.*, the concentration that for each drug induced 50% lethality in the MTT assay in HepG2 cells (Experimental section, Chapter 1). A fixed mixture ratio design was employed to produce a wide range of concentrations of this mixture. Additionally, other mixture designs were tested, aiming at mimicking more realistic circumstances relative to the *in vivo* situation. These included a mixture where the same individual components (MDMA, METH, 4-MTA and *d*-AMP) were mixed at individually ineffective concentrations (the EC_{01} value of each individual drug was chosen), and a third mixture where MDMA was present at a fixed ('realistic') concentration and the remaining components were changed over a wide range of concentrations, as would be expected if they were present as impurities in *ecstasy* tablets (Experimental section, Chapter 1). Nevertheless, we acknowledge that due to the overwhelming number of complex mixtures with variable compositions that could potentially occur in the organism after simultaneous ingestion of these drugs, even the most 'realistic' combination would suffer from a certain lack of realism. Despite not being able to mimic exactly the real mixtures an individual is exposed to, due to all the possible chemicals ingested and the impossibility of quantifying the doses ingested of each one, our approach was very effective at validating the system and allowing proof of concept. Furthermore, if we see mixture effects with these relatively simplistic mixtures, it is highly likely that similar (or even worse) combination effects occur in 'real life' scenarios. In all tested situations, these amphetamines acted together to generate additive effects.

We next continued our studies by applying this experimental protocol to the HepG2 cells incubated at a higher temperature (40.5 ºC), which mimics the hyperthermic state of the liver that occurs in human intoxications (Experimental section, Chapter 2). Primary rat hepatocytes were also tested at both physiological and hyperthermic incubation conditions (Experimental section, Chapter 3). All the obtained data showed notable additive effects between the tested amphetamine drugs. Overall, we found strong evidence that the effect of amphetamine mixtures can be correctly predicted based on the detailed knowledge of the toxicity of individual components, both in primary rat hepatocytes and in HepG2 cells, either at physiological (37 ^{o}C) or hyperthermic (40.5 ^{o}C) conditions, proving the applicability of the CA predicting model to situations similar to those occurring in humans. Although extensive data from other areas of toxicological interest, specially ecotoxicology, had already shown that the accurate modelling of the toxicity of the individual mixture constituents is critical to obtain good estimations of joint combined toxicity (Altenburger et al. 2000 ; Backhaus et al.

2000a; Faust et al. 2001; Faust et al. 2003; Scholze et al. 2001), we produced the first reports on the drug abuse field of research. Our group was the first to demonstrate that the responses of multi-component mixtures of amphetamines might be accurately anticipated by the concept of *CA*. CA computations fitted perfectly with the experimental outcomes of combinations of MDMA and the three other often co-ingested amphetamines (METH, 4-MTA and *d*-AMP). These results consistently indicated that severe and potentially lethal interactions among amphetaminic drugs are to be expected when these drugs are taken concomitantly. Our investigations provided sound experimental evidence supporting what is frequently observed with the human intoxications, *i.e.*, increased severity and lethality of the intoxication when more than one amphetamine derivative is ingested (Byard et al. 1998; CDC 2010; Forrest et al. 1994; Kunsdorf-Wnuk et al. 2005; Liechti et al. 2005). It also explains, at least in part, why it is often impossible to interpret the severity of the intoxication based upon the blood and/or tissue levels of the drugs that are measured after the intoxication or even *post-mortem* (Milroy 2011). It is now clear that these additive mixture effects must be taken into consideration. To efficiently predict such effects is a powerful tool not only to understand, but also to prevent toxicity by making misinformed users aware of the increased risks that they are exposed to, when ingesting 'amphetamine cocktails'.

Because MDMA generates extremely reactive metabolites *in vivo*, these have long been suspected to contribute to the mechanism of toxic action of the drug (Barbosa et al. 2012; Barenys et al. 2012; Capela et al. 2006; Carvalho et al. 2002b; Carvalho et al. 2004a; Carvalho et al. 2004b; Carvalho et al. 2004c; Ferreira et al. 2013; Gollamudi et al. 1989; Patel et al. 1991). Consequently, the concepts described above were also applied to predict joint effects that could occur in the liver cells after MDMA metabolism. For this purpose, and using the least metabolically competent in *vitro model*, the HepG2 cells, to minimize the interference of the metabolites that are produced by the cellular metabolism of MDMA, we incubated these cells with MDMA and its main human metabolites: MDA, *α*-MeDA, and *N*-methyl-*α*-MeDA, in an attempt to mimic the *in vivo* bioactivation expected after MDMA intake (Experimental section, Chapter 4). Again, additive joint effects could be observed and the *CA* model provided the most accurate estimations of the mixture toxicity. With this set of experiments we, for the first time, provided experimental evidence that supported the hypothesis that, even when produced at low concentrations, these metabolites contribute significantly to the overall toxic effect of the drug (Experimental section, Chapter 4). Even if MDMA is the only drug involved in the intoxication, mixture effects between the parent drug

and its oxidative metabolites, particularly *α*-MeDA and *N*-methyl-*α*-MeDA, cannot be overlooked when hazard implications of *ecstasy* intake are considered.

The ability of the drugs to elicit maximal responses (100% cell death) is an important criterion to obtain prediction curves that cover a suitable range of effects. For mathematical reasons, the concept of *CA* cannot predict mixture effects that exceed those of the mixture component with the lowest maximal effect. This issue was of special concern when addressing the combination toxicity of MDMA with its metabolites, since the interference with the optical density readings of the MTT assay that occurred at highest concentrations of the tested metabolites prevented obtaining full concentration-response curves that reached maximal effects (100% lethality). Despite incomplete additivity predictions potentially hindering the comparison between the competing expectation concepts (Rajapakse et al. 2001), due to the robustness of our *CA* estimations and to the excellent discrimination achieved between both *CA* and *IA* models, we could overcome these limitations.

It is interesting to note that in all studied cases, *IA* predicted a lower toxicity for the tested mixtures relative to *CA*, consistently underestimating the mixture toxicity. This supports the notion that the *CA* model is a pragmatic and realistic worst-case model, capable of ensuring an adequate level of protection when predicting the toxicity of amphetamines mixtures. This assumption agrees well to a recent opinion approved by the Scientific Committees of the European Commission (2012) on the toxicity of chemical mixtures.

To a large extent, the great consistency between *CA* predictions and experimental observations could be attributed to the high reproducibility of the MTT assay. It remains to be seen whether the methodology employed here can be successfully applied to bioassays that produce less reproducible data, such as those used in more complex *in vitro* or even in *in vivo* systems. For practical reasons, *CA* and *IA* have been mostly applied either to biochemical *in vitro* (Ohlsson et al. 2010; Payne et al. 2001; Rajapakse et al. 2002; Rajapakse et al. 2004; Silva et al. 2002; Silva et al. 2011) or to unicellular *in vivo* (Huang et al. 2011; Villa et al. 2012; Zhou et al. 2010) models. Due to their functional simplicity, it might be alleged that these models may not reflect the outcomes of more complex living systems, such as the entire organism (Barata et al. 2007). Notwithstanding, a few studies have contributed to our understanding of mixture effects at increasing levels of biological complexity (Christiansen et al. 2008; Correia et al. 2007; Goncalves et al. 2008). Hass *et al.*(2007) assessed joint effects of three androgen receptor antagonists (vinclozolin, flutamide and procymidone) on male sexual differentiation in rats after *in utero* and postnatal exposures. The authors found that effects of a mixture of similarly acting anti-androgens, which individually appears to exert

only small effects, may in concert induce marked responses that were predicted fairly accurately on the basis of the potency of the individual mixture components by using the *dose addition* concept. Christiansen and collegues (2009) investigated the effects of mixtures chemicals widely present in food, consumer products, and the environment in inducing irreversible demasculinization and malformations of sex organs among male rat offspring. In relation to some hallmarks of disrupted male sexual development, the combined effects were *dose additive* (Christiansen et al. 2009). Strikingly, the effect of combined exposure to the selected chemicals on malformations of external sex organs was synergistic, and the observed responses were greater than would be predicted from the toxicities of the individual chemicals (Christiansen et al. 2009). These outcomes are highly relevant to human risk assessment and evaluations that ignore the possibility of combination effects may lead to considerable underestimations of risks associated with exposures to chemicals that disrupt male sexual differentiation (Christiansen et al. 2009). An important task will be to further investigate whether our findings hold true for biological effects at higher levels of cellular organisation, *e.g*. at the organism level.

Despite our success in predicting the joint effect of amphetamines, a more important question remained to be answered: can these mixtures produce significant effects, even when the individual drugs are present at low, ineffective concentrations?

1.2. Mixture outcomes at low-effect concentrations of individual components

The relevance of the *in vitro* investigations of the toxicity of amphetamine-like designer drugs of abuse has often been questioned due to the high concentrations of the test drugs that are necessary to produce a positive response in the cytotoxic endpoints that are most frequently tested. This prompted us to investigate an important hypothesis: Can amphetamines, even at low ineffective concentrations, act together to produce significant responses? In order to address this issue we measured the joint toxic action when each amphetamine was present at levels that individually provoked unnoticeable effects, *i.e.*, indistinguishable from those of untreated controls. For this purpose, it was necessary to estimate, as accurately as possible, low effect concentrations of all tested agents. The EC_{01} was determined by interpolation to the best-fit cytotoxicity curve of MDMA, METH, *4*-MTA and *d*-AMP, since this has been considered the most accurate approach (Scholze and Kortenkamp 2007; Silva et al. 2007).

Also, such low concentrations could not be tested individually, as they do not produce effects above the limit of detection of the assay (they could not be differentiated from controls) and therefore could not be associated with a measurable effect. The only way to accurately estimate the effects associated with such concentrations was to derive them from the fits. Then, a more realistic mixture was tested by adding all individual components in proportion to their EC_{01} concentrations. The observed cytotoxic effect greatly exceeded that expected by the simple sum of the effects of the individual components. These additive effects were, once again, accurately predicted by the CA model.

Silva and co-workers (2002) tested multicomponent mixtures of eight xenoestrogens of environmental relevance and found that estrogenic agents are able to act together to produce substantial mixture effects even though each chemical was present at levels well below its NOEC and EC01. Also Brian *et al.*(2007) investigated the ecological significance of mixture effects at low-effect concentrations by assessing the combined effect of estrogenic chemicals on the reproductive performance of fish. Data from these experiments provide evidence of the capacity for chemicals to act together to affect reproductive performance, even when each component is present below the threshold of detectable effects (Brian et al. 2007). Christiansen and collegues (2008) observed that doses of anti-androgens, which appear to induce no hypospadias when judged on their own, may induce a very high frequency of hypospadias in rats when they interact in concert with other anti-androgens. Similar to our findings, these results stress the great limitations of focusing on single substances rather than on combinations. Had we tested the chemicals alone, we would have not detected any measurable toxicity. Thus, when dealing with mixture exposures, the assumption that individual drugs constitute negligible risk when present at low, ineffective levels in humans is most likely inappropriate. From our data, we can anticipate that if every amphetamine contributes additively to the overall effect, identifying the potential risks associated with these multiple drug exposures becomes highly dependent on the number of the drugs present in the organism.

These conclusions also emphasize that pursuing synergistic combination effects has been unnecessary. Instead, additive combination effects are of utmost importance and need urgent attention when considering the conceivable risks that amphetamines, and other drugs of abuse, may pose for humans (Silva et al. 2002).

1.3. Deviations from additivity

The strengths of the CA and IA models lie mainly on their relative simplicity and consistent applicability in environmental and human toxicology. Yet, some relevant weaknesses must be noted. Firstly, these two general principles do not contemplate pharmacokinetic interactions between the components of the mixture. So, enhanced (synergistic) or weakened (antagonistic) combined effects that occur with respect to either model expectations (CA and IA) need to be considered independently. In our studies, a slight deviation from the CA prediction model was observed when the HepG2 cells were exposed towards higher mixture concentrations, particularly at high effect levels (Experimental section, Chapters 1 and 2). Because the model does not take into account potential pharmacokinetic interactions between chemicals it seemed plausible that the amphetamines could be competing in the mixture for their shared metabolic routes (Caldwell et al. 1972; Carvalho et al. 2012; delaTorre et al. 2000a; Ewald et al. 2005; Kraemer and Maurer 2002; Kuwayama et al. 2012). Unexpectedly, an increase in MDA formation when MDMA was combined with the other amphetamines was observed, instead of the anticipated decrease in the oxidative metabolic pathways that remained unaltered (Experimental section, Chapter 1). Because MDA has been shown to be less toxic than the catechol metabolites, its increased formation could explain the aforementioned weak antagonistic effects. It is not possible, with the present data, to understand why such an increase would occur. However, one can speculate that the incubation period is long enough to allow for CYP induction to occur. If this is the case, then a preferential overexpression of CYP2B6 that catalyses the *N*-demethylation in detriment of the *O*-demethylenation pathway that produces the highly reactive metabolites can account for the observed effects. On the other hand, high concentrations of amphetamines will likely inhibit CYP2D6, the main CYP isoform involved in the *O*-demethylenation (Carvalho et al. 2012). Yet, the accurate pharmacokinetic interactions between amphetaminic drugs are still not fully understood and it is possible that additional factors are involved in the deviations observed, thus requiring further investigation.

Secondly, each of these concepts is based on assumptions on the primary mode of action of each of the components of the mixture, *i.e.*, whether they act in a dissimilar (IA) or similar (CA) way, but this information is recurrently unavailable. The toxicity mechanisms of the studied amphetamines are still not completely understood, but even in light of the limited information available, identifying the similarities in the mechanisms of action of the components of the amphetamines' mixture is a complex issue in practice. So how can we

decide whether a drug acts in a similar or dissimilar way to one another? Herein lie the uncertainties, as the term of similar action can be interpreted in different ways and the criteria used to define similar or dissimilar action of mixture components are not well defined (Berenbaum 1989; Greco et al. 1995). At one extreme of the spectrum of opinions, similar action has been suggested to be based upon each drug competitively and reversibly interacting with an identical molecular target site, by identical toxic intermediates – in a narrow sense we could say similar mechanisms (Pöch 1993). The opposite mechanistic standpoint is described by the view that 'similar action' should be taken to mean the induction of phenomenological similar effects, *e.g.* cell killing (Berenbaum 1989; Borgert et al. 2004; Faust et al. 2003). This particular toxicological endpoint can encompass a very broad range of chemicals being considered as similar.

So, while the mechanistic basis for the selection of *CA* and *IA* for a given mixture appears appealing (similar *vs.* dissimilar action), it has not yet been established how 'similar' the actions need to be before *CA* can be selected as the most proper model. Mechanistic studies of the individual compounds and the mixtures are, therefore, needed to shed light on the interactions that may or may not exist at the molecular level.

2. Understanding the mechanisms underlying the joint action of amphetamines

As mentioned above, a common feature for many drugs is that they exhibit a great variety of pathways that can be ignited to provoke cell death. In many cases, these drugs have known sites of action but an exact understanding of the mechanism of action is lacking. Following on from our findings at both physiological and hyperthermic conditions, we have seen how predictions based upon *CA* gave consistently better reflections of the observed mixture effects for all tested four-component combinations of amphetamines. These empirical observations would suggest that these drugs work in a similar fashion. We then set out to understand the mechanisms underlying the observed joint effects of the amphetamines.

2.1. Dissecting hepatocyte death pathways

An exploration of the modes of action of each individual drug and their combination was developed in a two-stage approach. The first was to explore whether there was a common activation by all single and combined drugs in the process of inducing cell death, either by apoptosis or necrosis. Thus, our initial question was: Are all the drugs, and their mixture, able to induce apoptosis/necrosis, and, if so, would this meet the similarity criterion required for *CA*? The second stage was to move towards the more restrictive definition of 'similar action' and to prove whether all drugs exhibited the same specific mechanisms of action in initiating apoptosis/necrosis. This was achieved by examining the cellular energetic status and the activation of a number of important signalling factors within the apoptotic pathway, namely, the relative expressions of *BCL-2*, *BCL-$_{XL}$*, *BAX*, *BIM*, *BAD*, *PUMA* and *BID* genes. The levels of PARP/cleaved PARP, Bak, PUMA, Bim (EL, S and L isoforms), Bid/cleaved Bid and Bax were also assessed. Ultimately, and since the tested drugs induced differential expression of anti- and pro-apoptotic BCL-2 family members, we further tested mitochondrial permeability impairment.

The relative contribution of apoptosis or necrosis to amphetamines-induced liver injury remains controversial, as evidence of both types of death has been described for hepatocytes (Cerretani et al. 2011; Kamijo et al. 2002; Marinkovic et al. 2011; Sano et al. 2009; Upreti et al. 2011). Apoptosis is an ATP-dependent cell death program comprising chromatin condensation, nuclear disintegration, cell contraction, blebbing, and formation of apoptotic bodies enclosing nuclear or cytoplasmic material (Kerr et al. 1972). Necrosis is typically the consequence of an acute metabolic perturbation with ATP depletion and often characterized by cytoplasmic swelling, dilation of organelles, and mechanical rupture of the cellular membrane (Edinger and Thompson 2004). In principle, the nature and length of cellular damage determine if cells die by apoptosis, necrosis, or other mechanisms. In several circumstances, a variety of deleterious stimuli prompt apoptosis, but the same stimuli can trigger necrosis under more severe conditions (Pontes et al. 2008b). The outcomes of the present research (Experimental section, Chapter 5) revealed that the cell death mode was dependent upon the exposure temperature. At physiological conditions (37 ^{o}C), the elicited HepG2 mortality occurred preferentially by apoptosis, while at 40.5 ^{o}C cell death occurred mainly through necrosis (Experimental section, Chapter 5). Cell lethality was exacerbated with increasing incubation period that equally affected both apoptotic and necrotic cell death. Accordingly, the ATP intracellular stores revealed no significant alterations when drugs

where tested at a physiological incubation temperature, a condition that favours the occurrence of apoptosis, an energetic-dependent process. On the other hand, incubations at 40.5 ^{o}C provoked a marked ATP depletion for all drug treatments. These findings suggest that the rise in incubation temperature rendered the cells unable to maintain cell homeostasis and, consequently, leading to necrosis. Due to the swelling and rupture of intracellular organelles, most notably the mitochondria, and ultimately, due to the plasma membrane breakdown accompanied by cell lysis, the cytoplasmic contents, including lysosomal enzymes, are released into the extracellular space. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response. This has been corroborated by frequent reports on liver toxicity following amphetamines exposure at high environmental temperatures. Most of these reports denote portal and sinusoidal inflammation (Marinkovic et al. 2011; Sano et al. 2009), periportal and lobular necrosis (Brauer et al. 1997; Ibranyi and Schonleber 2003; Marinkovic et al. 2011; Sano et al. 2009), stasis (Marinkovic et al. 2011), intralobular haemorrhage (Marinkovic et al. 2011), increase of liver enzymes serum activity (Marinkovic et al. 2011), and fatty degeneration (Sano et al. 2009) accompanied by inflammation (Sano et al. 2009).

The pro-apoptotic action of MDMA was already demonstrated in isolated rat hepatocytes (Montiel-Duarte et al. 2002) and in liver cells maintained in culture (immortal hepatic stellate HSC cell line) (Montiel-Duarte et al. 2004). Montiel-Duarte and co-workers (2002) demonstrated pro-apoptotic effects upon exposure to MDMA in freshly isolated rat hepatocytes and in immortalised liver cells (hepatic stellate HSC cells), as shown by chromatin condensation of the nuclei and accumulation of oligonucleosomal fragments in the cytoplasm. These observed effects correlated with decreased levels of the anti-apoptotic factor bcl-$_{XL}$, release of cyt *c* from the mitochondria and activation of caspase-3 (Montiel-Duarte et al. 2002). MDMA induced further PARP proteolysis in HSC cells (Montiel-Duarte et al. 2002). Additional studies from the same group attempted to scrutinize the role played by oxidative stress in the apoptotic response caused by MDMA on HSC cells (Montiel-Duarte et al. 2004). MDMA-induced apoptosis of HSC was accompanied, but not caused by oxidative stress (Montiel-Duarte et al. 2004). The results obtained suggest the involvement of a metabolic derivative of MDMA on its apoptotic process (Montiel-Duarte et al. 2004). These authors also observed an MDMA-induced activation of NF-κB that played a protective role against the apoptotic response (Montiel-Duarte et al. 2004). Also, Upreti *et al*. (2011) found that MDMA induces oxidative modification of many cytosolic proteins accompanied by increased oxidative stress and activation of c-Jun N-terminal protein kinase and p38 kinase.

Since these protein kinases phosphorylate anti-apoptotic Bcl-2 protein, their activation may promote apoptosis in MDMA-exposed tissues (Upreti et al. 2011). Cerretani *et al.* (2011) obtained evidence that MDMA-induced apoptosis in liver cells is accompanied by oxidative stress (loss of GSH homeostasis, decreases antioxidant enzyme activities, and lipoperoxidation). This correlation between MDMA-induced oxidative stress/apoptosis raises two major possibilities: (i) MDMA may prompt apoptosis by causing intracellular stress, and/or (ii) MDMA may increase the susceptibility to apoptosis induced by the extrinsic pathway (Cerretani et al. 2011). One possible mechanism for the later would be the depletion of GSH, which has been suggested to sensitize to tumor necrosis factor alpha (TNF-α)-induced extrinsic cell death (Cerretani et al. 2011; Montiel-Duarte et al. 2004; Nagai et al. 2002). In fact, Cerretani and co-authors (2011) hypothesised that the mitochondria are the central executioners for TNF-α-induced hepatocyte apoptosis, as supported by their observations of activation of pro-apoptotic (Bid, SMAC/DIABLO) and inhibition of anti-apoptotic (NF-κB, Bcl-2) proteins (Cerretani et al. 2011).

In the present work (Experimental section, Chapter 5) a thorough investigation of the apoptotic mechanisms triggered by the tested drugs (alone or in combination) showed the involvement of the BCL-2 superfamily members. BCL-2 is a family of interrelated proteins that rule mitochondrial or intrinsic pathway of apoptosis by means of the interaction of its proapoptotic and pro-survival members. Besides, BCL-2 members are also involved in the enhancement of the death signal triggered by the extrinsic death receptor pathway (mediated by cell surface receptors upon extracellular stimuli). So far, about 20 mammalian family related proteins were identified and are classically divided into three subfamilies: (i) Bcl-2 pro-survival subfamily (including the proteins Bcl-2 and Bcl-$_{XL}$), (ii) Bax-like pro-apoptotic subfamily (comprises Bax and Bak), and (iii) BH3-only pro-apoptotic subfamily (Garcia-Saez 2012). These proteins are crucial controllers of the permeabilization of the outer membrane of mitochondria that promotes the release of cyt *c* and other apoptotic factors to the cytosol, activating the downstream effector caspase-3/-7 cascade. As this is considered an irreversible point in the programmed cell death, these proteins play a pivotal role in deciding whether a cell lives or dies.

Our results evidenced a decline in *BCL-2* and *BCL-$_{XL}$* mRNA levels (Experimental section, Chapter 5). These genes code for the respective antiapoptotic proteins, Bcl-2 and Bcl-$x_{L,}$ which by binding apoptotic peptidase activating factor 1 (Apaf-1), prevent its association with Caspase-9. In turn, this caspase, when activated by cyt *c* release from the mitochondria, activates the effector caspase cascade (Fig. 6). Conversely, death inducers (Bax, Bak and

BH3-only proteins) help to dissipate the membrane potential of mitochondria and therefore make it more permeable for cyt *c* release. The BH3-only family members include the p53 upregulated modulator of apoptosis (Puma) that also plays a critical role in the p53 tumor suppressor pathway; Bim (which major isoforms Bim_{EL}, Bim_{L} and Bim_{S} are generated by alternative splicing and generally only transiently expressed during apoptosis), Bad and Bid. These proteins usually promote apoptosis by binding to and antagonizing anti-apoptotic Bcl-2 family members through BH3 domain interactions.

Synchronized with antiapoptotic transcripts downregulation, we also observed an upregulation of *BAX*, *BIM*, *BAD* (only for MDMA), *PUMA* and *BID* genes (Experimental section, Chapter 5). Additionally, the elevation of Bax, cleaved Bid, Puma, Bak and Bim (EL, S and L isoforms) at protein level was also confirmed (Experimental section, Chapter 5). Growing evidence suggest that Bid, a p22 protein usually confined to the cytosol of the cell as an inactive precursor, in response to apoptotic signalling, is cleaved by caspase-8 at internal Asp60 site resulting in one major p15 and two minor p13 and p11 fragments. Cleaved or truncated Bid (tBid) interacts with Bax, mainly present in cell cytosol, forcing it to undergo a conformation shift and further insert into the outer mitochondrial membrane (Fig. 6). Some studies indicate that p15 truncated form also translocates to the mitochondrial outer membrane as an integral membrane protein (Li et al. 1998; Luo et al. 1998; Yin et al. 1999). This induces the opening of the mitochondrial voltage-dependent anion channel or, alternatively, forms an oligomeric pore on mitochondrial membrane, promoting the leakage of apoptogenic factors, such as cyt *c* (Fig. 6). Translocation of tBid is also associated with cyt *c* efflux from the mitochondria, by affecting Bax/Bak oligomerization.

Since the apical caspase-8 is activated by cell surface death receptors, such as Fas and TNF, it is plausible that extrinsic signalling pathway mediates, at least in part, the apoptotic response triggered by amphetamines in HepG2 hepatocytes. Supporting this conviction are the results presented by Capela and co-workers (2012) that demonstrated that caspase-8 activation mediates programmed cell death of primary cultures of hippocampal neurons exposed to amphetamine derivatives.

As noted above, some of these apoptotic markers involved in the observed HepG2 cell death were already described in studies addressing the toxicity mechanisms of the amphetamines (Beauvais et al. 2011; Cerretani et al. 2011; Chetsawang et al. 2012; Krasnova et al. 2005; Shao et al. 2012). However, to the best of our knowledge, we have for the first time linked the Puma, Bim and Bak markers to the apoptosis induced by amphetamine drugs of abuse.

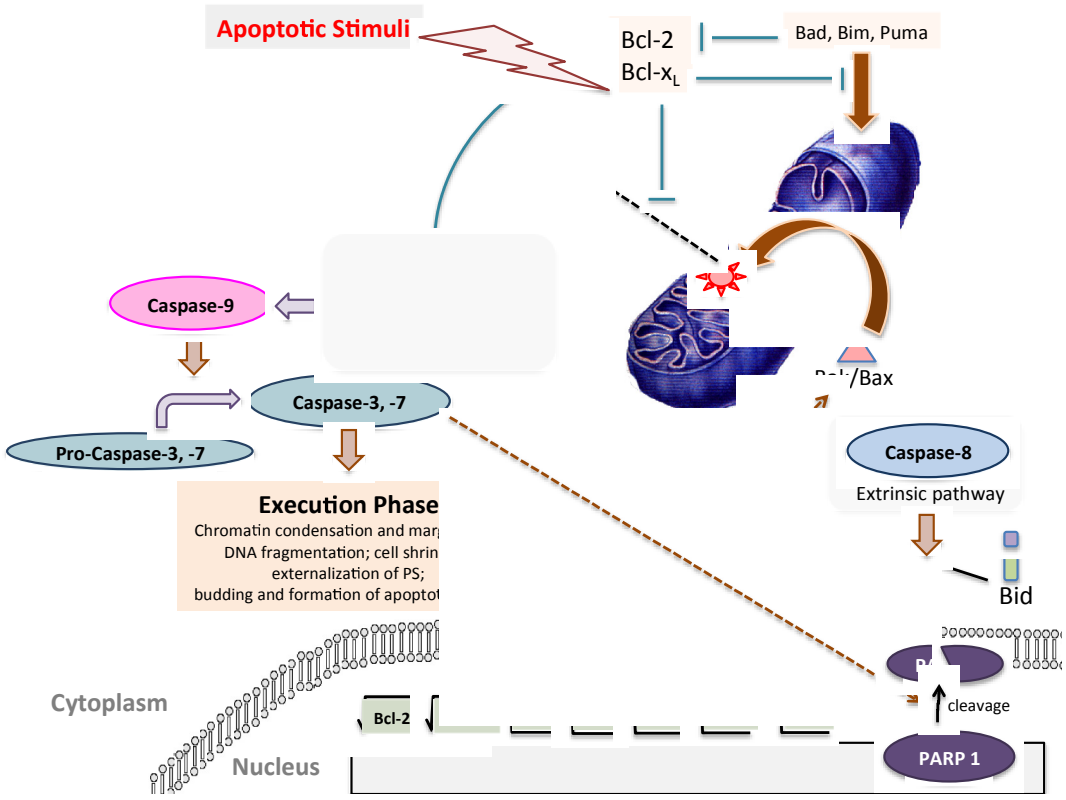

Figure 6. Schematic representation of putative molecular mechanisms involved in apoptosis evoked by amphetamines and their combinations, in HepG2 cells. Programmed cell death can be provoked by diverse stimuli from inside and outside the cell. Incoming signals, such those impelled by cytotoxic drugs, recurrently derive from nucleus and, in most cases, encourage expression of proapoptotic BCL-2 members and suppress antiapoptotic *BCL-2* and *BCL-$_{XL}$* expression. The sophisticated integration of the BCL-2 family antagonic members governs cell death, particularly at the level of mitochondrial membrane permeability. The mitochondrial control by these proteins is of utmost importance as it permits the release of cytochrome *c* (cyt *c*) from the intermembrane space to cytosol due to formation of a channel in the outer mitochondrial membrane. Bax and/or Bak form the pore after tBid activation, while Bcl-2 and Bcl-$_{XL}$ inhibit its formation. The BH3-only family members, such as Puma, Bim, Bad and Bid, are vital initiators of apoptosis. They unleash the apoptotic cascade by inactivating the protective function of the anti-apoptopic members and by activating the Bax/Bak-like pro-apoptotic BCL-2 family members. Once cyt *c* is released into the cytosol it binds with apoptotic peptidase activating factor 1 (Apaf-1) and ATP, which then bind to pro-caspase-9 to form a caspase-activating signaling complex, known as apoptosome. The apoptosome cleaves the pro-caspase-9 to its active form caspase-9, which in turn activates the effector caspase-3 and -7 that execute apoptosis: phosphatidylserine (PS) is exposed to the outer leaflet of cytoplasmic membrane, cell shrinks, death substrates are cleaved (*e.g.*, Poly (ADP-ribose) polymerase, PARP), the chromatin condenses, cellular membrane is blebbing and finally the cell is disintegrated into condensed membrane-enclosed assemblies, known as apoptotic bodies. Blue lines indicate inhibition in the pathway while brown arrows indicate activation. ATP, Adenosine-5'-triphosphate.

Additional data showed that time-dependent caspase-3/-7 activation but not mitochondrial membrane potential ($\Delta\psi_m$) disruption accompanied the observed amphetamine-induced apoptosis. Also, cell dismantling was confirmed by the observed poly PARP proteolysis (Experimental section, Chapter 5).

Considering all the parameters evaluated, no relevant differences were detected when cells were exposed to the equieffective concentrations of the drugs individually and in combination, corroborating the *CA* assumption that amphetamines combination effects are additive and that the whole mixture components acted in a similar way to prompt HepG2 cell death.

These results provide a new understanding on the hepatocyte death pathways prompted by the tested amphetamines and show that these pathways also operate when the amphetamines are combined. It also became clear that cell death is highly dependent on temperature and time of exposure stimuli.

2.2. Hyperthermia severely aggravates the risks associated with amphetamines abuse

From all the experiments performed, both at physiological and hyperthermic incubation conditions, it can be concluded that hyperthermia is a crucial determinant of toxicity of all the individual and combined amphetamines. It has already been proposed that the exposure of hepatocytes to amphetamines under hyperthermic conditions triggers multiple cellular signalling pathways, leading to the disruption of cellular homeostasis that may finally culminate in cell death (Gabai and Sherman 2002).

From our studies, one of the most compelling evidences of the detrimental influence of high incubation temperature on the cytotoxicity of the amphetamines, both individually and in combination, was the increased steepness of the slopes of the concentration–response curves that was observed. This means that very small increases in the concentrations of each drug/mixture prompted cytotoxic responses that rapidly shifted from imperceptible effects to maximal mortality, at 40.5 ºC. Of note, several studies have shown that an increase in body temperature is *per se* directly related with hepatotoxic damage, further exacerbating the amphetamines-related liver toxicity (Carvalho et al. 1997; Carvalho et al. 2001; Skibba and Gwartney 1997; Skibba et al. 1991). Among other factors that could explain such an influence, metabolism may be at play. Our data shows that the toxicity elicited by

amphetamines under hyperthermia was significantly aggravated in the most competent metabolic model, the rat primary hepatocytes when compared to the HepG2 cells (Experimental section, Chapter 3). Overall, these data indicate that hyperthermia significantly narrows the 'safety range' of amphetamines and their mixtures, highlighting the increased risks that *ecstasy* abusers are exposed to, in the corresponding drug abuse scene.

As noted before, MDMA-induced hyperthermia may be at least partly responsible for the hepatotoxicity that is observed *in vivo*, as liver injury is often accompanied by a hyperthermic state (Andreu et al. 1998; Broening et al. 1995; Chadwick et al. 1991; Coore 1996; Gahlinger 2004; Green et al. 2003; Hall and Henry 2006; Henry et al. 1992; Malberg and Seiden 1998; Mallick and Bodenham 1997; Mechan et al. 2002; Milroy et al. 1996; Nash et al. 1988; Schmidt et al. 1990; Screaton et al. 1992; Vanden Eede et al. 2012). Also, the hepatocellular damage observed in cases of poisoning with MDMA is, in many aspects, similar to that produced by hyperthermia in isolated hepatocytes (Santos-Marques et al. 2006). Skibba *et al.* (1991) studied the heat-induced hepatotoxicity by perfusing isolated rat livers with four solutions at different temperatures (from 37 ^{o}C to 43 ^{o}C) and observed a temperature-dependent increase in oxidative stress, purine catabolism and cell death rates. Consequently, it can be argued that oxidative stress plays a role in the toxicity linked with elevated temperatures.

In vitro and *in vivo* studies have shown that the elevation of incubation temperature of isolated rat hepatocytes enhances the hepatotoxic effects of MDMA, particularly the effects closely related to oxidative stress, such as decrease in the levels of intracellular GSH, increased glutathione disulfide (GSSG) and lipid peroxidation (Beitia et al. 2000; Carvalho et al. 2002a; Johnson et al. 2002; Ninkovic et al. 2004). Pourahmad and collegues (2010) reported MDMA-induced cytotoxicity, reactive oxygen species formation, $\Delta\psi_m$ decline and lysosomal membrane leakage in isolated rat hepatocytes at physiological temperature, and these effects on the oxidative stress markers were further exacerbated at 41 ^{o}C. Antioxidants, reactive oxygen species scavengers, and inhibitors of CYP2D6 reduced all markers of MDMA-induced hepatocyte oxidative stress (Pourahmad et al. 2010). Carvalho *et al.* (2002a) obtained some evidence of oxidative stress (as expressed by GSH depletion, lipid peroxidation, decreased catalase activity, decreased glutathione peroxidase activity, vacuolation of the hepatocytes, presence of blood clots and loss of typical hepatic cord organization), after the administration of an acute intraperitoneal dose of MDMA to mice that were placed at a high ambient temperature (30+/-2 ^{o}C). The same authors observed that a rise in incubation temperature from 37 ^{o}C to 41 ^{o}C increased oxidative stress in freshly isolated

mouse hepatocytes, reflected by increased lipid peroxidation, GSH depletion, and consequent loss of cell viability (Carvalho et al. 2001). These findings together indicate that hyperthermia acts as a pro-oxidant aggressive condition that worsens de pro-oxidant effects of amphetamines. We, therefore, pursued our mechanistic studies by evaluating the ability of the tested amphetamines to produce oxidative damage, when tested individually and in combination.

2.3. Oxidative-modifications elicited in HepG2 Cells

There is overwhelming evidence supporting the role of oxidative stress in amphetamines-induced damage. Several studies have shown the involvement of oxidative stress in hepatocellular toxicity induced by MDMA, *in vivo* and *in vitro* (Beitia et al. 1999; Beitia et al. 2000; Carvalho et al. 2002a; Johnson et al. 2002). Beitia and co-workers (1999) observed a marked decrease in the intracellular levels of GSH and cellular bioenergetics on isolated rat hepatocytes. The decrease of hepatic glutathione levels was also observed *in vivo* in rats (Beitia et al. 2000) and in mice that were administered with MDMA (Carvalho et al. 2002a; Johnson et al. 2002). Since the intracellular GSH is a crucial defence for the inactivation of toxic electrophiles, including ROS and RNS (Wu et al. 2004), low hepatic levels of GSH may be an initial step in the hepatotoxic action of MDMA. Cerretani *et al.* (2011) also observed MDMA-induced loss of GSH homeostasis, decreases antioxidant enzyme (GR and glutathione peroxidase or GPx) activities, and lipidperoxidation in rat liver. Others have corroborated these results (Ninkovic et al. 2004).

Our research also revealed a concentration-dependent disturbance in the pro-oxidant/antioxidant cellular status, in favour of the former, when HepG2 cells were exposed individually to the tested drugs and to their mixture (Experimental section, Chapter 2).

Despite the general agreement on the involvement of oxidative stress in amphetamines-elicited toxicity, the source of ROS and RNS is still a matter of debate. Generally, it has been postulated that the generation of oxidative/nitrosative stress can arise as a result of (i) the activity of the mitochondrial respiratory chain, (ii) depletion of antioxidants, (iii) redox cycles formed between *o*-quinones and their semiquinones derived from the metabolism of the catecholamines and of the amphetamines, (iv) the combination of produced metabolites and GSH, and (v) hyperthermia (Carvalho et al. 2012; Pontes 2009). The

produced reactive species interact with and oxidize vital cellular macromolecules such as DNA, proteins, and lipids (Pacher et al. 2007). The modification of these macromolecules has deleterious effects on their physiological functions.

Our results agree well with reports that show that the amphetamines, directly or through the production of reactive metabolites, are able to increase the generation of ROS and RNS, in several systems (Kaushal et al. 2012; Quinton and Yamamoto 2006; Shenouda et al. 2009). Similarly to the *o*-quinones resulting from the oxidation of endogenous catecholamines, also oxidation of catechol metabolites of MDMA (*N*-methyl-*α*-methyldopamine and *α*-methyldopamine) results in the formation of aminochromes adducts with glutathione and cysteine (Figure 1) (Carvalho et al. 2012; Pontes 2009). The reactivity of the formed compounds, the involvement in redox cycles accompanied by the formation of ROS and RNS and the ability to induce oxidative stress when intracellular antioxidant defences are depleted, are putative mechanisms for the occurrence of cellular damage (Carvalho et al. 2012; Pontes 2009). Carvalho *et al* (2004b) have demonstrated that cytotoxicity of the metabolite *N*-methyl-*α*-methyldopamine in isolated rat hepatocytes was higher than that of MDMA under the same experimental conditions, and that oxidative stress played a role on the achieved effects. In another study by the same authors, it was also demonstrated the occurrence of decreased levels of intracellular GSH and ATP, inhibition of antioxidant enzymes (such as GPx, glutathione reductase or GR, glutathione *S*-transferase or GST) and cell death by exposing isolated rat hepatocytes to *α*-methyldopamine (Carvalho et al. 2004a). Additionally, these reports evidenced the production of adducts of *α*-methyldopamine with glutathione (Carvalho et al. 2004a). Taken together, results from these studies showed that one of the consequences of MDMA initial metabolism in the expression of hepatic cytotoxicity appears to be the disruption of antioxidant homeostasis, which can result in loss of important intracellular functions and trigger the cascade of events that leads to irreversible cell damage (Carvalho et al. 2012; Carvalho et al. 2004a; Carvalho et al. 2004b; Pontes 2009).

Also, the heat-stimulation *per se* has been implicated in the increased flux of free radicals in the cells, such as superoxide and H_2O_2 (Flanagan et al. 1998). The mechanism is not yet understood, but it appears to involve the mitochondrial chain of electron transport. In fact, mitochondria is considered the main source of ROS, but other enzymes, such those belonging to CYP P450 system, are also known to produce ROS/RNS following MDMA exposure (Darvesh et al. 2005; Gow et al. 2004; Moon et al. 2008; Zheng and Laverty 1998). Certainly, the decline of total glutathione content instigated by amphetamines may leave

hepatocytes more susceptible to damage, as changes in the intracellular thiol-disulfide (GSH/GSSG) homeostasis are considered key determinants in the cellular redox balance. It has been vastly reported that glutathione has not only the ability to directly scavenge reactive species of both oxygen and nitrogen (acting as an oxidant neutralizing), but it also plays a crucial protective role against cellular injury favouring the regeneration of lipid peroxidase and tocopheryl-radical activities (Di Mascio et al. 1991).

The GSH depletion reported in our studies (Experimental section, Chapter 2) has already been demonstrated in primary hepatocyte systems (Beitia et al. 1999; Carvalho et al. 2001; Pontes et al. 2008b) and might be explained by: (i) the increased oxidation of GSH to GSSG by ROS and RNS whose formation is also increased, (ii) by the formation of adducts with reactive catechol metabolites, or (iii) by the decreased synthesis of GSH as a result of the inhibition of the activity of the enzymes of synthesis. This disruption of thiol homeostasis may result in a boost of free reactive species that are efficient in promoting loss of protein function and initiating a cascade of events leading to mitochondrial electron transport impairment, with subsequent exhaustion of ATP supplied and cell death. In accordance, the dramatic depletion of intracellular stores of ATP was particularly notorious under hyperthermia (Experimental section, Chapter 2).

Mitochondrial dysfunction was also confirmed by the loss in $\Delta\psi_m$, at 40.5 ^oC. These results are in agreement with reports showing that amphetamines induce oxidative stress in mitochondria (Alves et al. 2007; Brown et al. 2005; Burrows et al. 2000). Custódio *et al.* (2010) compared the direct influence of MDMA and 4-MTA on rat liver mitochondrial function (MPT), mitochondrial oxidative stress, and mitochondrial bioenergetics, and showed that 4-MTA was more potent than MDMA in decreasing the sensitivity of liver mitochondria to MPT, and that higher concentrations of 4-MTA were highly toxic to mitochondria. 4-MTA simultaneously increased H_2O_2 production and uncoupled and inhibited mitochondrial respiration. Increased production of ROS and RNS usually results in production of more potent ONOO·-, which interacts with cellular macromolecules such as DNA, proteins, and lipids to oxidize these components (Pacher et al. 2007). The modification of these macromolecules has deleterious effects on their physiological functions. Evidence demonstrated that the activities of mitochondrial aldehyde dehydrogenase (ALDH), 3-ketoacyl-CoA thiolases, and ATP synthase were significantly inhibited following MDMA exposure, by oxidative inactivation, which most likely contributes to mitochondrial dysfunction and subsequent liver damage in MDMA-exposed rats (Moon et al. 2008). This oxidative injury may affect mitochondrial energy processes (Krebs cycle/oxidative

phosphorylation and electron transport chain) causing damage to the membranes and/or mitochondrial proteins (*e.g.* NADH dehydrogenase, cyt *c* reductase, and cyt *c* oxidase) (Seitz and Stickel 2006). Also, oxidative damage to the mitochondria due to excess of reactive species can trigger intracellular signaling that ends up in hepatotoxicity. In particular, our studies provide evidence supporting that these drugs and their combination induce increases in proapoptotic proteins, Bax, cleaved Bid, Puma, Bak and Bim, and decreases in antiapoptotic gene expressions of *BCL-2* and *BCL-$_{XL}$*, which result in the activation of the downstream apoptotic cascade, characterized, among others, by activation of caspases-3/-7, and the breakdown of several structural cellular proteins, such as PARP.

Nonetheless, it is still unknown whether the generation of ROS in hepatocytes incubated with MDMA depends on either disturbance of the mitochondrial respiratory chain or on the redox cycles formed between ortho-quinones and their semiquinones derived from MDMA metabolism (Carvalho et al. 2004b; Hiramatsu et al. 1990). In fact, it is not clear whether MDMA directly uncouples mitochondrial respiration. In spite of the fact that it has been established that MDMA-mediated hyperthermia occurs by UCP3-mediated action, recent studies demonstrated that the effects of MDMA on respiratory uncoupling in liver mitochondria are modest (Rusyniak et al. 2004). Moreover, uncoupling proteins, particularly UCP3, although widely present in other tissues, are not highly expressed in hepatocytes and this may be the reasoning for the absence of effects in the liver (Carvalho et al. 2012; Larrouy et al. 1997).

The mitochondrial impairment that we observed has already been noted in several investigations. Song *et al.* (2010) reported MDMA-induced oxidation of several mitochondrial proteins involved in protein folding (chaperone proteins), oxidative phosphorylation, energy production, anti-oxidant defense, fat metabolism, electron transport, etc. In addition, Moon and coworkers (2008) demonstrated that the activities of certain enzymes such as NADH-ubiquinone oxidoreductase (complex I), ATP synthase (complex V), ALDH2, and 3-ketoacyl-CoA thiolase (involved in ATP production via the fat degradation pathway) were significantly inhibited in MDMA-exposed liver, suggesting that hepatocytes may not properly convey many cellular functions and eventually undergo cell death processes. Inactivation of these key mitochondrial enzymes correlated with increased H_2O_2, decreased ATP level, accumulation of lipid peroxides such as malondialdehyde, and triglyceride fat accumulation, which most likely leads to mitochondrial dysfunction and subsequent liver damage (Carvalho et al. 2012; Moon et al. 2008). Nakagawa and co-workers (2009) showed that the exposure of isolated hepatic mitochondria to MDMA causes

mitochondrial impairment and induction of the MPT accompanied by mitochondrial depolarization and depletion of ATP through uncoupling of oxidative phosphorylation (Nakagawa et al. 2009). Beitia and co-workers (1999) showed that MDMA induced a concentration- and time dependent ATP depletion. Carvalho *et al* (2004b) observed a severe ATP depletion subsequent to massive GSH depletion after hepatocytes incubation with MDMA metabolite *N*-Me-*α*-MeDA, suggesting that altered thiol homeostasis may also contribute to mitochondrial dysfunction (Carvalho et al. 2004b). GSH, in conjunction with GPx/GR is responsible for the elimination of cellular H_2O_2 and organic peroxides. Thus, depletion of GSH and/or decreased activity of these enzymes may compromise this pathway and thereby allow H_2O_2 to accumulate at toxic levels. It must be stressed that hepatic mitochondria are especially vulnerable to this effect since they lack the H_2O_2 metabolizing enzyme, catalase (Carvalho et al. 2012; DeLeve and Kaplowitz 1991; Pontes 2009).

Despite some reports of lipid peroxidative damage induced by MDMA as a consequence of oxidative stress (Skibba et al. 1990), we failed to find significant alterations in the lipoperoxidation profile under our experimental settings. Still others have reported hyperthermia-related lipid peroxidation in perfused rat liver (Skibba et al. 1991) and in freshly isolated rat hepatocytes (Carvalho et al. 1997; Carvalho et al. 2001).

An overall analysis of our mechanistic data on oxidative stress, energetic status, and mitochondrial dysfunction indicated that hepatocytes exposure to the drugs, either individually or in mixture, at equieffective concentrations, showed no significant differences in the elicited responses. This further suggests that there was, in fact, a similarity in the way these agents performed in each assay and supports the validity of the *CA* premise.

PART V – FINAL CONCLUSIONS

Experimental testing of mixture toxicity is hampered by the overwhelming number of possible mixtures that an individual, or a population, might be exposed to. Therefore, predictive approaches capable of estimating the effect of mixtures on the basis of the known toxicities of their individual components are an imperative. The two prevailing theoretical concepts of CA and IA, largely used to appraise mixture effects on a variety of fields, such as aquatic toxicology and cancer chemotherapy, were never applied to evaluate the combination effects of drugs of abuse. We were the first to apply these principles to evaluate joint effects between MDMA and three other amphetamines that are known to be concomitantly ingested with the drug, in the polydrug abuse settings. Ultimately, the work conducted under the scope of the present dissertation allowed us to conclude that:

1. CA is a suitable model to accurately estimate the joint cytotoxic effects of four amphetamines – MDMA, METH, 4-MTA and *d*-AMP – on HepG2 liver cells, using information on the individual drug components. This was true for a few different mixture designs, including mixtures with individual constituents combined at: (i) equipotent concentrations (to avoid different individual contributions to the mixture outcomes), (ii) concentrations simulating 'realistic' intoxication scenarios (with MDMA as the main constituent and the other amphetamines as contaminants), and, (iii) very low concentration of all the mixture components, which individually yield no significant toxic effects.

2. With basis on the current approach, the joint toxicological effect of all four amphetamines could be classified as additive.

3. According to the theoretical approach, our findings strongly suggest that remarkable mixture effects occur, even when each single component is present in the mixture at concentrations that individually elicit irrelevant cytotoxic effects. These observations emphasize the risk that the consumers of 'rave pills' are exposed to when ingesting an illicit *ecstasy* pill (often contaminated with other amphetaminic products obtained from uncontrolled synthetic processes).

4. The predictions according to IA proved to be inadequate for the evaluation of the joint effects of the tested amphetamines in our *in vitro* system. Taken together, these observations lead to the conclusion that these drugs of abuse work in a similar way to generate the overall mixture effects.

5. All former conclusions hold true also at conditions that more closely represent the *in vivo* situation. Notable additive effects that conformed well with CA where observed, even at very low and individually ineffective concentrations, when similar experiments were conducted under hyperthermia (40.5 ^{o}C), and also with primary rat hepatocytes, which are regarded as metabolically more competent.

6. Additionally, MDMA and its main human metabolites MDA, *α*-MeDA and *N*-Me-*α*-MeDA, interacted additively to produce substantial effects at both physiological and hyperthermic temperature settings, suggesting that the metabolism of MDMA has dangerous repercussions to liver cells, even when the metabolites are present at seemingly negligible concentrations.

7. In some of the tested mixture designs, a small deviation from additivity was observed at high effect levels. A possible metabolic interaction might be at play at these higher mixture concentrations, contributing to these small divergences from additivity. Nonetheless, these concentrations are not representative of those attained *in* vivo and therefore, unlikely to be relevant, when considering real intoxication scenarios.

8. Mechanistic evaluation on the pathways underlying cell death provoked by individual and combined amphetamines revealed that the switch of the cell death mode from apoptosis to necrosis depends on both temperature and duration of exposure. Under less hostile incubation settings, apoptosis was favoured and involved the synchronized inactivation of the protective function of anti-apoptopic and the activation of pro-apoptotic BCL-2 family members. To the best of our knowledge, this is the first time that Puma, Bim and Bak have been linked with the toxicity induced by amphetamines. Additionally, time-dependent caspase-3/-7 activation, PARP proteolysis, but not $\Delta\psi_m$ disruption across mitochondrial membrane, mediated amphetamine-related apoptosis, under our experimental conditions. At an increased incubation temperature, similar to that elicited *in vivo* by amphetamines (40.5 ^{o}C), necrosis rather than apoptosis was

observed, accompanied by a marked impairment of the cellular energetic status and lysosomal membrane injury.

9. At hyperthermia (40.5 ºC), very small increases in the concentrations of each drug/mixture prompted cytotoxic effects that quickly shifted from roughly undetectable to maximal mortality. The decline of antioxidant defences, the dramatic depletion of ATP intracellular stores, and the $\Delta\psi_m$ disruption were particularly exacerbated under these hyperthermic settings, suggesting a strong correlation with the observed toxicity.

10. Primary hepatocytes revealed greater susceptibility to individual and combined amphetamine toxic action when compared to HepG2 cells, especially under hyperthermia. Admitting that primary cells are metabolically more competent than the HepG2 cell line, this might render the cells more vulnerable when they are exposed to substances that are metabolised into harmful metabolites, particularly when subject to heat stress, a condition that has already been shown to improve metabolic efficiency.

The research presented herein clearly demonstrates that additive interactions among amphetaminic drugs greatly contribute to their *in vitro* toxicity in liver cells. An overview of the mechanisms most likely to be involved in these effects is depicted in Figure 6. Significantly, our work highlights the limitations of the traditional emphasis on the effects of single agents and stresses the importance of studying amphetamines in combination, when evaluating their impact on human health. Validation of the presented data in more complex models deserves further experimental investigation, but overall, it can be concluded from our studies that risk assessments that overlook the joint action of amphetamine-like drugs will almost certainly lead to significant underestimations of risk.

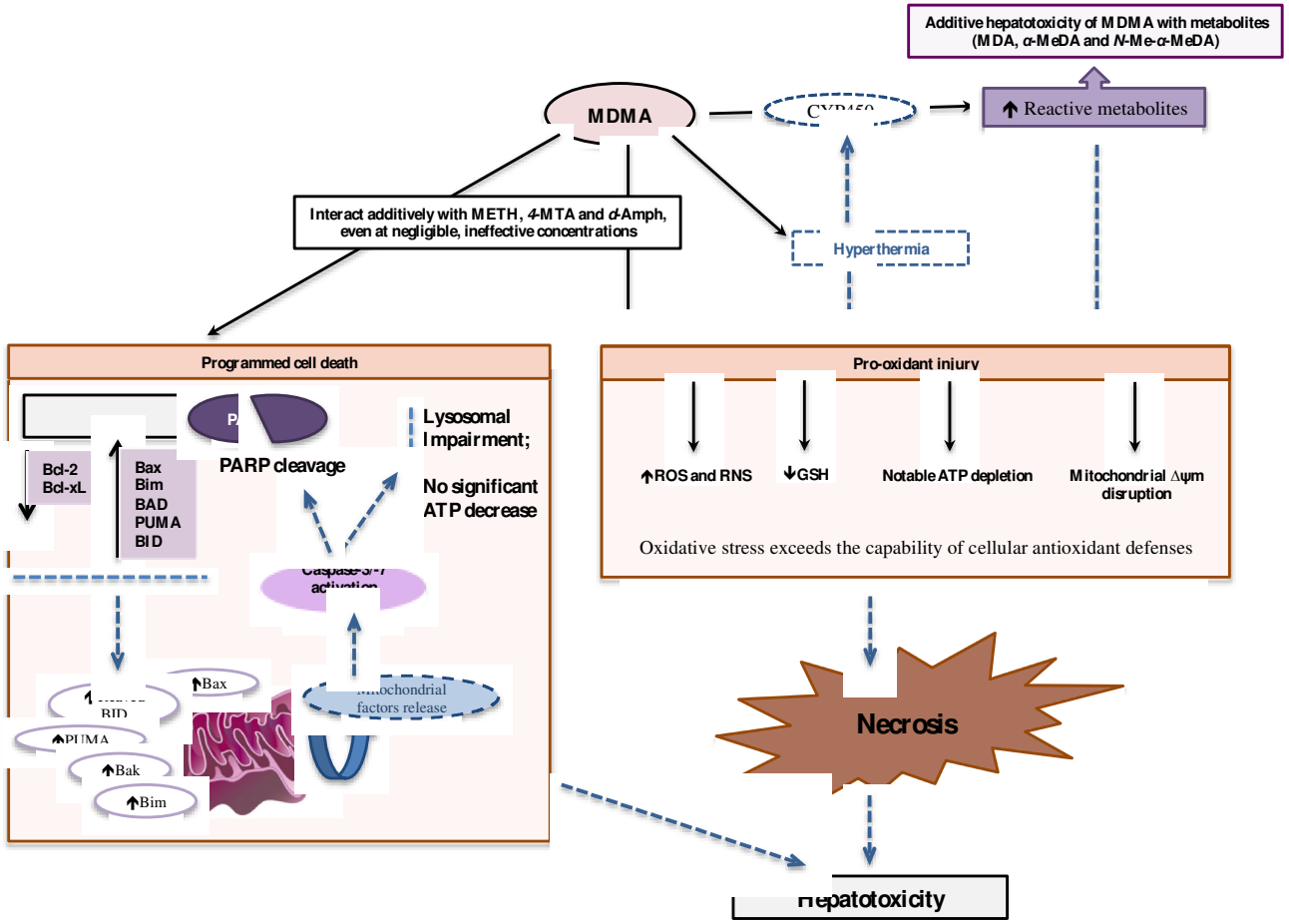

Figure 7. Overview of the mechanistic pathways triggered *in vitro* by MDMA, METH, 4-MTA, *d*-AMP and their mixture in liver cells.

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