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Evaluation of the influence of the metabolism on the cytotoxicity of 3-methylmethcathinone (3-MMC), commonly used as a drug of abuse

Thesis submitted to the Faculty of Medicine, University of Porto for the degree of Master in Forensic Science, under the supervision of Doctor Helena Maria Ferreira Carmo, Doctor Diana Dias da Silva and Professor Doctor Maria de Lourdes Bastos.

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Aos meus pais que sempre me apoiaram.
À minha irmã Cristina e avó Ermelinda.
À memória do meu Avô.
Ao Júlio, por estar sempre ao meu lado.

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Oral communications

1. Bárbara Ferreira, Rita Rebelo, Patrícia Moreira, Félix Carvalho, Maria de Lourdes Bastos, Diana Dias da Silva, Helena Carmo (2017) The new psychoactive substance 3-methylmethcathinone (3-MMC) induces hepatotoxicity toward primary rat hepatocytes by triggering intrinsic and extrinsic apoptosis pathways. XLVII Annual Meeting of Portuguese Society for Pharmacology, XXXV Annual Meeting of Clinical Pharmacology and XVI Annual Meeting of Toxicology. February 2–4. Coimbra, Portugal.

2. Bárbara Ferreira, Rita Rebelo, Patrícia Moreira, Félix Carvalho, Helena Carmo, Diana Dias da Silva, Maria de Lourdes Bastos (2017) 3-Methylmethcathinone (3-MMC) elicits oxidative stress and apoptosis in primary rat hepatocytes. IJUP 2017 – 10. º Encontro de Jovens Investigadores da U.PORTO. February 8–10. Porto, Portugal.

Poster communications

3. Bárbara Ferreira, Diana Dias da Silva, Rita Rebelo, Patrícia Moreira, Félix Carvalho, Maria de Lourdes Bastos, Helena Carmo (2017) Cytotoxicity of 3-methylmethcathinone (3-MMC or metaphedrone) in primary rat hepatocytes. II Annual Meeting of Portuguese Society for Forensic Sciences (APCF, Associação Portuguesa de Ciências Forenses). March 30–31. Porto, Portugal.

The abstracts of the communications and the poster of the third communication are presented in annex.

Resumo

Nos últimos anos, tem-se observado um aumento na produção de novas drogas que, por serem substâncias novas no mercado, são vendidas e consideradas como "drogas legais". Por esta razão, os consumidores, erradamente, consideram-nas seguras para consumo. No entanto, têm sido recentemente reportados vários casos de intoxicações e mortes por drogas legais. Um exemplo destas drogas é a 3-metilmetcatinona (3-MMC ou metafedrona). Esta droga, que pertence à família das catinonas sintéticas, foi introduzida no mercado com o objetivo de substituir a 4-metilmetcatinona (4-MMC ou mefedrona), que já é considerada uma droga ilegal. Com o aumento do consumo da 3-MMC, a avaliação da sua toxicidade tornou-se imprescindível. A maioria dos dados sobre os efeitos deletérios da 3-MMC provêm dos poucos relatos de casos clínicos, mas é necessária uma pesquisa mais aprofundada sobre as suas propriedades farmacológicas e toxicológicas para avaliar o potencial nocivo da droga. De facto, devido à escassez de informação sobre a droga: é de grande importância verificar os efeitos tóxicos em diversos órgãos-alvo, uma vez que não existem dados consistentes publicados, bem como descobrir as vias metabólicas, e a relevância do metabolismo da 3-MMC nos seus efeitos toxicológicos. Por estes motivos, os nossos objetivos foram: (i) estudar os mecanismos subjacentes à sua toxicidade e (ii) caracterizar o perfil metabólico da 3-MMC, investigando a possível relação entre ambos.

Como o fígado é um dos principais locais do metabolismo das drogas e, por isso, um alvo relevante para os seus efeitos deletérios, escolhemos os hepatócitos primários de rato como modelo para estudos *in-vitro*. Para este fim, foram isolados hepatócitos primários de ratos Wistar pelo método de perfusão com colagenase. Estes foram semeados e expostos durante 24h a várias concentrações da 3-MMC (31 nM – 10 mM), de forma a obter um perfil citotóxico completo. A morte celular foi avaliada por três métodos: (i) pelo ensaio de redução do brometo de 3-(4,5-dimetil-tiazol-2-il)-2,5-difeniltetrazólio (MTT); (ii) pelo ensaio da incorporação do corante vermelho neutro (NR); e (iii) pelo ensaio de libertação de lactato desidrogenase (LDH). Depois de expor as células às concentrações de 1 µM, 10 µM, 100 µM, e 500 µM de 3-MMC durante 24 h, foram avaliados alguns mecanismos de ação responsáveis pela citotoxicidade. Nomeadamente, através da medição da produção de espécies reativas de oxigénio e azoto (ROS/RNS), de glutationa reduzida (redGSH) e oxidada (GSSG), de trifosfato de adenosina (ATP), alterações ao potencial de membrana da mitocôndria ($\Delta\Psi m$) e a atividade das caspases-3, -8, -9. Finalmente, avaliámos os efeitos dos inibidores do citocromo P450 (CYP) na toxicidade da 3-MMC. Relativamente aos ensaios de

viabilidade, observou-se toxicidade a concentrações mais baixas no lisossoma (NR NOEC 312.5 µM), do que aquelas necessárias para causar dano na mitocôndria (MTT NOEC 379.5 µM) e na membrana citoplasmática (LDH NOEC 1.04 mM). De uma maneira geral, observámos um decréscimo dependente da concentração nas defesas antioxidantes (redGSH) paralelo com um aumento de GSSG. Verificou-se uma tendência, dependente da concentração, para o aumento da produção de ROS/RNS que se tornou particularmente significativo a partir da concentração de 10 µM. Nesta concentração, as atividades das caspases-3, -8 e -9 foram significativamente elevadas, mas não se observaram diferenças nas concentrações mais altas, indicando que nessas concentrações devem intervir outros mecanismos. Não foram observadas alterações significativas no potencial de membrana da mitocôndria ($\Delta\Psi m$), que é importante para a síntese de ATP e, por isso, para a apoptose. Em concordância com estes resultados, a 3-MMC apenas interferiu significativamente com o estado energético da célula em concentrações superiores a 10 µM. Os nossos resultados também demonstraram que a inibição da CYP2D6 diminuiu a toxicidade da 3-MMC, enquanto que a inibição da CYP2E1 exacerbou os efeitos deletérios da 3-MMC (este efeito foi apenas observado para concentrações superiores a 1.20 mM).

A caracterização do perfil metabólico foi realizada para os hepatócitos primários de rato através da adaptação de um método de cromatografia gasosa acoplada a um detetor de massa (GC-MS) para identificação e quantificação dos analitos. O método otimizado mostrou alta sensibilidade e desempenho adequado para a identificação e quantificação da 3-MMC. Em testes preliminares, o método apresenta linearidade, limites de deteção e quantificação, ausência de interferências e uma recuperação da extração aceitável para a análise das amostras selecionadas. Os resultados deste estudo são bastante preliminares, uma vez que apenas pudemos identificar e quantificar o composto-pai nas amostras analisadas; a identificação e a quantificação dos metabolitos da 3-MMC não foram realizadas. De uma forma geral, a baixas concentrações, a inibição da CYP2E1, da CYP2D6 e a inibição geral do CYP450, diminuíram a extensão metabólica. Uma hipótese plausível é que as vias alternativas que biotransformam a droga quando estas enzimas não estão disponíveis, possam sofrer o fenómeno de saturação. Apesar de preliminar, a nossa metodologia demonstrou resultados promissores, no entanto, é essencial a validação do método e a análise de um número maior de amostras.

Em geral, os resultados envolvem o metabolismo e o stress oxidativo na hepatotoxicidade da 3-MMC, que parece ser desencadeada tanto por mecanismos apoptóticos intrínsecos quanto extrínsecos. Em concentrações mais elevadas de 3-

MMC, podem estar a intervir outras vias de morte celular, como por exemplo, a necrose ou a autofagia, necessitando de mais investigação.

Palavras-chave: 3-metilmetcatinona (3-MMC) – Novas substâncias psicoativas (NPS) – Catinonas sintéticas – Toxicidade – Stress oxidativo – Metabolismo – Apoptose

Abstract

Recently, there has been an increase in the production of new psychoactive substances (NPS). Due to the novelty of these drugs, they are usually not under legal control, and are therefore considered licit drugs. For this reason, users mistakenly consider them as safe for consumption. However, there are several reports of cases of intoxications and deaths by NPS. An example of such a drug is 3-methylmethcathinone (3-MMC or metaphedrone). This drug, which belongs to the synthetic cathinones family, was introduced recently in the market to replace 4-methylmethcathinone (4-MMC) that was made illegal. With the increase in consumption of 3-MMC, its toxicity assessment has become mandatory. Most data on the deleterious effects of 3-MMC come from few case reports of intoxications, but further investigation on its toxicological properties is therefore required to evaluate the harmful potential of the drug. Indeed, by virtue of the scarce available information on the drug: it is of great importance to clearly ascertain the toxic effects on diverse target organs, as consistent data published on the subject is absent, as well as to reveal metabolic pathways and their relevance for the toxicological effects of 3-MMC. In addition, it is also highly relevant to study pharmacokinetics in different animal models and to scrutinize the signalling pathways triggering the known detrimental toxicological effects. For these reasons, our goals were: (i) to study the mechanisms underlying the toxicity of 3-MMC and (ii) to characterize the metabolic profile of 3-MMC, investigating a potential relationship between metabolism and toxicity.

As the liver is one of the main sites of drug metabolism, and therefore a relevant target for their deleterious effects, we chose the primary rat hepatocytes as an *in-vitro* model. For this purpose, primary hepatocytes of Wistar rats were isolated by a two-step collagenase perfusion, cultured, and exposed for 24 h to several concentrations of the drug (31 nM to 10 mM), selected to provide a complete cytotoxic profile. Cell death was assessed through three assays: (i) the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (ii) the neutral red (NR) uptake assay and (iii) lactate dehydrogenase (LDH) leakage assay. Afterwards, some of the mechanisms that contributed to the putative cytotoxicity were also assessed by measuring the intracellular reactive species of oxygen and nitrogen (ROS/RNS), reduced (GSH) and oxidized (GSSG) glutathione, adenosine triphosphate (ATP), alterations to the mitochondrial membrane potential ($\Delta\Psi m$), and to caspase-3, -8 and -9 activities, after exposing cells at 1 µM, 10 µM, 100 µM, and 500 µM of 3-MMC for 24 h. Finally, we evaluated the modulatory effects of cytochrome P450 (CYP) inhibitors on the toxicity of 3-MMC. 3-MMC-induced toxicity was perceived in the lysosome at lower

concentrations (NR NOEC 312.5 µM) than those needed to cause damage to mitochondria (MTT NOEC 379.5 µM) and cytoplasmic membrane (LDH NOEC 1.04 mM). Overall, we observed a concentration-dependent shortage in antioxidant defences (redGSH) with a concurrent increase of GSSG. A significant concentration-dependent increase of ROS/RNS was at concentrations higher than 10 µM. At this concentration, caspase-3, -8, and -9 activities were significantly elevated, but no differences were observed at higher concentrations, indicating that at these concentration levels other mechanisms should intervene. No significant alterations were observed in the ΔΨm, which is important for ATP synthesis, and therefore for energy-dependent apoptosis. In line with these finding, 3-MMC only significantly interfered with the cell energetic status at concentrations higher than 10 µM. Our data also demonstrated that CYP2D6 inhibition diminished 3-MMC toxicity, while on the contrary CYP2E1 inhibition exacerbated the deleterious effects of 3-MMC (but this effect was only observed at concentrations higher than 1.20 mM).

The characterization of the metabolic profile was performed for the primary rat hepatocytes by adapting a gas chromatography-mass spectrometry (GC-MS) method for identification and quantification of compounds. The optimized method showed high sensitivity and adequate performance for the identification and quantification of 3-MMC. In the preliminary tests, the method showed linearity, detection and quantitation limits, lack of interferences and acceptable extraction efficiency for the analysis of selected samples he results in this study are very preliminary because we could only identify and quantify the parent drug in the samples tested; identification and quantification of 3-MMC metabolites was not performed. Overall, at low concentration levels, inhibition of CYP2E1, CYP2D6 and general inhibition of CYP450, increased metabolic extent. For high concentrations, the metabolism diminished, when CYP2E1 and CYP3A4 were inhibited. We hypothesised that alternative metabolic pathways that biotransform the drug when these enzymes are not available could suffer saturation. Although preliminary, our methodology showed promising results, but it is essential to validate the method and to increase the number of samples analysed.

Overall, our data implicates the metabolism and the oxidative burst in the hepatotoxicity of 3-MMC, which seems to be triggered both by intrinsic and extrinsic apoptotic mechanisms. At higher drug concentrations, other cell death pathways might be at play, such as necrosis, deserving further investigation.

Keywords: 3-methylmethcathinone (3-MMC) – New psychoactive substance (NPS) – Synthetic cathinones – Toxicity – Oxidative stress – Metabolism – Apoptosis

Thesis layout

This thesis is divided into 6 chapters. The first chapter is a general introduction that comprises the current state of the art on 3-methylmethcathinone (3-MMC). The second chapter encompasses the main objectives of this thesis. The third chapter consists of the description of the experimental work and methodologies used in the realization of the work conducted in the ambit of this thesis. The forth chapter comprises the results obtained and the respective discussion. The fifth chapter contains the main conclusions from the conducted work, as well as potential future studies. The sixth chapter contains the bibliography cited.

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List of abbreviations

25I-NBOMe	2-(4-Iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine
2-FMA	2-Fluoromethamphetamine
3,4-DMMC	3,4-Dimethylmethcathinone
3-MMC	3-Methylmethcathinone or metaphedrone
4-FMC	4-Fluoromethcathinone or flephedrone
4-MEC	4-Methylethcathinone
4-MMC	4-Methylmethcathinone or mephedrone
4-MTA	4-Methylthioamphetamine
5-APB	5-(2-Aminopropyl)benzofuran
5-MAPB	1-(Benzofuran-5-yl)-*N*-methylpropan-2-amine
6-APB	6-(2-Aminopropyl)benzofuran"
ABT	1-Aminobenzotriazole
Ac-DEVD-pNA	N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide
Ac-IETD-pNA	Acetyl-Ile-Glu-Thr-Asp p-nitroaniline
Ac-LEHD-pNA	Ac-Leu-Glu-His-Asp-p-nitroanalide
AH-7921	3,4-Dichloro-N-{[1-(dimethylamino)cyclohexyl]methyl}benzamide
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
β-NADH	β-Nicotinamide adenine dinucleotide
β-NADPH	β-Nicotinamide adenine dinucleotide
Bpm	Beats per minute
BSA	Bovine serum albumin
C6G	Codeine-6-glucuronide
$CaCl_2.2H_2O$	Calcium chloride dihydrate
CAS	Chemical abstracts service
$C_2H_3NaO_2.3H_2O$	Sodium acetate trihydrate
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

CK	Creatine kinase
cLogP	Partition coefficient
Cmax	Maximal concentration
CO_2	Carbon dioxide
$CuSO_4.5H_2O$	Copper(II) sulfate pentahydrate
CV	Coefficient of variation
CYP2D6	Cytochrome P450 2D6
CYP2E1	Cytochrome P450 2E1
CYP3A4	Cytochrome P450 3A4
CYP450	Cytochrome P450
DCF	2',7'-Dichlorofluorescein
DCFH	2',7'-Dichlorodihydrofluorescein
DCFH-DA	2',7'-Dichlorodihydrofluorescein diacetate
DGAV	Direção Geral de Alimentação e Veterinária
DIC	Disseminated intravascular coagulation
DMSO	Dimethyl sulfoxide
DTNB	5,5′-Dithiobis(2-nitrobenzoic acid)/Ellman's Reagent
DTT	Dl-dithiothreitol
ΔΨM	Mitochondrial membrane potential
EC50	Half maximal effective concentration
EDDP	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EGTA	Ethylene glycol tetraacetic acid
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
FBS	Fetal bovine serum
GC-MS	Gas chromatography–mass spectrometry
GHB	Gamma-hydroxybutyric acid
GR	Glutathione reductase
GS	Glasgow score
GSSG	Oxidised glutathione
HBSS	Hank's balanced salt solution

HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
HEPES	2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HFBA	Heptafluorobutyric anhydride
IR	Infrared
IS	Internal standard
IS1	4-Hydroxy-3-methoxybenzylamine hydrochloride
IS2	Phenylpropanolamine
Katelin	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
$KHCO_3$	Potassium bicarbonate
$KNaC_4H_4O_6.4H_2O$	Potassium sodium tartrate
LC-HRMS	Liquid chromatography–high resolution mass spectrometry
LC-MS/MS	Liquid chromatography tandem-mass spectrometry
LDH	Lactate dehydrogenase
LOD	Limit of detection
LOEC	Lowest observed effect concentration
LOQ	Limit of quantitation
MDA	3,4-Methylenedioxyamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MDPV	Methylenedioxypyrovalerone
MEM	Minimum essential medium
MeOH	Methanol
$MgSO_4$	Magnesium sulfate
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
MSD	Mass selective detector
MSTFA	N-Methyl-N-trimethylsilyl-trifluoroacetamide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MXE	Methoxetamine
N_2	Nitrogen

Na_2HPO_4	Sodium phosphate dibasic
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide
NaH_2PO_4	Monosodium dihydrogen orthophosphate
NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide
NMR	Nuclear magnetic resonance
NOEC	No observed effect concentration
NPS	New psychoactive substances
NR	Neutral red
pKa	Logarithmic acid dissociation constant
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
R^2	Coefficient of determination
redGSH	Reduced glutathione
RLS	Reaction level scale
RNS	Reactive species of nitrogen
ROS	Reactive species of oxygen
SEM	Standard error of the mean
SPE	Solid-phase extraction
STRIDA	Swedish early-warning system on NPS
TBME	*Tert*-butylmethylether
TFAA	Trifluoroacetic anhydride
tGSH	Total glutathione
TMRE	Tetramethylrhodamine ethyl ester
TNB	5-Thio-2-nitrobenzoic acid

Chapter 1

General Introduction

1.1. Introduction

Cathinone is a natural psychoactive component found in the leaves of the *Catha edulis* plant, known as *khat* (Valente et al. 2014). *Khat* grows wild in the Horn of Africa and in the Southwest of the Arabian Peninsula and is usually consumed by chewing the fresh leaves, in sessions where men gather together and consume it (Valente et al. 2014). As the routes for transportation and distribution improved, the availability and use of khat leaves spread to Western countries (Griffiths et al. 2010; Valente et al. 2014). Cathinone is referred to as a natural amphetamine (1-phenylpropan-2-amine) due to their close chemical structure (it corresponds to the β-keto analogue of amphetamine) and similar effects (Carvalho et al. 2012).

The first synthetic cathinones were synthetized in the late 1920's for medical purposes and began being sold as recreational drugs in the 2000's (Brandt et al. 2011; Coppola and Mondola 2012). Methcathinone (ephedrone) and 4-methylmethcathinone (4-MMC or mephedrone) were the first synthetic cathinones produced (Valente et al. 2014). These drugs are often sold as "bath salts" or "plant food" (Valente et al. 2014) and labelled "not for human consumption" or "research chemical" to circumvent drug abuse laws (Dargan et al. 2011). These and other frequently abused synthetic cathinones are shown in Table 1.

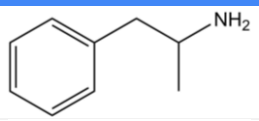
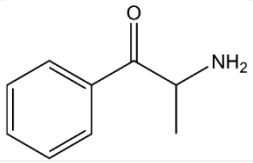
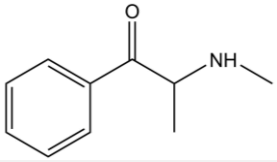
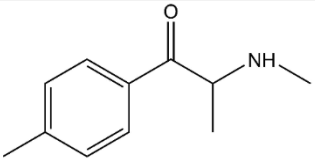
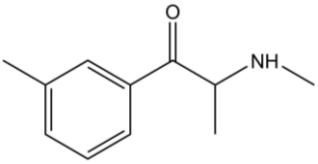
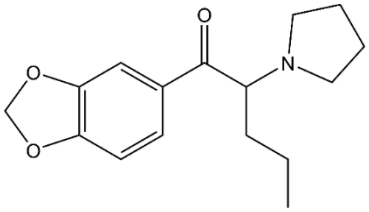
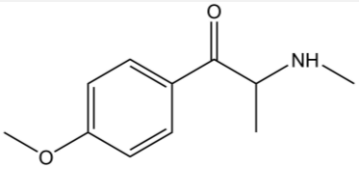
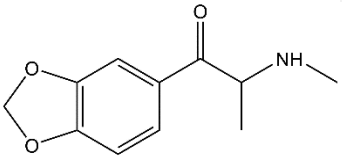
4-MMC is one of the most popular synthetic cathinones and its abuse was first detected in 2007 (EMCDDA 2010b). Users of 4-MMC describe its effects as similar to those of 3,4-methylenedioxymethamphetamine (MDMA) but with stronger feelings of craving (Zawilska 2014). The most frequently used routes of administration are insufflation (snorted) and oral (referred to as "bombing", i.e. the ingestion of capsules or powder wrapped in cigarette paper) (Zawilska 2014). As its consumption increased, toxicity reports also peaked and as a result, several countries banned 4-MMC. As a consequence, the market quickly evolved for a new generation of synthetic cathinones, with the appearance of new structurally-related derivatives (Shimshoni et al. 2015). In this context, 3-methylmethcathinone (3-MMC) first appeared in Sweden in 2012 as a "legal drug" to substitute for 4-MMC (Backberg et al. 2015), and was mostly sold on the internet as a research chemical. Like other synthetic cathinones, 3-MMC has no known therapeutic use (WHO 2016).

This novel designer drug is usually found in the form of white powder or crystals. Users describe its effects as similar to, but less potent and intense than those of MDMA and mephedrone (Adamowicz et al. 2016a). Repeated administration in a single

session is a common consequence of the short duration of its desired effects and this may be attributed to the short half-life of 3-MMC (Shimshoni et al. 2015).

Presently, 3-MMC is a controlled substance in several European countries, such as Sweden, France, Germany, Czech Republic, Poland and UK (Adamowicz et al. 2016a; Backberg et al. 2015; Jamey et al. 2016; Shimshoni et al. 2015). In Portugal, the list of controlled substances was updated in 2012 (Lei nº 13/2012, de 26 de Março), which included thereafter 4-MMC, 3-MMC and isomers. The drug is also controlled in some countries outside of Europe, namely Israel and USA (Shimshoni et al. 2015).

Table 1 Chemical structures of amphetamine, natural cathinone, and principal synthetic derivatives.

Compound	Chemical structure
Amphetamine	
Cathinone	
Methcathinone (ephedrone)	
4-methylmethcathinone (mephedrone; 4-MMC)	
3-methylmethcathinone (metaphedrone; 3-MMC)	
Methylenedioxypyrovalerone (MDPV)	
4-methoxymethcathinone (methedrone)	
3,4-methylenedioxy-*N*-methylcathinone (methylone)	

1.2. Physicochemical properties and analytical methods for identification

Chemically, synthetic cathinones are analogues of the natural cathinone, which in turn is closely related to the amphetamines, with the addition of a ketone group at the β-position of the amino alkyl chain, which is attached to the phenyl ring (Valente et al. 2014). 3-Methylmethcathinone, also known as 2-(methylamino)-1-(3-methylphenyl)propan-1-one, is a ring substituted cathinone with a methyl group attached to the third carbon atom of the phenyl ring (Zuba and Adamowicz 2016). Two enantiomers of 3-MMC exist, R-3-MMC and S-3-MMC, due to the chiral centre at the C-2 carbon of the propane side chain (WHO 2016). Similarly to cathinone, the S form of 3-MMC is expected to be more potent than the R form (WHO 2016) but this hypothesis awaits confirmation.

As depicted in Fig.1, 3-MMC is available as a free base or as a hydrochloride salt that can be synthetized through the reaction of 3-methylbenzaldehyde (1) with ethyl magnesium bromide followed by oxidation with pyridinium chlorochromate on silica gel, and bromination with hydrobromic acid/hydrogen peroxide to obtain the bromo ketone (4). Then, the reaction with ethanolic methylamine will produce the 3-MMC free base (5). The free base can be converted into 3-MMC hydrochloride salt (6) by adding ethereal hydrogen chloride. The purity of 3-MMC can vary according to the purification steps performed during its synthesis (Power et al. 2011). Additional chemical properties of 3-MMC are presented in Table 2.

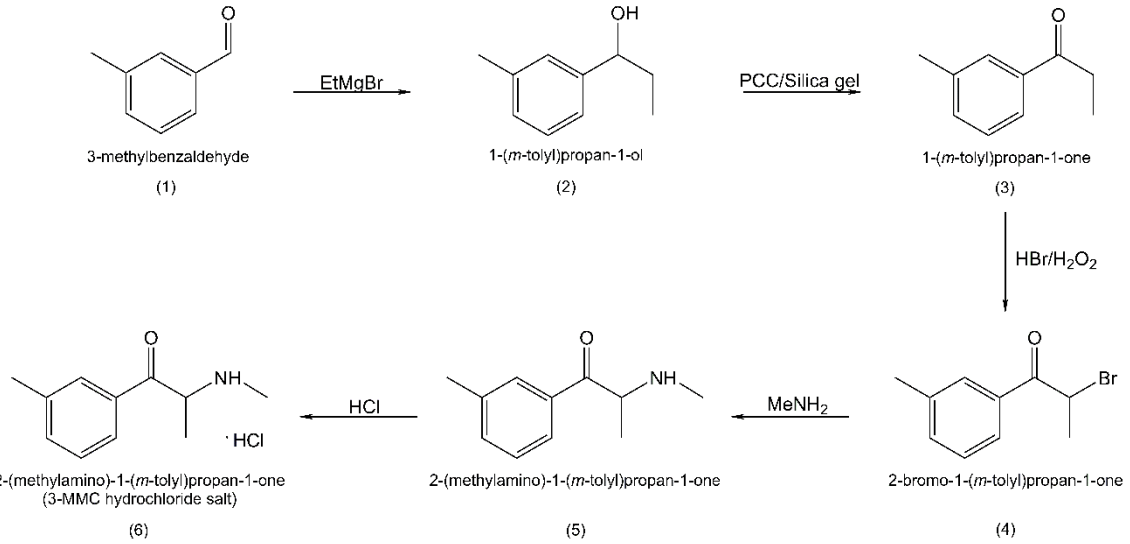


Fig. 1 Synthesis of 3-methylmethcathinone (3-MMC). EtMgBr – ethyl magnesium bromide; PCC/Silica gel – pyridinium chlorochromate on silica gel; HBr – hydrobromic acid; H_2O_2 – hydrogen peroxide; $MeNH_2$ – methylamine; HCl – hydrogen chloride. Adapted from Power et al. (2011).

Table 2 Physicochemical properties of 3-methylmethcathinone (3-MMC) hydrochloride salt.

	3-MMC (hydrochloride salt)
Chemical formula	$C_{11}H_{15}NO.HCl$
Molecular mass (g/mol)	213.7
Melting point (ºC)	193.2
Boiling point (ºC) (at 760 mm Hg)	280.5
pKa	7.84
Solubility	Soluble in HBSS Slightly soluble in ethanol, dimethyl sulfoxide, and dimethyl formamide
Partition coefficient (cLogP)	1.86
Chemical Abstracts Service (CAS) number	1246816-62-5
Other chemical names	3-methylmethcathinone; 3-methyl-N-methylcathinone; metaphedrone; 2-(methylamino)-1-(3-methylphenyl)-1-propanone

HBSS – Hank's Balanced Salt Solution. Adapted from Shimshoni et al. (2015) and WHO (2016)

There are several reports of the presence of cathinones in seized drug products and a number of analytical methods have been therefore developed for their identification, both in the seized materials and in the biological fluids of intoxicated individuals. In 2011, Power et al. (2011) synthetized and analysed 3-MMC through gas chromatography–mass spectrometry (GC-MS), infrared (IR) and nuclear magnetic resonance (NMR). In 2013, Christie et al. (2014) demonstrated that Raman spectrometry can distinguish between cathinones regioisomers, including 3-MMC. This is of great importance for the identification of drugs at airport security, where a fast and reliable identification is needed. Another great advantage of this method is the portability of the Raman microscope. In 2014, Strano Rossi et al. (2014) described an analytical approach to identify different types of new psychoactive substances (NPS) in seized material using GC-MS, liquid chromatography–high resolution mass spectrometry (LC-HRMS), and NMR. The most frequently used method for the identification of 3-MMC is liquid chromatography with different detectors, such as liquid chromatography tandem-mass spectrometry (LC-MS/MS) (Adamowicz et al. 2016a; Backberg et al. 2015; Maas et al. 2015; Shimshoni et al. 2015; Zuba and Adamowicz 2016) and LC-HRMS (Frison et al. 2016). The second most frequently used method is GC-MS (Adamowicz et al. 2014; Institoris et al. 2015; Jamey et al. 2016; Maas et al.

2015; Odoardi et al. 2016; Zuba and Adamowicz 2016). Regarding the GC-MS methodology, Zuba and Adamowicz (2016) suggest that it is better fitted for analysis of seized drugs. As the GC-MS method is less sensitive than the LC-MS/MS method, the latter is more suitable for toxicological analysis of biological specimens (Zuba and Adamowicz 2016). The LC-HRMS method is one of the most specific and sensitive methods for hair sample analysis. The NMR method is mostly used when there is a need to distinguish between isomers (Odoardi et al. 2016; Strano Rossi et al. 2014) and can also be used for determining the purity of a substance. The identification of substances is crucial in forensics and for such purpose the combination of different methods provides a more reliable identification of the substance (Zuba and Adamowicz 2016).

1.3. Appearance, patterns of consumption and prevalence

Like most “legal highs”, 3-MMC is usually sold online in the form of off-white crystals or white powder with a smell similar to lycorine (Jamey et al. 2016). It is labelled as a “research chemical” and/or “not for human consumption” (Institoris et al. 2015). The price ranges from 11 € to 35 € per gram (Eurochemicals 2016; get-RC 2016). Similarly to 4-MMC, consumption mostly occurs through insufflation (snorting) and orally (Adamowicz et al. 2016a; Backberg et al. 2015; Jamey et al. 2016; Sande 2016; Shimshoni et al. 2015), but the intravenous injection of the drug has also been reported (Adamowicz et al. 2016a; Backberg et al. 2015; Jamey et al. 2016).

In Germany, Marillier et al. (2017) analysed the purity of product samples donated by identified NPS users. From the 31 samples collected, 8 were positively confirmed for the presence of 3-MMC, although only 7 users admitted to be aware that the drug bought was 3-MMC. Those 8 samples had a range of purity between 51 % and 88 %. Reports from users of 3-MMC indicate drug administration doses often ranging from 50–250 mg, but higher amounts were occasionally described (up to 500 mg), with repeated administrations to prolong the desired effects adding up to ingestions that could reach 2 g in a single session (Adamowicz et al. 2016a). This scenario is similar to that of 4-MMC, in which case, for the same reason, the users take between 150-250 mg in repeated administrations, resulting in ingestions of 0.5–1 g in a single session (Shimshoni et al. 2015).

Studies conducted in Poland, Sweden, Hungary and Germany concluded that the users were mostly males with ages ranging from 17–50 years old (Adamowicz et al. 2016a; Backberg et al. 2015; Institoris et al. 2015; Romanek et al. 2017). In Slovenia,

other investigations found no gender differences in the drug consumption patterns and, although the age of the respondents included in the sample ranged from 15–40 years old, most of them were still attending school (Sande 2016).

The prevalence of these drugs is difficult to measure because little data is available and the existing information may not be accurate; questionnaires may not be reliable, as all too often consumers do not tell the truth about their consumption habits or do not know what they are buying. The same applies in case of intoxications without analytical confirmation. Some patterns of consumption are summarized in Table 3.

From 2012 to 2013, Institoris et al. (2015) sampled suspected drug abusers in Hungary. Among the 1959 individuals in Budapest and the 472 individuals in South-East Hungary (Csongrád County) that were confirmed positive for drug use, 3-MMC was detected in 0.97 % and 5.07 %, respectively (Institoris et al. 2015). In Germany, from 2010 to 2016, 81 intoxications with synthetic cathinones were reported by Romanek et al. (2017). From those, 13 tested positive for 3-MMC, 8 in 2014 and 5 in 2015. The slight decrease observed in the detected number of cases might be a consequence of the implementation of the German narcotics law in December 2014 (Maas et al. 2017), although the small size of the sample precludes robust conclusions.

An impressive report by Adamowicz et al. (2016b) presents the results of the clinical drug-related cases analysed in the Institute of Forensic Research (Krakow, Poland) from 2012 to 2014. Succinctly, 3-MMC was detected in 50 of the 112 cases that were positive for NPS, being the most prevalent drug. Eleven cases occurred in 2013 and 39 cases in 2014, supporting the increasing interest in the drug (Adamowicz et al. 2016b). The drug prevalence reported in this study is higher than that reported by Institoris et al. (2015). This could be explained by the small sample size in the Polish study, which may result in sample bias. Another explanation might be that the sample from Poland only includes NPS, while the sample from Hungary includes both classic drugs and NPS.

From May to October 2014, Sande (2016) employed a questionnaire to 249 drug users, in Slovenia. From those, 67.9 % of the users said that they had tried 3-MMC, while one-third of users admitted having used the drug in the past month (Sande 2016). As this questionnaire was performed online, it leaves out NPS consumers that do not have internet access.

The interest in a particular drug can also be measured in online forums. Ledberg (2015) studied the interest in 8 NPS before and after scheduling. The author used the data extracted from the *Flashback Forum*, a Swedish Internet discussion forum, and measured the interest by counting the number of posts per day, 180 days before and after the respective drug being prohibited. For 3-MMC, the interest before scheduling

hit a maximum around 80 posts per day (mean of 20 post per day) and around 5 posts per day after scheduling (mean of 3.1 posts per day) (Ledberg 2015). This is consistent with the pattern of analytically confirmed exposure to 3-MMC found in the Swedish STRIDA project (Swedish early-warning system on NPS), that decreased when the substance was legally framed (Backberg et al. 2015). Drug forums may also be important to obtain information on substance dose reported by users (Ledberg 2015), which may be of great importance to adopt measures in case of intoxications or to plan a study on the toxicity of the selected drug.

Table 3 Patterns of consumption of 3-methylmethcathinone (3-MMC).

Year	Location	n	Age (years)	Individuals consuming 3-MMC (%)	Consumed dose (g)	Reference
2012 – 2013	Budapest	1959	18 – 50	1.0		Institoris et al. (2015)
	Csongrád Country	472		5.1		
2012 – 2014	Sweden	786	17 – 49	6.4	0.5 – 2	Backberg et al. (2015)
2012 – 2014	Poland	112	16 – 50	44.6		Adamowicz et al. (2016b)
2014	Slovenia	249	15 – 40 [1]	67.9	1.5	Sande (2016)
2010 – 2016	Southern Germany	81	17 – 49	16.0		Romanek et al. (2017)

[1] Mostly students

1.4. Biological effects

Most information on biological effects in humans derives from case reports of intoxicated individuals that are admitted to the emergency room, from online questionnaires, or self-reports of consumers of 3-MMC, but even these data are scarce.

The desired effects of 3-MMC are euphoria, excitement, high energy levels, stimulation (rushing), happiness, awareness of senses, and appreciation of music (Adamowicz et al. 2016a; Adamowicz et al. 2014). As with amphetamines and other stimulant drugs of abuse, the social effects consist of improved social skills and feelings of empathy (Adamowicz et al. 2016a) but chronic abuse may trigger deterioration of relationships with family, partner and friends (Sande 2016).

Among the adverse effects of 3-MMC are verbosity, stuttering, fatigue, agitation, aggression, uncoordinated movements, and tachycardia (Adamowicz et al. 2016a).

Concentration difficulties, tingling in the arms and legs, hyponatremia, diaphoresis, seizures, hyperthermia, and rhabdomyolysis have been known to occur but with lower frequency (Backberg et al. 2015; Sande 2016).

Other effects that have been reported include hypertension, dilated pupils, hallucinations, fear, anxiety, depression, disorientation, slurred speech, strange behaviour, unsteady gait, and staggering but these effects could not be unequivocally attributed to 3-MMC as other substances were co-ingested (Adamowicz et al. 2016a; Backberg et al. 2015; Sande 2016). Maas et al. (2015) described the case of a user driving under the influence of methadone, 3-MMC, lorazepam and, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) who presented deficient concentration, delayed reaction time, problems with balance and coordination, impaired fine motor skills, washed out pronunciation, and disorientation, which cannot be attributed to 3-MMC alone. Reported case reports from fatal and non-fatal intoxications are shown in Tables 4 and 5.

1.5. Pharmacokinetics

Little data is available on the pharmacokinetics of 3-MMC. To the best of our knowledge no human studies are documented in the literature and only one study using animals is available (Shimshoni et al. 2015). The pharmacokinetic features of 3-MMC are described below and based on the work of Shimshoni et al. (2015) in pigs. Although this animal study is the only one so far reported, caution should be taken when interpreting the results due to the small sample size (n = 6) (Shimshoni et al. 2015).

1.5.1. Absorption

In this study, 3-MMC was only administered orally and through intravenous injection. After a single oral dose of 3 mg/kg of the hydrochloride salt form of the drug, the peak plasma concentration of 3-MMC (C_{max} = 27 µg/L) occurred at 5–10 min (Shimshoni et al. 2015). Only 7 % of the drug was absorbed, after oral administration. Absorption mostly occurred in the first 12 min (corresponding to more than 80 % of the absorbed drug). The low oral bioavailability observed may explain the preferred use of 3-MMC through insufflation (Shimshoni et al. 2015). This may indicate that 3-MMC suffers first-pass metabolism before reaching systemic circulation (Shimshoni et al. 2015). After a single intravenous dose of 0.3 mg/kg in saline of the hydrochloride salt form of the drug, almost none of the drug was detected after 4 h (Shimshoni et al. 2015).

Table 4 Case reports of non-fatal intoxications involving 3-MMC.

Year	Local	Cases (n)	Sex/Age (years)	Intoxication symptoms	Drug Concentration	Reference
2012 - 2014	Sweden	50	M (n = 38); F (n = 12)/ 17-49	Tachycardia (≥ 100/min); Agitation; Hypertension (systolic blood pressure ≥ 140 mmHg); Reduced level of consciousness (RLS > 2); Dilated pupils; Hallucinations; Hyponatremia (136 mmol sodium/L); Diaphoresis; Seizures; Clinical significant hyperthermia (39 °C); Rhabdomyolysis (25 IU CK/L, 3000 µg myoglobin/L)	0.002–1.5 µg/mL of 3-MMC[s]; 0.007 – 290 µg/mL of 3-MMC[u]	Backberg et al. (2015)
2012 - 2014	Poland	112	M (n = 102); F (n = 10)/ 16 - 50	Verbosity; Slurred speech; Strange behaviour; Unsteady gait; Staggering	< 0.001–1.6 µg/mL of 3-MMC[b]	Adamowicz et al. (2016b)
2013 - 2015	Poland	95	M (n = 89); F (n = 6)/ 17 – 50	Gaiety; Verbosity; Stuttering; Fatigue; Agitation; Aggression; Uncoordinated movements; Tachycardia (100 bpm)	0.003–0.2 µg/mL of 3-MMC[b]	Adamowicz et al. (2016a)
2015 - 2016	France	3	F/22	Confusion; Somnolence; Myosis; GS = 10	0.3 µg/mL of 3-MMC[p]; 0.02 µg/mL of 5-MAPB[p]; 0.1 µg/mL of methadone	Turcant et al. (2017)
			M/35	Agitation; Hypertension; Chest pain; Tachycardia	0.007 µg/mL of 3-MMC[p]; 2.4 µg/mL of 3-MMC[u]	
			M/29	Hallucinations; Tachycardia	0.2 µg/mL of 3-MMC[b]	

Table 4 Case reports of non-fatal intoxications involving 3-MMC (continuation).

Year	Local	Cases (n)	Sex/Age (years)	Intoxication symptoms	Drug Concentration	Reference
2015 - 2016	France	3	M/23	Seizures; GS = 3	1.6 µg/mL of 3-MMC^{p}; 0.9 µg/mL of 4-MEC^{p}; 1.2 µg/mL of MXE^{p}; 141 µg/mL of 3-MMC^{u}; 61 µg/mL of 4-MEC^{u}; 39 µg/mL of MXE^{u}	Turcant et al. (2017)
			M/37	Agitation; Mydriasis; Tachycardia	0.06 µg/mL of 3-MMC^{p}; 0.2 µg/mL of 4-MEC^{p}; 0.01 µg/mL of MEX^{p}; 0.1 µg/mL of MDMA^{p}; 13 µg/mL of 3-MMC^{u}; 85 µg/mL of 4-MEC^{u}; 0.9 µg/mL of MEX^{u}; 5.6 µg/mL of MDMA^{u}; 0.3 µg/mL of MDA^{u}	
			M/30	Coma; Myosis; Bradypnea; GS = 3-5	0.2 µg/mL of 3-MMC^{p}; 200 µg/mL of GHB^{p}; 41.6 µg/mL of 3-MMC^{u}; 685 µg/mL of GHB^{u}	
2015	Germany	1	M/34	Deficient concentration; Delayed reaction time; Problems with balance and coordination; Impaired fine motor skills; Washed out pronunciation; Disorientation	0.1 µg/mL of methadone^{s}; 0.006 µg/mL EDDP^{s}; 0.03 µg/mL of lorazepam^{s}; 0.04 µg/mL of 3-MMC^{s}	Maas et al. (2015)
2016	Italy	1	M		0.03 µg/mL of 3-MMC^{ph}	Frison et al. (2016)
2014 - 2016	Germany	2	F/26	Depressive mood; Pupil with slow reaction to light	0.04 µg/mL of 3-MMC^{s} 0.01 µg/mL of 3-MMC^{s}	Maas et al. (2017)

RLS – Reaction level scale; CK – Creatine kinase; EDDP – 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; GS – Glasgow score; 5-MAPB – 5-(2-Methylaminopropyl)benzofuran; 4-MEC – 4-Methylethcathinone; MXE – methoxetamine; MDMA – 3,4-Methylenedioxymethamphetamine; MDA – 3,4-methylenedioxyamphetamine; Bpm – beats per minute

^{s} Serum; ^{u} Urine; ^{b} Blood; ^{p} Plasma; ^{ph} Pubic hair

1.5.2. Distribution

3-MMC had a total clearance of 199 L/h and a volume of distribution of 240 L (Shimshoni et al. 2015). The total clearance determined in this study represents more than the sum of liver and kidney blood flow (88 L/h) in pigs that weight 30-40 kg (Shimshoni et al. 2015), which can mean that there are additional elimination sites or mechanisms besides hepatic and renal metabolism. The large volume of distribution may be due to low protein binding and probably due to active transportation into the tissue compartment (Shimshoni et al. 2015).

1.5.3. Metabolism

The metabolism of 3-MMC is not fully disclosed, but based on information obtained from the analysis by LC-HRMS of pubic hair it is known that 3-methylepedrine and 3-methylnorephedrine are metabolites of the drug (Frison et al. 2016). A hypothetical metabolic human pathway was proposed and presented in Fig. 2.

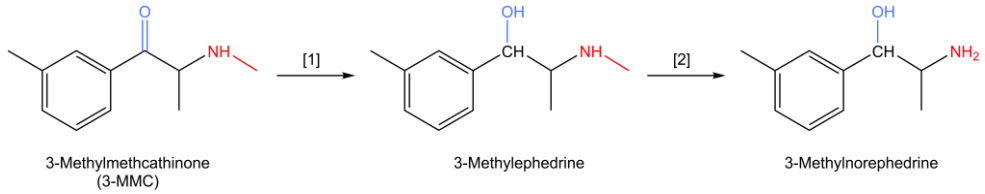


Fig. 2 Hypothetic metabolism of 3-methylmethcathinone (3-MMC) into 3-methylnorephedrine through β-keto-reduction [1] followed by N-demethylation [2] into 3-methylnorephedrine. Adapted from Frison et al. (2016).

The relevance of the metabolism for the pharmacological and toxicological profile of 3-MMC is yet to be revealed. Similar to 3-MMC, the metabolic pathway of 4-MMC is not fully known in humans (Khreit et al. 2013). It is known that nor-mephedrone, nor-dihydro mephedrone, hydroxytolyl mephedrone, and nor-hydroxytolyl mephedrone, 4-carboxymephedrone and 4-carboxydihydromepedrone are metabolites of 4-MMC excreted in urine (Ribeiro et al. 2012).

1.5.4. Excretion

In pigs, the pharmacokinetics of 3-MMC appears to follow a one-compartment model (Shimshoni et al. 2015). The elimination half-life of 3-MMC was 0.83 h, corroborating the values achieved for total clearance and distribution volume. Most of the dose was excreted after 4 h of administration, either when administered through injection or orally (Shimshoni et al. 2015). The elimination rate constant of 3-MMC was 0.84 h^{-1} (Shimshoni et al. 2015). This rapid elimination rate may justify the short psychoactive effects and, consequently, the need to repeat oral administrations during consumption sessions (WHO 2016).

1.6. Toxicity

1.6.1. Lethal case reports

Few fatal intoxications have been reported due to the consumption of 3-MMC. The cases reported so far were thirteen and all occurred in Europe (WHO 2016). The available information about these cases is summarized in Table 5.

In Sweden, two deaths occurred from 2012 to 2014; both patients co-ingested other drugs (Backberg et al. 2015). In the UK, one individual committed mechanical suicide under the influence of 3-MMC, venlafaxine, and an unknown substance (suspected of being cocaine and/or an amphetamine) (Elliott 2016). Suicide has already been associated with the use of other synthetic cathinones (Elliott and Evans 2014). In Poland, 5 fatal cases involving 3-MMC were described (Adamowicz et al. 2016a). The first case involved 3-MMC and MDMA. The second case involved 3-MMC and 5-APB (5-(2-Aminopropyl)benzofuran). The third case also involved 3-MMC and 5-APB, but different concentrations of the drugs were quantified in blood (Tables 5 and 6). The fourth case involved 3-MMC and 25I-NBOMe (2-(4-Iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine). The last case involved 3-MMC and tramadol at therapeutic concentration (Adamowicz et al. 2016a). In Norway, Karinen et al. (2014) described the fatal intoxication of a young man with AH-7921 (3,4-Dichloro-N-{[1-(dimethylamino)cyclohexyl]methyl}benzamide), 2-FMA (2-fluoromethamphetamine), 3-MMC, codeine, codeine-6-glucuronidie, and acetaminophen.

Only two deaths were attributed to 3-MMC alone. One of these deaths was reported in Italy (Rojek et al. 2015). The other one was described by Bottinelli et al. (2016) and occurred in France. All the other fatal cases reported involved the administration of the drug in association. Accordingly, Bottinelli et al. also described two deaths in which 3-MMC was detected along with other drugs; in the first one, 3-MMC was co-ingested with alcohol, paracetamol, paroxetine, sildenafil, and 4-methylethcathinone (4-MEC) (2016); in the second case, the death of a 32-year old individual was attributed to 3-MMC and GHB (2017). These two drugs were also involved in the cause of death of a 69-year old man, in France (Jamey et al. 2016).

The majority of the deaths involving 3-MMC (8 deaths out of 13) occurred with the co-ingestion of other drugs (Adamowicz et al. 2016a; Adamowicz et al. 2014; Backberg et al. 2015; Bottinelli et al. 2017; Bottinelli et al. 2016; Jamey et al. 2016; Karinen et al. 2014). Of these, 5 deaths were attributed to drugs used for medical purposes (Adamowicz et al. 2016a; Bottinelli et al. 2016; Jamey et al. 2016; Karinen et

al. 2014). In the two deaths that involved 3-MMC alone, blood concentrations of 0.249 µg/mL (Bottinelli et al. 2016) and 4.4 µg/mL (Rojek et al. 2015) were reported. Because the number of fatal cases is relatively small, and because only two of these cases involved 3-MMC alone, it is not possible to draw any conclusion about potentially toxic and/or lethal 3-MMC concentrations. Also, *postmortem* findings are highly influenced by the circumstances of the intoxication, time elapsed from drug intake, and whether life support measures were adopted or not. For these reasons, the reported drug blood/tissue concentrations are highly discrepant among intoxicated individuals (Table 5 and 6).

1.6.2. Mechanisms of toxicity

There are no studies elucidating the mechanisms of toxicity of 3-MMC. In the study conducted by Shimshoni et al. (2015) in pigs, no changes in serum biochemical markers were found after the administration of 3-MMC. Some of these markers were cholesterol, creatinine, total protein, and urea. The authors also stated no drug-related mortality or morbidity and did not detect any gross pathological or abnormal histopathological findings (Shimshoni et al. 2015). The tissues analysed were the heart, skeletal muscles (diaphragm, intercostal, triceps, and others), lungs, pancreas, liver, kidney, spleen, brain and small intestine.

In the literature, the toxicological data regarding synthetic cathinones is scarce. It is expected that some of the mechanisms are similar to those of amphetamines, as cathinone and synthetic cathinones have similar structures (Valente et al. 2014). From the few information available for synthetic cathinones, it is noted that 4-MMC produces concentration-dependent cytotoxicity and impairment of mitochondrial respiration in SH-SY5Y cells at concentrations of 1 mM and 2 mM, respectively (Hollander et al. 2015). By virtue of the similarity between both drugs, one can hypothesise that 3-MMC could likely induce the same effects (Valente et al. 2014).

It is well known that amphetamines cause hyperthermia, which can increase the formation of ROS and RNS (reactive species of oxygen and nitrogen, respectively) species (Dias da Silva et al. 2014a). As 3-MMC also causes hyperthermia, it is also likely to occur formation of ROS and RNS. Backberg et al. (2015) described a fatal case where the patient was hyperthermic after 20 h of administration (40.9 ºC), an effect that persisted even after sedation, external cooling and administration of cold fluids. The patient also had metabolic acidosis and rhabdomyolysis, which compromised his renal function. Six days after the admission the patient died (Backberg et al. 2015).

Table 5 Deaths related to 3-methylmethcathinone (3-MMC).

Year	Local	Cases (n)	Sex/Age (years)	Cause of death/ autopsy findings	*Postmortem* toxicological analysis	Reference
2012 – 2014	Sweden	2	M	Polydrug abuse	3-MMC: 0.002 µg/mL [s]; 0.09 µg/mL [u]. Small concentration of ethanol metabolites [u].	Backberg et al. (2015)
			M/25	Polydrug abuse		
2013	UK	1		Mechanical suicide	1.1 µg/mL of 3-MMC [fb]; 1.6 µg/mL of venlafaxine [fb]; 2.8 µg/mL O-desmethylvenlafaxine [fb]	Elliott (2016)
2013 – 2015	Poland	4	M	Polydrug abuse	< 0.001 µg/mL of 3-MMC; 0.03 µg/mL of MDMA	Adamowicz et al. (2016a)
				Polydrug abuse	0.02 µg/mL of 3-MMC; 0.1 µg/mL of 5-APB [1]; 3.4 mg/g of Katelin [s]	
			M	Acute respiratory failure caused by massive pneumonia/ Multiple organ failure	0.01 µg/mL of 3-MMC [b]; 0.003 µg/mL of 25I-NBOMe [b]	
					0.003 µg/mL of 3-MMC [b]; 0.6 µg/mL of tramadol [b]	
2014	Norway	1	M/early twenties	Poly drug abuse Oedematous lungs	0.002 µg/mL of 3-MMC [pwb]; 0.4 µg/mL of AH-7921 [pwb]; 0.4 µg/mL of codeine [pwb]; 0.8 µg/mL C6G [pwb]; 19 µg/mL of acetaminophen [pwb]; 0.007 of µg/mL 2-FMA [pwb]	Karinen et al. (2014)
2014	Poland	1	M/20	Polydrug abuse	1.6 µg/mL of 3-MMC [b]; 5.6 µg/mL of 5-APB [b]	Adamowicz et al. (2014)
2015	Italy	1		3-MMC intoxication	4.4 µg/mL of 3-MMC [b]	Rojek et al. (2015)

Table 5 Deaths related to 3-methylmethcathinone (3-MMC) (continuation).

Year	Local	Case (n)	Sex/Age (years)	Cause of death/ Autopsy findings	*Postmortem* toxicological analysis	Reference
2016	France	2	M/thirties	Polydrug abuse 3-MMC intoxication	0.08 µg/mL of 3-MMC [b]; 180 µg/mL of alcohol [b]; 0.1 µg/mL of paracetamol [b]; 0.1 µg/mL of paroxetine [b]; 0.08 µg/mL of sildenafil [b]; 0.1 µg/mL of 4-MEC [b] 0.2 µg/mL of 3-MMC [b]	Bottinelli et al. (2016)
2016	France	1	M/69	Polydrug abuse	3-MMC: 0.33 µg/mL [pb]; 0.9 µg/mL [cb]; 29.0 µg/mL [u]; 1.5 µg/mL [gc]; 0.6 µg/mL [bl]; 206.7 ng/mg [h]. GHB [1]: 576 µg/mL [pb]; 630 µg/mL [cb]; 719 µg/mL [u]; 2671 µg/mL [gc]; 909 µg/mL [bl]; 96.3 ng/mg [h]. Pseudoephedrine: 0.03 µg/mL [pb]; 0.08 µg/mL [cb]; 1.1 µg/mL [u]; 0.1 µg/mL [gc]; 0.05 µg/mL [bl]; 0.2 ng/mg [h].	Jamey et al. (2016)
2016	France	1	M/32	3-MMC intoxication	3-MMC: 0.2 µg/mL [pb]; 0.6 µg/mL [cb]; 3.0 µg/mL [vh]; 29.7 µg/mL [u]; 1.3 µg/mL [bl]. GHB: 35.7 µg/mL [pb]; 25.0 µg/mL [cb]; 8.8 µg/mL [u]; 12.4 µg/mL [bl].	Bottinelli et al. (2017)
2014 – 2015	Germany	1		Cardiac arrest/Cerebral oedema/Brain death		Romanek et al. (2017)

3-MMC – 3-Methylmethcathinone; MDMA – 3,4-Methylenedioxymethamphetamine; 5-APB – 1-Benzofuran-5-ylpropan-2-amine; katelin – potassium chloride; 25I-NBOMe – 2-(4-Iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine; AH-7921 – 3,4-Dichloro-N-{[1-(dimethylamino)cyclohexyl]methyl}obenzamide; C6G – Codeine-6-glucuronide; 2-FMA – 2-Fluoromethamphetamine; 4-MEC – 4-Methylethcathinone; GHB – γ-Hydroxybutyric acid;
[pwb] Peripheral whole blood; [b] Blood; [pb] Peripheral blood; [s] Stomach; [cb] Cardiac blood; [u] Urine; [gc] Gastric content; [bl] Bile; [h] Hair; [fb] Femoral blood; [vh] Vitreous humor

In comparison, the hyperthermia caused by MDMA is frequently responsible for fatal complications, including rhabdomyolysis, acute renal failure, disseminated intravascular coagulation (DIC), multiple organ failure, and acidosis (Henry et al. 1992; Kalant 2001; Kendrick et al. 1977). Some of these events were observed in the fatal case described by Backberg et al. (2015). This may suggest that 3-MMC shares some of the mechanisms of toxicity with MDMA.

1.7. Factors affecting the stimulant/toxicological effects

1.7.1. Dose/concentration

There are no studies in humans with 3-MMC that describe the relation between the administered dose and the elicited stimulant/toxicological effects. The only study containing the dose information was reported by Backberg et al. (2015) and describes the clinical features observed in patients positive for 3-MMC. Nevertheless, by virtue of the broad interval of doses mentioned (0.5–2 g), no associations between these and the reported symptoms could be established. The same applies to the blood concentrations found.

In the three-year review of Adamowicz et al. (2016b) mentioned above, the data collected from medical and police notes concerning 50 cases involving 3-MMC was divided in two groups: in the first group, information of the cases in which 3-MMC was detected alone was considered; in the second group, 3-MMC was detected along with other drugs. Regarding the first group, the blood concentration range observed for asymptomatic users was higher (0.01 µg/mL – 0.2 µg/mL) than that achieved for individuals presenting 3-MMC-induced symptoms (0.01 µg/mL – 0.02 µg/mL). This was attributed to the fact that symptomatic individuals were mostly first time users, while the same was not observed for asymptomatic users. In the second group, the same tendency was observed (users with no symptoms: 0.001 µg/mL – 0.2 µg/mL; users with symptoms: 0.001 µg/mL – 0.06 µg/mL) but in this case results were more difficult to be interpreted, since polydrug abuse was at play. This will be further discussed below.

Table 6 Blood concentrations of 3-methylmethcathinone (3-MMC) in fatal and non-fatal intoxications.

Dose (g)	Concentration (µg/mL)	Concentration *post-mortem* (µg/mL)	Intoxication	Reference
		0.002	Fatal	Karinen et al. (2014)
		1.6	Fatal	Adamowicz et al. (2014)
0.5 – 2	0.002 – 1.5		Non-fatal	Backberg et al. (2015)
	0.04		Non-fatal	Maas et al. (2015)
	< 0.001 – 1.6		Non-fatal	Adamowicz et al. (2016b)
	0.003 – 0.2		Non-fatal	Adamowicz et al. (2016a)
		< 0.001	Fatal	
		0.02	Fatal	
		0.01	Fatal	
		0.003	Fatal	
	0.02		Non-fatal	
		0.3	Fatal	Jamey et al. (2016)
		0.08	Fatal	Bottinelli et al. (2016)
		0.2	Fatal	
		4.4 *	Fatal	Rojek et al. (2015)
		1.1	Fatal	Elliott (2016)
		0.2	Fatal	Bottinelli et al. (2017)

In cases marked with *, the only drug involved was 3-methylmethcathinone (3-MMC).

1.7.2. Route of administration

Stimulant/toxic effects are dependent on the route of administration. As mentioned above, the preferred route of administration is through insufflation, which may be explained by the low oral bioavailability (Shimshoni et al. 2015). Rapid onset of the effects and cultural preferences may also help explain the use of this administration route. After insufflation, the first effects appear between 20–30 min and the peak effects occur after 50 min, lasting up to 3–4 h, which is why users repeat administration (two or three times, every 2 h) to prolong the effects (Adamowicz et al. 2016a). This is similar to 4-MMC, for which the first effects appear 15–45 min after administration, and last up to 4–5 h (Shimshoni et al. 2015).

1.7.3. Polydrug abuse

Frequently, 3-MMC is consumed with other drugs and it is difficult to describe how this abuse pattern affects the induced stimulant/toxic effects (Adamowicz et al. 2016a) because there are drug-drug interactions that are unknown. Accordingly, most of the symptoms reported cannot be attributed to 3-MMC alone, although it is usual that polydrug users have less severe effects than those who only consume 3-MMC (Adamowicz et al. 2016b). This can be explained due to the phenomenon of tolerance, as it is common that polydrug users are frequent drug abusers.

1.7.4. Interaction with therapeutic drugs

There are five cases described in the literature involving 3-MMC and therapeutic drugs, which were previously mentioned and are presented in Table 5. Overall, it is difficult to draw conclusions on the emerging interactions because all too often there are other drugs of abuse present. Contrary to the cases in which other types of drugs were detected, pharmaceuticals are present at very low levels.

Adamowicz et al. (2016a) briefly described two fatal cases with 3-MMC and therapeutic drug interactions. Among the co-abused drugs were tramadol and potassium chloride (Katelin) (Table 5).

Karinen et al. (2014) described a fatal case with 3-MMC, codeine, and acetaminophen. The man was prescribed a drug combination of codeine and acetaminophen (30 mg codeine/400 mg acetaminophen) after a minor traffic accident. He took 6 of these pills and some 3-MMC powder he bought online. The results from toxicological analysis of peripheral whole blood can be found in Table 5. Although codeine and acetaminophen were prescribed in therapeutic doses and low concentrations were found in blood, other non-therapeutic drugs were taken and may have also contributed for the fatality (Karinen et al. 2014).

Bottinelli et al. (2016) briefly described a case with 3-MMC, acetaminophen, sildenafil and paroxetine (Table 5). Jamey et al. (2016) described a case of 3-MMC and pseudoephedrine. The toxicological analysis revealed 0.3 µg/mL of 3-MMC, 576 µg/mL of GHB (gamma-hydroxybutyric) and 0.03 µg/mL of pseudoephedrine (Jamey et al. 2016). As the concentration of pseudoephedrine is much lower than the concentration of 3-MMC and GHB, the author concluded that it was likely that the death occurred because of the interaction of 3-MMC and GHB. It was hypothesized that the trace of pseudoephedrine was derived either from a clinical treatment or from the illicit

manufacture of 3-MMC, since pseudoephedrine is also a precursor in methamphetamine and methcathinone synthesis (Jamey et al. 2016).

1.7.5. Environmental conditions

When the consumption of the drug occurs in nightclubs, where the atmosphere is hot and crowded and the ventilation bad, it is expected a much more severe detrimental effect, as these conditions aggravate the hyperthermia and diaphoresis elicited by 3-MMC (Backberg et al. 2015). Accordingly, a body temperature as high as 40.9 ºC was observed in an individual who took the drug in a hot environment.

This is consistent with what is known for the related drug MDMA, it was reported that when the environmental temperature was below 22 ºC the rats tended to develop hypothermia, and when the temperature was above 28 ºC, the hyperthermia became life-threatening (Capela et al. 2009).

1.7.6. Individual characteristics of the consumer

There are several characteristics of the consumer that will influence the drug-associated risks (Carvalho et al. 2012). The main characteristics of the consumer that may increase the susceptibility to the acute effects of the drug are age, ethnicity, metabolic polymorphisms, and gender (Carvalho et al. 2012). To the best of our knowledge, there is no human study that relates these characteristics with the effects caused by synthetic cathinones. Since cathinone and synthetic cathinones have similar structures to amphetamines, it is expected that the influence of these factors to be similar (Valente et al. 2014).

1.7.6.1. Age

The consumption of NPS is mainly associated with younger age groups (EMCDDA 2016). It is known that age can affect the expression of enzymes involved in the metabolism of drugs (Parkinson et al. 2004). It is also known that adolescents have a higher metabolic rate of the enzymes involved in the metabolism (Parkinson et al. 2004), thus having reduced sensitivity to the psychoactive effects of certain drugs, for example, amphetamines (Carvalho et al. 2012). For this reason, it is expected that younger individuals consume more drug than older individuals to obtain the same

desired effects (Wiley et al. 2008). This might mean that they are more susceptible to intoxications.

1.7.6.2. Metabolism and enzymatic polymorphisms

Polymorphic expression of metabolic enzymes is known to highly influence the metabolism and toxicity of amphetamines. For example, for MDMA, it is known that CYP2D6 is a major enzyme involved in the oxidative metabolism (Carvalho et al. 2012). Therefore, it is expected that individuals carrying deficient alleles for the enzyme to be more susceptible for acute toxic effects (Carvalho et al. 2012). As CYP2D6 has various variants in different populations, the ethnicity of a user is expected to affect the toxicity of amphetamines (Ramamoorthy et al. 2002; Tucker et al. 1994).

1.7.6.3. Ethnicity

Ethnicity can influence the effects of drugs because of genetic variation, different dietary habits and lifestyle factors (Carvalho et al. 2012). As mentioned above, mutations on the genetic code can be the reason for the increase of stimulant/toxic effects, depending on the genotype (Haile et al. 2009). Some of these genetic polymorphisms can profoundly affect the expression and/or activity of membrane transporters and other proteins, as such that intervene in the metabolism of the drug, impacting the toxicological expression of the drug (Carvalho et al. 2012).

1.7.6.4. Gender

The gender can also affect the stimulant/toxic effects of a drug. It is known that women are more susceptible to the toxic effects of MDMA (Liechti et al. 2001; Parkinson et al. 2004), probably because they have a lower metabolic capacity (Carvalho et al. 2012). However, men seem more susceptible to hyperthermia and therefore to the toxic effects of amphetamines than women, thus experiencing more severe symptoms (Miller and O'Callaghan 1994). This most likely occurs because men have a higher metabolic rate and higher drug consumption patterns (EMCDDA 2010a).

Chapter 2

Objectives of the thesis

By virtue of the increasing frequency of use of synthetic cathinones, research to elucidate their toxicological effects is of utmost importance, as the vast majority of these drugs rarely endure safety and quality control procedures. In addition, as soon as a substance becomes prohibited, new derivatives are synthesized to fill its gap, which frequently results in numerous severe adverse events and even deaths. With the intense rate of production of NPS, it is difficult to follow their emergence in the drug scene with satisfactory knowledge on the mechanisms of toxicity. Notwithstanding, this information is vital to elucidate users about the serious risks involved in the consumption of such substances.

Since oral ingestion is the major administration route for these stimulants, the liver represents one of the main targets of their toxicity, largely due to the first passage effect. Also, due to the important role of liver in metabolism, it is possible that the drug may undergo bioactivation with production of more toxic metabolites, compromising the functionality of the organ and, therefore, the overall health of the abuser.

In this context, and since there is a significant lack of information on the underlying mechanisms of toxicity of 3-MMC but its consumption has been increasing, the current study aimed to:

i) Analyse the potential *in vitro* hepatoxicity of 3-MMC, using primary rat hepatocytes;

ii) Elucidate the contribution of the metabolism by different isoforms of the cytochrome P450 to the observed toxicity;

iii) Clarify the toxicological mechanisms involved in the observed hepatotoxic effects, by evaluating a range of oxidative stress, mitochondrial integrity, and energetic balance endpoints, and the mode of cell death.

iv) Characterize the metabolic profile of 3-MMC by gas chromatography-mass spectrometry (GC-MS) for the identification and/or quantification of 3-MMC and metabolites.

Chapter 3

Materials and methods

3.1. Cytotoxic profile of 3-methylmethcathinone (3-MMC)

3.1.1. Chemicals

The hydrochloride salt of 3-MMC was purchased online from the Sensearomatic website (http://sensearomatic.net, currently unavailable), in March 2016. Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich (Lisbon, Portugal) and all the cell culture reagents from Gibco® (Alfagene, Lisbon, Portugal).

3.1.2. Animals

Male Wistar Han rats with a body weight of 150–250 g were purchased from Charles River Laboratories (Barcelona, Spain). The animals, kept under controlled temperature (20 ± 2 °C), humidity (40–60 %), and lighting (12 h-light/dark cycle) conditions, were fed with standard rat chow and tap water *ad libitum*. Isolation of hepatocytes was always conducted between 8:00 and 10:00 a.m. Surgical procedures were performed after rat anaesthesia and analgesia induced by an intraperitoneal injection of a combination of 20 mg/kg xylazine (Rompun® 2 %, Bayer HealthCare, Germany) and 100 mg/kg ketamine (Clorketam® 1000, Vétoquinol, France), and maintained through inhalation of isoflurane vapour (IsoVet® 1000 mg/g, B. Braun VetCare, Germany). This study was approved by the local committee for the welfare of experimental animals and performed in accordance with national legislation by investigators accredited for laboratory animal use by the national authority *Direção Geral de Alimentação e Veterinária* (DGAV), under the terms of the Law No. 1005/92, of 23rd October, Article 3, paragraph b, subparagraph iii).

3.1.3. Isolation of primary rat hepatocytes

Isolation of hepatocytes was performed using a modified two-step perfusion of the liver, as previously described by Moldeus et al. (1978), with some modifications. The liver was perfused *in situ* via the *vena portae*, firstly with a sterile EGTA-buffer at 37 °C, for approximately 10–15 min. The chelator promotes the cleavage of the hepatic desmosomes through calcium sequestration. The EGTA-buffer consisted of 248 mL glucose solution (9 g/L d-glucose), 40 mL Krebs-Henseleit-buffer (60 g/L NaCl, 1.75 g/L KCl, and 1.6 g/L KH_2PO_4; adjusted to pH 7.4), 40 mL HEPES-buffer I (60 g/L HEPES; adjusted to pH 8.5), 30 mL MEM non-essential amino acid solution (100x), 30 mL MEM

Amino Acids (10x) solution, 4 mL glutamine solution (7 g/L l-glutamine), and 1.6 mL EGTA-solution (47.5 g/L EGTA added of NaOH until total dissolution; adjusted to pH 7.6). Subsequently, hepatic collagen was hydrolysed by liver perfusion for 10–15 min with a sterile collagenase buffer, at 37 °C. The collagenase buffer consisted of 155 mL glucose solution, 25 mL Krebs-Henseleit-buffer, 25 mL HEPES-buffer I, 15 mL MEM non-essential amino acid solution (100x), 15 mL MEM amino acids (10x) solution, 10 mL of $CaCl_2$ solution (19 g/L $CaCl_2.2H_2O$), 2.5 mL glutamine solution, and 50 mg of collagenase type IA from *Clostridium histolyticum* (dissolved immediately before use). After perfusion, the liver was dissected, removed from the animal, and dissociated in a sterile suspension-buffer. This buffer consisted of 124 mL glucose solution, 20 mL Krebs-Henseleit-buffer, 20 mL HEPES-buffer II (60 g/L HEPES; adjusted to pH 7.6), 15 mL MEM non-essential amino acid solution (100x), 15 mL MEM amino acids (10x) solution, 2 mL glutamine solution, 1.6 mL $CaCl_2$ solution (19 g/L $CaCl_2.2H_2O$), 0.8 mL $MgSO_4$ solution (24.6 g/L $MgSO_4.7H_2O$), and 400 mg bovine serum albumin (BSA). The obtained suspension was purified by three low-speed centrifugations at 250 g, for 5 min, at 4 °C. The viability of isolated hepatocytes was always above 85 %, as assessed by the trypan blue exclusion method.

3.1.4. Culture of primary rat hepatocytes

A suspension of 5 x 10^5 viable cells/mL in complete culture medium was seeded onto the central 60 wells of 96-well plates (5 x 10^4 cells/well) or onto 6-well plates (1 x 10^6 cells/well) pre-coated with collagen. Complete cell culture medium consisted of William's E medium (Sigma-Aldrich, Lisbon, Portugal) supplemented with 10 % FBS, 5 µg/mL insulin solution from bovine pancreas (Sigma-Aldrich, Lisbon, Portugal), 50 nM dexamethasone (Sigma-Aldrich, Lisbon, Portugal), 1 % antibiotic solution (100 U/mL penicillin, 100 µL/mL streptomycin), 100 µg/mL gentamicin, and 0.25 µg/mL amphotericin B. After seeding, primary rat hepatocytes were incubated overnight at 37 °C, in an atmosphere of 5 % CO_2, to promote cell adhesion. On the next day, the cells were exposed to the test drug.

3.1.5. Drug exposures

Stock solutions of 3-MMC were extemporaneously prepared in Hank's balanced salt solution (HBSS) and further diluted (at least 13x) in exposure medium (i.e. complete cell culture medium without FBS). For the cell viability experiments, isolated primary rat hepatocytes were exposed to a large interval of 3-MMC concentrations [0.3

μM – 10 mM]; all the other assays performed to elucidate the mechanisms responsible for the elicited hepatotoxicity were conducted at four test 3-MMC concentrations: 1 μM, 10 μM, 100 μM and 500 μM. These concentrations were chosen to allow the investigation of the toxicological mechanisms triggered by the drug at different effect levels. None of them was able to induce significant mortality ($p>0.05$), precluding interference with results on the endpoints to be evaluation. In addition, the lowest concentration (10 μM) is in line with the blood concentration found in a fatal intoxication (Adamowicz et al. 2016b). All drug incubations were performed for 24 h, at 37 ºC, in a humidified, 5 % CO_2 atmosphere. Solvent and negative controls were always carried out, parallel to drug incubations. Comparison between solvent (HBSS) and negative controls (cells treated with cell culture medium) showed no statistically significant differences ($p > 0.05$).

3.1.6. Exposure to inhibitors of CYP450 metabolism

Stock solutions of the inhibitors metyrapone (CYP2E1), quinidine (CYP2D6), ketoconazole (CYP3A4) and 1-aminobenzotriazole (ABT; general CYP450) were prepared in dimethyl sulfoxide (DMSO) and further diluted in exposure medium (ensuring that the final concentration of DMSO did not exceed 0.05 %). One hour before exposing the primary rat hepatocytes to 3-MMC, the cells were incubated with 500 μM of metyrapone, 10 μM of quinidine, 1 μM of ketoconazole and 1 mM of ABT.

3.1.7. Cell viability by the MTT reduction assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was performed in a 96-well plate to evaluate the mortality triggered by 3-MMC in primary rat hepatocytes. This assay measures cell viability indirectly through the succinate dehydrogenase activity, an indicator of metabolically active mitochondria. The protocol used was previously described by Dias da Silva et al. (2014b). With the purpose of further analysing the metabolic profile of the drug, after 24 h-incubations of 3-MMC, the culture medium was aspirated and stored frozen at -80 ºC. Then, 100 μL of a fresh solution of 1 mg/mL MTT (prepared in HBSS) was added to the attached cells, which were further incubated for 3 h, at 37 ºC. After this, the MTT solution was aspirated and the formazan crystals, formed after MTT reduction by mitochondrial reductases and retained inside the cells, were dissolved with 100 μL of DMSO. After 15 min shaking, the absorbance was measured in a multi-well plate reader BioTek

Synergy™ HT (BioTek Instruments, Inc.) at 550 nm. As the MTT is a photosensitive dye, the entire procedure was done under light protection. The data, obtained in triplicates of three independent experiments, were normalised to positive (1 % Triton X-100) controls and results were graphically presented as percentage of negative controls.

3.1.8. Cell viability by the LDH leakage assay

Cytoplasmic membrane integrity, evaluated through the lactate dehydrogenase (LDH) leakage assay, also offers a feasible indication of cell viability. As LDH is a cytoplasmic oxidoreductase, its presence in the extracellular medium is indicative of alterations in membrane permeability and consequently in cell integrity. The enzyme catalyses the reversible conversion of pyruvate to lactate, in the presence of nicotinamide adenine dinucleotide (NADH), which is in turn oxidized into NAD^+. At the end of the incubation period, 10 µL of cell exposure medium was transferred into a 96-well plate and added with 200 µL of freshly prepared 0.15 mg/mL β-NADH solution. Immediately before the absorbance reading, 25 µL of 2.5 mg/mL sodium pyruvate solution were added to start the reaction. Both β-NADH and sodium pyruvate solutions were prepared in potassium phosphate buffer (0.05 M KH_2PO_4, pH 7.4). The kinetics of the oxidation of NADH to NAD^+ was followed by reading the absorbance at 340 nm, every 16 seconds, for 3 minutes, using an automatic plate reader Power Wave XTM (BioTek Instruments, Inc.). The data, obtained in six replicates of three independent experiments, were normalised to the negative controls and results were graphically presented as percentage of positive (1 % Triton X-100) controls.

3.1.9. Cell viability by the NR inclusion assay

To provide supplementary cell viability data, we performed the neutral red (NR) uptake assay. This assay is based on the ability of viable cells to incorporate and bind the weak cationic dye NR, which penetrates into the cells by non-ionic diffusion, accumulating in lysosomes by interaction with anionic sites in the lysosomal matrix. At the end of the 24 h-incubations, the exposure medium of the primary hepatocytes seeded onto 96-well plates was replaced by 50 µg/mL NR solution, prepared in HBSS. The cells were incubated at 37 ºC in a humidified, 5 % CO_2 atmosphere for 3 h, allowing the lysosomes of viable cells to take up the dye. Thereafter, the cells were carefully washed twice with 200 µL of HBSS to eliminate extracellular dye, and lysed with 100 µL of lysis solution (50 % ethanol : 1 % glacial acid acetic : 49 % water). The

absorbance was measured at 540 nm in a multi-well plate reader BioTek Synergy™ HT (BioTek Instruments, Inc.). The data, obtained in triplicates of four independent experiments, were normalised to positive (1 % Triton X-100) controls and results were graphically presented as percentage of negative controls.

3.1.10. Measurement of intracellular reactive oxygen (ROS) and nitrogen (RNS) species

The production of ROS and RNS was evaluated as reported by Dias da Silva et al. (2014a). Primary rat hepatocytes seeded onto 96-well plates were incubated with 10 µM 2',7'-dichlorofluorescin diacetate (DCFH-DA), for 30–60 min, at 37 ºC, immediately before the hepatocyte drug incubations. As DCFH-DA is a lipophilic sensitive probe, it readily penetrates the cells producing by hydrolysis 2',7'-dichlorodihydrofluorescein (DCFH), which is further oxidized by intracellular ROS and RNS to the green fluorescent 2',7'-dichlorofluorescein (DCF). A 4 mM DCFH-DA stock solution was prepared in DMSO and stored at -20 ºC in an opaque airtight container. Immediately before each experiment, the working concentration was made up in fresh culture medium (ensuring that the final concentration of DMSO did not exceed 0.05 %). The cells were then rinsed twice with HBSS and incubated, at 37 ºC, with 3-MMC test concentrations. A blank (no cells) and a negative control (no test drugs) were also included in the plate. After a 24h incubation, fluorescence was measured at 485 nm excitation and 530 nm emission, on a fluorescence multi-well plate reader (BioTek Synergy™ HT, BioTek Instruments, Inc.). The data, obtained in three independent experiments run in triplicate, were normalised to the blanks and results were graphically presented as percentage of negative controls.

3.1.11. Sample preparation for measurement of intracellular total glutathione (tGSH), oxidised glutathione (GSSG) and adenosine triphosphate (ATP)

After drug exposures, primary rat hepatocytes seeded onto 6-well plates were rinsed with HBSS and added of 300 µL 5 % $HClO_4$ (w/v) per well. After 20 min at 4 ºC, the cells were scrapped and the obtained suspension was centrifuged at 16,000 g for 10 min at 4 ºC. The supernatants were collected and kept at -80 ºC until determination

of total glutathione (tGSH), oxidised glutathione (GSSG) and adenosine triphosphate (ATP). The obtained pellet was suspended in 1 M NaOH and used for protein quantification, through the Lowry assay.

3.1.12. Measurement of intracellular total glutathione (tGSH) and oxidized glutathione (GSSG)

Determination of tGSH and GSSG was made through the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)-GSSG reductase-recycling assay, as previously described (Dias da Silva et al. 2014a). The recycling assay for tGSH is a sensitive and specific enzymatic procedure. Reduced glutathione (redGSH) is oxidized by DTNB to give GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB). GSSG is reduced to redGSH by the action of the highly specific glutathione reductase (GR) and NADPH. Briefly, for tGSH determination, the supernatants of samples, blank, and standards were neutralized with 300 µL of 0.76 M $KHCO_3$ (until there was no formation of CO_2 following vortex mixing). The samples were then centrifuged for 10 min at 16,000 g (4 °C), and 100 µL of each supernatant were added to a 96-well plate, followed by 65 µL of a freshly prepared reagent solution. The reagent solution consists of 0.69 mM β-Nicotinamide adenine dinucleotide 2′-phosphate reduced (β-NADPH) and 4 mM DTNB, prepared in water. Samples were incubated for 15 min at 30 °C. Then, 40 µL of 10 U/mL GR solution freshly prepared in phosphate buffer (71.5 mM NaH_2PO_4, 71.5 mM Na_2HPO_4, 0.63 mM EDTA, pH 7.5) were added to each well. The formation of TNB was followed for 3 min, every 10 seconds, at 405 nm, using a multi-well plate reader (Power Wave XTM, BioTek Instruments, Inc.).To determine intracellular GSSG, 2-vinylpyridine was used to block reduced glutathione (redGSH). Accordingly, previous to sample neutralization with $KHCO_3$, 10 µL of 2-vinylpyridine were added per each 200 µL of supernatant (samples, blank, and standards) and mixed for 1h, on ice. Then, GSSG was determined as described for tGSH.

The amount of redGSH was calculated by subtracting GSSG from the tGSH, i.e. redGSH = tGSH–(2xGSSG). The GSSG and redGSH standard solutions were prepared in 5 % $HClO_4$; the concentrations ranged between 0.25–8 µM for GSSG and between 0.25–15 µM for GSH. Results from three independent experiments were normalised to the amount of protein of the sample and presented as nmol per mg of protein.

3.1.13. Assessment of mitochondrial membrane potential (ΔΨM)

The mitochondrial integrity was determined through the evaluation of tetramethylrhodamine ethyl ester (TMRE) inclusion, as described by Dias da Silva et al. (2014a). As TMRE is a cell permeable fluorescent dye positively charged, it labels active mitochondria by accumulation, a consequence of their relative negative charge. A 2 mM TMRE stock solution was initially prepared in DMSO and stored at -20 °C, protected from light. Immediately before each experiment, the working concentration was made up in fresh culture medium (ensuring that the final concentration of DMSO did not exceed 0.05 %). After the 3-MMC incubation period, the exposure medium was replaced by fresh medium containing 2 µM TMRE, and primary hepatocytes seeded onto 96-well plates were further incubated at 37°C, for 30 min, in the dark. The cells were washed twice with HBSS and the fluorescence was quantified on a fluorescence multi-well plate reader (BioTek Synergy™ HT, BioTek Instruments, Inc.) set to 544 nm excitation and 590 nm emission. Since TMRE is photosensitive, all steps were performed under light protection. The data, obtained in triplicates of three independent experiments, were normalised to the blanks and results were graphically presented as percentage of negative controls.

3.1.14. Measurement of intracellular adenosine triphosphate (ATP)

Determination of ATP was made through a bioluminescence assay that measures the light formed in the oxidation of luciferin, catalysed by luciferase in the presence of ATP, as described by Dias da Silva et al. (2014b). After neutralization of the thawed acidic supernatants with an equal volume of 0.76M $KHCO_3$, the samples were centrifuged for 10 min at 16,000 g, at 4 °C. Then, 75 µL of the neutralized supernatants, standards, or blank (5 % $HClO_4$, w/v) were transferred in triplicate to 96-well white plates and then added of 75 µL of the luciferin/luciferase solution. Luciferin/luciferase solution consisted of 0.15 mM luciferin, 300,000 light units of luciferase from *Photinus pyralis* (American firefly), 50 mM glycine, 10 mM $MgSO_4$, 1 mM Tris, 0.55 mM EDTA, and 1 % BSA (pH 7.6). Using a luminescence multi-well plate reader (BioTek Synergy™ HT, BioTek Instruments, Inc.), the emitted light intensity of the samples was evaluated and compared with an ATP standard curve (31.25–1000 µM) performed within each experiment. Data from three independent experiments were normalized to the protein content, and results were expressed as nmol per mg of protein.

3.1.15. Determination of protein

The protein content in the samples for normalisation of ATP and GSH/GSSH assays was measured through the method of Lowry et al. (1951). Accordingly, 50 µL of each sample, standard, or blank were transferred into a 96-well plate, in triplicate, and added of 100 µL reagent A (14.7 mL of 2 % Na_2CO_3, 0.15 mL of 2 % $KNaC_4H_4O_6.4H_2O$, and 0.15 mL of 1 % $CuSO_4.5H_2O$, extemporaneously prepared). The plate was incubated in the dark for 10 min, at room temperature, followed by the addition of 100 µL of reagent B (Folin and Ciocalteu's phenol reagent, diluted 15x in purified water). The plate was incubated for 10 min, under light protection, at room temperature, and the absorbance was measured at 750 nm in a 96-well microplate reader (Power Wave XTM, BioTek Instruments, Inc.). Protein standards (31.25–500 µg/mL) were prepared with BSA in 1 M NaOH.

3.1.16. Determination of caspase-3, caspase-8 and caspase-9

After 24 h-incubations of hepatocytes seeded onto 6-well plates with 3-MMC at 37 °C, the cell treatment medium was discarded (two wells were used per each treatment condition) and added of 75 µL of lysis buffer (50 mM HEPES, 0.1 mM EDTA, 0.1 % CHAPS, 1 mM DTT, pH 7.4). Following incubation at 4 ºC, for 30 min, cells were scrapped and collected into 2 mL centrifuge tubes. After homogenization, samples were centrifuged at 16,000 g for 10 min, at 4 °C. Fifty µL of the supernatant (cytoplasmic fraction) were transferred into wells of a 96-well plate and then added of 200 µL of assay buffer (100 mM NaCl, 50 mM HEPES, 1 mM EDTA, 0.1 % CHAPS, 10 % glycerol, 10 mM DTT, pH 7.4). To start the reaction, 5 µL of caspase-3 (Ac-DEVD-pNA; 4mM in DMSO), caspase-8 (Ac-IETD-pNA; 10mM in DMSO), or caspase-9 (Ac-LEHD-pNA; 10mM in DMSO) peptide substrate were added and incubated at 37 °C for 24 h, protected from light. The caspase-induced release of the p-nitroanilide moiety of the substrate was assessed by measuring absorbance at 405 nm, in a multi-well plate reader (BioTek Synergy™ HT, BioTek Instruments, Inc.), as previously described (Dias-da-Silva et al. 2015). The absorbance of blanks (non-enzymatic control) was subtracted from each value of absorbance and the data normalized to the amount of protein of each sample. The protein content in the cytoplasmic fraction was quantified using the Bio-Rad DC protein assay kit, as described by the manufacturer, and BSA solutions were used as standards (31.25 µg/mL – 1 mg/mL). Results from three

independent experiments were normalised to the amount of protein in each sample and expressed as percentage of negative controls.

3.1.17. Statistical analysis

The normalized MTT, LDH, and NR assays data were fitted to the dosimetric Logit model, which was chosen based on a statistical goodness-of-fit principle: $y = \boldsymbol{\theta_{max}}/\{1 + \exp[-\boldsymbol{\theta_1} - \boldsymbol{\theta_2} * \log(\boldsymbol{x})]\}$, where $\boldsymbol{\theta_{max}}$ is the maximal observed effects, $\boldsymbol{\theta_1}$ is the parameter for location, $\boldsymbol{\theta_2}$ is the slope parameter and $\boldsymbol{x}$ is the concentration of test drug. To compare concentration-response curves, overlapping of the 95 % confidence intervals was used and, additionally, the extra sum-of-squares F test. The Lowest Observed Effect Concentrations (LOECs) and No Observed Effect Concentrations (NOECs) were calculated by testing a trend in concentration effects against control by applying hypothesis-testing procedures (unpaired t tests) (Silva et al. 2007), which allowed the estimation of LOECs. Consequently, the next lower tested concentrations could be designated as NOEC values. Results from determination of ROS/RNS, GSH/GSSG, Δψm, caspase -3, -8, -9 activities, and ATP were presented as mean ± standard error of the mean (SEM) from three independent experiments. Normality of the data distribution was assessed by the Kolmogorov–Smirnov, D'Agostino & Pearson and Shapiro–Wilk normality tests. Statistical comparisons between groups were performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Solvent and negative control values were compared by the Student's unpaired t test. In all cases, significance was accepted at p values < 0.05. All statistical calculations were performed using GraphPad Prism software, version 7.0 (GraphPad Software, San Diego, CA, USA).

3.2. Metabolic profile of 3-MMC by Gas-Chromatography–Mass Spectrometry (GC–MS)

3.2.1. Chemicals

As mentioned before, the hydrochloride salt of 3-MMC was purchased online from the Sensearomatic website (http://sensearomatic.net, currently unavailable), in March, 2016. Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich (Lisbon, Portugal) and all the cell culture reagents from Gibco® (Alfagene, Lisbon, Portugal). Cell culture samples were prepared and used as mentioned in sections 1.4–1.7. Methanol (MeOH) and acetonitrile, both HPLC grade,

were purchased from Honeywell (Muskegon, USA). Dichloromethane (LC-MS grade), ammonium hydroxide (NH_4OH, 32 %), potassium dihydrogen phosphate (KH_2PO_4, analytical grade) and sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$, analytical grade) were obtained from Merck Co (Darmstadt, Germany). Hydrochloric acid (HCl) (37 %) was obtained from Fisher Scientific (UK). Sodium hydroxide (NaOH) (analytical grade), sodium chloride (NaCl) (analytical grade), HCl (analytical grade), 2-propanol (analytical grade) and *n*-hexane (HPLC grade) were purchased from VWR international (Spain). All reagents used were of analytical grade. Solid-phase extraction (SPE) was performed using Oasis MCX® SPE 1 cm^3 (30 mg) columns obtained from Waters (Milford, Massachusetts) and the Supelco Visiprep™ SPE Vacuum Manifold from Sigma-Aldrich (St. Louis, Missouri).

3.2.2. Standard solutions and calibrators

Stock solutions of 3-MMC and internal standards (IS), 4-hydroxy-3-methoxybenzylamine hydrochloride (IS_1) and phenylpropanolamine (IS_2), were prepared at 1 mg/mL in MeOH and stored at -20ºC. All intermediate dilutions and working solutions were also prepared in MeOH.

Eight methods of extraction were tested. For the extraction methods 1, 3–8 (Table 7), working calibrators for cell culture medium at 125, 175, 250, 500 and 1000 ng/mL were prepared by adding 100 µL of each 3-MMC standard to 900 µL of exposure medium (see composition in section 1.5). For method 2 (Table 7), working calibrators were prepared by diluting a 500 ng/mL 3-MMC stock solution in the volume of exposure medium necessary to made up 500 µL; then 1.5 mL of 0.1 M phosphate buffer (pH 4.4) was added.

3.2.3. Biological specimens

As mentioned above, the culture medium from primary rat hepatocyte exposures to 3-MMC was aspirated before performing the MTT assay. These samples were stored frozen at -80 ºC until further use for GC-MS analysis.

3.2.4. Extraction

Several methods of extraction were tested for further selection of the method presenting lower limit of detection, better linearity and extraction efficiency. The extraction methods tested are described in Table 7.

After extraction, the sample was evaporated to dryness under nitrogen (N_2) stream, and then added with 50 µL of 500 ng/mL IS_1 or IS_2 and evaporated again.

Table 7 Methods of extraction tested.

Extraction method	Type of extraction	Protocol steps
1	SPE	1. Load the sample 2. Wash with 2 mL of HCl 0.1 M and 2 mL of MeOH 3. Elute with 2 mL of 5 % NH_4OH/MeOH solution
2	SPE	1. Condition with 2 mL of MeOH and 2 mL of H_2O (deionized) 2. Load the sample 3. Wash with 2 mL of H_2O (deionized), 2 mL of HCl 0.1 M, 2 mL of dichloromethane:methanol (70:30 v/v)1 and 2 mL n-hexane 4. Elute with 2 mL dichloromethane:2-propanol:NH_4OH (78:20:2 v/v/v)
3	Liquid-liquid	1. Adjust sample pH above 11 with 1 M NaOH 2. Add 1 mL of TBME 3. Vortex for 30 s, then centrifuge at 5600 g for 5 min 4. Transfer supernatant into a 4 mL vial 5. Repeat steps 2–4 twice
4	Liquid–liquid followed by SPE	1. After extracting with method 3, evaporate samples till dryness 2. Dissolve the residue in 3 mL of 0.2 M sodium acetate buffer (pH 5.2) 3. Load columns with 3 mL of 0.2 M sodium acetate buffer (pH 5.2) 4. Extraction with method 1
5	QuEChERS	1. Add 5 mL of acetonitrile to 1 mL of sample 2. Add 2 g of $MgSO_4$ anhydrous and 0.5 g of NaCl 3. Vortex for 30 s. Then, centrifuge at 5600 g for 3 min 4. Collect supernatant into a 4 mL vial
6	QuEChERS	1. Add 5 mL of MeOH to 1 mL of sample 2. Add 2 g of $MgSO_4$ anhydrous and 0.5 g of NaCl 3. Vortex for 30 s then centrifuge at 5600 g for 3 min 4. Transfer supernatant into a 4 mL vial
7	QuEChERS	1. Adjust the sample pH to 5 or11 2. Add 2 mL of ultra-pure H_2O and 5 mL of acetonitrile 3. Add 2 g of $MgSO_4$ anhydrous and 0.5 g of NaCl 4. Vortex for 30 s. Then, centrifuge at 5600 g for 3 min 5. Transfer supernatant into a 4 mL vial
8	QuEChERS	1. Adjust the sample pH to 5 or 11 2. Add 3 mL of TBME 3. Add 750 mg of $MgSO_4$ anhydrous and 100 mg of NaCl 4. Vortex for 30 s. Then centrifuge at 5600 g for 3 min 5. Transfer supernatant into a 4 mL vial

SPE – Solid-phase extraction; HCl - Hydrochloric acid; MeOH – Methanol; NH_4OH – Ammonia solution; H_2O – Water; NaOH – Sodium hydroxide; TBME - *Tert*-butylmethylether; QuEChERS - Quick, Easy, Cheap, Effective, Rugged, and Safe; $MgSO_4$ – Magnesium sulfate; NaCl – Sodium chloride

The method 2 was adapted from Margalho et al. (2016); the methods 1, 3, and 4 were adapted from Moreira (2015); the method 5 adapted from Almada et al. (2016).

3.1.1. Derivatization

Three methods of derivatization were tested, as described in Table 8. The N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) derivatization procedure used was previously described by Magalhães (2015). The trifluoroacetic anhydride (TFAA) derivatization procedure used was previously described by da Silva et al. (2010). The heptafluorobutyric anhydride (HFBA) derivatization procedure was adapted from Jamey et al. (2016).

For the methods of extraction 5, 7 and 8, an additional step for the removal of the derivatization reagent HFBA was made, as previously described by Petrarca et al. (2017). Briefly, before dissolving the residue in ethyl acetate, it was dissolved in 1 mL of phosphate buffer solution (pH 7.4) and 3 mL of dichloromethane. After agitating by vortex, the dichloromethane phase (bottom layer) was collected, dehydrated with 100 mg of $MgSO_4$ anhydrous and evaporated to dryness under a stream of N_2. Finally, the residue was dissolved in 100 µL of ethyl acetate, and 1 µL was submitted to GC-MS analysis.

Table 8 Derivatization procedures tested.

Derivatization reagent	Derivatization procedure
MSTFA	1. Add 60 µL of MSTFA to the dry residue 2. Vortex for 30 s 3. Heat at 80 ºC for 30 min 4. Cool to room temperature
TFAA	1. Add 50 µL of ethyl acetate and 50 µL of TFAA to the dry residue 2. Vortex for 30 s 3. Heat at 70 ºC for 30 min 4. Cool to room temperature and evaporate till dryness under N_2 stream 5. Dissolve the residue in 100 µL of ethyl acetate
HFBA	1. Add 50 µL of ethyl acetate and 50 µL of HFBA to the dry residue 2. Vortex for 30 s 3. Heat at 60 ºC for 30 min 4. Cool to room temperature and evaporate till dryness under N_2 stream 5. Dissolve the residue in 100 µL of ethyl acetate

MSTFA – N-methyl-n-(trimethylsilyl)trifluoroacetamide; TFAA – Trifluoroacetic anhydride; HFBA – Heptafluorobutyric acid

3.1.2. Interferences

In order to evaluate the presence of chromatographic interferences for all tested methods, blank medium samples were prepared in parallel and analysed at the retention times of 3-MMC and IS. The chromatograms of spiked samples were then compared with the chromatograms obtained with the blank medium samples.

3.1.3. Chromatographic conditions and equipment

The GC-MS analysis was carried out using a HP 6890 series gas chromatograph (Agilent, Little Falls, DE, USA) equipped with Agilent 5973 Network MSD (Mass selective detector). A capillary column Rxi-5sil (30 m x 0.25 mm x 0.25 µm) from RESTEK was employed for the separation. The software used in the control, acquisition and data treatment was Agilent MSD Productivity ChemStation for GC and GC/SD Chemstation from Agilent Technologies (Santa Clara, CA, USA). Helium C-60 (Gasin, Portugal), at a constant flow rate of 1 mL/min, was used as the carrier gas. The injection of 1 µL of derivatized extracts was manually done in the splitless mode at 270 ºC. The column oven temperature was maintained at -80 ºC for 1 min, and then raised to 290 ºC at 25 ºC/min, and held at 290 ºC for 5.6 min, with a total run of 15 min. This method was adapted from Adamowicz et al. (2014) and from Zuba and Adamowicz (2016). The transfer line temperature was 280 ºC, and the electron energy was 70 eV. During the first 4 min, the ionization was maintained off to avoid solvent overloading. Data were collected at m/z 40-500. Mass spectra acquisition was performed between 4 and 15 min after the injection of the sample. Full scan mode allowed the detection of all ions and was used for the characterization of the spectra and identification of compounds; selected ion monitoring (SIM) mode allowed quantification of compounds. The ions selected for monitoring were chosen based on the specificity and abundance on the mass spectrum.

The identification of each analyte in the samples was made by comparing the retention times and the mass spectra (relative abundance of the ions) of the standards with those of the peaks obtained when injecting the samples under the same chromatographic conditions (see Tables 11 and 12 of section 4.2.3. of Chapter 4 – Results and discussion).

3.1.4. Quantitative determination of 3-methylmethcathinone in samples

3.1.4.1. Limit of detection (LOD) and limit of quantification (LOQ)

In this study, the limit of detection (LOD) and limit of quantitation (LOQ) were determined based on the signal-to-noise ratio. A signal-to-noise ratio of 3 and 10 is considered acceptable for estimating the LOD and LOQ, respectively. The 125 ng/mL working calibrator was progressively diluted (factor of 2) with cell culture medium to determine those limits.

3.1.4.2. Linearity studies

Working calibrators at 125, 175, 250, 500 and 1000 ng/mL were prepared and stored at -20 ºC. Regression curves were obtained by plotting the peak-area ratio between each analyte and the IS against analyte concentrations. Linearity was evaluated by the coefficient of determination (R^2). At least three independent curves were obtained.

3.1.4.3. Extraction efficiency

The extraction efficiency was tested at three different concentrations of 3-MMC (12.5, 25 and 50 ng/mL), performing four replicates for each concentration. One set of samples was spiked with the analytes to the elution/supernatant of the samples before evaporation to dryness (non-extracted samples). To the other set of samples, the analytes were added before extraction (extracted samples). In both set of samples the IS (IS_1 or IS_2) was added after evaporation to dryness followed by evaporation to dryness again. Extraction recovery (%) was calculated comparing the peak area ratios of analyte to IS for extracted and non-extracted samples [Extraction recovery (%) = mean peak area ratio of extracted samples / mean peak area ratio of non-extracted samples) x 100].

Chapter 4

Results and discussion

4.1. Cytotoxic profile of 3-MMC

4.1.1. Lysosome was the most sensitive organelle to 3-methylmethcathinone (3-MMC)–induced toxicity, followed by mitochondria and by the cytoplasmic membrane

Cellular viability was evaluated by three different assays (LDH, NR, and MTT assay) after the exposure of primary rat hepatocytes to a range of 3-MMC concentrations that enabled obtaining complete cytotoxic profiles (concentration-dependent responses from 0 % to 100 % of mortality). The comparison of the obtained results provided information about the relative sensitivity of the target organelles, when the cell was exposed to 3-MMC. As shown in Fig. 3, 3-MMC-induced toxicity was perceived in the lysosome at lower concentrations (NR LOEC 379.5 µM) than those needed to cause damage to mitochondria (MTT LOEC 531.2 µM) and cytoplasmic membrane (LDH LOEC 1.32 mM). Accordingly, also the potency of 3-MMC for provoking cell death was distinct among the different assays (Table 10; NR EC_{50}) 1.36 mM; MTT EC_{50} 1.68 mM; LDH EC_{50} 3.13 mM).

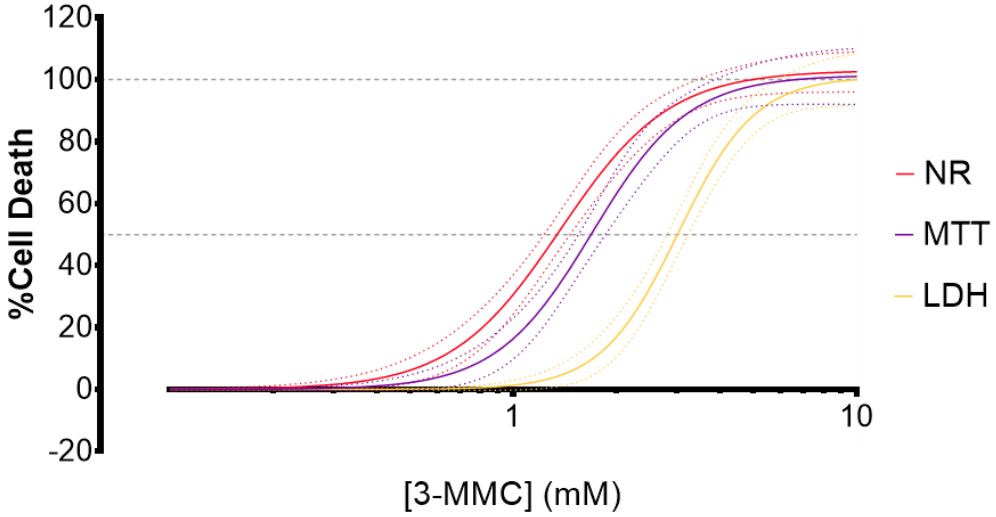


Fig. 3 Cell death after 24 h-exposures at 37 ºC of primary rat hepatocytes to 3-methylmethcathinone (3-MMC), obtained in the lactate dehydrogenase leakage assay (LDH, data displayed in yellow), neutral red uptake assay (NR, data displayed in red), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium reduction assay (MTT, data displayed in purple), as indirect measures of cell viability. Data are presented as percentage of cell death relative to the negative controls and are from at least three independent experiments performed in triplicate. Curves were fitted to the dosimetric Logit model (parameters displayed in Table 10). The dotted lines are the upper and lower limits of the 95 % confidence interval of the best estimate of mean responses. Differences between the best fits were deemed statistically significant, as their corresponding 95 % confidence belts did not overlap. The dashed grey lines represent 50 % and 100 % effect.

By comparison with data available in the literature, 3-MMC is generally less damaging to the mitochondria than most cathinones and other substituted amphetamines, as shown in Table 9. The exceptions detected were 4-fluoromethcathinone (4-FMC or flephedrone; EC_{50} 2.21 mM) and 6-(2-aminopropyl)benzofuran (6-APB; EC_{50} 1.94 mM) that revealed slightly lower cytotoxicities in primary rat hepatocytes (Bravo 2016). In spite of the apparent closely related chemical structure, the toxicities of 3,4-dimethylmethcathinone (3,4-DMMC; EC_{50} 160 µM) and 4-MEC (EC_{50} 840 µM) were significantly much higher than that for 3-MMC (EC_{50} 1.68 mM) (Bravo 2016; Valente et al. 2016). This is in accordance with reports of users that refer the effect of 3-MMC as less potent and intense than 4-MMC (Adamowicz et al. 2016a; Sande 2016).

Also, the damage caused by 3-MMC to the cytoplasmic membrane was lower than that provoked by methylenedioxypyrovalerone (MDPV; EC_{50} 2.06 mM) and MDMA (EC_{50} 1.99 mM) but slightly higher than that for methylone (EC_{50} 3.20 mM). Nevertheless, these differences might be justified by the fact of using of different cell models (MDPV, MDMA and methylone were tested in dopaminergic SH-SY5Y neuron-like cells). In regard of the damage to the lysosome, no data was found in the literature for comparison.

Table 9 Estimated EC_{50} values (mM) for each compound in three different cytotoxic assays: lactate dehydrogenase leakage assay (LDH), neutral red uptake assay (NR), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium reduction assay (MTT). The MTT EC_{50} values are estimated in primary rat hepatocytes and the LDH EC_{50} values are estimated in dopaminergic SH-SY5Y neuron-like cells. Adapted from Bravo (2016), Valente et al. (2016) and Valente et al. (2017b).

Drug Class	Drug	MTT	LDH	NR
Substituted Cathinone	Butylone	1.21		
	Buphedrone	1.57		
	3,4-DMMC	0.16		
	4-FMC	2.21		
	MDPV	0.76	2.06	
	Pentedrone	0.66		
	4-MEC	0.84		
	Methylone	1.26	3.20	
Other amphetamine-related drugs	6-APB	1.94		
	5-APB	0.96		
	MDMA	1.07	1.99	

3-MMC – 3-methylmethcathinone; 3,4-DMMC – 3,4-dimethylmethcathinone; 4-FMC – 4-fluoromethcathinone; MDPV – methylenedioxypyrovalerone; 4-MEC – 4-methylethcathinone; 6-APB – 6-(2-aminopropyl)benzofuran; 5-APB – 5-(2-aminopropyl)benzofuran; MDMA – 3,4-Methylenedioxymethamphetamine

Results for 3-MMC obtained in the current study were 1.68 mM in the MTT assay, 3.13 mM in the LDH assay and 1.36 mM in the NR assay, after 24 h of exposure in primary rat hepatocytes.

4.1.2. Cytochrome P450 (CYP450) metabolism impacts hepatotoxicity elicited by 3-methylmethcathinone (3-MMC)

To estimate the role of metabolism through the main CYP450 enzymes in the hepatotoxicity caused by 3-MMC, primary rat hepatocytes were treated with specific inhibitors of its different isoforms, namely CYP2D6, CYP2E1 and CYP3A4; a general inhibitor of CYP450 was additionally used. Results are represented in Fig. 4 and Table 10.

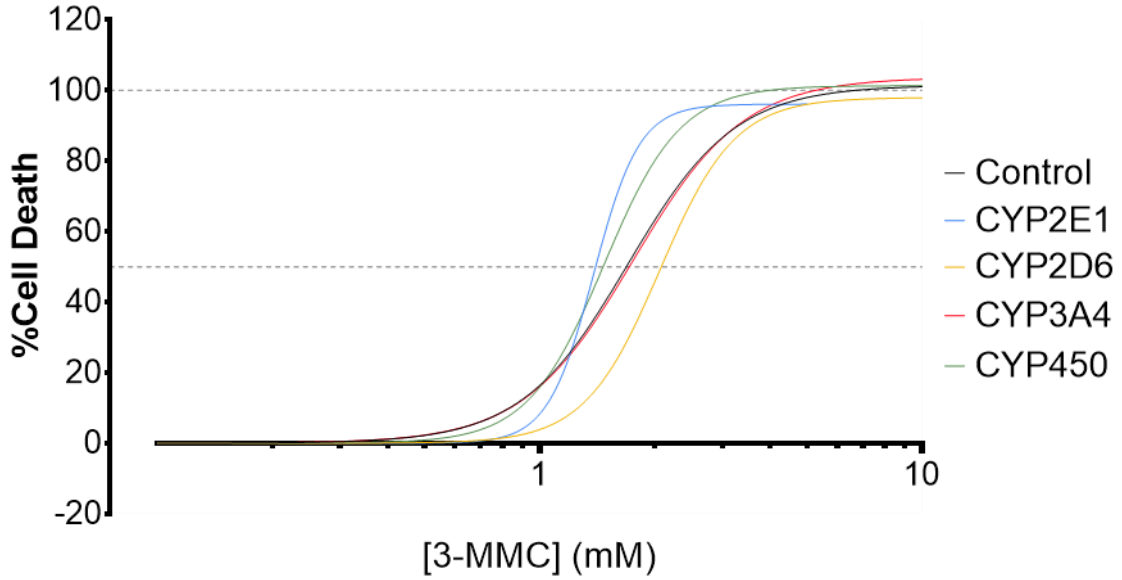


Fig. 4 Cell death obtained in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay, as an indirect measure of cell viability, after 24 h-exposures at 37 ºC of primary rat hepatocytes to 3-methylmethcathinone (3-MMC), in the presence (coloured solid lines) or absence (black solid line) of different inhibitors of cytochrome P450 isoforms (CYP). Data are presented as percentage of cell death relative to the negative controls and are from three independent experiments performed in triplicate. Curves were fitted to the dosimetric Logit model (parameters displayed in Table 10). The dashed grey lines represent 50 % and 100 % effect. Metyrapone was used as specific inhibitor of the isoform CYP2E1 (purple solid line); quinidine was used as specific inhibitor of the isoform CYP2D6 (green solid line); ketoconazole was used as specific inhibitor of the isoform CYP3A4 (orange solid line); and 1-aminobenzotriazole (ABT) was used as general inhibitor of cytochrome P450 (blue solid line).

Each inhibitor concentration was selected in order to induce inhibition without eliciting cytotoxicity, as already optimized for our experimental settings (cellular model and viability assay) by Dias-da-Silva et al. (2015). When treated with metyrapone (CYP2E1 inhibitor), the concentration-response curve (EC_{50} 1.40 mM) was shifted to the right, at the lower 3-MMC concentration range, indicating lower toxicity, compared with the control. This suggests that metabolism of 3-MMC through CYP2E1 is hepatotoxic at concentrations up to ~ 1.20 mM, which could indicate either a bioactivation of 3-MMC through this enzyme or the drug entering in an alternative metabolic pathway that results in higher toxicity. For the latter case, we further hypothesised the saturation of the enzymes involved in this substitute route, since an

inversion of the cytotoxic profile was observed (the inhibition of CYP2E1 increased cell death) at concentrations higher than ~ 1.20 mM.

Table 10 Estimated parameters for the Logit model (best-fit regression) of 3-methylmethcathinone (3-MMC) in the different viability assays, after 24 h-incubation at 37 ºC of primary rat hepatocytes, in the presence or absence of different isoforms of cytochrome P450 (CYP) inhibitors.

Assay	CYP inhibitor	Parameters for the Logit regression model			EC_{50} (mM)	NOEC (mM)	LOEC (mM)	*p* value
		θ_1[a]	θ_2[b]	θ_{max}[c]				
MTT	No inhibitor	-1.65	7.14	101.40	1.68	0.38	0.53	-
	CYP 3A4	-1.69	6.93	103.70	1.71	0.53	0.64	p = 0.9758 (vs. no inhibitor)
	CYP 2E1	-2.36	16.70	96.08	1.40	0.74	1.04	p = 0.0031 (vs. no inhibitor)
	CYP 2D6	-3.19	10.15	97.96	2.08	1.04	1.25	p = 0.0005 (vs. no inhibitor)
	CYP 450	-1.68	10.00	101.30	1.46	0.74	1.04	p = 0.0288 (vs. no inhibitor)
LDH	No inhibitor	-4.44	8.96	100.20	3.13	1.04	1.31	p < 0.0001 (vs. MTT no inhibitor)
NR	No inhibitor	-0.93	6.62	102.30	1.36	0.31	0.38	p = 0.0061 (vs. MTT no inhibitor)

Metyrapone at 500 µM was used as specific inhibitor of the isoform CYP2E1; quinidine at 10 µM was used as specific inhibitor of the isoform CYP2D6; ketoconazole at 1 µM was used as specific inhibitor of the isoform CYP3A4; and 1-aminobenzotriazole (ABT) at 1 mM was used as general inhibitor of CYP450. LDH, lactate dehydrogenase leakage. NR, neutral red uptake. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium reduction. NOEC, no observed effect concentration. EC_{50}, concentration of 3-MMC that gives half-maximal response, i.e. 50 % effect, in the respective assay. [a]Location parameter. [b]Slope parameter. [c]Maximal effect, expressed as % cell death (data scaled between 0 and 100 % cell death, corresponding to negative controls and positive controls, respectively). Comparisons between curves (θ_{max}, θ_1 and θ_2) were performed using the extra sum-of-squares F-test. p values lower than 0.05 were considered statistically significant.

On the other hand, when CYP2D6 was inhibited by quinidine (EC_{50} 2.08 mM), higher 3-MMC concentrations were needed to induce similar levels of cell death, compared to the control (EC_{50} 1.68 mM). This suggests that CYP2D6 might be responsible for the bioactivation of the drug, as this also occurs for other amphetamine derivatives. Accordingly, it is known that MDMA undergoes CYP2D6-mediated bioactivation with formation of catechols, which are highly reactive molecules that enter in redox cycles, Moreover, *in vitro* studies suggest that 4-MMC is mainly metabolized through CYP2D6 and therefore co-administration with other substances might alter

toxicity of the drug (Pedersen et al. 2013). Since the drug is an isomer of 4-MMC, one could speculate that the metabolization of both cathinones is similar. Although our findings contradict this assumption and more likely support bioactivation of 3-MMC through this CYP450 isoform.

Inhibition of CYP3A4 with ketoconazole produced results undistinguishable from the control. This isoform has a major role in the detoxification of several drugs (Zanger and Schwab 2013) and its function is highly redundant. Accordingly, when CYP3A4 is inhibited, for example by competition with other substances, the drugs such as 3-MMC undergo biotransformation through other metabolic pathways. Overall, it seems that 3-MMC metabolism through CYP450 is detoxifying at biologically relevant concentrations, as CYP450 general inhibition with ABT only increased cell death from ~ 1.03 mM on (EC_{50} 1.71 mM).

To the best of our knowledge, there is no further information regarding 3-MMC metabolism.

4.1.3. 3-Methylmethcathinone (3-MMC) increases intracellular reactive oxygen (ROS) and nitrogen (RNS) species

An increase in the production of reactive species (ROS and RNS) can be due to several physiological processes that occur in the cell, the most preponderant of them consists on the activity of the mitochondrial electron transport chain. Since the cell has defence mechanisms against production of reactive species, oxidative stress will only occur when there is an imbalance between the prooxidants and the antioxidants (in favour of the oxidants). Accordingly, a rise in the ROS/RNS burden causes the decrease of antioxidant protection and will ultimately result in failure to repair oxidative damage. As represented in Fig. 5, ROS and RNS production following exposure to 3-MMC increased with the increasing concentrations of the drug. Although at 1 µM, 3-MMC already induced an increase in ROS/RNS levels by 25 %, this augment only presented statistical significance ($p < 0.05$) when 3-MMC was present at higher concentrations (≥ 10 µM).

The formation of ROS and RNS is one of the mechanisms responsible for the toxicity of amphetamines, in primary rat hepatocytes (Dias da Silva et al. 2014a) and it was also reported for other cathinones, but at higher concentrations (Valente et al. 2016). Valente et al. (2016) showed that 4-MEC induced a significant increase in this stress parameter from 400 µM on. Similar levels of disturbance in production ROS and

RNS also occurred for 3,4-DMMC, 4-FMC and MDMA at concentrations as higher as 142 µM, 1.7 mM, and 1 mM, respectively (Bravo 2016; Dias da Silva et al. 2014a).

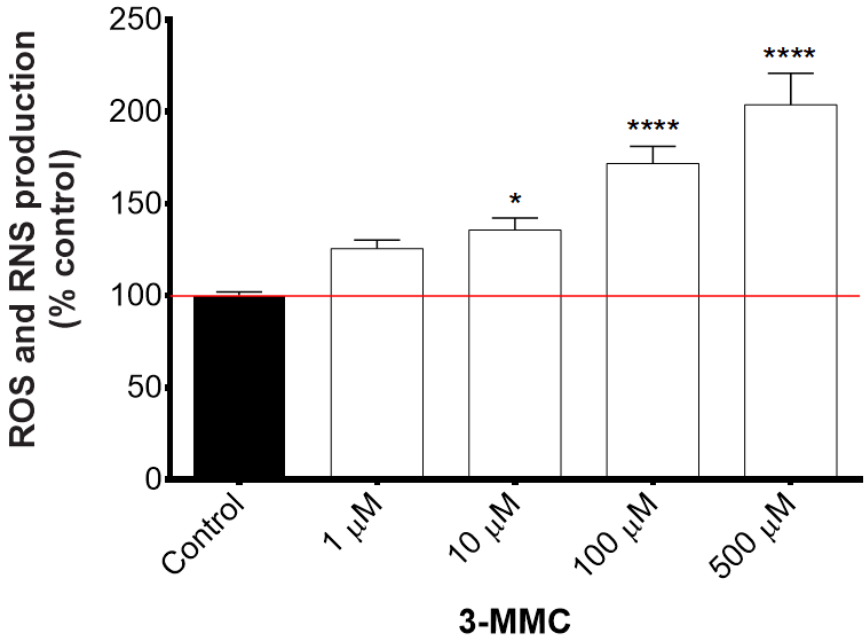


Fig. 5 Production of reactive species of oxygen (ROS) and nitrogen (RNS), measured through the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) assay, in rat primary hepatocytes after 24-h incubation with 3-methylmethcathinone (3-MMC), at 37 °C. Results are expressed as percentage control ± standard error of the mean (SEM) from three independent experiments, run in triplicate. Statistical comparisons were made using one-way ANOVA/Dunnett's post hoc test. *p < 0.05; ****p < 0.0001, versus control.

4.1.4. Intracellular levels of reduced (redGSH) and oxidized glutathione (GSSG) are affected by 3-methylmethcathinone (3-MMC)

Glutathione is an important antioxidant that prevents cell damage induced by ROS/RNS. Therefore, the evaluation of redGSH and GSSG levels in primary rat hepatocytes exposed to 3-MMC was used to provide complementary information on the redox cell status.

As depicted in Fig. 6, the increase in the ROS and RNS production is consistent with the redGSH depletion observed herein. Accordingly, the results from our study indicate that a significant decline in redGSH was observed for all concentrations of 3-MMC tested ($p < 0.01$, ANOVA/Dunnett's), with the exception of 10 µM. This might mean that, at this concentration, the cell is trying to respond to the insult triggered by the drug by activating pro-survival mechanisms. At the two highest concentrations (100 µM and 500 µM), however, the capacity of cell response was exceeded and a decline

in the redGSH, which is consistent with the reduction of the redGSH/GSSG ratio and the increase in the production on ROS and RNS, was observed.

Despite a slight 3-MMC-induced increase in the intracellular levels of GSSG, compared to control incubations (4.59 ± 0.26 nmol GSSG/mg protein), there were no significant alterations on this parameter, except when the drug was tested at the highest concentration. Accordingly, when 3-MMC was incubated at 500 µM, it induced an increase in GSSG to 8.24 ± 1.93 nmol GSSG/mg protein (Fig. 6, $p < 0.05$, ANOVA/Dunnett's). Cells exposed to high levels of oxidative stress, accumulate GSSG with reduction of the redGSH/GSSG ratio, an evidence of oxidative stress. Accordingly, our data also show a significant decrease of redGSH/GSSG ratio after all cell treatments (Fig. 6, $p < 0.0001$, ANOVA/Dunnett's).

Some compounds, such as MDMA, methylone and MDPV, can suffer extensive hepatic metabolism into catechols and be further oxidized to very unstable orthoquinones (Pedersen et al. 2013; Strano-Rossi et al. 2010; Valente et al. 2017b). These molecules can enter into redox chain reactions, inducing massive formation of ROS. When glutathione is conjugated with these orthoquinones, it produces glutathione-S-yl-adducts, which are still redox active and thus can be readily oxidized and further reduced by a second molecule of glutathione, resulting in 2,5-bis-(glutathione-S-yl)-conjugates. Formation of such conjugates might justify the discrepancies observed regarding the decrease of redGSH which was not compensated by the GSSG increase. The cell extrusion of the GSSG formed is also a possible explanation for this observation.

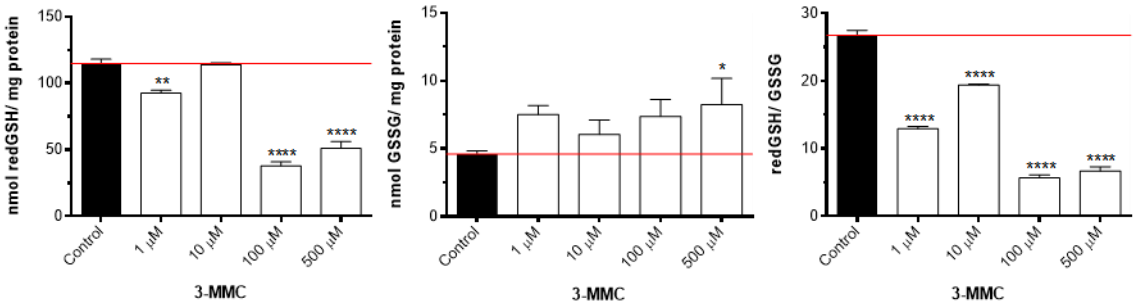


Fig. 6 Intracellular contents of reduced (redGSH) and oxidized glutathione (GSSG), and intracellular redGSH/GSSG ratio in rat primary hepatocytes after 24 h-incubation with 3-methylmethcathinone (3-MMC). Results are expressed as mean ± standard error from the mean (SEM) from four independent experiments. Statistical comparisons were made using one-way ANOVA/Dunnett's post hoc test. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$, versus control.

4.1.5. 3-Methylmethcathinone (3-MMC) does not alter mitochondrial membrane potential

The mitochondria are involved in apoptosis through the propagation of death signals originated from the inside (intrinsic apoptotic pathway) or the outside (extrinsic apoptotic pathways) of the cell. A significant loss in the mitochondrial inner membrane potential impairs oxidative phosphorylation, which causes decrease in energy and promotes the release of apoptotic factors (such as caspases), leading to death (Dias da Silva et al. 2013).

The mitochondrial integrity was determined by the evaluation of TMRE inclusion, as described by Dias da Silva et al. (2014a). As shown in Fig. 7, although TMRE inclusion slightly decreased when primary hepatocytes were exposed to all concentrations tested of 3-MMC, this alteration was not statistically significant, compared to control. These results were in line with the absence of depolarization of the inner mitochondrial membrane for low concentrations of several amphetamine derivatives. Accordingly, mitochondrial potential disruption was only observed at concentrations higher than 1.8 mM for MDMA, 900 µM for 4-MTA (4-methylthioamphetamine), and 2.2 mM for d-amphetamine, in HepG2 immortalised hepatocytes. For methamphetamine, no effect was observed in the referred study (the drug was tested up to 3 mM) (Dias da Silva et al. 2014a).

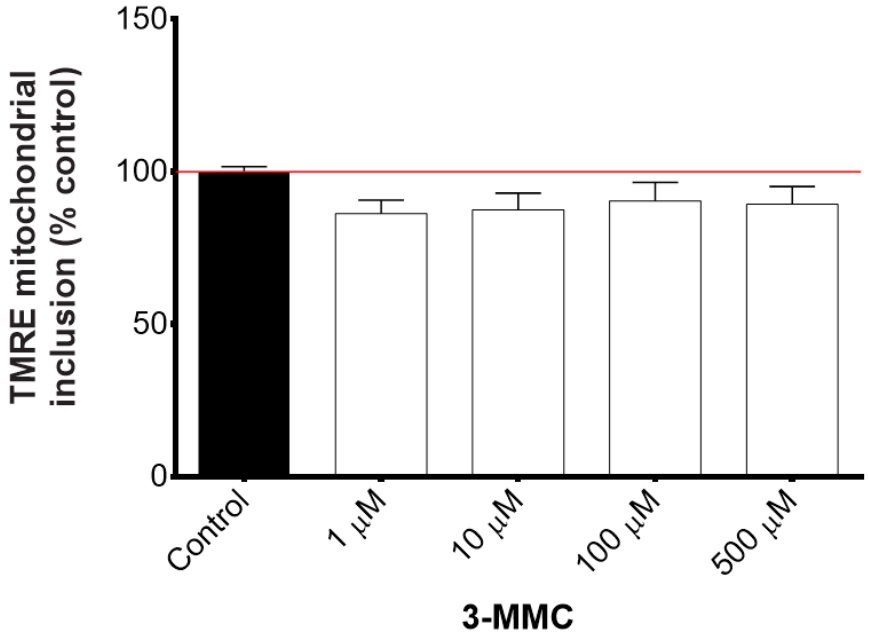


Fig. 7 Mitochondrial membrane potential (ΔΨm) measured as TMRE incorporation in mitochondria of rat primary hepatocytes after 24-h incubations with 3-methylmethcathinone (3-MMC), at 37 °C. Results are expressed as percentage control ± standard error of the mean (SEM) from three independent experiments, run in triplicates. No statistical significant differences to control were observed (one-way ANOVA/Dunnett's post hoc test).

4.1.6. Intracellular energetic status of hepatocytes was compromised by 3-methylmethcathinone (3-MMC) at high drug concentrations (≥ 100 µM)

Maintenance of mitochondrial membrane potential is tightly related to several mitochondrial processes, including ATP synthesis. In fact, the mitochondria are the main source of ATP, which is used by cells to execute energy-requiring processes. This means that ATP generated in the mitochondria is essential to maintain the integrity of the cell.

When cells were treated with 1 µM of 3-MMC, ATP was slightly higher (12.23 %) than the control (Fig. 8), supporting the energy-dependent pro-survival mechanisms mentioned above. At higher concentrations (≥ 10 µM) cell energetic stores decreased; this alteration was statistically significant from 100 µM on (Fig. 8, $p < 0.05$, ANOVA/Dunnett's). This is in accordance with the significant depletion of redGSH and the significant increase in the formation of ROS and RNS.

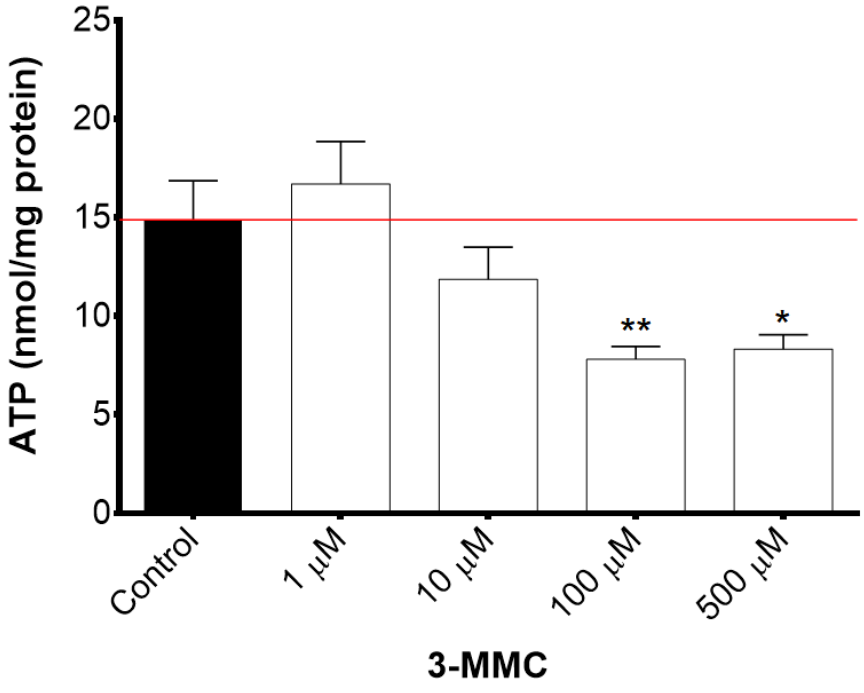


Fig. 8 Intracellular ATP levels in primary rat hepatocytes after 24 h-incubation with 3-methylmethcathinone (3-MMC), at 37 °C. Results are expressed as mean ± standard error of the mean (SEM) from four independent experiments. Statistical comparisons were made using one-way ANOVA/Dunnett's. *p < 0.05; **p < 0.01, versus control.

4.1.7. 3-Methylmethcathinone (3-MMC) at 10 µM activates pro-apoptotic caspase-3, -8, and -9

As a response to the oxidative injury, the mitochondria undergo extensive changes, such as the permeabilization of the mitochondrial membrane with consequent release of pro-apoptotic proteins, as cytochrome *c*. Once in the cytosol, cytochrome *c* triggers a complex signalling cascade, characteristic of the intrinsic mechanisms of apoptosis, with the activation of caspase-9 and, subsequently, the executioner caspase-3. This leads to the cleavage of intracellular substrates, causing morphological and biochemical changes that culminate in cell death. The activation of apoptosis also occurs through the extrinsic pathway. The binding of xenobiotics to death receptors located in the surface of cell membrane activates this mitochondria-independent pathway, with subsequent activation of caspase-8, and further direct activation of the executioner caspases (such as caspase-3) or indirect activation of the intrinsic apoptotic pathway (McIlwain et al. 2013).

The caspase-3, -8 and -9 activities were evaluated and the results are presented in Fig. 9. The activity of the three caspases was maximal when cells were treated with 10 µM of 3-MMC. At this concentration level, capase-3 activity almost duplicated (189.2 ± 21.9 %), while caspase-8 (148.1 ± 13.8 %) and caspase-9 (155.3 ± 13.5 %) activities were approximately 50 % higher than the activity in controls. At higher concentrations (100 µM and 500 µM) caspase activities suffered a drastic decrease to levels close to controls. This decrease is consistent with the observed reduction of ATP and suggests that the hepatotoxicity observed at these concentration levels must follow other forms of cell death, such as necrosis, as described before for amphetamines in HepG2 cells (Dias da Silva et al. 2013). Further investigation is required to support these assumptions and ascertain the pathways involved. The concentration of 3-MMC needed to provoke significant alteration in caspases activation in the current study (10 µM) was much lower than that observed for 4-MEC (1.6 mM) in the same cell model (Valente et al. 2016).

The regulation of the intrinsic pathway of apoptosis is made by pro-apoptotic and pro-survival (antiapoptotic) regulators belonging to the BCL-2 family of proteins. The pro-apoptotic BCL-2 factors are also implicated in the enhancement of the extrinsic death receptor pathway. Some proteins from this family are responsible, for instance, for the permeabilization of the outer membrane of the mitochondria. Dias da Silva and collaborators (2013) realised a thorough investigation on the apoptotic mechanisms

underlying toxicity of four amphetamine derivatives (MDMA, METH, 4-MTA and d-amph) and showed a decline in antiapoptotic BCL-2 and BCL-XL mRNA levels, with concurrent upregulation of the proapoptotic 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. According to our observations, additional results from the authors showed time-dependent caspase-3/-7 activation, but not ΔΨM disruption, mediated amphetamine-induced apoptosis, in HepG2 cells after 24 h exposures. The authors also confirmed cell dismantling by poly (ADP-ribose) polymerase (PARP) proteolysis. Given the structure and chemical similarity, it is possible that comparable mechanisms could be activated for 3-MMC but this hypothesis awaits experimental confirmation.

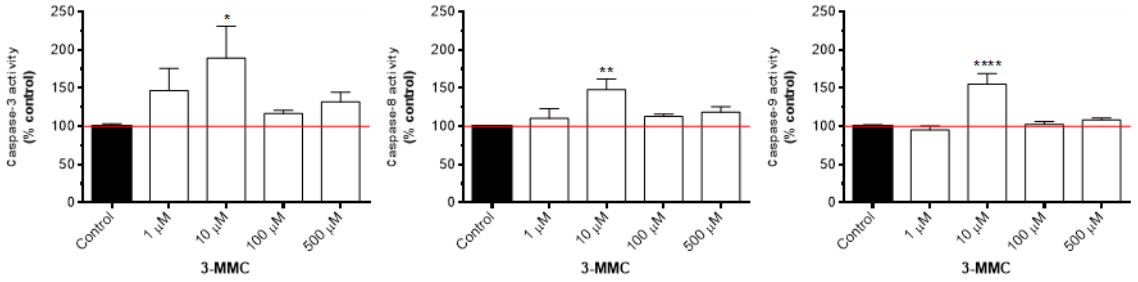


Fig. 9 Caspase-3, -8, and -9 activities in primary rat hepatocytes after 24-h incubations with 3-methylmethcathinone (3-MMC), at 37 °C. Results are expressed as percentage of control ± standard error of the mean (SEM) from three independent experiments. Statistical comparisons were made using Kruskal–Wallis one-way analysis of variance/Dunnett's post hoc test. *p < 0.05; **p < 0.01; ****p < 0.0001, versus control.

4.2. Quantification of 3-methylmethcathinone (3-MMC) by Gas Chromatography–Mass Spectrometry (GC–MS)

4.2.1. Extraction and evaluation of interferences

As mentioned above, studies on 3-MMC are scarce and most information derives from case reports. Herein, we proposed to evaluate the impact of 3-MMC metabolism on the cytotoxicity elicited by the drug. This type of investigation requires a very robust method with high sensitivity and wide linearity ranges for the quantification of the drug and its main metabolites in biological samples. So, the first part of this work consisted on the optimization of a GC-MS methodology suitable to address these issues. We tested several extraction methods and the addition of two different IS until we achieved adequate LOD and LOQ, linearity, and extraction efficiency.

The first method was adapted from that used for quantifying MDMA and piperazines in our laboratory (da Silva et al. 2010; Moreira 2015). In this method, we tested the derivatization with TFAA and IS_1. Nevertheless, the peak of 3-MMC overlapped with IS_1, as seen in Fig. 10. We therefore tested the same extraction method IS_2, but the extraction efficiency for this IS was below 80 %.

The method 2 was adapted from Margalho et al. (2016), who used it for cathinones. Derivatizing the extracts with TFAA produced recovery values that were above the acceptable criteria of 100 ± 20 % for this parameter. When these extracts were derivatized with HFBA, linearity was poor (R^2 = 0.9765) and also variability among replicates was noted. This method was deemed unfit for our quantification.

The methods 3 and 4 had been previously used for extracting piperazines in cell culture medium (Moreira 2015). We tested both methods simultaneously with addition of IS_1 and derivatization with HFBA. When comparing the chromatograms obtained from both methods, there were almost no differences (Fig. 11) but the extraction efficiency of method 4 was below 80 %. The method 3 showed good reproducibility (coefficient of variation, CV, below 5 %) and good linearity in both extracted (R^2 = 0.9887) and non-extracted (R^2 = 0.9998). However, the extraction efficiency was extremely low for the smallest concentration (~ 50 %) and, at the other concentrations, again variability between replicates was noted. Therefore, these methods also had to be discarded.

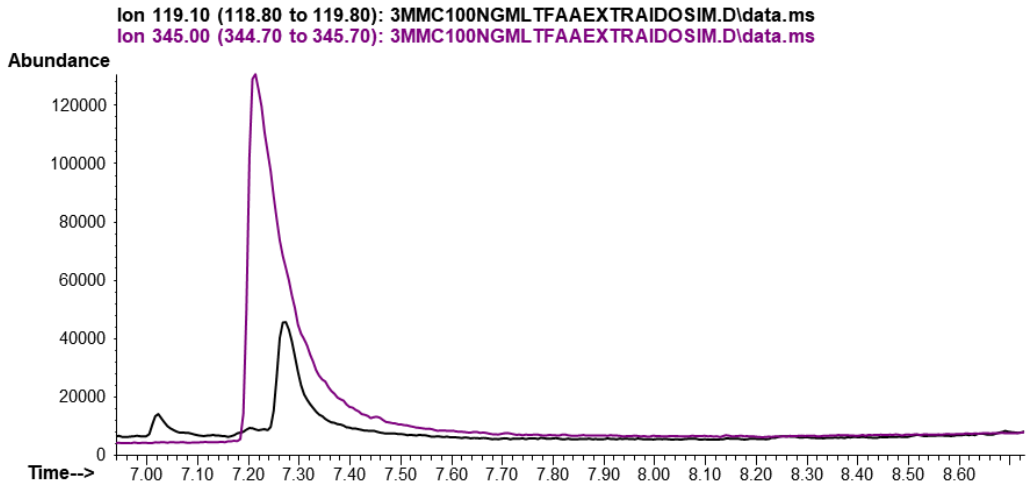


Fig. 10 Chromatogram obtained by using method of extraction 1, with 4-hydroxy-3-methoxybenzylamine hydrochloride (IS_1), and derivatization with trifluoroacetic anhydride (TFAA). Ion 119 corresponds to 3-MMC peak and ion 345 corresponds to IS_1. We can clearly see an overlap of the ions of 3-MMC and IS_1.

We then tested methods 5 and 6 simultaneously. Method 6 was rejected because during the evaporation until dryness under N_2 flow, many residues were retained in the vial and derivatization was not possible due to the sample turbidity. Method 5 was tested using IS_1 and HFBA as the derivatizing agent. The extraction efficiency was below 80 %. Removing the excess of derivatizing reagent before re-injecting the

sample did not improve the quality of the chromatograms (Fig. 12) and the method was discarded.

Then, we tested methods 7 and 8 with HFBA derivatization and IS_1 with the additional step of the removal of the derivatization reagent. For both methods, we adjusted the sample either to pH < 5 or pH > 11 aiming to obtain, by comparison, a decision on which one produced a better response (higher peak). However, all samples tested had an interference at the time of retention of 3-MMC (Fig. 13). We then decided to test only method 8 with the pH adjusted to < 5 or > 11, and without the removal of the derivatizing reagent, to see if the interference still occurred. However, this did not solve the problem, because the interference persisted (Fig. 14). Then we decided to test method 8 again with IS_2 and without removing the excess of derivatization reagent.

This last method was chosen to analyse the samples because it showed good LOD and LOQ, linearity and extraction efficiency as described below.

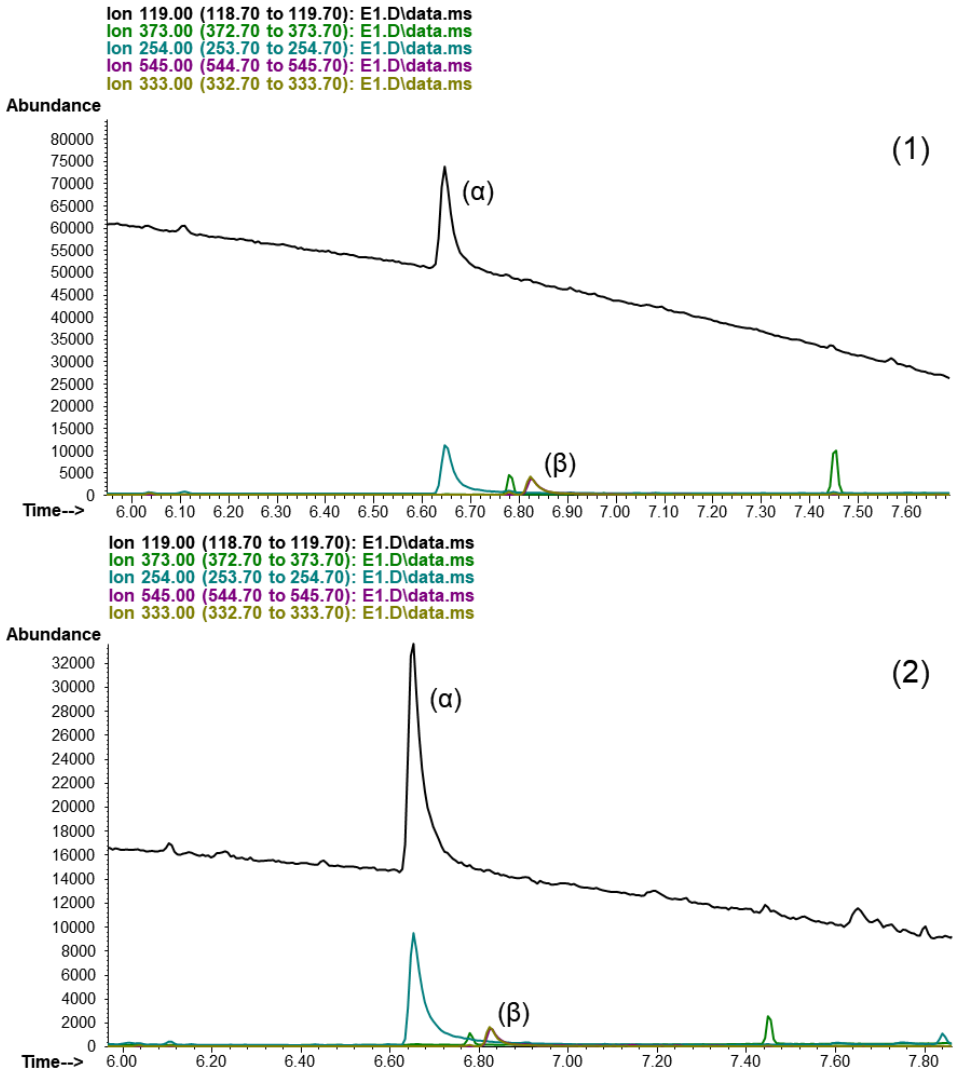


Fig. 11 Chromatograms obtained when extraction was performed with method 3 (1) and 4 (2) with 4-hydroxy-3-methoxybenzylamine hydrochloride (IS_1), and derivatization with heptafluorobutyric acid (HFBA). Ions 119, 373, and 254 correspond to 3-MMC peak (α) and ions 545, and 333 correspond to IS_1 (β).

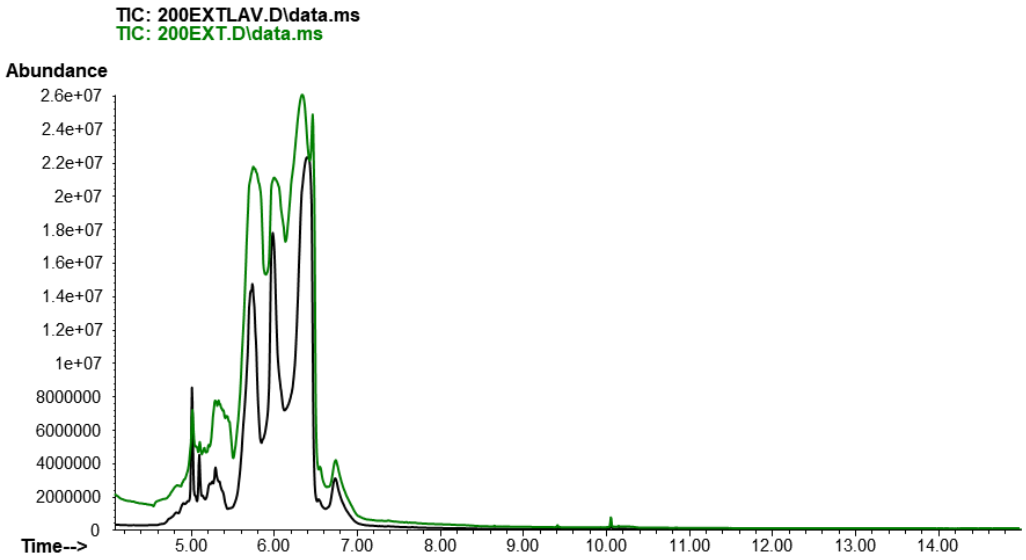


Fig. 12 Overlaid total ion chromatograms of method 5, with (green) and without (black) removal of excess of the derivatization reagent.

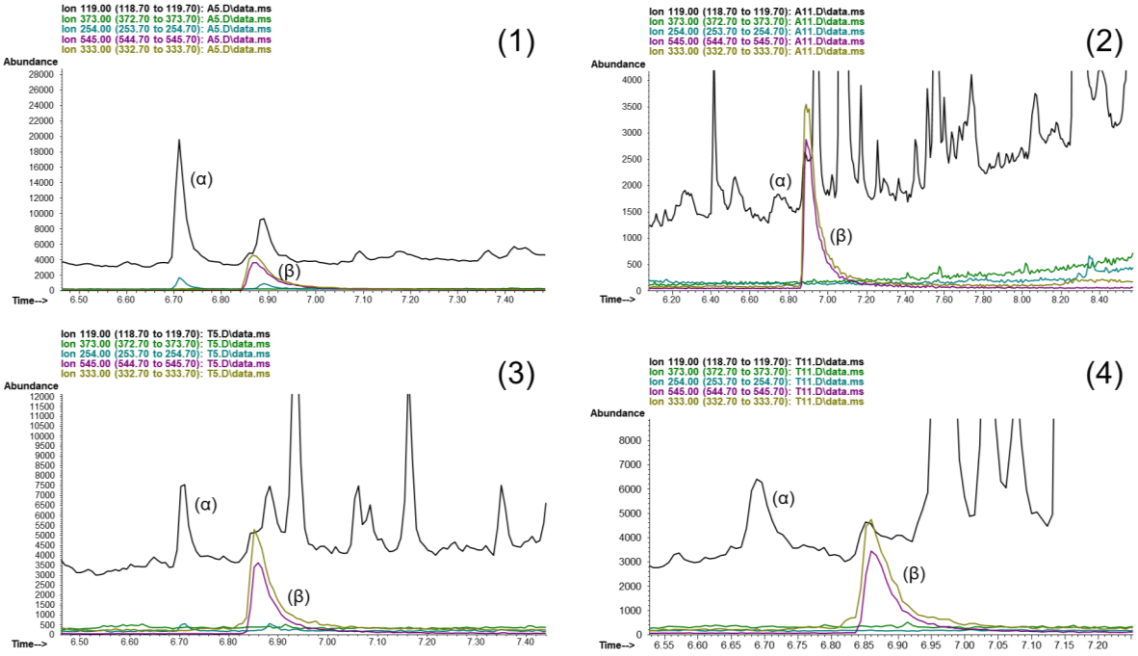


Fig. 13 Chromatograms obtained from injection of an extracted blank sample with method 7 and 8 with 4-hydroxy-3-methoxybenzylamine hydrochloride (IS_1), derivatization with heptafluorobutyric acid (HFBA), and removal of the excess derivatizing reagent. Interference peak (α) and IS_1 peak (β). (1) Method 7 with pH < 5; (2) Method 7 with pH > 11; (3) Method 8 with pH < 5; (4) Method 8 with pH > 11.

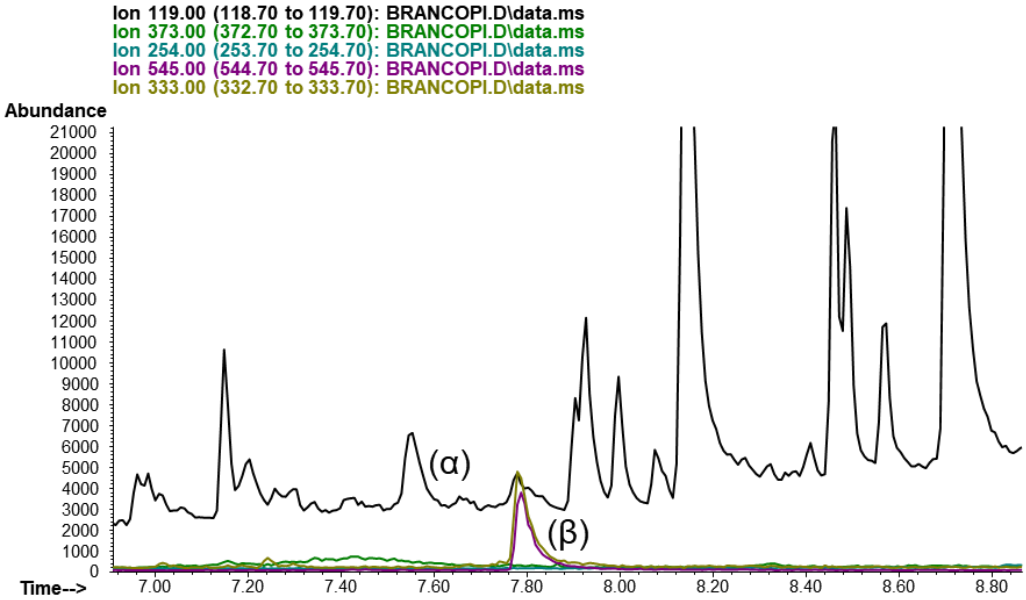


Fig. 14 Chromatogram obtained from injection of an extracted blank sample with method 8, with 4-hydroxy-3-methoxybenzylamine hydrochloride (IS_1), and derivatization with heptafluorobutyric acid (HFBA). Interference peak (α) and IS_1 peak (β).

4.2.2. Derivatization

When performing a GC analysis, the compounds of interest need to be volatile and stable at the high temperatures required for the analysis. However, some compounds are not directly suitable to GC analysis due to its poor volatility and/or thermal instability. In these cases, chemical derivatization is necessary. Derivatization can also be performed to enhance the chromatographic efficiency (peak resolution and separation) or even increase detectability. According to the literature, derivatization of 3-MMC with HFBA (Jamey et al. 2016) and acetic anhydride (Bottinelli et al. 2017) had been used previously. Based on this and on the reagent availability at the laboratory, we decided to test three different derivatization reagents (MSTFA, TFAA and HFBA). Derivatization with TFAA and HFBA consists on an acylation reaction and with MSTFA on a silylation. The general mechanism of the three derivatization procedures are illustrated in Fig. 15.

Contrary to derivatization with MSTFA, derivatization with TFAA and HFBA produced satisfactory results. However, derivatization with HFBA allowed detection of lower concentrations, compared to derivatization with TFAA. For these reasons, the chosen derivatizing reagent was HFBA. The proposed fragmentation pattern of both IS_1 and IS_2, and 3-MMC are presented in Fig.16.

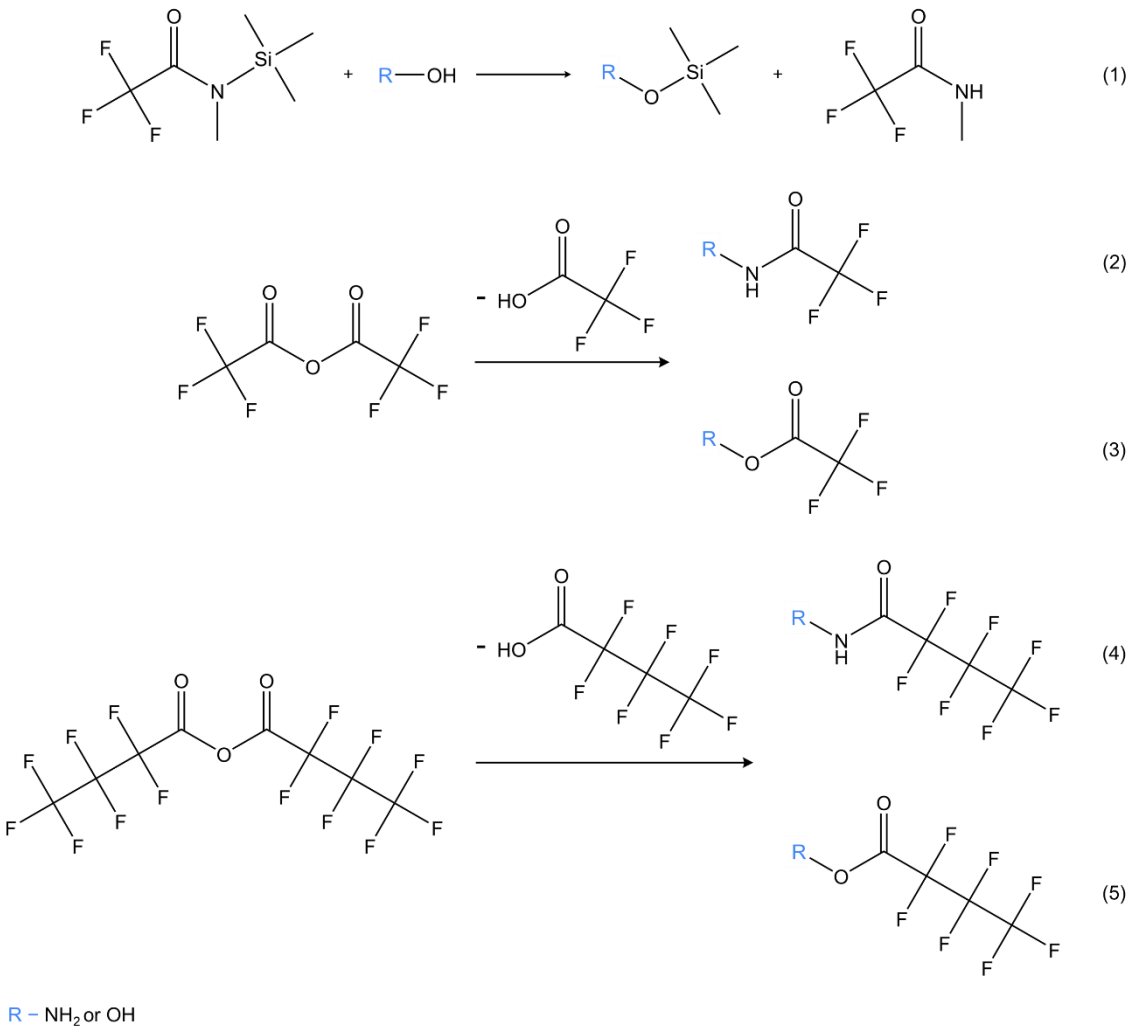


Fig. 15 General mechanisms for derivatization with – N-methyl-n-(trimethylsilyl)trifluoroacetamide (MSTFA) (1), trifluoroacetic anhydride (TFAA) (2,3) and heptafluorobutyric acid (HFBA) (4,5).

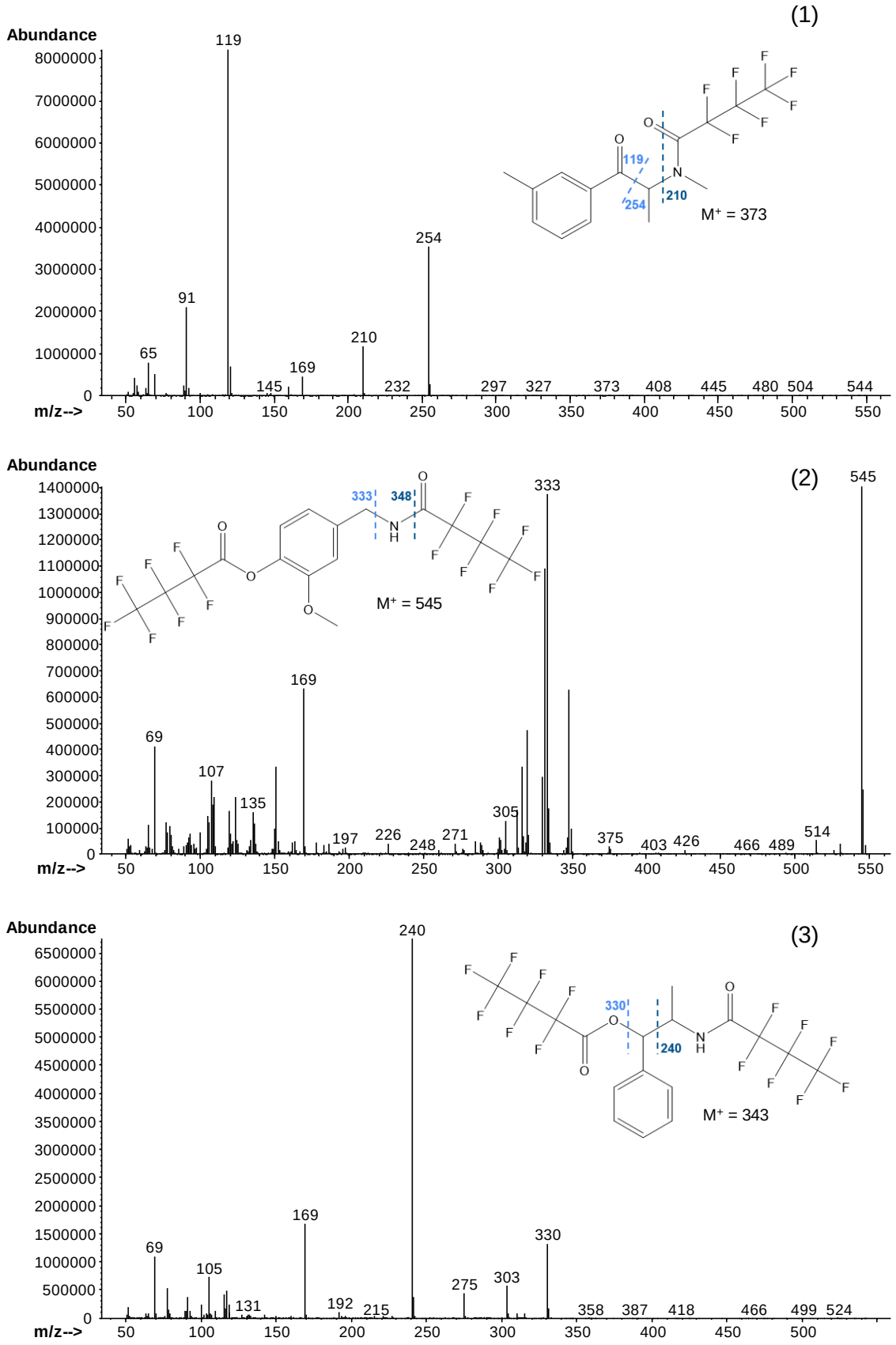


Fig. 16 EI mass spectra of 3-methylmethcathinone (3-MMC) (1), 4-hydroxy-3-methoxybenzylamine hydrochloride (IS_1) (2) and phenylpropanolamine (IS_2) (3) and proposed fragmentation pattern after derivatization with heptafluorobutyric acid (HFBA).

4.2.3. Chromatographic conditions and equipment

The GC-MS method was adapted from Adamowicz et al. (2014) and from Zuba and Adamowicz (2016). The analysis performed with the method described above (section 3.2.7. Chromatographic conditions and equipment of Chapter 3 – Materials and methods), resulted in good chromatograms with good resolution, the absence of co-eluted peaks and an adequate time of analysis. The selected ions for monitorization are presented in Table 11. The observed retention times are presented in Table 12.

Table 11 Selected ions for monitoring.

Compound	TFAA ions		HFBA ion	
	Quantification	Identification	Quantification	Identification
3-MMC	119	273, 154, 120	119	373, 254, 210
IS_1	345	248, 232	545	348,333
IS_2	140	230, 203, 117	240	330,275

3-MMC – 3-methylmethcathinone; IS_1 – 4-hydroxy-3-methoxybenzylamine hydrochloride; IS_2 – phenylpropanolamine; TFAA – trifluoroacetic anhydride: HFBA – heptafluorobutyric acid

Table 12 Retention times for 3-methylmethcathinone (3-MMC) and internal standards (IS).

Compound	Retention time (minutes)	
	TFAA	HFBA
3-MMC	7.18	6.70
IS_1	7.19	6.87
IS_2	5.24	5.55

3-MMC – 3-methylmethcathinone; IS_1 – 4-hydroxy-3-methoxybenzylamine hydrochloride; IS_2 – phenylpropanolamine; TFAA – trifluoroacetic anhydride: HFBA – heptafluorobutyric acid

4.2.4. Quantitative determination of 3-methylmethcathinone (3-MMC) in samples

After choosing the method 8 for the extraction, the IS_2, and the derivatizing reagent (HFBA), we performed some additional tests to estimate the LOD and LOQ, the linearity, and the recovery. Then, we analysed the samples, i.e. the supernatants collected from exposures of primary hepatocytes to several 3-MMC concentrations, during the cytotoxicity experiments.

4.2.4.1. Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) were determined based on the signal-to-noise ratio. To establish the LOD and LOQ, the 125 ng/mL working calibrator was progressively diluted (factor of 2) with cell culture medium. The LOD was 3.5 ng/mL and the LOQ was 7 ng/mL.

4.2.4.2. Linearity studies

The calibration curves obtained showed good linearity (R^2 = 0.9980 and R^2 = 0.9975) in the concentration range tested (12.5 – 100 ng/mL) (Fig. 17).

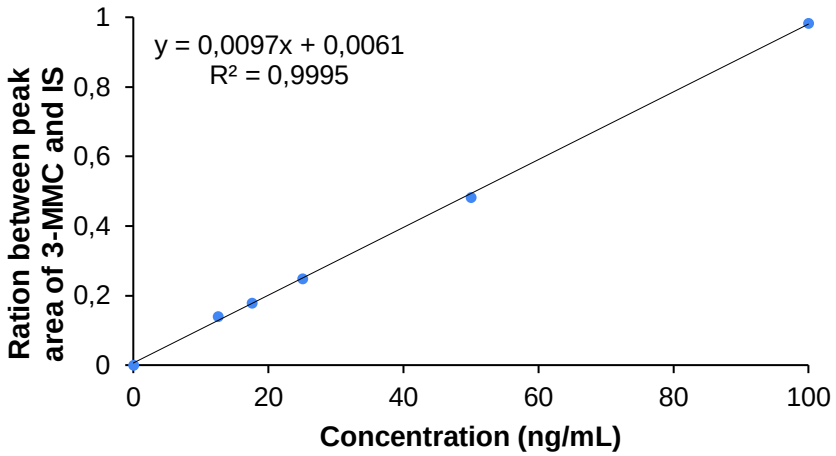


Fig. 17 Representative calibration curve for 3-methylmethcathinone (3-MMC).

4.2.4.3. Extraction efficiency

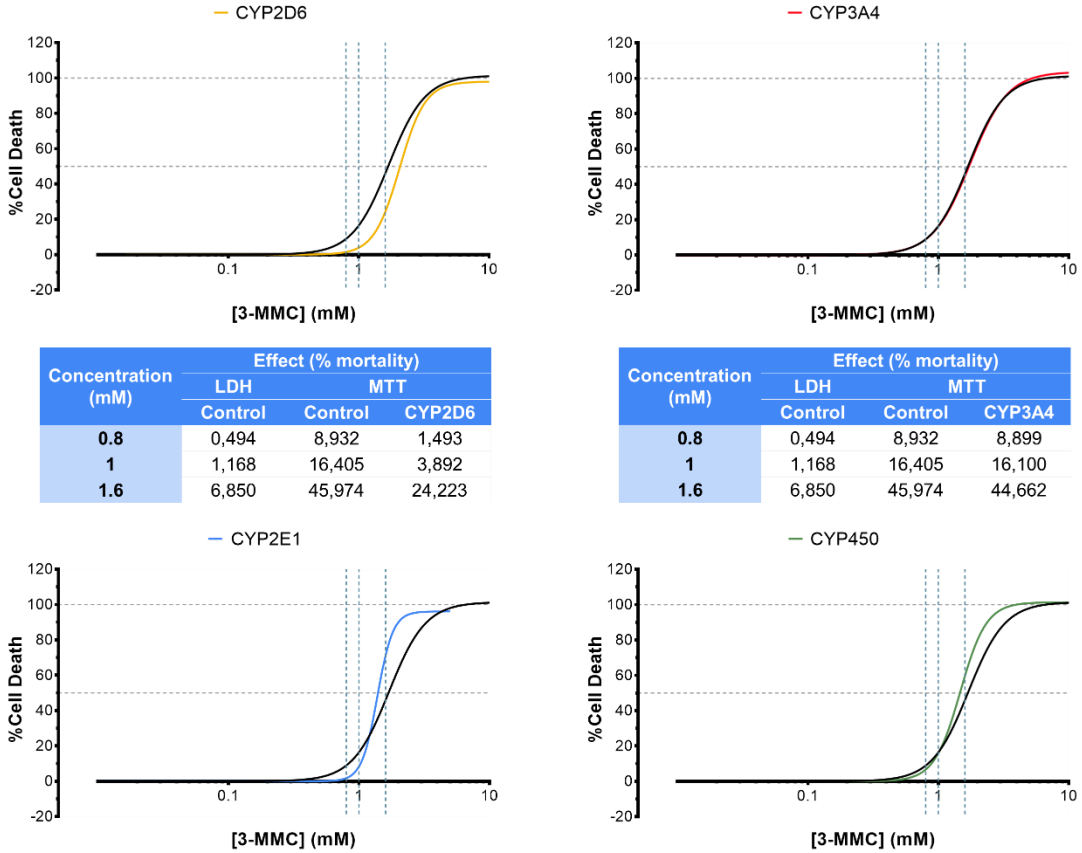
The extraction efficiencies [Extraction efficiency (%) = (mean peak area ratio of extracted samples / mean peak area ratio of non-extracted samples) x 100] were determined at three different levels of concentration. The results are presented in Table 13, and showed recovery values that were at the acceptable criteria of 100 ± 20 % for this parameter.

Table 13 Extraction efficiencies (%) for the method of extraction 8 (n = 2).

Concentration (ng/mL)	Extraction efficiency (%)
12.5	85
25	94
50	96

4.2.4.4. Quantification of 3-methylmethcathinone (3-MMC) in samples selected from the cytotoxicity experiments

Based on the complete cell death profiles obtained in the cytotoxicity experiments for 3-MMC (individually or co-incubated with metabolism inhibitors; Fig. 18), we chose the samples from primary hepatocyte incubations at 0.8 mM, 1 mM and 1.6 mM of 3-MMC. At these concentrations, the cytotoxicity profiles of 3-MMC, with or without CYP450 inhibition, showed significant differences and, therefore, it would be interesting to understand whether these differences were accompanied by a different extension in the metabolism of 3-MMC.



Concentration (mM)	Effect (% mortality)		
	LDH	MTT	
	Control	Control	CYP2D6
0.8	0,494	8,932	1,493
1	1,168	16,405	3,892
1.6	6,850	45,974	24,223

Concentration (mM)	Effect (% mortality)		
	LDH	MTT	
	Control	Control	CYP3A4
0.8	0,494	8,932	8,899
1	1,168	16,405	16,100
1.6	6,850	45,974	44,662

Concentration (mM)	Effect (% mortality)		
	LDH	MTT	
	Control	Control	CYP2E1
0.8	0,494	8,932	1,776
1	1,168	16,405	8,327
1.6	6,850	45,974	71,206

Concentration (mM)	Effect (% mortality)		
	LDH	MTT	
	Control	Control	CYP450
0.8	0,494	8,932	6,692
1	1,168	16,405	15,914
1.6	6,850	45,974	59,689

Fig. 18 Cytotoxic profiles of 3-methylmethcathinone (3-MMC) following inhibition of CYP2D6 (yellow), CYP3A4 (red), CYP2E1 (blue) and CYP450 (green), in comparison with no metabolic inhibition (black line). The tables contain information on the effect (% of mortality) elicited by each test concentration, in the presence or absence of the respective inhibitor, as assessed by the lactate dehydrogenase (LDH) leakage assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.

Table 14 Amount of 3-methylmethcahinone (3-MMC) metabolized.

Concentration (mM)	Control		CYP2D6 Inhibition		CYP3A4 Inhibition		CYP2E1 Inhibition		CYP450 Inhibition	
	Met	Viab	Met	Viab	Met	Viab	Met	Viab	Met	Viab
0.8	78.9	81.8	86.0	-	100.5	96.7	89.9	95.4	82.5	91.6
1	77.5	94.7	81.8	-	84.6	94.4	84.1	71.9	93.7	91.4
1.6	94.1	76.4	94.6	-	89.4	36.3	78.3	8.0	95.5	37.3

All values are in percentage (%). The percentage of metabolization was calculated as follows: % metabolism = (concentration of 3-MMC to which cells were exposed to / concentration of 3-MMC detected by GC-MS) x 100. Met – metabolization; Viab – viability.

In the absence of metabolic inhibition (controls), an increase in the extent of metabolism was observed with increasing concentrations of 3-MMC (78.9 % at 0.8 mM and 94.1 % at 1.6 mM), denoting no apparent saturation of normal 3-MMC metabolic pathways.

When CYP2E1 was inhibited, at the low concentration range (up to approximately 1.20 mM), the toxicity diminished (Fig. 18) and the metabolism of 3-MMC seemed to increase (Fig. 19). Taken together, these observations appear to indicate that the drug followed more efficient alternative metabolic pathways (higher metabolic rate) and that the degradation of 3-MMC diminished the toxicity (metabolism through these alternative pathways contributed to the drug detoxification). Nevertheless, at higher concentrations, an inversion of the cytotoxic profile was noted. Accordingly, an enhancement of the toxicity occurred. Also, at this concentration level an apparent reduction of metabolism was observed, compared to that at lower concentrations (Figs. 18 and 19). This might be due to the saturation of the alternative metabolic pathways (for instance, through other CYP450 isoforms).

When inhibiting CYP2D6, the toxicity decreased but only a slight increase in the extent of 3-MMC metabolism occurred, compared to controls, particularly at the lower concentrations tested (Table 14 and Fig. 19). Metabolism though this isoform seems to be toxifying, as previously observed for the amphetamine-derivative MDMA, in cardiomyocytes (Carvalho et al. 2004). Accordingly, the opening of the methylenedioxy ring of MDMA and its metabolite 3,4-methylenedioxyamphetamine (MDA) occurs through CYP2D6 and originates two catechol metabolites, N-methyl-α-methyldopamine (N-Me-α-MeDA) and α-methyldopamine (α-MeDA), respectively. The presence of the catechol group confers high reactivity to these metabolites, which easily oxidize, giving the corresponding o-quinones. The oxidation of quinones may also cause the formation of aminochromes, whose subsequent oxidation leads to the production of polymers of melanin type. The cycles of reduction-oxidation (redox) associated with this metabolic

conversion result in the formation of ROS/RNS, which similarly to what occurs with quinones, might attack relevant intracellular nucleophilic groups, such as protein sulfhydryl groups e.g. GSH), resulting in significant modification of macromolecules, including proteins, lipids and deoxyribonucleic acid (DNA) (Carvalho et al. 2012).

On the contrary, when CYP3A4 was inhibited, the increase of 3-MMC concentration apparently reduced the drug metabolism, compared to controls. However, this alteration did not seem to have a significant impact, as no effect on the cytotoxic profile of 3-MMC was noted upon CYP3A4 inhibition (Fig. 18; Table 14).

Finally, when inhibiting the whole CYP450, the metabolism apparently increased, in comparison with the control, mostly at the low concentrations tested (Table 14). However, at these concentrations, no difference in toxicity was noted, when compared with the control (Fig. 19). Surprisingly, the difference in toxicity was only noted at the higher concentration range (from 1.03 mM on; Fig. 18), for which metabolism through CYP450 seemed to be detoxifying. If the whole CYP450 is inhibited, the metabolism might be occurring though other enzymes. For instance, through catechol-O-methyltransferase (COMT), as described for the hepatic metabolism of MDMA (Dias da Silva et al. 2013b). One could therefore speculate that the metabolism of 3-MMC is also regulated through the latter enzyme.

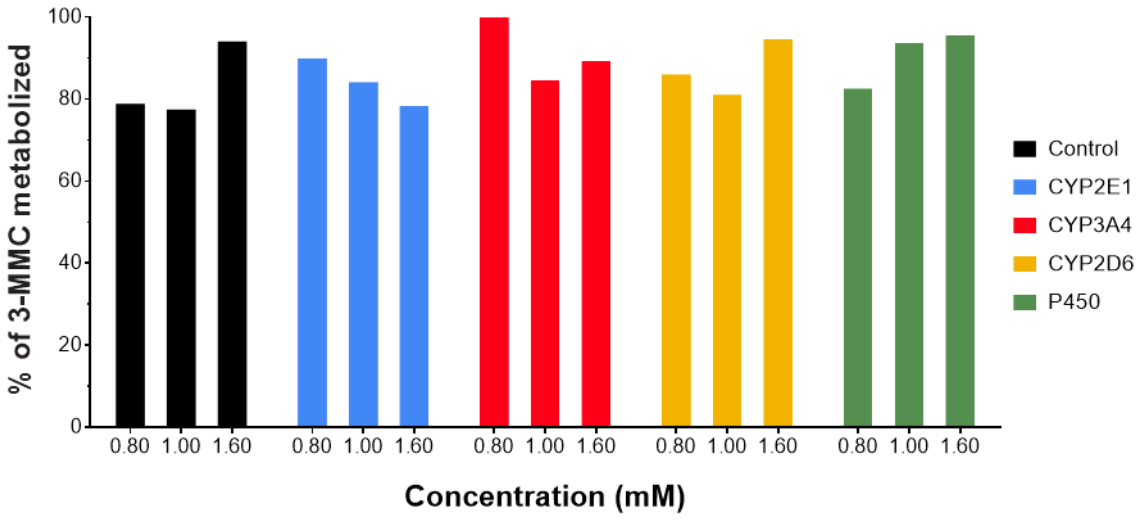


Fig. 19 Percentage of 3-methylmethcathinone (3-MMC) metabolized when CYP2D6 (yellow), CYP3A4 (red), CYP2E1 (blue) and CYP450 (green) are inhibited and when no inhibition is made (black).

Chapter 5

Conclusion and future perspectives

With the increase in consumption and the lack of toxicological studies on 3-MMC, its toxicity assessment has become mandatory. For the first time, the *in vitro* hepatotoxic effects of 3-MMC have been demonstrated. This synthetic cathinone induced oxidative stress, loss of mitochondrial membrane potential, GSH and ATP depletion, increased GSSG, as well as caspase-3, -8, and -9 activation. Drug metabolism through CYP2D6 might be involved in the toxicological effects of 3-MMC, while CYP2E1 might be responsible for detoxification, particularly at high concentrations (above 1.20 mM). Bioactivation of 3-MMC by CYP2D6 and, for the low concentration range, by CYP2E1 is involved in the observed toxicity. The results of the GC-MS also showed comparable results.

Overall, data presented herein allowed us to conclude that:

i) Hepatotoxicity of 3-MMC might be triggered both by mitochondria-dependent (intrinsic) and mitochondria-independent (extrinsic) pathways of apoptotic cell death;

ii) At higher drug concentrations, other cell death pathways might be at play, such as necrosis;

iii) Oxidative stress and alterations on energy homeostasis might be mediating the observed effects;

iv) Bioactivation of 3-MMC by CYP2D6 and CYP2E1 (at concentrations lower than 1.20 mM) might be involved in the observed toxicity;

v) The metabolism might also occur through other enzymes besides CYP450.

The present study is a starting point in the research concerning 3-MMC and, therefore, further investigation is required to elucidate users that should be alert of the risks associated with the abuse of this substance. Future perspectives may potentially involve the following work:

i) To clarify the other pathways of cell death (necrosis and autophagy);

ii) To evaluate other stress markers, such as quantification of intercellular calcium;

iii) To explore the role of hyperthermia in the toxicity of this drug;

iv) To validate the significance of *in vitro* data presented in this thesis through *in vivo* studies;

v) To further develop and validate a method for GC-MS identification and quantification of both 3-MMC and its metabolites.

Chapter 6

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Annex

Communications

THE NEW PSYCHOACTIVE SUBSTANCE 3-METHYLMETHCATHINONE (3-MMC) INDUCES HEPATOTOXICITY TOWARD PRIMARY RAT HEPATOCYTES BY TRIGGERING INTRINSIC AND EXTRINSIC APOPTOSIS PATHWAYS

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Introduction: In the recent years, there has been an increase in the production of new psychoactive substances (NPS). As some NPS are still considered legal in some countries, users mistakenly consider them as safe for consumption. However, several cases of intoxications and deaths related to NPS have been recently reported (1). An example of such a drug is 3-methylmethcathinone (3-MMC or metaphedrone). This drug belongs to the synthetic cathinones family and was recently introduced in the market to replace 4-methylmethcathinone (4-MMC or mephedrone), which became illegal. There is some evidence of 3-MMC being as dangerous as 4-MMC (2) but information on 3-MMC toxic profile is profoundly scarce.
Aims: As the liver is one of the main sites of drug metabolism, and therefore a relevant target for their deleterious effects, the aim of this project was to assess the hepatotoxicity of 3-MMC.
Material and methods: For this purpose, primary hepatocytes of Wistar rats were isolated by collagenase perfusion, cultured, and exposed for 24 h to several concentrations of the drug, selected to provide a complete cytotoxic profile. Cell death was assessed through three assays, namely the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, lactate dehydrogenase (LDH) leakage assay, and the neutral red (NR) uptake assay. Afterwards, some of the mechanisms that contributed to the putative cytotoxicity were also assessed by measuring the intracellular reactive species of oxygen and nitrogen (ROS/RNS), alterations to the mitochondrial membrane potential ($\Delta\Psi m$), and to caspase activities, after exposing cells at 1 μM, 10 μM, 100 μM, and 500 μM of 3-MMC. Finally, we evaluated the modulatory effects of cytochrome P450 (CYP) inhibitors on the toxicity of 3-MMC.
Results: 3-MMC-induced toxicity was firstly perceived in the lysosome (NR NOEC 312.5 μM), next in the mitochondria (MTT NOEC 379.5 μM), and finally in the cytoplasmic membrane (LDH NOEC 1.04 mM). A significant concentration-dependent increase of ROS/RNS was observed from 10 μM on. At this concentration, caspase -3, -8, and -9 activities were significantly elevated, but no differences were observed at higher concentrations, indicating that at these levels other mechanisms should intervene. No significant alterations were observed in the $\Delta\Psi m$, which is important for ATP synthesis, and therefore for energy-dependent apoptosis. CYP 2D6, 2E1, and 3A4 inhibitors diminished 3-MMC toxicity.
Conclusions: Overall, our data point to a role of metabolism in the hepatotoxicity of 3-MMC, which seems to be triggered both by intrinsic and extrinsic apoptotic mechanisms. At higher drug concentrations, other cell death pathways might be at play, such as necrosis or autophagy, deserving further investigation.

1 - EMCDDA *European Drug Report* 2016; 2 - Adamowicz P et al. *Journal of Analytical Toxicology* 2016; 40(4):272-6.

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3-Methylmethcathinone (3-MMC) elicits oxidative stress and apoptosis in primary rat hepatocytes

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3-Methylmethcathinone (3-MMC or metaphedrone) is a new psychoactive synthetic cathinone that has been recently deemed responsible for several cases of intoxications and deaths, as a consequence of its use in recreational settings. As the liver is one of the main sites of metabolism of drugs, the aim of this study was to assess the hepatotoxicity of 3-MMC.

For this purpose, primary hepatocytes of Wistar rats were isolated by collagenase perfusion, cultured and exposed for 24 h to several concentrations of the drug, selected to provide a complete cytotoxic profile. Cell death was assessed through three assays, namely the MTT reduction assay, LDH leakage assay, and the neutral red (NR) uptake assay. Afterwards, some of the mechanisms that contributed to the putative cytotoxicity were also assessed by measuring the intracellular ROS and RNS, the alterations to mitochondrial membrane potential, and to caspase -3, -8, and -9 activities, after exposing cells at 1 μM, 10 μM, 100 μM, and 500 μM of 3-MMC.

3-MMC-induced toxicity was firstly perceived in the lysosome (NR NOEC 312.5 μM), next in the mitochondria (MTT NOEC 379.5 μM), and finally in the cytoplasmic membrane (LDH NOEC 1.04 mM). An increase of ROS and RNS was observed in a concentration-dependent manner, statistically significant from 10 μM on. At this concentration, caspase -3, -8, and -9 activities were significantly elevated, but no differences were observed at higher doses, indicating that at these levels other mechanisms should intervene. No significant alterations were observed in the mitochondrial membrane potential, which is important for ATP synthesis, and therefore for energy-dependent apoptosis.

Overall, our data indicate that hepatotoxicity of 3-MMC is triggered both by intrinsic and extrinsic apoptotic mechanisms but, at higher drug concentrations, other cell death pathways might be at play, such as necrosis or autophagy, deserving further investigation.

POSTER

CITOTOXICITY OF 3-METHYLMETHCATHINONE (3-MMC OR METAPHEDRONE) IN PRIMARY RAT HEPATOCYTES

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Introduction: The XXI century has been witnessing a change in paradigm of drug abuse, through a steadily increase in the production of new psychoactive substances (NPS). As they are still legal in some countries, users mistakenly consider them safe. However, there are several reports of intoxications and deaths by NPS [1]. The synthetic cathinone 3-methylmethcathinone (3-MMC or metaphedrone) was recently introduced in the market to replace the illegal drug 4-methylmethcathinone (4-MMC or mephedrone). There is evidence of 3-MMC being as dangerous as 4-MMC to humans [2] but information on the toxicity mechanisms is scarce, demanding investigation.
Aims: As the liver is one of the main sites of cathinones' metabolism, and therefore a relevant target for their deleterious effects, the aim of this project was to assess the mechanistic pathways of toxicity of 3-MMC in primary rat hepatocytes.
Material and methods: Primary hepatocytes of Wistar rats were isolated by collagenase perfusion, cultured, and exposed for 24 h to the drug at a concentration range [from 31 nM to 10 mM] selected to provide a complete cytotoxic profile. Cell death was assessed through three assays, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, lactate dehydrogenase (LDH) leakage assay, and the neutral red (NR) uptake assay. Some of the mechanisms that could contribute to the observed cytotoxicity were also assessed by measuring the intracellular reactive species of oxygen and nitrogen (ROS/RNS), reduced (GSH) and oxidized (GSSG) glutathione, adenosine triphosphate (ATP), alterations to the mitochondrial membrane potential ($\Delta\Psi m$) and to caspase activities, after exposing cells to 1 µM, 10 µM, 100 µM, and 500 µM of 3-MMC. Finally, the modulatory effects of cytochrome P450 (CYP) inhibitors on the toxicity of 3-MMC were also evaluated.
Results: 3-MMC-induced toxicity was perceived in the lysosome at lower concentrations (NR NOEC 312.5 µM), compared to mitochondria (MTT NOEC 379.5 µM) and to cytoplasmic membrane (LDH NOEC 1.04 mM). A significant concentration-dependent increase of ROS/RNS was observed from 10 µM on. At this concentration, caspase -3, -8, and -9 activities were significantly increased, but no differences were observed at higher concentrations, indicating that at this level other cell death mechanisms may intervene. No significant alterations were observed in the $\Delta\Psi m$, which is important for ATP synthesis, and therefore for energy-dependent apoptosis. ATP intracellular content slightly increased at 1 µM and significantly decreased at the highest concentrations (100 µM and 500 µM). We observed a concentration-dependent decrease in antioxidant defences (GSH) with a concurrent increase of GSSG. CYP2D6 and 2E1 inhibition diminished 3-MMC toxicity, but for CYP2E1 this effect was only observed for concentrations up to 1.3 mM.
Conclusions: Overall, our data point to a role of metabolism in the hepatotoxicity of 3-MMC, which seems to be triggered both by intrinsic and extrinsic apoptotic mechanisms. At higher drug concentrations, other cell death pathways might be at play, such as necrosis or autophagy, deserving further investigation.

References:

1. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). European Drug Report 2016: Trends and Developments. European Monitoring Centre for Drugs and Drug Addiction, Lisbon. 2016
2. Adamowicz P, Gieron J, Gil D, Lechowicz W, Skulska A, Tokarczyk B. 3-Methylmethcathinone-Interpretation of Blood Concentrations Based on Analysis of 95 Cases. Journal of Analytical Toxicology 40:272-276, 2016

CYTOTOXICITY OF 3-METHYLMETHCATHINONE (3-MMC OR METAPHEDRONE) IN PRIMARY RAT HEPATOCYTES

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Introduction

The XXI century has been witnessing a change in paradigm of drug abuse, through a steadily increase in the production of new psychoactive substances (NPS). As they are still legal in some countries, users mistakenly consider them safe. However, there are several reports of intoxications and deaths by NPS [1]. The synthetic cathinone 3-methylmethcathinone (3-MMC or metaphedrone) was recently introduced in the market to replace the illegal drug 4-methylmethcathinone (4-MMC or mephedrone). However, there is evidence of 3-MMC being as dangerous as 4-MMC to humans [2] though information on the toxicity mechanisms is scarce, demanding further investigation.

Aim

As the liver is one of the main sites of cathinones' metabolism, and therefore a relevant target for their deleterious effects, the aim of this study was to assess the pathways of toxicity of 3-MMC in primary rat hepatocytes.

Material and methods

Primary hepatocytes of Wistar rats were isolated by collagenase perfusion, cultured, and exposed for 24 h to the drug at a concentration range [from 31 nM to 10 mM] selected to provide a complete cytotoxic profile. Cell death was assessed through three assays, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, lactate dehydrogenase (LDH) leakage assay, and the neutral red (NR) uptake assay. Some of the mechanisms that could contribute to the observed cytotoxicity were also assessed by measuring the intracellular reactive oxygen and nitrogen species (ROS/RNS), reduced (GSH) and oxidized (GSSG) glutathione, adenosine triphosphate (ATP), alterations to the mitochondrial membrane potential (ΔΨm) and to caspase activities, after exposing cells to 1 µM, 10 µM, 100 µM, and 500 µM of 3-MMC. The modulatory effects of cytochrome P450 (CYP) inhibitors on the toxicity of 3-MMC were also evaluated.

Results

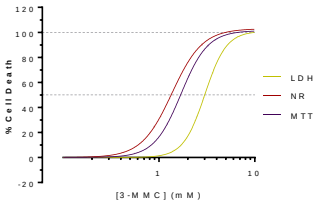

Figure 1 – Concentration–response curves obtained with the MTT reduction assay, LDH leakage assay and NR uptake assay.

Table 1 – NOEC and EC_{50} of MTT reduction assay, LDH leakage assay and NR uptake assay

NOEC (mM)			EC_{50} (mM)		
NR	MTT	LDH	NR	MTT	LDH
0.31	0.38	1.04	1.34	1.68	3.00

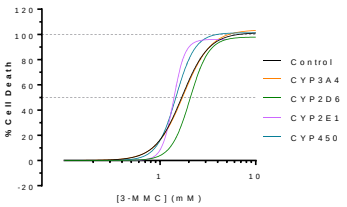

Figure 2 – Effect of the CYP 3A4, 2D6, 2E1 and general CYP450 inhibitors on the metabolism of 3-MMC

Results

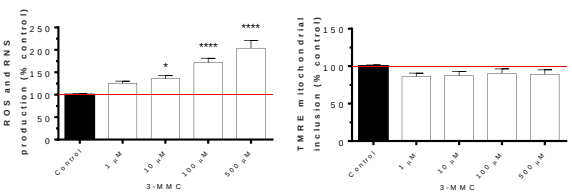

Figure 3 – Effect of 3-MMC on ROS and RNS production (left) and on the mitochondrial membrane potential, as measured by (TMRE) incorporated within the mitochondria (right)

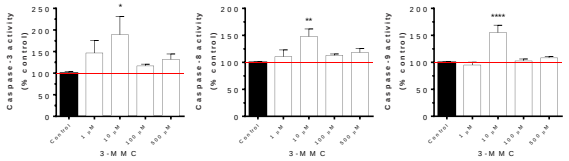

Figure 4 – Caspase -3, -8 and -9 activation after exposure to 3-MMC for 24h

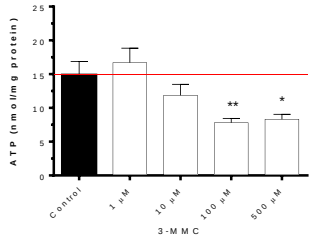

Figure 5 – Effect of 3-MMC on ATP intracellular content

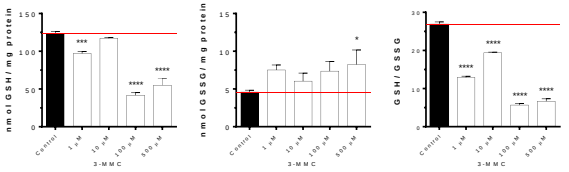

Figure 6 – Effect of 3-MMC on the total (tGSH) and reduced glutathione (redGSH), and on GSH/GSSG ratio

Conclusions

Overall, metabolism seems to be implicated in the hepatotoxicity of 3-MMC, which is suggested to be triggered both by intrinsic and extrinsic apoptotic pathways. At higher drug concentrations, other cell death mechanisms might be at play, such as necrosis or autophagy, deserving further investigation.

References

1. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). European Drug Report 2016: Trends and Developments. European Monitoring Centre for Drugs and Drug Addiction, Lisbon. 2016
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