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ASSESSMENT OF NEURONAL CYTOTOXICITY OF JWH-073 AND JWH-250

Work submitted by

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Dedicated to each deep breath my mom Heloísa and my father Pompeu had to bear my behavior these last few years, to the people of the Amazonas region, my home, and my proud to be South-American native

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Thank you God for being by my side always and everywhere.

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Thank you sis, Naísa Karla, and bro, Renanzinho, for never stop loving me.

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“O Homem que deseja ser Cientista e à Ciência dedicar seu tempo e amor tem ao menos três certezas: a de que morrerá um dia (como todo mundo), a de que não ficará rico (como quase todo mundo) e a de que se divertirá muito (como pouca gente).

(Prof. Newton Freire, Departamento de Genética, UFPR)

Certificate of originality

This is to certify that I am responsible for the work submitted in this thesis and that the work is original and not copied or plagiarised from any other source, except as specified in the acknowledgements and in references. Neither the thesis nor the original work contained therein has been previously submitted to any institution for a degree.

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Name: Carlos Victor Montefusco Pereira
Date: 15 July 2014

Abstract

Synthetic cannabinoids from marijuana herbal blends like ‘Spice’ and ‘K2’ are drawing the attention of drug of abuse organizations, including the UNODC[1], the EMCCDA[2] and emergency hospital all over the world. This concern rises from clinical episodes of psychotropic effects that go beyond the regular range of marijuana and THC – namely, panic attacks, psychosis, catatonia, addiction and withdrawal symptoms. Our study addressed two emergent synthetic cannabinoids (napthtoylindoles) denominated JWH-073 and JWH-250 that are currently detected on ‘Spice’-like products, in order to observe their cell toxicity profile on neuronal cells in vitro model (SH-SY5Y). Using 0.2% DMSO as negative control, MTT and LDH results revealed that within concentrations of 1, 5, 10, 25, 37.5 and 50 µM, JWH-250 is identified as ‘toxic’ in a statistically significant manner at higher concentrations. This work did not detect any statistically significant toxicity from JWH-073. This data suggests to extend these studies on new synthetic cannabinoids to neuronal cells with increased concentrations, as well as the application of assays assessing apoptosis (conditions and signalling), neuronal function and activity (as cell membrane potential assay) within differentiated cells as neurons and glia. At the same time, the evaluation of herbal mixtures of more than one cannabinoids and plant types is advisable in order to understand synergic effects.

Keywords: Synthetic cannabinoids. Forensic toxicology. Illicit drugs. Napthtoylindoles. Phenylacetylindoles. Marijuana.

[1] United Nations Office on Drugs and Crime.
[2] European Monitoring Centre for Drugs and Drug Addiction

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List of abbreviations

Δ9THC - delta-9-tetrahydrocannabinol

µM - micromolar

ATCC - American Type Culture Collection

ANOVA – Analysis Of Variance

AEA - N-arachidonylethanolamide; anandamide

cAMP - Cyclic adenosine monophosphate

CB - cannabinoid receptors

CDC – Center for Diseases Control

CNS - Central Nervous System

CP - cyclohexylphenol

DEA - Drug Enforcement Administration

DMEM - Dulbecco's Modified Eagle's Medium

DMSO – dimethylsulfoxide

DNA - Deoxyribonucleic acid

EMCDDA - European Monitoring Centre for Drugs and Drug Addiction

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EU – European Union

GABA - Gamma-AminoButyric Acid

GC-MS - gas chromatography – mass spectrometry

GHB - gamma hydroxy butyrate

GPCR - G protein-coupled receptors

g/mol – grams per mol

GRK – G protein-coupled receptors kinases

h – hours

H^+ - hydrogen ion

HEK-293 - Human Embryonic Kidney 293 cells

HU210 - Hebrew University 210

IC_{50} - inhibitory concentration 50%

INCB - International Narcotic Control Board

JWH – John W. Huffman

LCMS - liquid chromatography – mass spectrometry

LC-MS/TOF - liquid chromatography – mass spectrometry/time of flight

LDH – Lactate desidrogenase

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

mTOR - mammalian target of rapamycin

mg/kg – milligrams per quilo

mg/g – milligrams per gram

mL – millilitre

NAD^+ - Nicotinamide adenine dinucleotide

$NADHH^+$ - Nicotinamide Adenine Dinucleotide - Hydrogen (reduced)

ng/mL – nanograms per millilitre

nM - nanomolar

NPS - novel psychoactive substances

OD – optical density

pen strep – penicillin streptomycin

pg/mg – pictograms per milligrams

PKB - protein kinase B

PPAR - peroxisome proliferator-activated receptor

S.D. – standard deviation

SC - synthetic cannabinoid

TB – Trypan blue

THC – Tetrahydrocannabinol

TRP - transient receptor potential cation channel

US – United States

UHPLC-MS/MS - ultra high performance liquid chromatography-tandem mass spectrometry

UV – ultraviolet

UK – United Kingdom

UNODC - United Nations Office on Drugs and Crime

WHO - World Health Organization

Content

1. Introduction

1.1. Background

In 2013, a 31 year old female was brought to the Emergency Department, following a three stories fall from her apartment's fire escape. She was unresponsive and, at the scene, was diagnosed as a 3 on the Glasgow Coma Scale[3]: she could still open her eyes in response, but she uttered words and had abnormal reactions to pain stimulus. The woman was intubated with no need to use general anaesthesia. Her radiographic data showed a large subdural hematoma, facial fractures, pelvic fractures, liver laceration and elbow fracture. On day 10 of hospitalisation, the patient died from her traumatic injuries.

According to her husband, on that evening, while rehearsing for a play, she ingested a "pot brownie". Shortly after, she began to feel "weird" and called her husband at work for help; when he got home she expressed her intention to jump off the balcony. He gave her water and induced vomiting and physically restrained her to the bed twice afterwards. She however broke free and jumped.

The medical doctors reasoned that marijuana does not normally cause such symptoms and while the patient's urine routine toxicology was negative for cannabinoids, further testing was performed with gas chromatography – mass spectrometry (GC-MS), searching for delta-9-tetrahydrocannabinol (Δ9THC) and other products of THC or marijuana metabolism. Those were all negative, however, the patient's ante mortem serum was tested with liquid chromatography – mass spectrometry/time of flight (LC-MS/TOF). A new compound never detected in patients before was found in her serum; it was JWH-175, one of the many emerging members of the synthetic cannabinoid (SC) family. This case was reported by Dr. Armenian, a medical doctor from the Emergency Department of the Hospital-University of California, at San Francisco (Armenian, 2014).

Marijuana derives from the *Cannabis sativa* plant, which expresses around 420 chemical compounds, of which eighty terpeno-compounds are specifically found in *C. sativa* and are named phytocannabinoids (Mechoulam and Hanus, 2000). Utmost consideration has been dedicated to Δ9THC, the most psychotropic constituent of marijuana, but Izzo *et al.* (2009) presented a picture (Figure 1) of pharmacological activities of other

[3] The Glasgow Coma Scale provides efficient recording of the state of conscience of a patient in which assesses motor, verbal and eye responses. It is used a textbook practice on Emergency Medicine. (CDC, 2003)

phytocannabinoids: as cannabinol, cannabidiol, delta-9-tetrahydrocannabivarin, cannabichromene, cannabigerol, delta-9-tetrahydrocannabinolic acid and cannabidiolic acid.

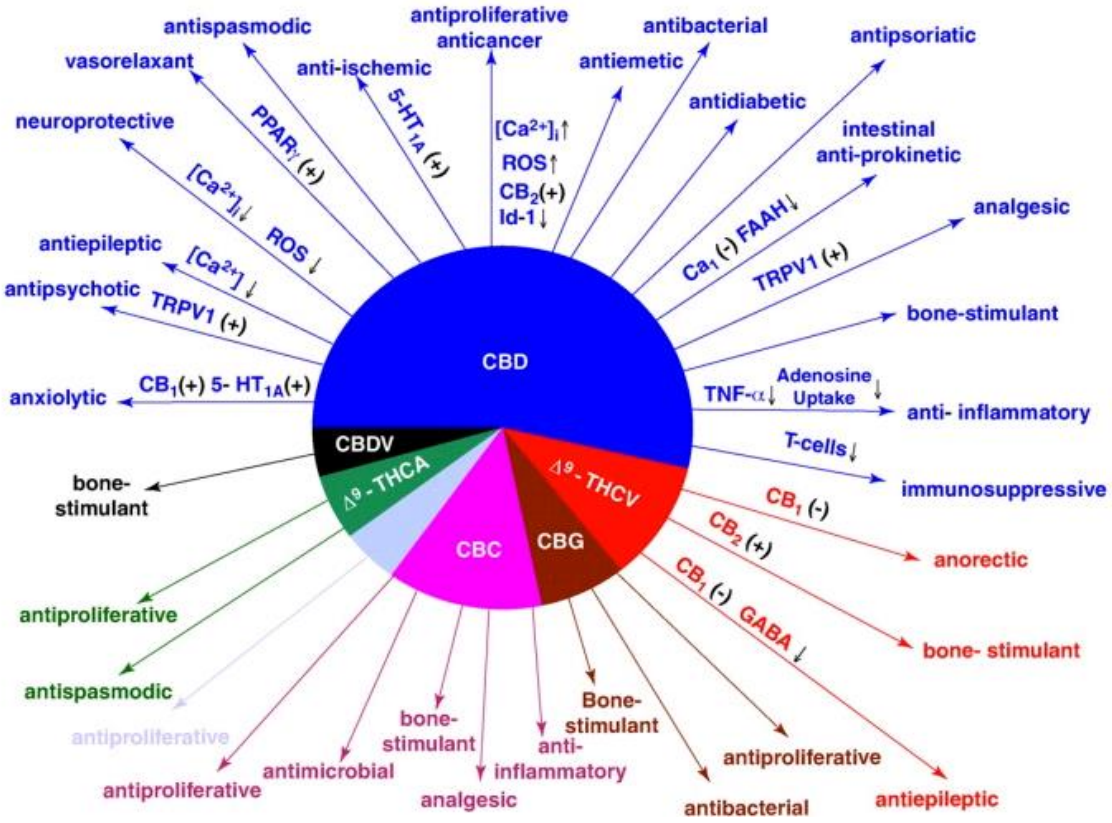

Figure 1. Pharmacological activities of non-psychotropic cannabinoids (and its suggested mechanisms of action). (Izzo *et al.*, 2009)

These compounds are associated with binding G protein-coupled receptors (GPCR), denominated CB1 and CB2 (cannabinoid receptors). This discovery implied the existance of endogenous compounds as ligands, usually referred to as endocannabinoids (DiMarzo, 2008). Figure 2 shows how the literature categorises these elements among the ligands and cannabinoid compounds.

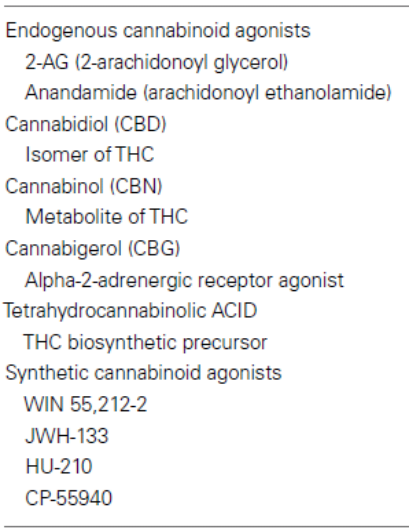

Figure 2. Types of cannabinoids. (Greydanus *et al.*, 2013)

The cannabinoid system consists of Central Nervous System (CNS) cannabinoid receptors and their endogenous ligands or triggering molecules that bind to a target protein site. Tetrahydrocannabinol (THC) is the main active principle in marijuana. In the 1980s, researchers began to synthesise THC analogues and look for marijuana metabolites (Greydanus *et al*., 2013). These studies led to the development of new classes of bicyclic cannabinoids (as CP55,940), the aminoalkylindoles (as WIN55,212-2), among others; the present study is focusing on members of the naphthoylindoles class.

The JWH[4] type of synthetic cannabinoids belongs to naphthoylindole class. The physical and psychological effects of JWH cannabinoids are similar – but stronger – to those of Δ9THC. In fact, it has been stated that JWH binds 4x more than THC to the CB1 and 10x more to the CB2 receptor (Wintermeyer *et al.* 2010).

Dr. Huffman intended to explore the analgesic potential properties of CB1 agonists, but by describing and publishing JWH synthesis he *de facto* primed the production of this synthetic drug family.

In 2008, forensic investigators in Germany and Austria were able to detect JWH-018, a synthetic cannabinoid as a primary compound of the recreational drug in "Spice" (in fact, according to the German press, these products were being sold since 2002 - Frankfurter Rundschau, 2008). These so-called "Spice"-like products are herbal blends sprayed with synthetic cannabinoids drugs – sometimes even more than one SC. This addition is not described on any label or part of the product, which is commercialized with several different

[4] After John W. Huffman, whi first synthesized these compounds at Clemson University.

brand names: K2, Spice Gold or Silver, Yucatan Fire, and several others. After 2008, various cannabinoids were detected in marijuana blends.

The use by humans of novel psychoactive substances (NPS), such as synthetic cannabinoids, instead of traditional illegal substances – (e.g. cannabis) is a fairly recent practice (UNODC, 2013). However, the majority of European countries list prohibited/controlled synthetic cannabinoids and their commercialisation is being scrutinised by the authorities. These drugs are hard to deal with from the point of view of their legality. They are labeled as `legal highs`, an umbrella term impliying that they have not yet been "reviewed" by legislators, therefore being indirectly legal. This is then an area characterised by limited data with unknown risks. (EMCDDA, 2009)

Legislators are delayed on evaluating the danger of NPS, but emergency rooms see an increased number of cases. Izzo *et al.* (2009) showed a summary of the first agonists of cannabinoid system and the way they could modify our physiology, when in fact, these have the characteristic of being non-psychoactive drugs. This has suggestive implications on the effect of psychotropic, psychostimulant agonists of CB1 and 2 such as synthetic cannabinoid compounds.

About 20 case studies with full medical and toxicological assessment are published in the literature and two of them are summarized here. In 2012, a 16-yr old was submitted at the emergency room with altered mental status and was hospitalised overnight in catatonic state after smoking "K2"; a 18-yr old was described as agitated, aggressive and had profuse sweating (Cohen *et al.*, 2012) after using "Spice". In 2013, a fatal case was described in which postmortem blood showed the presence of JWH-018 (0.1 ng/mL) and JWH-122 (0.3 ng/mL) together with amphetamine. The conclusion of this report alerted that synthetic cannabinoids are more potent and effective than THC, potentially leading to life-threatening situations (Schaefer *et al.*, 2013).

From the emergency room to the basic sciences laboratory, there are studies evaluating the toxicity of SC in cell culture. The tests to determine the direct cell toxicity of synthetic cannabinoids is an emerging field and reports have demonstrated that naphthoylindoles synthetic cannabinoids (JWH-018, 073, 122, 210 and of one benzoylindole, AM-694) damaged the cell membranes of buccal (TR146) and breast (MCF-7) derived cells. In addition, no cytotoxic responses were seen in assays which assess the mitochondrial damage, protein synthesis or lysosomal damage (Koller *et al.*, 2013). On human NG 108-15 (neuroblastoma-glioma) cell lines, it was found that all three of cyclohexylphenol (CP)

bicyclic classes of synthetic cannabinoids tested, were cytotoxic in a concentration-dependent manner. They further found that cell death was mediated by the CB1 receptors and not the CB 2 receptors (Tomiyama & Funada, 2011). Another study demonstrated that all 3 types of cannabinoids (endo, phyto and synthetic) can induce apoptosis in cells, even in the absence of CB receptors (Athanasiou *et al.*, 2007).

There is an emerging body of evidence that these compounds are being pursued with the original intention of developing new therapeutics. This establish another field of research on synthetic cannabinoids. There are numerous pieces of evidence of non-toxic potential use of SC on the recovery of myelination in multiple sclerosis neuronal models (Arévalo-Martín, 2003; Downer, 2011) and still onto the analgesic effects for cancer patients (Reynolds, 2013). The paradox is what some SC do possess therapeutical use but the abuse of other SC in spiked marijuana can cause human intoxication. Therefore, toxicological assays are paramount to know how harmful these new psychoactive substances can be.

With the spreading use of marijuana nowadays (plant consumption; pharmaceuticals for cancer-related symptoms – Bar-Sela *et al.*, 2013; epilepsy – Devinsky *et al.*, 2014; obesity – Le Foll *et al.*, 2013; schizophrenia – Robson *et al.*, 2014; improved appetite and sense of taste – Brisbois *et al.*, 2011) and the near future possibility of marijuana legalization worldwide, there is a chance of illegal markets investing in synthetic cannabinoids for their potency and addictive potential. Toxicological assays are vital to assess the harm potential of these novel psychoactive substances.

1.2. Cannabinoid system, receptors and types of chemicals

CB1 is the first receptor cloned from the cannabinoid system by Matsuda *et al.* (1990), using a library of rat complementary DNA. CB1 has been acknowledged for behavioural alterations caused by tetrahydrocannabinol (Monory *et al.*, 2007), possibly also on humans (Huestis *et al.*, 2001). Munro *et al.* (1993) discovered CB2, a second receptor, which according to Chiou *et al.* (2013) share 44% global identity (68% for the transmembrane domains) with CB1. Altogether, both CB1 and CB2 are GPCR that engage onto the Gi/o class (guanine nucleotide-binding protein, alpha inhibiting activity) of G proteins and are considered agonist leading to the blockage of adenylyl cyclase and following reduction of Cyclic adenosine monophosphate (cAMP) (Howlett *et al.*, 2002).

Cell biologists found that CB1 can stimulate the initiation of mitogen activated protein kinases signalling, (Daigle *et al.*, 2008) influencing cell movement, growth and diversification (Derkinderen et al., 2003). With continued stimulation, the CB1 receptor is regulated by desensitisation and internalisation of receptors (Hsieh *et al.*, 1999). In fact, desensitisation is thought to be regulated by kinases of GPCR (so called GRK2 and GRK3) and β-arrestin (Premont & Gainetdinov, 2007). While other molecular pathways like protein kinase B (PKB or Akt) and mammalian target of rapamicin (Manning and Cantley, 2007) are induced by CB1 receptor, CB1 agonists, like WIN55212-2 (one of the first synthetic cannabinoids) was shown to be involved in the rodent and human brain of cocaine addiction (Alvaro-Bartolome *et al.*, 2011). The CB1 receptor was also found intracellularly in mitochondrial membranes (Benard *et al.*, 2012), while on transfected HEK-293 cells CB1 is inside intracellular vesicles and plasma membrane (Leterrier, et al., 2004).

The CB1 receptor has also been characterised on neuronal cells, including its involvement on the inhibition of voltage gated Ca^{+} channels and activation of K^{+} channels (Mackie *et al.*, 1995). It has been found to reduce cellular excitability and the chances of neurotransmitter release (Shen *et al.*, 1996), as well as detaining inhibitory and excitatory synapses, as found with glutamatergic and GABA terminals (Chevaleyre *et al.*, 2006).

Overall, CB1 activity affects neurotransmission, but mostly for its predominance in crucial locations in the human body. Mackie (2005) described the expression of CB1 in the central nervous system, particularly in axon terminal and primarily at the cortex (anterior cingulate, limbic area), cerebellum, hippocampus, amygdala, basal ganglia, hypothalamus and not often in the brain stem – helping cannabinoids to have low toxicity if accidental (Herkenham *et al*., 1990). Since the CB2 receptor is predominantly located on immune cells (Galiegue *et al*., 1995), but also in the CNS as in microglia, spinal cord and brainstem (Van Sickle, *et al.*, 2005), it was suggested as a novel target for pain and inflammation management (Onaivi *et al.*, 2012).

Cannabinoid activity (mainly endocannabinoids) is not restricted to the CB1/CB2 receptors: other receptors like the transient receptor potential cation channel (TRP) and the peroxisome proliferator-activated receptor (PPAR) have been investigated as potential cannabinoid binding sites (Pertwee *et al.*, 2010). Studies on synthetic cannabinoids, knockout mice and genetic tools have uncovered new sites of activity that can lead to the discovery and classification of new cannabinoid receptors to be defined (Hajos *et al*., 2001; Breivogel *et al*., 2001)

1.3. Synthetic cannabinoids precedent

Endocannabinoids are part of marijuana-related research, whilst AEA (arachidonic acid moiety, N-arachidonylethanolamide), was entitled “anandamide” - according to Sanscrit for “bliss” -, a range of pharmacological tools and new synthetic cannabinoids have been established. The classification of cannabinoid compounds is settled with five categories, including the classical (for instance, Δ9THC and HU210 – developed by Dr. Mechoulam at Hebrew University), non-classical (as CP-55,940), indoles (WIN 55,212), eicosanoids (mostly endogenous as AEA and 2-arachidonylglycerol) and pharmacological antagonists (AM251 and AM630, developed by Pfizer) (Devane *et al.*, 1992). And its structures are presented below:

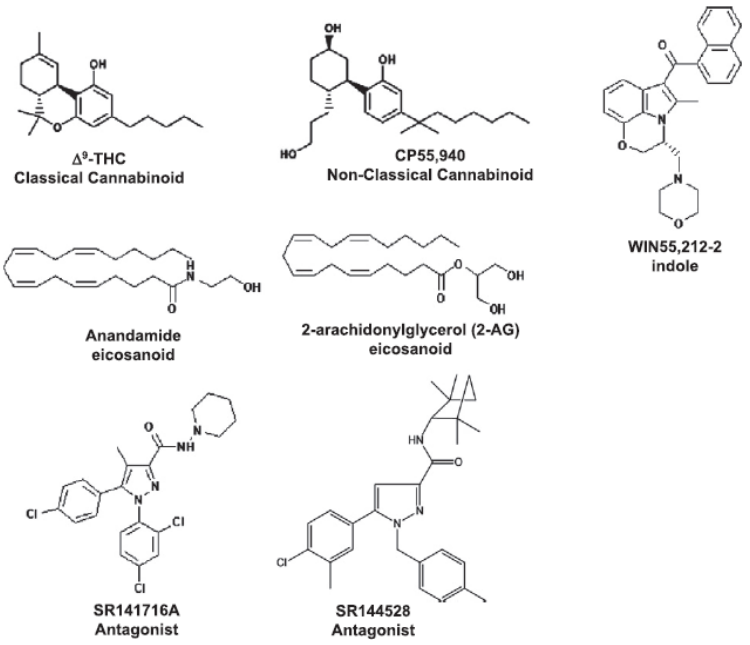

Figure 3. Structure classes of cannabinoids (Console-Bram *et al.*, 2012)

However, synthetic cannabinoids can have its own classification according to the chemical structure and Bretteville-Jensen *et al.* (2013) provides grouping as presented in Figure 4 that contemplates the important difference and resemblances of each category.

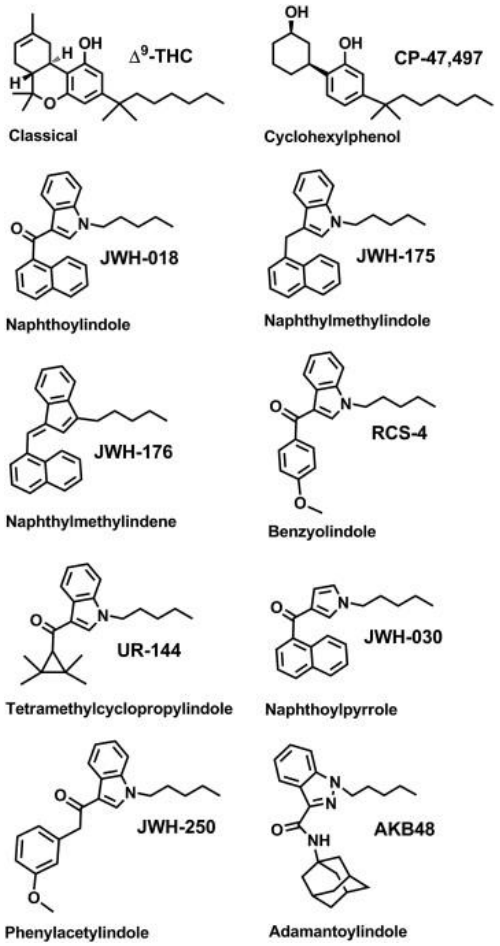

Figure 4. Structures of representative synthetic cannabinoids categories, and representative compound, commonly found in "Spice/K2" products (taken from Fattore and Fratta, 2011).

Gaoni and Mechoulam (1964) first exposed Δ9THC as the utmost psychoeffective constituent in marijuana and from that point, numerous additional typical and non-typical cannabinoids have been examined for medicine usage. However, structure-activity relationship study of Δ9THC shows cannabinoid pharmacological properties for any chemical with affinity for the cannabinoid receptor. This presents a challenge in producing a single effect selectivity of a cannabinoid drug. (Compton *et al.*, 1993)

Marijuana alternatives, mistakenly advertised as "incenses", are recent on the market of drugs of abuse (Seely *et al.*, 2011) and are frequently known as "K2" or "Spice" are mixed with synthetic cannabinoids that own cannabis-like effects (Auwarter *et al*., 2009). The International Narcotic Control Board (INCB) mentions that there is an increase of manufacture, together with market of the novel 'designer drugs' and their availability, it is becoming uncontralable (INCB, 2011).

Currently, we are not aware of the entire chemical content of these new drugs, their acute or chronic toxicity, what can be translated into a worrisome obstacle to public health. For instance, in the United Kingdom, some first 'legal highs' (to be noted: piperazines, "spice" and mephedrone) were already deliberated by the Misuse of Drugs Act of 1971, nonetheless, the introduction of legal boundaries over these drugs presented little changes on the drug scenario and, at least online, the banned drugs continue to be sold as new brands. These new brands are commercialized as greater products, and, as licit options to the banned drugs (Ramsey *et al*., 2010). We are not aware of the new brands' content, if it comprises of new synthetic and legal compounds or if it possesses the amount of illegal compounds that do explain the link of a number of related deaths (Baron *et al.*, 2011; EMCCDA, 2011). In Europe, the EU Early Warning System has received 81 new psychoactive substance in 2013, which 29 were synthetic cannabinoids, as presented on Figure 5, that shows constancy of NPS apprehensions all over Europe.

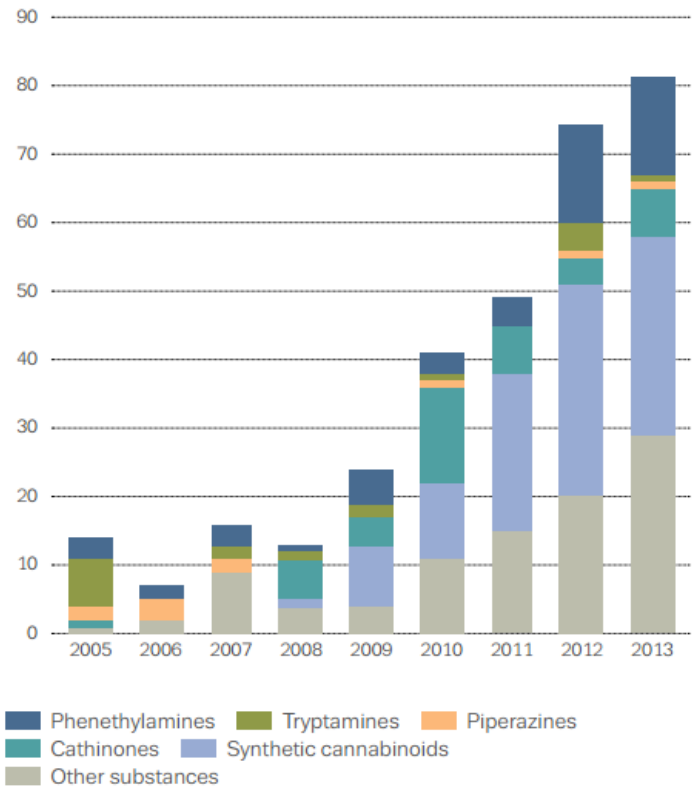

Figure 5. NPS notification from member countries of the EU Early Warning system (EMCDDA, 2014)

There is then a confusing perception of safety to some of the users (Sheridan and Butler, 2010), when actually there is no information on the psychology or behaviour consequences from the use in humans, especially for drugs like the synthetic cannabinoids (EMCCDA, 2009). In fact, products, when labelled, not always show its real content (or more worrisome, the manufactures could not even tell the content), even for products with the same name and brand (Davies *et al.*, 2010). In the end, users are having their health comprimised when exposed to unknown drugs of unidentified concentrations and even frequent users of the same product could be purchasing a different drug or a more potent one. This possibility can be extended to the concern of drugs interaction and the effects of metabolites in human physiology. Although, some research is being done to unravel toxicity cases (Salmner *et al.*, 2010), the current challenge puts the non-identification or association of adverse effects of these drugs by clinicians at emergency rooms (Smith *et al.*, 2011), together with the difficulty in identifying the drugs, the new ones and unique compounds (Houston, 2011).

Meanwhile, a minor quantity is necessary in order to provoke a result and the minimum amount that can be bought is of one gram. Consumers will still go to hospital departments across the world, and medical doctors will need prompt information on these new drugs, their consequences and risks. Daily, the professionals, who are in contact with NPS cases, suffers with the scarcity of scientific and medical data. Investigation in this field is not all consolidated, medical clinical cases do not contemplate the reality and amount of episodes at the various hospitals and the health system of countries have not yet develop a central database that combines toxicology and forensics (Boyce, 2011).

Contributing to the risk scenario, the absence of safety guidance (how to use, overdosage, adverse effects) on the online market of NPS drugs is justified by putting labels as “not for human consumption”. This act does not stop the consumers use and it represents an attempt from the manufactures to avoid health vigilance and regulations, bringing the chance of adding anything to the herbal blend. When there is the application of legal prohibition, the effect is not only the supply and demand of the products, but instead it leads an invitation to chemists from the illegal market to change the molecular structure of drugs. This action is creating alternatives that do not fit into the control of one chemical compound, but it is evolving the discovery of stronger chemicals than the ones before. The concept of harm is discussed as the legality of new compounds is confusing to scientifically “calculate” the harm to fill in the legislation and it is constantly being argued (Rolles and Measham, 2011).

Even though synthetic cannabinoids were designed to provide insights into the cannabinoid organization (Huffman *et al*., 1994), numerous synthetic cannabinoids turned out as drugs of abuse, apparently as these could present advantages resembling marijuana; to mention, the non-appreciation and awareness of drug legislation or detectability on conventional drug-urine exams (Seely *et al.*, 2012). From that, studies endorse comparable physiological reactions from 'Spice' and cannabis use (Zimmermann *et al.*, 2009, this paper describes a patient case that was undertaking cannabis-like withdrawal and tolerance symptoms subsequently the stop of 'Spice' use). Disturbingly, the usage of K2 (one of the synthetic cannabinoids commercial product) has a significant occurrence of serious adverse effects, that are not usually described with marijuana (tachycardia with hypertension, anxiety attacks, seizures, psychosis and hallucinations (Harris & Brown, 2013).

With the occurrence of synthetic cannabinoids in commercial herbal blends from 2008, various research cores have use liquid or gas chromatography with mass spectrometry (LCMS; GC-MS) in order to point out its presence on the content of 'K2/Spice' (Lindigkeit *et al.*, 2009). Remarkably, Δ9THC was not found in the analyses of 'Spice' samples, proposing that the physiological impact of these substances were happening because of the synthetic cannabinoid 'cutting agents' (Auwarter *et al*., 2009). The investigating group that used LC/GCMS and identified synthetic cannabinoids did find ones as JWH-018, JWH073 and CP-47,497 in a concurrent presence as components of the marijuana products. The prediction nowadays is that more new compounds will be encountered to be sprayed on 'Spice' and it is already true to findings of JWH398 and JWH250 in Germany and the UK (Vardakou *et al.*, 2010).

1.4. JWH-073 (1-butyl-1H-indole-3-yl)-1-naphthalenyl-methanone)

Dresen *et al.* (2010) mention the initial cannabimimetics had succumbed to new, yet similar, synthesised ones that represents an escape of the European legislation assessing and prohibiting JWH and CP's; while Lindigkeit *et al.* (2009) places JWH-073 (JWH-018 homolog, using instead a butyl radical) as a substitute to the prevalent JWH-018 (structural comparison below).

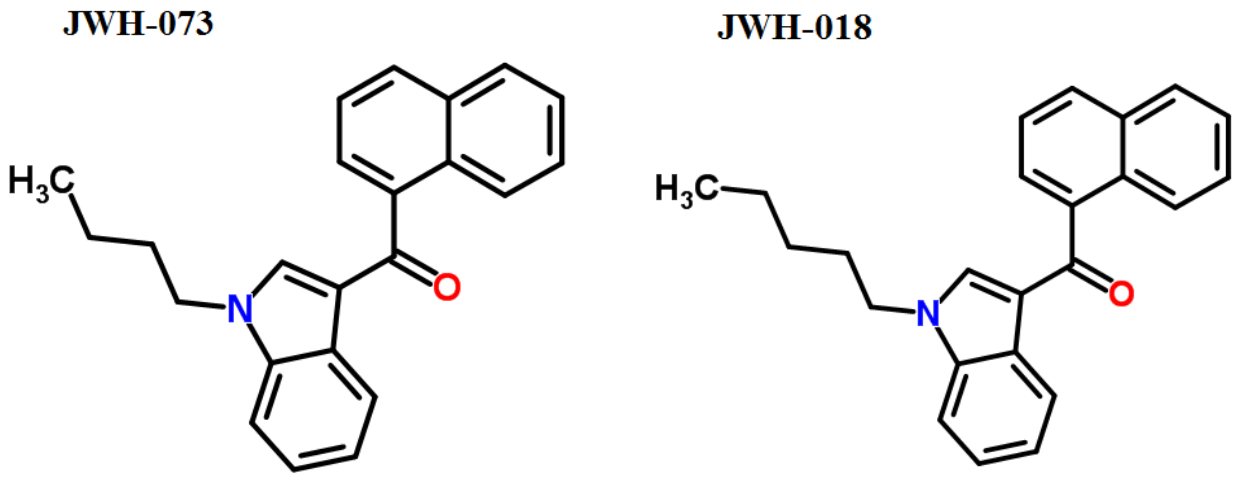

Figure 6. Structural comparison for JWH073 (1-Butyl-1H-indol-3-yl)(1-naphthyl)methanone, $C_{23}H_{21}NO$) and JWH018 (1-Naphthyl(1-pentyl-1H-indol-3-yl)methanone, $C_{24}H_{23}NO$) (Adapted from JWH-018 and JWH-073, ChemSpider, 2014)

November 24th of 2010 was the date of ban of JWH-073 and other four synthetic cannabinoids, by including these compounds as Schedule I (meaning, high potential for abuse, no accepted medical use, lack of accepted safety) of the Substance Act from the DEA (Drug Enforcement Administration) in the United States (Young *et al*, 2012). This action is already a consequence from intoxication cases, although it pushes the DEA to figure out toxicology in order to describe and explain why the ban has happened. JWH-073 in high concentration on synthetic marijuana is considered unlikely, or more as an additive or impurity of producing JWH-018, but also, there is a chance for JWH-073 detection as consequence of metabolism of AM-2201, a newer synthetic cannabinoid, or from the decarboxylation of JWH-018 (Hutter, 2013).

JWH-073 is described to possess increased efficacy, nearly, 5-fold greater than Δ9THC (Brents *et al.,* 2012) and, although, JWH-018 and JWH-073 are scheduled as narcotics in Germany, this does not mean these compounds have been banned from the commercial products. In fact, a study using new method of LC-MS (Kneisel and Auwater, 2012) identified JWH-073 in serum of patients from emergency rooms and cases of criminal investigation on levels of 7.1 ng/mL, in an average of 0.85 ng/mL. As far as cases of intoxication are concerned, JWH-073 has been described to be involved on Cannabinoid hyperemesis syndrome (Hopkins and Gilchrist, 2013) or cardiotoxicity (Young *et al.*, 2012).

Based on a study assessing the potential of CB1 agonists (Atwood *et al.*, 2011), JWH-073 inhibits neurotransmission (IC_{50} of 49.4 nM) by reducing excitatory postsynaptic currents in hippocampal neurons; however it is less potent resembling JWH-018 (IC_{50} of 14.9 nM). Together with neural inhibition, the issue of tolerance has been put onto the synthetic

cannabinoids and the main assessment is through receptor sensitisation and internalisation; in which, JWH-073 is considering for Atwood *et al*. (2011) to produce slower internalisation (about 105 minutes) than JWH-018 (37 minutes). However, Wu *et al.* (2008) describes faster desensitisation for JWH-073, instead of JWH-018. In the end, the studies provide inferences that needed to be assessed *in vivo*, but it can be strong evidence on clinical reports of withdrawal and tolerance cases (as described on Zimmermann *et al.*, 2009).

In a structure-affinity study of indoles synthetic cannabinoids (Aung *et al*., 2000), there was the observation of influence of changes in the N-1 alkyl length of the chain on CB1/CB2 binding. For JWH-018, the kinetic values are CB1: 9.00 ± 5.00 and CB2: 2.94 ± 2.65 nM, while for JWH-073, the values for CB1: 8.90 ± 1.80 and CB2: 38.0 ± 24.0 nM (while Δ9THC had values of CB1: 40.7 ± 1.7 and CB2: 36.4 ± 10 nM). These values and the increase of the alkyl chain shows a decrease of binding the bulkier the compound is, but interestingly presents JWH-073 low binding, gathering important data for the reflect on human physiology. That can be evidence to explain results as JWH-073 having the same discriminatory effects as Δ9THC in rhesus monkeys (Ginsburg *et al*., 2012), hypo motion, stillness, anti-nociception, and hypothermia in mice (Hruba *et al.*, 2012).

Concerning cytotoxicity, JWH-073 has been evaluated in a range of 75-100 μM, to be toxic to cell lines from hepatoma, buccal epithelium and mammary tissue, using lactate desidrogenase assay (only at 100 μM), but this same drug was not considered toxic with XTT assay that assess mitochondrial function. There was evaluation of genotoxic effects with positive toxic results of JWH-073 (also at 100 μM) in buccal cells (remembering that JWH are smoked and buccal cells are one of the first ones affected) and hepatoma cells. (Koller *et al.*, 2013)

1.5. JWH-250 (2-(2-Methoxyphenyl)-1-(1-pentyl-1H-indol-3-yl)ethanone)

Korean market for synthetic cannabinoids still has focus on JWH-018 or JWH-073, however, in the last years, new compounds have appeared in seized synthetic marijuana, as JWH-250 and the AM chemicals (Jang *et al.*, 2014) and this same situation has been reported in other countries (JWH-250 detected: Turkey [Gurdal *et al.*, 2013]; Italy [Gattardo and Tagliaro, 2011]; United States [NORCHEM, 2012]; Norway [Tuv *et al.*, 2014]).

In fact, JWH-250 is mentioned to be first detected in Germany, on 2009, and it is classified as a phenylacetylindole (see Fig. 7) with receptor CB1 affinity of Ki = 11nM; KI= 33nM for CB2 (Huffman *et al.*, 2005; Dargan *et al.*, 2011), and, according to the UNODC (2011), it is considered controlled substance in Austria (Oct 2010), Denmark (Mar 2010), Germany (2011), Japan (Sep 2010), Lithuania (May 2009), Romania (Feb 2010), Switzerland (Dec 2010) and the USA (Jul 2012, DEA 2013).

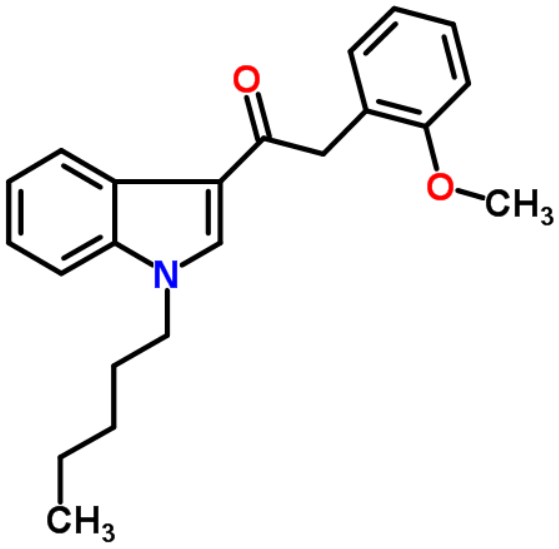

Figure 7. Structural molecule for JWH250 2-(2-Methoxyphenyl)-1-(1-pentyl-1H-indol-3-yl)ethanone, $C_{22}H_{25}NO_2$ (Adapted from JWH-250, ChemSpider, 2014)

For this person, several methodologies of JWH-250 detection have been developed, even with the difficulties in detecting new synthetic cannabinoids, new ways of screening have been researched, including hair detection by ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) that showed real cases enclosing 4.8-83.4 pg/mg (Salomone *et al.*, 2014). While at the emergency departments, cases of intoxication in which JWH-250 is present (in a mix of other cannabinoids) shows symptoms as:

- In 38% of the cases, symptoms as nervousness, impatience, acute psychosis, and hallucinations, and, also, light and external stimulus hypersensitivity and panic reactions (Hermanns-Clausen *et al.*, 2013)
- In 18% of the cases, nervousness, confusion, hallucinations. (Lonati *et al.*, 2012)
- Lethargy, catalepsy, damage of vision and speech. (Westerbergh and Hulten, 2011)

However these symptoms are from episodes in which JWH-250 was not the only synthetic cannabinoid involved and the detected levels of this drug on human serum was of 0,1-0.4 ng/mL, providing intoxication signs in 6 to 24h (WHO, 2014). In JWH-250 smoking self-studies (product '8-Ball', a mix of JWH-250, JWH-019, JWH-081 and RCS-4 in approximately 10 mg/g), levels of this drug reached the blood as 10 ng/ml (after 20 minutes) and 10 ng/ml in oral fluid, while reached a peak of 140 ng/ml of its 4-hydroxylated metabolite in urine 1 hour after smoking (Adams and Logan, 2011). A review from the Expert Committee on Drug Dependence (WHO, 2014) points out that there is no study on dependence data or abuse potential and "No pre-clinical safety data are available about the toxicity, reproductive impact and mutagenic/carcinogenic potential of JWH-250".

1.6. Study rationale

This study intends to assess the cellular toxicity of JWH-073 and JWH-250, synthetic cannabinoids identified as drugs of abuse. The cell lineage model is SH-SY5Y, a human-derived neuroblastoma-glioma line. This cell line shows adherent behaviour and possesses the ability to differentiate along the neuronal type of cells (La Quaglia and Manchester, 1996).

Cell toxicity is initially evaluated with the metabolic reduction of 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Via endocytosis or protein facilitation, MTT enter cells and is reduced, by mitochondrial enzymes, to produce a purple compound (formazan). This compound is generally not permeable to membranes, therefore accumulating inside live cells. Dissolution of formazan crystals in the cells releases a purple product that is identified using a spectrophotometer. The cells' capability of reducing MTT signals mitochondrial physiology and therefore an indication of cell viability. (Maioli *et al*, 2009)

Cell death is then assessed focusing on the impairment of plasma membrane. The LDH assay measures lactate dehydrogenase as a steady cytoplasmic enzyme which is present in all cells. If the membrane is impaired, LDH diffuses to the culture medium. The LDH reaction is evaluated by a colorimetric assay, in two steps: first, NAD^+ is reduced to $NADHH^+$, then, via LDH and with the reaction mixture of the assay kit (containing diaphorase enzyme), an H^+ is transferred to a tetrazolium salt molecule reduced to formazan product. Formazan here is then a marker for cell death. (Chan *et al*., 2013)

1.7. Research questions, Aim and Objectives

1.7.1. Key research questions/hypotheses

- Are JWH-073 and JWH-250 cytotoxic to neuronal cells? To what extent of dose dependency?
- If cytotoxic, do these chemicals act on cell metabolism and cytoplasmatic leakage?
- Can CB1 and CB2 receptors facilitate synthetic cannabinoids binding and possibly affect their toxicity?

1.7.2. Aim

To test neuronal toxicity of JWH-073 and JWH-250 on SH-SY5Y cell lineage.

1.7.3. Objectives

- Test neuronal toxicity focusing on mitochondrial damage with the MTT assay
- Test neuronal toxicity focusing on the release of cytoplasmatic content with the LDH assay
- Explore the use of SH-SY5Y human neuroblastoma cells, as cell models of toxicology for synthetic cannabinoids.

2. Experimental

2.1. Reagents and Equipment

All JWH were acquired from Lipomed, Switzerland. Stock solutions of JWH 018 (molecular mass = 341.5 g/mol), JWH 073 (molecular mass = 327.4 g/mol) and JWH 250 (molecular mass = 335.2 g/mol) were prepared to the final volume of 1ml and concentration of 25000 µM in pure sterile DMSO. The groups of samples applied were one blank (no cells, all other MTT components), one negative control (cells, 0.2% DMSO, no JWH)[5], one positive control (cells, 1% Triton X-100, no JWH), 6 concentrations of each JWH (1, 5, 10, 25, 37.5, 50 µM)

MTT (Thiazolyl Blue Tetrazolium Bromide; Sigma-Aldrich, Portugal; 0.5 mg/ml) solution was prepared extemporanely in each time to read a new plate. For that, knowing the amount of wells to be used on the assay, we weighted in a semi analytical scale (Sartorius, Germany) of pure MTT to be dissolved on sterile conditions inside the vertical laminar flow.

LDH (Lactate desidrogenase Kit, Clontech, USA) was prepared according to manufacturer's instructions for 100 tests.

For cell culture and assays, we used a laminal flow hood (Scanlaf, Mars Safety Class 2), T25 culture plastic flasks (25 cm², volume of 60ml, VWR, Belgium), Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Portugal) supplemented with 10% fetal bovine serum and 1% pen strep + glutamine (both from Gibco, Portugal), a cell culture incubator (Water jacket incubator, ShelLab, USA), an inverted light microscope (Zeiss, Germany), Phosphate Buffered Saline (Gibco, Portugal), TrypLE™ Express (1X; Life Technologies, Portugal), a Neubauer hemocytometer chamber (Hirschmann EM Techcolor, 0.0025 mm^2, depth 0.1 mm) centrifuge machine (Sigma 3-16PK, Portugal), Trypan Blue dye (Sigma, Portugal), 96-well microplates (Corning Inc., USA).

2.2. Cell Culture

Using nitrile non-powdered gloves and disposable lab coats, the laminal flow hood was sterilized with 70% ethanol, UV was applied for 15 minutes then the blower was turned on, each time before and after every use. Before handling all culture materials, analyst hands

[5] In order to investigate more on DMSO toxicity effects, we added a second negative control of 0.4% DMSO (5.2 µl of pure DMSO + 1294.8 µl supplemented DMEM

were sprayed with 70% ethanol, then the same procedure for every materials that need to be inside the hood and other safety protocols were followed.

SH-SY5Y cell line (human neuroblastoma-glioma) was obtained as a gift from Dr Tiago Outeiro, from the Cell and Molecular Neuroscience Unit of the Institute of Molecular from Lisbon, Portugal. The cell lines were stored in vials at -80°C and after cultivation were used for the experiments. This cell line was cultivated in T25 culture plastic flasks (25 cm^2, volume of 60ml, VWR, Belgium) in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum and 1% pen strep + glutamine, incubated under standard conditions (37 °C, humidified atmosphere, 5% CO_2).

Passages with changing of media occurred every 3–4 days; whilst proliferation was observed every 24h using an inverted light microscope. When the cultures reached confluence of 70-80%, cells were washed with Phosphate Buffered Saline, detached with TrypLE™ Express – acting for one minute under standard incubation conditions -, centrifuged, the supernatant was discard, the cells were re-suspended in 5 mL of DMEM and subcultured or proceed for counting. This counting was determined by exclusion of Trypan Blue dye before drug exposure growth and cell viability was of 90 % in the untreated cells.

For drug exposure, cells were seeded into 96-well microplates. Drug exposures were started after 24 h of each subculture. JWH 073 and 250 (2.6 µL from stock solutions) were dissolved in 100 % DMSO, as vehicle, then diluted with supplemented DMEM (final concentration of 0.2% of DMSO) and added to cells to a final concentration of 1, 2, 5, 10, 25, 37.5 and 50 µM (as presented on Table 1 – Appendices).

2.3. Trypan blue (TB) counting

TB counting measurement was described before (Reeb, 1992). Forty microliters of TB solution and 10 µL of cell suspension were used. The suspension was loaded into a Neubauer hemocytometer chamber with cover slip and scored with a light microscope at 40×, using quadrants 1, 5 and 4 (vide Figure 15). Cells that stained blue were scored as nonviable. Measurements were considered as follow:

No. of viable cells = [(Viable cells from Q1 + Viable cells from Q4 + Viable cells from Q5)/3] x 5 (Dilution factor) x 10^4 (one well's size on the Neubauer chamber)

No. of NON-viable cells = [(Non-Viable cells from Q1+ Non-Viable cells from Q4+ Non-Viable cells from Q5)/3] x 5 x 10^4

Viability (%) = (No. of viable cells/ No. of viable cells + No. of NON-viable cells) x 100

2.4. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay: optimization and cytotoxicity assay

According to ATCC (2011), in order to define the ideal number of cells per well of the 96 well plate, trials must be done to measure the number of cells to be used. Cell suspension was obtained from T25 flasks and re-suspended at $6.2x10^5$ cells per ml. From this starting concentration, serial dilutions of cells in culture medium were seeded in a 96-well microplate (vide Figure x – Appendix), within a range of $1,5x10^4$ to $5x10^5$ cells per ml ($1,5x10^4$, $2x10^4$, $2.5x10^4$, $3x10^4$, $3.5x10^4$, $4x10^4$, $4.5x10^4$, $5x10^4$, $1x10^5$, $1.5x10^5$, $2x10^5$, $2.5x10^5$, $3x10^5$, $3.5x10^5$, $4x10^5$, $4.5x10^5$, $5x10^5$ cells per ml). The selection of the cell density range for MTT was based on the reports about its IC_{50} in the literature ($3x10^4$ - Cernaiani, 2008; $4x10^4$ - Kim, 2010; $1x10^4$ - Wang, 2011; $1x10^5$ - Tai, 2011) and our preliminary experiments.

The dilutions were done using the last concentration ($5x10^5$) calculated to obtain 700 µl (triplicates; each well fits 200 µl) of each concentration (vide Table 2 and 3 for microplate scheme and summary of cell density obtention). On triplicates, 200 µL of the dilution samples were added into wells of a 96-well plate, including control wells of medium alone to provide the blanks for absorbance readings, then proceed to incubation under appropriate conditions for the cell line for 24 hours. From this step, proceed to MTT assay normally (to be described below), then after the formation of crystals, read absorbance in an UV spectrophotometer (Biorad, USA). Blanks will show values of zero (± 0.1). Defining the mean values from triplicate readings, we could subtract the mean value for the blank and use the MTT reduction formula (to be presented below).

MTT assay (described by Maioli, 2009) itself starts after cell trypsinization, when cells were again counted with Trypan Blue method to achieve a concentration of 3×10^4 cells/well ($1x10^5$ cells per ml defined from the cell optimization assay; area of each well was 0.32 cm^2), into to a sterile 96-well microplate. As each well contains 200 µl, it was calculated the amount of volume to apply $1x10^5$ cells/ml, plus the difference was complete with supplemented DMEM medium. After 24 hours of microplate culture, the medium was removed and substituted with 200 µl of drug medium, which contains JWH 073 and 250 on concentrations of 1, 5, 10, 25, 50 and 100 µM; or blank-vehicle control (0.2% DMSO) or 1% TritonX-100 as positive control. After 24h of incubation, the resulted media from each well was collected to

perform LDH assay. New 100 µl of DMEM was applied to wash drug-exposed cells on the microplate, then removed before MTT adding. Previously MTT solution (200µl; 0.5mg/ml) was added to each well, and the plates were incubated in the dark for 2,5 h at 37°C/5% CO_2 (this incubation will allow formazan crystals formation). Dissolve crystals with 200µl of pure DMSO and carefully homogenising the liquid and perform absorbance readings at 595nm, calculate mean, standard deviation and error (%) of the samples and plot as dose range. This experiment was repeated in independent sample at least three times. For calculations, use the equation:

% reduced MTT = 100 x (mean of the sample / mean of the negative control)

2.5. Lactate Desidrogenase (LDH) assay

With the same microplate cell culture from MTT assay (already exposed to JWH and controls), we collect 150 µL of media samples of each well carefully, without disturbing the microplate bottom, into an eppendorf. We performed centrifugation of the tubes at 1000g/3min, and, without disturbing the cell pellet, transferred 100 µl of supernatant into a new 96-well plate. After, add 100 µl of the Reaction Mixture (freshly prepared; LDH kit from Clontech, USA) to each well, we incubated the plate at room temperature for 30 min, protecting from light, to allow the enzymatic reaction to take place. Then, absorbance was read at 490 nm and cytotoxicity was measured with the formula below. The experiment used six replicates and it was performed three times independently.

Cytotoxicity (%) = 100 x [(Triplicate Absorbance – Negative control) / (Positive Triton-X Control - Negative control)]

2.7. Statistical Analysis

Data is presented as the mean of the % MTT or % LDH ± S.D. for, at least, 3 independent experiments for MTT and one experiment for LDH assay. MTT results compiled from all independent experiments and evaluated for outlier detection (Grubbs, Sigma rule, Inner and Outer Fence Rule). As data was considered non-parametric, we used Kruskal-Wallis 1-way ANOVA test. LDH results were evaluated by F-test to find out variances behaviour, then Student's T-test. The statistical methods were achieved using Tanagra software, version 12.0.1 or Microsoft Excel 2010 – Analysis ToolPak, using a confidence interval of 95%.

3. Results

3.1. MTT Optimization

Since Mossmann (1983) introduced the MTT assay, adjustments were done to suit different laboratories, different analysts and other variable as time of incubation. It is indeed important to adapt the assay to the laboratory, cell lineage as well as to the analyst methodologies. This will provide the best results in the given set-up but will necessarily need to be reflected in the results interpretation. In addition, these factors do influence on results' interpretation. Before proceeding with the assay, preliminary laboratory work was performed in order to identify the cell concentration most suitable to study proliferation and death as a function of drug exposure. Appendices tables reports the detail of this preparatory phase. The exponential growth phase was defined from a search on the literature that provide a range of $3x10^3$ to $1x10^5$ cells per well. Assays here were done in three replicates of each cell density.

Previous calibration used bovine serum to establish detection limits for the spectrophotometer used in this work's MTT assays. These limits were found to be between 0.7 and 1.2 in optical density (OD). Optimally, an optical density of approximately 1 should therefore be picked, leaving enough leeway to observe both the stimulus and the inhibition of cell. This OD allows us to have measurements on both the stimulus and inhibition of cell proliferation. Figure 8 reports the study to correlate the cell culture absorbance at 595 nm with the optimized cell concentration was of $3x10^4$ cells per well. As each well of the microplate contains 200 µL, the equivalent concentration will be $1x10^5$ cells/ml. This is defined as optimized cell number to be used on all following MTT assays.

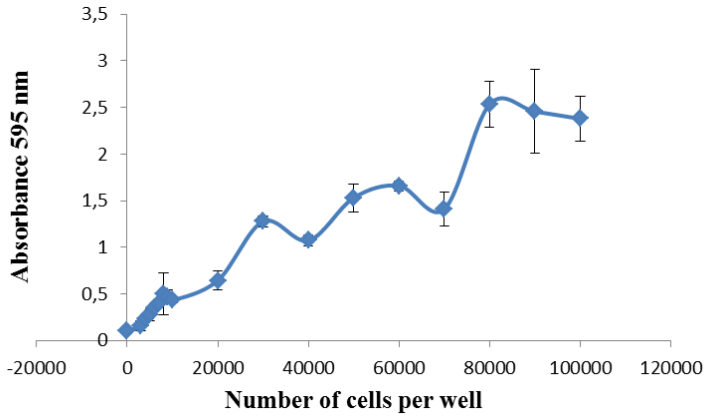

Figure 8. Optimization for MTT assay. Without drug exposure and yielding an optical density at 595nm of 1.2, the concentration of $3x10^4$ cells per well ($1,5x10^5$ cells per ml) was selected for cytotoxicity assays.

3.2. JWH 073 MTT Assay

The MTT method was applied to SH-SY5Y neuroblastoma cell line to JWH-073 (range of 1-50 µM), in parallel to a positive control of 1% Triton X-100 and a negative control of 0.2% DMSO. Drug exposure was performed after 24h of culture growth on a 96-wells microplate and then again incubated for more 24h. The fractional reduction in the SH-SY5Y cell culture was determined through optical density measurements, as detailed in the experimental section 2.4.

Fig. 9 shows the results for control samples as well as the exposure of the culture to a range of JWH-073 concentrations. Triton X-100 effectively worked as a positive control on cell non-viability (97 % ± 0.8 of MTT toxicity resembling negative control, $p < 0.05$, Kruskal-Wallis), mainly due to its characteristics as detergent and protein extractor (Stowe *et al*, 1995). Applying the same statistical treatment (see Appendices), none of the concentration applied of JWH-073 affected the colture significantly with respect to the negative control.

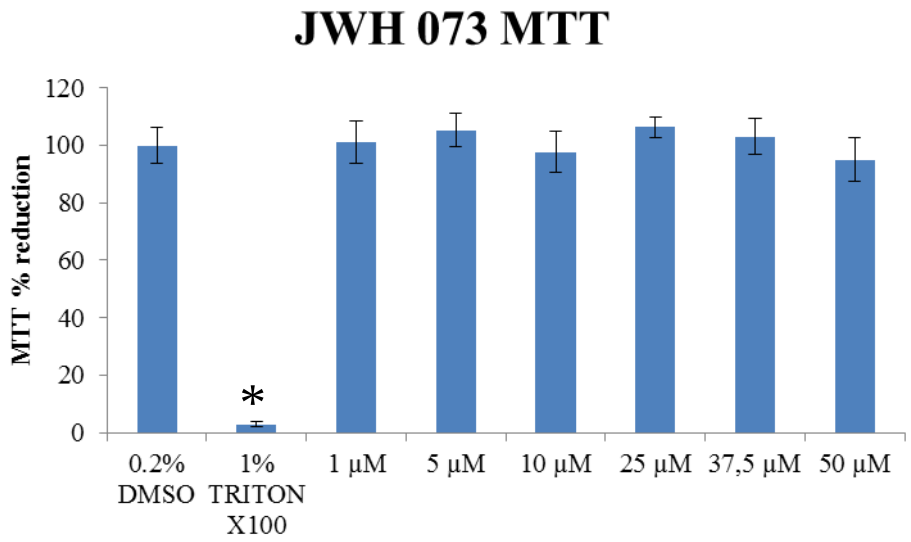

Figure 9. MTT results of SH-SY5Y exposure to JWH-073 (95% confidence interval, Kruskal-Wallis, n=3 independent assays of 6 replicates each).

3.4. JWH 250 MTT Assay

MTT was also performed exposing SH-SY5Y neuroblastoma cell line to JWH-250 in the same manner as mentioned before for JWH-073.

Fig. 10 show results for a range of JWH-250 concentrations and control samples. Tritox X-100 effectively worked as a positive control on cell non-viability (84% ± 14.8 of MTT toxicity resembling negative control, $p < 0.05$, Kruskal-Wallis). Applying statistical treatment (see Appendices), there was statistical significance for the concentration of 50 µM applied of JWH-250 showing a MTT reduction of 40.1% ± 15.8 and the concentration of 37.5 showing MTT reduction of 20.4 % ± 13 µM with respect to the negative control (0.2% DMSO).

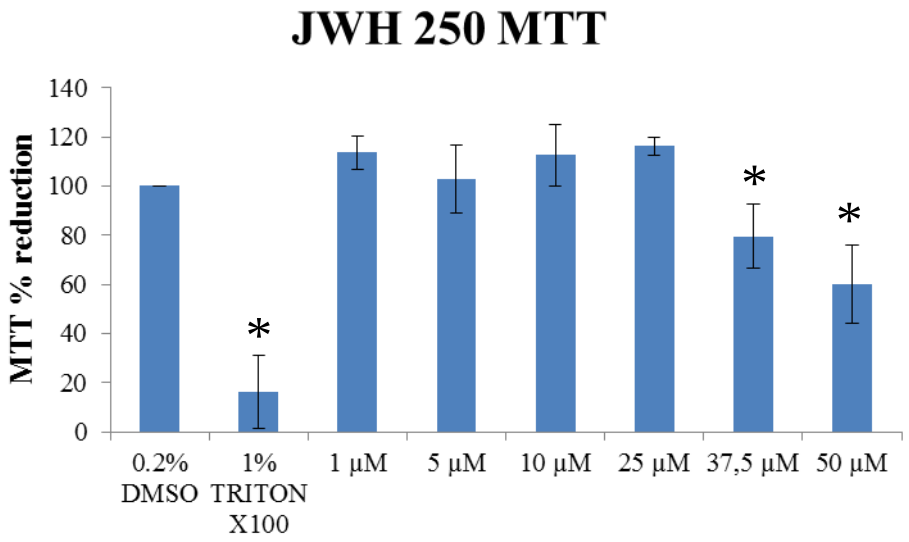

Figure 10. MTT results of SH-SY5Y exposure to JWH-250 ($p < 0.05$, Kruskal-Wallis, n=3 independent assays of 6 replicates each).

3.5. JWH 073 LDH Assay

In the range of *in vitro* assays that use enzymes as cellular markers of death, the choice in literature is usually between glucose-6-phosphate dehydrogenase, adenylate kinase and lactate dehydrogenase (LDH). Commercial kits to detect these enzymes are developed and available; however the stability of enzymes in between and within the assays is an issue. LDH is the one assay preferred on the detection of leakage of cytoplasmatic content that leads to cell death.

Samples for the LDH assay were collected from the MTT assay tray before applying the MTT test. JWH-073 (range of 1-50 µM), positive control of 1% Triton x100 and negative control of 0.2% DMSO were used. Drug exposure was performed after 24h of culture growth on a 96-wells microplate. On the determination of chemicals' effect in SH-SY5Y culture, the optical density was obtained in which a calculation (see Experimental section 2.5) was done to obtain the percentage of LDH leakage detection.

Fig. 11 show results to JWH-073 range and controls. DMSO (0.2%) effectively worked as a negative control on the LDH assay (6% ± 4.3). Applying statistical treatment (see Appendices for data with mean and standard deviation – Table 6, F-test – Table 7 - and T-test for equal variances – Table 8), the concentrations of 37.5 and 50 µM were significantly different than the negative control and were considered 'inducers of cell death' with LDH leakage of 29 ± 12 % and 45 ± 11 %, respectively (while positive control, 1% Tritox X-100, showed 93.6 ± 1.9 %, compared to negative control).

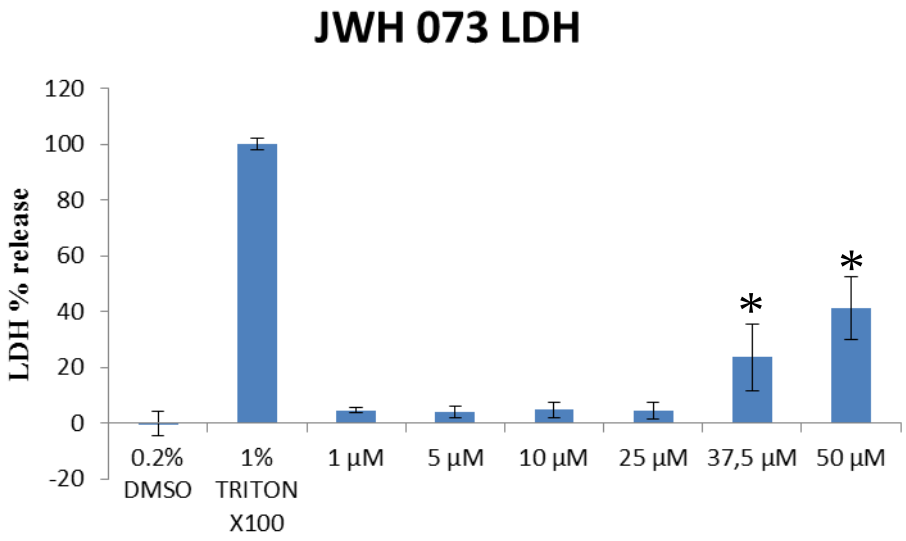

Figure 11. LDH results of SH-SY5Y exposure to JWH-073 ($p < 0.02$, Student's T test, n=1).

3.6. JWH 250 LDH Assay

LDH was also performed exposing SH-SY5Y neuroblastoma cell line to JWH-250 in the same manner as mentioned before for JWH-073. Fig. 12 show results to JWH-250 range and controls. DMSO (0.2%) effectively worked as a negative control on the LDH assay (-2 ± 2.8 %). Applying statistical treatment (see Appendices for data with mean and standard

deviation – Table 9, F-test – Table 10 - and T-test for equal variances), the concentrations of 25 and 50 µM were significantly different than the negative control and were considered 'inducers of cell death' with LDH leakage of 9 ± 3.1 % and 9 ± 3.4 %, respectively (while positive control, 1% Triton X-100, showed 93.1 ± 11.6 %, compared to negative control).

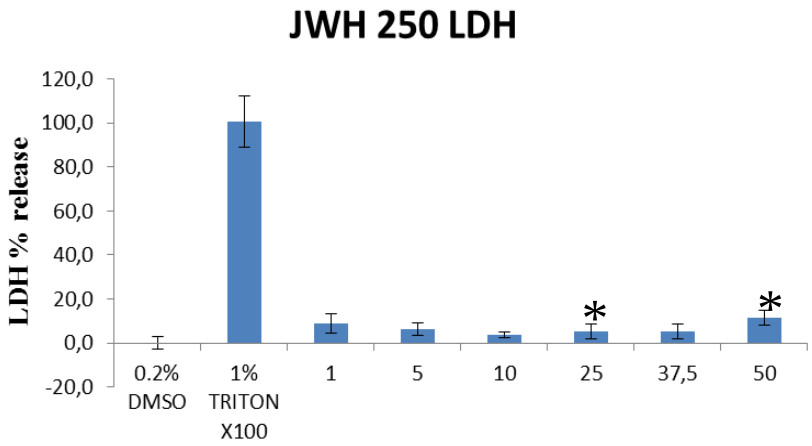

Figure 12. LDH results of SH-SY5Y exposure to JWH-250 ($p < 0.0006$, Student's T test, n=1).

3.8. JWH comparison

In order to observe tendencies for both JWH synthetic cannabinoids, both MTT and LDH were assayed and JWH-073 and JWH-250 were studied as a function of drug concentration. Figure 15 reports the four line plots, comparing the reduction/release fractions as a function of concentration. It shows a correlation of MTT and LDH results in order to support each other. Both JWH have similar MTT 'behavior', although LDH demonstrates the statistical significance of JWH-073 toxicity on concentration of 37.5 and 50 µM. Nonetheless, futher characterization could be interesting, together with standard cannabinoids as THC, JWH-018 or WIN 55,212-2.

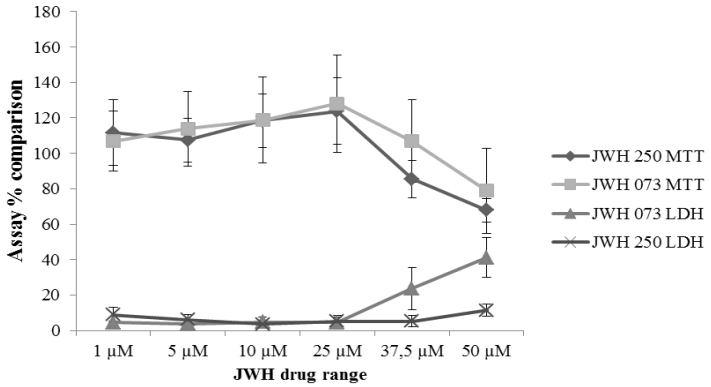

Figure 13. Assay comparison and observation of tendencies for JWH-250 and JWH-073.

4. Discussion

Synthetic cannabinoids present as potent drugs, as far as 0.5 to 5 mg is considered a dose that can lead to psychoactive effects (Tuv *et al.*, 2014), what presents as a challenges to forensic scientists throughout the world to provide insight into new methods of detection in biological matrices, while THC has been described as a strong impairment agent on cognition and motor skills, as driving (Ramaekers *et al*, 2000). In fact, an Australian report described driving accidents to have a prevalence of 10.8% of Cannabis use and 13,5% on fatal cases (Longo *et al.*, 2000; Drummer *et al*., 2003). Concerning synthetic cannabinoids, these are considered stronger drugs then THC (Griffiths *et al.*, 2010) and clinicians are step by step describing the symptoms of a synthetic cannabinoids' intoxication that includes euphoria, panic attacks, restlessness and anxiety (Bebarta *et al.*, 2012).

The epidemiology of synthetic cannabinoids' use is yet to be clarified and organizations as the EMCDDA and the UNODC have provided efforts on it, however, local scenarios of epidemiologic research are needed. Together with clinical reports, that can provide material for biological analysis, there have been large surveys on drug users' behaviour, preferences and reactions. One example is the group of Winstock *et al.* (2011) assessing the British dance scene population, with a survey resulting on a prevalence of 13% of 'Spice' users. A second example is a survey describing use of 'Spice' among US American college students with a rate of 9% (Hu et al., 2011), while athletes are another segment investigated, Heltsley *et al.* (2012) performed 5956 urine screening and found synthetic cannabinoids in 4.5%.

The amount of side effects from the use of synthetics it is translated on the chemical analysed of the herbal blends of 'Spice'-products. Zuba *et al*. (2011) mentions that most frequently the blends do contain more than on synthetic cannabinoid and analysis from blood-work cases reveal intoxication mediated by more several synthetic cannabinoids; what is confirmed as 50% of the episodes from a series of toxicological cases performed by Yeakel and Logan (2013). Besides, the blends not only contain synthetic cannabinoids, but some do enclose known drugs and it can be detected as methamphetamine, benzodiazepines and THC itself – with other cases including GHB and codeine (Tuv *et al*., 2014). This entire scenario contributes to intoxication of multiple symptoms.

Studies here and elsewhere reviewed show correlation of synthetic cannabinoids to psychotic episodes, however, the elements of direct evidence need to be scrutinized by

Toxicology. Remembering that 'Spice'-products do vary intra and inter batches and it generates fractions of more than one JWH or other synthetic cannabinoid. The comprehension of polysubstances can lead to misunderstanding on inferences of toxicological effects. It is interesting to investigate these new drugs on its own specificity of influence on human physiology, but also to consider that the heterogeneity of compounds in one package of 'Spice' and its overall contribution to adverse neurological effects.

That is one of the reasons why this study here chose SH-SY5Y cell lineages to be applied. In addition, clinical reports are finally arising, as the one from Bernson-Leung *et al.* (2014), in which it identifies two cases of stroke symptoms on healthy individuals after experiencing synthetic cannabis for the first time.

SH-SY5Y as an option of *in vitro* model of toxicity focused on Δ9-THC been demonstrated as an inhibitor of the production of nucleic acids and proteins on neuronal cell lines, from human and mouse, but, also to influence on the cell membrane and cell growth (Lew, 1996). In fact, Δ9THC has an emphasis on nerve cells, which type can be originated from neuroblastoma cultures (La Quaglia and Manchester, 1996). The SH-SY5Y has the most utilized cell line human-derived on toxicity studies, with more than 4900 hits on PubMed and it has been used for *in vitro* toxicology focusing on neurodegenerative disease, as Alzheimer's (Harvey *et al*., 2012), as Parkinson's (Choong and Say, 2011) and, for oxidative stress mediated by drugs (Halliwell, 2006), including on the test of cannabinoids toxicity or potential for therapy. These cells are considered easy to cultivate (Cheung et al., 2009) and are applied on the study or neuronal development (Radio *et al*., 2008). In addition, SH-SY5Y has been profiled to express enzymes as tyrosine- (Chen *et al.*, 2013) and dopamine hydrolase (Ou et al., 1998). To complete information, the study of Sanfeliu *et al.* (1999) compared SH-SY5Y with primary culture of neuronal cells and found similar behaviour when exposed to toxicants, what brings advantage on the use of readily available immortal culture of SH-SY5Y, instead of having access primary human tissue sources. Altogether, it presents a good tool for in *vitro* investigation and that is why it was a chosen cell line to be used in the European Union project for acute systemic toxicity "Acutox" that is reliably correlated to *in vivo* assays (Gustafsson *et al.*, 2010).

We proposed to characterise the properties of synthetic cannabinoids, JWH-073 and JWH-250 as acute neurotoxicant chemicals on SH-SY5Y neuroblastoma cell line. This neurotoxicity was assessed via assays involving the activity of cell mitochondria, the leakage of cytoplasmatic content and on the cell diameter distribution for apoptotic stages.

4.1. JWH-073 results

MTT do rely on reducing a colouring reagent via a dehydrogenase enzyme to be functional in a live cell; this can be a measurement of cell viability. This test is also considered easy-going, safe and to possess good reproducibility, what makes it useful for either test of viability and cytotoxicity. These properties make it a common initial step in the toxicological assessment. MTT is frequently used as one of the method measuring the activity of mitochondria in live cells, while mitochondrial NADH converts MTT to formazan and this later as water-insoluble, it forms crystal needles of purple that can be readily dissolved by an organic solvent (in our case, DMSO). Altogether, MTT depends highly on mitochondrial state and not entirely on the cell itself.

LDH, as a marker of cell membrane damage, provided results that increased our suspicious to re-investigate concentrations of 50 µM and higher. Our MTT results do not show statistically significant evidence of toxicity for JWH-073 at the concentrations considered, however some caveats need to be taken into account. First, a total number of 18 replicates (6 on 3 independent assays) increased the uncertainty of the measurement and lead to interpretation difficulty, mainly on the last concentration (50 µM) of this drug. Cells acquiring genetic changes at each subculture – that can lead to differential growth or adaptation to the culture -, the need to use cell clones from different batches of culture flasks and the analyst handling of pippeting procedures are matters to be considered when reflecting on uncertainty.

Secondly, JWH-073 differs only by one methyl group from JWH-018 (the SC with greater binding energy to CB1, what can reflect its toxicity) and this similarity can be useful in comparing degrees of toxicity. However, the issue here lies on the expectative of obtaining comparable results to the chemical match of these two synthetic cannabinoids.

In fact, JWH-018 and JWH073 were assessed by Koller *et al.* (2013) and concluded to be cytotoxic (concentrations higher the 75-100 µM), but in cell lines of buccal (TR146) and mammary tissue (MCF-7) cells, as opposed to the neuroblastoma cells used in this study. However, we must remember neither of the cell lines used by Koller *et al.* possess CB1 receptor and could not allow binding of JWH drugs , although there could be other effective receptors that are currently being investigated, as TRP and PPAR). To overcome the receptor issue, Tomiyama and Funada (2014) used mice forebrain culture to test JWH-018 and it

showed toxicity of 10-30 µM of drug. Again, here it can be discussed that forebrain culture are way more sensitive than immortalized cell lines as SH-SY5Y, yielding higher toxicities.. In fact, this same study puts in test other synthetic cannabinoids (HU-210, AM-2201, and MAM-2201), belonging to the differenct chemical classes rather than the JWH category. The Tomiyama and Funada study found all these cannabinoids to be cytotoxic, suggesting that chemical differences of cannabinoids do not matter as all these are considered toxic on these specific cells.

Following this lead,, Atwood *et al.* (2011) tested JWH-073 on primary culture of hippocampal cells and analyse the influence on neurotransmitter release. They do not find this drug to be as effective as JWH-018. This shows the sensitivity of primary culture to synthetic cannabinoids, is functional rather than toxicity-driven: besides cellular death, functional impairment needs to be assessed as another dimension.

When investigating complex systems rather than cells, Ginsburg *et al*. (2012) find that, JWH073 effectively mimics THC psychotropic effects in rhesus monkey in a concentration of 0.058 mg/kg in only 1 hour of exposure with intravenous administration, while JWH-018 was found to be more effective (0.013 mg/kg) in 2 hours. Although this study is important, we must consider realistic routes of administration when discussing drug dose effect. JWH is is usually self-administered through smoking. Marshell *et al.* (2014) compared both the smoking and intravenous routes, in which at 100 mg / 30L air of JWH-073 mimicked 50% of THC psychotropic effect, while JWH-018 induced 80% of effect at the same concentration. Also Poklis *et al.* (2012) exposed mice to 'Magic Gold' marijuana smoke (containing a mix of JWH-018, JWH-073 and JWH-398) and found JWH-073 levels in blood, after 20 minutes, were of 67-244 ng/ mL. However, in brain tissue the detected levels of JWH-073 were 412-873 ng/g. This data puts the brain as an important distribution site or point of accumulation of JWH-073: the brain is in fact fat-rich and is capable of biotransforming xenobiotics.

Our results do not show significant toxicity for neuronal cell line. This is not inconsistent with literature: toxic effects of JWH-073 alone haven't been observed on humans, who are usually exposed to admixtures of different JWH compounds. Hermanns-Clausen *et al.* (2013) mention a case of intoxication from the "lava red" synthetic marijuana. Their study revealed concentrations of several cannabinoids in the subject urine: JWH-210 (2.5 ng/ml), JWH-073 (0.11 ng/ml) and JWH-015 (< 0.1 ng/ml). There are not described cases of intoxication of JWH-073 itself and most commercial products do contain more than

one synthetic cannabinoid (Lindigkeit *et al.*, 2009). This shows the necessity of assessing synergy of effects (toxic or not) of synthetic marijuana combinations.

4.2. JWH-250 results

The Critical Review Report on JWH-250 from the World Health Organization (2014) mentions "*No pre-clinical safety data are available about the toxicity, reproductive impact and mutagenic/carcinogenic potential of JWH-250*". In this workhere we do provide initial toxicological assessment of this drug. The use of a neuronal cell line is instrumental to clarify the role of JWH-250 in psychopathological symptoms and in episodes of intoxication including confusion, hallucinations and convulsions (Papanti *et al.*, 2013; Lonati *et al.*, 2012).

JWH-250 show in this study at concentrations of 37.5 and 50 µM for MTT and of 25 and 50 µM for LDH. There are so far few reported cases of contamination of marijuana products with JWH-250, involving serum levels of 0.1-1.1 ng/mL, with effects in 6-24 hours (Kneisel *et al*., 2012). Our study shows a toxic effect of 50 µM of JWH-073 on SH-SY5Y neuronal cell line of 40.1% ± 15 (MTT) and 9 ± 3.4 % (LDH), in an exposure of 24 hours. This relatively small effect is however statistically significant and must be considered in perspective of potentially higher concentrations and longer exposures. Further studies are needed to understand its effect on neuronal function.

The study of Hermann-Clausen *et al.* (2013) presents JWH-250 as part of marijuana combination that provided acute intoxications, mainly together with JWH-081, JWH-018, JWH-122, THC and benzodiazepines. This variety of co-participants in the marijuana herbal blend suggests the need further investigations on the basal toxicity of JWH-250. Like in the JWH-073 case, synergy may play an important qualitative and quantitative role in adverse effects.

From the chemical point of view, JWH-250 is considered as a 2-substituted 1-pentyl-3-phenylacetylindoles which suggests less binding affinity to CB1 receptor compared to naphtoylindoles (as JWH-018), however greater affinity to CB1 and CB2, when compared to 3-substituted compounds (Huffman *et al*., 2005). In addition, Compton *et al*. (1992) describes 3-phenylacetylindoles as having activity on the cessation of movement, analgesia and hypothermia in mice, which are common effects of synthetic cannabinoids.

Mitochondrial effects of these drugs - as evaluated through MTT may be an important focal point: Athasaniou *et al.* (2007) conjecture a direct action of synthetic cannabinoid on mitochondria, which have a role in:

- the metabolism of brain aging (Chakrabarti *et al.*, 2011)

- the release of synaptic vesicles (Ivannikov *et al.*, 2013)

- working memory of monkeys (which Hara *et al.*, 2013, correlate with mitochondria morphology)

- the dynamics of DNA mutations and their interplay with energetic fluctutations (Picard and McEwen, 2014)

Results coming from experiments with SH-SY5Y allow to expand the approach to differentiated cells (recalling that SH-SY5Y neuroblastoma cells can be differentiated using all-trans-retinoid acid into neurons), including glial (astrocytes, microglia, oligodendrocytes), neuronal (neurons from different regions of the brain and that release different types of neurotransmitters) and nerve cells. These studies can be complemented by the use of primary cultures from brain segments (motor cortex, hippocampus, limbic system involved with addiction, etc.) and nerves from the peripheral system.

In fact, the use of MTT and LDH techniques would allow inferences on viability and death (or any type of cell), and could be expanded to assess energetic metabolism, uptake of glucose, oxidative stress, homeostasis of calcium. Emphasis can be put on measurements of neuronal type of cells as electrical activity, release of neurotransmitters, migration of axons, activation of specific receptors and channels, the chance of excitoxicity, the influence on the interaction between neurons and glial cells. Moreover, it can be expanded to the development of the nervous system in activities that include cell proliferation, movement, apoptosis signalling, commitment with neural cell type, and also, on the influence on progenitor cells, the growth of neurites, activation of glia (for instance, brain microglial cells on inflammatory states), the impact on myelin production and electrophysiology. (Suñol *et al*., 2008)

5. Conclusions

This work focused on the evaluation of neuronal cytotoxicity (SH-SY5Y neuroblastoma-glioma cell line) of two synthetic cannabinoids, JWH-250 and JWH073. Results show evidences of toxicity for only one of the cannabinoids, JWH-250 in a concentration of 50 µM, via alterations on mitochondrial activity – measured through MTT –

and the release of cytoplasmatic content on the results of LDH assay. Altogether, this confirms that phenylacetylindoles can have toxic effect , reflecting its binding strength to the CB1 receptor. SH-SY5Y cells possess CB1 receptors, but do not show clear evidence of heightened syntetic cannabinoid uptake in our preliminary study. Our results based on the SH-SY5Y cell line are definitely encouraging. These cells have proven to be a good model for cell toxicity in the nervous system. Their use in further characterizing the cannabinoids considered in this study - as well as other psychotropic susbtances - is definitely a promising line of research on the impact of a continuously evolving synthetic drug market.

6. Recommendations for further work

This dissertation is one of the first results in our laboratory on the use of neuronal culture model and the first characterization of JWH-250 on cell toxicity. The results presented here are an initial assessment of toxicological properties of these synthetic cannabinoids. These results clearly indicate the need to expand these drugs' *in vitro* evaluation dalso independently from the cell model used. Our findings stress the importance of furthering the understanding of the influence of synthetic cannabinoids on apoptosis and its signalling mechanisms as caspases, Bcl/Bax, Akt or mTOR pathway. The drugs' biotransformation needs to be addressed too, with special focus on mono-hydroxylated metabolites of other cannabinoids with stronger activity.

Our study will benefit from a study in a broader range of damages such as the effect on the genetic and hormonal levels (potentially linking the action of cannabinoids to the activation of CB1 and CB2 receptors). From the point of view of cannabinoid receptors, it is misleading to consider them the only mediators for psychotropic effects of cannabinoids: new receptors are in need to be discovered and classified, and other already known receptors and channels could be potentially involved with cannabinoid drugs. In addition, the concerning aspect of the abuse and dependence degrees of synthetic cannabinoids calls for further investigation of receptors desensitization and regulation.

Cannabinoids admixtures (such as the ones from 'Spice', 'K2' and other synthetic marijuana), as well as admixtures of psychotropic phytoderivatives must also be better represented on toxicological tests in order to understand their synergies. A clear approach to the analysis of these admixtures and metabolites is primordial to understand the reflection and effects on human physiology.

The resulting condition of SC intake requires more attention on the data availability. Information from poison centres and emergency department need to be systematically shared and approached through analytical biochemistry and laboratory techniques for identification, quantification, comprehension and publication of new intoxication cases. Altogether, the present work contributes to the broad evolving of SC neurotoxicology. y. New assays, as well as studies focusing on the electrical activity, should be targeted on neuronal cell types to better model and understand their effect on cellular and organic physiology. At the same time, progress is needed in assessing *in vivo* effects through models such as rats and monkeys, complementing the case studies based on drug users behaviour.

7. References

Adams WR, Logan BK. (2011) *Missouri K2 Administration Study, raw data*. Available from http://www.nmslabs.com/uploads/PDF/Pharm%20of%20Synthetic%20Cann%20021712.pdf [Accessed 01 July 2014].

Alvaro-Bartolome M, La Harpe R, Callado LF, Meana JJ, Garcıa-Sevilla JA. (2011) Molecular adaptations of apoptotic pathways and signalling partners in the cerebral cortex of human cocaine addicts and cocaine-treated rats. *Neuroscience*, 196 1–15.

American Type Culture Collection – ATCC. (2011) *MTT Cell Proliferation Assay - Instruction Guide*. Available from http://www.atcc.org/~/media/DA5285A1F52C414E864C966FD78C9A79.ashx [Accessed 4 jun 2014].

Armenian, P. (2014) *Week 4 (Stimulants) - Part 6 - Synthetic Cannabinoids with Dr. Roy Gerona. Slides presentation from the course "Poisonings in the Home and Community: Assessment and Emergency Response"*. [online] San Francisco: University of California, San Francisco. Available from https://www.coursera.org/course/poisonings [Accessed 27 May 2014].

Arévalo-Martín A, Vela JM, Molina-Holgado E, Borrell J, Guaza C. (2003) Therapeutic action of cannabinoids in a murine model of multiple sclerosis. *Journal of Neurosciences*, 23(7) 2511-6.

Athanasiou, A., Clarke, A.B., Turner, A.E., Kumaran, N.M., Vakilpour, S. (2007) Cannabinoid receptor agonists are mitochondrial inhibitors: A unified hypothesis of how cannabinoids modulate mitochondrial function and induce cell death. *Biochemical and Biophysical Research Communications*, 364 (1) 131-137.

Atwood BK, Lee D, Straiker A, Widlanski TS, Mackie K. (2011) CP47,497-C8 and JWH073, commonly found in 'Spice' herbal blends, are potent and efficacious CB1 cannabinoid receptor agonists. *European Journal of Pharmacology*, 659 (2-3) 139-45.

Aung, M.M., Griffin, G., Huffman, J.W.,Wu, M., Keel, C., Yang, B., Showalter, V.M., Abood,M. E.,Martin, B.R. (2000) Influence of the N-1 alkyl chain length of cannabimimetic indoles upon CB(1) and CB(2) receptor binding. *Drug Alcohol Dependence*, 60 (1) 133–140.

Auwarter V, Dresen S, Weinmann W, Muller M, Putz M, Ferreiros N (2009). 'Spice' and other herbal blends: harmless incense or cannabinoid designer drugs? *Journal of Mass Spectrometry*, 44 (1) 832–837.

Baron M, Eile M, Eile L. (2011) Analysis of legal highs do they contain what it says on the tin? *Drug Test Analysis*, 3 (1) 576-81.

Bar-Sela G, Vorobeichik M, Drawsheh S, Omer A, Goldberg V, Muller E. (2013) The medical necessity for medicinal cannabis: prospective, observational study evaluating the treatment in cancer patients on supportive or palliative care. *Evidence-based Complementary and Alternative Medicine*. 2013 (1) 510392.

Bebarta VS, Ramirez S, Varney SM. (2012) Complication of spice use in a deployedcombat setting-seizure while on duty. *The American Journal on Addictions*, 21 (5) 496–497.

Benard G, Massa F, Puente N, Lourenc¸o J, Bellocchio L, Soria-Gomez E, Matias I, Delamarre A, Metna-Laurent M, Cannich A, Hebert-Chatelain E, Mulle C, Ortega-Gutierrez S, Martın-Fontecha M, Klugmann M, Guggenhuber S, Lutz B, Gertsch J, Chaouloff F, Lopez-Rodrıguez ML, Grandes P, Rossignol R, Marsicano G (2012) Mitochondrial CB1 receptors regulate neuronal energy metabolism. *Nature Neurosciences*, 15 (1) 558–564.

Bernson-Leung ME, Leung LY, Kumar S. (2014) Synthetic cannabis and acute ischemic stroke. *Journal of Stroke and Cerebrovascular Diseases*, 23 (5) 1239-41.
Boyce N. (2011) Health warnings for people who use heroin. *Lancet*, 377 (1) 193-4.
Brents LK, Gallus-Zawada A, Radominska-Pandya A, Vasiljevik T, Prisinzano TE, Fantegrossi WE, Moran JH, and Prather PL. (2012) Monohydroxylated metabolites of the K2 synthetic cannabinoid JWH-073 retain intermediate to high cannabinoid 1 receptor (CB1R) affinity and exhibit neutral antagonist to partial agonist activity. *Biochemical Pharmacology*, 83 (1) 952–961.
Bretteville-Jensen AL, Tuv SS, Bilgrei OR, Fjeld B, Bachs L. (2013) Synthetic cannabinoids and cathinones: prevalence and markets. *Forensic Science Review*, 25 (1) 7–26.
Breivogel CS, Griffin G, Di Marzo V, Martin BR. (2001) Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. *Molecular Pharmacology*, 60 (1) 155–63.
Brisbois TD, de Kock IH, Watanabe SM, Mirhosseini M, Lamoureux DC, Chasen M, MacDonald N, Baracos VE, Wismer WV. (2011) Delta-9-tetrahydrocannabinol may palliate altered chemosensory perception in cancer patients: results of a randomized, double-blind, placebo-controlled pilot trial. *Annals of Oncology*, 22 (9) 2086-93.
Center of Diseases Control (CDC), Mass Casualties. (2003) Glasgow Coma Scale. Available from http://www.bt.cdc.gov/masscasualties/pdf/glasgow-coma-scale.pdf [Accessed 1 July 2014].
Chakrabarti S, Munshi S, Banerjee K, Thakurta IG, Sinha M, Bagh MB. (2011) Mitochondrial Dysfunction during Brain Aging: Role of Oxidative Stress and Modulation by Antioxidant Supplementation. *Aging Diseases*, 2 (3) 242-56.
Chan FK, Moriwaki K, De Rosa MJ. (2013) Detection of necrosis by release of lactate dehydrogenase activity. *Methods of Molecular Biology*, 979 (1) 65-70.
Chen L, Huang E, Wang H, Qiu P, Liu C. (2013) RNA interference targeting a-synuclein attenuates methamphetamine-induced neurotoxicity in SH-SY5Y cells. *Brain Research,* 1521 (1) 59–67.
Cheung YT, Lau WK, Yu MS, Lai CS, Yeung SC, So KF, et al. (2009) Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. *Neurotoxicology*, 30 (1) 127–35.
Chevaleyre V, Takahashi KA, Castillo PE. (2006) Endocannabinoid mediated synaptic plasticity in the CNS. *Annual Review of Neurosciences*, 29 (1) 37–76.
Choong CJ, Say YH. (2011) Neuroprotection of a-synuclein under acute and chronic rotenone and maneb treatment is abolished by its familial Parkinson's disease mutations A30P A53T and E46K. *Neurotoxicology*, 32 (1) 857–63.
Cohen J, Morrison S, Greenberg J, Saidinejad M. (2012) Clinical Presentation of Intoxication Due to Synthetic Cannabinoids. *Pediatrics*, 129 (4) 1064-7.
Console-Bram L, Marcu J, Abood ME. (2012) Cannabinoid receptors: nomenclature and pharmacological principles. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, 38 (1) 4-15.
CLONTECH. (2007) LDH Cytotoxicity Detection Kit - User Manual. Available from http://www.clontech.com/xxclt_ibcGetAttachment.jsp?cItemId=17508 [Accessed 1 July 2014].
Compton DR, Rice KC, De Costa BR, Razdan RK, Melvin LS, Johnson MR, Martin BR. (1993) Cannabinoid structure-activity relationships: correlation of receptor binding and in vivo activities. *Journal of Pharmacology and Experimental Therapeutics*, 265 (1) 218-26.

Daigle TL, Kearn CS, Mackie K. (2008) Rapid CB1 cannabinoid receptor desensitization defines the time course of ERK1/2 MAP kinase signaling. *Neuropharmacology*, 54 (1) 36–44.

Dargan PI, Hudson S, Ramsey J, Wood DM. The impact of changes in UK classification of the synthetic cannabinoid receptor agonists in 'Spice'. *International Journal of Drug Policy*, 22 (4) 274-7.

Davies S, Wood DM, Smith G, et al. (2010) Purchasing legal highs on the internet is there consistency in what you get? *QJM*, 103 (1) 489-93.

Drug Enforcement Agency (DEA). (2013) Establishment of drug codes for 26 substances. Final rule. United States Department of Justice, *Federal Register*, 78 (3) 664-6.

Derkinderen P, Valjent E, Toutant M, Corvol JC, Enslen H, Ledent C et al. (2003) Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. *Journal of Neurosciences*, 23 (1) 2371–2382.

Devinsky O, Cilio MR, Cross H, Fernandez-Ruiz J, French J, Hill C, Katz R, Di Marzo V, Jutras-Aswad D, Notcutt WG, Martinez-Orgado J, Robson PJ, Rohrback BG, Thiele E, Whalley B, Friedman D. (2014) Cannabidiol: Pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. *Epilepsia*, 55 (6) 791-802.

Di Marzo, V. (2008) Targeting the endocannabinoid system: to enhance or reduce? *Nature Reviews Drug Discovery*, 7 (1) 438–455.

Dresen S, Ferreirós N, Pütz M, Westphal F, Zimmermann R, and Auwärter V. (2010) Monitoring of herbal mixtures potentially containing synthetic cannabinoids as psychoactive compounds. Journal of Mass Spectrometry, 45 (1) 1186–1194.

Drummer OH, Gerostamoulos J, Batziris H, Chu M, Caplehorn JR, Robertson MD, Swann P. (2003) The incidence of drugs in drivers killed in australian roadtraffic crashes. *Forensic Sciences International*, 134 (2–3) 154–162.

European Monitoring Centre for Drugs and Drug Addiction (2009) *Understanding the 'Spice' phenomenon*. EMCDDA, Luxembourg. Available from http://www.drugsandalcohol.ie/12597/1/Understanding_the_Spice_phenomenon.pdf [Accessed 11 February 2014].

European Monitoring Centre for Drugs and Drug Addiction. (2011) *Europol 2010 Annual Report on the Implementation of Council Decision 2005/387/JHA*. Lisbon: EMCDDA, 2011.

European Monitoring Centre for Drugs and Drug Addiction (2013) *Perspectives On Drugs: Synthetic cannabinoids in Europe*. EMCDDA, Luxembourg. Available from http://www.emcdda.europa.eu/topics/pods/synthetic-cannabinoids [Accessed 11 February 2014].

European Monitoring Centre for Drugs and Drug Addiction (2014) *European Drug Report: trends and development*. EMCDDA, Luxembourg. Available from http://www.emcdda.europa.eu/edr2014 [Accessed 25 June 2014].

Fattore L, Fratta W. (2011) Beyond THC: The New Generation of Cannabinoid Designer Drugs. *Frontiers in Behavioral Neuroscience*, 21 (Sep) 5-60.

Frankfurter Rundschau (2008) Gefährlicher Kick mit "Spice". 15 December. Available from http://www.fr-online.de/rhein-main/frankfurt-gefaehrlicher-kick-mit--spice-,1472796,3375090.html [Accessed 1 July 2014].

Galiegue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, et al. (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *European Journal of Biochemistry*, 232 (1) 54-61.

Gaoni YM and Mechoulam R (1964) Isolation, structure and partial synthesis of an active constituent of hashish. *Journal of the American Chemical Society*, 86 (1) 1646–1647.
Gattardo R, Tagliaro F. (2011) I cannabinoidi sintetici, una nuova classe di sostanze d'abuso: Problemi e prospettive analitiche. *Italian Journal on Addiction*, 1 (5-6) 17-22.
Ginsburg BC, Schulze DR, Hruba L, and McMahon LR. (2012) JWH-018 and JWH-073: delta-9-tetrahydrocannabinol-like discriminative stimulus effects in monkeys. *Journal of Pharmacology and Experimental Therapeutics*, 340 (1) 37–45.
GOMES PR, MIGUEL FB, OLIVEIRA ME, FERREIRA VV, GUIMARÃES DSM, LIMA AB, BARBOSA CS, OLIVEIRA MA, ALMEIDA MV, VIANA GHR, COURI MRC, VAROTTI FP. (2014) Síntese e avaliação da atividade antimalárica de compostos derivados da curcumina. Química Nova, 37 (3) 492-496.
Greydanus, D.E., Hawver, K., Greydanus, M.M., Merrick, J. (2013) Marijuana: Current Concepts. *Frontiers in Public Health*, 1 (42) 1-17.
Griffifths, P., Sedefov, R., Gallegos, A., Lopez, D. (2010) How globalization and marketinnovation challenge how we think about and respond to drug use: 'Spice' a case study. *Addiction*, 105 (6) 951–953.
Gustafsson H, Runesson J, Lundqvist J, Lindegren H, Axelsson V, Forsby A. (2010) Neurofunctional endpoints assessed in human neuroblastoma SH-SY5Y cells for estimation of acute systemic toxicity. *Toxicology and Applied Pharmacology*, 245 (2) 191-202.
Halliwell B. (2006) Oxidative stress and neurodegeneration: where are we now? *Journal of Neurochemistry*, 97 (6) 1634–58.
Hajos N, Ledent C, Freund TF. (2001) Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. *Neuroscience*, 106 (1) 1–4.
Hara Y, Yuk F, Puri R, Janssen WG, Rapp PR, Morrison JH. (2014) Presynaptic mitochondrial morphology in monkey prefrontal cortex correlates with working memory and is improved with estrogen treatment. *Proceedings of the National Academy of Sciences of the United States of America*, 111 (1) 486-91.
Harris CR and Brown A. (2013) Synthetic cannabinoid intoxication: a case series and review. *The Journal of Emergency Medicine*, 44 (1) 360–366.
Harvey BS, Ohlsson KS, Mååg JL, Musgrave IF, Smid SD. (2012) Contrasting protective effects of cannabinoids against oxidative stress and amyloid-β evoked neurotoxicity in vitro. *Neurotoxicology*, 33 (1) 138-46.
Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, et al. (1990) Cannabinoid receptor localization in brain. *Proceedings of the National Academy of Sciences of the United States of America*, 87 (1) 1932-6.
Hermanns-Clausen M, Kneisel S, Szabo B, Auwärter V. (2013) Acute toxicity due to the confirmed consumption of synthetic cannabinoids: Clinical and laboratory findings. *Addiction*, 108 (3) 534–544.
Heltsley R, Shelby M.K, Crouch DJ, Black DL, Robert TA, Marshall L, Bender CL, Depriest AZ, Colello M.A. (2012) Prevalence of synthetic cannabinoids in U.S. athletes: initial findings. *Journal of Analytical Toxicology*, 36 (8) 588–593.
Hopkins CY, Gilchrist BL. (2013) A case of cannabinoid hyperemesis syndrome caused by synthetic cannabinoids. *The Journal of Emergency Medicine*, 45 (4) 544-6.

Houston, S. (2011) Detecting Bath Salts. *Forensic Magazine*, 25 Oct. Available from http://www.forensicmag.com/news/2011/10/detecting-bath-salts [Accessed 1 July 2014]
Howlett AC. (2002) The cannabinoid receptors. *Prostaglandins & Other Lipid Mediators*, 68–69 (1) 619–631.
Hruba L, Ginsburg BC, McMahon LR. (2012) Apparent inverse relationship between cannabinoid agonist efficacy and tolerance/cross-tolerance produced by Δ^9-tetrahydrocannabinol treatment in rhesus monkeys. *Journal of Pharmacology and Experimental Therapeutics*, 342 (3) 843-9.
Hsieh C, Brown S, Derleth C, Mackie K. (1999) Internalization and recycling of the CB1 cannabinoid receptor. *Journal of Neurochemistry*, 73 (1) 493–501.
Hu X, Primack BA, Barnett TE, Cook RL. (2011) College students and use of K2: an emerging drug of abuse in young persons. *Substance Abuse Treatment, Prevention, and Policy*, 6 (1) 16.
Hudson S, Ramsey J, King L, Timbers S, Maynard S, Dargan PI, and Wood DM. (2010) Use of high-resolution accurate mass spectrometry to detect reported and previously unreported cannabinomimetics in "herbal high" products. *Journal of Analytical Toxicology*, 34 (1) 252–260.
Huffman JW, Dong D, Martin BR, Compton DR. (1994) Design, synthesis and pharmacology of cannabimimetic indoles. *Bioorganic & Medicinal Chemistry Letters*, 4 (1) 563–566.
Huffman JW, Szklennik PV, Almond A, Bushell K, Selley DE, He H, Cassidy MP, Wiley JL, Martin BR. (2005) 1-Pentyl-3-phenylacetylindoles, a new class of cannabimimetic indoles. *Bioorganic & Medicinal Chemistry Letters*, 15 (18) 4110-3.
Huestis MA, Gorelick DA, Heishman SJ, Preston KL, Nelson RA, Moolchan ET et al. (2001) Blockade of effects of smoked marijuana by the CB1-selective cannabinoid receptor antagonist SR141716. *Archives of General Psychiatry*, 58 (4) 322–328.
Hutter M, Moosmann B, Kneisel S, Auwärter V. (2013) Characteristics of the designer drug and synthetic cannabinoid receptor agonist AM-2201 regarding its chemistry and metabolism. *Journal of Mass Spectrometry*, 48 (7) 885-94.
Iarmarcovai G, Ceppi M, Botta A, Orsiere T, Bonassi S. (2008) Micronuclei frequency in peripheral blood lymphocytes of cancer patients: A meta-analysis. *Mutation Research*, 659 (3) 274–283.
International Narcotic Control Board (INCB), United Nations (2011) *Annual Report*. New York: United Nations. Available from http://www.unodc.org/documents/southasia/reports/2011_INCB_ANNUAL_REPORT_english_PDF.pdf [Accessed 1 July 2014]
Ivannikov MV, Sugimori M, Llinás RR. (2013) Synaptic vesicle exocytosis in hippocampal synaptosomes correlates directly with total mitochondrial volume. *Journal of Molecular Neurosciences*, 49 (1) 223-30.
Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. (2009) Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. *Trends in Pharmacological Science*, 30 (10) 515-27.
Jang M, Yang W, Shin I, Choi H, Chang H, Kim E. (2014) Determination of AM-2201 metabolites in urine and comparison with JWH-018 abuse. *International Journal of Legal Medicine*, 128 (2) 285–294.

JWH-018; Chemspider ID 8558143 [Online]. Royal Society of Chemistry. Available from http://www.chemspider.com/Chemical-Structure.8558143.html [Accessed 1 July 2014].
JWH-073; Chemspider ID 8647081 [Online]. Royal Society of Chemistry. Available from http://www.chemspider.com/Chemical-Structure.8647081.html?rid=25be5200-5a88-4928-94e7-15da999b0e54 [Accessed 1 July 2014].
JWH-250; Chemspider ID 23256117 [Online]. Royal Society of Chemistry. Available from http://www.chemspider.com/Chemical-Structure.23256117.html?rid=8de49798-0086-40f6-8834-5ae9831f8c6c [Accessed 1 July 2014].
Kneisel S, Auwärter V. (2012) Analysis of 30 synthetic cannabinoids in serum by liquid chromatography-electrospray ionization tandem mass spectrometry after liquid-liquid extraction. *Journal of Mass Spectrometry*, 47 (7) 825-35.
Koller, V.J., Zlabinger, G.J., Auwärter, V., Fuchs, S., Knasmueller, S. (2013) Toxicological profiles of selected synthetic cannabinoids showing high binding affinities to the cannabinoid receptor subtype CB1. *Archives of Toxicology*, 87 (7) 1287–97.
La Quaglia MP, Manchester KM. (1996) A comparative analysis of neuroblastic and substrate-adherent human neuroblastoma cell lines. *Journal of Pediatric Surgery*, 31 (2) 315-8.
Le Foll B, Trigo JM, Sharkey KA, Le Strat Y. (2013) Cannabis and Δ9-tetrahydrocannabinol (THC) for weight loss? *Medical Hypotheses*, 80 (5) 564-7.
Lee HK, Choi EB, Pak CS. (2009) The current status and future perspectives of studies of cannabinoid receptor 1 antagonists as anti-obesity agents. *Current Topics in Medicinal Chemistry*, 9 (6) 482-503.
Leterrier C, Bonnard D, Carrel D, Rossier J, Lenkei Z. (2004) Constitutive endocytic cycle of the CB1 cannabinoid receptor. *The Journal of Biological Chemistry*, 279 (34) 36013–36021.
Lew GM. (1996) Tau protein after delta-9-tetrahydrocannabinol in a human neuroblastoma cell line. *General Pharmacology*, 27 (7) 1141-3.
Lindigkeit R, Boehme A, Eiserloh I, Luebbecke M, Wiggermann M, Ernst L, and Beuerle T. (2009) Spice: a never ending story? *Forensic Sciences International*, 191 (1-3) 58–63.
Longo MC, Hunter CE, Lokan RJ, White JM, White MA. (2000) The prevalence of alcohol, cannabinoids, benzodiazepines and stimulants amongst injured drivers and their role in driver culpability: Part i: the prevalence of drug use in drive the drug-positive group. *Accident Analysis & Prevention*, 32 (5) 613–622.
Lonati D, Buscaglia E, Vecchio S, Giampreti A, Petrolini VM, Mazzoleni M, Chiara F, Aloise M, Manzo L, Valli A, Rocchi L, Papa P, Rolandi L, Rimondo C, Seri C, Serpelloni G, Locatelli CA. (2012) Cannabinoidi e catinoni sintetici: aspetti clinici. *16th Congresso Società Italiana Tossicologia*, Messina, 21-23 March, Messina, Italy. Società Italiana Tossicologia Available from http://www.sitox.org/congresso_12/congresso_abs_view.php?id=132 [Accessed 01 July 2014].
Mackie K, Lai Y, Westenbroek R, Mitchell R. (1995) Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *Journal of Neurosciences*, 15 (10) 6552–6561.
Mackie K. (2005) Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handbook of Experimental Pharmacology*, (168) 299–325.

Maioli, E., Torricelli, C., Fortino, V., Carlucci, F., Tommassini, V., Pacini, A. (2009) Critical Appraisal of the MTT Assay in the Presence of Rottlerin and Uncouplers. *Biological Procedures Online*, 11 (1) 227-40.

Manning BD, Cantley LC. (2007) AKT/PKB signaling: navigating downstream. *Cell*, 129 (7) 1261–1274.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*, 346 (6284) 561-4.

Marshell R, Kearney-Ramos T, Brents LK, Hyatt WS, Tai S, Prather PL, Fantegrossi WE. (2014) In vivo effects of synthetic cannabinoids JWH-018 and JWH-073 and phytocannabinoid Δ9-THC in mice: Inhalation versus intraperitoneal injection. *Pharmacology Biochemistry and Behavior*, 124 (C) 40-47.

Meulenberg C, Cemazar M. (2014) Low-magnification image analysis of Giemsa stained, electroporation and bleomycin treated endothelial monolayers provides reliable monolayer integrity data. *Toxicology in Vitro*, 28 (4) 502-509.

Mir A, Obafemi A, Young A, and Kane C. (2011) Myocardial infarction associated with use of the synthetic cannabinoid K2. *Pediatrics*, 128 (6) 1622–1627.

Mechoulam, R. and Hanus, L. (2000) A historical overview of chemical research on cannabinoids. *Chemistry and Physics of Lipids*, 108 (1-2) 1–13.

MEDCHEM. (2000) Giemsa stain. Available from http://www.med-chem.com/pages/lab_procedures/pdf/giemsa_blood_stain.pdf [Accessed 4 June 2014].

Monory K, Blaudzun H, Massa F, Kaiser N, Lemberger T, Schutz G et al. (2007) Genetic dissection of behavioural and autonomic effects of delta(9)-tetrahydrocannabinol in mice. *PLoS Biology*, 5 (10) 269.

Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65 (1-2) 55-63.

Muller H, Sperling W, Kohrmann M, Huttner HB, Kornhuber J, Maler JM. (2010) The synthetic cannabinoid Spice as a trigger for an acute exacerbation of cannabis induced recurrent psychotic episodes. *Schizophrenia Research*, 118 (1-3) 309–310.

Mundy, W.R., Radio, N.M., Freudenrich, T.M. (2010) Neuronal models for evaluation of proliferation in vitro using high content screening. *Toxicology*, 270 (2) 121-130.

Munro S, Thomas KL, Abu-Shaar M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature, 365 (6441) 61-5.

Norchem Newsletter. (2012) Drug trends in the criminal justice market. No:001. Available from http://www.norchemlab.com/wp-content/uploads/2011/08/NOR-Newsletter-v6.pdf [Accessed 01 July 2014].

Nutt D, King L, Phillips L. (2010) Drug harms in the UK: a multicriteria decision analysis. *Lancet*, 376 (9752) 1558-65.

Onaivi ES, Ishiguro H, Gu S, Liu Q-R. (2012) CNS effects of CB2 cannabinoid receptors beyond neuro-immuno-cannabinoid activity. Journal of Psychopharmacology 26 (1) 92–103.

Ou XM, Partoens PM, Wang JM, Walker JH, Danks K, Vaughan PF, et al. (1998) The storage of noradrenaline, neuropeptide Y and chromogranins in and stoichiometric release from large dense cored vesicles of the undifferentiated human neuroblastoma cell line SH-SY5Y. *International Journal of Molecular Medicine*, 1 (1) 105–12.

Papanti D, Schifano F, Botteon G, Bertossi F, Mannix J, Vidoni D, Impagnatiello M, Pascolo-Fabrici E, Bonavigo T. (2013) "Spiceophrenia": a systematic overview of "spice"-related psychopathological issues and a case report. *Human Psychopharmacology*, 28 (4) 379-89.
Pertwee RG, Howlett AC, Abood ME, Alexander SP, Di Marzo V, Elphick MR, et al. International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2. *Pharmacological Reviews*, 62 (4) 588–631.
Picard M, McEwen BS. (2014) Mitochondria impact brain function and cognition. *Proceedings of the National Academy of Sciences of the United States of America*. 111 (1) 7-8.
Premont RT, Gainetdinov RR. (2007) Physiological roles of G protein coupled receptor kinases and arrestins. *Annual Review of Physiology*, 69 (1) 511–534.
Radio NM, Mundy WR. Developmental neurotoxicity testing in vitro: models for assessing chemical effects on neurite outgrowth. *Neurotoxicology*, 29 (1) 361–76.
Ramaekers JG, Robbe HW, O'hanlon J.F. (2000) Marijuana, alcohol and actualdriving performance. *Human Psychopharmacology*, 15 (7) 551–558.
Ramsey J, Dargan PI, Smyllie M, et al. (2010) Buying legal recreational drugs does not mean that you are not breaking the law. *QJM* 103 (1) 777-83.
Reeb BA. Dye exclusion test for bone marrow viability. (1992) In: Areman EM, Deeg HJ, Sacher RA, eds. *Bone marrow and stem cell processing: a manual of current techniques*. New York: F.A. Davis Company, 403-4.
Reynolds TD, Osborn HL. The use of cannabinoids in chronic pain. British Medical Journal Case Reports, 26 (1) 2013.
Robson PJ, Guy GW, Di Marzo V. (2014) Cannabinoids and schizophrenia: therapeutic prospects. *Current Pharmaceutical Design*, 20 (13) 2194-204.
Rolles S, Measham F. (2011) Questioning the method and utility of ranking drug harms in drug policy. *International Journal of Drug Policy*, 22 (4) 243-6.
Salmner EM, Foley PL, Lauder GD, et al. (2010) A harmless high? *Lancet* 376 (9742) 742.
Salomone A, Luciano C, Di Corcia D, Gerace E, Vincenti M. (2014) Hair analysis as a tool to evaluate the prevalence of synthetic cannabinoids in different populations of drug consumers. *Drug Test Analysis*, 6 (1-2) 126-34.
Sanfeliu C, Cristòfol R, Torán N, Rodríguez-Farré E, Kim SU. (1999) Use of human central nervous system cell cultures in neurotoxicity testing. *Toxicology In Vitro*, 13 (4-5) 753-9.
Schaefer N, Peters B, Bregel D, Kneisel S, Auwärter V, Schmidt PH, Ewald AH (2013). A fatal case involving several synthetic cannabinoids. *Toxichem Krimtech*, 80 (special issue) 248-251.
Schneir AB, Cullen J, and Ly BT. (2011) "Spice" girls: synthetic cannabinoid intoxication. *The Journal of Emergency Medicine*, 40 (3) 296–299.
Simmons J, Cookman L, Kang C, and Skinner C. (2011) Three cases of "spice" exposure. *Clinical toxicology (Philadelphia, Pa.)*, 49 (5) 431–433.
Seely KA, Prather PL, James LP, and Moran JH. (2011) Marijuana-based drugs: innovative therapeutics or designer drugs of abuse? *Molecular Interventions*, 11(1) 36–51.
Seely KA, Lapoint J, Moran JH, and Fattore L. (2012) Spice drugs are more than harmless herbal blends: a review of the pharmacology and toxicology of synthetic cannabinoids. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 39 (1) 234–243.

Shen M, Piser TM, Seybold VS, Thayer SA. (1996) Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. *Journal of Neurosciences,* 16 (1) 4322–4334.

Sheridan J, Butler R. (2010) They're legal so they're safe right? *International Journal of Drug Policy,* 21 (1) 77-81.

Smith CPT, Cardile AP, Miller M. (2011) Bath salts as a "legal high". *The American Journal of Medicine,* 124 (1) 7-8.

Sultan, H. (2013) *Toxicological Profile of JWH-018 and JWH-018 N-(3-hydroxypentyl) metabolite in the HEK 293 T Cell Line*. MSc. Instituto Superior de Ciências da Saúde Egas Moniz.

Suñol C., Babot Z., Fonfría E., Galofré M., García D., Herrera N., Iraola S., Vendrell I. (2008) Studies with neuronal cells: from basic studies of mechanisms of neurotoxicity to the prediction of chemical toxicity. *Toxicology In Vitro,* 22 (5) 1350-5.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, et al. (2000) Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis*, 35 (1) 206–221.

Tomiyama K, Funada M. (2014) Cytotoxicity of synthetic cannabinoids on primary neuronal cells of the forebrain: the involvement of cannabinoid CB1 receptors and apoptotic cell death. *Toxicology and Applied Pharmacology,* 274 (2014) 17–23.

Tomiyama, K. and Funada, M. (2011) Cytotoxicity of synthetic cannabinoids found in "Spice" products: the role of cannabinoid receptors and the caspase cascade in the NG 108-15 cell line. *Toxicology Letters*, 207 (1) 12–7.

Tuv SS, Krabseth H, Karinen R, Olsen KM, Øiestad EL, Vindenes V. (2014) Prevalence of synthetic cannabinoids in blood samples from Norwegian drivers suspected of impaired driving during a seven weeks period. *Accident Analysis & Prevention*, 62 (1) 26-31.

United Nations Office on Drugs and Crime – UNODC. (2011) *Synthetic Cannabinoids in Herbal Products*. New York: United Nations, 2011. Available from https://www.unodc.org/documents/scientific/Synthetic_Cannabinoids.pdf [Accessed 1 July 2014].

United Nations Office on Drugs and Crime – UNODC. (2013) *World Drug Report 2013*. New York: United Nations, 2013. Available from http://www.unodc.org/unodc/secured/wdr/wdr2013/World_Drug_Report_2013.pdf [Accessed 01 July 2014].

Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, et al. (2005) Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science,* 310 (5746) 329-32.

Vardakou I, Pistos C, Spiliopoulou, C. (2010) Spice drugs as a new trend: mode of action, identification and legislation. *Toxicology Letters*, 197 (3) 157–162.

Vos M, Lauwers E, Verstreken P. (2010) Synaptic mitochondria in synaptic transmission and organization of vesicle pools in health and disease. *Frontiers in Synaptic Neuroscience*, 22 (2) 139.

Westerbergh J, Hulten P. (2011) Novel Synthetic Cannabinoids, CRA13, JWH-015, JWH-081 and JWH-210 – Detected in a Case Series. *Clinical Toxicology*, 49 (3) 197–269.

World Health Organization (WHO). (2014) *JWH-250, Critical Review Report*. Expert Committee on Drug Dependence, Thirty-sixth Meeting, Geneva, 16-0 June 2014. Available from http://www.who.int/medicines/areas/quality_safety/4_11_review.pdf [Accessed 01 July 2014].

Wintermeyer, A., Möller, I., Thevis, M., Jübner, M., Beike, J., Rothschild, M.A., Bender, K. (2010) In vitro phase I metabolism of the synthetic cannabimimetic JWH-018. *Analytical and Bioanalytical Chemistry*, 398 (5) 2141–53.

Winstock AR, Mitcheson LR, Deluca P, Davey Z, Corazza O, Schifano F. (2011) Mephedrone, new kid for the chop? *Addiction*, 106 (1) 154–161.

Wu DF, Yang LQ, Goschke A, Stumm R, Brandenburg LO, Liang YJ, Höllt V, Koch T. (2008) Role of receptor internalization in the agonist-induced desensitization of cannabinoid type 1 receptors. *Journal of Neurochemistry*, 104 (4) 1132–1143.

Yeakel JK, Logan BK. (2013) Blood synthetic cannabinoid concentrations in cases of suspected impaired driving. *Journal of Analytical Toxicology*, 37 (8) 547-51.

Young AC, Schwarz E, Medina G, Obafemi A, Feng SY, Kane C, Kleinschmidt K. (2012) Cardiotoxicity associated with the synthetic cannabinoid, K9, with laboratory confirmation. *The American Journal of Emergency Medicine*, 30 (7) 1320.e5-7.

Zimmermann US, Winkelmann PR, Pilhatsch M, Nees JA, Spanagel R, Schulz K. (2009) Withdrawal phenomena and dependence syndrome after the consumption of "spice gold". *Deutsches Ärzteblatt international Bundesärztekammer*, 106 (27) 464–467.

Zuba D, Byrska B, Maciow M. (2011) Comparison of "herbal highs" composition. *Analytical and Bioanalytical Chemistry*, 400 (1) 119–126.

8. Appendices

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	BLANK	NEG CTRL 0.2% DMSO	NEG CTRL 0.2% DMSO	0.4% DMSO	0.4% DMSO	POS CTRL 1% TritonX-100	1uM	5uM	10uM	25uM	37.5uM	50uM
C	BLANK	NEG CTRL 0.2% DMSO	NEG CTRL 0.2% DMSO	0.4% DMSO	0.4% DMSO	POS CTRL 1% TritonX-100	1uM	5uM	10uM	25uM	37.5uM	50uM
D	BLANK	NEG CTRL 0.2% DMSO	NEG CTRL 0.2% DMSO	0.4% DMSO	0.4% DMSO	POS CTRL 1% TritonX-100	1uM	5uM	10uM	25uM	37.5uM	50uM
E	BLANK	NEG CTRL 0.2% DMSO	NEG CTRL 0.2% DMSO	0.4% DMSO	0.4% DMSO	POS CTRL 1% TritonX-100	1uM	5uM	10uM	25uM	37.5uM	50uM
F	BLANK	NEG CTRL 0.2% DMSO	NEG CTRL	0.4% DMSO	0.4% DMSO	POS CTRL 1% TritonX-100	1uM	5uM	10uM	25uM	37.5uM	50uM
G	BLANK	NEG CTRL 0.2% DMSO	NEG CTRL 0.2% DMSO	0.4% DMSO	0.4% DMSO	POS CTRL 1% TritonX-100	1uM	5uM	10uM	25uM	37.5uM	50uM
H												

Table 1. Scheme for cell MTT/LDH assay on a 96-wells microplate.

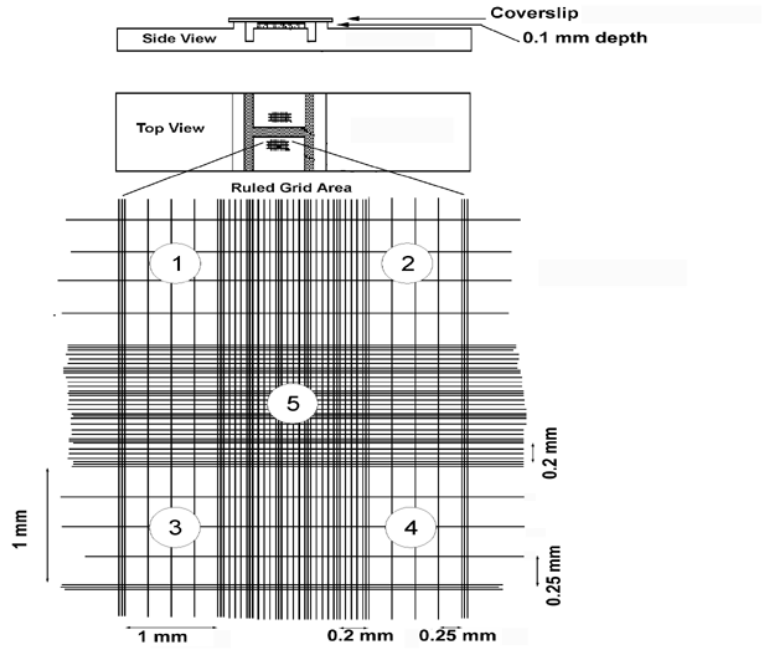

Figure 14. Graphic view of the Neubauer chamber. Counting was performed at quadrants 1, 5 and 4, then average from it divided by 3, multiplied by 5 (as the dilution factor) and 104 (possible amount on cells in 1cm² of the quadrants).

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	3x103	4x103	5x103	6x103	7x103	8x103	9x103	1x104	2x104	3x104
B	0	0	3x103	4x103	5x103	6x103	7x103	8x103	9x103	1x104	2x104	3x104
C	0	0	3x103	4x103	5x103	6x103	7x103	8x103	9x103	1x104	2x104	3x104
D												
E	4x104	5x104	6x104	7x104	8x104	9x104	1x105					
F	4x104	5x104	6x104	7x104	8x104	9x104	1x105					
G	4x104	5x104	6x104	7x104	8x104	9x104	1x105					
H												

Table 2. Scheme for cell optimization on a 96-wells microplate.

Sample	Number of cells per well	Concentration (cells per ml)	Volume of intermediate solution (concentration x final volume of 700/ int. sol conc of 500000)	Volume of DMEM
1	0	0	0	700
2	0	0	0	700
3	3000	15000	21,0	679,0
4	4000	20000	28,0	672,0
5	5000	25000	35,0	665,0
6	6000	30000	42,0	658,0
7	7000	35000	49,0	651,0
8	8000	40000	56,0	644,0
9	9000	45000	63,0	637,0
10	10000	50000	70,0	630,0
11	20000	100000	140,0	560,0
12	30000	150000	210,0	490,0
13	40000	200000	280,0	420,0
14	50000	250000	350,0	350,0
15	60000	300000	420,0	280,0
16	70000	350000	490,0	210,0
17	80000	400000	560,0	140,0
18	90000	450000	630,0	70,0
19	100000	500000	700,0	0,0

Table 3. Scheme for obtaining required cell density for cell optimization on a 96-wells microplate.

Independent assay	0.2% DMSO	1% TRITON X100	1 µM	5 µM	10 µM	25 µM	37,5 µM	50 µM
1	0,19	0,034	0,163	0,198	0,223	0,241	0,216	0,032
	0,191	0,015	0,277	0,176	0,148	0,142	0,095	-0,019
	0,264	0,038	0,181	0,208	0,172	0,184	0,159	-0,005
	0,298	0,04	0,293	0,238	0,224	0,268	0,116	0,097
	0,245	0,036	0,14	0,199	0,228	0,162	0,173	0,04
	0,372	0,072	0,35	0,273	0,255	0,213	0,252	0,283
2	0,404	0,001	0,501	0,54	0,656	0,745	0,701	0,593
	0,472	0,019	0,469	0,565	0,677	0,644	0,542	0,447
	0,536	0,045	0,608	0,704	0,611	0,735	0,572	0,388
	0,425	0,011	0,552	0,677	0,561	0,752	0,553	0,436
	0,469	-0,003	0,561	0,547	0,524	0,673	0,651	0,53
	0,451	0,02	0,524	0,599	0,848	0,774	0,588	0,584
3	0,198	-0,003	0,228	0,339	0,445	0,253	0,415	0,636
	0,286	-0,023	0,282	0,252	0,233	0,228	0,304	0,296
	0,346	-0,021	0,168	0,229	0,266	0,24	0,401	0,44
	0,244	-0,031	0,237	0,365	0,155	0,252	0,236	0,395
	0,398	-0,032	0,421	0,349	0,254	0,423	0,292	0,428
	0,251	-0,02	0,33	0,209	0,132	0,19	0,309	0,385

Table 4. Absorbance data minus blank from MTT results of JWH-073.

	0.2% DMSO	1% TRITON X100	1 µM	5 µM	10 µM	25 µM	37,5 µM	50 µM
Assay 1	99,98839	-8,84401	96,68059	101,149	72,4234	84,69708	113,5678	143,454
Assay 2	100	3,373232	116,6123	131,7374	140,6239	156,8009	130,8306	108,016
Assay 3	100	14,23077	90	82,82051	80,12821	77,5641	64,80769	33,30769
MTT %	99,99613	2,919997	101,0976	105,2356	97,72516	106,354	103,0687	94,9259
SD	6,270899	0,848126	7,396174	5,746983	7,158542	3,676826	6,331426	7,608587

Table 5. Means of each independent assay, final mean equals the calculation of % MTT and standard deviation (SD) to JWH-073.

JWH 073 – FIRST MTT ASSAY

0.2% DMSO	1% TRITON X100	1 µM	5 µM	10 µM	25 µM	37,5 µM	50 µM
0,19	0,034	0,163	0,198	0,223	0,241	0,216	0,032
0,191	0,015	0,277	0,176	0,148	0,142	0,095	-0,019
0,264	0,038	0,181	0,208	0,172	0,184	0,159	-0,005
0,298	0,04	0,293	0,238	0,224	0,268	0,116	0,097
0,245	0,036	0,14	0,199	0,228	0,162	0,173	0,04
0,372	0,072	0,35	0,273	0,255	0,213	0,252	0,283

Univariate Outlier Detection 1

Parameters

Parameters	
Grubbs' p-value	0,0500
Multiple of sigma	3,0
Use Fences	1
Show outliers	0
Filtering Parameters	
Remove outliers	1
Use sigma	0
Use inner fence	0
Use outer fence	1

Results

Univariate Outliers Detection

Detailed results for each variable

Variable	Grubbs Stat.	Sigma rule			Inner Fence rule			Outer Fence Rule		
-	Cut : 1,8871	L.B	U.B	Detected	L.B	U.B	Detected	L.B	U.B	Detected
0.2% DMSO	1,6203	0,0526	0,4674	0	0,0305	0,4585	0	-0,1300	0,6190	0
1% TRITON X100	1,7802	-0,0162	0,0945	0	0,0250	0,0490	2	0,0160	0,0580	2
1 M	1,3772	-0,0187	0,4867	0	-0,0320	0,4880	0	-0,2270	0,6830	0
5 M	1,6637	0,1113	0,3193	0	0,1380	0,2980	0	0,0780	0,3580	0
10 M	1,5097	0,0884	0,3282	0	0,0880	0,3120	0	0,0040	0,3960	0
25 M	1,3819	0,0577	0,3457	0	0,0435	0,3595	0	-0,0750	0,4780	0
37,5 M	1,4115	-0,0090	0,3460	0	-0,0340	0,3660	0	-0,1840	0,5160	0
50 M	1,9012	-0,2627	0,4053	0	-0,1580	0,2500	1	-0,3110	0,4030	0

Removed outliers (if option activated) : 2

Computation time : 47 ms.
Created at 15/07/2014 18:12:23

0.2% DMSO	1% TRITON X100	1 µM	5 µM	10 µM	25 µM	37,5 µM	50 µM
0,19	0,034	0,163	0,198	0,223	0,241	0,216	0,032
0,191		0,277	0,176	0,148	0,142	0,095	-0,019
0,264	0,038	0,181	0,208	0,172	0,184	0,159	
0,298	0,04	0,293	0,238	0,224	0,268	0,116	0,097
0,245	0,036	0,14	0,199	0,228	0,162	0,173	0,04
0,372		0,35	0,273	0,255	0,213	0,252	0,283

Results

Univariate Outliers Detection

Detailed results for each variable

Variable	Grubbs Stat.	Sigma rule			Inner Fence rule			Outer Fence Rule		
-	Cut : 1,4812	L.B	U.B	Detected	L.B	U.B	Detected	L.B	U.B	Detected
0.2% DMSO	1,3115	0,1137	0,3848	0	0,1222	0,3763	0	0,0270	0,4715	0
1% TRITON X100	1,1619	0,0293	0,0447	0	0,0290	0,0450	0	0,0230	0,0510	0
1 M	1,4535	-0,0096	0,3981	0	0,0232	0,3653	0	-0,1050	0,4935	0
5 M	1,4561	0,1546	0,2669	0	0,1617	0,2598	0	0,1250	0,2965	0
10 M	1,4950	0,1320	0,2915	0	0,1548	0,2688	0	0,1120	0,3115	0
25 M	1,1036	0,0663	0,3612	0	0,0507	0,3768	0	-0,0715	0,4990	0
37,5 M	1,2129	0,0423	0,2897	0	0,0520	0,2800	0	-0,0335	0,3655	0
50 M	1,3557	-0,0789	0,1634	0	-0,0627	0,1473	0	-0,1415	0,2260	0

Removed outliers (if option activated) : 0

JWH 073 – SECOND MTT ASSAY

0.2% DMSO	1% TRITON X100	1 µM	5 µM	10 µM	25 µM	37,5 µM	50 µM
0,404	0,001	0,501	0,54	0,656	0,745	0,701	0,593
0,472	0,019	0,469	0,565	0,677	0,644	0,542	0,447
0,536	0,045	0,608	0,704	0,611	0,735	0,572	0,388
0,425	0,011	0,552	0,677	0,561	0,752	0,553	0,436
0,469	-0,003	0,561	0,547	0,524	0,673	0,651	0,53
0,451	0,02	0,524	0,599	0,848	0,774	0,588	0,584

Univariate Outlier Detection 1

Parameters

Parameters	
Grubbs' p-value	0,0500
Multiple of sigma	3,0
Use Fences	1
Show outliers	0
Filtering Parameters	
Remove outliers	1
Use sigma	0
Use inner fence	0
Use outer fence	1

Results

Univariate Outliers Detection

Detailed results for each variable

Variable	Grubbs Stat.	Sigma rule			Inner Fence rule			Outer Fence Rule		
-	Cut : 1,8871	L.B	U.B	Detected	L.B	U.B	Detected	L.B	U.B	Detected
0.2% DMSO	1,6740	0,3224	0,5966	0	0,3545	0,5425	0	0,2840	0,6130	0
1% TRITON X100	1,7173	-0,0360	0,0670	0	-0,0275	0,0485	0	-0,0560	0,0770	0
1 M	1,4779	0,3893	0,6823	0	0,4110	0,6510	0	0,3210	0,7410	0
5 M	1,4180	0,3966	0,8141	0	0,3520	0,8720	0	0,1570	1,0670	0
10 M	1,7675	0,3036	0,9887	0	0,3870	0,8510	0	0,2130	1,0250	0
25 M	1,5135	0,5689	0,8721	0	0,5545	0,8705	0	0,4360	0,9890	0
37,5 M	1,6071	0,4148	0,7875	0	0,4060	0,7980	0	0,2590	0,9450	0
50 M	1,2772	0,2419	0,7508	0	0,2140	0,8060	0	-0,0080	1,0280	0

Removed outliers (if option activated) : 0

JWH 073 – THIRD MTT ASSAY

0.2% DMSO	1% TRITON X100	1 µM	5 µM	10 µM	25 µM	37,5 µM	50 µM
0,198	-0,003	0,228	0,339	0,445	0,253	0,415	0,636
0,286	-0,023	0,282	0,252	0,233	0,228	0,304	0,296
0,346	-0,021	0,168	0,229	0,266	0,24	0,401	0,44
0,244	-0,031	0,237	0,365	0,155	0,252	0,236	0,395
0,398	-0,032	0,421	0,349	0,254	0,423	0,292	0,428
0,251	-0,02	0,33	0,209	0,132	0,19	0,309	0,385

Univariate Outlier Detection 1

Parameters

Parameters	
Grubbs' p-value	0,0500
Multiple of sigma	3,0
Use Fences	1
Show outliers	0
Filtering Parameters	
Remove outliers	1
Use sigma	0
Use inner fence	0
Use outer fence	1

Results

Univariate Outliers Detection

Detailed results for each variable

Variable	Grubbs Stat.	Sigma rule			Inner Fence rule			Outer Fence Rule		
-	Cut : 1,8871	L.B	U.B	Detected	L.B	U.B	Detected	L.B	U.B	Detected
0.2% DMSO	1,5119	0,0672	0,5071	0	0,0910	0,4990	0	-0,0620	0,6520	0
1% TRITON X100	1,7841	-0,0531	0,0097	0	-0,0475	-0,0035	1	-0,0640	0,0130	0
1 M	1,6137	0,0112	0,5441	0	0,0750	0,4830	0	-0,0780	0,6360	0
5 M	1,1956	0,0860	0,4950	0	0,0490	0,5290	0	-0,1310	0,7090	0
10 M	1,7808	-0,0852	0,5802	0	-0,0115	0,4325	1	-0,1780	0,5990	0
25 M	1,9562	0,0210	0,5077	0	0,1905	0,2905	2	0,1530	0,3280	1
37,5 M	1,3131	0,1202	0,5322	0	0,1285	0,5645	0	-0,0350	0,7280	0
50 M	1,8244	0,0913	0,7687	0	0,3025	0,5225	2	0,2200	0,6050	1

Removed outliers (if option activated) : 2

0.2% DMSO	1% TRITON X100	1 µM	5 µM	10 µM	25 µM	37,5 µM	50 µM
0,198	-0,023	0,228	0,339	0,233	0,253	0,415	0,44
0,286	-0,021	0,282	0,252	0,266	0,228	0,304	0,395
0,346	-0,031	0,168	0,229	0,155	0,24	0,401	0,428
0,244	-0,032	0,237	0,365	0,254	0,252	0,236	0,385
0,398	-0,02	0,421	0,349	0,132		0,292	
0,251		0,33	0,209			0,309	

Univariate Outliers Detection

Detailed results for each variable

Variable	Grubbs Stat.	Sigma rule			Inner Fence rule			Outer Fence Rule		
-	Cut : 1,4812	L.B	U.B	Detected	L.B	U.B	Detected	L.B	U.B	Detected
0.2% DMSO	1,2314	0,0797	0,4573	0	0,0785	0,4585	0	-0,0640	0,6010	0
1% TRITON X100	1,0341	-0,0434	-0,0101	0	-0,0458	-0,0077	0	-0,0600	0,0065	0
1 M	1,2957	0,0881	0,3694	0	0,1057	0,3518	0	0,0135	0,4440	0
5 M	1,0430	0,0985	0,4940	0	0,0733	0,5192	0	-0,0940	0,6865	0
10 M	1,4429	0,0773	0,3767	0	0,0950	0,3590	0	-0,0040	0,4580	0
25 M	1,2970	0,2080	0,2785	0	0,2062	0,2802	0	0,1785	0,3080	0
37,5 M	1,2180	0,0853	0,5927	0	0,0630	0,6150	0	-0,1440	0,8220	0
50 M	1,0690	0,3334	0,4906	0	0,3240	0,5000	0	0,2580	0,5660	0

Removed outliers (if option activated) : 0

Figure 15 (compilation). Outlier detection tests for JWH-073 MTT data.

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9961	15,0	5,0000	Kruskal-Wallis	3,857143	0,049535
		1% TRITON X100	3	2,9200	6,0	2,0000	KW (corr.ties)	3,970588	0,046302
		All	6	51,4581	21,0	3,5000			

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9961	12,0	4,0000	Kruskal-Wallis	0,428571	0,512691
		1	3	101,0976	9,0	3,0000	KW (corr.ties)	0,441176	0,506555
		All	6	100,5469	21,0	3,5000			

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9961	9,0	3,0000	Kruskal-Wallis	0,428571	0,512691
		5	3	105,2356	12,0	4,0000	KW (corr.ties)	0,441176	0,506555
		All	6	102,6159	21,0	3,5000			

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9961	12,0	4,0000	Kruskal-Wallis	0,428571	0,512691
		10	3	97,7252	9,0	3,0000	KW (corr.ties)	0,441176	0,506555
		All	6	98,8606	21,0	3,5000			

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9961	12,0	4,0000	Kruskal-Wallis	0,428571	0,512691
		25	3	106,3540	9,0	3,0000	KW (corr.ties)	0,441176	0,506555
		All	6	103,1751	21,0	3,5000			

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9961	9,0	3,0000	Kruskal-Wallis	0,428571	0,512691
		37,5	3	103,0687	12,0	4,0000	KW (corr.ties)	0,441176	0,506555
		All	6	101,5324	21,0	3,5000			

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9961	9,0	3,0000	Kruskal-Wallis	0,428571	0,512691
		50	3	94,9259	12,0	4,0000	KW (corr.ties)	0,441176	0,506555
		All	6	97,4610	21,0	3,5000			

Computation time : 15 ms.
Created at 15/07/2014 18:50:21

Figure 16 (compilation). Kruskal-Wallis tests for JWH-073 MTT data.

Independent assay	0.2% DMSO	1% TRITON X100	1 µM	5 µM	10 µM	25 µM	37,5 µM	50 µM
1	0,668	0,11	0,727	0,488	0,779	0,74	0,455	0,293
	0,667	0,122	0,746	0,601	0,514	0,721	0,391	0,246
	0,593	0,108	0,804	0,49	0,488	0,838	0,547	0,338
	0,477	0,108	0,782	0,716	0,578	0,75	0,71	0,373
	0,658	0,094	0,545	0,606	0,735	0,851	0,349	0,521
	0,702	0,143	0,496	0,69	0,858	0,356	0,368	0,335
2	0,456	0,125	0,454	0,464	0,52	0,396	0,489	0,347
	0,383	0,132	0,456	0,409	0,442	0,487	0,355	0,277
	0,322	0,116	0,403	0,388	0,45	0,445	0,366	0,276
	0,353	0,089	0,554	0,384	0,486	0,489	0,291	0,277
	0,336	0,092	0,415	0,48	0,476	0,442	0,303	0,31
	0,404	0,12	0,421	0,549	0,491	0,559	0,323	0,257
3	0,563	0,005	0,622	0,383	0,674	0,635	0,35	0,188
	0,544	-0,001	0,623	0,478	0,391	0,598	0,268	0,123
	0,475	-0,01	0,686	0,372	0,37	0,72	0,429	0,22
	0,365	-0,004	0,67	0,604	0,466	0,638	0,598	0,261
	0,566	0,002	0,453	0,514	0,643	0,759	0,257	0,429
	0,578	0,019	0,372	0,566	0,734	0,232	0,244	0,211

Table 6. Absorbance data minus blank from MTT results of JWH-250.

	0.2% DMSO	1% TRITON X100	1 µM	5 µM	10 µM	25 µM	37,5 µM	50 µM
Assay 1	100,00	18,19	108,90	95,38	104,97	113,04	74,90	55,94
Assay 2	100,00	29,90	121,49	118,63	127,11	120,27	94,37	77,37
Assay 3	99,99	0,36	110,83	94,36	106,04	115,88	69,42	46,33
MTT %	100,00	16,15	113,74	102,79	112,71	116,39	79,56	59,88
SD	0,00	14,88	6,78	13,73	12,48	3,64	13,11	15,90

Table 7. Means of each independent assay, final mean equals the calculation of % MTT and standard deviation (SD) to JWH-250.

JWH 250 – FIRST MTT ASSAY

0.2% DMSO	1% TRITON X100	1	5	10	25	37,5	50
0,563	0,005	0,622	0,383	0,674	0,635	0,35	0,188
0,544	-0,001	0,623	0,478	0,391	0,598	0,268	0,123
0,475	-0,01	0,686	0,372	0,37	0,72	0,429	0,22
0,365	-0,004	0,67	0,604	0,466	0,638	0,598	0,261
0,566	0,002	0,453	0,514	0,643	0,759	0,257	0,429
0,578	0,019	0,372	0,566	0,734	0,232	0,244	0,211

Univariate Outlier Detection 1

Parameters

Parameters	
Grubbs' p-value	0,0500
Multiple of sigma	3,0
Use Fences	1
Show outliers	0
Filtering Parameters	
Remove outliers	1
Use sigma	0
Use inner fence	0
Use outer fence	1

Results

Univariate Outliers Detection

Detailed results for each variable

Variable	Grubbs Stat.	Sigma rule			Inner Fence rule			Outer Fence Rule		
-	Cut : 1,8871	L.B	U.B	Detected	L.B	U.B	Detected	L.B	U.B	Detected
0.2% DMSO	1,4757	0,0248	0,4192	0	-0,0110	0,4290	0	-0,1760	0,5940	0
1% TRITON X100	1,6856	-0,0361	0,0588	0	-0,0330	0,0470	0	-0,0630	0,0770	0
1	1,8686	0,1689	0,5301	0	0,2270	0,4510	1	0,1430	0,5350	0
5	1,4783	0,1532	0,5361	0	0,1570	0,5170	0	0,0220	0,6520	0
10	1,5208	0,2650	0,4880	0	0,2740	0,4660	0	0,2020	0,5380	0
25	1,6262	0,2205	0,5169	0	0,2570	0,4810	0	0,1730	0,5650	0
37,5	1,9409	0,0240	0,4830	0	0,1195	0,3475	1	0,0340	0,4330	0
50	1,7195	0,0670	0,3124	0	0,0870	0,2870	0	0,0120	0,3620	0

Removed outliers (if option activated) : 0

JWH 250 – SECOND MTT ASSAY

0.2% DMSO	1% TRITON X100	1	5	10	25	37,5	50
0,456	0,125	0,454	0,464	0,52	0,396	0,489	0,347
0,383	0,132	0,456	0,409	0,442	0,487	0,355	0,277
0,322	0,116	0,403	0,388	0,45	0,445	0,366	0,276
0,353	0,089	0,554	0,384	0,486	0,489	0,291	0,277
0,336	0,092	0,415	0,48	0,476	0,442	0,303	0,31
0,404	0,12	0,421	0,549	0,491	0,559	0,323	0,257

Univariate Outliers Detection

Detailed results for each variable

Variable	Grubbs Stat.	Sigma rule			Inner Fence rule			Outer Fence Rule		
-	Cut : 1,8871	L.B	U.B	Detected	L.B	U.B	Detected	L.B	U.B	Detected
0.2% DMSO	1,8260	0,2685	0,7619	0	0,3385	0,7025	0	0,2020	0,8390	0
1% TRITON X100	1,7397	-0,0278	0,0314	0	-0,0175	0,0185	1	-0,0310	0,0320	0
1	1,5553	0,1872	0,9548	0	0,1275	0,9955	0	-0,1980	1,3210	0
5	1,2452	0,2023	0,7701	0	0,1085	0,8405	0	-0,1660	1,1150	0
10	1,1988	0,0767	1,0160	0	-0,0335	1,0985	0	-0,4580	1,5230	0
25	1,9360	0,0314	1,1626	0	0,4150	0,9030	1	0,2320	1,0860	0
37,5	1,7528	-0,0537	0,7690	0	-0,0010	0,6870	0	-0,2590	0,9450	0
50	1,8351	-0,0725	0,5498	0	0,0785	0,3705	1	-0,0310	0,4800	0

Removed outliers (if option activated) : 0

JWH 250 – THIRD MTT ASSAY

0.2% DMSO	1% TRITON X100	1	5	10	25	37,5	50
0,668	0,11	0,727	0,488	0,779	0,74	0,455	0,293
0,667	0,122	0,746	0,601	0,514	0,721	0,391	0,246
0,593	0,108	0,804	0,49	0,488	0,838	0,547	0,338
0,477	0,108	0,782	0,716	0,578	0,75	0,71	0,373
0,658	0,094	0,545	0,606	0,735	0,851	0,349	0,521
0,702	0,143	0,496	0,69	0,858	0,356	0,368	0,335

Univariate Outliers Detection

Detailed results for each variable

Variable	Grubbs Stat.	Sigma rule			Inner Fence rule			Outer Fence Rule		
-	Cut : 1,8871	L.B	U.B	Detected	L.B	U.B	Detected	L.B	U.B	Detected
0.2% DMSO	1,6548	0,1473	0,5027	0	0,2035	0,4635	0	0,1060	0,5610	0
1% TRITON X100	1,8056	0,0241	0,1775	0	0,0585	0,1345	1	0,0300	0,1630	0
1	1,5783	0,0863	0,4804	0	0,0600	0,4920	0	-0,1020	0,6540	0
5	1,3158	0,2516	0,4051	0	0,2440	0,4200	0	0,1780	0,4860	0
10	1,5953	0,2295	0,4351	0	0,2240	0,4240	0	0,1490	0,4990	0
25	1,4208	0,2145	0,4432	0	0,2125	0,4245	0	0,1330	0,5040	0
37,5	1,1434	0,1528	0,5122	0	0,1330	0,5330	0	-0,0170	0,6830	0
50	1,4702	0,2186	0,4607	0	0,2245	0,4685	0	0,1330	0,5600	0

Removed outliers (if option activated) : 0

Figure 17 (compilation). Outlier detection tests for JWH-250 MTT data.

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9978	15,0	5,0000	Kruskal-Wallis	3,857143	0,049535
		1% TRITON X100	3	16,1507	6,0	2,0000	KW (corr.ties)	3,857143	0,049535
		All	6	58,0743	21,0	3,5000			

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9978	6,0	2,0000	Kruskal-Wallis	3,857143	0,049535
		1	3	113,7397	15,0	5,0000	KW (corr.ties)	3,857143	0,049535
		All	6	106,8688	21,0	3,5000			

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9978	12,0	4,0000	Kruskal-Wallis	0,428571	0,512691
		5	3	102,7922	9,0	3,0000	KW (corr.ties)	0,428571	0,512691
		All	6	101,3950	21,0	3,5000			

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9978	6,0	2,0000	Kruskal-Wallis	3,857143	0,049535
		10	3	112,7057	15,0	5,0000	KW (corr.ties)	3,857143	0,049535
		All	6	106,3517	21,0	3,5000			

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9978	6,0	2,0000	Kruskal-Wallis	3,857143	0,049535
		25	3	116,3949	15,0	5,0000	KW (corr.ties)	3,857143	0,049535
		All	6	108,1963	21,0	3,5000			

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9978	15,0	5,0000	Kruskal-Wallis	3,857143	0,049535
		37,5	3	79,5629	6,0	2,0000	KW (corr.ties)	3,857143	0,049535
		All	6	89,7804	21,0	3,5000			

Kruskal-Wallis 1-way ANOVA 1

Parameters

Parameters	
Sort results	no

Results

Attribute_Y	Attribute_X	Description					Statistical test		
		Value	Examples	Average	Rank sum	Rank mean	Statistics	Value	Proba
rating	group	0.2% DMSO	3	99,9978	15,0	5,0000	Kruskal-Wallis	3,857143	0,049535
		50	3	59,8783	6,0	2,0000	KW (corr.ties)	3,857143	0,049535
		All	6	79,9380	21,0	3,5000			

Figure 18 (compilation). Kruskal-Wallis tests for JWH-250 MTT data.

Replicates	0.2% DMSO	1% TRITON X100	1 µM	5 µM	10 µM	25 µM	37,5 µM	50 µM
1	0,033	1,292	0,126	0,101	0,106	0,136	0,452	0,721
2	0,118	1,275	0,133	0,16	0,18	0,18	0,225	0,401
3	0,091	1,239	0,148	0,122	0,13	0,088	0,409	0,59
Mean	0,081	1,269	0,136	0,128	0,139	0,135	0,362	0,571
SD	0,043	0,020	0,008	0,022	0,028	0,031	0,121	0,113
LDH %	-0,028	99,972	4,602	3,928	4,854	4,517	23,653	41,218
SD for LDH	4,343	1,978	0,822	2,156	2,756	3,111	12,058	11,311

Table 8. Raw data from LDH results of JWH-073

Teste F: duas amostras para variâncias

	0.2% DMSO	*1% TRITON X100*
Média	0,080666667	1,268666667
Variância	0,001886333	0,000732333
Observações	3	3
gl	2	2
F	2,575785162	
P(F<=f) uni-caudal	0,279658859	
F crítico uni-caudal	19	

Teste F: duas amostras para variâncias

	0.2% DMSO	*1 µM*
Média	0,080667	0,135667
Variância	0,001886	0,000126
Observações	3	3
gl	2	2
F	14,9314	
P(F<=f) uni-caudal	0,062769	
F crítico uni-caudal	19	

Teste F: duas amostras para variâncias

	0.2% DMSO	*5 µM*
Média	0,080667	0,127667
Variância	0,001886	0,000894
Observações	3	3
gl	2	2
F	2,109206	
P(F<=f) uni-caudal	0,321626	
F crítico uni-caudal	19	

Teste F: duas amostras para variâncias

	0.2% DMSO	*10 µM*
Média	0,080667	0,138667
Variância	0,001886	0,001425
Observações	3	3
gl	2	2
F	1,323433	
P(F<=f) uni-caudal	0,430398	
F crítico uni-caudal	19	

Teste F: duas amostras para variâncias

	25 µM	*0.2% DMSO*
Média	0,134666667	0,080666667
Variância	0,002117333	0,001886333
Observações	3	3
gl	2	2
F	1,122459799	
P(F<=f) uni-caudal	0,471151445	
F crítico uni-caudal	19	

Teste F: duas amostras para variâncias

	37,5 µM	*0.2% DMSO*
Média	0,362	0,080667
Variância	0,014539	0,001886
Observações	3	3
gl	2	2
F	7,707546	
P(F<=f) uni-caudal	0,114843	
F crítico uni-caudal	19	

Teste F: duas amostras para variâncias

	50 µM	*0.2% DMSO*
Média	0,570667	0,080667
Variância	0,02588	0,001886
Observações	3	3
gl	2	2
F	13,71992	
P(F<=f) uni-caudal	0,067935	
F crítico uni-caudal	19	

F < F crit, equal variances
F > F crit, unequal variances
All equal variances, perform T-test for equal variances.

Table 9 (Compilation). F-tests for LDH results of JWH-073

Teste T: duas amostras com variâncias iguais

	0.2% DMSO	*1 µM*
Média	0,080667	0,135667
Variância	0,001886	0,000126
Observações	3	3
Variância agrupada	0,001006	
Hipótese de diferença de média	0	
gl	4	
Stat t	-2,12343	
P(T<=t) uni-caudal	0,050476	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,100952	
t crítico bi-caudal	2,776445	

Teste T: duas amostras com variâncias iguais

	0.2% DMSO	*5 µM*
Média	0,080667	0,127667
Variância	0,001886	0,000894
Observações	3	3
Variância agrupada	0,00139	
Hipótese de diferença de média	0	
gl	4	
Stat t	-1,54378	
P(T<=t) uni-caudal	0,09876	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,19752	
t crítico bi-caudal	2,776445	

Teste T: duas amostras com variâncias iguais

	0.2% DMSO	*10 µM*
Média	0,080667	0,138667
Variância	0,001886	0,001425
Observações	3	3
Variância agrupada	0,001656	
Hipótese de diferença de média	0	
gl	4	
Stat t	-1,74568	
P(T<=t) uni-caudal	0,0779	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,1558	
t crítico bi-caudal	2,776445	

Teste T: duas amostras com variâncias iguais

	0.2% DMSO	*1% TRITON X100*
Média	0,080667	1,268667
Variância	0,001886	0,000732
Observações	3	3
Variância agrupada	0,001309	
Hipótese de diferença de média	0	
gl	4	
Stat t	-40,2103	
P(T<=t) uni-caudal	1,14E-06	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	2,29E-06	
t crítico bi-caudal	2,776445	

Teste T: duas amostras com variâncias iguais

	25 µM	*0.2% DMSO*
Média	0,134667	0,080667
Variância	0,002117	0,001886
Observações	3	3
Variância agrupada	0,002002	
Hipótese de diferença de média	0	
gl	4	
Stat t	1,478174	
P(T<=t) uni-caudal	0,106717	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,213435	
t crítico bi-caudal	2,776445	

Teste T: duas amostras com variâncias iguais

	37,5 µM	*0.2% DMSO*
Média	0,362	0,080667
Variância	0,014539	0,001886
Observações	3	3
Variância agrupada	0,008213	
Hipótese de diferença de média	0	
gl	4	
Stat t	3,80211	
P(T<=t) uni-caudal	0,009535	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,019069	
t crítico bi-caudal	2,776445	

Teste T: duas amostras com variâncias iguais

	50 µM	*0.2% DMSO*
Média	0,570667	0,080667
Variância	0,02588	0,001886
Observações	3	3
Variância agrupada	0,013883	
Hipótese de diferença de média	0	
gl	4	
Stat t	5,093248	
P(T<=t) uni-caudal	0,003508	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,007016	
t crítico bi-caudal	2,776445	

P bi caudal > 0,05 accept H0, means are equal
P bi caudal < 0,05 reject H0, means are unequal and statistically different

Table 10 (Compilation). Student's T-tests for LDH results of JWH-073

Replicates	0.2% DMSO	1% TRITON X100	1 µM	5 µM	10 µM	25 µM	37,5 µM	50 µM
1	-0,061	1,009	0,078	0,035	0,017	0,121	0,02	0,131
2	-0,025	0,813	0,123	0,084	0,033	0,095	0,075	0,105
3	0,022	1,142	0,002	0,003	-0,004	0,037	-0,012	0,041
Mean	-0,021	0,988	0,068	0,041	0,015	0,084	0,028	0,092
SD	0,029	0,117	0,044	0,029	0,013	0,032	0,032	0,034
LDH %	-0,052	100,881	8,848	6,148	3,615	5,237	5,237	11,315
SD for LDH	2,889	11,667	4,378	2,889	1,289	3,156	3,156	3,422

Table 11. Raw data from LDH results of JWH-250

Teste F: duas amostras para variâncias

	1% TRITON X100	*0.2% DMSO*
Média	0,988	-0,021333
Variância	0,027391	0,0017323
Observações	3	3
gl	2	2
F	15,81162	
P(F<=f) uni-caudal	0,059483	
F crítico uni-caudal	19	

Teste F: duas amostras para variâncias

	1 µM	*0.2% DMSO*
Média	0,067667	-0,02133
Variância	0,00374	0,001732
Observações	3	3
gl	2	2
F	2,15913	
P(F<=f) uni-caudal	0,316543	
F crítico uni-caudal	19	

Teste F: duas amostras para variâncias

	0.2% DMSO	*5 µM*
Média	-0,02133	0,040667
Variância	0,001732	0,001664
Observações	3	3
gl	2	2
F	1,040857	
P(F<=f) uni-caudal	0,48999	
F crítico uni-caudal	19	

Teste F: duas amostras para variâncias

	0.2% DMSO	*10 µM*
Média	-0,02133	0,015333
Variância	0,001732	0,000344
Observações	3	3
gl	2	2
F	5,030978	
P(F<=f) uni-caudal	0,165811	
F crítico uni-caudal	19	

Teste F: duas amostras para variâncias

	25 µM	*0.2% DMSO*
Média	0,084333	-0,021333
Variância	0,001849	0,0017323
Observações	3	3
gl	2	2
F	1,067539	
P(F<=f) uni-caudal	0,483667	
F crítico uni-caudal	19	

Teste F: duas amostras para variâncias

	37,5 µM	*0.2% DMSO*
Média	0,027667	-0,02133
Variância	0,001936	0,001732
Observações	3	3
gl	2	2
F	1,11776	
P(F<=f) uni-caudal	0,472197	
F crítico uni-caudal	19	

Teste F: duas amostras para variâncias

	50 µM	*0.2% DMSO*
Média	0,092333	-0,02133
Variância	0,002145	0,001732
Observações	3	3
gl	2	2
F	1,238407	
P(F<=f) uni-caudal	0,446746	
F crítico uni-caudal	19	

F < F crit, equal variances; F > F crit, unequal variances

Table 12 (Compilation). F-tests for LDH results of JWH-250.

Teste T: duas amostras com variâncias iguais

	1% TRITON X100	*0.2% DMSO*
Média	0,988	-0,02133
Variância	0,027391	0,001732
Observações	3	3
Variância agrupada	0,014562	
Hipótese de diferença de média	0	
gl	4	
Stat t	10,24412	
P(T<=t) uni-caudal	0,000256	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,000512	
t crítico bi-caudal	2,776445	

Teste T: duas amostras com variâncias iguais

	1 µM	*0.2% DMSO*
Média	0,067667	-0,02133
Variância	0,00374	0,001732
Observações	3	3
Variância agrupada	0,002736	
Hipótese de diferença de média	0	
gl	4	
Stat t	2,083776	
P(T<=t) uni-caudal	0,052785	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,105571	
t crítico bi-caudal	2,776445	

Teste T: duas amostras com variâncias iguais

	5 µM	*0.2% DMSO*
Média	0,040667	-0,02133
Variância	0,001664	0,001732
Observações	3	3
Variância agrupada	0,001698	
Hipótese de diferença de média	0	
gl	4	
Stat t	1,842578	
P(T<=t) uni-caudal	0,069591	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,139182	
t crítico bi-caudal	2,776445	

Teste T: duas amostras com variâncias iguais

	0.2% DMSO	*10 µM*
Média	-0,02133	0,015333
Variância	0,001732	0,000344
Observações	3	3
Variância agrupada	0,001038	
Hipótese de diferença de média	0	
gl	4	
Stat t	-1,39363	
P(T<=t) uni-caudal	0,117935	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,235869	
t crítico bi-caudal	2,776445	

Teste T: duas amostras com variâncias iguais

	25 µM	*0.2% DMSO*
Média	0,084333	-0,02133
Variância	0,001849	0,001732
Observações	3	3
Variância agrupada	0,001791	
Hipótese de diferença de média	0	
gl	4	
Stat t	3,058131	
P(T<=t) uni-caudal	0,018864	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,037727	
t crítico bi-caudal	2,776445	

Teste T: duas amostras com variâncias iguais

	37,5 µM	*0.2% DMSO*
Média	0,027667	-0,02133
Variância	0,001936	0,001732
Observações	3	3
Variância agrupada	0,001834	
Hipótese de diferença de média	0	
gl	4	
Stat t	1,401208	
P(T<=t) uni-caudal	0,116883	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,233767	
t crítico bi-caudal	2,776445	

Teste T: duas amostras com variâncias iguais

	50 µM	*0.2% DMSO*
Média	0,092333	-0,02133
Variância	0,002145	0,001732
Observações	3	3
Variância agrupada	0,001939	
Hipótese de diferença de média	0	
gl	4	
Stat t	3,161612	
P(T<=t) uni-caudal	0,017066	
t crítico uni-caudal	2,131847	
P(T<=t) bi-caudal	0,034131	
t crítico bi-caudal	2,776445	

P bi caudal > 0,05 **accept H0, means are equal**
P bi caudal < 0,05 **reject H0, means are unequal and statistically different**

Table 13 (Compilation). Student's T-tests for LDH results of JWH-250.