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Cytotoxiciy test of cathinones in a human kidney cell model

Abstract

The number of new psychoactive substances (NSP) is continuously growing, completely changing the recreational drug market. Of this wide variety of new substances, a great emphasis is placed on synthetic cathinones. These drugs are known as "bath salts" and appeared on the market as legal substitutes for illicit substances, such as 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) or cocaine. The use of these substances is often considered harmless, but has actually been linked to several cases of toxicity and deaths. Most toxicological studies to date involve the use of liver or brain in vitro models, with little toxicological information on renal damage.

The kidney is one of the excretory organs of these drugs and is therefore exposed to toxicity, this work covers the preliminary study of nephrotoxicity caused by cathinones. For this, a model of human kidney cells (the immortalized cell line HK-2) was exposed for 24h to a wide range of concentrations (0.01-10 mM) of two synthetic cathinones (methylone and MDPV) and the cytotoxicity was evaluated by the assay of reduction of MTT.

Keywords: synthetic cathinones, kidney cells, cytotoxicity, MTT test, viability

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Introduction

New psychoactive substances (NPS) have appeared on the European market as "legal alternatives" to internationally controlled substances in recent years. By 31st October 2020, the EU Early Warning System has monitored more than 820 substances since 1997.[1] The number of NPS introduced to the drug market have increased dramatically in 2008. Between 2008 and 2018 around 90% of these compounds have been identified, having the maximum number of NPS notified in 2014-2015 with approximately 100 substances.[2–4] However, probably due to efforts to control NPS in Europe, as well as legal changes in countries of origin to restrict production, the number of substances introduced into the European illicit market has decreased in recent years, with 53 substances notified for the first time in 2019 and 38 substances in 2020. Despite this downward trend, around 400 previously reported NPS have been detected in the European market each year since 2015.[1] Due to their constant chemical modification and rapid introduction on the market, they are not being controlled by international control mechanisms.[5] They make up a wide range of compounds, such as synthetic cannabinoids, stimulants, opioids and benzodiazepines. Therefore, they are marketed as an alternative to already legislatively controlled illicit drugs, usually labelled under name of "legal highs", "designer drugs" or "research chemicals". In other cases, they are destined for minority groups who want to test them to see their potential novel effects.[4] This produces a high-risk to public health and a challenge to drug policies. Synthetic cathinones, often named "bath salts" or "plant feeders", are a subgroup of NPS. They were introduced into the illicit market in 2004 first time as replacements for stimulant drugs, such as 3,4-methylenedioxymethamphetamine (MDMA) or cocaine.[4,6] According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) report, 138 cathinones have been monitored by the end of 2018. In addition, most seizures of NPS are dominated by synthetic cannabinoids and cathinones in Europe, approximately 77% of all seizures reported in 2018.[4,7] Synthetic cathinones are derived from of cathinone (*S*-(-)-2-amino-1-phenyl-1-propanone), a natural stimulant present in *khat* leaves plant (*Catha edulis Forsk*) and a β-ketone analogue of amphetamine, both from phenethylamine family. Structurally, synthetic cathinones are formed by a phenethylamine core, with an alkyl group attached to α position and a ketone group attached to β carbon, together with additional substitutions.[8,9] The most popular synthetic cathinones used as drugs of abuse include 3,4-methylenedioxypyrovalerone (MDPV) and methylone.[10–13] Consumption of these amphetamine-like stimulants can produce adverse effects as tachycardia, vasoconstriction, hypertension, hyperthermia, respiratory distress, spams, chest pain, hepatic dysfunction, acute kidney injury, coma and death.[9,14,15] Even so, toxicological information on the possible harmful effects of these types of compounds remains scarce. Synthetic cathinones exert their stimulating effects by modifying the synaptic concentration of catecholamines in the central nervous system.[16,17] MDPV has the ability to block the reuptake of dopamine, acting as a selective inhibitor of dopamine transporter activity (DAT). On the other hand, methylone behaves as a non-selective inhibitor of monoamine transports, which induces the release mediated by the transporter of dopamine (DA), norepinephrine (NE) and serotonin (5-HT), similar to the actions of MDMA.[9,17–20] Studies at the cellular level to evaluate the neurotoxic potential of synthetic cathinones were evaluated in cultured human dopaminergic SH-SY5Y cells.[12,20,21] Otherwise, the liver is a major target for many amphetamine-like stimulants. Several *in vitro* hepatotoxicity studies have been described using different hepatic cell lines or primary hepatocytes of different species to determine the possible cytotoxic effect of synthetic cathinones,[15,22,23] or amphetamines.[23,24] In general, most of the data obtained by these studies show the similarities between the toxic events caused by synthetic cathinones such as MDPV, and amphetamines such as MDMA. This suggests a correlation in the toxicity mechanism of

these drugs.[15,20,21] In addition, synthetic cathinones induced the loss of cell viability in a concentration-and time-dependent manner, showing neurotoxicity[20,21] or hepatotoxicity.[15,22]

The kidney is one of the major organs of excretion and is exposed to a greater proportion of drugs and chemicals, which can be excreted unchanged or in the form of metabolites that may be more toxic than the parent compound.[25]The glomerular filtrate is concentrated in the proximal tubules of kidneys by reabsorption of essential molecules.[26] Proximal tubes are the place predisposed to injury by drugs, especially when those normally eliminated through kidneys. It is therefore important screening and understanding of the potential toxicity of drugs cause in this place to minimizes risks of nephrotoxicity.[27] The adverse effects produced by synthetic cathinones described in the scientific literature include hyperthermia or rhabdomyolysis, leading to acute renal injury.[28–31] However, the cellular mechanisms behind the nephrotoxic effects of synthetic cathinones are not yet known. Therefore, this work aimed to carry out a preliminary study to evaluate *in vitro* the cytotoxic potential of two cathinones in human kidney cells, derived from proximal tubules of kidneys (Hk-2).

Material and methods

Materials and reagents

RPM1-1640 medium, Triton X-100 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Heat-inactivated fetal bovine serum (FBS), antibiotic mixture of penicillin/streptomycin (10,000 U/ml/10,000 mg/ml) and trypsin 0.25%-EDTA were obtained from GIBCO Invitrogen (Barcelona, Spain). All other chemicals of analytical grade were purchased from Merck (Darmstadt, Germany). Hydrochloride salts of methylone and MDPV were purchased online from the Sensearomatic website (http://sensearomatic.net, currently unavailable). The salts were fully characterized by mass spectrometry, NMR and elemental analysis (data not shown), and purity was >98%. Hk-2 cells (human kidney cell line) was obtained from the American Type Culture Collection (ATCC®, VA, USA).

Cell culture

HK-2 cells were cultured in 75 cm^2 canted-neck tissue culture flasks in RPM1-1640 medium (pH 7.4), supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (10000 U mL^{-1}/10000 µg mL^{-1}). Cells were maintained in a humidified incubator, at 37 °C, with 5% CO_2. Cells were sub-cultured at approximately 80% confluence over a maximum of 10 passages. For this, the medium was removed, the cells were washed with 1 mL of trypsin/EDTA 0.25% and another 2 mL trypsin/EDTA 0.25% was added to the flask and incubated at 37 °C for 5 minutes. Once the cells were detached from the surface of the flask, trypsin/EDTA was inactivated with fresh culture medium (1:4) and the suspension was passed to a culture flask and/or plate.

Stock and working solutions

Stock solutions of methylone and MDPV were prepared individually in sterile water by weighing the corresponding compounds at 50 mM and stored at -20 °C. At the time of the experiments, stock solutions were diluted in serum-free RPMI medium until the concentrations of 0.02, 0.1, 0.2, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 20 mM. Hk-2 cells were exposed to concentrations of 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8 and 10 mM, by dilution (1:2) in wells with serum-free culture medium.

Exposition of drugs and MTT reduction assay

Briefly, Hk-2 cells were seeded in 96-wells plates, at a density of 1 x 10^4 cell per well. Peripheral wells on the plate were filled with sterile water to prevent evaporation and concentration of test solutions. Twenty-four hours after seeding, the medium was gently aspirated and the cells were incubated with the test drugs, methylone and MDPV, individually, in a humidified air atmosphere containing 5% CO_2, at 37 °C. The concentrations were selected to cover the whole effect range, from undetectable effects (when compared with negative controls) to 100% mortality. Each plate included six replicates of negative controls (i.e. serum-free culture medium without test agents) and six replicates of positive controls (full media with 1% Triton X-100) (Figure 1.1a). After 24 h of exposure to drugs, at 37 °C, the medium was removed followed by the addition of 100 µL of fresh serum-free culture medium containing 0.5 mg/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Plates were incubated at 37 °C, for 2h. Finally, the cell culture medium was aspirated and the formed intracellular formazan crystals dissolved in 200 µL of 100% DMSO Figure 1.1b and measured on the spectrophotometer. The spectrophotometric analysis was run at 550 and 690 nm using a multi-wells plate reader (Stat Fax 3200, Awareness Technology, USA). Data were obtained from at least five (MDPV) and six (methylone) independent experiments, with each test plate containing three replicates of 16 increasing concentrations of the tested individual drugs.

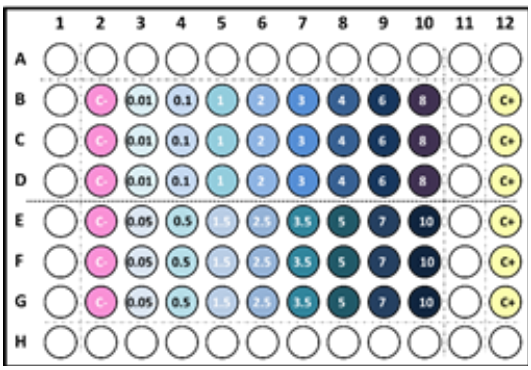

Figure 1A Seeding of Hk-2 cells and exposure to concentrations of methylone and MDPV (in mM). The negative control is serum-free culture medium (*C- in the pink circle*) and positive control is 2% Triton (*C+ in yellow circle*).

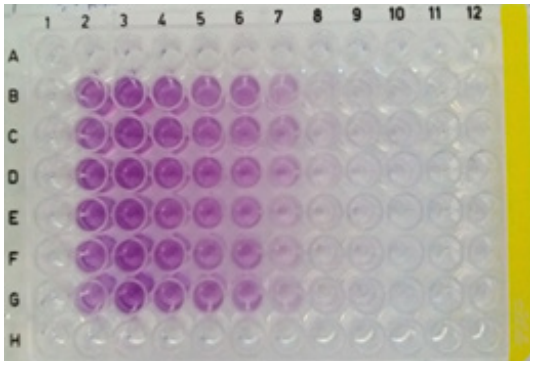

Figure 1B An example of the MTT assay for methylone.

Statistical analysis

Curves of normalized MTT assay data were adjusted and analyzed to the dosimetric logit model based on a goodness-fit approach,[32] following the equation:

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$$y = \frac{\theta min + (\theta max - \theta)}{1 + \exp[-\theta 1 - \theta 2 * \log(x)]}$$

where θ_{min} and θ_{max} represent the minimal and maximal observed effects, respectively x is the concentration of test compound, θ_1 is the parameter for location and θ_2 is the slope parameter. MTT data were obtained from a least five or six independent experiments, performed in triplicate, and are presented including the 95% confidence intervals (CI) of the mean values and as mean ± standard error of the mean (SEM). Statistical comparisons were performed by one-way analysis of variance (ANOVA) following by Dunn´s multiple comparison test. Significance was accepted at $p<0.05$. Statistical analysis was performed using Graph Pad Prism 7 (version 7.01) for Windows.

Results

The potential cytotoxic effects of cathinones derivatives were screened *in vitro* in the human renal cells (Hk-2 cell line). Hk-2 is an immortalized cell line retaining functional and phenotypic characteristics of normal adult human proximal tubular epithelium, which is suitable for the study of nephrotoxic agents such as drugs of abuse.[27] Hk-2 cells were exposed to different concentrations of cathinones for 24 h. Cell viability was indirectly evaluated through the assessment of mitochondrial function (*MTT reduction assay*) by reducing the tetrazolium salts. Briefly, in MTT reduction assay, yellow tetrazolium MTT is reduced by metabolically active cells (mitochondrial reductase enzymes) to generate reducing equivalents, such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means (550 and 690 nm) (Figure 2).

In this work, it was observed by the MTT assay, that both individual cathinone derivatives tested, induced cell death in a concentration-dependent manner. The data were obtained through five (MDPV) or six (methylone) independent experiments, using independently prepared serial dilutions of all tested compounds. The cytotoxicity dose-response curves for each of the tested drugs, including the upper and lower 95% CI, are displayed in Figure 3. Several parameters were determined, namely the EC_{50}, the lower and upper limits of the curve, as well as the Hill Slope. Cathinones showed variable potencies according to their structure.

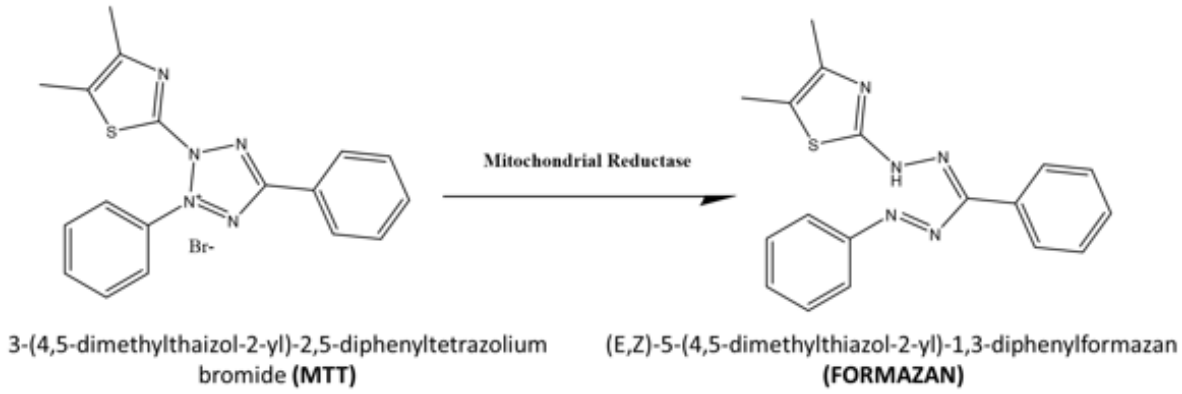

Figure 2 MTT reduction reaction.

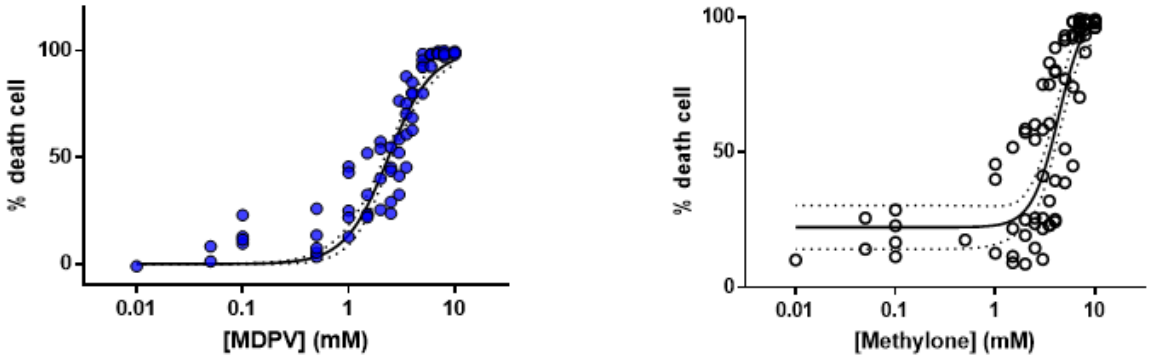

Figure 3 Complete concentration-response curves obtained with the MTT reduction cytotoxicity assay for MPDV and methylone, using Hk-2 cell line after 24 h incubations at 37 °C. The blue and open circles represent data from five and six independent experiments for MDPV and methylone, respectively. The solid lines represent the regression model (logit) and the dotted lines represent the corresponding 95% CI. The *blue line* shows the EC_{50} and EC_{max} for each response curve.

Significance differences were observed in the studied compounds. Methylone presents an EC_{50} value of 4.35 mM, whereas MDPV presents an EC_{50} value of 2.377 mM. Therefore, methylone decreased the viability of Hk-2 cells in a concentration-dependent manner, inducing a significant decrease in cell viability at a concentration of 5 mM. MDPV also decreased the viability of Hk-2 cells in a concentration-dependent manner, instead induce a significant decrease in cell viability at a concentration of 1 mM or higher, being more cytotoxic than methylone (Figures 4 &5).

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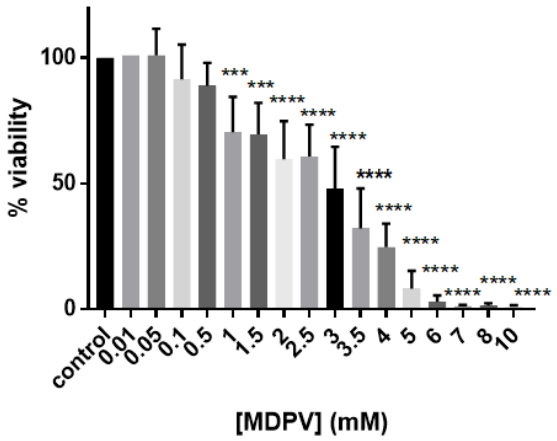

Figure 4 Effects of MDPV on MTT reduction in Hk-2 cells. Cells were exposed for 24 h, at 37 °C, to 0.01–10 mM MDPV concentrations. Results are presented as mean ± SEM from at least five independent experiments, performed in triplicate (***$p < 0.001$, ****$p < 0.0001$ versus control).

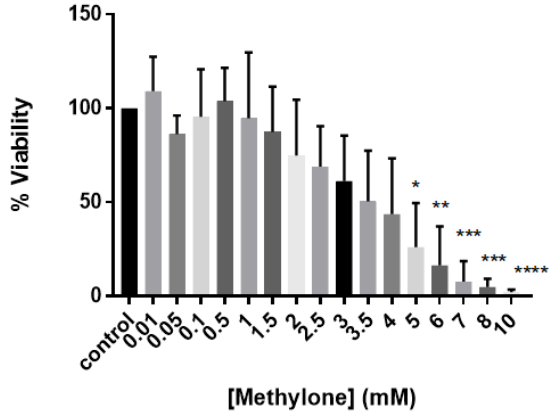

Figure 5 Effects of Methylone on MTT reduction in Hk-2 cells. Cells were exposed for 24 h, at 37 °C, to 0.01–10 mM methylone concentrations. Results are presented as mean ± SEM from at least six independent experiments, performed in triplicate (*$p < 0.1$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ versus control).

Discussion

Potential harms arising from the use of cathinones are currently scarce. Cathinones are used as an alternative for long-studied amphetamines, due to chemical similarity. Thus, similar pharmacodynamics and toxicological mechanisms for β-keto amphetamine-derivative drugs could be expected.[33] In addition, research into the toxicological mechanism responsible for kidney injury due to drug nephrotoxicity has been limited and unclear.[27]

This work provides a preliminary step in the evidence of nephrotoxicity by methylone and MDPV in human kidney cells (Hk-2). The results showed that methylone was the least cytotoxic compound in this *in vitro* model, whereas MDPV was a more potent substance. Methylone (3,4-methylenedioxymethcathinone) is a substituted cathinone analogue of 3,4-methylenedioxymethamphetamine (MDMA, commonly known as "ecstasy"). It differs from MDMA in a ketone group in the beta position of the phenethylamine core, located in methylone. Due to the presence of the β-keto group, methylone has a lower log P value (-0.396) compared with MDMA (2.05), which indicates lower lipophilicity. In another way, MDPV has a pyrrolidine ring and a nitrogen atom attached to three carbon atoms composing a tertiary amino group in the structure causing high lipophilicity (log P 2.43) and creating a less polar molecule more able to cross the blood-brain barrier.[16,34] In addition, a longer α-alkyl chain causes higher lipophilicity, hence, MDPV exhibits a higher plasma concentration than methylone after dosing the same amount of the pure substance.[9,14] Therefore, results are in accordance with those found in previous studies for cathinones derivatives and some other amphetamine-like derivatives,[15,24,35,36] carried out in hepatic *in vitro* cell models. In the same way, the data obtained in this investigation are in agreement with those found in subsequent published study of these cathinones in Hk-2 cells.[27] These authors obtained similar results in terms of potency, despite having obtained slightly higher EC_{50} for methylone (EC_{50} 6.31 vs 4.35) and MDPV (EC_{50} 3.83 vs 2.37).

On the other hand, the concentrations used in this *in vitro* study were selected to cover a wide cytotoxic effect range. However, these concentrations exceeded those found in biological samples, which are usually in the low micromolar range.[37–40] Even so, the concentrations used can be considered physiological relevant, due to the unique property of the renal tubular epithelium of concentrating urine and its components, including drugs, which makes the kidney particularly susceptible to toxicity. According to this, recently published data[41] showed that the concentration found in urine samples are commonly higher than those found in blood. In addition, the actual levels of synthetic cathinones detected in urine samples range from 0.2 to than 500 µg/mL, which is relatively high considering the EC values analyzed here (2.4-31180 µg/mL). In addition, due to their high lipophilicity, these cathinones have a high volume of distribution, which means that concentrations of alpha-pyrrolidinophenone derivatives are not directly related to blood levels.In some postmortem studies, methylone[40] and MDPV[42,43] concentrations were found higher in tissues such as brain, liver and kidneys than in blood. Furthermore, postmortem concentrations can be lower than after drug intake due to metabolic procedure and postmortem redistribution. Methylenedioxy-types cathinones with a secondary amine have the highest central/peripheral blood ratios, so they are susceptible to postmortem redistribution. Due to the instability of beta-keto amphetamines, the distribution of cathinones postmortem is complicated. Therefore, the concentrations of these cathinones were found higher in hepatocytes[35,44] and renal[27] than those found in blood.

Conclusion

The results obtained from this study revealed that nephrotoxicity occurred at relatively high concentrations of the tested cathinones compared to the low micromolar concentrations found in blood or urine samples. In addition, the same concentration range used in these experiments was similar to that used in *in vitro* toxicity studies with cathinones and amphetamines derivatives, e.g. MDMA, and hepatocytes or renal cells (Hk-2) as a model cellular. Therefore, the results are in accordance with those obtained for methylone and MDPV in hepatocytes and renal cells (HK-2), establishing the kidney as a target organ for nephrotoxicity by synthetic cathinones.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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