LISBON ADDICTIONS 2019

LISBON CONGRESS CENTRE
23 -25 OCTOBER 2019
THIRD EUROPEAN CONFERENCE ON ADDICTIVE BEHAVIOURS AND DEPENDENCIES

THE IN VITRO HEPATOTOXICITY OF THE NEW RECREATIONAL DRUG 3-METHYLMETHCATHINONE (3-MMC) INVOLVES AUTOPHAGY AND APOPTOSIS

Rita Roque Bravo*, Tomás Duarte de Almeida, Maria João Valente, Bárbara Ferreira, Rita Rebelo, Félix Carvalho, Maria de Lourdes Bastos, Helena Carmo, Diana Dias da Silva*

UCIBIO, REQUIMTE, Laboratory of Toxicology, Faculty of Pharmacy, University of Porto, Rua Jorge Viterbo Ferreira, 228, Porto, 4050-313, Portugal.
* e-mails: dsilva@ff.up.pt; rita.rdsb@gmail.com

INTRODUCTION AND AIMS

3-Methylmethcathinone (3-MMC) has become one of the most popular recreational drugs worldwide after the ban of mephedrone, and it has been associated with several reports of intoxications and fatal cases. Current knowledge of the effects and toxicity of 3-MMC is mainly based on user reports and clinical intoxications, while pre-clinical toxicological data are scarce. As the liver is a target organ due to its preponderant role in metabolism of toxicants, the aim of this study was to assess the potential hepatotoxicity of 3-MMC, by evaluating its in vitro toxicity to primary rat hepatocytes (PRH), the influence of metabolism in the drug's toxicity and the underlying toxicological mechanisms.

METHODS

Primary rat hepatocytes (PRH) were exposed for 24 h to 3-MMC [from 31 nM to 10 mM]. Cell death was assessed through the MTT reduction assay, the LDH leakage assay, and the NR uptake assay; and data were fitted to the Logit regression model. The mechanisms that contributed to the putative cytotoxicity were also assessed by measuring oxidative stress parameters, alterations to mitochondrial function, and activation of cell death pathways, after exposing cells to 1 µM, 10 µM, 100 µM, and 500 µM of 3-MMC. Finally, the modulatory effects of cytochrome P450 (CYP) inhibitors on the toxicity of 3-MMC were also evaluated.

RESULTS

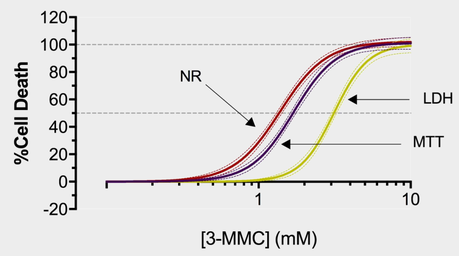

Figure 1. Cell death after 24 h-exposures at 37 ºC of primary rat hepatocytes to 3-methylmethcathinone (3-MMC), obtained in the lactate dehydrogenase leakage assay (LDH, data displayed in yellow), neutral red uptake assay (NR, data displayed in red), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium reduction assay (MTT, data displayed in purple), as indirect measures of cell viability. Data are presented as percentage of cell death relative to the negative controls and are from four independent experiments performed in triplicate. Curves were fitted to the dosimetric Logit model. The dotted lines are the upper and lower limits of the 95 % confidence interval of the best estimate of mean responses. The dashed grey lines represent 50 % and 100 % effect.

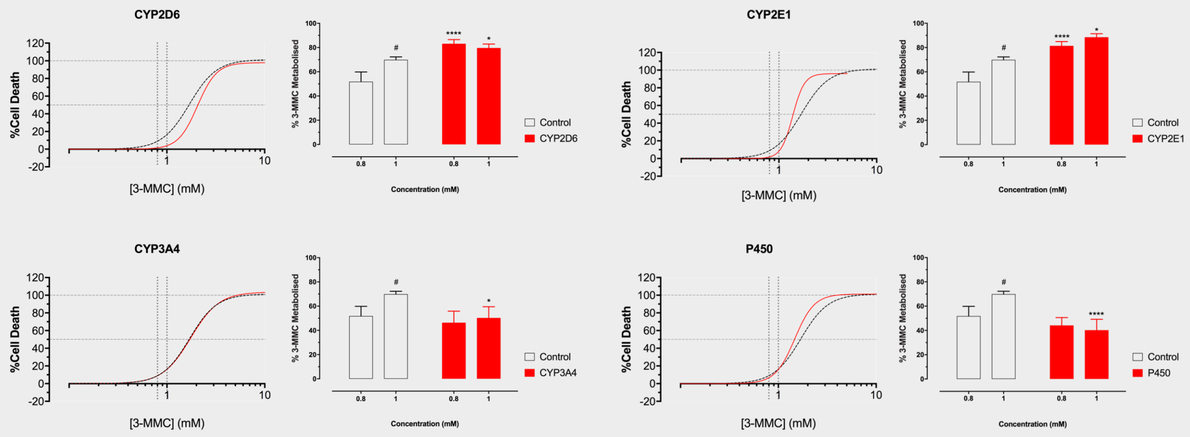

Figure 2. *Left side of each panel:* Cell death obtained in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay, as an indirect measure of cell viability, after 24 h-exposures at 37 ºC of primary rat hepatocytes to 3-methylmethcathinone (3-MMC), in the presence (red solid curves) or absence (black dashed curves) of different inhibitors of cytochrome P450 isoforms (CYP). Data are presented as percentage of cell death relative to the negative controls and are from four independent experiments performed in triplicate. Curves were fitted to the dosimetric Logit model (parameters displayed in Table 1). The dashed grey lines represent 50 % and 100 % effect. *Right side of each panel:* Metabolism of 3-MMC determined by GC–MS when tested at 800 µM or 1 mM, in the presence (red bars) or absence (white bars) of P450 specific and non-specific inhibitors. Data are mean ± SEM and were obtained from four independent experiments run in duplicate. Metyrapone was used as inhibitor of the isoform CYP2E1; quinidine was used as inhibitor of the isoform CYP2D6; ketoconazole was used as inhibitor of the isoform CYP3A4; and 1-aminobenzotriazole (ABT) was used as general inhibitor of cytochrome P450. Statistical comparisons were made using one-way ANOVA/Dunnett's post hoc test. *$p < 0.05$; ****$p < 0.0001$, *versus* control at the same concentration; #$p < 0.05$, *versus* control at 800 µM.

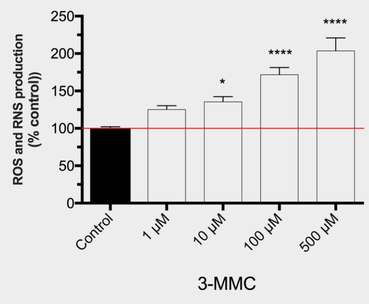

Figure 3. Production of oxygen and nitrogen reactive species (ROS and RNS), measured through the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) assay, in rat primary hepatocytes after 24-h incubation with 3-methylmethcathinone (3-MMC), at 37 °C. Results are expressed as percentage control ± standard error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

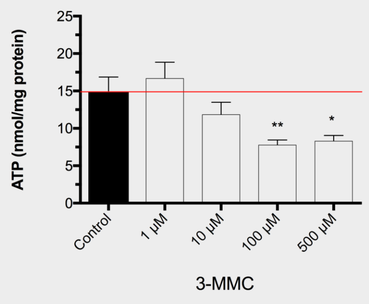

Figure 4. Intracellular ATP levels, measured through the luciferin-luciferase assay, in primary rat hepatocytes after 24 h-incubation with 3-methylmethcathinone (3-MMC), at 37 °C. Results are expressed as mean ± standard error of the mean (SEM) from four independent experiments. Statistical comparisons were made using one-way ANOVA/Dunnett's. *$p < 0.05$; **$p < 0.01$, *versus* control.

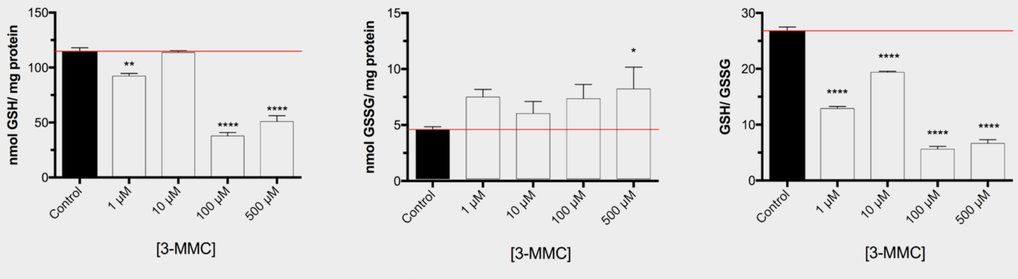

Figure 5. Intracellular contents of reduced (GSH) and oxidized glutathione (GSSG), measured through the DTNB-GSSG reductase recycling assay, and intracellular GSH/GSSG ratio in rat primary hepatocytes after 24 h-incubation with 3-methylmethcathinone (3-MMC). Results are expressed as mean ± standard error from the mean (SEM) from four independent experiments. Statistical comparisons were made using one-way ANOVA/Dunnett's post hoc test. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$, *versus* control.

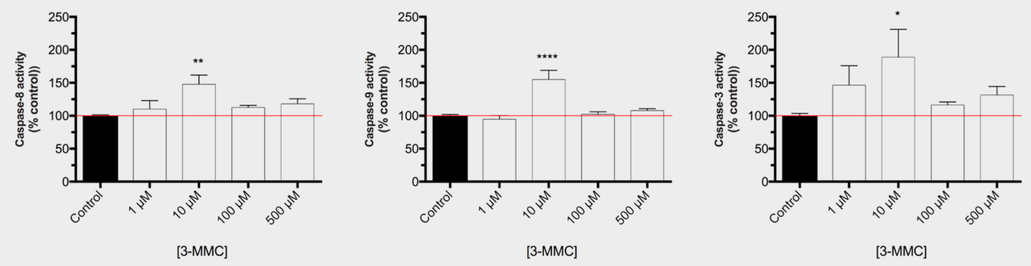

Figure 6. Caspase-3, -8, and -9 activities, measured in the cytoplasmic fraction through a colorimetric assay, in primary rat hepatocytes after 24-h incubations with 3-methylmethcathinone (3-MMC), at 37 °C. Results are expressed as percentage of control ± standard error of the mean (SEM) from four independent experiments. Statistical comparisons were made using one-way ANOVA/Dunnett's post hoc test. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$, *versus* control.

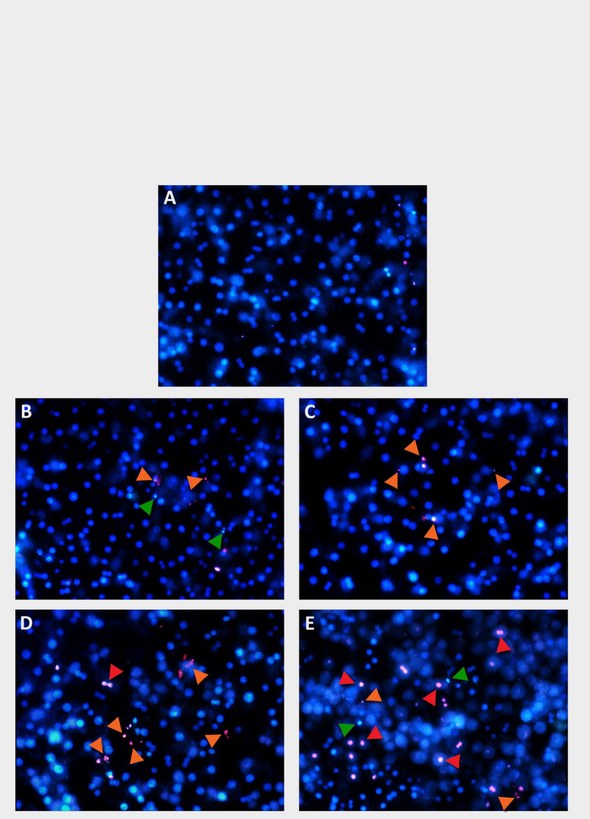

Figure 7. Hoechst 33342/propidium iodide staining of primary rat hepatocytes after exposure to control cells (A) or 3-methylmethcathinone (3-MMC) at 1 µM (B), 10 µM (C), 100 µM (D) and 500 µM (E) for 24 h, at 37 ºC. Red arrows indicate necrotic cells, green arrows indicate early apoptotic cells and orange arrows indicate late apoptotic cells. Original magnification, 200×.

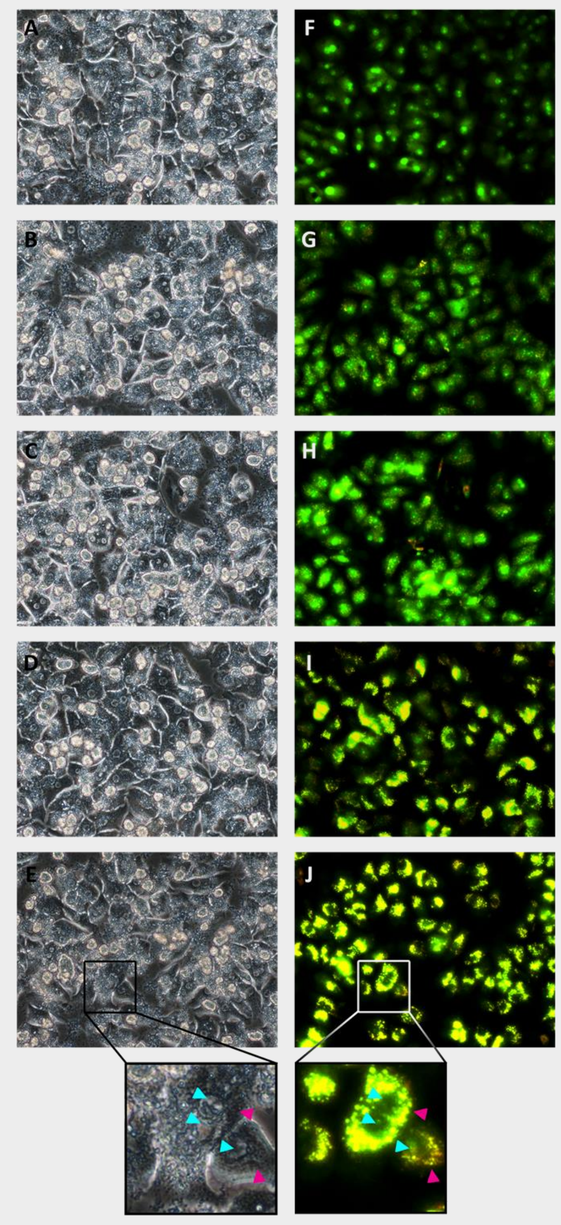

Figure 8. Representative phase contrast (A, B, C, D and E) and fluorescence (F, G, H, I and J) micrographs of primary hepatocytes stained with acridine orange after exposure to 3-methylmethcathinone (3-MMC). Cells treated with 1 µM (B, G), 10 µM (C, H), 100 µM (D, I) and 500 µM (E, J) of 3-MMC for 24 h present acidic vesicular organelles stained in yellow (red arrow), which are absent in control cells (A, F).

CONCLUSIONS

Overall, our data point to a role of metabolism in the hepatotoxicity of 3-MMC, which appears to be associated to both autophagic and apoptotic mechanisms. The current work is a first attempt to better understand the toxicology of 3-MMC.

Conflicts of Interest: The authors declare that they have no conflict of interest

Funding: FEDER funds through the COMPETE 2020–POCI and FCT in the framework of the project **POCI-01-0145-FEDER-029584**

BIBLIOGRAPHY Tyrkko E, Pelander A, Ketola RA and Ojanpera I (2013) In silico and in vitro metabolism studies support identification of designer drugs in human urine by liquid chromatography/quadrupole-time-of-flight mass spectrometry. *Analytical and bioanalytical chemistry* **405**:6697-6709. Shima N, Katagi M, Kamata H, Matsuta S, Nakanishi K, Zaitsu K, Kamata T, Nishioka H, Miki A, Tatsuno M, Sato T, Tsuchihashi H and Suzuki K (2013) Urinary excretion and metabolism of the newly encountered designer drug 3,4- dimethylmethcathinone in humans. *Forensic Toxicology* **31**:101-112.