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## 3-FLUOROMETHCATHINONE, A STRUCTURAL ANALOG OF MEPHEDRONE, INHIBITS GROWTH AND INDUCES CELL CYCLE ARREST IN HT22 MOUSE HIPPOCAMPAL CELLS

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Recently, the abuse of recreational drugs has become an important problem in many countries. Among these psychoactive substances are synthetic cathinones, a group of compounds derived from the alkaloid cathinone, that have gained widespread popularity. Many cathinones have demonstrated neurotoxic effects. The aim of this study was to examine the effects of 3-fluoromethcathinone, a structural analog of mephedrone, on HT22 mouse hippocampal cells. Cell viability was assessed using the sulforhodamine B assay. Flow cytometry was used to study the cell cycle distribution. We found that 3-fluoromethcathinone inhibits growth of HT22 cells. Our results also revealed that it induces G<sub>0</sub>/G<sub>1</sub>-phase cell cycle arrest. To our knowledge, this is the first study to demonstrate the cytotoxic action of 3-fluoromethcathinone. Our findings suggest that abuse of this cathinone derivative may not be without risk.

**Key words:** 3-fluoromethcathinone, cathinones, recreational drugs, legal highs, mouse hippocampal cells

### INTRODUCTION

Recently, synthetic cathinones have gained widespread popularity as a new group of recreational drugs. Synthetic cathinones are derivatives of cathinone (*Fig. 1B*), an alkaloid found in leaves of *Catha edulis* (khat) (1). These compounds are structurally related to phenylalkylamines such as amphetamine (*Fig. 1A*) and act as central nervous system stimulants (2-5). They promote the release and/or inhibit the reuptake of monoamine neurotransmitters such as serotonin, dopamine and noradrenaline. Several cathinone derivatives such as methcathinone, bupropion or pyrovalerone have been used as active pharmaceutical ingredients (6, 7). Recent data confirm the growing popularity of nonmedical use of cathinones (6, 8-12). They are used for their psychostimulant effects and entertainment purposes. 'Designer' cathinones are mostly encountered as amorphous or crystalline powders, tablets and capsules. In recent years, they have been marketed in 'head shops' or *via* the internet and have often been advertised as 'legal highs'. These products are intentionally mislabelled and distributed as 'plant food', 'plant feeders', 'research chemicals' or 'bath salts' (6, 9, 13, 14). They are often labelled with warnings 'not for human consumption' or 'for research purposes only'. Psychological and behavioural effects of synthetic cathinones reported by users include general stimulation, euphoria, improved mood, mild empathogenic effects, sociability, talkativeness, awareness of senses and enhanced music appreciation (6, 13, 15-19). The most common adverse effects according to users' reports are tachycardia, hypertension, hyperthermia, insomnia, agitation, hallucinations/delusions and

confusion (6, 13, 15-30). Cathinones are often combined with other psychoactive substances, such as cannabis, MDMA (3,4-methylenedioxymethamphetamine) as well as alcohol (6). There are numerous reports of poisonings and fatality cases related to synthetic cathinones (16-30). Most of them resulted from mephedrone (4-methylmethcathinone, *Fig. 1C*) use (18, 19, 21, 23, 24, 26-28). Distribution of many synthetic cathinones has been legally controlled in some countries (7, 13). However, their new derivatives have been continuously developed to circumvent control mechanisms (13, 26, 31). At the end of the last decade, especially in 2009-2010, the most popular synthetic cathinone in European Union countries was mephedrone (10, 13). Once it became illegal, its structural analogs appeared on the market as its legal alternatives. Among them were naphyrone (naphthylpyrovalerone), flephedrone (4-fluoromethcathinone) or 3-fluoromethcathinone (*Fig. 1D*) (30-34).

Synthetic cathinones have important implications for public health. There is limited data about their biological activity. Thus, potential users of cathinones are exposed to substances of unknown health risk. There are very few reports in the literature regarding 3-fluoromethcathinone (20, 34-39). It was found to inhibit noradrenaline and dopamine uptake and to release noradrenaline, dopamine and serotonin (39). There are only a few reports on its metabolism in human liver microsomes, rat urine and rabbit liver slices (36, 37). Since many cathinones have been demonstrated to exert neurotoxic effects (40-44), we hypothesized that 3-fluoromethcathinone may also be neurotoxic. Therefore, the aim of the study was to examine the effects of 3-fluoromethcathinone on HT22 mouse hippocampal cells viability *via* cellular protein determination with the

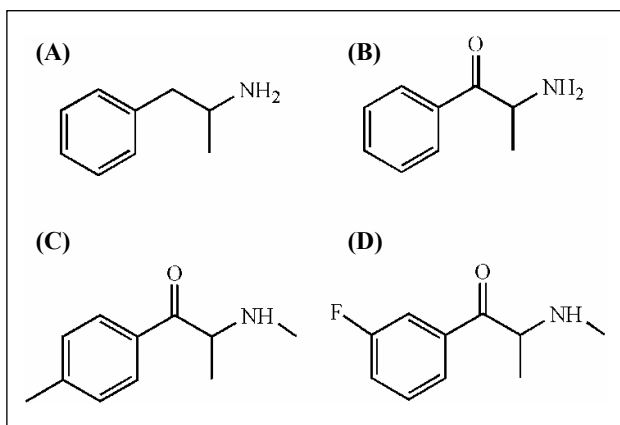


Fig. 1. Chemical structure of (A) amphetamine, (B) cathinone, (C) mephedrone, (D) 3-fluoromethcathinone.

sulforhodamine B assay and cell cycle distribution using flow cytometry.

## MATERIALS AND METHODS

### Chemicals

3-fluoromethcathinone was purchased from LGC Standards (UK). Its stock solutions were prepared using sterile physiological saline solution and diluted to indicated concentrations before use. Sulforhodamine B, RNase A and propidium iodide (PI) were obtained from Sigma-Aldrich (USA).

### Cell culture

The immortalized mouse hippocampal neuronal HT22 cell line was kindly provided by Professor M. Wozniak (Department of Medical Chemistry, Medical University of Gdansk, Poland). HT22 cells were maintained in CO<sub>2</sub> incubator, at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, USA), supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, USA), 100 IU/ml penicillin (Sigma-Aldrich, USA) and 100 µg/ml streptomycin (Sigma-Aldrich, USA).

### Sulforhodamine B assay

HT22 cells were seeded in 24-well plates (7×10<sup>5</sup> cells per well) and allowed to attach for 24 hours. Next, cells were exposed to 3-fluoromethcathinone for 24 hours. Control cells were incubated for 24 hours in the presence of solvent (physiological saline). After treatment, TCA (trichloroacetic acid, final concentration: 10% w/v) was added and cells were incubated at 4°C for 1 hour. Thereafter, supernatants were removed and cells were rinsed several times with deionized water. Plates were air dried (20 min, room temperature) and cells were then incubated with 0.4% (w/v) sulforhodamine B solution (sulforhodamine B was dissolved in 1% acetic acid) for 20 min at room temperature. Supernatants were removed and cells were then rinsed several times with 1% acetic acid to remove the unbound dye. Plates were air dried (20 min, room temperature) and the incorporated sulforhodamine B was then released from cells with 10 mM Tris base solution (pH 10.5). Absorbance was measured at a wavelength of 570 nm using a microplate reader (ELx800; BioTek Instruments, Inc., USA).

### Cell cycle analysis

After treatment, cells were collected, washed with cold phosphate buffered saline (PBS) and fixed in ice-cold 70% ethanol at -20°C overnight. Next, cells were washed with cold PBS, suspended in staining solution (50 µg/ml PI and 25 µg/ml DNase-free RNase A in PBS) and incubated in the dark at 37°C for 30 min. After incubation, flow cytometry analyses were performed (Becton Dickinson FACSCalibur, USA).

### Statistical analysis

Statistical analysis was performed using Statistica 9 software (StatSoft, Poland). Data are expressed as means ± S.D. Each experiment was repeated at least three times. Statistical differences between samples were evaluated using the non-parametric Mann-Whitney U test. Differences were considered significant at p < 0.05.

## RESULTS

### Effect of 3-fluoromethcathinone on cell viability

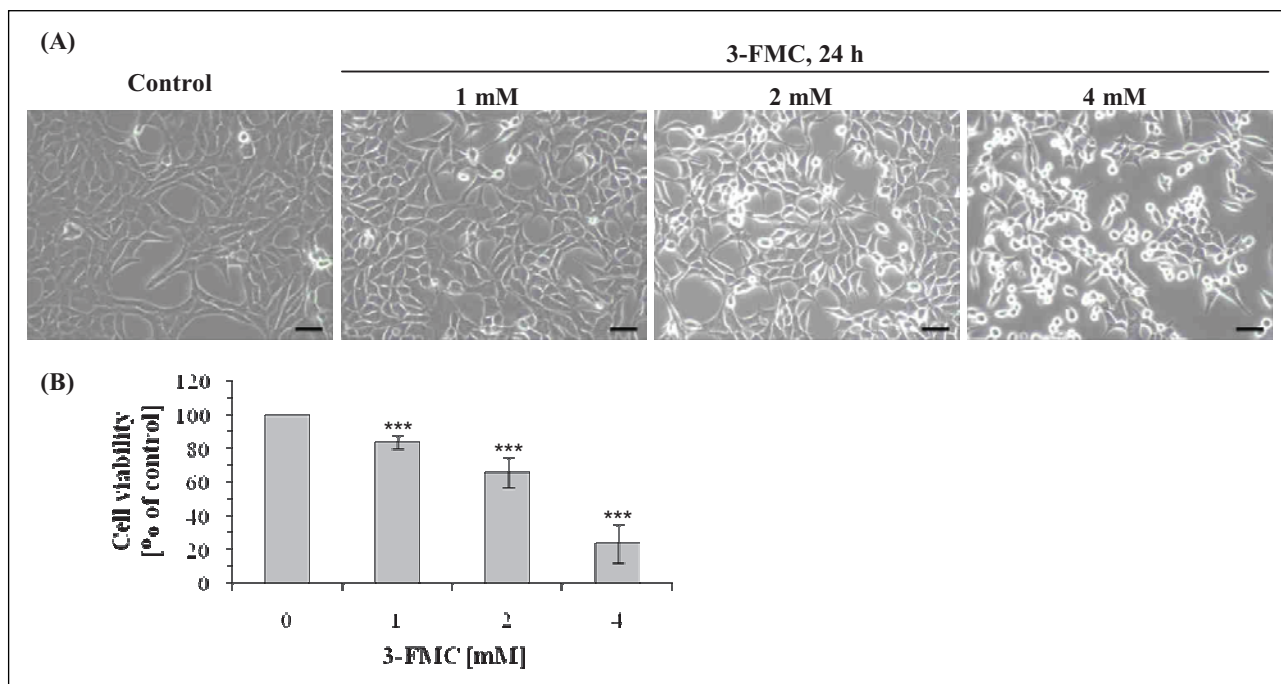
Our results showed that 3-fluoromethcathinone (3-FMC) at millimolar concentrations reduced viability of HT22 hippocampal cells (Fig. 2A, 2B). Compared to control (untreated cells), 1 mM 3-FMC reduced cell viability by about 16% (Fig. 2B). 2 mM and 4 mM 3-FMC caused about 34% and 76% reduction of cell growth, respectively (Fig. 2B). Light microscopy micrographs showed a concentration-dependent increase in the number of cells that were detached from the culture plate surface (Fig. 2A).

### Effect of 3-fluoromethcathinone on cell cycle progression

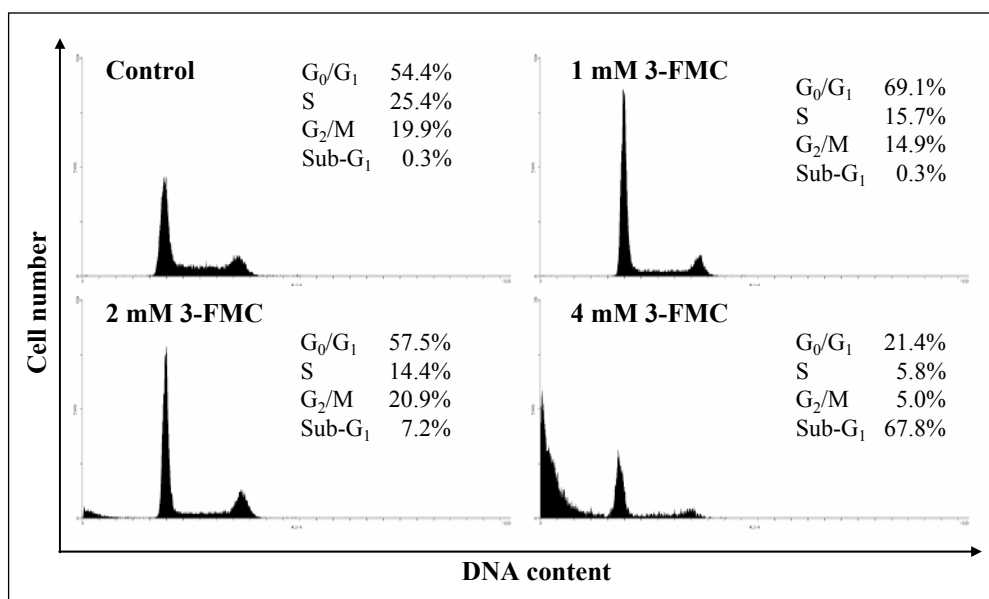
The cell cycle analysis revealed that 24 hours of incubation of HT22 cells with 1 mM 3-FMC led to the G<sub>0</sub>/G<sub>1</sub>-phase arrest (Fig. 3). Accumulation of cells in the G<sub>0</sub>/G<sub>1</sub> phase was accompanied by a corresponding decrease in the number of cells in S and G<sub>2</sub>/M fractions (Fig. 3). Compared to control, treatment of cells with 2 mM and 4 mM 3-FMC for 24 hours resulted in an increase in the number of cells in the sub-G<sub>1</sub> fraction (Fig. 3). This effect was negligible in the case of 2 mM 3-FMC, but was prominent in the case of 4 mM 3-FMC.

## DISCUSSION

Many recreational drugs, including synthetic cathinones have been reported to exert neurotoxic and other adverse effects (6, 13, 40, 41, 45-49). There is evidence suggesting that MDMA, an active component of 'Ecstasy', is toxic to brain serotonin neurons (45-47). Methylone, a cathinone derivative structurally similar to MDMA, was reported to cause selective depletion of serotonin and its transporter levels in the rat brain, indicative of neurotoxicity (40). Serotonin transporters play a crucial role in neurotransmission (50-52). Mephedrone was found to reduce working memory performance in animal models (40). There is also evidence of impaired memory after mephedrone use in humans (53). Mephedrone was found to be a substrate for the serotonin transporter (4). Hadlock *et al.* demonstrated decreases in hippocampal serotonin transporter function and serotonin level after repeated mephedrone administrations in rats (41). In contrast to this finding, however, other *in vivo* studies indicated that mephedrone did not alter monoamine neurotransmitters levels in rats and mice (4, 40). It was also found that it did not



**Fig. 2.** Effect of 3-FMC on viability of HT22 cells. (A) Phase-contrast images of HT22 cells. Cells were incubated in the absence (control) or presence of 3-FMC for 24 hours. Data are representative of three independent experiments in duplicates. *Bar* 50  $\mu$ m. (B) Sulforhodamine B assay. HT22 cells were incubated in the absence (control) or presence of 3-FMC for 24 hours. Data are presented as means  $\pm$  S.D. of four independent experiments,  $n=12$  ( $n$  – number of samples per each experimental point).



**Fig. 3.** Effect of 3-FMC on cell cycle distribution of HT22 cells (flow cytometry analysis, PI staining). HT22 cells were treated in the absence (control) or presence of 3-FMC for 24 hours. Data represent one of three independent experiments, which gave similar results.

cause neurotoxicity to dopamine nerve endings of the striatum and serotonin nerve endings of the hippocampus in mice (43, 44). It is noteworthy that 3-fluoromethcathinone was found to affect rotorod performance and impair locomotor coordination and balance in a functional observation battery in mice (38). Its structural analogs were shown to have effects on serotonergic neurons (54). Pharmacological examination of 3-trifluoromethylmethcathinone revealed that it was 10-fold more potent than methcathinone as an uptake inhibitor and a releasing agent at the serotonin transporter (54).

To our knowledge, there are no studies regarding *in vitro* toxicity of 3-fluoromethcathinone. In the present study, we demonstrate for the first time that 3-FMC at millimolar

concentrations inhibits growth of HT22 mouse hippocampal cells. HT22 cell line has been widely used as an *in vitro* neuronal model (55-59). HT22 cells resemble proliferating neuronal precursor cells. After treatment with 3-FMC we observed concentration-dependent decrease in viability of HT22 cells. This effect was the smallest at 1 mM and the most prominent at 4 mM 3-FMC concentration. Noteworthy, Bredholt *et al.* demonstrated that 0.1 mM cathinone and 0.1 mM cathine had no cytotoxic effects on human peripheral blood leukocytes (60). Moreover, amphetamine decreased viability of rat cortical neurons (61). Its IC<sub>50</sub> concentration estimated after 24 hours of treatment was 1.40 mM. 24 hours - exposure to 0.3 mM methamphetamine significantly reduced proliferation of rat

hippocampal neural progenitor cells. (62). The mechanism underlying 3-FMC cytotoxicity needs to be elucidated. Noteworthy, oxidative stress has been suggested to be involved in toxicity of some recreational drugs (62-66).

Our results revealed that 1 mM 3-FMC induced G<sub>0</sub>/G<sub>1</sub>-phase cell cycle arrest, whereas 2 mM and 4 mM 3-FMC did not. The possible explanation may be that after 24 hours of treatment, this effect occurs over a very narrow concentration range of 3-FMC. To our knowledge, there are no reports regarding effects of this cathinone derivative on the cell cycle distribution. However, the G<sub>0</sub>/G<sub>1</sub>-phase cell cycle arrest was also observed after exposure of normal human oral keratinocytes and fibroblasts to khat extract - a source of cathinone and its derivatives (67). We found that at 4 mM concentration of 3-FMC a prominent, statistically significant increase in the number of cells in the sub-G1 fraction appeared, indicative of apoptotic DNA cleavage (68). However, additional studies are needed to confirm whether 3-FMC induces apoptosis in HT22 cells (69). Interestingly, khat extract and its purified constituents cathinone and cathine were found to induce apoptosis in HL60 human leukemia cells (70). Moreover, khat extract was also shown to induce apoptosis in normal human oral keratinocytes and fibroblasts (71). Amphetamine, methamphetamine, methylenedioxyamphetamine, and methylenedioxymethamphetamine ('Ecstasy') reduced proliferation and induced apoptotic cell death in rat neocortical neurons *in vitro* (72). These effects were studied after 96 hours of treatment and were more pronounced at 0.5 mM and higher concentrations of the drugs (72). Amphetamine induced apoptosis in rat cortical neurons (61). Apoptosis was also detected in rat hippocampal neural progenitor cells after treatment with methamphetamine (62). Moreover, 24 hours exposure of N9 murine microglial cells to methamphetamine at relatively high concentrations 1 mM, 2 mM and 4 mM led to a significant increase of population of apoptotic cells (73). Our results revealed that after 24 hours of treatment the effect of 2 mM 3-FMC on the number of cells in the sub-G1 fraction was negligible. However, it may probably become more prominent after longer time of treatment (74). 1 mM 3-FMC did not significantly increase the population of cells in the sub-G1 fraction. However, this effect may appear after prolonged incubation with the drug as a consequence of the G<sub>0</sub>/G<sub>1</sub>-phase cell cycle arrest (75). Although 3-FMC caused concentration-dependent decrease in viability of HT22 cells, results of the cell cycle analysis suggest that at different concentrations effects of this drug on the cell cycle may vary. Probably, at different concentrations its mechanism of action varies or shows different time-dependency pattern.

Noteworthy, many cathinones were reported to be used at relatively high doses (6, 13, 15). For instance, according to users' self-reports during the typical mephedrone 'session' a total of 0.5–1.0 g of the drug is taken. Acute mephedrone intoxications that required hospitalisation were reported to involve doses ranging from 0.1–7.0 g (15). In comparison to amphetamine analogs higher doses of cathinones are necessary to produce similar psychostimulatory effects (13). To prolong drugs' effects users take several doses, thereby increasing the risk of overdose. In cases of fatal mephedrone intoxications, mephedrone concentrations measured in blood of the deceased were 0.13 mg/L (28), 0.23 mg/L (28), 0.5 mg/L (76), 0.98 mg/L (28), 2.24 mg/L (28), 3.3 mg/L (77), 5.1 mg/L (23), 5.5 mg/L (19), 22 mg/L (77). Mephedrone concentrations in urine of the deceased were 186 mg/L (23) and 198 mg/L (76). There are no reports in the literature regarding doses of 3-FMC or fatal intoxications with this drug. Our results revealed that 3-FMC exerted significant effects on viability and cell cycle distribution of HT22 mouse hippocampal cells at relatively high concentrations. They are much higher than concentrations of

mephedrone detected in blood of the deceased (19, 23, 28, 76, 77). Probably, they cannot be reached in the brain *in vivo*. Thus, 3-FMC exhibited relatively low cytotoxicity against HT22 cell line. However, additional studies on different experimental models should be performed to improve our knowledge about effects of this drug.

In summary, we have demonstrated cytotoxic effects of 3-fluoromethcathinone. Further studies are required to explain the mechanisms of its action. However, our findings suggest caution when ingesting 3-FMC. Its abuse may not be without risk.

*Acknowledgements:* This work was supported by grant no. MN-93 and grant no. MN 01-0039/08 from the Medical University of Gdansk (Gdansk, Poland).

Conflict of interests: None declared.

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Received: November 8, 2013

Accepted: February 10, 2014

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