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Acute effects of methcathinone and manganese in mice: A dose response study

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ABSTRACT

An intravenously injectable illicit drug made by mixing pseudoephedrine, potassium permanganate, vinegar and water, yielding methcathinone (Mcat) and manganese (Mn), induces an extrapyramidal syndrome with parkinsonism, dystonia, gait and balance disorders similar to manganism. Although the cause of the syndrome is largely attributed to Mn, the interaction of the drug's individual components is not known and the role of Mcat is possibly underestimated. Aim of the present study was to analyze dose-dependent behavioral effects of the mixture and its two main active components Mcat and Mn in an acute setting and determine the lethal doses of each substance.

Three groups of C57BL/6 mice were injected intraperitoneally with (1) the drug mixture containing 10, 25, 50, 100 or 150 mg of Mcat and respectively 1.6, 3.8, 6.9, 17.1 and 22.6 mg of Mn per kilogram of body weight; (2) 10, 25, 50, 100, 150, 200 or 300 mg of racemic Mcat/kg of body weight; (3) $MnCl_2$ 10, 25 or 50 mg/kg of body weight. Locomotor activity of the animals, various signs and time of death were recorded.

Lower doses (10 and 25 mg/kg) of Mcat had a clear motor activity stimulating effect and this was clearly dose-dependent. High doses of Mcat produced epileptic seizures in 74% of the animals and became lethal with the highest doses. Similarly, the mixture had a clear dose-dependent stimulating effect and the higher doses became lethal. The LD_{50} of the pseudoephedrine mixture was 110.2 mg of Mcat/kg and for pure Mcat 201.7 mg/kg. Mn did not prove to be lethal in doses up to 50 mg/kg, but had a strong dose dependent inhibitory effect on the animals' behavior. Our data reveal that both Mn and Mcat have a significant role in the toxicity of the mixture.

1. Introduction

During the last 20 years, a drug induced extrapyramidal syndrome has been described mostly in Eastern European countries [1, 2] with cases also reported in North America and Western European countries [3, 4]. Although not very widespread, it has a devastating effect on the social and physical functioning of its sufferers.

The clinical syndrome is caused by an illicit drug composition made by adding potassium permanganate and vinegar to common cold remedies containing pseudoephedrine. The drug is then injected intravenously multiple times a day, generating an immediate short-lasting amphetamine-like "high" driving further abuse of the mixture. After several months of continuous injections, most users develop an irreversible extrapyramidal syndrome described by marked hypokinesia, dysarthria, dystonia and postural instability with early falls. This syndrome closely resembles manganism - a neurological condition caused by excessive environmental exposure to manganese (Mn) [5]. Despite evident symptoms of extrapyramidal damage, the syndrome, like manganism, does not respond to usual antiparkinsonian treatment and remains a lifelong cause of disability to the vast majority of the influenced population.

As a byproduct of the chemical reaction, the mixture contains a large amount of two potent neurotoxic substances – methcathinone (Mcat) and Mn. Due to the similarities between manganism and the aforementioned

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syndrome, the cause has been largely attributed to Mn. This environmentally abundant metal is known to accumulate in the basal ganglia leading to significant damage of several monoaminergic systems and harming basal ganglia function [6]. During active drug use the subjects have an increased blood concentration of manganese as well as T1 hyperintense lesions of the basal ganglia visible on magnetic resonance imaging. Both of these alterations will normalize after cessation of drug use while the clinical syndrome remains unchanged [2].

Mcat (also named as ephedrone) is a drug from the amphetamine class of substances. It can be synthesized by oxidizing ephedrine or pseudoephedrine with potassium permanganate under acidic conditions. The reduction product of these substances is methamphetamine (METH) [7]. Mcat has a dose and enantiomer dependent dopamine depleting effect in the *striatum* (STR) of rodents [8], as well as several other deleterious effects on different neurotransmitter systems. There is increasing evidence suggesting an increased risk for movement disorders associated with psychostimulant use [9].

Presently there is a lack of literature concerning the Mcat/Mn mixture to serve as the basis for further preclinical studies. Thus the aim of this study was to determine and describe the acute dose related effects (lethal doses, motor behavior) of the mixture and its main components in mice in order to establish an animal model of the neurological syndrome in future studies.

2. Materials and methods

This study was approved by the National Board of Animal Experiments of Estonian Ministry of Agriculture, permission number 124, 04.12.2008. Funding for the study was provided by grant PUT1239 from the Estonian Research Council.

2.1. Animals

Male C57BL/6 mice (Harlan Laboratories B.V., Venray, The Netherlands), aged four months, were housed in groups of 6 in plastic housing containers with open tops. The animals were kept in vivarium conditions (constant room temperature and humidity) on a 12 h light/dark cycle with free access to water and food. The animals received a standard laboratory animal feed (R-70, LabFor, Sweden). This feed has a manganese (Mn) content of 68 mg/kg of feed. The health status, cage bedding and nutrition were checked once per day. Prior to injections, the mice were weighed and randomly assigned into treatment groups of 14 animals each.

2.2. Drugs and dose-response curve

The methcathinone and manganese containing mixture (Mcat/Mn) was prepared as follows: 48 pills of Sudafed (GlaxoSmithKline Pharmaceuticals, Poznan, Poland) containing a total of 2880 mg of pseudoephedrine hydrochloride ([(+)-(1S,2S)-2-methylamino-1-phenylpropan-1-ol, $C_{10}H_{15}NO$, CAS 90-82-4, MW 165.22]) were stripped of the coating layer, mixed with 4.8 ml (96 drops) of vinegar ([30% acetic acid, $C_2H_4O_2$], AS JAPS M.V.M., Estonia) and 4 grams of potassium permanganate ($KMnO_4$, UAB "Valentis", Vilnius, Lithuania) and were then added to 88 ml of boiling tap water. The mixture was stirred until the uncoated pills were dissolved and then cooled rapidly in a cold water bath. The clear top layer was filtered through a filter paper to remove any visible debris.

Mcat content of the solution was measured (see below) and the resulting solution was further diluted with 0.9% saline (Sodium Chloride 0.9%, B. Braun Melsungen AG, Melsungen, Germany) in order to deliver 10, 25, 50, 100 and 150 mg of Mcat per kilogram of animal body weight (groups Mcat/Mn 1 – Mcat/Mn 5, see Table 1). The Mn content of each dilution was measured by atomic absorption spectrometry (see below). This method of drug preparation closely resembles the one used by addicts [1].

Racemic Mcat ([(*RS*)-2-(methylamino)-1-phenylpropan-1-one hydrochloride, $C_{10}H_{13}NO$ HCl, MW 199.68], Sigma-Aldrich Corp., St. Louis, MO, USA) was dissolved in sterile 0.9% saline (Sodium Chloride 0.9%, B. Braun Melsungen AG, Melsungen, Germany) in order to deliver 10, 25, 50, 100, 150, 200 and 300 mg of Mcat per kilogram of animal body weight (groups Mcat 1 – Mcat 7, Table 1).

Manganese chloride ($MnCl_2$ x 4H2O, Sigma-Aldrich Corp., St. Louis, MO, USA) was diluted with sterile 0.9% saline in order to deliver either 10, 25 or 50 mg of $MnCl_2$ per kilogram of animal body weight (groups Mn 1- Mn 3, Table 1).

All control groups received sterile 0.9% saline.

All injections were given intraperitoneally with a 0.3 ml injection volume. For the purposes of easier comparison to Mcat, Mcat/Mn groups are labelled according to the amount of Mcat in the mixture. All doses are listed in Table 1.

After completion of all necessary experimental work the animals were euthanized. Animals were euthanized using an intraperitoneal injection of Sodium Thiopenthal (300 mg/kg). When death of the animal was confirmed, the brain was removed and flash-frozen in liquid nitrogen and then stored at -80C for any further studies.

2.3. Motor activity recording

Locomotor activity was tested immediately after the injections during a 30 min period in photoelectric motility boxes. Each box measuring 448 × 448 × 450 mm was connected to a computer (ActiMot/MoTil, TSE Technical & Scientific Equipment GmbH, Germany). Illumination of the boxes was approximately 400 lux. Each animal was placed in the center area of the box singly and time in locomotion (in seconds), distance travelled (in meters), time on rear feet (in seconds) and time spent in the

Table 1
Concentrations of substances according to groups.

Mcat/Mn			Methcathinone (Mcat)		Manganese (Mn)	
Group	Methcathinone concentration (mg/kg)	Manganese concentration (mg/kg)	Group	Methcathinone concentration (mg/kg)	Group	Manganese concentration (mg/kg)
Control	0	0	Control	0	Control	0
Mcat/Mn 1	10	1.6	Mcat 1	10	Mn 1	10
Mcat/Mn 2	25	3.8	Mcat 2	25	Mn 2	25
Mcat/Mn 3	50	6.9	Mcat 3	50	Mn 3	50
Mcat/Mn 4	100	17.1	Mcat 4	100		
Mcat/Mn 5	150	22.6	Mcat 5	150		
			Mcat 6	200		
			Mcat 7	300		

center of the box (in seconds) were recorded. Center of the box was defined as 60% of the centermost floor area. Seizures, stereotypical behavior and time of death were recorded manually during the 30 min period.

2.4. Methcathinone content analysis

Mcat content of the non diluted Mcat/Mn solution was analyzed. The Acquity UPC^2 system from Waters Corporation, Milford, USA, was equipped with a binary solvent delivery pump, an autosampler, a column oven and a back pressure regulator. The qualitative analysis was performed at 40°C using an Acquity UPC^2 HSS C_{18} SB column (100 mm × 3.0 mm, 1.8 μm; Waters, Milford, MA, USA). The mobile phase flow rate was maintained at 2.0 mL/min with a gradient elution (eluent A, CO_2; eluent B, methanol with 0.1% formic acid). The gradient program was started with 2% of component B, then, a linear gradient was programmed from 2% to 50% for 1.00 min, held for 0.20 min followed by a linear gradient down to 2% B in 0.30 min, and finally it was held for 1.50 min which allowed ionic liquids to elute out from the instrument. Isocratic solvent was ethanol at a flow rate of 0.3 mL/min. The back pressure was set at 1900 psi and the injection volume was 1.0 μL.

Mcat was identified by using a Waters Xevo TQ-S mass spectrometer (Milford, MA, USA). The data acquisition was in the positive ion electrospray ionization (ESI) mode. The desolvation gas was nitrogen, and the collision gas was argon (0.25 mL/min). The data acquisition range was *m/z* 25–575. The capillary voltage was 1.0 kV, the cone voltage was 32.0 V, and the source offset was 58 V. The source temperature was 150 °C and the desolvation temperature was 550 °C with the desolvation gas flow rate of 600.0 L/h. The cone gas flow was 150.0 L/h. The nebulizer gas flow was at 4.9 bar. MS data were collected using two separate scan functions. The first scan function was set at low collision energy (5 eV), which provided parent ions, and the second scan function was set at high collision energy (12 eV) which provided fragment ions (*m/z* 145.88) of parent ions (*m/z* 164.08) (multiple reactions monitoring (MRM)) from standard Mcat purchased from Sigma AB (Stockholm, Sweden). The dwell time for each function was set at 0.08 s. Data were acquired and analyzed with Waters MassLynx v4.1 software. Identification was done by comparing retention time and standard fragments obtained from an authentic Mcat. Finally the quantification was accomplished using an external calibration curve. The sample analysis was performed in triplicates to test the repeatability, and the results are acceptable. All data collected in centroid mode were obtained using Masslynx NT4.1 software (Waters Corp., Milford, MA USA).

2.5. Mn content analysis

Mn levels in the Mcat/Mn samples were determined by electrothermal atomic absorption spectrometry (ETAAS) as described by Truus et al. [31] Spectra AA 220Z atomic absorption spectrometer (Varian, Mulgrave, Australia) equipped with a side-heated GTA-110Z graphite atomizer, a Zeeman-effect background correction, and an integrated autosampler was used. Graphite tubes with coating and platforms made of pyrolytic graphite were used. Argon of 99.998% purity (AGA, Helsinki, Finland) was used as the purge gas. The stock atomic spectroscopy standard solution (1000 mg/L^{-1}) of Mn was gradually diluted with 4% HNO3 before use. Water was purified to 18.2 M Ω cm^{-1} resistivity using a Milli-Q water purification system (Millipore, Bedford, USA). The total volume of the solution pipetted into the atomizer was 0.2 mL (0.1 mL of the sample solution plus 0.1 mL of the modifier solution). At least two replicates were measured for each solution.

2.6. Statistical analysis

For the survival analysis we used the groups with lethal outcomes. Survival proportions for these groups were calculated using the Kaplan-Meier method with log rank test for curve comparison. LD50 values were calculated from the dose response data using a sigmoidal dose-response curve and three parameter logistic regression analysis.

Motility data was analyzed using one-way analysis of variance with Dunnett's test for groups comparison and prior D'Agostino and Pearson normality test with the cutoff p value of 0.05. All toxin groups were compared to the control group. P value of <0.05 was considered statistically significant. Analysis was carried out using Prism 6 software (GraphPad Software Inc., LaJolla, CA, USA).

3. Results

3.1. Dose-dependent effects

The Mcat concentration in the non diluted Mcat/Mn mixture was 2.64 ± 0.02 mg/ml. Mn concentration in the various dilutions of Mcat/Mn solution is shown in Table 1.

Main locomotor results are reported by substance in Figs. 1, 2, and 3. In general, all substances had a dose dependent effect on the motor activity of the animals. Mcat and Mcat/Mn had a strong stimulating effect at lower doses (groups Mcat 25 mg/kg, Mcat 50 mg/kg and Mcat/Mn 25 mg/kg and 50 mg/kg), causing a significant increase in motor activity. Behavior altering effects of all active substances were evident within the first 5 min of the injection. The highest doses of all substances resulted in significant decrease of motor activity as well as exploratory behavior of the animals. At the same time the intermediate doses had no significant effect on motor activity. High doses of Mn resulted in significantly decreased motor activity (Figs. 3A and 3B).

All injected substances also influenced exploratory behavior of the animals. Mcat/Mn had the most profound and consistent effect on the time the animals spent in the center of the box and on rear feet. The effect of Mcat was less uniform. Mn had a significant effect on exploratory behavior only at the highest doses.

3.2. Survival analysis

Higher doses of Mcat and Mcat/Mn were lethal. Mortality after administration of 100 mg/kg of Mcat/Mn was 35.7% (5/14) and after 150 mg/kg it was 92.9% (13/14). Injection of 200 and 300 mg/kg of Mcat was lethal in 57.1% (8/14) and 100% (14/14) of the cases respectively. One animal died after administration of 100 mg/kg of Mcat (Fig. 4). Calculated LD_{50} for Mcat/Mn was 110.2 mg/kg and for Mcat it was 201.7 mg/kg. Mn was not lethal at doses up to 50 mg/kg.

3.3. Behavioral analysis

Seizure activity and stereotypical movements were recorded. Mcat had the strongest epileptogenic effect. Most animals in groups Mcat 150 mg/kg (7/14, 50%), Mcat 200 mg/kg (11/14, 78%) and Mcat 300 mg/kg (13/14, 93%) developed generalized tonic-clonic seizures within a few minutes after the injection. With Mcat/Mn, seizures occurred in all animals with lethal outcome in groups Mcat/Mn 100 mg/kg (13/14, 92.9%) and Mcat/Mn 150 mg/kg (5/14, 35.7%). Injections of Mn were not epileptogenic throughout the dose range.

Stereotypical behavior included repetitive movements with front legs and continuous attempts to chew the box floor. This kind of behavior was mostly observed in group Mcat 100 mg/kg (10/14, 71%) but also in group Mcat 150 mg/kg (4/14, 29%). With the higher doses, all animals developed marked difficulties of movement.

High doses of Mn (group Mn 50 mg/kg) did not provoke any seizures or stereotypical behaviour. Instead, these animals (14/14, 100%) were incapable of moving around for a mean duration of 22.7 min after the injection. This effect was considerably less pronounced with lower doses.

4. Discussion

With this study we demonstrate the acute effects of a “home made”

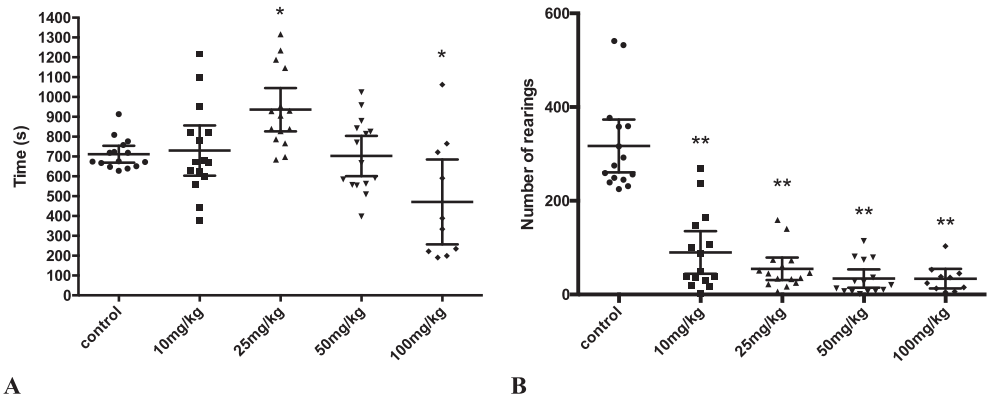


Fig. 1. Effects of Mcat/Mn on time in motion (A) and number of rearings (B) grouped by methcathinone dose. Presented values are means with 95% confidence interval. * signifies $p < 0.05$ and ** signifies $p < 0.01$. The 150 mg/kg group has been excluded from the analysis and in the 100 mg/kg group only the surviving animals were included.

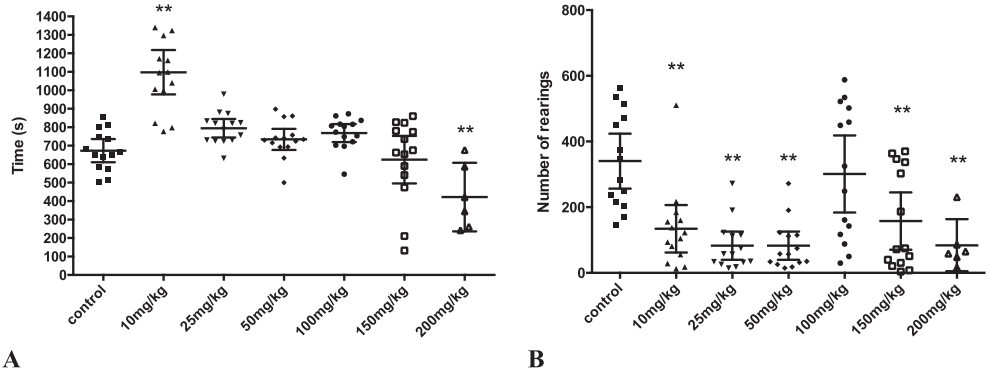


Fig. 2. Effect of Mcat on time in motion (A) and number of rearings (B). Values presented are means with 95% confidence interval. * is $p < 0.05$ and ** is $p < 0.01$. The 300 mg/kg group has been excluded from analysis and in the 200 mg/kg group only the surviving animals were included in the analysis.

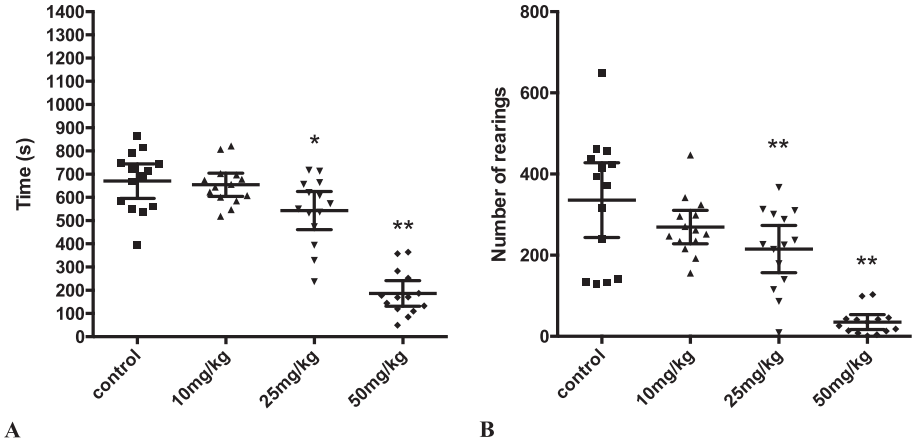
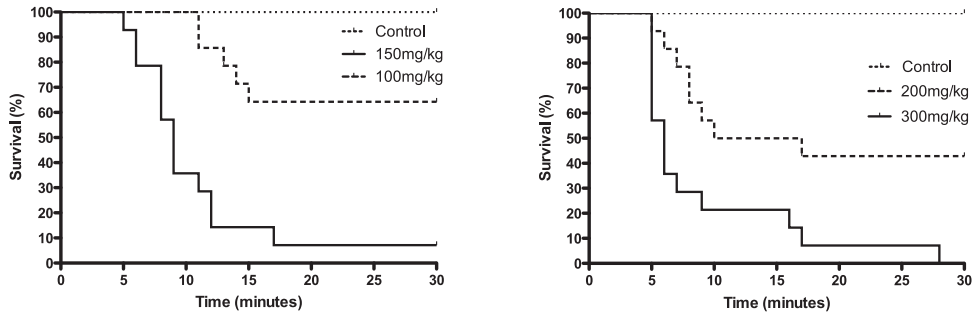


Fig. 3. Effect of Mn on time in motion (A) and number of rearings (B). The values presented are means with 95% confidence interval, all groups are compared to the control group. * is $p < 0.05$ and ** is $p < 0.01$.

narcotic mixture containing Mcat and Mn as the main active components and compare it to the effects of the two components separately.

Both, Mn and Mcat are potent neurotoxic substances. Mn toxicity continues to be a widely researched topic in part due to its apparent association with an irreversible extrapyramidal syndrome (see the excellent review by O'Neal and Zheng [10]). Mn is an essential element for normal cellular functioning. If administered in excessive doses, it accumulates in mitochondria and leads to cellular damage in the basal ganglia with the main target areas being *substantia nigra*, *striatum* and *globus pallidus* [11]. Uptake of Mn is mediated largely by divalent metal transporter 1 (DMT1), which is widely expressed in *striatum*, *substantia nigra pars reticulata* and less in *globus pallidus* and *substantia nigra pars compacta* [12]. Although mechanisms of Mn toxicity are not precisely known, the key components are shown to be the production of reactive oxygen species (ROS), induction of mitochondrial dysfunction [13], protein aggregation [14] and interference with neurotransmitter levels



A B

Fig. 4. Survival curves after administration of Mcat/Mn (A) and Mcat (B). In both groups comparison of survival curves with logrank test shows significant difference between curves ($p < 0.0001$).

and regulation [15]. Dopaminergic system appears to be specifically sensitive compared to other neurotransmitters. Mn toxicity is dependent on its oxidation state, with Mn^{3+} being a more potent toxin than Mn^{2+} [16].

The role of Mn in the toxic properties of the Mcat/Mn is stressed by the fact that when during preparation potassium permanganate is substituted with sodium dichromate, sulfuric acid, acetone or toluene, the drug users avoid the extrapyramidal syndrome [17]. Several experiments have found that in rodents chronic low-level manganese exposure is associated with impaired motor function already at doses below 10 mg/kg [18]. A study by Yang *et al.* [19] shows that intrastriatal administration of $MnCl_2$ also has an acute effect on locomotor activity of mice. We were able to confirm that a single dose of $MnCl_2$ significantly impairs locomotor and exploratory activity of mice. 50 mg/kg of Mn exceeds the amount of Mn given as part of the Mcat/Mn mixture and was not lehtal. Data from literature has shown that cumulative Mn doses of up to 5300 mg/kg can be tolerated.

We observed a dose dependent effect of racemic Mcat and Mcat/Mn containing S(-)methcathinone on the locomotor activity of mice. Racemic Mcat has a strong stimulating effect at 10 mg/kg, whereas equivalent stimulating effect is reached at 25 mg/kg of Mcat as part of the mixture. A notable difference in lethal doses between Mcat (200 mg/kg) and Mcat/Mn (150 mg/kg of Mcat) is observed. The reason for this phenomenon is unclear. A previous study by Gatch *et al* [20] has shown similar results with Mcat, although a lehtal dose was not reached. Increased locomotor activity of animals has also been shown in a study by Anneken *et al* [30], where doses of racemic Mcat up to 80 mg/kg increased both movement time as well as stereotypy time. Mcat belongs to the phenylisopropylamines group of substances. It is the *N*-monomethylated derivate of cathinone. It can be easily synthesized by oxidizing pseudoephedrine with potassium permanganate. In rats racemic Mcat demonstrates more potent activity in stimulus generalization tests compared to racemic cathinone or amphetamine and also induces release of radioactivity form [^{3}H]dopamine prelabeled caudate tissue similar to amphetamine and METH [21]. Two stereoisomers of Mcat are available. Structure activity relationship studies have shown S(-)methcathinone to be more potent than R(+)methcathinone as a locomotor stimulant with the racemic compound fitting between the two [22].

Amphetamines are known to deplete striatal dopamine [23] and damage dopaminergic nerve terminals [24]. In addition, serotonin (5-HT) levels and 5-HT transporter density are decreased in METH abusers. Although analogy of the structure-activity relationship between Mcat and *N*-monomethylated amphetamine derivates exists, they are not completely identical [25]. Psychostimulants are neurotoxic towards the dopaminergic system. A PET study by McCann *et al.* [26] shows reduced striatal binding of a highly specific dopamine transporter (DAT) ligand [11C]WIN-35,428 in abstinent Mcat users – a feature similar to subjects suffering from Parkinson's disease (PD). Additional common pathological changes shared by psychostimulant abuse and PD have been identified. These include METH induced cytoplasmic inclusions in *substantia nigra pars compacta* neurons, inhibition of the ubiquitin-proteasome system due to faulty signalling cascades [27], involvement of specific G-protein coupled receptor (GPCR) signaling mediator proteins like β-arrestin [28] and several others [29].

As a shortcoming of the study, the data does not give a clear understanding about the reasons why the drugs were lethal. We did not measure the cardiovascular effects or alterations in body temperature, which could both significantly alter survival after Mcat use. Effects at the cellular level were also not studied at this time. Mice were selected due to the fact that they have been proven to be an effective and informative model system as demonstrated by numerous studies concerning movement disorders. This model allows for assessment of behavioural effects as well as structural changes in brain tissue, although the results can not necessarily be directly related to humans.

Although in previous studies the toxicity of Mcat/Mn was justified solely by the presence of Mn, our study supports a pivotal role of Mcat in the mixture. This is further supported by the aforementioned findings from several studies. Mcat is the drug with clear psychostimulating activity and has a relatively small activity window – doses 10 times higher than the most effective dose become lethal. We conclude that general toxicity of Mcat may be present already at lower doses and therefore we can not exclude cytotoxicity of the most active dose. Characterisation of the cytotoxic properties of Mcat and Mcat/Mn is already the scope of further studies. Current results also provide information for dose selection in further studies.

Declarations

Author contribution statement

Andres Asser: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sulev Koks, Pille Taba: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ursel Soomets: Performed the experiments; Analyzed and interpreted the data.

Anton Terasmaa, Martin Sauk, Mall Eltermaa, Piret Piip: Performed the experiments.

Kumari Ubhayasekera: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jonas Bergquist: Conceived and designed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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