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Toxicological evaluation of 5-methoxy-2-aminoindane (MEAI): Binge mitigating agent in development

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ABSTRACT

5-Methoxy-2-aminoindane (MEAI) is a psychoactive compound of the aminoindane class, which in recent years has been recreationally used by many people, who reported of a mild euphoric, alcohol-like tipsy experience and reduced desire to consume alcoholic beverages. In the light of these observations it was decided to progress MEAI through a preliminary drug development route and evaluate the acute and subacute toxicity of MEAI administrated orally to Sprague Dawley rats, as well as to determine potential in-vitro cytotoxic and mutagenic effects using state-of-the-art protocols. Furthermore, the interaction of MEAI at the highest non-toxic concentration (100 mg/L) with ethanol at cytotoxic levels of 6% and 7.5% was explored, in order to identify possible additive or synergistic effects.

MEAI showed a good safety profile in rats at 10 and 30 mg/kg body weight, corresponding to the human doses of 1.6 mg/kg and 4.8 mg/kg body weight, respectively. Cytotoxic effect was demonstrated using concentrations of 500 and 1000 mg/L with calculated IC_{50} value of 368.2 mg/L for rat brain striatum primary neurons and 403.1 mg/L for human primary healthy hepatocytes. The combination of 6% or 7.5% ethanol with 100 mg/L MEAI revealed no statistically significant increase of cytotoxic effect. Further studies, especially long term chronic and addictive behavior studies, are required in-order to assess MEAI safety profile.

1. Introduction

Alcohol consumption presents a growing problem worldwide, which some believe may have already overtaken tobacco in terms of overall health and social care costs (Gitto et al., 2016; Nutt et al., 2010). The harms of alcohol overconsumption in the USA alone were estimated to cost almost $250 billion in 2010 (Sacks et al., 2015). Excessive and/or prolonged alcohol consumption results in undesired physiological and psychological adverse effects, including short-term, effects such as gastric irritation, anxiety disorders and other excitable states, and longer-term effects such as cirrhosis, fatty liver disease, cardiomyopathy and dementia (Gitto et al., 2016).

The scale of the problem constituted a major driving force in exploring various drug classes as potential alcohol substitutes, lacking the hazardous side effects, while maintaining alcohol's pleasant properties (Nutt, 2013; Slezak, 2014). The aminoindane derivatives are novel psychoactive substances exerting some empathogenic and mild psychostimulant effects that might provide an alternative form of relaxation to alcohol (Brandt et al., 2013; Haadsma-Svensson et al., 1994; Sainsbury et al., 2011; Simmler et al., 2014). The pharmacological profile of ring-substituted aminoindane derivatives can be generally classified as monoamine transport inhibitors exerting to some extent serotonin releasing activities through the serotonin transporter (Brandt et al., 2013; Simmler et al., 2014).

5-Methoxy-2-aminoindane (MEAI or Chaperon) is a psychoactive compound of the aminoindane class with a mechanism of action described by one patent as being possibly mediated by binding to the dopamine D_3 receptor (Haadsma-Svensson et al., 1994). In recent years MEAI has been recreationally used by many people, mainly in the UK, who report a mild euphoric, alcohol-like tipsy experience (Nutt, 2013; Slezak, 2014). Typically recreational users ingest orally MEAI at doses of up to 1–2 mg/kg body weight in a session and report an onset of effect of up to 4 h with reduced desire to consume alcoholic beverages. This observation has led us to hypothesize that MEAI could potentially be used as a binge mitigation agent, that could impart a feeling of satisfaction, satiety or contentedness and regulate or even discourage binge drinking. According to the recreational MEAI users, no serious adverse events were reported, although one person didn't enjoy the experience. In light of these observations it was decided to progress MEAI through a

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preliminary toxicity study, providing the foundation for a possible future comprehensive traditional preclinical study in rodents and non-rodent species, to be conducted under GMP conditions according to the industry guidelines (OECD, 1998). Hence, this paper reports the outcome of such a preliminary toxicology study the objective of which was to preclinically evaluate the acute and subacute toxicity of MEAI administrated orally to Sprague Dawley (SD) rats, as well as to determine potential in-vitro cytotoxic and mutagenic effects using state-of-the-art protocols.

2. Materials and methods

2.1. Reagents

MEAI (97.0% pure) was obtained from Vanamali Organics Pvt Ltd (Hyderabad, India) and its purity confirmed by standard analytical methods utilizing HPLC/UV and ^{3}H-NMR. Physicochemical properties (Table 1) were determined according to the methods published by Zimmerman (1986). Melting point was determined using Mettler Toledo FP62 instrument (Greifense, Switzerland), while cLog P was theoretically calculated by utilizing ACDlabs software (Toronto, Canada). Ultraviolet (UV) spectrum of MEAI hydrochloride dissolved in water was recorded with diode-array UV-detector, Agilent Technologies Deutschland GmbH, Waldbronn, Germany. MEAI hydrochloride solutions for oral administration were prepared in pyrogen free sterile 0.9% saline solution immediately before administration (Teva, Israel).

2.2. Formulation of MEAI for oral gavage

For the Acute maximum tolerated dose (MTD) study, a stock solution of 0.1 mg/L in pyrogen free sterile water for injection was prepared immediately before administration. The preparation of dosing solutions of 0.01 mg/L and 0.001 mg/L for dose level of 100 and 10 mg/kg body weight (bw) was achieved by diluting the stock solution with sterile water for injection. Sterile water for injection served as a vehicle for the control group, administered orally at a volume of 0.01 L/kg. For the subacute toxicity study, MEAI dosing solutions of 9000 mg/L, 3000 mg/L and 1000 mg/L were freshly prepared in sterile water for injection prior to oral administration for the dose levels of 90 mg/kg, 30 mg/kg and 10 mg/kg, respectively.

2.3. Animal management

Animal handling was performed according to the guidelines of the National Institute of Health (NIH) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals were housed in polyethylene cages (maximum 3 rats/cage) measuring 42.5 × 26.5 × 18.5 cm, with a stainless steel top grill facilitating administration of pelleted food and drinking water in a plastic bottle; bedding: steam sterilized clean paddy husk (Harlan Laboratories, Indianapolis, US). Bedding material was changed along with the cage at least twice a week. All animals were acclimatized 5 days prior to study onset. Animals were fed a commercial rodent diet ad libitum (Teklad Global 18% Protein rodent Diet, Harlan Laboratories, Indianapolis, US) and had free access to acidified drinking water (pH between 2.5 and 3.5) obtained from the municipality supply. Animals were housed under standard laboratory conditions, and enclosures were air conditioned and filtered (HEPA F6/6) with an adequate fresh air supply (minimum 15 air changes/h). Animals were kept in a climate controlled environment with a temperature range of 20–24 °C and a relative humidity range of 30–70% with a 12 h light and 12 h dark cycle. Animals were inspected on arrival and found to be fit for the study. Animals were inspected daily for any signs of morbidity or mortality.

Table 1
Physico-chemical properties of MEAI hydrochloride.

Physico-chemical property[a]	MEAI value ± SE
cLog P[a]	1.23 ± 0.3
MW[b] (g/mol)	199.68
Melting point (°C)	230 ± 2
Water solubility as hydrochloride (mg/mL)	>100
Ethanol solubility as hydrochloride (mg/mL)	9

[a] Theoretically calculated by utilizing ACDlabs software (Toronto, Canada).
[b] Calculated molecular weight (MW).

2.4. Ethical animal use

This study was performed in compliance with The Israel Animal Welfare Act and following The Israel Board for Animal Experiments approval No. IL-15-07-233.

2.5. Acute maximum tolerated dose (MTD)

The principle of the study was based on standard MTD procedure, which uses the minimum number of animals to generate sufficient information on the acute toxicity of MEAI (OECD, 2001). In brief, a total of 24 SD rats (12 female and 12 male rats) were utilized. At the study initiation the rats were 8 weeks old and the average (±standard deviation) male and female bw at study initiation were 272.9 ± 8.6 g and 187.4 ± 6.5 g, respectively. The animals were divided into 4 dosing groups (1000, 100, 10 mg/kg bw or vehicle) randomly upon reception, of three female and three male rats. The number and gender of the animals were based on OECD 423 guidelines (OECD, 2001). Dosing was performed in a staggered fashion by oral gavage, each increase in dose pending on the outcome of the previous dose. Each test item dose was administrated to 3 female and 3 male rats. The administration was performed orally at a dose volume of 10 mL/kg. The lag time between the increasing doses was approximately 1 h. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention given during the first 4 h), and daily thereafter, for a total of 14 days. Observations were performed for any changes in skin, fur, eyes, mucous membranes, respiratory, occurrence of secretions and excretions (e.g. diarrhea) and autonomic activity (e.g. lacrimation, salivation, piloerection, pupil size, unusual respiratory pattern). Changes in gait, posture and response to handling, as the presence of bizarre behavior, tremors, convulsions, sleep and coma were also included. All observed abnormalities, toxic signs, moribund condition and pre-terminal deaths were recorded. Body weight was monitored one day after animal arrival, prior to MEAI administration and twice a week thereafter. Animals were sacrificed by carbon dioxide asphyxiation and gross pathology was performed examining the major tissue and organ systems. All animals were subjected to gross necropsy. All gross lesions in organs and major tissue were documented. Selected organs (kidney, liver, brain, spleen, lungs, and heart) of the highest dosage group, namely 1000 mg/kg, were harvested and fixed in buffered 4% formaldehyde for further histological evaluation. Tissues were embedded in paraffin, sectioned at a 5 μm thickness and stained with haematoxylin and eosin.

2.6. Subacute repeated dose toxicity study

Subacute toxicity of MEAI following five-day repeated oral gavage administration was evaluated at four dose levels of 0, 10, 30 and 90 mg/kg bw in male and female SD rats during 14 day follow-up. The study variables and endpoints included daily mortality and morbidity monitoring, twice weekly bw measurements, daily clinical signs observation and the gross pathology and histopathology at termination. The initial dose levels and study design were based on previous knowledge on the MTD toxicity study outcome. A total of 40 rats (20 female and 20 male rats) were utilized. The animals were divided randomly into 4 groups of five-female and five male rats. The number and gender of the animals was based on OECD 407 guidelines (OECD, 2008). Each

test item dose was administrated for 5 consecutive days to 5 female and 5 male rats. The administration was performed orally at a dose volume of 0.01 L/kg. Dosing was performed in an escalating fashion, each increase in dose pending on the outcome of the previous dose. Animals were observed individually at least once during the first 60 min after dosing and daily thereafter, for a total of 14 days. Observations were performed for any changes in skin, fur, eyes, mucous membranes, respiratory, occurrence of secretions and excretions (e.g. diarrhea) and autonomic activity (e.g. lacrimation, salivation, piloerection, pupil size, unusual respiratory pattern). Changes in gait, posture and response to handling, as the presence of bizarre behavior, tremors, convulsions, sleep and coma were also included. All observed abnormalities, toxic signs, moribund condition and pre-terminal deaths were recorded. Body weight was monitored one day after animal arrival, prior to the first administration of MEAI and twice a week thereafter. Individual body weight changes were calculated. Animals were sacrificed by carbon dioxide asphyxiation and gross pathology was performed. All animals were subjected to gross necropsy and histopathology examination. All gross lesions in organs and major tissue were documented. Selected organs (liver, kidney, spleen, lungs, heart, and brain) and organs with gross pathology abnormality were harvested and fixed in buffered 4% formaldehyde for optional further histological evaluation. Tissues were embedded in paraffin, sectioned at a 5 μm thickness and stained with haematoxylin and eosin.

2.7. Stability study

A stability study of MEAI for the duration of 7 days at −20 °C and at room temperature was established by spiking MEAI into sterile water for injection at low (1 mg/L) and high (100 mg/L) concentrations of six replicates each, according to the recommended guidelines reported by Hartmann et al. (Hartmann et al., 1998). Concentration ratios between samples at day 0 and samples after 7 days storage at −20 °C or room temperature of 90–110% with 90% confidence interval within 85–115% have been regarded as acceptable criteria for stability. MEAI was analyzed by an in-house validated method according to the guidelines defined by the European Union (European Commission, 2002). Briefly, 1 mL water for injection was basified by adding 50 μL of aqueous 1 M Ca_2CO_3 and vortexed for 30 s. MEAI was then extracted with 4 mL ethyl acetate, vortex mixed for 30 s, followed by centrifugation at 4000*g* for 5 min. The upper organic phase was subjected to GC-MS analysis. The quantitative analysis of MEAI was made on a model 7890A gas chromatograph equipped with a single quadruple 5975C VL-MSD, nitrogen phosphorus detector and a J&W Megabore 5% phenyl-95% methyl silicone capillary column (0.25 μm * 15 m * 0.25 mm). The injection volume was 1 μL set at splitless mode. The helium carrier gas in the column was maintained at a constant flow of 1 mL min^{-1}. The temperature program was as follows: injector temperature, 220 °C; initial temperature, 80 °C for 0 min; gradient of 15 °C/min until 180 °C, maintained for 1 min; gradient of 30 °C/min to 300 °C, maintained at 300 °C for 1 min. The MS parameters were set as follows: source temperature, 230 °C; transfer line, 230 °C; positive ion monitoring, EI-MS (70 eV). Major MEAI fragments were 163 (M^+), 146, 135 m/z. MEAI was eluted with a retention time of 8.18 min.

2.8. Blood samples for complete blood count (CBC) and biochemistry analysis

Animals were fasted (food only) overnight on Day 14 prior to blood collection. At study termination (Day 15) after light anesthesia with carbon dioxide inhalation, two blood samples were drawn from all animals directly from the retro orbital sinus. Samples for complete blood count were collected in potassium-EDTA tubes, and analyzed within 2 h from collection using a hematology analyzer (Advia 120, Siemens Medical Solutions Diagnostics GmbH, formerly Bayer HealthCare GmbH, Erfurt, Germany). Samples for serum chemistry were collected in plain tubes with gel separators. Samples were centrifuged within 30 min from collection, and harvested sera were immediately analyzed using a wet chemistry auto analyzer (Cobras Integra 400 Plus, Roche, Mannheim, Germany, at 37 °C) and reagents supplied by the manufacturer (Roche, Mannheim, Germany).The parameters determined for complete blood count and serum biochemistry are summarized in the Supplemental section, Table S1–S3.

2.9. Bacterial reverse mutation test

The bacterial reverse mutation test was performed at the Institute Pasteur de Lille (IPL) using the plate incorporation methodology as described by Ames et al. (1975) and in accordance with the OECD 471 (1997) guidelines. The following technique was performed for each of the five *Salmonella typhimurium* strains (TA1535, TA1537, TA100, TA98 and TA102) with or without metabolic activation of MEAI by S9 rat liver homogenate fraction: 0.1 mL of a bacterial suspension from a culture agitated overnight at 37 °C, with or without the addition of 0.5 mL of the rat S9 mix metabolic activation system, was incubated with 0.1 mL MEAI dissolved in sterile water, giving the final doses ranging from 0.0015–5 mg/plate. The mixture was subsequently added to 2 mL of top agar to which 10% of 0.5 mM biotin histidine solution, maintained in a state of superfusion at 45 °C, had been added. The mixture was then pre-incubated while being stirred at 37 °C for 60 min prior to adding soft agar and being spread out in a Petri plate. Subsequently, the content of each tube was spread out in a Petri plate containing 20 mL of minimal agar. Three plates were used per dose. The plates were then incubated at 37 °C for approximately 44 h. At the end of the expression time, colonies of revertants were counted for each plate. At the same time, solvent controls (0.1 mL solvent/plate) were performed under the same conditions using 6 plates per solvent. Appropriate positive reference controls were also performed as depicted in Table S4 (Supplemental section). The highest analyzable MEAI dose for mutagenicity determination was 1.5 mg/plate and 5 mg/plate, in the absence and presence of metabolic activation, respectively, due to bacterial toxicity response (see below). The identification of a positive response was based on at least 2-fold (TA98, TA100, and TA102) or 3-fold (TA1535 and TA1537) increase in the mean revertants/plate accompanied by a dose-response to increasing concentrations of the test substance. The results are expressed as the mean number of revertants per plate and for each MEAI dose. The induction ratio was calculated as: = mean revertants number per treatment plate/mean revertants number per solvent control plate.

The S9 rat liver homogenate was prepared at the IPL according to the method described by Ames et al. (1975) in rat male Sprague Dawley. The components of the S9 fraction (per mL) included 0.315 mL water, 0.5 mL of 0.2 M NaH_2PO_4/Na_2HPO_4 (pH 7.4), 0.005 mL of 1 M glucose-6-phosphate, 0.04 mL of 0.1 M NADP, 0.02 mL of 1.65 M KCl, 0.02 mL 0.4 M $MgCl_2$ and 0.1 mL of S9 homogenate.

MEAI was considered mutagenic at a specific dose only if all of the below listed criteria have been met: 1. At least one of the test doses exhibits a biologically significant increase (2 fold or 3 fold increase in the mean number TA98, TA100,TA102 and TA1535 and TA1537 strains, respectively) compared with the concurrent negative control 2. The increase is dose-related. 3. The mean numbers of revertants of the test doses are outside the distribution of the negative control data (OECD, 1997). A toxicity test on each *Salmonella typhimurium* strain (TA1535, TA1537, TA100, TA98 and TA102) with or without metabolic activation of MEAI by S9 fraction was performed concurrently to the main mutagenicity test by microscopic examination of the background lawn according to the OECD guidelines (1997). In the absence of metabolic activation, the highest dose of 5 mg/plate induced strong bacterial toxicity and therefore the next highest dose of 1.5 mg/plate and its serial dilutions were utilized for the bacterial reverse mutation test. In the presence of metabolic activation, the highest dose of 5 mg/plate and

its serial dilutions of up to 1.5 mg/plate were utilized, due to only slight bacterial toxicity observed at 5 mg/plate.

2.9.1. Cytotoxicity assay on rat brain striatum primary neurons and human primary hepatocytes.

Rat brain striatum primary neurons were cultivated in the recommended medium (Bulletkit™, Primary Neuron Growth Medium, Lonza, Basel, Switzerland). The Bulletkit™ contains a 200 mL bottle of Primary Neuron Basal Medium (Lonza, Basel, Switzerland) and Primary Neuron Growth Medium (Lonza, Basel, Switzerland). The fully supplemented culture medium was prepared according to the manufacturer's instructions. It contained 2 mM L-glutamine (Lonza, Basel, Switzerland), 50 mg/L gentamicin–37 μg/L amphotericin (Lonza, Basel, Switzerland 4) and 2% NSF-1 (Neural Survival Factor-1) (Lonza, Basel, Switzerland). Following thawing, the cells were seeded in poly-D-lysine coated 96-well microtiter plate (0.2 ml/well) at a density of 8×10^4 cells/well. The cells were incubated for 4 h in an incubator (37 °C, 5% CO_2). Next, the medium was removed and fresh, pre-warmed culture medium was added. The cells were then incubated (37 °C, 5% CO_2) for four days. Cell death occurred during the first few days after plating and debris were observed (normal process). After 4 days in culture, the cells formed a neurite network and by the 7th day, debris was minimal. An initial medium change occurred at Day 5. Wells containing culture medium without cells were also prepared and used for background determination. On Day 7, the culture medium was aspirated from the 96-microtiter plate. Then, the medium was replaced on all plates by 0.1 mL of either, MEAI (final concentrations of 0.01, 0.1, 0.5, 1, 5, 10, 50, 100, 500 and 100 mg/L in PBS), ethanol (0.1, 2, 6, 7.5%), combined MEAI (100 mg/L) - ethanol (6 or 7.5%) mixture or the appropriate negative controls (PBS), diluted in culture medium. The plates were incubated for 24 h (37°C, 5% CO_2) before the MTS-assay. Wells designated for positive control (complete cell death) were treated by lysis solution (2% triton solution) 15 min before the colorimetric assay for assessing cell metabolic activity as a measure for cytotoxicity (MTS-assay) was performed. The human primary hepatocytes were processed according to the Manufacturer's instructions (Triangle Research Labs, North Carolina USA). Three different media were used: MCHT50 (thawing medium), MP250 (plating medium) and MM250 (maintenance medium). After thawing, the cells were seeded in Collagen type I coated 96-well microtiter plate (0.1 mL/well) at a density of 5×10^4 cells/well using the human hepatocyte plating medium. The cells were incubated for 5 h in an incubator (37 °C, 5% CO_2). Next, the medium was removed and pre-warmed maintenance medium was added. Wells containing maintenance medium without cells were also prepared and used for background determination. After 24 h, the maintenance medium was aspirated from the 96-microtiter plate. Then, the medium was replaced on all plates by 0.1 mL of either, MEAI (final concentrations of 0.01, 0.1, 0.5, 1, 5, 10, 50, 100, 500 and 100 mg/L in PBS) or the appropriate negative controls (PBS), diluted in culture medium. The plates will be incubated for 24 h (37 °C, 5% CO_2) before the MTS assay.

Wells designated for positive control (complete cell death) were treated by lysis solution (2% triton) 15 min before MTS assay. MTS assay was performed following Manufacturer's instructions (CellTiter 96® AQueous One Solution Reagent, Promega, WI, USA). In brief, MTS reagent was thawed at room temperature or using a water bath at 37 °C. MTS reagent (20 μL) was directly added into each well of the 96-microtiter plate containing the samples in 100 μL of media. Next, plates were incubated for 2 h in a humidified atmosphere (37 °C, 5% CO_2). After the appropriated incubation time, the 96-microtiter plate was read by an ELISA reader at 490 nm. The average optical density of read blank wells was subtracted from each reading

2.9.2. Statistical analysis.

Wherever applicable, numerical results were presented as means and standard deviation or standard error of the mean. Data for males and females were analyzed separately. Descriptive statistics and group comparisons of data were performed using statistical analysis program (GraphPad Prism version 5.00 for Windows, GraphPad Software, San-Diego California USA). After testing data for normality and equal variance, the appropriate parametric or non-parametric test was performed followed by appropriate post-hoc analysis. A probability of 5% ($p \leq 0.05$) was regarded as statistically significant. Data from the bacterial reverse mutation test was analyzed by means of Dunnett's method allowing the comparison of the mean value for each dose to the mean value for the corresponding solvent control.

3. Results and discussion

To the best of our knowledge, there is a paucity of information regarding the toxicological profile of aminoindane related compounds (Simmler et al., 2014). Animal studies have focused solely on comparing the effects of aminoindanes with amphetamines and related drugs in order to elucidate their pharmacological effects, particularly with regard to dopamine and serotonin pathway (Brandt et al., 2013; Sainsbury et al., 2011; Simmler et al., 2014). Hence, the significance of the present study lays in the characterization of the toxicological profile of MEAI, psychoactive representative of the aminoindane class.

3.1. Physicochemical properties of MEAI

Physicochemical properties of MEAI hydrochloride are summarized in Table 1.

3.2. Stability study

Concentration ratios between samples at day 0 and samples after 7 days storage at −20 °C or room temperature were 99.1 % ± 1.2 and 98.9% ± 1.6, respectively. Hence, MEAI was found stable at −20 °C and room temperature (25 °C) for 7 days at the lower (1 mg/L) and upper (100 mg/L) concentration range according to the acceptance criteria specified in the methods and materials section.

3.3. Acute maximum tolerated dose (MTD)

The present MTD study was utilized to identify adverse effects of a substance which result from a single high dose exposure of MEAI in a short period of time (10 mg/kg, 100 mg/kg and 1000 mg/kg). The adverse effects were monitored within 14 days of the administration of MEAI.

No abnormal clinical signs were observed in the groups treated with vehicle and 10 mg/kg MEAI at all-time points. All animals (3 male and 3 female mice) in the 100 mg/kg MEAI dose group displayed transient adverse clinical signs that lasted for one day post dosing. The major adverse clinical signs observed in these animals included: tremor, straub tail, dyspnea, piloerection, hunched back, decreased motor activity and salivation. On Day 2, peri orbital staining (POS) and peri nasal staining (PNS) were observed in this dose level. These adverse clinical signs were resolved spontaneously on Day 3 and no abnormal clinical signs were observed until study termination. No gross pathology abnormalities were detected in the groups treated with vehicle, 10 mg/kg and 100 mg/kg MEAI at the scheduled termination day (Day 15).

The aforementioned major clinical signs observed at 100 mg/kg MEAI are commonly reported for murine laboratory animals in distress, which also might be indicative of transient neurotoxic response (Hamm et al., 2006). The common clinical signs that have been observed during the toxicological assessment might provide us with a clue regarding the organ, which is most likely involved in eliciting those responses. For instance decreased spontaneous motor activity and ataxia are usually the result of depressed central nervous system function elicited by the drug. Tremors and convulsions on the other hand are involuntary movements resulting from neuromuscular and/or central nervous system excitation.

Moreover, piloerection and salivation are associated with drugs effects on the autonomic nervous system.

All of these observed clinical signs in rats are clearly an indication for MEAI's neurotoxic effects at the higher end doses. The clinical signs of piloerection, tremor, Straub tail and hunched back, observed in the present study, were occasionally reported for the serotonin syndrome in laboratory rodents, which have been primarily associated with 5-HT_{1A} receptor activation (Haberzettl et al., 2013, 2014, 2015). However, the most frequently observed responses in serotonin syndrome in rats, namely forepaw treading, hind limb abduction and head weaving were completely absent in the present study, indicating a more complex mode of toxicity involving both excitatory as well as inhibitory neurotransmitters. Still, in extrapolating to humans, a possible hazardous serotoninergic response at toxic doses of MEAI cannot be ruled out, and additional studies will be required to address this issue.

Since dopamine and noradrenaline agonists as well as parasympathomimetics have been demonstrated to induce similar clinical signs reported herein, the attribution of clinical signs to a specific mode of toxicity is not possible (Haberzettl et al., 2013). Taken all together, it would seem generally advisable to monitor for signs of neurotoxicity such as tremor, motor incoordination, agitation, seizures and possibly elevated body temperature, in case of suspected MEAI overdosing during human recreational usage.

All animals in the 1000 mg/kg MEAI dose group (3 male and 3 female mice) died within 30–120 min post dosing. Tremors, Straub tail, dyspnea, piloerection, decreased motor activity and convulsions were the major adverse clinical signs observed in these animals before death. Enlarged lungs, whitish spleen and surface white spots on the liver were the only gross pathology abnormalities observed post-mortem in the animals that died from the group treated with 1000 mg/kg. However, no treatment related histopathological changes were observed during the study in the 1000 mg/kg group as compared to control. The discrepancy between the lack of histopathological evidence of drug injury and high mortality rate in acute toxicity studies is not surprising, since usually a prolonged drug administration is required for the manifestation of significant pathological and histopathological findings.

Weight loss is a common nonspecific finding to assess general health status of laboratory animals (Hamm et al., 2006). As shown in Fig. 1, group average body weight gain in the groups treated with 10 and 100 mg/kg MEAI was comparable to the matched vehicle group, in male and female rats, at all-time points until study termination (following two-way ANOVA and Bonferroni's post-hoc analysis).

Based on the above findings and under the conditions of this study, it is concluded that single oral administration of MEAI, at a dose level of 10 mg/kg in male and female rats, was well tolerated and did not cause any significant adverse clinical effects compared to vehicle treatment.

3.4. Subacute repeated dose toxicity study

The present sub-chronic toxicity study was performed in-order to characterize the adverse effects resulting from a repeated-dose study for five consecutive days. The adverse effects were monitored within 14 days of the administration of MEAI. Since 100 mg/kg of MEAI in the acute toxicity study resulted in severe adverse effects, the following dosages were utilized in order to assess the no-observed adverse effect level (NOAEL): 10 mg/kg, 30 mg/kg and 90 mg/kg.

No abnormal clinical signs were observed in male and female rats from the groups treated with vehicle and 10 mg/kg MEAI at all-time points. In the 30 mg/kg dose groups, one male animal (out of five) displayed diarrhea on Day 2, and two female animals (out of five) displayed decreased motor activity on Day 1 after dosing and one animal displayed piloerection on Day 2 after second dosing. These mild adverse clinical signs were resolved spontaneously on subsequent days and no abnormal clinical signs were observed until study termination.

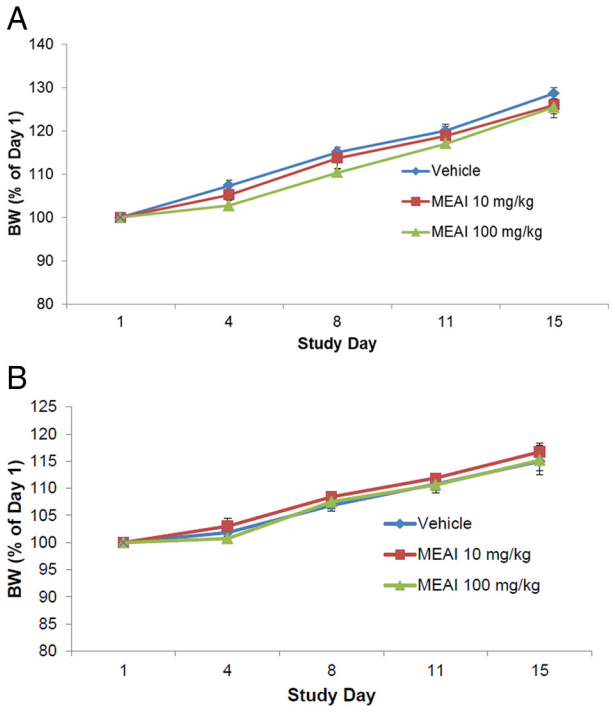

Fig. 1. Group average body weight gain (% of Day 1) in male rats (A) and female rats (B).

In the 90 mg/kg MEAI dose groups, all animals displayed transient adverse clinical signs that lasted for five days during dosing. These adverse clinical signs were resolved spontaneously on the next day. It seems that the adverse clinical signs were more severe after the first dosing and less severe after the third dosing, as if the animals developed some kind of tolerance. In these groups, the major adverse clinical signs observed in male and female animals post dosing included: on Day 1, tremor, straub tail, dyspnea, piloerection, decreased motor activity, lacrimation, PNS and salivation; on Days 2 and 3, dyspnea, salivation and piloerection; on Day 4, salivation and piloerection; on Day 5, piloerection and lacrimation in female rats only. These adverse clinical signs were resolved spontaneously on Day 6 and no abnormal clinical signs were observed until study termination. On Day 3, one male animal was euthanized on humane ground. This animal displayed dyspnea, cyanosis, decreased motor activity, tremor, piloerection and salivation up to one hour after dosing and before euthanization. Post mortem gross pathology examination revealed that the animal had a lot of clear transudate in the thoracic cavity. The stomach contained dark mucosa. The kidneys were dark colored probably as a result of circulatory failure. The reason for death could not be established and was attributed most likely to aspiration and not related to MEAI treatment. The adverse effect profile observed in the 90 mg/kg group was similar to the clinical signs reported in the acute toxicity test in the 100 mg/kg group.

In male rats, group average body weight gain was observed in all groups (Fig. 2a). Statistical analysis of the data using two-way ANOVA followed by Bonferroni's post-hoc analysis revealed that the average body weight gain of the group treated with 90 mg/kg oral MEAI was found to be significantly attenuated starting on Day 4 until termination on Day 15, compared to the vehicle treated group. This was considered as a minor undesirable effect, indicative of reduced general wellbeing. However, in female rats, group average body weight gain was comparable in all oral MEAI treated groups compared to the vehicle group at all-time points, until study termination (Fig. 2b). Statistical analysis of the data using two-way ANOVA showed that none of the various treatments exhibited statistical significance at any time point, following Bonferroni's post-hoc analysis. Consequently, it seems that male rats are more sensitive to MEAI at the 90 mg/kg dose than female rats.

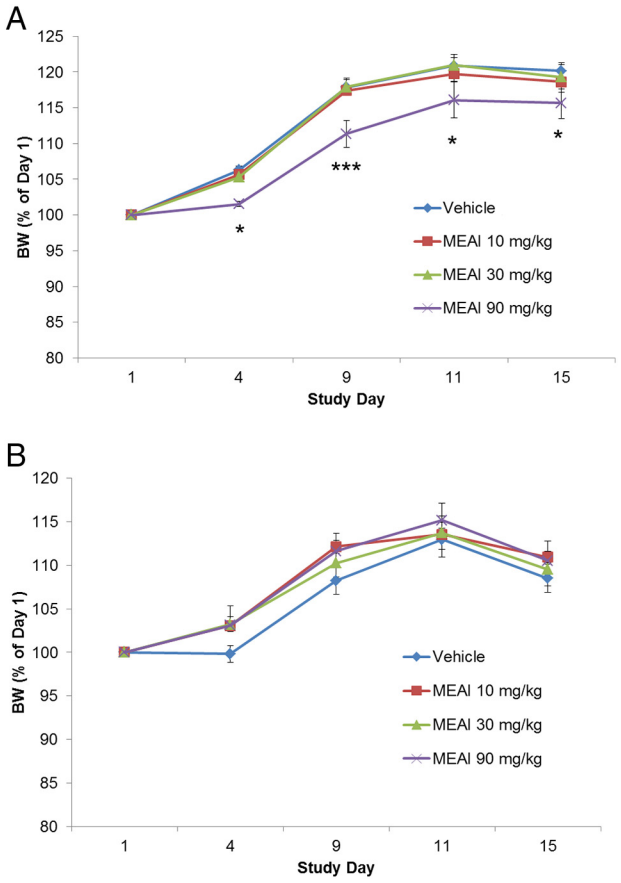

Fig. 2. Group average body weight gain (% of Day 1) measurements in male (A) and female rats (B). Statistical analysis by two-way ANOVA followed by Bonferroni post-hoc comparisons against control group (vehicle): $^{*}p < 0.05$; $^{***}p < 0.001$.

No gross histopathological abnormalities related to MEAI treatment were detected in all male and female groups at the scheduled termination day (Day 15) as compared with the control group. Based on the above findings and under the conditions of this study, it is concluded that five day oral administration of MEAI, at a dose level of 10 and 30 mg/kg in male and female rats, was well tolerated and did not cause any significant adverse clinical effects compared to vehicle treatment.

3.5. Clinical chemistry and hematology

A comparison of the complete blood count values between the treatment groups (10, 30 and 90 mg/kg) in males and females to the control group, revealed no significant changes, which indicate a lack of toxicity on the hematopoietic system after five days of subacute MEAI exposure (Supplemental section, Table S1–S2). No abnormal biochemical markers were observed in male and female rats from the groups treated with 10 mg/kg and 30 mg/kg MEAI as compared to the control group (Table 2 and Table 3). These results together with the pathological and histopathological finding indicate lack of liver and kidney toxicities in the 10 mg/kg and 30 mg/kg treatment groups in the subacute toxicity study.

However, in the 90 mg/kg dosing group in both females and males MEAI significantly induced a 66% and 90% increase in serum triglyceride levels respectively as compared to the control group by the end of the study (Day 15; Table 2 and Table 3). The mean total triglyceride values in males (118 ± 21) and females (99 ± 18.6) exceeded significantly ($p < 0.05$) the normal range value for rats of the same species and age group, indicating a meaningful adverse clinical finding of MEAI at 90 mg/kg (Giknis and Clifford, 2006). Due to lack of liver histopathological and pathological findings as well as normal serum total cholesterol, total protein and serum liver enzymes determined in all of the treatment groups, liver toxicity as the major cause can be reasonably excluded. Therefore, the clinically observed hypertriglyceridemia at highest dose group is most probably the result of induced metabolic disturbance at the level of adipose tissue, rather than direct interference with liver fatty acid metabolism.

3.6. Cytotoxicity assay on rat brain striatum primary neurons and human primary hepatocytes

Since MEAI has been reported to exert psychoactive effects in humans, we considered that neuronal tissue constitutes a principal target organ for potential MEAI toxicity. Furthermore, as the liver is the principal organ involved in the biotransformation of xenobiotics this is also a target organ for drug toxicity. Consequently, we have used both primary neuron and hepatocyte cell cultures for assessing potential neuro- and hepatotoxic effects.

Reduction in cell viability at 24 h was calculated in percent relative to vehicle (PBS)-treated cells group (Fig. 3). Cell viability reduction of more than 30% (represented by a dashed line) compared to control was considered a cytotoxic effect (Fig. 3). Increasing concentrations of MEAI (0.01–1000 mg/L) demonstrated reduction of cell viability on rat brain striatum primary neurons only when the neurons were treated with 500 and 1000 mg/L (Fig. 3). The calculated IC_{50} value of MEAI was 368.2 mg/L.

Similar to the neurons, increasing concentrations of MEAI (0.01–1000 mg/L) on human primary hepatocytes demonstrated cytotoxic effect only when hepatocytes were treated with 500 and 1000 mg/L (Fig. 4). The calculated IC_{50} value of MEAI, namely 403.1 mg/L, was comparable to the IC_{50} determined in primary neurons.

To the best of our knowledge, no published reports are currently available regarding the cytotoxic effect of aminoindane derivatives and analogues in primary neurons and primary hepatocytes. Notwithstanding, MEAI's large IC_{50} values in primary neurons and hepatocytes as compared to the IC_{50} values of cathinone and MDMA derivatives and analogues in various in-vitro cytotoxicity assays, clearly point at MEAI's relatively low cytotoxic properties (Dias da Silva et al., 2013; Milhazes et al., 2006; Nakagawa et al., 2009). Notwithstanding, caution needs to be exercised in extrapolating the low neurotoxicity outcome observed in rats to humans, as certain differences in monoaminergic receptor/transporter affinities and expression as well as in metabolic capabilities exist between human and rat neuronal cells (Moratalla et al., 2015).

3.7. Viability assessment of rat brain striatum primary neurons treated with ethanol in combination with MEAI.

Since MEAI is intended to reduce binge ethanol consumption in combination with alcoholic beverages, in the present assay the interaction of MEAI at the highest non-toxic concentration (100 mg/L) with ethanol at cytotoxic levels of 6% and 7.5% was explored, in order to identify possible additive or synergistic effects. At 0.1 and 2% ethanol was non-toxic, while at 6% and 7.5% ethanol reduced cell viability to 52 and 5% respectively (Fig. 5). Examination of combination of 6% ethanol with 100 mg/L MEAI revealed no significant change in the cytotoxic effect as compared to 6% ethanol treatment. Interestingly, combination of 7.5% ethanol with 100 mg/L MEAI displayed higher levels of viability in comparison to 7.5% ethanol treatment. These results were however not statistically significant ($P = 0.183$ compared between the two treatment groups, using a T-Test). Based on the aforementioned results, MEAI has no additive or synergistic effect to ethanol. Although not statistically significant, it seems that MEAI at 100 mg/L has a certain antagonistic effect to ethanol.

Further studies are warranted to confirm and explore MEAI's potential antagonistic properties in relation to ethanol neuro-cytotoxicity.

Table 2
Serum biochemical markers of female rats at day 15 after 5 days of daily oral drug administration (10, 30 and 90 mg/kg) vs. control group.[a]

Biochemical marker[b]	Control male ($n = 5$)	10 mg/kg ($n = 5$)	30 mg/kg ($n = 5$)	90 mg/kg ($n = 5$)	Statistical Analysis: one-way repeated ANOVA (p value, F-value)	Dunnette post-hoc multiple comparison test against control (q value and p value)
Alb (g/dL)	4.9 ± 0.1	4.8 ± 0.2	4.8 ± 0.2	4.8 ± 0.3	NS[c]	–
ALP (U/L)	87.7 ± 10.0	83.2 ± 13.9	85.6 ± 15.5	90.3 ± 19.4	NS	–
ALT (U/L)	34.4 ± 2.4	33.4 ± 3.8	43.2 ± 6.2	42.4 ± 5.4	NS	–
Amylase (U/L)	1100.0 ± 245.2	1158.8 ± 230.6	1075.7 ± 185.3	1013.8 ± 215.2	NS	–
AST (U/L)	84.3 ± 12.3	82.6 ± 7.0	85.2 ± 6.4	82.6 ± 5.3	NS	–
Bil (mg/dL)	0.06 ± 0.0	0.03 ± 0.0	0.03 ± 0.0	0.05 ± 0.0	NS	–
Ca^{2+} (mg/dL)	10.6 ± 0.2	10.6 ± 0.4	10.9 ± 0.3	10.9 ± 0.2	NS	–
Cholesterol (mg/dL)	104.2 ± 19.8	114.4 ± 23.0	122.6 ± 7.5	111.2 ± 18.8	NS	–
CK (U/L)	240.8 ± 104.3	301.0 ± 125.3	283.8 ± 137.6	292.8 ± 91.1	NS	–
CL (mmol/L)	98.5 ± 1.2	98.1 ± 1.1	96.9 ± 1.8	96.5 ± 1.3	NS	–
Creatinine (mg/dL)	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	NS	–
Glucose (mg/dL)	96.3 ± 5.8	92.0 ± 3.7	91.9 ± 7.8	87.1 ± 8.1	NS	–
K (mmol/L)	6.3 ± 0.2	6.3 ± 0.2	6.5 ± 0.3	6.6 ± 0.2	NS	–
Na (mmol/L)	142.7 ± 0.5	142.2 ± 1.0	143.2 ± 1.7	142.9 ± 1.4	NS	–
Phosphate (mg/dL)	7.3 ± 0.8	7.3 ± 0.5	7.8 ± 0.5	8.2 ± 0.7	NS	–
TP(g/dL)	6.8 ± 0.1	6.7 ± 0.4	6.6 ± 0.3	6.6 ± 0.4	NS	–
TG (mg/dL)	60.3 ± 8.5	75.6 ± 23.6	74.5 ± 19.7	99.1 ± 18.6	$P = 0.03$; F = 3.8	Control vs. 90 mg/kg group is significant at $p < 0.05$; q = 3.3
Urea (mg/dL)	34.6 ± 6.5	35.6 ± 9.9	32.8 ± 4.7	33.1 ± 12.3	NS	–

[a] Biochemical analysis was performed at the Biochemistry Laboratory, The Veterinarian Hospital, The Hebrew University, Bet-Dagan, Israel.
[b] Alb, albumin; ALP, alkaline phosphatase; ALT, alanine transferase; AST, aspartate aminotransferase; Bil, total bilirubin; GGT, gamma glutamyl transferase; CK, creatinine kinase; TP, total protein, TG, triglyceride.
[c] NS, non-significant at $\alpha = 0.05$.

Table 3
Serum biochemical markers of male rats at day 15 after 5 days of daily oral drug administration (10, 30 and 90 mg/kg) vs. control group ($n = 5$).[a]

Biochemical marker[b]	Control male ($n = 5$)	10 mg/kg ($n = 5$)	30 mg/kg ($n = 5$)	90 mg/kg ($n = 5$)	Statistical analysis: one-way repeated ANOVA (P value, F-value)	Dunnette post-hoc multiple comparison test against control (q value and p value)
Alb (g/dL)	3.8 ± 0.7	3.3 ± 0.2	4.5 ± 0.2	4.4 ± 0.1	NS[c]	–
ALP (U/L)	127.9 ± 8.8	108.7 ± 16.2	136.1 ± 13.4	136.1 ± 17.6	NS	–
ALT (U/L)	48.1 ± 4.7	42.4 ± 4.8	52.2 ± 9.3	51.7 ± 9.4	NS	–
Amylase (U/L)	1930.7 ± 654.8	1670.0 ± 175.5	1883.5 ± 245.9	1911.2 ± 313.8	NS	–
AST (U/L)	93.5 ± 15.7	79.4 ± 5.0	89.4 ± 10.8	90.8 ± 7.1	NS	–
Bil (mg/dL)	0.03 ± 0.0	0.01 ± 0.0	0.02 ± 0.0	0.04 ± 0.0	NS	–
Ca^{2+} (mg/dL)	10.2 ± 0.9	9.5 ± 0.5	10.8 ± 0.3	10.8 ± 0.1	NS	–
Cholesterol (mg/dL)	93.6 ± 23.2	79.8 ± 11.1	126.0 ± 12.5	124.2 ± 13.5	NS	–
CK (U/L)	436.4 ± 290.5	153.8 ± 18.0	358.4 ± 266.5	229.0 ± 130.7	NS	–
CL (mmol/L)	95.0 ± 5.1	88.7 ± 1.8	97.6 ± 2.1	97.0 ± 0.7	NS	–
Creatinine (mg/dL)	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	NS	–
Glucose (mg/dL)	73.1 ± 11.2	69.8 ± 2.1	89.8 ± 6.1	86.6 ± 5.2	NS	–
K (mmol/L)	6.7 ± 0.4	6.1 ± 0.3	6.5 ± 0.4	6.8 ± 0.4	NS	–
Na (mmol/L)	140.6 ± 8.4	132.9 ± 3.3	143.7 ± 1.5	143.2 ± 1.1	NS	–
Phosphate (mg/dL)	8.3 ± 0.9	7.6 ± 0.2	8.8 ± 0.5	8.3 ± 0.2	NS	–
TP(g/dL)	5.7 ± 1.0	5.0 ± 0.3	6.8 ± 0.6	6.6 ± 0.3	NS	–
TG (mg/dL)	62.2 ± 21.6	75.1 ± 23.7	95.1 ± 14.9	118.8 ± 21.4	$P = 0.005$; F = 6.4	Control vs. 90 mg/kg group is significant at $p < 0.05$; q = 4.1
Urea (mg/dL)	26.3 ± 2.1	22.4 ± 2.3	27.9 ± 2.5	28.3 ± 2.9	NS	–

[a] Biochemical analysis was performed at the Biochemistry Laboratory, The Veterinarian Hospital, The Hebrew University, Bet-Dagan, Israel.
[b] Alb, albumin; ALP, alkaline phosphatase; ALT, alanine transferase; AST, aspartate aminotransferase; Bil, total bilirubin; GGT, gamma glutamyl transferase; CK, creatinine kinase; TP, total protein, TG, triglyceride.
[c] NS, non-significant at $\alpha = 0.05$.

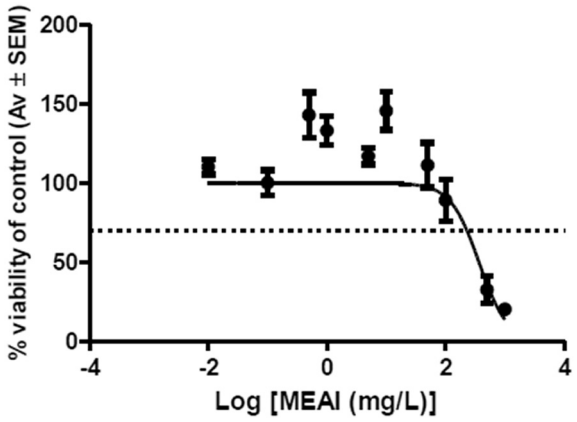

Fig. 3. Viability assessment of rat brain striatum primary neurons treated with serial concentrations of MEAI. Cell viability reduction of >30% (represented by a dashed line) compared to control was considered a cytotoxic effect. Statistical analysis was carried out using GraphPad Prism software.

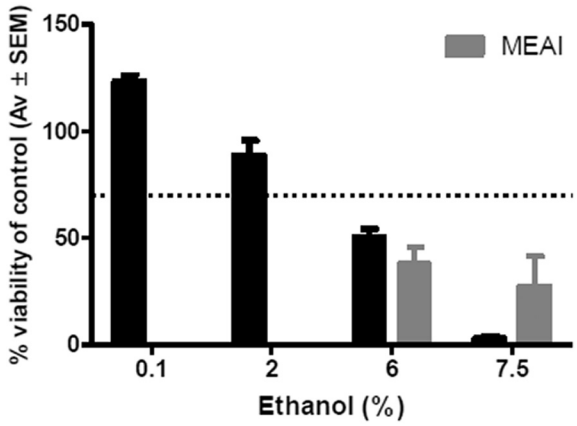

Fig. 5. Viability assessment of rat brain striatum primary neurons treated with ethanol in combination with MEAI (100 mg/kg).

3.8. Bacterial reverse mutation test

The test results are summarized in Table 4. In this assay, no statistically significant increases in the mean number of revertants/plate were noted in the *Salmonella typhimurium* strains TA1535 and TA1537 either with or without metabolic activation and in strains TA98, TA100 and TA102 in absence of metabolic activation (Table 4). MEAI was thus considered as not mutagenic under all of these conditions.

Following metabolic activation however, strain TA98 revealed statistically significant increases in the number of revertants at the highest doses tested of 1.5 and 5 mg/plate with induction ratios of 1.7 and 3, respectively, *i.e.* above the threshold for a biologically significant response at the highest dose of 5 mg/plate (set at 2 in this strain). Furthermore, the value for the mean number of revertants at this dose (5 mg/plate) was higher than the upper bound of historical data for negative control (*i.e.* 76.3 *vs.* 58). Hence, MEAI was considered as mutagenic in this condition, though from a mechanistic point of view, MEAI induced frameshift mutations, only after metabolic activation.

In strain TA100 in the presence of metabolic activation with pre-incubation (Table 4), a statistically significant increase in the number of revertants was observed at the intermediary dose tested of 1.5 mg/plate with an induction ratio of 1.5, *i.e* below the threshold ratio for a biologically significant response (set at 2 in this strain). Moreover, the value for the mean number of revertants at this dose was within the intervals of historical data for negative control (*i.e.* 146 *vs.* 54–166). Hence, MEAI was thus considered as not mutagenic under this condition. In strain TA102 in presence of metabolic activation with pre-incubation (Table 4), a statistically significant increase in the number of revertants was observed at the lowest dose tested of 0.0015 mg/plate with an induction ratio of 1.3, *i.e* clearly below the threshold ratio for a biologically significant response (set at 2 in this strain). Moreover, the value for the mean number of revertants at this dose was within the intervals of historical data for negative control (*i.e.* 172.7 *vs.* 105–436). MEAI was thus considered as not mutagenic in this condition. In conclusion, a slight mutagenic activity was revealed in strain TA98 and only at the highest dose tested (5 mg/plate) with metabolic activation. In return, no mutagenic activity was revealed in any of the other conditions.

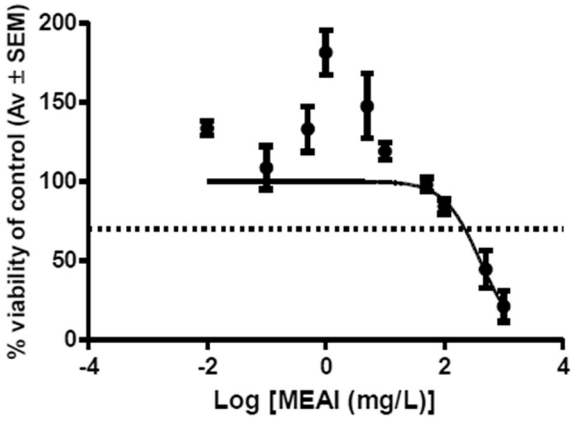

Fig. 4. Viability assessment of human primary hepatocytes treated with serial concentrations of MEAI. Cell viability reduction of >30% (represented by a dashed line) compared to control was considered a cytotoxic effect. Statistical analysis was carried out using GraphPad Prism software.

3.9. Extrapolation to human recreational MEAI consumption

The extrapolation of animal dosage to human equivalent dose of recreational drugs is presently an unexplored area with no explicit guidelines. Therefore, we have adopted the industry guidelines for estimating the maximum active safe starting dose in initial future human trials (Guidance for industry, 2005). The lowest tested dose in the present study of 10 mg/kg bw induced no observable clinical adverse effects and therefore can be regarded as the NOAEL. Converting the NOAEL in rats to human equivalent dose (HED) based on normalization to body surface area, yields a dosage of 1.6 mg/kg bw, or 96 mg for 60 kg person (Guidance for industry, 2005). According to the industry guidelines, a safety factor should be applied to the HED, which accounts for several intra- and interspecies uncertainties and variabilities (Guidance for industry, 2005). Applying the default safety factor of 10, would result in a maximum safe starting dose of 0.16 mg/kg, a dosage regarded as pharmacologically inactive (Guidance for industry, 2005). However, based on the reports of recreational users, the lowest recreationally active dosage lacking significant adverse effects was 70 mg for an average adult, hence a potential maximum safe and recreationally active starting dose could be 1 mg/kg bw (Nutt, 2013; Slezak, 2014). The middle (30 mg/kg) and the highest doses (90 mg/kg) used in the subacute studies are equivalent to the human doses of 4.8 mg/kg and 14.5 mg/kg, respectively (Guidance for industry, 2005). At 30 mg/kg only one male and two female rats displayed mild and transient adverse effects expressed as piloerection and mild decreased motor activities. Consequently, one might argue that human equivalent doses of up to 4.8 mg/kg are not expected to result in severe adverse effects. Notwithstanding, a more reliable estimation can only be achieved after conducting additional preclinical safety studies for a longer duration of time in rodents and non-rodent animal models. Based on recreational human experience and the present

Table 4
Mean number of revertants determined in the *Salmonella typhimurium* reverse mutation test following exposure to MEAI with and without rat metabolic activation with preincubation. The mean values were analyzed by Dunnett's method allowing the comparison of the mean value for each dose to the mean value for the corresponding solvent control.

	Treatment	Doses (μg/plate)	TA1535		TA1537		TA98		TA100		TA102	
			Mean revertants/plate ± SD[a] (induction ratio[b])	*P* value (Duttnett's test)	Mean revertants/plate ± SD (induction ratio)	*P* value (Duttnett's test)	Mean Revertants/plate ± SD (induction ratio)	*P* value (Duttnett's test)	Mean revertants/plate ± SD (induction ratio)	*P* value (Duttnett's test	Mean revertants/plate ± SD (induction ratio)	*P* value (Duttnett's test
Without metabolic activation by S9-mix[d]	Solvent	0	15.5 ± 5	–	6.7 ± 3	–	17.2 ± 3.7	–	109.0 ± 7	–	124.0 ± 12	–
	MEAI	1.5	12.3 ± 5 (0.8)	NS[c] (1.0)	7.7 ± 4 (1.1)	NS (0.3)	19.0 ± 4.6 (1.1)	NS (0.5)	112.0 ± 8 (1.0)	NS (0.4)	142.0 ± 16 (1.1)	NS (1.6)
		5	15.7 ± 4 (1.0)	NS (0.1)	6.7 ± 3.5 (1.0)	NS (0.04)	19.3 ± 3.1 (1.1)	NS (0.6)	111.3 ± 17 (1.0)	NS (0.2)	118.7 ± 9 (1.0)	NS (0.5)
		15	18.3 ± 5 (1.2)	NS (0.8)	4.7 ± 0.6 (0.7)	NS (0.8)	15.0 ± 3 (0.9)	NS (0.6)	116. 0 ± 20 (1.1)	NS (0.8)	122.7 ± 15 (1.0)	NS (0.1)
		50	20.0 ± 6 (1.3)	NS (1.2)	7.3 ± 2.5 (1.1)	NS (0.3)	16.3 ± 8.6 (0.9)	NS (0.5)	114.0 ± 7 (1.0)	NS (0.6)	138.0 ± 16 (1.1)	NS (1.3)
		150	13.7 ± 1 (0.9)	NS (0.45)	6.7 ± 5.1 (1.0)	NS (0.3)	15.0 ± 6.9 (0.9)	NS (0.7)	104.7 ± 9 (1.0)	NS (0.5)	147.3 ± 14 (1.2)	NS (2.1)
		500	14.5 ± 0.7 (0.9)	NS (0.19)	5.7 ± 2.1 (0.9)	NS (0.3)	13.0 ± 1.7 (0.8)	NS (1.2)	120.0 ± 10 (1.1)	NS (1.3)	90.0 ± 12 (0.7)	NS (3.5)
		1500	19.7 ± 2 (1.3)	NS (1.26)	5.3 ± 2.1 (0.8)	NS (0.5)	16.3 ± 4.9 (0.9)	NS (0.3)	94.3 ± 8.5 (0.9)	NS (1.9)	64.7 ± 17 (0.5)	<0.01 (6.7)
Without metabolic activation by S9-mix	Solvent	0	10.3 ± 3	–	8.2 ± 4.2	–	25.3 ± 4	–	95.3 ± 14	–	138.0 ± 12	–
	MEAI	1.5	8.7 ± 1 (0.8)	NS (0.59)	6.0 ± 0 (0.7)	NS (0.9)	22.0 ± 4.6 (0.9)	NS (1.0)	106.3 ± 10 (1.1)	NS (1.4)	172.7 ± 13 (1.3)	<0.01 (3.8)
		5	11.5 ± 2 (1.1)	NS (0.5)	6.7 ± 3.8 (0.8)	NS (0.7)	19.0 ± 6.2 (0.8)	NS (2.0)	102.3 ± 7 (1.1)	NS (0.9)	171.3 ± 27 (1.2)	NS (3.6)
		15	19.0 ± 3 (1.8)	NS (2.82)	7.3 ± 0.6 (0.9)	NS (0.2)	24.3 ± 4 (1.0)	NS (0.3)	102.3 ± 6 (1.1)	NS (0.9)	150.0 ± 9 (1.1)	NS (2.1)
		50	13.7 ± 2 (1.3)	NS (1.22)	6.3 ± 3.2 (0.8)	NS (0.9)	24.0 ± 2 (0.9)	NS (0.3)	90.0 ± 11 (0.9)	NS (0.6)	156.0 ± 7 (1.1)	NS (2.5)
		150	11.5 ± 2 (1.1)	NS (0.5)	5.7 ± 1.2 (0.7)	NS (1.2)	22.7 ± 6 (0.9)	NS (0.8)	124.7 ± 12 (1.3)	NS (3.5)	160.0 ± 9 (1.2)	NS (3.5)
		500	15.0 ± 4 (1.5)	NS (1.62)	6.0 ± 2.6 (0.7)	NS (1)	23.7 ± 6.8 (0.9)	NS (0.5)	118.0 ± 15 (1.2)	NS (2.7)	169.3 ± 15 (1.2)	NS (2.7)
		1500	14.0 ± 5(1.4)	NS (1.27)	14.0 ± 1 (1.7)	NS (2.7)	43.3 ± 6.4 (1.7)	<0.01 (4.5)	146.0 ± 14 (1.5)	<0.01 (5.8)	162.7 ± 12 (1.2)	NS (1.9)
		5000	19.0 ± 12 (1.8)	NS (2.54)	14.0 ± 3 (1.7)	NS (2.7)	76.3 ± 0.6 (3.0)	<0.01 (10.7)	73.7 ± 6 (0.8)	NS (2.8)	121.3 ± 2 (0.9)	NS (2.8)

[a] SD, standard deviation.
[b] Induction ratio: number of revertants in the treatment group/number of revertants in the negative control group.
[c] NS, non-significant at $\alpha = 0.05$.
[d] S9-mix, rat liver microsomal fraction prepared according to the method described by Ames et al. (1975) in male rat Sprague Dawley.

toxicological evaluation in rats, it seems that dosages of 1 mg/kg/day of MEAI in humans and 30 mg/kg/day in rats orally ingested over several h for a short period of time, are not expected to result in clinically acute severe adverse effects.

4. Conclusion

Based on the above findings and under the conditions of this study, it is concluded that single oral administration of MEAI to rats, at a dose level of 10 mg/kg was well tolerated. Furthermore, in the five-day oral administration of MEAI to rats at a dose level of 30 mg/kg, it was well tolerated and did not cause any significant adverse clinical effects nor histopathological, hematological or serum biochemical changes as compared to vehicle treatment. In the cytotoxicity test, cell viability was not affected in both rat brain striatum primary neurons and human primary hepatocytes using up to 100 mg/L MEAI. Cytotoxic effect was demonstrated using concentrations of 500 and 1000 mg/L with a calculated IC_{50} value of 368.2 mg/L for rat brain striatum primary neurons and 403.1 mg/L for human primary healthy hepatocytes. The combination of 6% or 7.5% ethanol with 100 mg/L MEAI revealed no statistically significant increase in cytotoxic effect. In the bacterial mutagenicity test, MEAI in the absence of metabolic activation was considered as not mutagenic. However, in the presence of metabolic activation, MEAI revealed a slight mutagenic activity only at the highest dose (5 mg/plate) and only in one *S. typhimurium* strain out of five.

In conclusion, MEAI showed a good safety profile at 10 and 30 mg/kg in rats, corresponding to the human doses of 1.6 mg/kg and 4.8 mg/kg, respectively. Further studies, especially long term chronic and behavior studies in rodents and non-rodent species, are required in-order to assess MEAI safety profile adequately, before a human trial can be commenced.

Conflict of interest

The authors state no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2017.01.018.

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