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Methoxetamine Induces Cytotoxicity in H9c2 Cells: Possible Role of p21 Protein (Cdc42/Rac)-Activated Kinase 1

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Abstract

The abuse of new psychoactive substances (NPS) is an emerging social problem. Methoxetamine, one of the NPS, was designed as an alternative to ketamine and it was considered an NPS candidate owing to its high addictive potential. However, cardiotoxicity of the phencyclidine analogue, methoxetamine, has not been extensively evaluated. P21 protein (Cdc42/Rac)-activated kinase 1 (*PAK-1*) is associated with the drug-induced cardiotoxicity and hypertrophy of cardiomyocytes. In the present study, we investigated the effects of methoxetamine on rat cardiomyocytes and *PAK-1*. Methoxetamine (at 10 μM) reduced cell viability and *PAK-1* mRNA levels in H9c2 cells. Methoxetamine treatment (100 μM) decreased the beating rate of primary cardiomyocytes. However, 100 μM methoxetamine-induced heart rate decline was less than 100 μM PCP- or ketamine-induced heart rate decline. Meanwhile, fingolimod hydrochloride (FTY720, 1 μM), a *PAK-1* activator, increased cell viability and inhibited hypertrophy induced by methoxetamine in H9c2 cells. These results suggest that methoxetamine may have harmful effects on the cardiovascular system through the regulation of the expression and function of *PAK-1*.

Keywords New psychoactive substance · Methoxetamine · Cardiac toxicity · *PAK-1* · FTY720

Introduction

New psychoactive substances (NPS) are widely abused and considered a global social problem. The use of NPS, known to cause serious problems, worldwide has increased rapidly [1, 2]. Furthermore, a variety of NPS have appeared in the market, and the actual use and harmful effects of NPS are much more common than what statistics reveal. NPS have diverse adverse effects including cardiovascular, neurological, gastrointestinal, and pulmonary effects. Most reports

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that describe NPS-induced toxicity have focused on retro- or prospectively analyzed cases of intoxication and interviews with drug users. The toxicity caused by these NPS needs to be elucidated. However, most preclinical research studies have investigated the dependence potential and neuropsychiatric effects of NPS.

Methoxetamine, an *N*-ethyl derivative of ketamine, was designed as an alternative to ketamine by a UK-based research chemist and it was considered an NPS candidate owing to its high addictive potential [3–5]. In 2014, methoxetamine was included in the list of NPS by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), categorized as a ketamine derivative [6]. There has been an increase in the number of reports regarding the abuse of methoxetamine in humans, which resulted in serious or even fatal outcomes [7, 8]. Despite the reports of misuse, methoxetamine is still not a controlled drug in many countries [6].

Cardiac hypertrophy accompanies many forms of heart disease, including ischemic disease, hypertension, heart failure, valvular disease, and malignant arrhythmia [9–11]. Therefore, it is essential to identify the molecular events involved in the hypertrophic process and the molecular targets, such as p21 (Cdc42/Rac)-activated kinase 1 (*PAK-1*) [11, 12]. Ras-related small G proteins, Cdc42, and Rac1 regulate *PAK-1*, which is a part of the family of serine-threonine protein kinases [13]. *Pak1* may be involved in the development of compensated cardiac hypertrophy and the transition to decompensation and failure of cardiac cells [14]. Recent data indicate that novel signaling in the heart involves *PAK-1* and *PAK-1* is involved in cardiac function and cardiotoxicity [15–17]. Furthermore, Human *Ether-à-go-go*-related gene (hERG) binding property is a putative biomarker for arrhythmias [18]. In the present study, we investigated the cardiotoxicity induced by methoxetamine in cardiomyocytes and hERG binding assay. We also examined the preventive effects of a *PAK-1* activator, fingolimod hydrochloride (FTY720), on the cardiotoxicity and hypertrophy induced by methoxetamine.

Materials and Methods

Animals

All experimental procedures were approved by the Animal Ethics Committee, National Institute of Food and Drug Safety Evaluation, and complied with the Guide for the Care and Use of Laboratory Animals (National Research Council, NRC, 1996). Neonatal ICR mice were obtained from the Ministry of Food and Drug Safety (AAALAC member, Osong, Republic of Korea) and maintained in animal facility. The animal holding rooms were maintained at a temperature of 21–24 °C and 40–60% relative humidity with a 12-h light/dark cycle. The animals received a solid diet and tap water ad libitum.

Materials

Methoxetamine, PCP (Phenylcyclohexyl piperidine), and ketamine were purchased from Cayman Chemical (Ann Arbor, MI, USA). E-4031 was purchased from Invitrogen (Carlsbad, CA, USA). The H9c2 (CRL-1446) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). FTY720 and the other routine chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Effects of Methoxetamine on Cell Viability

Cell viability was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instruction Sigma-Aldrich (St. Louis, MO, USA). The H9c2 and HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, ATCC) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 1% antibiotics/antimycotics (Invitrogen, Carlsbad, CA, USA) in an atmosphere consisting of 95% air and 5% CO_2, using standard culture methods. Cells were plated in a 96-well plate (corning #3599) (1×10^5 cells/mL, 100 µL/well). After overnight incubation at 37 °C, the medium was removed and replaced with fresh medium containing varying concentrations (0.1–500 µM) of methoxetamine for 16 h. The absorbance was measured by a microplate reader (SpectraMAX M5, molecular device, Sunnyvale, CA, USA). The viable cell value was calculated as a percentage of the value obtained from cells incubated with fresh medium only.

Pak1 mRNA Expression in H9c2 Cells

H9c2 cells (ATCC) were maintained in DMEM (ATCC) supplemented with 10% FBS (Invitrogen) and 1% antibiotics/antimycotics (Invitrogen) in 95% air and 5% CO_2 using standard culture methods. Cells were plated in a 6-well plate (2×10^5 cells/well). After overnight incubation at 37 °C to allow for cell attachment, the medium was removed and replaced with vehicle control (medium with 0.5% DMSO) or medium containing 10 µM of methoxetamine for 16 h. Complementary DNA synthesized and quantitative real-time PCR was performed as previously described [19]. The primers used were as follows: 5′-ACCATGGTGGGAACT CCATA-3′ (forward) and 5′-CATCTCAAGACAGCGGTT CA-3′ (reverse) for *PAK-1* and 5′-TGTCAAGCTCATTTC CTGGT-3′ (forward) and 5′-CTTACTCCTTGGAGGCCA

TG-3′ (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as an internal control.

hERG Fluorescence Polarization Assay

To assess the hERG inhibition caused by methoxetamine, a hERG fluorescence polarization assay was performed using a Predictor™ hERG fluorescence polarization assay kit (PV 5365, Invitrogen, Carlsbad, CA, USA) according to a previous report [20].

Beating Rate

Spontaneous beating cardiomyocytes were removed from neonatal ICR mouse hearts. Dissociated cells were placed in culture medium consisting of 0.05% collagenase and 10% newborn calf serum (Thermo Fisher Scientific, Waltham, MA, USA). Cells were plated on 35 mm collagen type 1 precoating plate and incubated at 37 °C in 95% O_2 and 5% CO_2. The neonatal cardiac cells began to beat spontaneously after 2 days in the culture. We measured the beating rate after 6 days from the day of the primary culture. Spontaneously beating cells from cardiomyocytes of neonatal ICR mouse were cultured and subjected to treatment with methoxetamine, PCP (Phenylcyclohexyl piperidine) and ketamine (0, 1, 10, and 100 μM) and were measured (at 32 ± 2 °C) using a video-based camera system (JP/CKX41SF, Olympus, Tokyo, Japan). The cells were recorded for 10 min and rate data were used alternately (1 min recording followed by 1 min non-recording).

Cytotoxicity of Methoxetamine in H9c2 Cells

Cell viability was measured using a LIVE/DEAD viability/cytotoxicity kit according to the manufacturer's instruction (L-3244, Thermo Fisher Scientific, Waltham, MA, USA). Cells were plated in a 96-well plate (1×10^5 cells/mL, 100 μL/well) for the assay. After overnight incubation, the medium was removed and replaced with fresh medium containing 100 μM of methoxetamine for 16 h. Cells were co-treated with FTY720 (Sigma-Aldrich, St. Louis, MO, USA) and methoxetamine. Cells were washed prior to the assay twice with phosphate-buffered saline (PBS, Gibco, Brooklyn, NY, USA) and 100 μL of PBS was added to each well. One hundred microliters of the combined LIVE/DEAD® assay reagents were added to each well and the cells were incubated for 30–45 min at room temperature in the dark, and the wells were covered with a lid. Following the incubation, cell viability was measured by a microplate-based cell cytometer (Celigo, Brooks, San Mateo, CA, USA). The data of live (% corrected) cells were used to analyze the cell viability.

Measurement of Hypertrophic Growth in H9c2 Cells

Hypertrophy was assayed by measuring the cell surface area of H9c2 cells as previously described [21]. H9c2 cells (5×10^4 cells/well in 24-well plate) were treated with methoxetamine or FTY720 for 16 h. The cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Fixed cells were rinsed with PBS and stained with 0.4% Crystal violet (BD Diagnostics, Sparks, MD, USA) for 20 min. The cells were then washed and images were obtained using a light microscope (JP/CKX41SF, Olympus). Three random photographs were taken from each of the four samples and the cell surface area was determined using ImageJ software (National Institutes of Health). The data shown represent the image analysis from two independent experiments.

Data Analysis

The data are presented as the mean ± standard error (S.E.) and the statistically significant differences between the drug-treated and vehicle-treated groups were analyzed using a Student's *t* test. In addition, a one-way, two-way, or two-way repeated measures analysis of variance (ANOVA) and the Bonferroni's test for equal variance data were performed, followed by Dunnett's rank test for non-equal variance data using SigmaPlot 13 software (Sigmaplot, Chicago, IL, USA). To estimate the half maximal inhibitory concentration (IC_{50}) values, all data were analyzed using Prism (GraphPad Software, San Diego, CA, USA).

Results

Methoxetamine Reduced Cell Viability in H9c2 Cells

To investigate the effect of methoxetamine on cell viability, H9c2 cells were treated with 0, 0.1, 1, 10, 100, or 500 μM methoxetamine for 16 h. Methoxetamine significantly reduced cell viabilities at 10, 100, and 500 μM (Fig. 1a). On the contrary, methoxetamine has no effect on HepG2 cell viability (Fig. S1). We further examined the effect of methoxetamine on ROS production in H9c2 cells. As shown in (Fig. S2) we found that methoxetamine has no effect on ROS production in H9c2 cells.

Methoxetamine Reduced *PAK-1* Expression in H9c2 Cells

To measure the expression levels of *PAK-1* in H9c2 cells, we analyzed the level of *PAK-1* mRNA by real-time quantitative RT-PCR. The average relative expression level of *PAK-1* (pak-1/glyceraldehyde-3-phosphate dehydrogenase) was

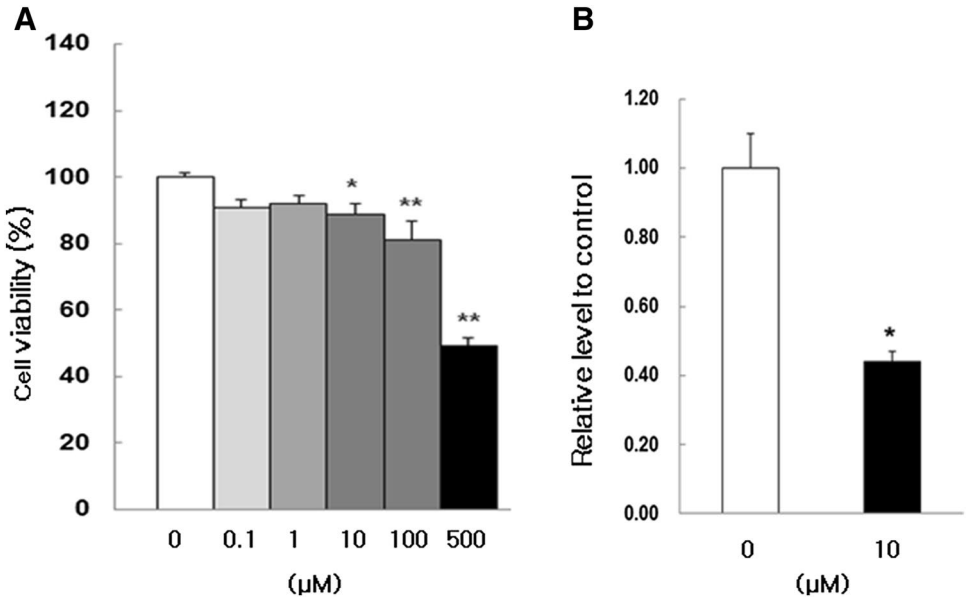

Fig. 1 Methoxetamine reduces H9c2 cell viability and *PAK-1* expression. **a** Effects on H9c2 cell viability. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after methoxetamine treatment for 16 h at the indicated concentrations. Data are shown as the means ± standard error (S.E.) of eight wells. Cell viability was expressed as a percentage of that at vehicle. $*p < 0.05$, $**p < 0.01$ versus each vehicle-treated group (by one-way ANOVA and Bonferroni's test). **b** Effects of methoxetamine on *PAK-1* expression in H9c2 cells. H9c2 cells were treated with methoxetamine (10 μM) for 16 h and quantitative real-time RT-PCR analysis of *PAK*-1 was performed. The expression levels were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in each sample. Data are shown as the means ± S.E. of different six wells. $*p < 0.05$, versus vehicle-treated group (Student's *t* test)

significantly lower in the methoxetamine-treated (10 μM, 16 h) group than in the vehicle-treated group (Fig. 1b).

hERG Channel Binding Property of E-4031 and Methoxetamine

To investigate the binding property of methoxetamine and E-4031 to the hERG channel, a hERG fluorescence polarization assay was performed. A concentration of 0.001–10000 μM of E-4031 and methoxetamine was added to each well. The fit model was used to calculate IC 50. The results showed that the IC_{50} of E-4031 (positive control) was 14 μM (Fig. 2a), whereas IC_{50} of methoxetamine was 417.2 μM (Fig. 2b).

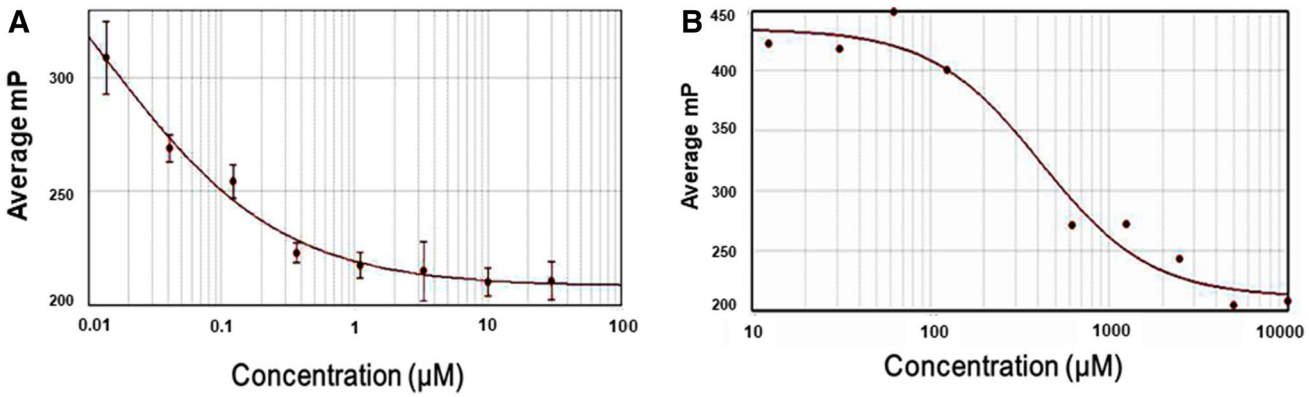

Fig. 2 Binding properties of methoxetamine and the beating rate of primary cultured cardiomyocytes. **a, b** Human *Ether-à-go-go*-related gene (hERG) channel binding properties of E-4031 (positive control) and methoxetamine, respectively. Concentration-response curve for E-4031 and methoxetamine and their IC_{50} values to the hERG channel. The IC_{50} for E-4031 and methoxetamine was (14 and 417.2 μM) respectively

Methoxetamine, PCP, and Ketamine Reduced the Beating Rates of Mice Cardiomyocytes

To investigate the effects of methoxetamine, PCP (Phenylcyclohexyl piperidine) and ketamine on the function of cardiomyocytes, we measured the beating rates of mice primary cardiomyocytes. The number of beats per minute was different between the vehicle-treated and methoxetamine, PCP and ketamine-treated cardiomyocytes. Methoxetamine treatment reduced the beating rate at concentration of 100 μM in cardiomyocytes (Fig. 3a). Likewise, PCP and Ketamine treatment reduced the beating at 10 and 100 μM in cardiomyocytes (Fig. 3b, c) respectively.

Effect of FTY720 on the Cytotoxicity and the Hypertrophy-Induced by Methoxetamine in H9c2 Cells

Methoxetamine (100 μM, 24 h) induced cytotoxicity in H9c2 cells, however, FTY720 (1 μM), a *PAK-1* activator, decreased this cytotoxicity (Fig. 4). Also, to verify the role of *PAK-1* in the hypertrophy induced by methoxetamine, we measured the cell surface area. As demonstrated in (Fig. 5), in H9c2 cells treated with methoxetamine (100 μM, 16 h), the cell size was significantly increased. However, FTY720 (1 μM), a *PAK-1* activator, inhibited this hypertrophy.

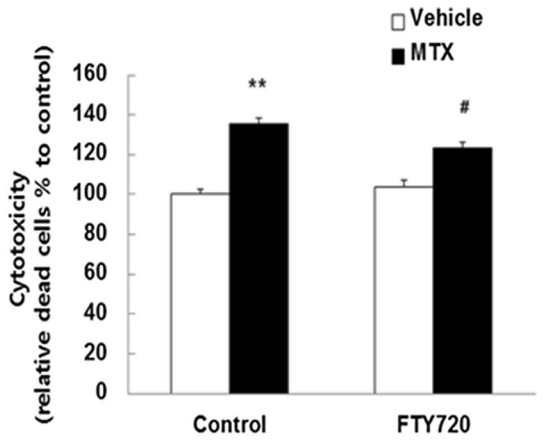

Fig. 4 Effects of fingolimod hydrochloride (FTY720), a *PAK-1* activator, on H9c2 cell death induced by methoxetamine. H9c2 cells were treated with fresh vehicle (DMEM) or an indicated substance (methoxetamine, 100 μM; FTY720, 1 μM) for 16 h. Data are shown as the means ± standard error (S.E.). $n = 4–6$, $^{**}p < 0.01$ versus control-vehicle, $\#p < 0.05$ versus control-methoxetamine (by two-way analysis of variance (ANOVA) and Bonferroni's test). *MTX* methoxetamine

Discussion

NPS can cause a variety of adverse effects including cardiovascular effects, such as tachycardia and arterial hypertension; neurological effects, such as headache, mydriasis, and light-headedness; and metabolic effects, such as

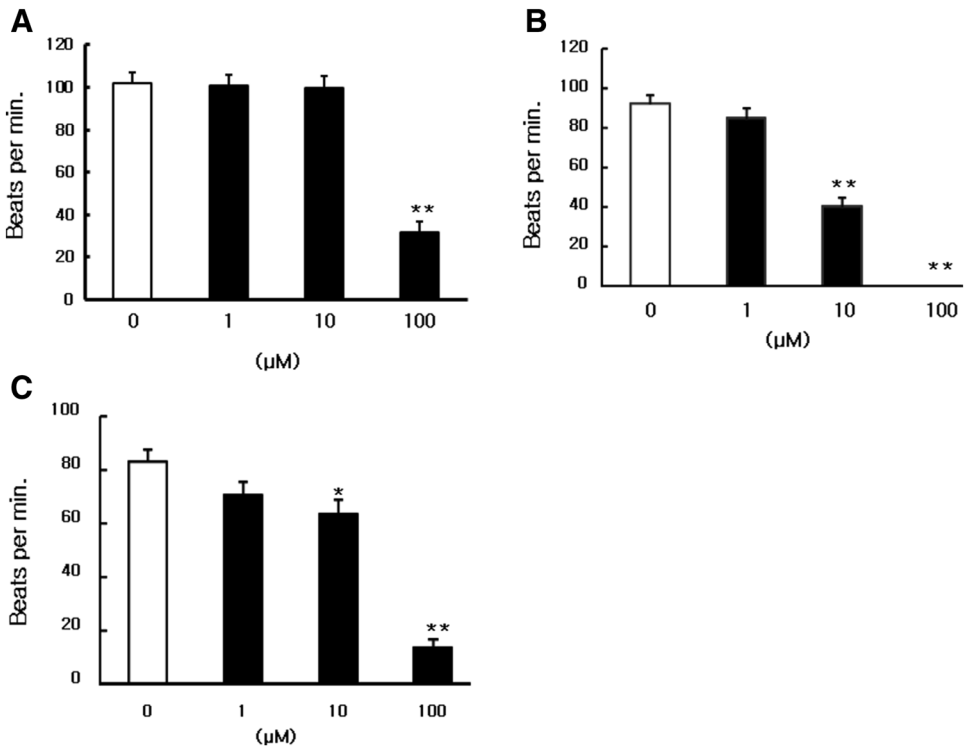

Fig. 3 Effects of Methoxetamine, PCP and Ketamine on the beating rate of primary cultured cardiomyocytes. Cardiomyocytes of neonatal ICR mouse were cultured and subjected to treatment with methoxetamine, PCP and ketamine (0, 1, 10, and 100 μM) respectively and were measured (at 32 ± 2 °C) using a video-based camera system (JP/CKX41SF, Olympus, Tokyo, Japan). **a** Methoxetamine treatment reduced the beating rate at concentration of 100 μM in cardiomyocytes, whereas PCP and Ketamine treatment reduced the beating at 10 and 100 μM in cardiomyocytes (**b** and **c**) respectively. Data are shown as the means ± standard error (S.E.) $^{*}p < 0.05$ and $^{**}p < 0.01$ versus vehicle-treated group (by one-way repeated measures analysis of variance (ANOVA) and Bonferroni's test)

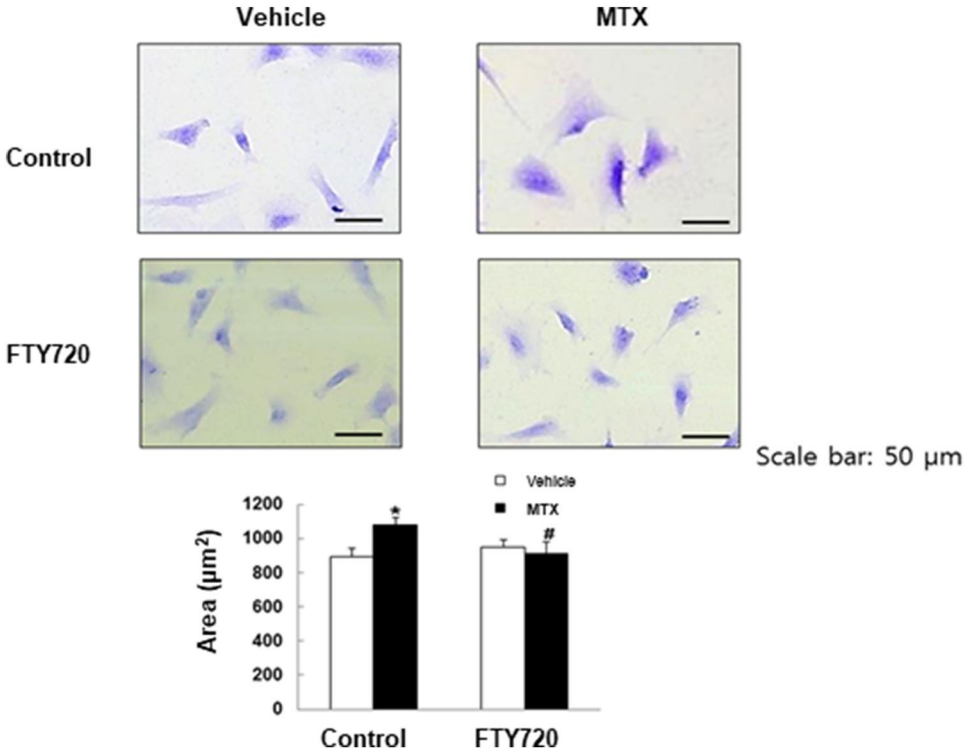

Fig. 5 Effect of fingolimod hydrochloride (FTY720), a *PAK-1* activator, on methoxetamine-induced hypertrophy in H9c2 cells. Representative photographs show the crystal violet stained cell surface area of H9c2 cells. Scale bar = 50 μm. The cell surface area measurement revealed that FTY720 (1 μM) treatment inhibited the hypertrophy induced by methoxetamine (100 μM) for 16 h. Data are shown as the means ± standard error (S.E.). $n = 97–129$ $*p < 0.05$ versus vehicle, $\#p < 0.05$ versus methoxetamine (by two-way analysis of variance (ANOVA) and Bonferroni's test). *MTX* methoxetamine

hyponatremia and hypokalemia [22, 23]. The abuse of synthetic psychoactive ketamine analogues is an emerging social problem. Ketamine analogues may possess potential harms associated with lack of pharmacological, clinical, and toxicological profiles [24]. Of particular importance is the cardiovascular toxicity caused by NPS [25]. Methoxetamine is a new, synthetic, psychoactive drug derived from ketamine [26, 27]. Since its debut in 2010, it has become a popular recreational drug especially among adolescents [28]. Ketamine stimulates the cardiovascular system and increases one's heart rate, systemic arterial pressure, and systemic vascular resistance [29, 30]. However, to our knowledge the effect of methoxetamine on the cardiovascular system is still unknown. It has been reported that blood concentration of methoxetamine was 0.09–0.20 mg/L (≈0.36–0.808 μM), in intoxication cases [5], also 0.45 mg/L (≈2 μM) in clinical case of acute methoxetamine poisoning [31] and 8.6 mg/L (≈35 μM) in accidental fatal intoxication [32]. In present study, treatment of 10 μM of methoxetamine for 16 h reduced cell viability and acute treatment of 100 μM of methoxetamine reduced beating rate of cardiomyocytes. Based on concentrations which was reported in clinical fatal intoxication cases, methoxetamine concentration above at least 10 μM may induce fatal clinical outcomes. Therefore, in our study, we measured the effect of methoxetamine at doses which are comparable to that of toxic cases of human.

Ketamine analogues such as PCP and methoxetamine are ligands for glutamate receptors [33]. Especially, mGluR1/5 is widely present in cardiac cells but not in liver cells [34]. Furthermore, mGluR1/5 is involved in activation of Erk1/2 which is mediated by *PAK-1* in H9c2 cells [35, 36]. These results suggest that activation of glutamate receptor induced by methoxetamine may play a role in the decreased cell viability of H9c2 cells not in HepG2 cells. In addition, treatment of methoxetamine resulted in hypertrophy in H9c2 cells. This effect was decreased when FTY720 was administered, which is known to activate *PAK-1*. *PAK-1* is associated with ventricular arrhythmias, which are related to heart failure [37]. Therefore, we asked if methoxetamine had adverse effect on beating rate of primary cultured cardiomyocytes. We demonstrated that methoxetamine as well as ketamine and PCP significantly reduced beating rate of mice cardiomyocytes. In addition, hERG binding property is a putative biomarker for arrhythmias [18]. It has been suggested that ketamine has low IC_{50} values in hERG assay [38]. Therefore, we examined whether methoxetamine has similar hERG binding property or not. Conversely, our present study showed that IC_{50} value of methoxetamine in H9c2 cells was 417.2 μM, indicating that methoxetamine has a low binding affinity to hERG. These results suggest that methoxetamine may not affect cardiac functions via the acute inhibition of ion current channels, such as IKr.

Nevertheless, *PAK-1* is known to be involved in the cardiovascular system, including the development of compensated cardiac hypertrophy, and the transition to the decompensation and failure of cardiac cells [15]. However, currently, it is unknown how *PAK-1* regulates the cardiac system in heart cells. It was reported that *PAK-1* enhanced

myofilament Ca^{2+} sensitivity in cardiomyocytes and is a natural inhibitor of Erk1/2 and a novel anti-hypertrophic signaling molecule upstream of PP2A [15, 16]. In addition, we previously reported that *PAK-1* is associated with cardiotoxicity induced by H1 antihistamines, such as terfenadine and astemizole, which are banned products due to their cardiotoxicity [17]. Furthermore, hypertrophy leads to cell death via apoptosis [39, 40] and methoxetamine may induce *PAK-1* reduction and hypertrophy, consequently increasing apoptosis-related gene (Fig. S3). Therefore, we suggest that *PAK-1* plays an important role in the cytotoxicity and hypertrophy induced by methoxetamine in H9c2 cells.

Conclusion

Treatment of methoxetamine reduced beating rate of primary cardiomyocytes and cell viability of H9c2 cells which is embryonic rat cardiomyocytes from embryonic BD1X rat heart tissue. *PAK-1* is associated with ventricular arrhythmias which are related to heart failure. Methoxetamine can induce activation of glutamate receptor. The mGluR1/5 is involved in activation of Erk1/2 which is mediated by *PAK-1*, in present study, treatment of methoxetamine resulted in hypertrophy in H9c2 cells but this effect was decreased by treatment of FTY720 which is known to activate *PAK-1*. Therefore, methoxetamine may have harmful effects on the cardiovascular system through the regulation of the expression and function of *PAK-1*.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical Approval All experimental procedures were approved by the Animal Ethics Committee, National Institute of Food and Drug Safety Evaluation, and complied with the Guide for the Care and Use of Laboratory Animals (National Research Council, NRC, 1996).

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