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Title Page

Potent Inhibition of Alcohol Self-Administration in Alcohol-Preferring Rats by a Kappa Opioid Receptor Antagonist

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Abbreviations: μ, mu opioid receptor; δ, delta opioid receptor; κ, kappa opioid receptor; NOP, nociceptin opioid receptor; nor-BNI, Norbinaltorphimine; GNTI, 5'-Guanidinonaltrindole; JDTic, (3*R*)-7-Hydroxy-N-[(2*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]-3-methylbutan-2-yl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide; DIPEA, Diisopropylethylamine; BOP, (dimethylamino) phosphonium hexa-fluorophosphate; DCM, dichloromethane, ESI, electrospray ionization; BALs, Blood Alcohol Levels; SGPT, serum glutamic-pyruvic transaminase; SGOT, serum glutamic oxaloacetic transaminase.

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Abstract Page

ABSTRACT

A substituted aryl amide derivative of 6-naltrexamine, compound **5**, previously shown to be a potent kappa opioid receptor antagonist, was used to characterize the physicochemical properties and efficacy to decrease alcohol self-administration in alcohol-preferring rats (P-rats) and binge-like P-rats. Previous studies showed that compounds closely related to **5** possessed good blood-brain barrier penetrating properties. Pharmacokinetic studies showed that **5** had acceptable bioavailability. In contrast to other kappa receptor antagonists (i.e., nor-BNI) compound **5** showed favorable drug-like properties and further studies were done. Safety studies showed that **5** was not hepatotoxic at doses 200-fold greater than an efficacious dose. The effect of **5** or naltrexone on the hepatotoxicity of thiobenzamide was investigated. In contrast to naltrexone that exacerbated thiobenzamide-mediated hepatotoxicity, **5** was observed to be hepatoprotective. Based on the physicochemical properties of **5**, the compound was examined in rat animal models of alcohol self-administration. The inhibition of ethanol self-administration by **5** in alcohol-dependent or alcohol-nondependent P-rats trained to self-administer a 10 % (w/v) ethanol solution, utilizing operant techniques showed very potent efficacy (i.e., estimated ED_{50} value 4-5 μg/kg). In a binge-like P-rat animal model, inhibition of alcohol self-administration by **5** had an estimated ED_{50} value of 8 μg/kg. The results suggest that **5** is a potent drug-like kappa opioid receptor antagonist of utility in alcohol cessation medications development.

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Introduction

Ethanol overuse is a serious public health disorder with significant social and economic consequences. In 1994, naltrexone, a pure opioid μ receptor antagonist with relatively low affinity for δ and κ receptors and no abuse potential (Tabakoff et al., 1983) (compound **1**, Scheme 1) was approved by the United States FDA for treatment of alcoholism. A number of studies suggest that alcohol interacts with endogenous opioid systems (Grisel et al., 1995; Gianoulakis et al., 1996). Antagonizing opioid receptors decreases the effects of alcohol-mediated pleasure-inducing endogenous opioids. By attenuating the positive reinforcing effects of alcohol consumption, opioid receptor antagonists have direct effects on alcohol-seeking behavior (Pastor et al., 2006). A decrease in alcohol consumption by antagonism of opioid receptors suggests direct effects of this reinforcement system and animal studies have shown that mu (μ)-, delta (δ)- and kappa (κ)-opioid receptors contribute to alcohol-induced reinforcement (Herz, 1997; Ulm et al., 1995).

Based on a number of clinical studies, naltrexone is effective in decreasing alcohol consumption in heavy drinkers (Pettinati et al., 2006) and for treating alcoholism (Bouza et al., 2004; Anton et al., 1999). However, naltrexone is not successful in treating all alcoholics and adverse effects including intolerable nausea (Croop et al., 1997) and hepatotoxicity (Mason et al., 1999, Mitchell et al., 1987) confound treatment of patients with liver disease. However, most reports (Brewer and Wong, 2004; Sax et al., 1994; Yen et al., 2006) suggest that naltrexone itself does not cause clinically significant hepatotoxicity. Relatively low bioavailability of naltrexone (Anton et al., 1999) and possibly genetic variability of the opioid receptors (Oslin et al., 2006) may explain the less than consistent efficacy of naltrexone (Roozen et al., 2006).

Thiobenzamide is a well-characterized hepatotoxin that causes centrolobular necrosis (Hanzlik et al., 1980; Hanzlik et al., 1978) and requires S-oxidative metabolic bioactivation for full expression of its hepatotoxicity (Cashman and Hanzlik, 1981; Hanzlik and Cashman, 1983). Hepatotoxicity of toxic doses of thiobenzamide is maximal 24 h after administration and thus can provide an excellent acute model system to examine the effect of **5** or naltrexone on the exacerbation or protection of hepatotoxicity.

In contrast to naltrexone, a more selective kappa opioid receptor antagonist is nor-BNI. Nor-BNI is effective at decreasing alcohol self-administration in small animals (Walker et al., 2011; Walker and Koob, 2008). Despite its promise, nor-BNI possesses very long-lasting effects (Horan et al., 1992) and is possibly unstable to oxidation (Osa et al., 2007). Like other long-acting kappa opioid antagonists such as GNTI and JDTic, nor-BNI has a very long time course of kappa opioid receptor antagonism (Munro et al., 2012). There is thus a need for a relatively fast-acting drug-like kappa opioid receptor antagonist that possesses appropriate pharmacokinetic and biodistribution properties consistent with a reversible drug.

Studies using rodent animal models have shown that naltrexone decreases alcohol self-administration (Benjamin et al., 1993; Stromberg et al., 2001), suggesting that these types of agents may prevent the reinforcing effects of alcohol consumption (Bouza et al., 2004). The alcohol preferring rat (P-rat) has been effectively used as a small animal model to study binge drinking (Li et al., 1987). In the P-rat, naltrexone (Ji et al., 2008; Gilpin et al., 2008; Biggs and Myers, 1997) and other opioids (Weiss et al., 1990) have been shown to be effective in decreasing alcohol self-administration. Nalmefene (Scheme 1), the 6-methylene analog of naltrexone, is a more potent κ opioid antagonist than naltrexone and is an effective antagonist of alcohol self-administration in outbred and P-rats (June et al., 2004; June et al., 1998).

Herein, we report on the evaluation of a potent κ-opioid antagonist as an alcohol self-administration cessation agent. Kappa opioid antagonists are anticipated to show a dual

action by inhibiting alcohol reinforcement and stimulating dopamine release to decrease craving. Compound **5** (Scheme 1) has been previously reported to decrease alcohol self-administration in Wistar rats. In this study, we extend the analysis to alcohol-preferring and binge-like P-rats. The results show that compound **5** is a very potent, relatively short-acting agent that decreases alcohol self-administration in P-rats and binge-like P rats. Compound **5** possesses good physicochemical properties and is very drug-like and, in contrast to naltrexone, protects from the hepatotoxicity of a potent hepatotoxin in rats. The rationale for our work was to develop a relatively short-acting drug-like κ-opioid antagonist by replacing the metabolically labile 6-keto moiety of naltrexone with an amide moiety, thus leading to an agent with potent pharmacological activity and potentially less hepatotoxicity.

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Methods

Chemicals. Naltrexone and nalmefene hydrochloride (compounds **1** and **2,** respectively) were obtained from Tyco Mallincrodt (St. Louis, MO). Compound **3**, 17-Cyclopropylmethyl-3, 14β-dihydroxy-4, 5α-epoxy-6β-[(4'-bromo) benzamido]morphinan- hydrochloride, and compound **5,** 17-Cyclopropylmethyl-3, 14β-dihydroxy-4, 5α-epoxy-6β-[(4'-trimethylfluoro)benzamido]morphinan-hydrochloride were synthesized as previously described (Ghirmai et al., 2009) (Scheme 1).

Diisopropylethylamine (DIPEA), (dimethylamino) phosphonium hexa-fluorophosphate (BOP), HBF_4, $Pd(OAc)_2$, tetrabutylammonium hydroxide, thiobenzamide, heparin and Supersac were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO) and were used as received. All of the solvents and buffers used were obtained in the highest grade commercially available from VWR (San Diego, CA).

General Procedures. Synthetic chemical reactions were run under a positive pressure of nitrogen with magnetic stirring at ambient temperature using oven-dried glassware unless otherwise indicated. Silica gel (230-400 mesh) was used for column chromatography. Dichloromethane (DCM) was dried by filtration through a column of neutral alumina and stored over activated 4 Å molecular sieves under nitrogen prior to use. All other solvents and reagents were used as received. ^1H NMR spectra were recorded at 300.0 MHz on a Varian Mercury 300 instrument. Chemical shifts were reported in ppm (δ) relative to $CDCl_3$ at 7.26 ppm. NMR spectra were recorded in $CDCl_3$. Mass spectra were obtained with a Hitachi spectrometer operating in the ESI mode. Analytical purities were

determined by reverse phase HPLC using a Hitachi D2500 Hitachi Chromato-integrator, an L-6000 Hitachi pump and an L-4200 UV-vis Hitachi detector (285 nm) using a reverse phase system (5 μm x 4.6 mm x 250 mm). The mobile phase was 20% 0.05 M tetrabutylammonium hydroxide and 80% methanol using isocratic elution at a flow rate of 1 mL/min. Analytical work for the pharmacokinetic studies was done at Microconstants, Inc., (San Diego, CA).

Animals. Animal work was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. Formal approval to conduct the experiments was obtained from the Institutional Animal Care and Use Committees (IACUCs) of the Human BioMolecular Research Institute and Behavioral Pharma, Inc. Animals were assigned randomly to experimental groups, allowed to acclimatize to the facilities for 1 week, and fed commercial rat chow and sterile distilled water *ad libitum*. For the studies with thiobenzamide, male Sprague Dawley rats weighing 300-400 g from Harlan (San Jose, CA) were used. For pharmacokinetic studies, canulated male Sprague Dawley rats (Harlan) weighing 250-300 g at the time of the experiment were housed individually and maintained in a temperature-controlled environment on a 12h:12h light cycle (off 7:30 am: on 7:30 pm). Except during testing, animals were given free access to food and water. Animals administered compounds via the oral route were deprived of food 10 h before the experiment. For toxicology studies, compound **5** was administered to male Sprague Dawley rats weighing 300-350 g (Harlan). Twenty-four h after the last dose of **5**, animals were killed, blood was obtained, centrifuged and serum was separated and frozen for analysis of serum clinical chemistry at IDEXX Laboratories (Sacramento, CA). For

alcohol self-administration studies, male alcohol-preferring Wistar rats (P-rats) 225-249 g were obtained from the University of Indiana (Indianapolis, IN) and were housed in groups of two or three and maintained in a temperature-controlled environment on a 12h:12h light cycle (off 7:30 am: on 7:30 pm). Except during behavioral testing, animals were given free access to food and water.

Chemical Synthesis.

6-β-(4'-Trifluoromethyl-2',3',5',6'-tetradeutrio)benzamido-14-hydroxy-17-(cyclopropylmethyl)nordesmorphine, compound 4. Compound **4** (Scheme 1) was synthesized by combining β-naltrexamine and 4-CF_3-benzoic acid-d_4 and BOP dissolved in anhydrous DCM followed by addition of DIPEA. After removal of the ester at the 3-position by treatment with potassium carbonate, **4** was obtained in quantitative yield and converted to its hydrochloride salt. The requisite 4-CF_3-benzoic acid-d_4 was obtained following a literature procedure for a non-deuterated analog (Watson et al., 2008). A mixture of K_2CO_3 (624 mg, 4.5 mmol, 1.5 equiv.), DCPP x HBF_4 (73.2 mg, 0.12 mmol, 4% mol) and $Pd(OAc)_2$ (13.5 mg, 0.06 mmol, 2% mol) were placed in an 8-dram vial. The vial was sealed with a septum and purged with Ar. A solution of 4-chloro-trifluoromethyl-toluene-d_4 (554 mg, 3 mmol) in DMSO (3 mL) and H_2O (108 μL, 108 mg, 6 mmol, 2 equiv.) was added via syringe. An atmosphere of CO was added to the vial and purged three times and run for 15 h at 100 ºC under a CO atmosphere. The mixture was cooled and diluted with 0.25 M NaOH aq. (65 mL) and extracted with DCM (2 x 25 mL). The aqueous layer was neutralized with 3 M HCl (10 mL) and extracted with Et_2O (3 x 50 mL). The combined organic material was dried over Na_2SO_4, filtered and

evaporated to give 4-CF_3-benzoic acid-d_4 as a white solid, 550 mg, in 94% yield.

Compound **4**-d_4 was obtained following a previously reported procedure (Ghirmai et al., 2008). ß-Naltrexamine (100 mg, 0.29 mmol), 4-CF_3-benzoic acid-d_4 (113.3 mg, 0.584 mmol, 2 equiv.) and BOP (258 mg, 0.584 mmol, 2 equiv.) was placed in anhydrous DCM (4 mL) and DIPEA (152 µL, 0.876 mmol, 3 equiv.) was added and the reaction was stirred overnight at rt to afford the ester-amide. After purification by flash chromatography (100% EtOAc) the ester-amide was dissolved in methanol and potassium carbonate was added. The mixture was stirred at rt for 3 hr and potassium carbonate was removed by filtration and the product was purified by PTLC ($CHCl_3$/MeOH) 20/1 to obtain in quantitative yield the desired product. The purity was > 98% on the basis of HPLC and LCMS.

^{1}H NMR ($CDCl_3$) δ 0.13 – 0.18 (m, 2H), 0.53 – 0.59 (m, 2H), 0.81 – 0.92 (m, 1H), 1.39 – 1.62 (m, 3H), 1.66 – 1.74 (m, 1H), 1.89 – 2.0 (m, 1H), 2.17 – 2.26 (m, 2H), 2.39 (d, J = 6.3 Hz, 2H), 2.65 (d, J = 9.9 Hz, 2H), 3.04 (d, J = 13.4 Hz, 1H), 3.16 (d, J = 5.2 Hz, 1H), 3.64 (d, J = 11.0 Hz, 1H), 4.08 – 4.18 (m, 2H), 4.63 (d, J = 5.24 Hz, 1H), 6.52 (d, J = 8.0 Hz, 1H), 6.67 (d, J = 8.0 Hz, 1H), 7.79 (d, J = 9.0 Hz, 1H). ESI/MS *m/z* = 518.95 [M+H]

Pharmacokinetic Studies.. The night before the oral pharmacokinetic study, the animals were fasted. Groups of two jugular cannulated rats were administered **5** hydrochloride by the i.v. route of administration (20 or 50 µg/kg, 1 ml/kg) or the oral route of administration (200 µg/kg, 2 ml/kg) in isotonic saline. For the i.v. study of **5**, blood was

taken at 5, 15, 30, 60, 120, 240 mins and 6 and 10 hours. For the oral study, blood was taken at 15, 30, 60, 120, 240 mins and 6 and 10 hours. Blood was combined with 2 IUs of heparin and immediately cooled to 4 ºC. Separated plasma was brought to a pH of 10 with ammonium hydroxide, 400 pg/ml of **4** was added as an internal standard and extracted with hexane/methyl-tert-butyl ether (3:1, v:v). After centrifugation at 13,000 rpm for 5 min, the organic fraction was collected and the solvent was removed with a stream of argon. The residue was reconstituted in water:acetonitrile:formic acid (80:20:0.1, v:v) and run isocratically in 0.1% formic acid in water, 0.1% formic acid in acetonitrile (60:40) using a Waters Acquity instrument and Waters XEVO tandem quadrupole detector (Waters, Milford, MA). An aliquot was analyzed by RP-HPLC using a Synergi Polar RP column (2.1 mm x 150 mm, 4 µm) maintained at 45°C. The mobile phase was nebulized using heated nitrogen in a Z-spray source/interface set to electrospray positive mode. The ionized compounds were detected using MS/MS and both compounds **4** and **5** had retention times of 2.7 min in the LCMS experiment. The standard curve was run between 20-20,000 pg/ml. The calibration curves were obtained by fitting the in-transformed peak height ratios of compound **4/5** and their log-transformed standard concentrations to an appropriate regression equation using MassLynx software (Waters). Pharmacokinetic data was determined using an in-house Excel Macro program for pharmacokinetic parameters.

In Vivo Hepatotoxicology Studies. Thiobenzamide was administered i.p. as a very fine suspension in corn oil (2 mmol/kg, 274 mg/kg, 4 ml/kg). Naltrexone hydrochloride (500 µg/kg, 1 ml/kg, i.p.) was administered in sterile saline. Compound **5** hydrochloride (20

µg/kg, 1 ml/kg, i.p.) was administered in sterile saline. On the day of the experiment, groups of six animals each were administered thiobenzamide or vehicle as a challenge dose. Twenty-four h after the challenge dose, treatments were administered. The compound treatments were as follows: vehicle, naltrexone (1.3 µmole/kg or 500 µg/kg) or compound **5** (0.036 µmole/kg or 20 µg/kg). Forty-eight h after administration of thiobenzamide or vehicle , the animals were killed and blood was collected in heparin-treated syringes, centrifuged and serum was immediately frozen. Serum was sent to IDEXX Laboratories and serum clinical values were obtained. The mean and standard deviation of the values were calculated and summarized in Table 2.

Operant Procedure for Oral EtOH and Supersaccharin Self-Administration Training. Ethanol or Supersac self-administration training was conducted in standard alcohol vapor chambers (La Jolla Alcohol Research, La Jolla, CA) located in sound-attenuated, ventilated cubicles. Two 35-ml syringes dispensed either EtOH, water or Supersac through plastic tubing into two stainless steel drinking cups mounted 4 cm above the grid floor and centered on the front panel of each chamber. Each drinking cup held 2 reinforcer deliveries (0.1 ml fluid/reinforcer). Two retractable levers were located 4.5 cm to either side of the drinking cups. Fluid delivery and recording of operant responses were controlled by a microcomputer.

Briefly, animals were trained to voluntarily self-administer 10% (w/v) EtOH (n=11) or Supersac (n=11) by the oral route using the saccharin fadeout method (Rassnick et al., 1993) and were tested for their response for EtOH or Supersac solution in a two-lever free choice situation. Once baseline EtOH and Supersac intake were achieved (i.e., when

responding across three consecutive days varied less than 20% and response rates corresponded to pharmacologically relevant blood alcohol levels (BALs)), dose response testing for compound **5** commenced. BALs were measured once per week but never immediately before or after testing as this was deemed too stressful to the animal. Typically, 2-3 days prior to testing BALs were obtained. BAL levels during these experiments were maintained at 150-200 mg percent. To allow for a complete dissipation of any carry-over effects, a one week washout period, where rats were re-baselined during daily 30 min operant sessions, occurred between testing of different doses.

Ethanol self-administration studies.

P-rats were divided into alcohol binge drinkers (n=11) and Supersac controls (n=11). Prior to two-bottle choice training, all rats were given an initial 2-hour training session during which they were allowed to drink Supersac in a single-bottle situation. Rats were allowed 30-min drinking sessions for 9-14 consecutive days before pharmacological manipulation occurred. Following baseline training, rats were injected subcutaneously (s.c.) with one of several doses of **5**, (0.00312, 0.00625, and 0.0125 mg/kg, 1 ml/kg) 30-min before two-bottle choice test sessions in a within-subjects Latin-square design. Rats were allowed to self-administer every day during this time, but were injected with test compound every other day. Rats were tested during their active cycle.

Data Analysis. Statistics, regression analyses and determination of ED_{50} values were done using GraphPad Prism® (version 4.02, GraphPad, San Diego, CA). Data of the

effect of opioids on thiobenzamide hepatotoxicity was expressed as mean +/- standard deviation (SD) and analyzed with the Student *t* test for the difference between two means with a Welch correction. Multiple means were analyzed by a randomized one-way analysis of variance. When the analysis indicated that a significant difference existed, the means of each group were compared by the Student-Newman-Keuls test. In the analyses, the level of significance chosen was $p < 0.05$. For alcohol and Supersac self-administration studies, data were simultaneously collected on-line from multiple operant chambers. Results of the operant procedure were reported as mean cumulative number of bar presses ± standard error of the mean (SEM) for ethanol or Supersac and normalized for body weight (i.e., g ethanol/kg body weight; ml Supersac/kg body weight). The effects of **5** on alcohol (g/kg) intake and Supersac (ml/kg) intake were analyzed by one-way repeated measures analysis of variance, with a dose of **5** as a within-subjects factor. In general, tests for homogeneity of variance were first conducted on the data. If the scores did not violate the assumption of homogeneity of variance, appropriate analyses of variance (ANOVA) were conducted. Data were analyzed using the StatView statistical package on a PC-Compatible computer. Mixed-design ANOVAs were used with test compound treatments as a within-subjects factor (i.e., repeated measures design for test compound treatment). A priori analysis examining individual test compound doses to vehicle control dose was conducted using paired *t*-tests. Significant test compound effects were defined as having $p < 0.05$ compared to vehicle-treated rats.

Results

The chemical synthesis of 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[(4'-trimethylfluoro)benzamido]morphinan (compound **5,** Scheme 1) was efficiently

accomplished as described previously (Ghirmai et al., 2009). As a standard for pharmacokinetic studies, a deuterated analog, compound **4,** was efficiently synthesized (Scheme 1). Thus, deuterated compound **4** was synthesized by combining β-naltrexamine, 4-CF_3-benzoic acid-d_4 and BOP dissolved in anhydrous DCM followed by addition of DIPEA and, after removal of the ester by treatment with potassium carbonate, **4** was obtained in quantitative yield.

As previously reported, compound **5** was evaluated in the presence of opioid receptors using a [^{35}S]GTPγS assay (Traynor and Nahorski, 1995). The [^{35}S]GTPγS binding data showed that compound **5** was a partial agonist at the μ-opioid receptor, and an antagonist of δ- and κ-opioid receptors (Ghirmai et al., 2009). In the presence of the NOP receptor, compound **5** had very low affinity and did not stimulate agonist-induced GTPγS binding. Compound **5** was found to potently decrease basal binding at NOP. Compound **5** was a high affinity compound that showed low or partial agonist activity in the GTPγS binding experiment and was tested for inhibition of agonist-induced GTPγS binding at each opioid receptor. Compound **5** produced potent inhibition at both κ- and NOP-receptors, modest inhibition at δ-receptor, but not at the μ-receptor. Compound **5** was shown to possess potent antagonism for the κ-opioid and NOP-receptors and was taken forward for *in vivo* studies. As described below, further kinetic analysis was done to characterize the pharmaceutical properties of compound **5**.

Metabolic Stability and Pharmacokinetics

As reported previously, the metabolic stability of compound **5** was examined in the presence of rat, mouse and human liver preparations plus the appropriate NADPH

generating system (Ghirmai et al., 2009). Compared to nalmefene, compound **5** was quite metabolically stable. In the presence of mouse or human liver microsomes, compound **5** possessed half life values in excess of 112 min and was judged to be quite metabolically stable. In the presence of rat liver microsomes, overall, **5** was somewhat less metabolically stable, but the half life values observed did not preclude evaluation of the compounds *in vivo*.

Evaluation of the inhibition of selective functional activity of CYP previously reported (Ghirmai et al., 2009) was done for **5** as a control on the apparent metabolic stability. The CYP enzyme assays were done using standard conditions as previously described (Denton et al., 2004). Compared to nalmefene, compound **5** possessed less inhibitory potency against the CYPs studied (i.e., CYP-3A4, -2B6, -2C9, -2C19 and -2D6). A possible exception was CYP2C19 that appeared to be more sensitive than nalmefene to inhibition by **5**. Because no significant inhibition of CYP was observed and based on the low plasma concentration of **5** observed (i.e., 2-8 ng/ml, Table 1) it is unlikely compound **5** inhibits CYP and alcohol metabolism in vivo at the doses used in this study. This is based on the well-recognized relationship (i.e., I/K_i) that predicts the potential for in vivo interaction (Wienkers and Heath, 2005). If I/K_i is greater than 1 then a significant interaction is predicted. In the case herein, the I/K_i ratio is = 0.0003 assuming a K_i of 10 μM. Therefore, no significant interaction is predicted. At the concentrations that are effective at decreasing alcohol self administration (i.e., 5-10 μg/kg), there is virtually no effect of **5** on CYP-mediated alcohol metabolism. Accordingly, compound **5** was advanced to pharmacokinetic studies.

***In Vivo* Studies with Compound 5.**

The pharmacokinetics (PK) of compound **5** was examined in male Sprague Dawley rats by the i.v. (i.e., 2 doses, 20 and 50 µg/kg) and oral (one dose, 200 µg/kg) routes of administration. The doses were chosen to mimic the situation in efficacy studies and still be above the lowest limit of detection (20 pg/ml in plasma) by LCMS-MS. Serum was extracted and analytes were determined by LCMS-MS. The data of Table 1 shows the PK parameters for compound **5**. The preliminary PK studies of the *para*-bromophenyl analog of **5** (i.e., compound **3**, Scheme 1) have been previously reported (Ghirmai et al., 2009) and are in general agreement with the results described below for **5**.

The hydrochloride salt of **5** was administered to two groups of three rats via the oral (200 µg/kg) or i.v. (20 µg/kg) routes of administration. After oral administration of **5**, the T_{max} was 120 min and the apparent $T_{1/2}$ was 3.4 h. After i.v. administration of **5**, the T_{max} was 5 min and the $T_{1/2}$ was 114 min. A summary of the pharmacokinetic parameters is listed in Table 1. The bioavailability was calculated at 11%. Previously, reported data showed that the brain tissue:plasma ratio of the closely related *para*-bromophenyl analog **3** (i.e., a ratio of 2.3:1) was adequate to proceed with *in vivo* studies (Ghirmai et al., 2009).

Before extensive efficacy studies were conducted, preliminary toxicology studies were undertaken to help establish the safety of **5**. Range-finding toxicology studies were done in male Sprague Dawley rats. Compound **5** was very well tolerated in rats. Doses as great as 4 mg/kg (oral) of **5** did not show any adverse effects and clinical chemistry analysis of plasma revealed no liver or kidney toxicity. A dose of 4 mg/kg of **5** is a dose that is 200-fold greater than an estimated efficacious dose. Chronic dosing of compound **5** for 7 days at a dose of 2 mg/kg (i.e., a dose that is 100-fold greater than an estimated

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efficacious dose) showed no signs of clinical toxicity on the basis of analysis of plasma clinical chemistry. Compared to rats treated with vehicle alone, 7 day dosing of compound **5** at 2 mg/kg caused no apparent liver or kidney toxicity.

Effect of compound 5 or Naltrexone on an animal model of acute hepatotoxicity

The effect of **5** or naltrexone on the relative hepatotoxicity of co-administered thiobenzamide to rats was determined. As shown in Table 2, thiobenzamide (2 mmole/kg, i.p.) produced significant hepatotoxicity at 48 hours post-administration compared with vehicle (i.e., 17.8-fold and 12.4-fold increase in hepatotoxicity, respectively) on the basis of serum SGPT and SGOT values. Administration of **5** (20 µg/kg, i.p.) 24 h after thiobenzamide (2 mmole/kg in corn oil, i.p.) showed a decrease in SGPT and SGOT values (i.e., almost a 4-fold and 0.4-fold, respectively, decrease in hepatotoxicity compared with thiobenzamide alone). In contrast, administration of naltrexone (500 µg/kg, i.p.) 24 h after thiobenzamide exacerbated the hepatotoxicity of thiobenzamide. Compared to thiobenzamide alone, administration of thiobenzamide and then naltrexone increased SGPT and SGOT levels over 21-fold and 17.8-fold, respectively. Compared to administration of naltrexone, administration of compound **5** twenty-four h after thiobenzamide significantly decreased hepatotoxicity of thiobenzamide (p = 0.0034). The hepatoprotective effect of **5** on thiobenzamide hepatotoxicity was statistically significant compared to the lack of any hepatoprotective effect of naltrexone on thiobenzamide hepatotoxicity (p = 0.0005). The hepatoprotective effect of **5** on thiobenzamide hepatotoxicity as judged by SGOT values was nearly statistically significant compared to the lack of any hepatoprotective effect of naltrexone on thiobenzamide hepatotoxicity (p = 0.055). There was no statistically significant

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difference of treatment by **5** or naltrexone on the toxicity of thiobenzamide on the basis of serum albumin or BUN values.

***In Vivo* Alcohol Self-Administration Studies**

Previously, we showed that **5** possessed potent effects on ethanol (EtOH) intake in non-dependent Wistar rats trained to self-administer a 10 % (w/v) ethanol solution, utilizing operant techniques (Ghirmai et al., 2009). As a positive control, nalmefene hydrochloride was also examined. Previous studies showed that **5,** naltrexone and nalmefene inhibited alcohol self-administration with ED_{50} values of 0.019, 0.5 and 0.040 mg/kg, respectively, in the Wistar rat model. Because compound **5** showed considerable potency at inhibition of alcohol self-administration it was studied further in alcohol-preferring rats (i.e., P-rats). We based the dose selection of **5** in P-rats on the outcome of the testing of compound **5** in non-dependent normal Wistar rats.

Results showed that P-rats voluntarily and orally self-administered amounts of alcohol to produce blood alcohol levels on average of 0.071 g % following 30-minute self-administration sessions. The average sweetened alcohol solution intake in P-rat vehicle controls during drug testing was 9.0 ml (1.5 g/kg) in the absence of food or water deprivation. Compound **5** was administered s.c. in a Latin Square Design dose range study and showed significant efficacy. A detailed study employing **5** from 0.003125 to 0.0125 mg/kg showed that the compound was efficacious at inhibiting sweetened alcohol self-administration in non-dependent (air exposed) and EtOH-dependent (ethanol vapor exposed) P-rats (Figure 1). Compound **5** pretreatment dose-dependently decreased intake of sweetened alcohol solution by P-rats (Figure 1). Analysis revealed that **5** at 0.00312, 0.00625 and 0.0125 mg/kg doses significantly suppressed alcohol intake in alcohol-

dependent P-rats ($p < 0.05$). Analysis revealed that **5** at 0.00625 and 0.0125 mg/kg doses significantly suppressed alcohol intake in alcohol-nondependent P-rats ($p < 0.05$) (Figure 1).

To test whether the effect of compound **5** was selective for sweetened ethanol, the effect of **5** on self-administration of water (Figure 2) was examined. Treatment with compound **5** did not have an overall effect on the self-administration of water compared with vehicle. In control alcohol-dependent P-rats that consumed water, analysis did not reveal any significant effect of compound **5** dose on water intake (Figure 2). In control alcohol-nondependent P-rats that consumed water, analysis did not reveal any significant effect of compound **5** dose on water intake except at the 0.0125 mg/kg dose (Figure 2). Data represented mean responses for EtOH following compound **5** (0.0-0.0125 mg/kg) administration in non-dependent controls (air exposed, n=8) and ethanol-dependent (EtOH vapor exposed, n=10) P-rats after 6 h withdrawal. Compound **5** produced decreases in EtOH self-administration at 0.00625 and 0.0125 mg/kg compared to air (white bars) and EtOH vapor-exposed ((black bars) vehicle controls; $*p < 0.05$) (Figure 1). The ED_{50} for compound **5** in EtOH-dependent (black bars) P-rats was estimated to be 0.0044 mg/kg, and in non-dependent rats (white bars) was estimated to be 0.05 mg/kg, using linear regression methods.

To further examine the effect of **5** on alcohol self-administration, compound **5** was examined on alcohol self-administration in Binge-like P-rats. The term Binge-like P-rats was used because the animals did not quite achieve BALs that are normally associated with Binge drinking P-rats (i.e., Binge-like P-rats attained 1.2-1.4 g/kg EtOH in a 30 min session

whereas Binge-P-rats generally achieve 1.5 g/kg EtOH in a 30 min session). Compound **5** was administered s.c. in a Latin Square Design dose range study and showed significant efficacy. Doses of **5** from 0.00312 to 0.0125 mg/kg showed that compound **5** inhibited Supersac-sweetened alcohol self-administration in Binge-like P-rats (Figure 3). Compared with vehicle, analysis showed that at all doses of **5** examined, compound **5** significantly suppressed Binge-like alcohol intake in P-rats ($p < 0.05$). The ED_{50} was estimated to be 0.008 mg/kg in Binge-like P-rats (Figure 3). To test whether the effect of compound **5** was selective for Supersac-sweetened ethanol, the effect of **5** on self-administration of Supersac (Figure 4) was examined. In control animals that only consumed Supersac, analysis did not reveal any significant effect of **5** for the doses examined on Supersac intake (Figure 4).

Next, the effect of **5** on alcohol self-administration in Binge-like Wistar rats was examined. Compound **5** was administered s.c. in a Latin Square Design dose range study and showed significant efficacy. Doses of **5** from 0.00312 to 0.0125 mg/kg showed that compound **5** inhibited Supersac-sweetened alcohol self-administration in Binge-like Wistar rats (Figure 5). Compared with vehicle, analysis showed that 0.00625 and 0.0125 mg/kg of **5**, compound **5** significantly suppressed Binge-like alcohol intake in Wistar rats ($p < 0.05$). The ED_{50} was estimated to be 0.012 mg/kg in Binge-like Wistar rats (Figure 5). To test whether the effect of compound **5** was selective for Supersac-sweetened ethanol, the effect of **5** on self-administration of Supersac was examined (Figure 6). In control animals that only consumed Supersac, analysis did not reveal any significant effect of **5** for the doses examined on Supersac intake except 0.0125 mg/kg (Figure 6).

Discussion

Replacement of the C-6 ketone group of naltrexone with an aryl amide substituent as in **5** afforded a compound that inhibited the self-administration of alcohol in P-rats and in Binge-like P rats. Compound **5** is a reversible, relatively short-acting kappa antagonist. Compared to nor-BNI, **5** is much more drug-like and much shorter-acting. Compound **5** is lipophilic (i.e., log P = 3.73) and based on its pharmacokinetics rapidly leaves the bloodstream and gets into the brain. Because **5** does not possess a propensity for auto-oxidation that nor-BNI shows, its residence time and duration of action in the brain is also considerably shorter. Consequently, the effect of **5** on opioid receptors (i.e., binding, receptor desensitization etc.) must be fundamentally different than for nor-BNI and other long-acting kappa antagonists. Animals treated with **5** showed no residual effects after 24 hours and appeared to be normal from a morphological and behavioral standpoint. Administration of a dose of **5** to rats 500-fold greater than a dose required for an ED_{50} dose for inhibition of alcohol self-administration did not show any detectable hepatotoxicity or renal toxicity or other toxicity. Chronic dosing of **5** in rats at 2 mg/kg for 7 days did not cause any detectable hepatotoxicity or other untoward clinical chemical abnormalities on the basis of analysis of plasma clinical chemical parameters taken at 7 days. The conclusion is that compound **5** is a relatively fast-acting opioid that is safe and relatively well-tolerated in small animals.

Compared to naltrexone (ED_{50} 500 μg/kg) or nalmefene (ED_{50} 40 μg/kg), compound **5** (ED_{50} 19 μg/kg) is a more potent inhibitor of alcohol self-administration in non-dependent normal Wistar rats (Ghirmai et al., 2009). Using P-rat and Binge-like P-rat animals herein we showed that **5** was even more efficacious at inhibiting alcohol self-administration (i.e., ED_{50} 4-5 μg/kg and ED_{50} 8 μg/kg, respectively). These data show

that under a variety of experimental conditions compound **5** is an effective antagonist of responding maintained by large amounts of alcohol. We attribute this increase in efficacy as due to potent κ opioid antagonism compared with naltrexone or nalmefene. As described above, it is also likely due to improved pharmaceutical properties of the compound and decreased interaction with the prominent CYP drug metabolizing system. It may be that attenuation of the inhibitory potency of **5** toward CYP (Ghirmai et al., 2009) contributes to the safety of **5**. Compared to naltrexone, **5** showed decreased interaction with CYP and this may in part explain some of the metabolic stability observed for **5** and related compounds (Ghirmai et al., 2009; MacDougall et al., 2004) as well as some of the hepatoprotective properties. Substitution of an aryl amide moiety at the C-6 position of ß-naltrexamine may also explain some of the hepatoprotective effects of **5**. For example, at a dose of naltrexone that represents the ED_{50} for inhibition of alcohol self-administration (i.e., ED_{50} 500 µg/kg), naltrexone exacerbates the hepatotoxicity of thiobenzamide in a rat model of hepatotoxicity. In contrast, at a dose of **5** that represents its ED_{50} (i.e., ED_{50} 20 µg/kg), compound **5** protects against the hepatotoxicity of thiobenzamide in rats challenged with thiobenzamide, a potent hepatotoxin. Exacerbation of the hepatotoxicity of thiobenzamide by naltrexone is of considerable concern because, generally, the liver of individuals that abuse alcohol is severely compromised. It may be that decreasing the affinity of opioid derivatives for metabolic enzymes and increasing the metabolic stability results in compounds with less potential for increasing hepatotoxicity.

In a previous study (Ghirmai et al., 2009) we showed that **5** decreased alcohol self-administration in normal Wistar rats. We proposed the mechanism of action of **5** was

working at least in part as a kappa opioid receptor antagonist. In good agreement with those results, we show herein that **5** is very effective to decrease alcohol self-administration in a Binge-like P-rat model as well as a Binge-like Wistar rat model. Moreover, the effect of **5** to decrease alcohol self-administration was selective because at efficacious doses, compound **5** did not have an effect on water or Supersac consumption. This is important because some opioid receptor antagonists decrease both ethanol and sucrose intake in rats (Pastor et al., 2006) or inhibit energy-rich food consumption (Reid et al., 1985). It may be that opioid receptor antagonists prevent central reward mechanisms that may share common neural substrates responsible for the development of alcohol dependence (Yeomans et al., 2002).

On the basis of previously published opioid receptor binding data, it appears that **5** works as an partial agonist at the μ receptor and an antagonist at the δ and κ opioid receptors. But the potency against the κ opioid receptor is much greater than that of the δ opioid receptor and at the concentration of **5** that is efficacious in vivo at inhibiting alcohol self-administration, we conclude κ is the pharmacologically prominent receptor. The finding from *in vivo* studies that **5** potently inhibits alcohol self-administration in P-rats and Binge-like Wistar rats supports the idea that antagonism of κ opioid receptors might be of utility for full alcohol cessation functional activity. However, compared to naltrexone, the *in vivo* efficacy of **5** may not only be dependent on interaction with the κ opioid receptor but also partial agonism of the μ opioid receptor. Presumably, the profile of opioid receptor binding coupled with the drug-like properties of **5** contributes to the optimal functional activity as an alcohol self-administration inhibition agent *in vivo*. This is in agreement with recent studies that show that an opioid with strong κ opioid receptor

antagonism albeit possessing some opioid agonism (i.e., nalmefene) (Bart et al., 2005) was more effective at inhibition of alcohol-self administration than an opioid with broad opioid receptor antagonism (i.e., naltrexone) (Walker and Koob, 2008). Consequently, compounds such as **5** and related agents may represent an exciting lead for developing the next generation of opioid compounds useful in the treatment of alcohol abuse.

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Authorship Contribution

Participated in research design: Cashman, Azar

Conducted experiments: Cashman, Azar

Contributed new reagents or analytical tools: Cashman

Performed data analysis: Cashman, Azar

Wrote or contributed to the writing of the manuscript: Cashman, Azar

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Footnote

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Legends for Figures and Scheme

Scheme 1. Chemical structures of compounds **1-5**.

Figure 1. Operant lever presses for ethanol by alcohol-dependent (black bars) and alcohol-nondependent (white bars) P-rats following injection of compound **5** doses (0, 0.00312, 0.00625, 0.0125 mg/kg). Operant tests occurred 6 hours following termination of vapor exposure (i.e., 6 hours withdrawal). *p < 0.05 significant difference from vehicle condition in alcohol-dependent or alcohol-nondependent control P-rats.

Figure 2. Operant lever presses for water by alcohol-dependent (black bars) and alcohol-nondependent (white bars) P-rats following injection of compound **5** (0, 0.00312, 0.00625, 0.0125 mg/kg). Operant tests occurred 6 hours following termination of vapor exposure (i.e., 6 hours withdrawal). *p < 0.05 significant difference from vehicle condition in alcohol-dependent or alcohol-nondependent control P-rats.

Figure 3. Mean ± SEM intake (g/kg) of Supersac sweetened (3% glucose + 0.125% saccharin) 10% (w/v) alcohol solution by P-rats in the alcohol binge-like group (n=12) following pretreatment with one of four doses of compound **5** (0, 0.00312, 0.00625, 0.0125 mg/kg). * p < 0.05, significant difference from vehicle condition.

Figure 4. Mean ± SEM Supersac intake (ml/kg) by Supersac controls P-rats (n=12) in the following pretreatment with one of four doses of compound **5** (0, 0.00312, 0.00625,

0.0125 mg/kg). Data revealed no non-specific effects of on fluid intake following pretreatment with compound **5.**

Figure 5. Mean ± SEM intake (g/kg) of Supersac sweetened (3% glucose + 0.125% saccharin) 10% (w/v) alcohol solution by Wistar rats in the alcohol binge-like group (n=12) following pretreatment with one of four doses of compound **5** (0, 0.00312, 0.00625, 0.0125 mg/kg). * $p < 0.05$, significant difference from vehicle condition.

Figure 6. Mean ± SEM Supersac (3% glucose + 0.125% saccharin) intake (ml/kg) by Supersac control Wistar rats (n=12) following pretreatment with one of four doses of compound **5** (0, 0.00312, 0.00625, 0.0125 mg/kg). *$p < 0.05$, significant difference from vehicle condition.

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Table 1. Pharmacokinetic parameters for lead compound **5**.

Route	Dose (µg/kg)	Cmax (pg/ml)	Tmax (hr)	AUC (pg x hr/ml)	CL/F (L/hr/kg)	T1/2 (hr)
iv	20	2230	0.08	1704	11.73	1.9
iv	50	7790	0.08	3559	14.05	1.5
Oral	200	89	2	578	346	3.4

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Table 2. Effect of Kappa Antagonism on the Hepatotoxicity of Thiobenzamide

Condition	Alkaline Phosphatase[1]	SGPT (ALT)	SGOT (AST)	Albumin	BUN
Control	227.3 ± 13.8	44.7 ± 8.7	82.3 ± 27.6	2.9 ± 0.1	23.3 ± 3.2
Thiobenzamide alone	150.5 ± 55.6*	798 ± 447.1*	1021 ± 775.8*	2.6 ± 0.3	66.2 ± 34.9*
Thiobenzamide + Compd **5**	122.5 ± 18.8	613.7 ± 349.2^{+}	993 ± 172.2	2.8 ± 0.4	43.2 ± 7.4
Thiobenzamide + Naltrexone	169 ± 84.5	1749.8 ± 245.1$^{\#}$	1461.8 ± 312.3	2.5 ± 0.2	57.8 ± 23.9

[1] Mean ± the standard deviation of values from six animals.

* Control vs. Thiobenzamide (274 mg/kg) Alone. The level of significance chosen was P-values < 0.05

+ Thiobenzamide (274 mg/kg) Alone vs. Thiobenzamide + Naltrexone (500 µg/kg). The level of significance chosen was P-values < 0.05

Thiobenzamide (274 mg/kg) + Compound **5** (20 µg/kg) vs. Thiobenzamide (274 mg/kg) + Naltrexone (500 µg/kg). The level of significance chosen was P-values < 0.05.

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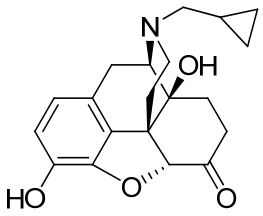
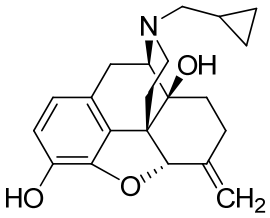
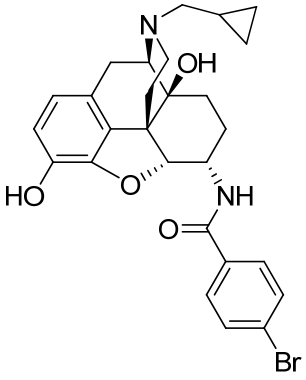
Scheme 1



1
Naltrexone



2
Nalmefene



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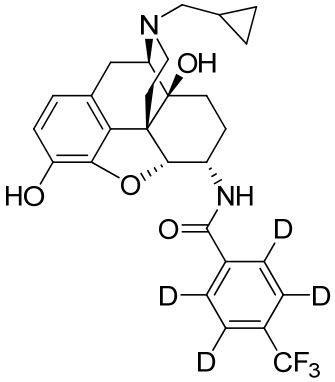

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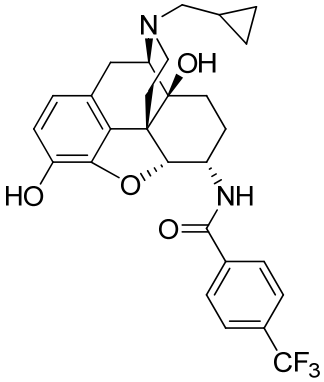

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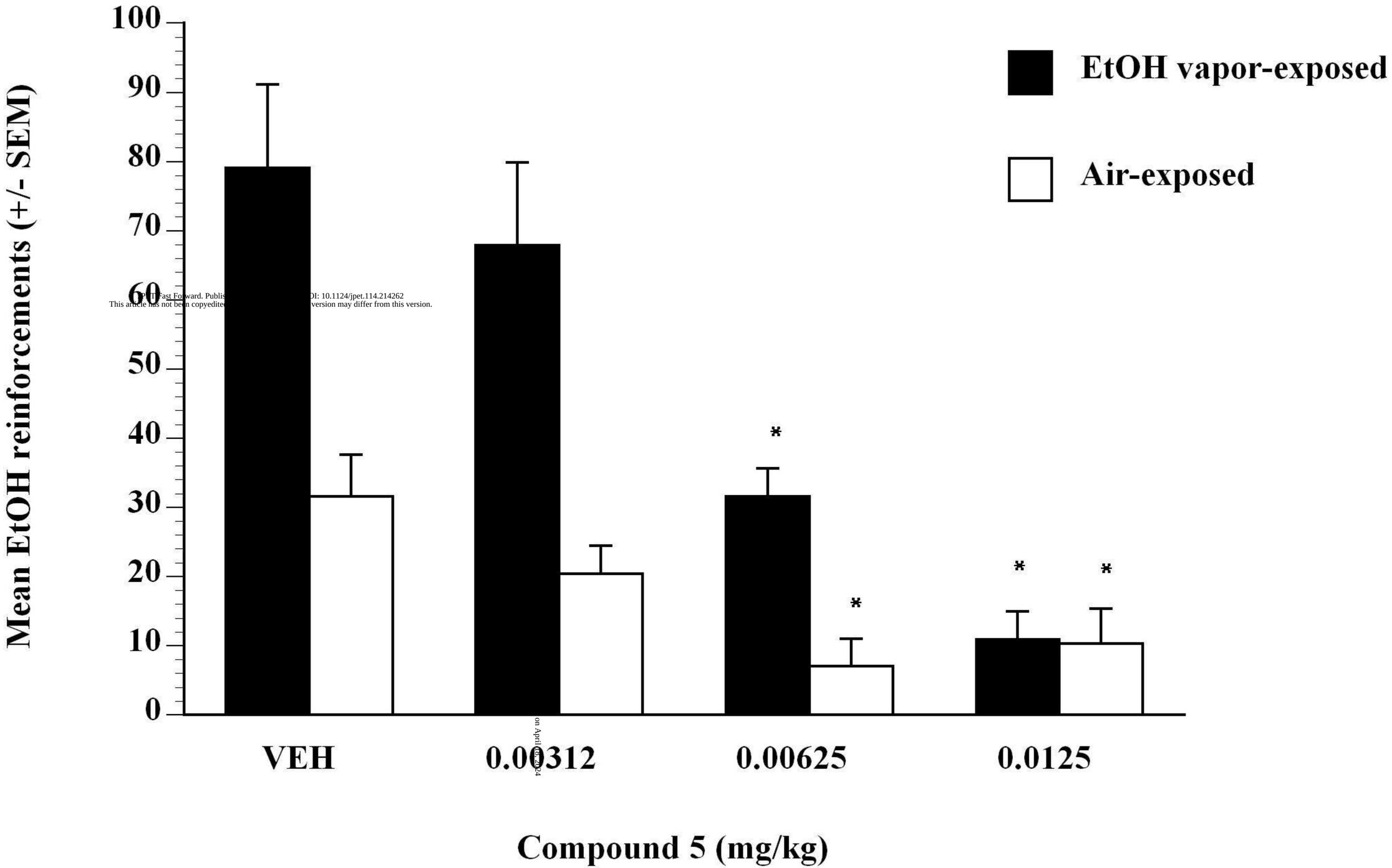

Figure 1

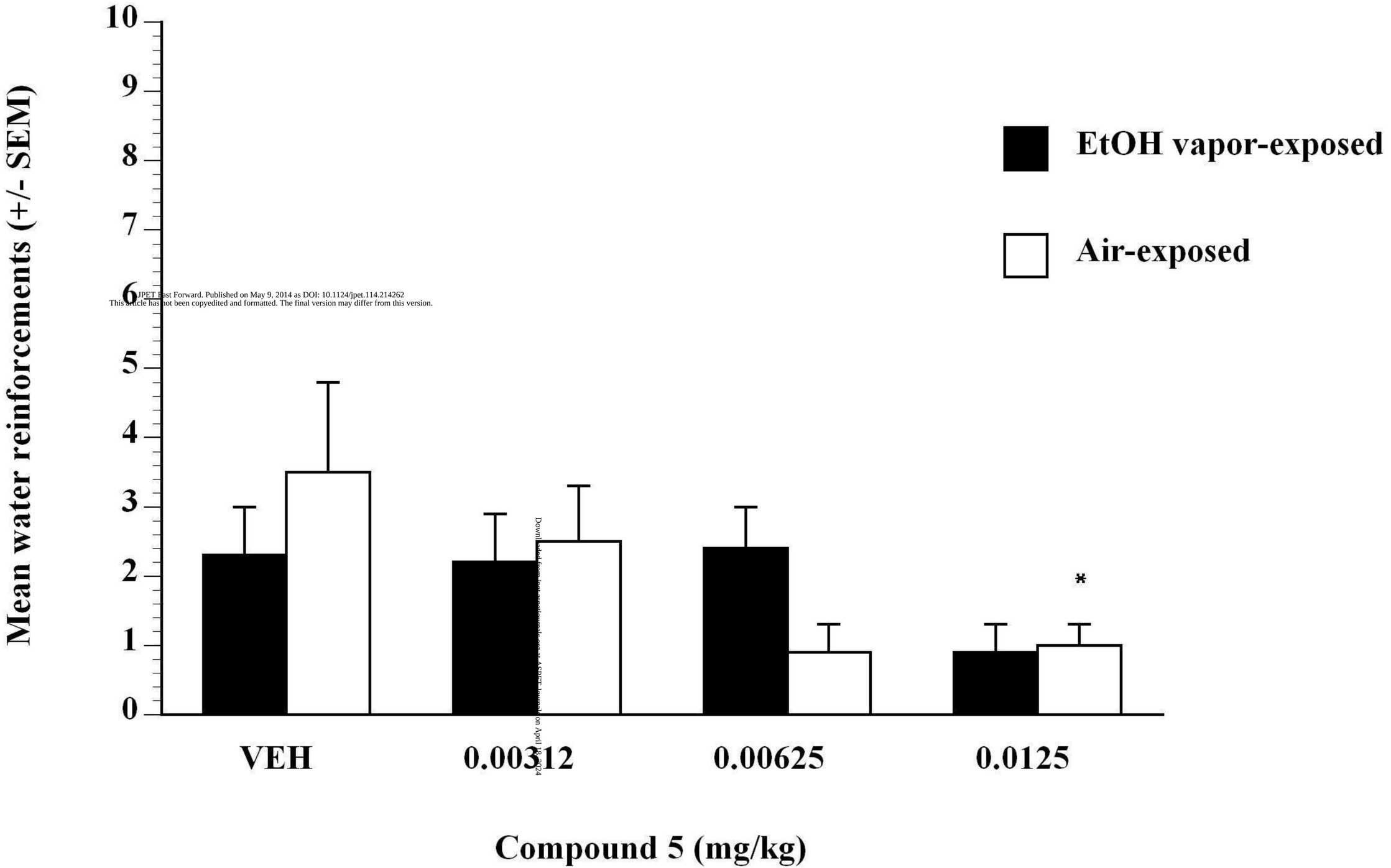

Figure 2

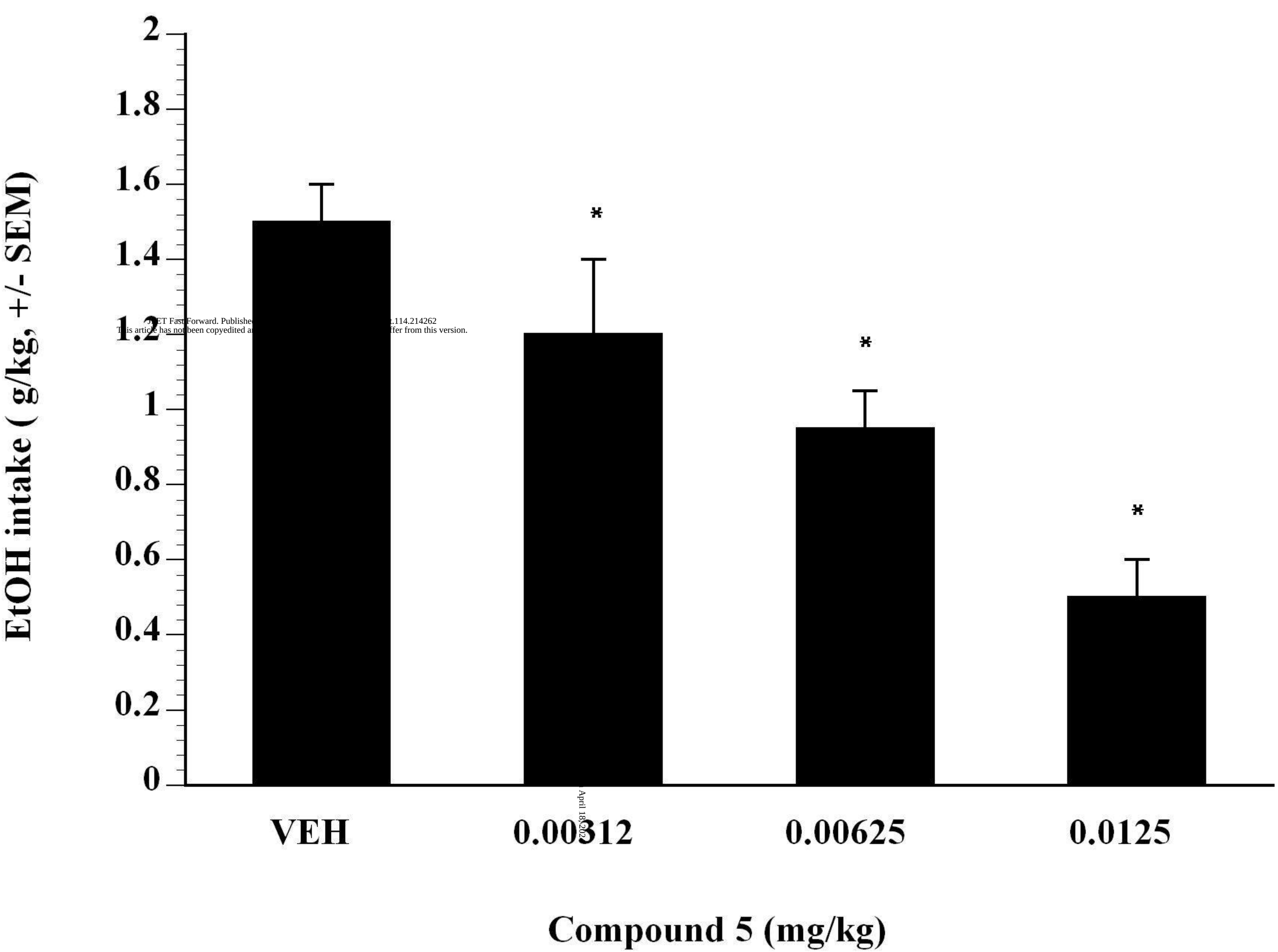

Figure 3

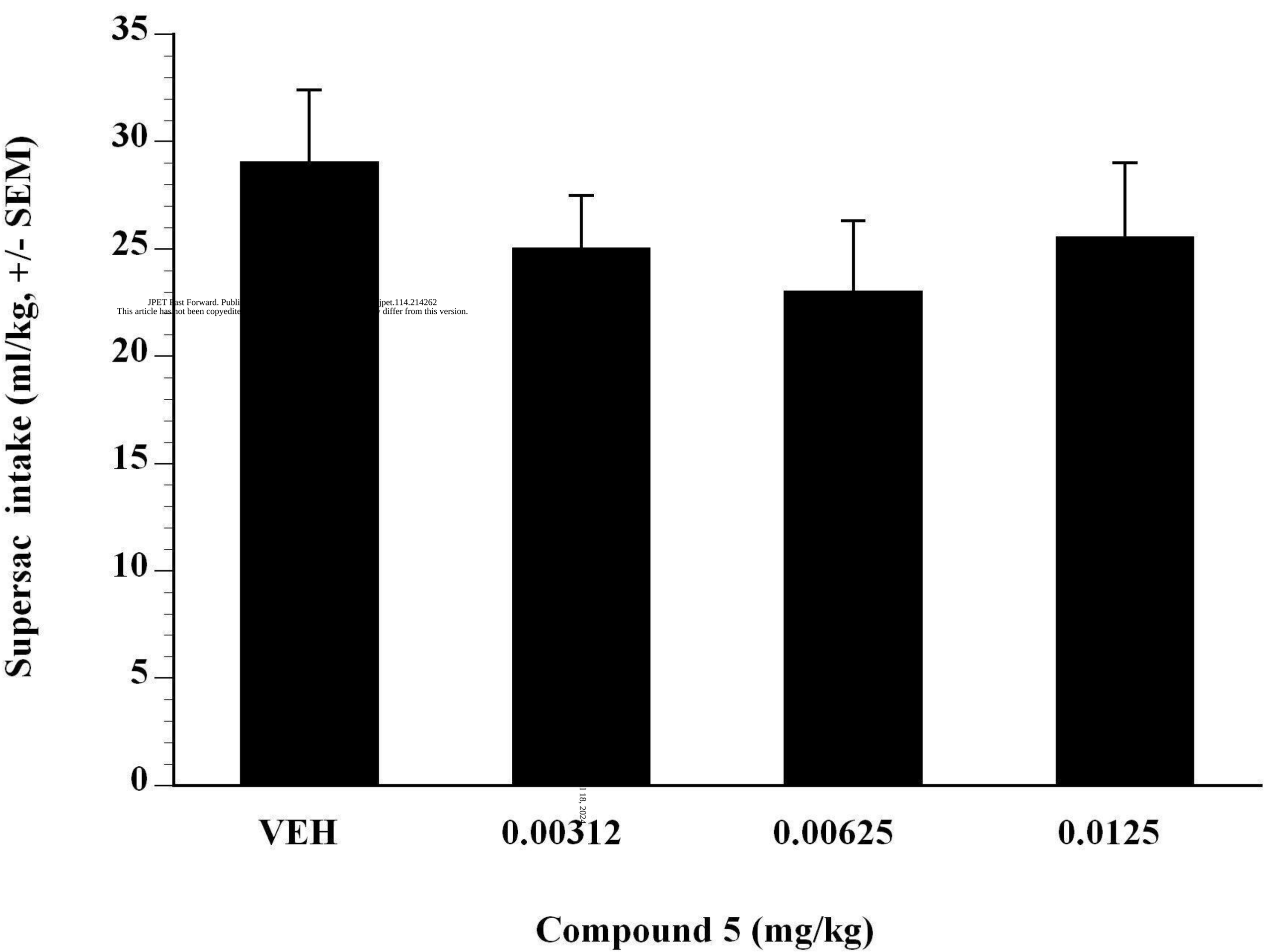

Figure 4

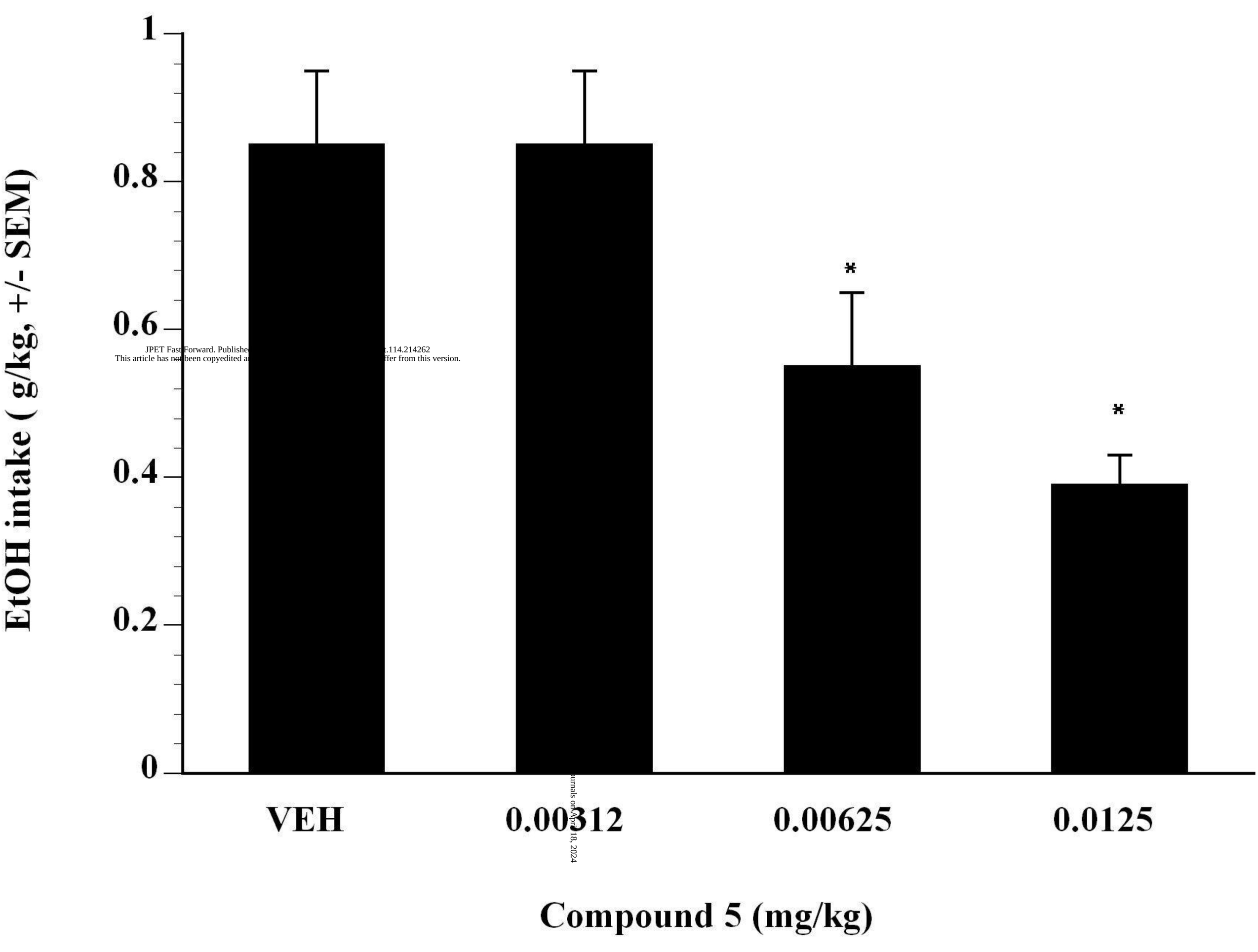

Figure 5

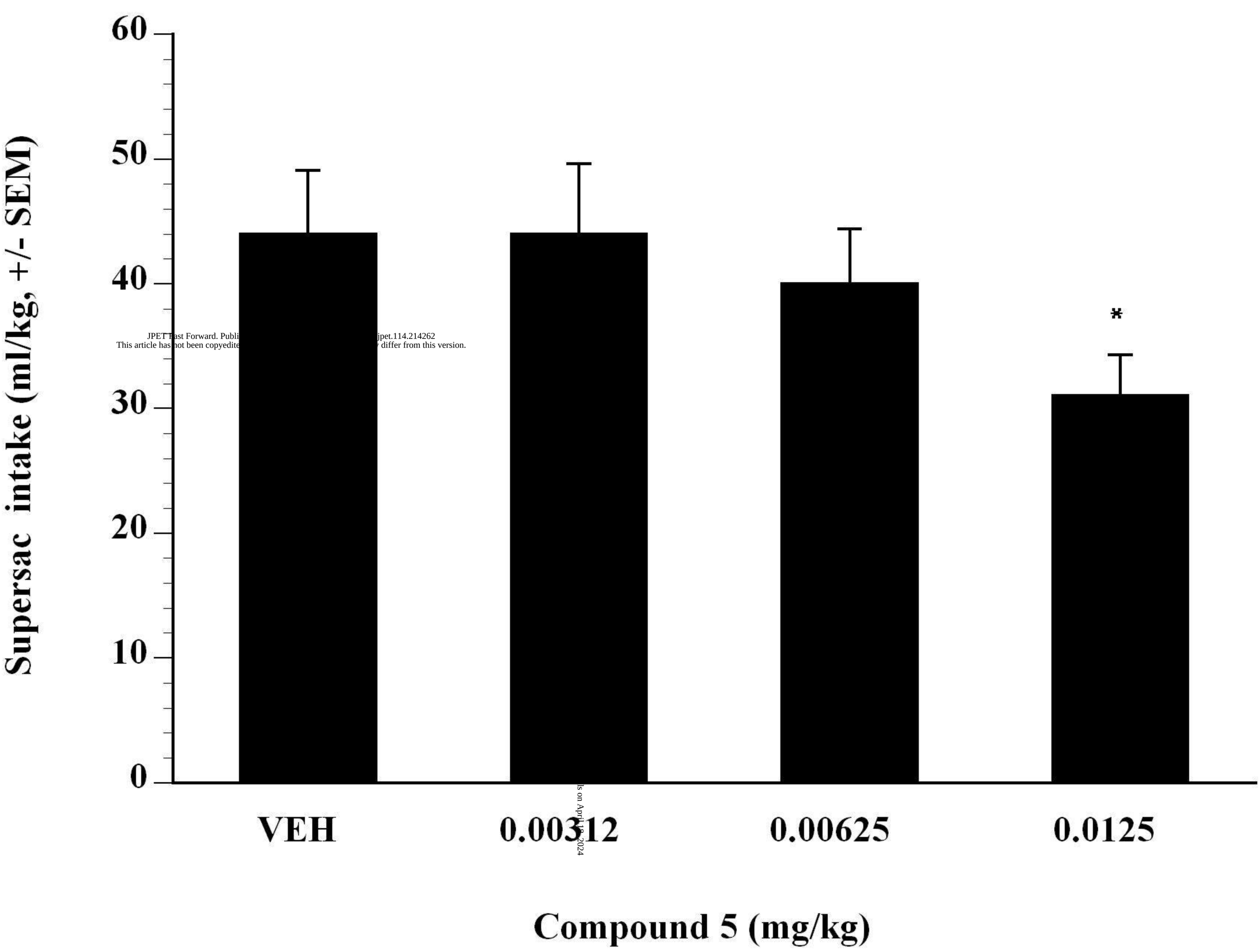

Figure 6