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Benzofuran Derivatives Substitute for the Discriminative Stimulus Effects of 3,4Methylenedioxymethamphetamine (MDMA) in Male Sprague-Dawley Rats

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BENZOFURAN DERIVATIVES SUBSTITUTE FOR THE DISCRIMINATIVE STIMULUS EFFECTS OF 3,4-METHYLENEDIOXYMETHAMPHETAMINE (MDMA) IN MALE SPRAGUE-DAWLEY RATS

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3, 4-Methylenedioxymethamphetamine (MDMA) is currently under evaluation in phase III clinical trials as medication-assisted therapy for post-traumatic stress disorder (PTSD) and is expected to be approved by the FDA for clinical use in the near future. MDMA is also a popular abused substance with risks for cardiovascular toxicity and neurotoxicity, particularly when misused at higher doses. Characterization of the behavioral and neurochemical effects of novel psychoactive substances is an essential step in the development of safer alternative therapeutic agents. Drug discrimination is a preclinical behavioral assay with pharmacological specificity for characterizing *in vivo* drug actions in the central nervous system. This study implemented rodent drug discrimination to characterize the enantiomers of 5-(2-methylaminopropyl) benzofuran (5MAPB), 5-(2- methylaminobutyl) benzofuran (5-MBPB), and 6-(2- methylaminobutyl) benzofuran, benzofuran derivatives with potential MDMA-like effects. Eight male Sprague Dawley rats were trained in a standard two-lever operant drug discrimination procedure under a fixed ratio 20 schedule of food reinforcement to discriminate MDMA (1.5 mg/kg) from saline. Once criteria for stimulus control were established, stimulus substitution tests were conducted with (RS) 5-MAPB, (R)-5-MAPB, (S)-5-MAPB, (R)-5-MBPB, (S)-5-MBPB, (R)-6-MBPB, and (S)-6-MBPB. All substances produced dose-dependent increases in MDMA-lever responding and full substitution at the highest dose assessed with minimal effects on response rate. The S- and R- enantiomers differed slightly in potency. These findings, considered together with *in vitro* neurochemical assays, indicate the benzofuran scaffold is a viable candidate for development of medications with MDMA-like therapeutic effects and reduced toxicities.

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by

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INTRODUCTION

3,4-methylenedioxymethamphetamine (MDMA), commonly known as “Molly” or “Ecstasy” among recreational users, is a phenethylamine derivative with structural similarities to the psychostimulant, methamphetamine and the hallucinogen, mescaline (Luethi & Liechti, 2018). The S (+) isomer of MDMA produces psychostimulant and empathogenic effects and the R (-) isomer exerts hallucinogenic effects (Kalant, 2001; Luethi & Liechti, 2018). MDMA inhibits reuptake and stimulates the release of serotonin (5-HT), dopamine (DA), and norepinephrine (NE). These monoamine neurotransmitters have been implicated in mood disorders and anxiety, and the therapeutic potential of MDMA has been a topic of interest for several decades (Parrott, 2007; Pentney, 2001).

Recently, MDMA-assisted psychotherapy has received considerable attention, and phase III clinical trials are ongoing for the treatment of post-traumatic stress disorder (PTSD). Individuals with PTSD have intrusive memories from a traumatic event, which invokes physical and emotional reactions, flashbacks, and negative thinking (Boeckel et al., 2017). The empathogenic and pro-social effects of MDMA reduce anxiety and facilitate conversation, which helps patients describe traumatic experiences (Carhart-Harris et al., 2014; Boeckel et al., 2017). In phase II clinical trials, symptom alleviation was assessed through the clinician administered PTSD scale (CAPS-IV). Results from two different studies indicate that PTSD symptom severity significantly decreased following MDMA assisted-psychotherapy (Jerome et al., 2020; Mithoefer et al., 2018).

Though preliminary results from clinical trials with MDMA are promising, there remain causes for concern. Recreational MDMA use has a well-documented history of neurotoxicity and cardiovascular issues (Mitchell et al., 2021; Simmler & Liechti, 2018). Both acute and chronic

MDMA use has negative consequences. Prolonged MDMA use results in depleted 5-HT receptor density, widespread 5-HT depletion in the cerebral cortex, and potential serotonergic neurotoxicity (Parrott, 2013). Neuroimaging studies in humans have also shown decreases in 5HT density in heavy MDMA users (Aguilar et al., 2020; Kish et al., 2000; Reneman et al., 2006).

Similarly, single, low doses of MDMA can produce serotonergic deficits as well (Mueller et al., 2013). Fluctuations in serotonergic functioning impacts cell activity, sleep, mood, and memory (Parrott, 2013). MDMA use can also lead to cardiovascular toxicity due to overstimulation of noradrenergic receptors (Simmler & Liechti, 2018). These harmful side effects of MDMA highlight the need for a similar but safer therapeutic alternative. Novel psychoactive substances (NPS) such as benzofurans are structurally similar to MDMA and can be explored as a medicinal option. Additionally, subjective reports detail similar hallucinogen and empathogenic effects following benzofuran use (Greene, 2013).

To further examine structure activity relationships between benzofuran derivatives and MDMA, this study employed the drug discrimination procedure. Drug discrimination is a well regarded *in vivo* preclinical behavioral assay that elucidates the neurochemical actions of novel and well known compounds. This paradigm has predictive utility and is often used to schedule the risk and abuse potential of drugs (Gauvin et al., 2018; Glennon & Young, 2011; Horton et al., 2013). The U.S. Food & Drug Administration has used drug discrimination studies to inform scheduling of drugs such as Lisdexamfetamine (Vyvanse) and Briveracetam (Briviact) (Gauvin et al., 2018). Through several drug discrimination experiments, Schechter (1987;1988) demonstrated that the interoceptive stimulus effects of MDMA are mediated through serotonergic and dopaminergic pathways. Using rats trained to discriminate MDMA, Harvey &

Baker (2016) determined that 5-HT release was a crucial aspect of mephedrone's stimulus effects. Moreover, Simmler & Lietchi (2018) concluded that MDMA-like NPSs have a low abuse liability based on their high selectivity for serotonin receptor transporters (SERT) and norepinephrine receptor transporters (NET) instead of dopamine receptor transporters (DAT). Taken together, drug discrimination is a trusted and useful paradigm to determine the mechanism of action and salient features of novel MDMA-like substances (Berquist et al., 2020; Schetcher, 1988).

Previous studies have assessed the pharmacology of (RS)-5-MAPB and its metabolite (5-APB) (Dolan et al., 2017; Fuwa et al., 2016; Shimshoni et al., 2017; Simmler & Lietchi, 2018). An *in vivo* microdialysis study in mouse striatum demonstrated that 5-MAPB increased extracellular concentrations of NE, 5-HT, and DA to a significantly greater extent in comparison to MDMA (Fuwa et al., 2016). Additionally, 5-MAPB is an agonist at 5-HT_{1A}, 5-HT_{2A} and $5HT_{2C}$ receptor sites (Rickli et al., 2015; Shimshoni et al., 2017; Oeri et al., 2021). To date, few published studies have evaluated benzofurans in the drug discrimination paradigm. Dolan et al. (2017) demonstrated that 4-APB and 6-APDB substituted fully in rats trained to discriminate MDMA. Whereas pharmacological specificity is a key strength of nonhuman drug discrimination, the aim of the present study is to characterize the (S)- and (R)- enantiomers of of three benzofuran derivatives, 5-MAPB, 6-MBPB, and 5-MBPB, for substitution in rats trained to discriminate MDMA. Based on the aforementioned findings reported by Dolan et al. (2017), it was predicted that these substances would fully substitute for MDMA.

METHODS

Subjects: Eight adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were individually housed in polycarbonate cages with corncob bedding in animal facilities

maintained at a constant temperature of (20 ± 2 °C) and humidity (50 ± 5%) and under a 12:12 light/dark cycle (lights on from 07:00 to 19:00 h). Animals were provided water *ad libitum* in home cages and fed restricted diets of commercial rodent chow (Purina®, Richmond, IN, USA) to maintain body weights at approximately 90% of free-feeding weights (380-460 g). All procedures were reviewed and approved by the Western Michigan University Institutional Animal Care and Use Committee and were in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals (National Research Council of the National Academies 2011).

Apparatus: All tests were conducted in eight three-lever, sound-attenuated operant conditioning chambers (ENV-001, Med Associates Inc., St. Albans, Vermont, USA), and controlled using Med-PC software (version IV, Med Associates Inc.). Food reinforcers consisted of 45 mg Dustless Precision Pellets® (Product# F0021, Bio-Serv Inc., Flemington, New Jersey, USA) dispensed from a pellet dispenser directly above the center lever.

Drugs: 3,4-Methylenedioxymethamphetamine (MDMA) was provided by the National Institute on Drug Abuse Drug Control Supply Program (Bethesda, MD) 5-(2-methylaminopropyl) benzofuran, 5-(2-methylaminobutyl) benzofuran, and 6-(2-methylaminobutyl) benzofuran were supplied by Tactogen (Menlo Park CA). All drugs were dissolved in 0.9% saline and injected intraperitoneally at a volume of 1 ml/kg. Doses were calculated based on the weight of the solid compound.

Preliminary Training: Subjects initially completed one 60-minute session to acclimate them to the operant chambers and food pellet delivery. During this session, no levers were present and pellets were dispensed on a 60-second fixed time interval. Following this session, rats received pellets in their home cage to reduce the novelty of the reinforcer. Subsequent training sessions

lasted 20 minutes and were conducted once per day 5-6 times a week. During preliminary lever press training, the center lever was extended, and reinforcement was delivered on a fixed ratio (FR) 1 schedule that was gradually incremented based on subject performance to an FR 20 schedule.

Errorless Training: Once subjects consistently responded on an FR 20 schedule, errorless training commenced. For these sessions, only one lever was extended during the session (i.e, center, left, or right lever). Subjects were given an intraperitoneal saline or 1.5 mg/kg of MDMA injection 15 minutes before each training session. Subjects 1-4 were reinforced for responses on the left lever following MDMA injections and for responses on the right lever after saline injections. Conditions were reversed for subjects 5-8. Responses were reinforced on a fixed ratio schedule that was incremented from FR 1 to FR 20 over 24 (±2) sessions based on subject performance. Errorless training sessions were conducted with each lever in the following order: V, V, D, D, V, D.

Discrimination Training: Drug (D) and vehicle (V) training sessions lasted 20 minutes and followed an alternating schedule of V, V, D, D, V, D. For these sessions, both the left and right levers were extended. Rats were trained until stimulus control was established. Specifically, they were required to complete a minimum of eight out of ten consecutive training sessions with 80% or greater correct lever responses on the first FR and for the remainder of each training session before substitution testing could begin.

Substitution Testing: Substitution tests were conducted with a range of MDMA doses (0.19, 0.38, 0.75. 1.5 mg/kg) and saline. Test sessions were conducted similar to training sessions, with the exception that no reinforcers were delivered upon completion 20 consecutive responses on either lever. Test sessions ended upon completion of an FR 20 or after 20 minutes elapsed,

whichever occurred first. Before subjects were tested for stimulus generalization with each test dose, they were required to meet the criteria for stimulus control on at least one V and one D training session. Both the racemic combination and the (R)- and (S)- enantiomers of 5-(2methylaminopropyl) benzofuran (0.15, 0.32, 0.68, and 1.2 mg/kg. I.P. 30 min) were assessed. Subsequently, (S)- and (R)-5-(2-methylaminobutyl) benzofuran (0.32,0.64,1.28, and 2.56 mg/kg, I.P. 30 min) were tested. Lastly, (S)- and (R)-6-(2-methylaminobutyl) benzofuran (0.32, 0.64,1.28, 2.56 mg/kg, I.P. 30 min) were assessed for substitution. The results of substitution tests were used to plot dose response curves for each compound.

Data Analysis: Sessions to criteria were denoted as the number of training sessions completed to meet stimulus control (criteria: 80% or higher for the minimum of eight out of ten sessions). Percent drug lever was calculated by dividing the number of responses emitted on the MDMA-associated lever by the total number of responses on both levers and multiplying by 100. Response rates were expressed as the number of responses emitted per second throughout the test session. For the dose-response curve, full substitution was considered 80% or greater lever responses on the MDMA associated lever; partial substitution was defined as responses between 20-79% on the MDMA paired lever; no substitution was 20% or less responses on the MDMA associated lever. Nonlinear regressions were performed on the percentage MDMA lever dose response curves to determine ED50s. For each test compound assessed, response rate was analyzed with a repeated measures analysis of variance. Statistical significance was determined at alpha of $p < 0.05$. All statistical and graphical analyses were performed using the GraphPad Prism version 7 software (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

All eight rats met the initial criteria for stimulus control in 34 (± 17.58) training sessions.

After stimulus control was established with MDMA, substitution tests were conducted with a range of MDMA doses to generate a dose response curve for comparison to each test compound. Figure 1 depicts the MDMA dose response curve. As displayed in this figure, MDMA produced a dose-dependent increase in MDMA-lever responses with full substitution following 0.75 mg/kg and 1.5 mg/kg. Response rate was not significantly different among MDMA doses or in comparison to saline control levels [F (4, 28) = 1.03, P=0.41]. The MDMA ED50 was 0.37 (Confidence Intervals (C.I.): 0.27 to 0.49).

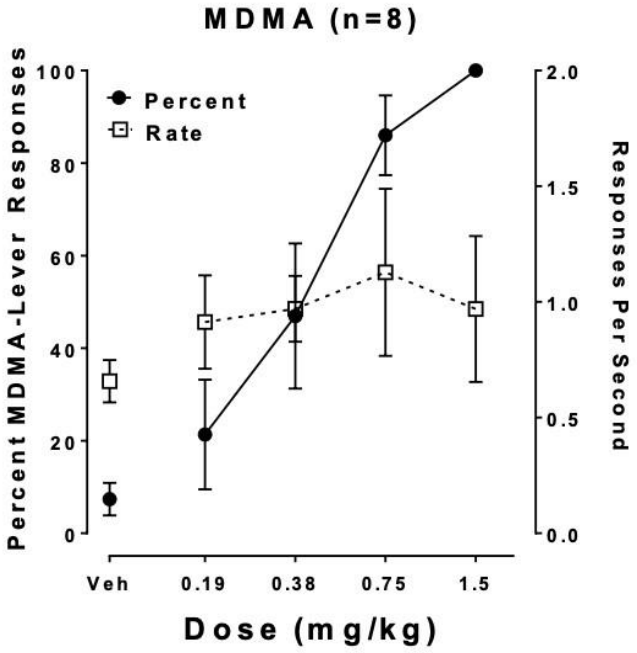

Figure 1. Dose response curve for MDMA. Percent MDMA-Lever Responses (closed symbols) is plotted on the left Y axis and response rate (open symbols) is plotted on the right Y axis. Points represent group means (± S.E.M.).

Figure 2 depicts the dose response curves generated from substitution tests with racemic 5-MAPB and each of its enantiomers. Each of these compounds produced a dose-dependent increase in MDMA-lever responses with full substitution following 0.6 mg/kg and 1.2 mg/kg. Additionally, (R)-5-MAPB was more potent than the other compounds, producing full

substitution at 0.3 mg/kg. The ED50s for (RS)-5-MAPB, (S)-5-MAPB and (R)-5-MAPB are 0.37 (C.I.: 0.27 to 0.49), 0.28 (C.I.: 0.20 to 0.39), and 0.20 (C.I.: 0.234 to 0.57), respectively.

Response rates were similar following treatment with racemic and (R)- 5-MAPB, while (S)-5MAPB produced more variability in response rates following higher doses. Differences in response rates were not statistically significant following (RS)-5-MAPB [F (4, 28) = 2.41, P=0.07] or (R)-5-MAPB [F (4, 28) = 1.38, P=0.26]. However, there was a statistically significant effect of (S)-5-MAPB on response rate [F (4, 28) = 3.04, P=0.03].

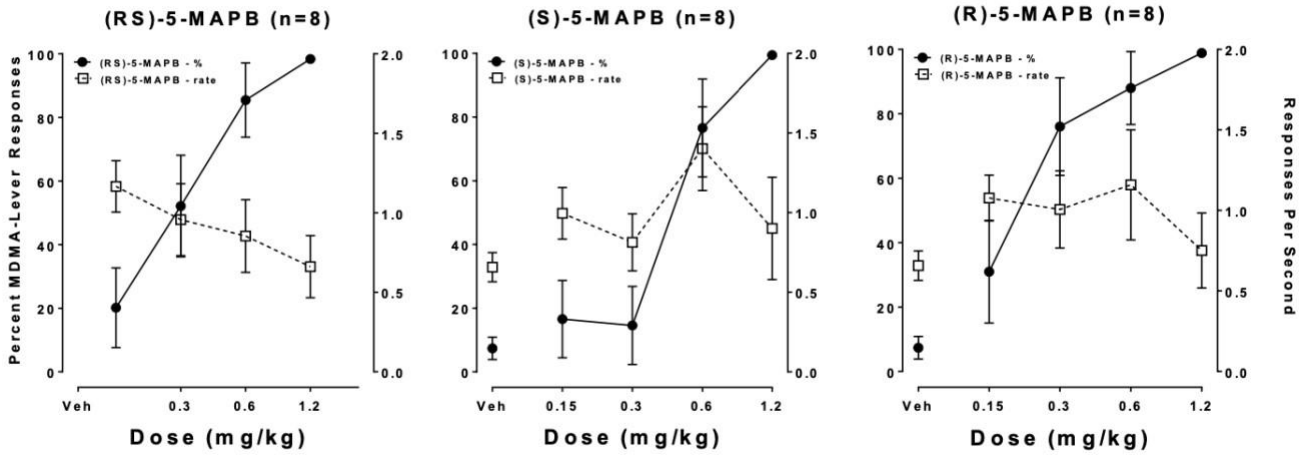

Figure 2. Dose response curves for (RS)-5-MAPB, (S)-5-MAPB, and (R)-5-MAPB. Percent MDMA-Lever Responses (closed symbols) is plotted on the left Y axis and response rate (open symbols) is plotted on the right Y axis. Points represent group means (± S.E.M.).

The dose response curves generated from substitution tests with the enantiomers of 5MBPB are displayed in figure 3. (S)-5-MBPB produced a dose-dependent increase in MDMA lever responses with full substitution following 1.28 mg/kg and 2.56 mg/kg. (R)-5-MBPB produced a dose-dependent increase in MDMA-lever responses with partial substitution at 1.28 mg/kg, and full substitution following 2.56 mg/kg. The ED50s for (S)-5-MBPB and (R)-5MBPB

are 0.35 (C.I.: 0.02 to 0.62) and 0.74 (C.I.: 0.44 to 1.14), respectively. Both (S)-5-MBPB and (R)-5-MBPB produced minimal effects on response rate compared to vehicle control. A repeated measures ANOVA on response rate indicated no statistically significant effect of (S)-5MBPB [F (4, 28) = 0.43, P=0.79] or (R)-5-MBPB [F (4, 24) = 0.26, P=0.90] on response rate.

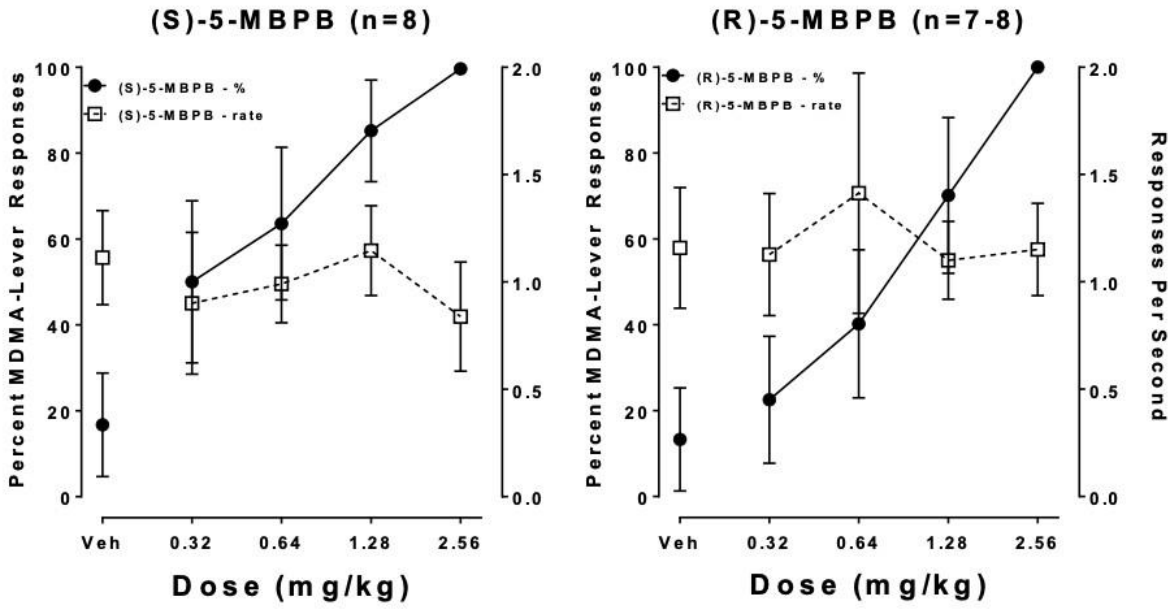

Figure 3. Dose response curves for (S)-5-MBPB, and (R)-5-MBPB. Percent MDMA-Lever Responses (closed symbols) is plotted on the left Y axis and response rate (open symbols) is plotted on the right Y axis. Points represent group means (± S.E.M.).

The dose response curves generated from substitution tests with the enantiomers of 6MBPB are displayed in figure 4. Each of these compounds produced a dose-dependent increase in MDMA-lever responses with full substitution following 1.28 mg/kg. However, differences in response rate were not statistically significant. The ED50 for (S)-6-MBPB and (R)-6-MBPB were 0.21and 0.45 (C.I.: 0.31 to 0.61), respectively. Due to the irregular shape of the (S)-6MBPB dose response function, the lower confidence interval could not be estimated by the nonlinear

regression model used. Response rates displayed a dose-dependent decrease with both enantiomers. These effects were not statistically significant for either (S)-6-MBPB [F (4, 28) = 1.09, P=0.38] or (R)-6-MBPB [F (4, 24) = 1.48, P=0.24].

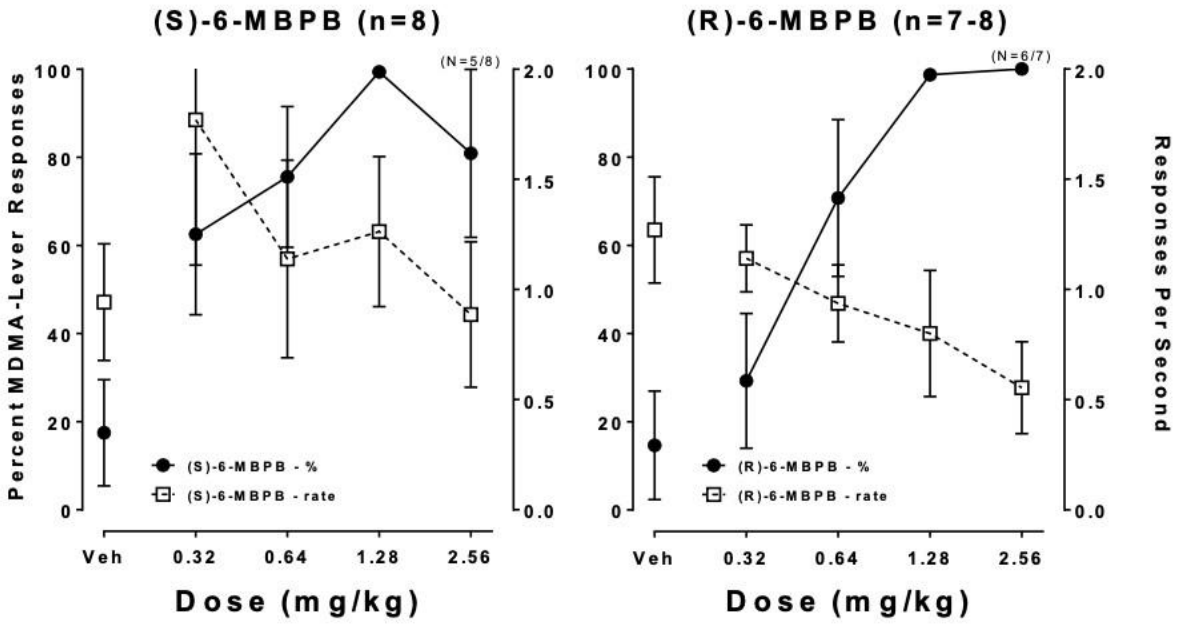

Figure 4. Dose response curves for (S)-6-MBPB, and (R)-6-MBPB. Percent MDMA-Lever Responses (closed symbols) is plotted on the left Y axis and response rate (open symbols) is plotted on the right Y axis. Points represent group means (± S.E.M.).

DISCUSSION

Current treatments for PTSD are ineffective for most patients (Shimshoni et al., 2017) and there is a significant need for new, effective treatments. MDMA-assisted psychotherapy has shown promise and is under further investigation in clinical trials. The structural similarities between NPS compounds, such as benzofurans, and MDMA have led to investigations into these molecules for medication development as potential alternative therapeutics to MDMA. Although safety and toxicity studies will be required prior to clinical evaluation of benzofurans, preclinical

studies on their potential efficacy are essential to the medication development process. Toward that objective, the current study utilized a well-established preclinical behavioral paradigm with pharmacological specificity. Results from the present study support that benzofuran derivatives produce similar discriminative stimulus effects to those of MDMA. All tested compounds produced dose-dependent increases in MDMA-lever responses and substituted fully for the interoceptive stimulus effects of MDMA at the highest dose administered without disrupting response rates. Notably, (R)-5-MAPB is more potent than its counterparts ((S)- and (RS)-), showing full substitution at a lower dose of 0.3 mg/kg, and the (S)- enantiomers of 5-MBPB and 6-MBPB were slightly more potent than their (R)- enantiomers.

This is the first study to examine the discriminative stimulus effects of the enantiomers of the benzofurans, 5-MAPB, 5-MBPB, and 6-MBPB. Based on past research on racemic 5-MAPB, the potency differences between (S)- and (R)-5-MAPB observed in the present study, may be mediated through differential activities at DAT, SERT or NET (Simmler & Liechti, 2018). Recent investigations on the pharmacological actions of these enantiomers are relevant to the current study. Specifically, the (S)- enantiomers for all of the benzofuran derivatives assessed in the current study, were found to be slightly more potent 5-HT releasers compared to the (R)-enantiomers [Baumann, personal communication]. Although similar potency differences were observed *in vivo* with the 5-MBPB and 6-MBPB enantiomers, this was not observed with the 5-MAPB enantiomers. Discrepancies between the current *in vivo* findings and Baumann's *in vitro* findings could be due to differences in pharmacokinetics (e.g., drug absorption, metabolism) yet to be evaluated.

The current study findings are supported by previous reports on the neurochemical similarities between 5-MAPB and MDMA. Fuwa et al. (2016) examined the effects of MDMA,

5-MAPB, and other benzofuran derivatives in mouse brains and found significant increases in monoamine levels. 5-MAPB increased monoamine levels significantly more than any other compound. Notably, at all doses the level of 5-HT was always far greater than DA levels. Fuwa et al. (2016) attributed the potency of 5-MAPB to its structure: the furan ring found in 5-MAPB is presumably responsible for increased extracellular monoamine levels compared to the dioxolane ring in MDMA. Fuwa et al. (2016) also compared monoamine levels following administration of 5-MAPB and its metabolite, 5-(2-aminopropyl) benzofuran (5-APB). 5-APB was a significantly more efficacious 5-HT releaser, and the increase in 5-HT was two times greater than that produced by 5-MAPB.

Although there are no published drug discrimination studies with which to directly compare the current study findings, Dolan et al. (2017) assessed 5-APB and 6-APDB in rats trained to discriminate MDMA, methamphetamine, cocaine or 2,5-dimethoxy-4-methylphenyl isopropylamine (DOM) from saline. They found that 5-APB fully substituted for MDMA and partially substituted for methamphetamine, cocaine, and DOM. The authors concluded that 5APB discrimination is mediated by both dopamine and serotonin (Dolan et al., 2017). To further assess the duration of action and active dose range of benzofuran compounds the researchers also measured the effects of these substances on locomotor activity in mice. Their findings indicated that 5-APB has a slightly different locomotor activity profile from MDMA, with 5-APB injected mice showing rapid increases in activity, while MDMA injected rats displayed delayed stimulation. The current findings are consistent with the conclusions made by both Fuwa et al. (2016) and Dolan et al. (2017) that 5-MAPB is neurochemically analogous to MDMA.

The observed potency of the (S) isomers of 5- and 6-MBPB closely aligns with the pharmacological profile of MDMA. With respect to discriminative stimulus effects, (S)-MDMA

is more potent than (R)-MDMA(Baker & Taylor, 1997 ; Schechter, 1987). In a study in which animals were trained to discriminate either MDMA enantiomer from saline, the serotonergic hallucinogens, DOM, lysergic acid diethylamide (LSD), and mescaline partially substituted in rats trained to discriminate (S)-MDMA, whereas the psychostimulants, amphetamine, and cocaine did not substitute for either isomer (Baker et al., 1995). In consideration of the pharmacological specificity of drug discrimination, these findings implicate serotonergic activities are more salient to the discriminative stimulus effects of (S)-MDMA than to those of (R)-MDMA, whereas dopaminergic actions are less relevant to the discrimination of either isomer.

As stated before, Baumann's *in vitro* assessment found that for both 5- and 6-MBPB, the (S)-enantiomer is a more potent 5-HT releaser than the (R)-enantiomer. Additionally, the (S)enantiomers of these compounds are potent DAT inhibitors and releasers, whereas the (R)enantiomers are DAT inhibitors, but they are not efficacious DA releasers (Baumann, personal communication). With the similarities in structure and neurochemical actions of 5-MBPB, and 6MBPB to those of MDMA, these benzofuran derivatives are presumably operating through serotonergic pathways and may produce effects comparable to serotonergic hallucinogens and less CNS stimulant effects.

The current study was limited to the investigation of selected benzofuran molecules with structural and pharmacological similarities to MDMA. Thus, other than saline, no negative controls were assessed in this study. In future studies, assessment of substances with distinct pharmacological actions, such as ketamine or morphine, to serve as a negative control would add confidence to the conclusion that the discriminative stimulus effects of benzofurans are mediated by similar neurochemical actions underlying MDMA's discriminative stimulus effects.

Additionally, studies in which rats are trained to discriminate selected benzofuran molecules would be invaluable to further assessment of these compounds.

In summation, the results obtained from the present study affirm that 5-MAPB, 5-MBPB and 6-MBPB and their enantiomers produce dose-dependent increases in MDMA-lever responding and fully substitute for MDMA at the highest dose assessed with minimal effects on response rate. Further research is needed to discern which monoamine transporters contribute to the discriminative stimulus effects of these benzofuran derivatives and their enantiomers. For example, assessment of selective serotonin or dopamine receptor antagonists would help differentiate the relative importance of DA versus 5-HT in the MDMA-like stimulus effects of benzofuran derivatives. Based on the structural and pharmacological similarities of MDMA to 5- and 6-MBPB, it would also be of interest to assess these compounds for stimulant effects. Objective assessments of locomotor activity, such as distance traveled and stereotypy measures, would be valuable to determine if the S- and R- enantiomers differ with respect to CNS stimulant effects. Nevertheless, the present study contributes to the growing literature on the *in vivo* pharmacological actions of benzofuran derivatives. Further evaluation of this class of molecules is warranted and may aid in development of novel medications that retain MDMA-like therapeutic effects with reduced toxicities.

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