

# Effects of “Legal X” Piperazine Analogs on Dopamine and Serotonin Release in Rat Brain

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**ABSTRACT:** 3,4-Methylenedioxymethamphetamine (MDMA) is a popular illicit drug that evokes transporter-mediated release of serotonin (5-HT) and dopamine (DA) from nerve cells. Recently, drug users have ingested combinations of the piperazine analogs, 1-benzylpiperazine (BZP) and 1-(*m*-trifluoromethylphenyl)piperazine (TFMPP), in an attempt to mimic the subjective effects of MDMA. In the present study, we compared neurochemical effects of MDMA, BZP, and TFMPP in rat brain. The ability of MDMA, BZP, and TFMPP to stimulate efflux of [<sup>3</sup>H]5-HT and [<sup>3</sup>H]MPP<sup>+</sup> (a DA transporter substrate) was determined *in vitro* using release assays in synaptosomes. The ability of these drugs to increase extracellular 5-HT and DA *in vivo* was assessed using intracranial microdialysis in nucleus accumbens. MDMA stimulated transporter-mediated release of 5-HT (EC<sub>50</sub> = 58 nM) and MPP<sup>+</sup> (EC<sub>50</sub> = 119 nM). BZP was a selective releaser of MPP<sup>+</sup> (EC<sub>50</sub> = 175 nM), whereas TFMPP was a selective releaser of 5-HT (EC<sub>50</sub> = 121 nM). MDMA injections (1 and 3 mg/kg, *i.v.*) increased dialysate 5-HT and DA in a dose-related manner, but actions on 5-HT were predominant. BZP (3 and 10 mg/kg, *i.v.*) elevated dialysate DA and 5-HT, while TFMPP (3 and 10 mg/kg, *i.v.*) elevated only 5-HT. The coadministration of BZP plus TFMPP (BZP/TFMPP) produced marked elevations in extracellular 5-HT and DA that mirrored the effects of MDMA. At the high dose of BZP/TFMPP (10 mg/kg, *i.v.*), the rise in dialysate DA exceeded the summed effects of the drugs alone. Our results support the hypothesis that the BZP/TFMPP combination mimics the neurochemical mechanism of MDMA, providing a basis for recreational use of these agents. Additionally, the findings suggest possible drug–drug synergism when piperazine drugs are coadministered at high doses.

**KEYWORDS:** 3,4-methylenedioxymethamphetamine (MDMA); serotonin (5-HT); dopamine (DA); piperazine analog; 1-benzylpiperazine (BZP); 1-(*m*-trifluoromethylphenyl)piperazine (TFMPP)

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## INTRODUCTION

3,4-Methylenedioxymethamphetamine (MDMA, or "Ecstasy") is a widely abused drug that displays a unique profile of psychotropic actions (reviewed in Ref. 1). Human subjects report that acute MDMA intoxication produces a pleasurable mix of stimulant-like and hallucinogen-like effects, accompanied by feelings of empathy and closeness toward others.<sup>2</sup> The mechanisms underlying the psychotropic actions of MDMA appear to involve the release of serotonin (5-HT), and possibly dopamine (DA), from nerve cells in the brain.<sup>3</sup> At the molecular level, MDMA serves as a substrate for 5-HT transporters (SERTs) and DA transporters (DATs), thereby triggering diffusion-exchange efflux of transmitter molecules from 5-HT and DA neurons.<sup>1,4</sup> A growing body of clinical evidence shows that prolonged experimentation with MDMA may cause 5-HT neurotoxicity and cognitive impairments in users.<sup>1,5</sup>

Given the potential risks associated with MDMA, some drug users have sought to identify legal and safe alternatives to MDMA. For example, piperazine analogs, such as 1-benzylpiperazine (BZP, or "A2") and 1-(*m*-trifluoromethylphenyl)piperazine (TFMPP, or "Molly"), have been referred to as "Legal E" or "Legal X" compounds on Internet Web sites.<sup>6</sup> Individuals in European countries have ingested the combination of BZP plus TFMPP (BZP/TFMPP) in an attempt to mimic the MDMA subjective experience.<sup>7</sup> Increased misuse of these compounds in the United States has prompted the Drug Enforcement Administration (DEA) to invoke emergency Schedule I classification of BZP and TFMPP, making possession of these compounds a criminal offense.<sup>8</sup>

It is well established that TFMPP is a nonselective 5-HT receptor agonist, with the drug exhibiting modest binding affinity (~100 nM) for 5-HT<sub>1</sub> and 5-HT<sub>2</sub> receptor subtypes.<sup>9</sup> However, similar to MDMA, TFMPP also displays presynaptic actions that include the stimulation of 5-HT release from neurons, as demonstrated *in vitro*<sup>10</sup> and *in vivo*.<sup>11</sup> There is limited information available on the molecular mechanism of BZP, but this drug appears to elicit amphetamine-like behavioral effects in rodents<sup>12</sup> and humans.<sup>13</sup> No scientific investigations regarding the neurobiology of BZP plus TFMPP (BZP/TFMPP) are available. Thus, the aim of the present study was to examine the effects of BZP and TFMPP on monoamine neurotransmission in rat brain. In particular, we compared the effects of MDMA, BZP, and TFMPP on transporter-mediated release of 5-HT and DA using *in vitro* and *in vivo* neurochemical methods. Our findings demonstrate that the BZP/TFMPP combination evokes release of 5-HT and DA from neurons, and this profile of action mimics the molecular mechanism of MDMA.

## METHODS

### *Animals*

Male Sprague-Dawley rats weighing 300–350 g were singly housed (lights on: 0700–1900 h) with food and water freely available. Rats were maintained in facilities accredited by the American Association of the Accreditation of Laboratory Animal Care, and the procedures described herein were carried out in accordance with

the Animal Care And Use Committee of the National Institute on Drug Abuse (NIDA) Intramural Research Program (IRP).

### *Drugs and Reagents*

(±)-3,4-Methylenedioxyamphetamine (MDMA) was generously provided by the NIDA Drug Supply Program, Rockville, MD. 1-Benzylpiperazine hydrochloride (BZP) and 1-(*m*-trifluoromethylphenyl)piperazine hydrochloride (TFMPP) were obtained from Research Triangle Institute, Research Triangle Park, NC. Sources of the reagents required for the *in vitro* release assays and the *in vivo* microdialysis methods were previously reported.<sup>14,15</sup>

### *In Vitro Release Methods*

Transporter-mediated release assays were carried out as previously described.<sup>14</sup> Rats were sacrificed by CO<sub>2</sub> asphyxiation. Tissue from caudate (for DAT assay), or from whole brain minus cerebellum and caudate (for SERT assay), was homogenized in ice-cold 10% sucrose containing 1 μM reserpine. For SERT-mediated release assays, [<sup>3</sup>H]5-HT was used as the radiolabeled substrate; 100 nM nomifensine and 100 nM GBR12935 were added to the sucrose solution to prevent uptake of [<sup>3</sup>H]5-HT into NE and DA nerve terminals. For DAT-mediated release assays, [<sup>3</sup>H]1-methyl-4-phenylpyridinium ([<sup>3</sup>H]MPP<sup>+</sup>) was used as the radiolabeled substrate; 100 nM desipramine and 100 nM citalopram were added to prevent uptake of [<sup>3</sup>H]MPP<sup>+</sup> into NE and 5-HT nerves. Synaptosomal preparations were incubated to steady state with 5 nM [<sup>3</sup>H]5-HT (60 min) or 5 nM [<sup>3</sup>H]MPP<sup>+</sup> (60 min) in Krebs-phosphate buffer (pH 7.4), plus 1 μM reserpine. After incubation to steady state, 850 μL of synaptosomes preloaded with [<sup>3</sup>H]ligand was added to polystyrene test tubes that contained 150 μL of test drug in assay buffer plus 1 mg/mL BSA. After 5 min ([<sup>3</sup>H]5-HT) or 30 min ([<sup>3</sup>H]MPP<sup>+</sup>), the release reaction was terminated by dilution with 4 mL wash buffer followed by rapid vacuum filtration. The retained tritium was counted by a Topcount liquid scintillation counter.

### *In Vivo Microdialysis Methods*

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) for surgery. Intracerebral guide cannulae were implanted in the nucleus accumbens of rats, and flexible catheters were implanted into the jugular vein. On the evening before an experiment, dialysis probes were inserted into guide tubes and extension tubes were attached to jugular catheters. Each rat was placed into its own plastic container and connected to a tethering system that allowed motor activity within the container. The microdialysis inflow and outflow tubing, as well as the catheter extension tubing, was connected to a fluid swivel. Probes were perfused *in situ* overnight at 1 μL/min. On the morning of the experiment, dialysate samples were collected at 20-min intervals. Samples were immediately assayed for DA and 5-HT by HPLC with electrochemical detection, as described below.<sup>15</sup> When three stable baseline samples were obtained, drug treatments were administered. All rats received two sequential i.v. drug injections; the first drug injection was given at time zero, followed by the second injection given 60 min later. Microdialysis samples were collected throughout the postinjection period for 2 h.

### *Analysis of Dopamine and Serotonin in Microdialysates*

Aliquots of dialysate (5  $\mu$ L) were injected onto a microbore HPLC C<sub>18</sub> column that was coupled to an amperometric detector. The mobile phase was pumped at a rate of 60  $\mu$ L/min. Chromatographic data were acquired on-line and exported to a software system for peak amplification, integration, and analysis. Standards of DA and 5-HT were run daily before dialysate samples, and standard curves were linear over a wide range of concentrations (1–1000 pg). Peak heights of unknowns were compared with peak heights of standards, and the lower limit of detection (3  $\times$  baseline noise) was 100 fg/5- $\mu$ L sample.

## RESULTS

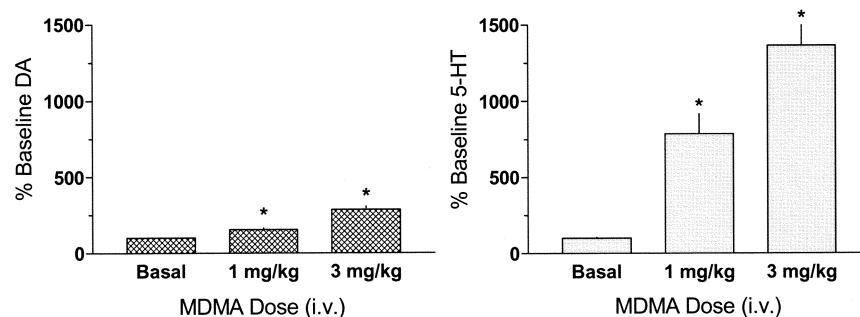
TABLE 1 summarizes the effects of MDMA, BZP, and TFMPP on the release of preloaded [<sup>3</sup>H]5-HT and [<sup>3</sup>H]MPP<sup>+</sup> from synaptosomes. MDMA released both [<sup>3</sup>H]substrates, with effects on 5-HT release (EC<sub>50</sub> = 58 nM) being slightly more potent than effects on MPP<sup>+</sup> release (EC<sub>50</sub> = 119 nM). The release of [<sup>3</sup>H]5-HT evoked by MDMA was antagonized by concurrent treatment with the 5-HT uptake blocker fluoxetine (10 nM), indicating that the 5-HT release was mediated by SERTs. Similarly, MDMA-evoked release of [<sup>3</sup>H]MPP<sup>+</sup> was antagonized by treatment with the DA uptake blocker GBR12909 (10 nM), indicating MPP<sup>+</sup> release involved DATs. BZP released [<sup>3</sup>H]MPP<sup>+</sup> via a DAT-mediated mechanism (EC<sub>50</sub> = 175 nM), but had no effects on [<sup>3</sup>H]5-HT release at doses up to 10  $\mu$ M. TFMPP released [<sup>3</sup>H]5-HT via a SERT-mediated mechanism (EC<sub>50</sub> = 121 nM), and this drug had no effects on [<sup>3</sup>H]MPP<sup>+</sup> release at doses up to 10  $\mu$ M.

FIGURE 1 shows the effects of i.v. administration of MDMA on *in vivo* release of DA and 5-HT in rat nucleus accumbens. MDMA produced dose-related elevations in the levels of DA ( $F_{2,18} = 6.38$ ;  $P < .01$ ) and 5-HT ( $F_{2,18} = 13.74$ ;  $P < .00001$ ), but the effects of the drug on 5-HT were always predominant. For example, at the 1.0-mg/kg dose, MDMA caused a 180% increase in DA and a 580% increase in 5-HT. The effects of BZP on dialysate transmitter levels are depicted in FIGURE 2. BZP stimulated a dose-related rise in dialysate DA ( $F_{2,18} = 19.82$ ;  $P < .00001$ ) and 5-HT ( $F_{2,18} = 8.69$ ;  $P < .002$ ) that was significant at the 3-mg/kg and 10-mg/kg doses. Interestingly, BZP evoked the release of DA and 5-HT with nearly identical potency and efficacy when assessed *in vivo*. As shown in FIGURE 3, TFMPP produced a mod-

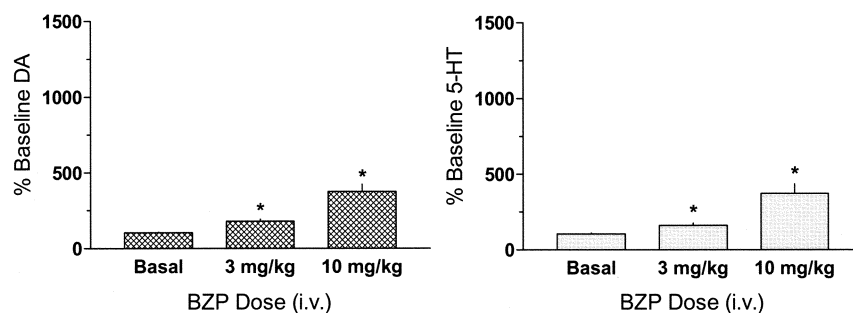
**TABLE 1** Effects of drugs on [<sup>3</sup>H]5-HT and [<sup>3</sup>H]MPP<sup>+</sup> release in rat brain synaptosomes

Drug	SERT activity, determined via [ <sup>3</sup> H]5-HT release EC <sub>50</sub> (nM)	SERT activity, determined via [ <sup>3</sup> H]MPP <sup>+</sup> release EC <sub>50</sub> (nM)
MDMA	58 $\pm$ 6.7	119 $\pm$ 7.8
BZP	>10,000	175 $\pm$ 12.7
TFMPP	121 $\pm$ 16.9	>10,000

NOTE: Data are expressed as mean  $\pm$  SD for three experiments performed in duplicate.



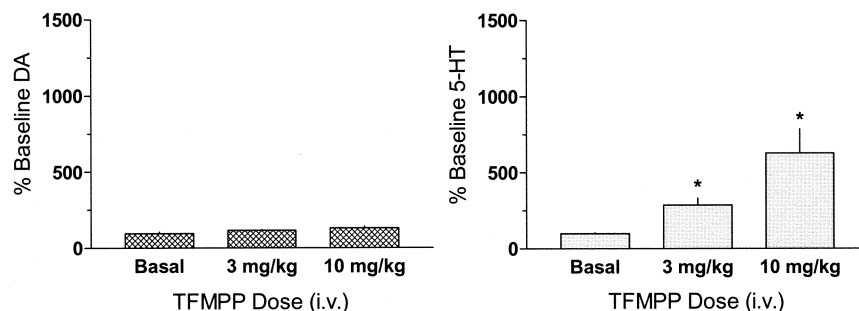
**FIGURE 1.** Effects of MDMA on extracellular levels of DA and 5-HT in rat nucleus accumbens. Rats received i.v. injections of 1 mg/kg MDMA at time zero followed by 3 mg/kg 60 min later. Data are expressed as % baseline for  $N = 7$  rats per group, with each bar representing the mean  $\pm$  SEM for peak effects measured 20 min after drug injections. \* $P < .05$  versus basal, Duncan's Multiple Range Test for *post hoc* comparisons.



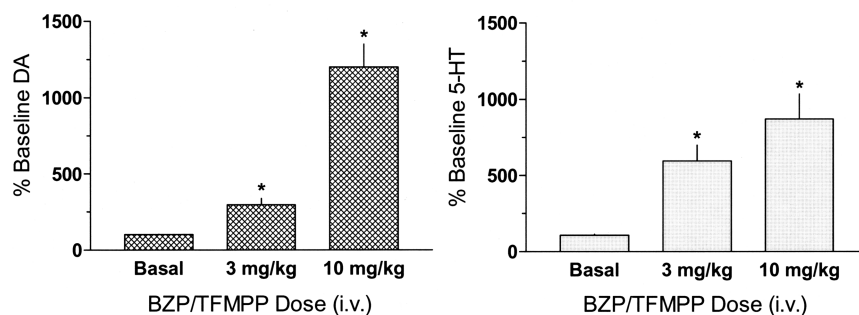
**FIGURE 2.** Effects of BZP on extracellular levels of DA and 5-HT in rat nucleus accumbens. Rats received i.v. injections of 3 mg/kg BZP at time zero followed by 10 mg/kg 60 min later. Data are expressed as % baseline for  $N = 7$  rats per group, with each bar representing mean  $\pm$  SEM for peak effects measured 20 min after drug injections. \* $P < .05$  versus basal, Duncan's Multiple Range Test for *post hoc* comparisons.

est and selective increase in dialysate 5-HT ( $F_{2,15} = 15.35$ ;  $P < .0002$ ). It is noteworthy that the effects of TFMPP on 5-HT release were much less potent and less efficacious when compared to MDMA (compare FIGS. 1 and 3).

The data illustrated in FIGURE 4 show that the BZP/TFMPP combination produced dramatic increases in extracellular DA ( $F_{2,18} = 43.23$ ;  $P < .00001$ ) and 5-HT ( $F_{2,18} = 38.67$ ;  $P < .00001$ ). At a 3-mg/kg dose, the effects of BZP/TFMPP on DA and 5-HT release resembled the effects of 1 mg/kg MDMA. When the higher 10-mg/kg dose was administered, BZP/TFMPP elevated dialysate levels of DA and 5-HT to 1280% and 870%, respectively; thus the effects of the combination on DA release were far greater than the additive effects of BZP (376%) and TFMPP (130%) given alone. It must be mentioned that the 10-mg/kg dose of BZP/TFMPP elicited seizures



**FIGURE 3.** Effects of TFMPP on extracellular levels of DA and 5-HT in rat nucleus accumbens. Rats received i.v. injections of 3 mg/kg TFMPP at time zero followed by 10 mg/kg 60 min later. Data are expressed as % baseline for  $N = 6$  rats per group, with each *bar* representing mean  $\pm$  SEM for peak effects measured 20 min after drug injections. \* $P < .05$  versus baseline, Duncan's Multiple Range Test for *post hoc* comparisons.



**FIGURE 4.** Effects of BZP plus TFMPP (BZP/TFMPP) on extracellular levels of DA and 5-HT in rat nucleus accumbens. Rats received i.v. injections of 3 mg/kg each of BZP and TFMPP (BZP/TFMPP) at time zero followed by 10 mg/kg 60 min later. Data are expressed as % baseline for  $N = 7$  rats per group, with each *bar* representing mean  $\pm$  SEM for peak effects measured 20 min after drug injections. \* $P < .05$  versus baseline, Duncan's Multiple Range Test for *post hoc* comparisons.

in 5 of the 7 rats tested. The seizures were short-lived and were followed by a brief period of ataxia; however, no rats died from the treatment.

## DISCUSSION

The main goal of the present study was to examine the neurochemical effects produced by BZP and TFMPP, when administered alone and in combination. A secondary aim was to compare the effects of these piperazine analogs with the popular "club drug," MDMA. Accumulating evidence suggests that the misuse of BZP and TFMPP is rising in the United States and abroad.<sup>6-8</sup> In the United States, for exam-

ple, law enforcement officials from federal, state, and local jurisdictions have reported a marked increase in the number of confiscated tablets containing BZP and TFMPP. Internet Web sites reveal personal accounts of drug users who are ingesting various combinations of piperazines in an attempt to reproduce the MDMA subjective experience. While the extent of misuse of piperazines is impossible to ascertain, the DEA has placed BZP and TFMPP into emergency Schedule I status, based on the potential for imminent hazard to public safety.<sup>8</sup>

The present *in vitro* data demonstrate that MDMA is a substrate-type releasing agent interacting at SERTs and DATs in nervous tissue, and the drug displays somewhat greater potency as a 5-HT releaser. Our findings agree with the work of others who showed that MDMA evokes transporter-mediated release of [<sup>3</sup>H]5-HT and [<sup>3</sup>H]DA from rat brain slices<sup>16</sup> and synaptosomes.<sup>17,18</sup> In a side-by-side comparison with MDMA, we found that BZP is a selective releaser of [<sup>3</sup>H]MPP<sup>+</sup>, whereas TFMPP is a selective releaser of [<sup>3</sup>H]5-HT. Similar to MDMA, the releasing activities of BZP and TFMPP are dependent upon DATs and SERTs, respectively. It is noteworthy that effects of BZP and TFMPP on [<sup>3</sup>H]monoamine release are less potent than the comparable effects afforded by MDMA. Our data support the findings of Pettibone and Williams<sup>10</sup> and Auerbach *et al.*,<sup>19</sup> who showed that TFMPP and other substituted piperazines can release endogenous 5-HT from rat brain slices at doses ranging from 0.3  $\mu$ M to 10  $\mu$ M.

The intracranial microdialysis data reported here show that MDMA is a powerful releaser of 5-HT and DA *in vivo*, with effects of the drug on 5-HT being more prevalent. The stimulatory actions of MDMA on transmitter release in the nucleus accumbens resemble the MDMA-induced increases in extracellular 5-HT and DA in the prefrontal cortex<sup>20</sup> and caudate nucleus<sup>21,22</sup> reported by others. Using the present experimental paradigm, the threshold dose of MDMA capable of stimulating concurrent 5-HT and DA release was 1 mg/kg, *i.v.*, a dose that has been reported to maintain self-administration behavior in rats.<sup>23,24</sup> BZP stimulated a parallel rise in extracellular DA and 5-HT when administered *in vivo*. The fact that BZP evoked 5-HT release *in vivo* was surprising, given that this drug is essentially inactive when tested in the *in vitro* [<sup>3</sup>H]5-HT release assay (see TABLE 1). TFMPP was a very selective 5-HT releaser *in vivo*, in accordance with the *in vitro* findings. Importantly, BZP and TFMPP were at least 3-fold less potent than MDMA in their ability to stimulate release of endogenous DA and 5-HT. Our microdialysis results with TFMPP agree with those of Auerbach *et al.*,<sup>11</sup> who demonstrated that *i.p.* administration of TFMPP produces elevations in dialysate levels of 5-HT in rat diencephalon. We have previously shown that the chloro-substituted analog of TFMPP, mCPP, stimulates 5-HT release *in vivo* via a SERT-mediated process.<sup>15,25</sup>

When BZP and TFMPP were administered together, we observed dramatic increases in extracellular levels of 5-HT and DA. At the low dose of BZP/TFMPP (3 mg/kg, *i.v.*), the combination evoked a release of 5-HT and DA that mimicked the neurochemical effects of low dose MDMA (1 mg/kg, *i.v.*). Thus, we have discovered a likely neurochemical mechanism underlying the evolving recreational use of BZP/TFMPP and the reported MDMA-like subjective experience produced by this drug combination. At the high dose of BZP/TFMPP (10 mg/kg, *i.v.*), extracellular DA was elevated to a much greater extent than the summed effects of BZP and TFMPP alone, suggesting a synergistic effect when the drugs are combined. Moreover, several rats receiving the high-dose combination developed seizures and subsequent ataxia. No

interactions between BZP and TFMPP were noted with respect to *in vitro* DA and 5-HT release (data not shown); this suggests the possibility that apparent synergism between BZP and TFMPP *in vivo* could be due to pharmacokinetic factors.

In conclusion, the present findings indicate that BZP and TFMPP are capable of releasing DA and 5-HT from neurons via mechanisms dependent upon DATs and SERTs, respectively. The monoamine-releasing actions of these piperazine analogs were consistently demonstrated using *in vitro* and *in vivo* methods. The ability of the BZP/TFMPP combination to produce simultaneous elevations in extracellular 5-HT and DA in the nucleus accumbens mirrors the known molecular mechanism of MDMA. Thus, the presynaptic actions of BZP/TFMPP might explain the evolving misuse of these agents in humans. Perhaps the most striking findings reported here are the apparent drug–drug synergism and adverse behavioral effects (i.e., seizures) associated with high-dose administration of BZP/TFMPP. The drug-induced seizures caused by BZP/TFMPP occurred at a dose that is just 3-fold greater than the threshold dose for biological activity, indicating a very narrow window of safety. Collectively, our findings suggest the potential for dangerous, possibly life-threatening, consequences if BZP and TFMPP are taken in combination by human drug users.

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