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# Estrogenic effects and their action mechanism of the major active components of party pill drugs

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## HIGHLIGHTS

- Benzylpiperazine (BZP) and trifluoromethylphenylpiperazine (TFMPP) stimulated cell proliferation in a dose-dependent manner.
- In an estrogen sensitive reporter gene assay, BZP and TFMPP significantly increased transcriptional activities of party pill drugs.
- PR and pS2 (ER-related genes) were significantly stimulated by BZP and TFMPP.
- BZP and TFMPP could have estrogenic activities related to the ER-mediated pathway.
- BZP and TFMPP did not show significant effects on weight increase in a rodent uterotrophic assay.

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## ABSTRACT

Benzylpiperazine (BZP) and trifluoromethylphenylpiperazine (TFMPP) are commonly used constituents of party pill drugs. They are reported to induce psychoactive effects such as euphoria and provide effects similar with other illicit drugs such as methylenedioxymethamphetamine (MDMA). A great deal of evidence has proven that party pills, as alternatives for MDMA, exert harmful effects on users. However, their toxicological effects have not been fully understood and endocrine disruptive effects are still unknown. In this study, we identified estrogenic effects of BZP and TFMPP by using in vitro and in vivo assays. BZP and TFMPP stimulated cell proliferation in a dose-dependent manner, while co-treatment with tamoxifen and BZP or TFMPP showed a decrease of $E_2$-induced cell proliferation. In an estrogen sensitive reporter gene assay, BZP and TFMPP significantly increased transcriptional activities of party pill drugs. In addition, ER-related genes, PR and pS2, were significantly stimulated by BZP and TFMPP. These results indicated that BZP and TFMPP could have estrogenic activities related to the ER-mediated pathway. Unlike the in vitro assay results, BZP and TFMPP did not show significant effects on weight increase in a rodent uterotrophic assay. However, further studies would be necessary to verify the estrogenic activities of BZP and TFMPP by a chronic exposure animal study.



## 1. Introduction

Party pill drugs have been recently emerged as recreational drugs found to have psychoactive effects and have become popular among young people. In some countries, party pill drugs, such as methamphetamine and methylenedioxymethamphetamine (MDMA or 'ecstasy'), have been perceived as safer alternatives and used as stimulants (Johnstone et al., 2007).

Benzylpiperazine (BZP) and trifluoromethylphenylpiperazine (TFMPP) are one of the synthetic phenylpiperazine analogs (Fig. 1). BZP and TFMPP are the main constituents in most party pill drugs with several minor ingredients such as guarana and vitamins (Butler and Sheridan, 2007). BZP has been reported to have amphetamine-like effects (Bye et al., 1973; Campbell et al., 1973), and to provoke stimulant effects through dopaminergic

*Abbreviations:* 5-HT, 5-hydroxytryptamine; ATCC, American type culture collection; BZP, benzylpiperazine; CD, charcoal-dextran; DMEM, Dulbecco's modified Eagles medium; $E_2$, estradiol; EEFs, estradiol equivalency factors; ER, estrogen receptor; ERE, estrogen receptor elements; EDTA, ethylenediaminetetraacetic acid; EtBr, ethidium bromide; FBS, fetal bovine serum; MDMA, methylenedioxymethamphetamine; LSD, lysergic acid diethylamide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PK/PD, pharmacokinetic/pharmacodynamic; PR, progesterone receptor; RPE, relative proliferation effect; RT, reverse transcription; SDS, sodium dodecyl sulfate; SRB, sulforhodamine B; TFMPP, trifluoromethylphenylpiperazine.

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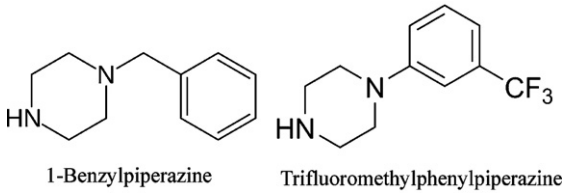


**Fig. 1.** Chemical structures of BZP and TFMPP.

(Tekes et al., 1987; Meririnne et al., 2006), serotonergic (Baumann et al., 2005), and noradrenergic neuronal transmissions (De Deurwaerdere et al., 1998). Moreover, TFMPP has been shown to have LSD (lysergic acid diethylamide)-like effects by acting on 5-HT (5-hydroxytryptamine, serotonin) receptors (Asarch et al., 1985; Alhaider et al., 1993).

These compounds exert elevated levels of both DA (dopamine) and 5-HT when they are taken together, which is similar with the effects of MDMA. This effect made these two compounds to be used in recreational settings in the late 1990s, and the safety and legislations of them have been required since then due to their potentials in causing harm and abuse. In the US, BZP and TFMPP were placed on Schedule I of controlled Substances Act in 2002. Even though TFMPP was removed from the schedule due to the lack of information about its effects on people, BZP was put into permanent schedule 1 in 2004 (FDA, 2008). Besides, BZP and TFMPP have been under strict control over the world including Greece, Denmark, UK and New Zealand. Before New Zealand had banned the use of party pill drugs, the prevalence and patterns of legal party pills usage were reported by Wilkins et al. (2006). According to this report, one in five of surveyed New Zealanders in the age range between 13 and 15 years (20.3%; $n = 2010$) had tried party pills at least once, and most of them were in their late teens or early twenties. Alarmingly, more than 40% of 18–19 year olds and one in six (16.3%; 11.7–22.3%) of 15–17 year olds had tried legal party pills.

People who are in the middle of adolescence are under the final stage of development. The reproductive system is regulated by steroid hormonal changes for successful adult reproductive functions during puberty as well as other developmental processes. For example, endocrine disrupting chemicals, including pesticides, industrial chemicals, phytochemicals, and pharmaceuticals, have potentials to interfere with the endocrine system in humans and have been implicated to produce adverse effects on pubertal maturation of both males and females (Blanck et al., 2000; Colon et al., 2000; Den Hond et al., 2002).

As there have been many scientific inquiries to determine the toxicity of BZP and TFMPP in party pills, several researches have reported harmful aspects on humans. However, they covered a narrow area of study and mainly focused on pharmacokinetic/pharmacodynamic (PK/PD) profiles or subjective effects including euphoria like other illicit drugs (Maurer et al., 2004; Jan et al., 2010; Lin et al., 2011) due to their usage purpose. However, the aspects of reproductive toxicities of party pills should not be underestimated considering the prevalence in adolescence and potential risks as endocrine disruptors. Marijuana is a good example of an abused drug for not only producing psychoactive effects but also for its estrogenic effects (Ashton, 2001; Brown and Dobs, 2002; Lee et al., 2006). Endocrine disrupting effects of BZP and TFMPP have not been discovered yet, thus we investigated the aspects of estrogenic effects and the interactions between estrogenic compounds and hormone receptors that accounted for most of the endocrine disrupting actions.

In this study, estrogenic effects and action mechanisms of BZP and TFMPP were evaluated using human breast cancer cells and immature female rats. To determine the involvement of estrogen receptors, diverse endpoints of the ER-mediated pathway were examined. First of all, we tested the binding affinities of BZP and TFMPP to ER-α and -β. Following this, the interaction between ligand–receptor complexes and estrogen responsive elements (ERE) in DNA was evaluated by a reporter gene assay with ERE-inserted luciferase vector. We also analyzed the induction of estrogen-dependent gene (pS2 and PR genes) expression by using RT-PCR (reverse transcription polymerase chain reaction) and conducted an estrogen-responsive cell proliferation assay with the estrogen-sensitive MCF7-BUS cells. Finally, we carried out an uterotrophic assay with immature female rats to identify the biological effects of BZP and TFMPP on pubertal maturation.

## 2. Materials and methods

### 2.1. Reagents

Powdered hydrochloride salt form of BZP and TFMPP were kindly offered from NFS (National Forensic Service) in Korea. The purities of both BZP and TFMPP were 98%. Stock solutions were prepared in dimethylsulfoxide (DMSO, Sigma–Aldrich, MO, USA). 17β-Estradiol ($E_2$) was purchased from Sigma–Aldrich (MO, USA).

### 2.2. Cell culture

The MCF7-BUS cell line, estrogen-sensitive human breast cancer cells, was kindly provided by Dr. Soto (Tufts University, MA, USA), and MCF-7 cells were obtained from the American Type Culture Collection (ATCC, VA, USA). Cells were grown in Dulbecco's modified Eagles medium (DMEM, Gibco BRL, NY, USA) supplemented with 5% fetal bovine serum (FBS, Hyclone, TU, USA), penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified incubator at 37 °C in a 5% $CO_2$/95% air atmosphere.

### 2.3. Animals

Female Crj:CD (SD) rats, 18 days old, were obtained from Orient Bio Co. Ltd. (Seoul, South Korea). The animals were allowed a 3-day acclimation period in the laboratory animal facility. The rats were provided with tap water and a commercial diet ad libitum. The animal room was maintained at a temperature of 24 ± 2 °C and a relative humidity of 50 ± 20%, with a 12 h (hour) light/dark cycle.

### 2.4. CDFBS preparation

In order to remove the estrogenic steroids from the serum, FBS was treated with 5% charcoal–0.5% dextran (Olea et al., 1996). The charcoal (acid washed, Sigma–Aldrich, MO, USA) was activated with cold sterile water immediately before use. The suspensions of 5% charcoal with 0.5% dextran T70 (Pharmacia LKB, NY, USA) were gentle stirring to coat charcoal by dextran, and centrifuged at 1000 × $g$ for 10 min. After aspirating the supernatant, the charcoal pellets were mixed with FBS and maintained in suspension by rolling at 6 cycles/min at 37 °C for 1 h. The suspension was centrifuged at 2000 × $g$ for 50 min, and the supernatant was filtered through a 0.45-μm filter (Nunc, NY, USA). The charcoal/dextran-treated FBS (CDFBS) did not show any significant cell proliferation in MCF-7 cells and was stored at −20 °C until use.

### 2.5. Cell viability test

To evaluate the cytotoxic effects and to exclude cytotoxic concentration ranges of BZP and TFMPP, we conducted cell viability test using the WST-1 (a sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay (Roche, Germany). The MCF7-BUS cells were seeded onto 96 well plates at a density of 3000 cells/well with 100 μl of medium and incubated for 24 h at 37 °C under 5% $CO_2$. After attachment, the cells were treated with 100 μl of BZP or TFMPP at 20–100 μM in triplicate. After 24 h of incubation, 100 μl of medium was removed and 10 μl of WST-1 reagent was added to each well. After the plates were incubated for 4 h, we measured the absorbance at the wavelength of 440 nm with reference wavelength of 690 nm using Versamax™ microplate reader (Molecular Devices, CA, USA).

### 2.6. Cell proliferation assay

The cell proliferation assay using the MCF7-BUS cells was carried out as described elsewhere (Oh et al., 2000; Perez et al., 1998). Briefly, the cells were harvested with 0.05% trypsin–0.53 mM EDTA·4 Na and resuspended in 5% FBS–DMEM. They were seeded onto 48 well plates at a density of 5000 cells/well and incubated in an atmosphere containing 5% $CO_2$ in air at 37 °C for 48 h. The cells were then treated with a medium containing BZP or TFMPP in triplicate at the indicated concentration in phenol-red free 10% CDFBS–DMEM. After incubating the cells for 144 h, the sulforhodamine B (SRB) assay was carried out to evaluate the extent of cell proliferation. Maximum cell proliferation was observed with $10^{-10}$ M $E_2$, and samples were