

Attenuation of benzodiazepine dependence in mice by a tri-substituted benzoflavone moiety of *Passiflora incarnata* Linneaus: A non-habit forming anxiolytic.

Kamaldeep Dhawan

Department of Drugs Control, Government of Haryana, Panchkula, India

Sanju Dhawan

University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, India

Sumit Chhabra

Department of Pharmaceutical Sciences, Maharishi Dayanand University, Rohtak, Haryana, India

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ABSTRACT. PURPOSE. A tri-substituted benzoflavone moiety (BZF) recently isolated from the methanol extract of aerial parts of the plant *Passiflora incarnata* Linneaus had exhibited encouraging results in countering the dependence produced by addiction-prone substances like morphine, nicotine, cannabinoids and ethyl alcohol, during the studies performed by the authors. Since the BZF moiety had exhibited significant anxiolytic properties at 10 mg/kg p.o. dose in mice, therefore, it was desirable to evaluate this potential phyto-moiety (BZF) for its own dependence-liabilities. It was also deemed viable to evaluate BZF moiety for its possible usefulness in countering the dependence-liabilities associated with the chronic use of benzodiazepines keeping in light their tremendous clinical use in the management of anxiety and insomnia. **METHODS.** Different groups of mice were administered BZF alone (10, 50 or 100 mg/kg, p.o.), and concomitantly with diazepam (20 mg/kg, p.o.) in a 21-days treatment regimen, followed by no treatments for the next 72-hours. The withdrawal effects in the form of ambulatory behavior of the treated animals were recorded on the 25th day using an Actophotometer. **RESULTS.** The BZF-alone (three doses) treated mice exhibited a normal ambulatory behavior on 25th day. Mice groups receiving co-treatments, i.e., BZF-diazepam concomitantly, also exhibited a normal ambulatory behavior in a dose-dependant manner, i.e., the higher dose of BZF (100 mg/kg) being more effective in countering the withdrawal effects of chronically administered diazepam than the lower doses (10 or 50 mg/kg). **CONCLUSIONS.** The studies revealed that the chronic administra-

tion of the BZF moiety (three doses), did not exhibit any dependence-liability of its own, even upon an abrupt cessation. Additionally, the BZF co-treatments with diazepam also prevented the incurrence of diazepam-dependence, which might be because of the aromatase enzyme inhibiting properties associated with the BZF moiety.

INTRODUCTION

Benzodiazepines (e.g., diazepam, nitrazepam, lorazepam, alprazolam) are the most frequently prescribed synthetic chemical drugs for a variety of conditions, particularly anxiety, depression, epilepsy and insomnia (1, 2). The prevalent prescription pattern of benzodiazepines is symptomatic which results in their potential overuse (3). Chronic use of benzodiazepines causes deterioration of cognitive functioning (4), physical dependence (5) and tolerance (6, 7). Besides addiction-liabilities, benzodiazepines adversely affect the respiratory, digestive and immune systems of the body (8). Since, the chronic treatment with benzodiazepines often proves more harmful in the longer run, there has been an increasing thrust worldwide to opt for safer and effective plant-derived anxiolytics and hypnotics mentioned in the traditional medical systems (9, 10). The plant *Passiflora incarnata* Linneaus (family Passifloraceae, synonyms: passionflower, maracuja, maypops) has been put to use as an anxiolytic, sedative, anti-convulsant, nervine and anti-spasmodic in the oriental system of healing in several countries since time immemorial (11, 12). The plant extract is popular homoeopathic medicine for anxiety and insomnia (13). Monographs of *P. incarnata* in the herbal pharmacopoeias of several countries mention the plant to be a safe and non-habit forming medicine for long term management of insomnia, anxiety, depression, epilepsy and morphine de-addiction (14-17).

Corresponding Author: Kamaldeep Dhawan, # 1068, 38-B, Chandigarh-160014, India. kdd@glide.net.in

In recent studies by the authors, a methanol extract of the leaves of *P. incarnata* was confirmed to possess anxiolytic (18, 19), anti-tussive (20), anti-asthmatic (21) and aphrodisiac properties (22) in experimental animals at a dose of 100 mg/kg *per oral*. The bioactive methanol extract was further subjected to bioactivity-directed-fractionation and standard chromatographic procedures to isolate a pure bioactive fraction from which a tri-substituted benzoflavone moiety (BZF) was isolated and its chemical identity was established using the standard spectroscopic techniques (23-25). Our subsequent pharmacological studies on the BZF moiety confirmed the significant usefulness of *P. incarnata* in countering the dependence and development of tolerance upon administration with substances like morphine (26), cannabinoids (27), nicotine (28) and ethanol (29) in mice at a dose-range of 10-50 mg/kg. A 30-days treatment with 10 mg/kg of the BZF moiety exhibited significant increase in the libido, sperm count, fertilization potential and the sexual virility in 2-years old male rats (30). The co-administration of 10 mg/kg dose of BZF along-with chronically administered (30-days treatment regimen) ethanol, nicotine, ethanol-nicotine combinations (31), as well as Δ^9 -tetrahydrocannabinol (32) prevented substance induced azospermia, mating efficiency and virility in male rats.

In the present study, the preventive effects of the BZF moiety of *P. incarnata* upon the diazepam dependence have been evaluated in mice. In addition to this, the BZF moiety has also been evaluated for its own dependence-liability in mice upon a chronic treatment-regimen (21-days) at three different doses (10, 50 and 100 mg/kg, *po*). One group of mice was given the 20 mg/kg dose of diazepam (*i.e.*, ten times of the anxiolytic dose of 2 mg/kg of diazepam in mice) for 21 days. The other groups were given BZF (10, 50 and 100 mg/kg) concurrently with the 20 mg/kg dose of diazepam for 21 days. No drug was administered for the next 72 hours. The ambulatory activity exhibited by each of the treated group was recorded 72 h after the administration of the last treatment on the 21st day (*i.e.*, on the 25th day). The ambulatory activity exhibited by different groups of mice reflects the physiological and physical manifestation of diazepam-dependence, abrupt-diazepam-deprivation oriented withdrawal effects, and the preventive effects of various doses of BZF upon development of diazepam-dependence and expression of diazepam-cessation withdrawal effects in mice (33, 34).

MATERIALS AND METHODS

Plant material

Aerial parts of *P. incarnata* were picked up in January 1999 from cultivated source Rati Ram Nursery at village Khurampur via Kalsia in district Saharanpur (Uttar Pradesh, India). The identity of the procured plant material was confirmed from the Department of Systematic Botany, Forest Research Institute, Dehradun, India (FRI). A voucher specimen (code no. 1325/2000) was deposited in the Herbarium-cum-Museum of the FRI.

Extraction, fractionation and isolation of the bioactive BZF

The aerial parts were dried in the shade and powdered (# 60 mesh size) and 100 g of the dried powder was Soxhlet extracted successively with petroleum ether (60-80°C), chloroform (Ranbaxy Laboratory Chemicals), methanol (sd Fine-Chem Limited) and distilled water. All these extracts were dried using a Buchi RE-121 Rotavapor (Buchi Laboratories-Technik AG, CH-9230 Flawyl/Schweiz, Switzerland) and were preserved in a vacuum desiccator containing anhydrous silica blue. The weight of various extracts after drying was calculated as: petroleum ether extract (6.8875 g), chloroform extract (8.2314 g), methanol extract (11.8787 g) and water extract (4.8876 g). The four different extracts of *P. incarnata* were suspended in a vehicle comprising simple syrup I.P. (Indian Pharmacopoeia 1996) and 1% w/w carboxymethylcellulose (CMC) as suspending agent. Five sets of doses *viz.*, 300, 200, 125, 100 and 75 mg/kg of each extract of *P. incarnata* were prepared by suspending the dried extracts in the vehicle under vigorous stirring to form a uniform suspension. The weight of the dried extracts was so adjusted as to administer 0.25 ml of the suspension of the extracts. Simple syrup containing CMC was used as control. Amongst the four extracts, only the methanol extract of *P. incarnata* showed a significant anxiolytic activity at a dose of 125 mg/kg. The remaining three extracts did not exhibit anxiolytic activity statistically comparable to that of the standard anxiolytic (diazepam, 2 mg/kg in vehicle, *p.o.*). The bioactive methanol extract was processed and purified further by resorting to bioactivity directed fractionation using column chromatographic procedures until a fraction, which exhibited significant anxiolytic activity at a dose of 10 mg/kg in mice, was obtained (23). This fraction (yield = 332 mg, 0.33%) gave positive tests for the presence of flavones (35). UV, LC-MS, GC-MS, IR, ¹H-NMR, ¹³C-NMR characterization studies have confirmed

the presence of a benzoflavone moiety (BZF), never reported from *P. incarnata* earlier, that has been accounted for the CNS effects of *P. incarnata* (23-25) by the authors. The exact structure and complete chemical identity of BZF is not being presented here due to patent considerations.

Animals

Swiss albino mice (either sex) procured from the Disease-Free Small Animals House, College of Veterinary Sciences, Haryana Agriculture University, Hisar, India, were bred at the Central Animal House of the Panjab University, Chandigarh. The mice were allowed standard laboratory feed and water *ad libitum*. Groups of ten mice (20-24 g) were used in all sets of experiments.

Treatment schedule and measurement of ambulatory activity

The eight groups of mice were subjected to the following treatment for 21 days as follows:

- 1 Diazepam treated group (20 mg/kg) **D**
- 2 BZF treated group (10 mg/kg) **P-10**
- 3 BZF treated group (50 mg/kg) **P-50**
- 4 BZF treated group (100 mg/kg) **P-100**
- 5 Diazepam + BZF co-treatment group (20 mg/kg and 10 mg/kg respectively) **DP-10**
- 6 Diazepam + BZF co-treatment group (20 mg/kg and 50 mg/kg respectively) **DP-50**
- 7 Diazepam + BZF co-treatment group (20 mg/kg and 100 mg/kg respectively) **DP-100**
- 8 Control group that received vehicle only **C**

All treatments were administered orally for 21 days at 09.00 hour daily. No treatment was given during the next 3 days. The locomotor (ambulatory) activity of all the treated groups was determined on 25th day using an Animal Activity Meter (Opto Varimex Mini, Columbus Instruments, Ohio, USA). An array of 15 infrared emitter/detector pairs, spaced at 2.5 cm intervals, measured the animal

activity along a single axis of motion, the digital data being displayed on the front panel meters as ambulatory activity. The animals were placed individually in a transparent plastic cage (29 cm x 22 cm x 22 cm) for 5 min and the ambulatory activity was recorded 72 hours after the administration of the last dose on the 21day, *i.e.*, on the 25th day (36).

Statistical analysis

The data expressed as mean \pm S.D. were analyzed by analysis of variance (ANOVA) followed by a Fischer LSD test, and *P* values < 0.05 were considered statistically significant (37).

RESULTS

Effect of chronic treatment with diazepam

Chronic administration produced dependence and withdrawal effects appeared when the drug was discontinued. On abrupt termination of diazepam after 21-days treatment, the animals show relapse of severe anxiety, evident from the significant increase in ambulatory activity. The maximum withdrawal effects (manifested by increase in ambulatory activity) were apparent 72 hours after the cessation of 21-days treatment with diazepam.

Effect of chronic treatments with BZF

Chronic administration of BZF (10, 50 and 100 mg/kg, *po*) for 21 days did not produce any dependence. After the discontinuation of 21-days treatment with the three doses of BZF, the pattern of ambulatory activity reflected a normal physiological behavior on the 25th day.

Effect of BZF on the expression of diazepam-dependence oriented withdrawal effects

The three doses of BZF (10, 50, 100 mg/kg) upon concurrent administration with 20 mg/kg diazepam, significantly blocked the expression of withdrawal effects of diazepam-dependence, in a dose dependent manner. The group DP-100 showed less ambulatory activity relative to the group DP-50 and DP-10 respectively on the 25th day.

DISCUSSION

In rodents, an abrupt cessation of a chronic treatment with a proven anxiolytic agent (e.g., ethanol or diazepam) is manifested in the form of increased anxiety (4, 38) that has a direct bearing on the locomotor behavior of the animals (34, 39, 40). In several studies on behavioral manifestation

of ethanol-dependence in mice, it has been hypothesized that an abrupt cessation of chronic treatment with ethanol leads to a relapse of anxiety, which is manifested in the form of increased ambulatory behavior in mice (29, 41, 42). The results of the present study with diazepam and BZF dependence also favor the same hypothesis. It is evident from Table 1 that administration of 10, 50 or 100 mg/kg doses of BZF for 21 days did not induce any dependence which is apparent from the normal ambulatory behavior of the BZF-treated mice, even after the cessation of BZF treatments for the next 3 days.

Table 1: Ambulatory activity scores recorded 3 days after administration of vehicle, diazepam, BZF, and diazepam-BZF combinations for 21 days

Treatment (mg/kg)	Ambulatory activity counts	Percent ambulatory activity counts relative to diazepam (PAAC)
C (vehicle 0.25 ml)	78.5 ± 5.43	34.4
D	229.0 ± 9.52*	100
P-10	82.1 ± 3.25	35.9
P-50	82.2 ± 4.41	35.9
P-100	80.9 ± 3.33	35.3
DP-10	177.2 ± 9.29*	77.4
DP-50	119.4 ± 7.71*	52.0
DP-100	80.0 ± 4.77	34.9

The data are expressed as Mean ± S.D., n = 10, *P < 0.05 versus Control. ANOVA followed by Fischer's LSD test. All treatments were administered orally and the maximum volume of each administration was 0.25 ml. The vehicle comprised simple syrup and 1% w/w carboxy-methyl-cellulose. Ambulatory activity scores were recorded for 5 min, 72 h after the last administration on the 21st day

The percent ambulatory activity counts (PAAC) after cessation of BZF-treatments, being statistically similar to the PAAC of the control group mice, further confirm that there are no physical symptoms of aggression or relapse of anxiety on 25th day, after the treatment with BZF for 21 days. Co-treatments of diazepam (20 mg/kg) concomitantly with the three doses of BZF for 21 days, also attenuate the development of diazepam dependence, in a dose-dependent-manner. The co-treatment of diazepam (20 mg/kg) with 100 mg/kg of BZF (DP-100) affords the complete suppression of the withdrawal-induced-anxiety, as the PAAC after this co-treatment (34.9) is statistically equivalent to that of the PAAC exhibited by the control group (34.4) of animals on the 25th day. The PAAC of the other co-treatments, i.e., DP-50 (20 mg/kg diazepam + 50 mg/kg BZF) and DP-10 (20 mg/kg diazepam + 10 mg/kg BZF) being 52.0 and 77.4 respectively.

Scientific studies have proved that chronic use of psychotropic substances like morphine (43), nicotine (44), ethanol (45), cannabinoids (46) and benzodiazepines (47-50) causes decline in blood-androgen levels depending upon the duration and quantity of the substance consumed. Benzodiazepines have also been reported to be implicated in male fertility and levels of testosterone upon chronic

treatments (51-56). Treatment with 20 mg/kg dose of diazepam for 21 days is postulated to induce a significant decrease in testosterone levels that is manifested in the form of the anxiogenic behavior of the diazepam-treated-mice (57). However, co-treatments with BZF and diazepam are hypothesized to prevent decline in blood-testosterone levels, especially in light of the similar studies performed by the authors where the BZF moiety has been reported to prevent chronic ethanol and nicotine-induced azospermia, sterility and decreased libido, in healthy male rats (30, 31).

Benzodiazepines and barbiturates have long been hypothesized to modulate receptors in the gamma amino-butyric acid (GABA) system (58). Over the past more than a decade, neuro-steroids (testosterone, progesterone, pregnanolone, dehydro-epi-androsterone and tetra-hydro-corticosterone, etc) have been acknowledged as the key brain chemicals which regulate the neuro-biology of various psychiatric disorders, including drug-dependence and tolerance (59, 60). The identification of a steroid binding site on the GABA receptors and its implication in the sedative actions of neuroactive steroids has been a landmark discovery which facilitated the better understanding of various behavioral changes during drug addiction and other mental disorders (61-63). The neuro-steroidal implications of the BZF moiety isolated from the plant *P. incarnata* have provided an interesting hypothesis in understanding the multifarious biological effects of this potent plant-derived phyto-moiety. The benzoflavone compounds are the strongest (64, 65) inhibitors of the liver microsomal enzyme aromatase - a member of cytochrome P-450 family (66). Aromatase (P-450 3A4 enzyme) metabolizes testosterone to estradiol (67, 68). A specific functional aromatase gene 'CYP 19' is necessary for the expression of aromatase enzyme in mice and human beings (69). Increased estrogen due to chronic benzodiazepine-administration increases the production of sex-hormone binding globulin (SHBG) (70). The SHBG binds testosterone and reduces the free testosterone in the blood. The increased estrogen levels cause the disruption of normal testicular production of testosterone and saturation of testosterone receptors in the hypothalamus in brain, thereby, reducing the signal sent to the pituitary gland which in turn reduces the secretion of LH. This ultimately reduces testosterone production in gonads (71). The testosterone levels in the plasma have an effect upon the gonadotropins [luteinizing (LH) and follicle-stimulating-hormone (FSH)], which regulate spermatogenesis and maturation of sperms (72). The

BZF moiety isolated from aerial parts of *P. incarnata* is hypothesized to prevent the decline in androgens (a) through its anti-aromatase function, and (b) by eliminating estrogen's negative feedback loop, in light of the various reports (72-74). Further, the BZF moiety can be speculated to inhibit the metabolic conversion of androgens (testosterone) to estrogens (estradiol), thus, increasing free testosterone and decreasing free estrogen (75). The role of free testosterone in regulating behavioral anxiety (57) and stress in human beings and animals has been well established and well documented during the last 10 years (76, 77). High testosterone accounts for normalization of behavioral changes, that otherwise appear, in most of the mental disorders. BZF moiety influences the testosterone synthesis and maintains the optimum levels of free testosterone. Normalization of testosterone levels means health, joviality, and a normal behavior.

The beneficial effect of the co-treatment of diazepam with BZF is therefore apparent, as there are practically very little withdrawal effects even upon chronic intake of diazepam. The BZF moiety of *P. incarnata* affords a useful anxiolytic agent being free from dependence-liabilities even on a long-term chronic use. Secondly, the concurrent administration of BZF and diazepam affords a useful combination therapy, as the BZF moiety also prevents the development of diazepam-dependence and the subsequent appearance of withdrawal effects. The BZF moiety from passion flower offers a very useful phyto-moiety in countering the menace of substance dependence at-least in preliminary animal studies.

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