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Characterization of 6-methoxyflavanone as a novel anxiolytic agent: A behavioral and pharmacokinetic approach

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ABSTRACT

Benzodiazepines are regularly prescribed for treatment of anxiety though there are side effects. Flavonoids have selective affinity for $GABA_A$ receptors implicated in anxiolytic-like activity in rodents, but are devoid of the unwanted side effects of benzodiazepines. In this study, 6-methoxyflavanone (6-MeOF), a positive allosteric modulator of γ-amino butyric acid (GABA) responses at human recombinant $GABA_A$ receptors, was evaluated for its behavioral profile in the elevated plus-maze as well as the staircase- plus and open-field tests in mice. In addition, the distribution of 6-MeOF in selected brain areas involved in anxiety (amygdala and cerebral cortex) was also examined using a validated high performance liquid chromatography ultraviolet detection (HPLC/UV) method. 6-MeOF (10, 30 and 50 mg/kg) exerted an anxiolytic-like effect, increasing entries and time spent in the open arm and the central platform, as well as head-dipping frequency in the mouse elevated plus-maze assay. It also decreased rearing incidence without suppressing the number of steps ascended in the staircase test. Whereas, in the open-field anxiety test, 6-MeOF had no effect on locomotor activity at lower doses, a decrease was observed at the highest dose (100 mg/kg). 6-MeOF additionally produced an anxiolytic-like increase in the time spent at the center of the open-field apparatus. These effects were preferentially antagonized by pentylenetetrazole (15 mg/kg). Furthermore, pharmacokinetic studies disclosed a rapid appearance of 6-MeOF in the plasma and discrete brain areas. Taken together, our findings suggest that 6-MeOF readily crosses the blood brain barrier (BBB) generating anxiolytic activity, mediated through the GABAergic system.

1. Introduction

Generalized anxiety disorder, social anxiety and panic disorder have long been recognized as being amongst the most commonplace disabling conditions in society (Association, 1994). Although selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) are considered as primary treatments for anxiety, benzodiazepines are still quite regularly prescribed despite having unwanted side effects (Davidson, 2009; Paladini et al., 1999). Benzodiazepines act as positive allosteric modulators *via* a subpopulation of γ-amino butyric acid receptors ($GABA_A$) increasing the frequency of chloride channel opening (Chebib and Johnston, 2000). In the brain, GABA tends to be the most abundant inhibitory neurotransmitter regulating different physiological phenomena including sleep, anxiety, memory formation and reward (Zeilhofer et al., 2009) and $GABA_A$ receptors are implicated as a major inhibitory element (Macdonald and Olsen, 1994). Accordingly, GABA inhibitory interneurons function *via* $GABA_A$ receptor subtypes that are involved in mediating behavior (Mohler et al., 2004).

A few years ago, a new family of ligands possessing a flavonoid nucleus, was unveiled by a research group in a quest for safer $GABA_A$ receptor modulators (Ognibene et al., 2008). These polyphenolic compounds (phenyl benzopyrones) have been found not only in plants but also in dietary components and they have been produced synthetically as well (Kempuraj et al., 2005). Flavonoids have been extensively examined for their peripheral actions, however a selective affinity for

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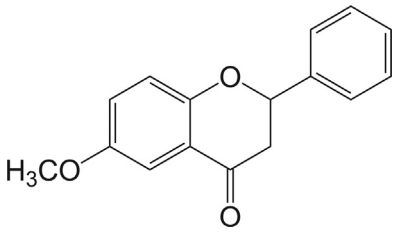

Fig. 1. Chemical structure of 6-methoxyflavanone (6-MeOF).

$GABA_A$ receptors has been reported in studies using rat and bovine brain membrane binding assays (Hong and Hopfinger, 2003). In conjunction with binding studies, behavioral investigations have also disclosed anxiolytic-like activity of flavonoids in rodents without unwanted benzodiazepine side effects (Griebel et al., 1999). Furthermore, a number of synthetic derivatives of natural flavonoids are known for their potent anxiolytic properties (Karim et al., 2011; Fernandez et al., 2008; Griebel et al., 1999).

Interestingly, a recent radioligand binding study of 6-MeOF (a synthetic flavanone, Fig. 1) has revealed that its molecular binding site is different from other known flavonoid modulators of $GABA_A$ receptors. Thus, it binds to a novel allosteric site independent of both high and low affinity benzodiazepine binding sites (Hall et al., 2014). Consequently, it acts upon γ-subunit containing $GABA_A$ receptors like diazepam, but is insensitive to flumazenil antagonism whilst inhibiting [^{3}H]-flunitrazepam binding at the same time. Hence, 6-MeOF as a $GABA_A$ receptor ligand, may conceivably exhibit an anxiolytic profile. Therefore, this study was devised to investigate such a propensity of activity *in vivo* utilizing different anxiety models. We also investigated the possible contribution of GABAergic mechanisms to the pharmacological activity of 6-MeOF by pretreatment with a designated $GABA_A$ receptor antagonist, pentylenetetrazole (PTZ) (Rattka et al., 2011). Additionally, high performance liquid chromatography (HPLC) coupled with UV detection was employed to determine the pharmacokinetic profile as well as the distribution pattern of 6-MeOF both in plasma and the brain areas (cerebral cortex and amygdala) involved in anxiety.

2. Materials and methods

2.1. Drugs and chemicals

6-Methoxyflavanone (> 95%, Sigma-Aldrich, USA), HPLC grade acetonitrile (99.9%) as well as methanol (99.9%) (Fisher Scientific, UK), diazepam (Valium, 10 mg/2 ml, Roche, Pakistan) and pentylenetetrazole (≥98%, Sigma Aldrich, UK) were employed. 6-MeOF was dissolved in a vehicle comprising of Tween 80 (1%), DMSO (5%) and saline (94%).

2.2. Animals

BALB/c mice (18–30 g) of both sex (equal numbers of males and females per group), bred and maintained in a controlled environment (temperature =22 ± 2 °C and relative humidity =60 ± 10%) on a 12:12 h day/night cycle (lights on at 7:00 am) in the animal house (Department of Pharmacy, University of Peshawar) were used. Food and water were provided *ad libitum* and both males as well females were included experimentally because previous studies have verified that mice of either sex may be employed in measures of anxiety (File, 2001). All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and according to the rules and ethics set forth by the Ethical Committee of the Department of Pharmacy, University of Peshawar. Approval for this study was granted with the registration number: 08/EC-15/Pharm (dated: April 10, 2015).

2.3. Behavioral analysis

Mice were housed in polycarbonate cages in groups of three per cage (dimensions 42.5×26.6×15.5 cm). All the behavioral tests were performed during the light phase between 8:00 am to 2:00 pm. On the test day, the mice were transferred to a dimly illuminated room (red light, 40 lx) 1 h prior to experimentation. Each experiment was performed on separate groups of animals (n =6) according to the guidelines of Animal (Scientific Procedures) Act 1986. The sample size was calculated according to the resource equation method (Festing and Altman, 2002). There are no previous reports on the pharmacological activity of 6-MeOF, so the dose range selected (10–100 mg/kg) was based on those of other synthetic flavonoids known to exert anxiolytic activity (Karim et al., 2011; Ognibene et al., 2008). Diazepam is widely used in the dose range 1.0–3.0 mg/kg as a standard in different anxiety tests, therefore 2.0 mg/kg was selected as mid-range (Karim et al., 2011; Fernandez et al., 2009). PTZ is considered non-convulsive and anxiogenic at doses lower than 30 mg/kg, so a 15 mg/kg dose was selected for the purpose of the current study (Hoeller et al., 2013; Rodgers et al., 1995).

2.4. Elevated plus-maze

The apparatus was comprised of two open arms (27×5×0.25 cm) and two closed arms (27×5×15 cm) extended from a central platform (5×5 cm) at a height of 40 cm above floor level (Macri et al., 2002). The animals were habituated in a dimly illuminated laboratory (red light, 40 lx) 1 h prior to testing. The mice were then placed on the intersection of the open and closed arms facing the open arm, 20 min post-treatment with vehicle, diazepam (2.0 mg/kg; i.p.) or 6-MeOF (10, 30, 50 and 100 mg/kg; i.p.). They were allowed to freely explore the maze for 5 min. All the experimental sessions were recorded with a digital camera (Cat's Eye IR IP Camera, Taiwan). Parameters including the percentage of entries and time spent in open arms (entries/time spent in open arms/ total entries/time ×100), time spent at the intersection/ center of the apparatus (s) and closed arm entries (animal entry with all the four paws into either closed arm) were considered as spatio-temporal. Other parameters incorporating head-dipping frequency (depressing head-shoulders at the edges of maze) and the frequency of rearing were considered under ethological measures (Rodgers et al., 1995; Pellow et al., 1985). In the drug combination study, PTZ (15 mg/kg; i.p.) was administered 30 min prior to drug administration. After each trial the apparatus was thoroughly swabbed with a wet paper towel (soaked in a mixture of ethanol, detergent and water) in order to remove any odor or residues (Karim et al., 2011).

2.5. Staircase test

Mice (18–24 g) were administered vehicle, 6-MeOF (10, 30, 50 and 100 mg/kg; i.p.) or diazepam (2.0 mg/kg; i.p.). In the drug combination experiments, PTZ (15 mg/kg; i.p.) was administered 30 min prior to drug treatment. Thirty min later, the number of steps ascended and rears by each animal were observed for 3 min using the staircase apparatus according to the methods described by Simiand et al. (1984). A step was considered to be ascended only if the criterion was met whereby an animal placed all four paws on the step.

2.6. Open-field test

Locomotor activity was evaluated in a box with dimensions of 50×40 cm, and divided into four equal quadrants by lines. Mice (22 ± 2 g) were administered 6-MeOF (10, 30, 50 and 100 mg/kg; i.p.) or diazepam (2.0 mg/kg; i.p.). In the drug combination experiments, PTZ (15 mg/kg; i.p.) was administered 30 min prior to drug treatment. Thirty min later, the animals were placed at the center of the recording apparatus and the number of lines crossed and time spent at the center