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Gender difference in epileptogenic effects of 2-BFI and BU224 in mice

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Abstract

Imidazoline I₂ receptors are involved in pain modulation and psychiatric disorders and its ligands may represent a new therapeutic strategy against pain and depression. In particular, 2-BFI and BU224 are two most widely studied I₂ receptor ligands and have antinociceptive and antidepressant-like activities in rodents. However, little is known of the toxicological effects and potential gender differences of these I₂ receptor ligands. This study examined the epileptogenic activities of 2-BFI and BU224 in male and female mice and also examined their underlying receptor mechanisms. 2-BFI (10–40 mg/kg, i.p.) and BU224 (10–40 mg/kg) produced epileptic seizures in a dose-related manner, as did the epileptogenic agent, pentylenetetrazole (PTZ, 15–60 mg/kg). However, female mice were significantly more sensitive than male mice in all the measures. The commonly used I₂ receptor antagonist, idazoxan (10 mg/kg), did not block the onset and magnitude of the epileptic seizures or lethality induced by 2-BFI and BU224. When studied in combination, PTZ potentiated the epileptogenic effect of 2-BFI and BU224. The lack of antagonism by idazoxan of the epileptogenic activities of 2-BFI and BU224 suggests that the epileptogenic effects of 2-BFI and BU224 are mediated by non-imidazoline I₂ receptors and that I₂ receptors remain a viable therapeutic target for neurological disorders such as pain.

Keywords

2-BFI; BU224; imidazoline I₂ receptor; idazoxan; seizure; mice

1. Introduction

Imidazoline receptors are a family of heterogeneous binding sites that are widely distributed and traditionally are thought to preferentially recognize compounds with an imidazoline moiety (Head and Mayorov, 2006; Regunathan and Reis, 1996). Over the years, consensus

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has been reached that there are at least three imidazoline receptor subtypes, each of which is involved in different functionalities. Specifically, I₁ receptors are involved in central control of blood pressure and glucose balance (Head and Mayorov, 2006; Sun et al., 2007) and a gene that encodes functional I₁ receptors (imidazoline receptor antisera-selected gene/Nischarin) has been identified (Sun *et al.*, 2007). I₃ receptors are generally thought to be involved in pancreatic insulin secretion and may be a potential target for the treatment of diabetes (Eglen et al., 1998).

Imidazoline I₂ receptors have been attracting increasing interest as a potential drug target for some neurological and psychiatric disorders (Li and Zhang, 2011). Continued medicinal chemistry efforts have led to several highly selective I₂ receptor ligands and subsequent pharmacological studies have revealed possible therapeutic utilities of some of these compounds (Hudson et al., 2003; Nikolic and Agbaba, 2012). For example, I₂ receptor ligands such as 2-BFI, BU224 and CR4056 have antinociceptive activities in various rodent models of acute and chronic pain (Ferrari et al., 2011; Li et al., 2011; Meregalli et al., 2012; Sampson et al., 2012). The same ligands also demonstrate robust antidepressant-like effects in both rats and mice (Finn et al., 2003; Hudson et al., 2003; Meregalli et al., 2012; Tonello et al., 2012). In addition, several lines of evidence support the neuroprotective role of I₂ receptor ligands (Han et al., 2012; Han et al., 2010) and suggest that this effect may be mediated by inducing anti-apoptotic proteins (Garau et al., 2012; Han et al., 2010). Combined, these results strongly suggest a broad range of therapeutic potentials of ligands targeting imidazoline I₂ receptors against some of the most treatment-resistant neurological and psychiatric disorders such as neuropathic pain and depression.

As an emerging new pharmacological target, although the evidence of therapeutic potentials is accumulating, it is surprising to see little data regarding the safety profiles of I₂ receptor ligands. In fact, relatively few studies have conducted complete dose-response relationships in the reported functional experiments (Li and Zhang, 2011; Li et al., 2011; Meregalli et al., 2012; Sampson et al., 2012; Tonello et al., 2012), and none has simultaneously evaluated the potential adverse effects of the several widely used I₂ receptor ligands such as 2-BFI and BU224. This study attempted to begin to understand the effects of two most widely studied I₂ receptor ligands, 2-BFI and BU224, in inducing central excitability by quantitative and qualitative measures of drug-induced seizures in mice.

2. Materials and methods

2.1 Subjects

Adult male and female C57/BL6 mice and ICR mice (n = 9–11/group) weighing 20–30g were used in these experiments. Mice were group housed at a constant temperature of 22°C and under a light cycle of 12-h light/12-h darkness (behavioral experiments were conducted during the light period) with free access to food and water and were allowed for at least 3 days of habituation before experimentations. For all epileptic seizure tests, animals were deprived of food overnight before tests. Animals were maintained and experiments were conducted in accordance with the Institutional Animal Care and Use Committee, Wuhan University and with the National Institute of Health, Guide for the Care and Use of Laboratory Animals (8th edition).

2.2 Behavioral procedure

The categories of evaluating the seizure magnitudes were detailed elsewhere (Racine, 1972). Clear acrylic cylinders (radius = 10 cm) were used as behavioral observation arenas. Mice were allowed 30 min to habituate to the test environment and then immediately after a drug injection, mice were placed individually into the cylinders and their behaviors were observed for 30 min. In the case that two drugs were studied in combination, the

pretreatment drug (PTZ or idazoxan) were administered 30 min prior to the test drug treatment. The magnitude of the seizures was rated according to the following scales: 0, no change in behavior; I, myoclonic jerks; II, minimum seizures, with convulsive wave through the body; III, fully developed minimal seizures, clonus of the head muscles and forelimbs, righting reflex present; IV, major seizures (generalized without the tonic phase); V, generalized tonic-clonic seizures beginning with running, followed by the loss of righting ability, then a short tonic phase (flexion or extension of fore and hind limbs) progressing to clonus of all four limbs that sometimes lead to the death of the animal. Although the start of myoclonic jerks is readily identifiable, grade V represents the most apparent seizure episode and lower grades (I–IV) are difficult to discern from one another, this study only reported qualitative data with grade V seizures. The time between the drug injection and the myoclonic jerks is defined as the latency of the seizure-onset time. All experiments were carried out between 8:00 and 13:00 h and mice were used only once.

2.3 Drugs

Pentylenetetrazole (PTZ) and idazoxan hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA), 2-BFI hydrochloride (2-(2-benzofuranyl)-2-imidazoline hydrochloride) and BU224 hydrochloride (2-(4, 5-dihydroimidazol-2-yl) quinoline hydrochloride) were synthesized according to the published procedure (Ishihara and Togo, 2007). All drugs were dissolved in 0.9% physiological saline. Drugs were injected at a volume of 1 ml/100 g body weight. All drugs were injected intraperitoneally.

2.4 Data analyses

Latency of the seizure onset, percentage of mice demonstrating grade V seizure and percentage of mice died within 30 min were the behavioral endpoints subjecting further analyses. For the quantitative data (latency, sec), data were presented as mean $\pm$ S.E.M and one-way analyses of variance (ANOVA) (three or more groups) followed by Newman-Keul's multiple comparison test or Student's *t* test (two groups) was conducted to detect between-group differences. Two-way ANOVA (gender $\times$ dose) was used to detect gender differences among the studied doses. For the qualitative data (% mice demonstrating grade V seizure and % mice died), a Chi-square test for trend was conducted to test whether the overall effect had a significant dose-dependent trend. If a significant trend was identified, Fisher's test was conducted to compare each dose with the control. When appropriate, the dose of drug that produced 50% lethality (LD₅₀) was estimated through regression analysis. A *P* < 0.05 was considered statistically significant.

3. Results

Epileptic seizure is an unnatural behavior and does not occur in normal healthy mice. In the cases of drug-induced seizures, seizure either does not occur or occurs within 10 min. Thus, although all mice were observed for 30 min after drug or vehicle injections, 10 min was used as the cutoff time for practical reasons. As compared to control group, 2-BFI (10–40 mg/kg) dose-dependently decreased the latency of seizure onset in both male and female C57/BL6 mice (*F* [3, 39] = 195.0, *P* < 0.0001) (top left, Fig. 1). Post hoc analysis revealed significant differences in groups receiving 20 mg/kg or 40 mg/kg 2-BFI in female mice and 40 mg/kg 2-BFI in male mice (*P* < 0.05 vs. control). In addition, the latency was significantly shorter in female than in male mice after 20 mg/kg or 40 mg/kg 2-BFI treatment (*P* < 0.05). There was a significant dose-dependent trend to induce grade V seizures (χ^2 [1] = 3.93, *P* < 0.05) in female but not in male mice, but Fisher's exact test failed to reach statistical significance for any dose when compared to control group (middle left, Fig. 1). All the mice survived after receiving a dose of 10–40 mg/kg 2-BFI (bottom left, Fig. 1). BU224 (10–40 mg/kg) also dose-dependently decreased the latency of seizure onset (*F* [3, 39] = 707.6, *P* < 0.0001)

(top center, Fig. 1). Post hoc analysis revealed significant differences in groups receiving 20 mg/kg or 40 mg/kg BU224 in female mice and 30 mg/kg or 40 mg/kg BU224 in male mice ($P < 0.05$ vs. control). In addition, the latency was significantly shorter in female than in male mice after 20 mg/kg or 40 mg/kg BU224 treatment ($P < 0.05$). There was a significant dose-dependent trend to induce grade V seizures ($\chi^2 [1] = 3.53$, $P < 0.05$) both in female and in male mice, and Fisher's exact test revealed significance in female mice receiving 40 mg/kg BU224 as compared to control group (middle center, Fig. 1). There was a significant dose-dependent trend to induce lethality ($\chi^2 [1] = 14.40$, $P < 0.001$). All mice survived after 10 and 20 mg/kg BU224 but all female mice died after receiving 40 mg/kg BU224 treatment ($P < 0.001$) (bottom center, Fig. 1). Regression analysis estimated that the LD₅₀ dose of BU224 was 28.3 mg/kg. As a control and as expected, PTZ (15–60 mg/kg) also dose-dependently decreased the latency of seizure onset in both female and male mice ($F [3, 39] = 2897.0$, $P < 0.0001$) (top right, Fig. 1). Post hoc analysis revealed significant differences in groups receiving 30 mg/kg or 45 mg/kg PTZ in female mice and 45 mg/kg or 60 mg/kg PTZ in male mice ($P < 0.05$ vs. control). In addition, the latency was significantly shorter in female than in male mice after 30 mg/kg or 45 mg/kg PTZ treatment ($P < 0.05$). There was a significant dose-dependent trend to induce grade V seizures ($\chi^2 [1] = 11.66$, $P < 0.001$), and Fisher's exact test revealed significance in female mice receiving 45 mg/kg PTZ and in male mice receiving 60 mg/kg PTZ as compared to control group (middle right, Fig. 1). PTZ dose-dependently increased lethality and reached significance at a dose of 45 mg/kg in female mice and 60 mg/kg in male mice ($P < 0.01$). Regression analysis estimated that the LD₅₀ dose of PTZ was 34.4 mg/kg in female mice and 55.6 mg/kg in male mice.

In order to understand the receptor mechanism underlying the epileptogenic effects of 2-BFI and BU224, a commonly used imidazoline I₂ receptor antagonist/adrenergic α_2 receptor antagonist idazoxan was studied alone or in combination with an epileptogenic dose of 2-BFI or BU224. At a dose of 10 mg/kg that is more than adequate to block central I₂ receptors in mice and rats (Li et al., 2011; Tonello et al., 2012), idazoxan alone did not induce seizure nor lead to lethality (Table 1). When studied in combination with 40 mg/kg 2-BFI, idazoxan also did not significantly modify any of the effects of 2-BFI in both female and male mice ($P > 0.05$) (Table 1). When studied in combination with 40 mg/kg BU224, idazoxan only slightly although significantly increased the latency of seizure onset in female mice ($t [10] = 3.70$, $P < 0.01$) but showed no significant effect in male mice (Table 1). Idazoxan had no significant effect on BU224-induced seizure magnitude (% mice showing grade V seizure) or lethality in mice with either gender.

Because both 2-BFI and BU224 induced seizures in mice, we then reasoned whether they had any interactions with a known epileptogenic agent, PTZ. When studied in combination with a smaller dose of PTZ (15 mg/kg in female mice and 30 mg/kg in male mice) that alone did not induce seizure, 40 mg/kg 2-BFI further significantly decreased the latency of seizure onset ($t [17] = 5.4$, $P < 0.001$ in female mice; $t [18] = 7.9$, $P < 0.001$ in male mice) (Table 1), suggesting a potentiating effect. This combination also further increased the number of mice demonstrating grade V seizure and increased lethality in male but not in female mice (Table 1). The combination of 30 mg/kg PTZ and 30 mg/kg BU224 in male mice also decreased the latency of seizure onset ($t [19] = 6.1$, $P < 0.05$). This combination did not lead to grade V seizure or lethality (Table 1).

In order to increase the generality of the current results, the same dose range of BU224 was also studied in a different strain of mice, ICR mice (Table 2). As compared to control group, BU224 (20–40 mg/kg) dose-dependently decreased the latency of seizure onset in both male and female ICR mice ($F [3, 39] = 167.0$, $P < 0.0001$) (Table 2). Post hoc analysis revealed significant differences in groups receiving 30 mg/kg or 40 mg/kg BU224 in male and 20 mg/kg or 40 mg/kg in female ICR mice ($P < 0.05$ vs. control). In addition, the latency was

significantly shorter in female than in male mice after 20 mg/kg BU224 treatment ($P < 0.05$). There was a significant dose-dependent trend to induce grade V seizures ($P < 0.05$) both in male and in female ICR mice, and Fisher's exact test revealed a statistical significance for 40 mg/kg BU224 both in male and in female ICR mice ($P < 0.05$) when compared to control group (Table 2). All the male mice died and 6 out of 10 female mice died after receiving a dose of 40 mg/kg BU224 (Table 2).

4. Discussion

The primary findings of the current study were that both 2-BFI and BU224 dose-dependently increased seizure episodes in two strains of mice and demonstrated a significant gender difference. To our knowledge, this is the first study that shows gender differences with imidazoline I_2 receptor ligands. In addition, when studied in combination with another epileptogenic agent, PTZ, the epileptogenic effects of 2-BFI and BU224 were either further potentiated or were not changed, demonstrating a largely additive interaction. These findings are significant because 2-BFI and BU224 have long been used as prototypic I_2 receptor ligands and I_2 receptors have been increasingly considered as a promising drug target for the treatment of such disorders as pain and depression (Hudson et al., 2003; Li and Zhang, 2011; Tonello et al., 2012). Such findings could suggest the potentially serious neurological toxicity accompanying therapeutic potentials and, if proved to be related to the target, the poor safety profile of I_2 receptor ligands. However, the lack of antagonism of 2-BFI- and BU224-induced seizures by idazoxan may suggest that the epileptogenic activities observed here are I_2 receptor-independent and ligand-specific but not a general effect attributable to I_2 receptors.

Historically, imidazoline I_2 receptors are non-adrenergic binding sites that bind 3H -idazoxan with high affinity and bind 3H -para-aminoclonidine and 3H -clonidine with much lower affinity (Regunathan and Reis, 1996). Subsequent efforts to discover novel I_2 receptor ligands usually aimed at improving the binding selectivity of compounds at I_2 receptors against I_1 receptors and adrenergic α_2 receptors (Eglen *et al.*, 1998). Due to practical reasons, the binding profiles of these compounds to other receptors are usually not explored or not reported because of the increased cost to screen a big array of receptors and the essentially exclusive research interest in I_2 receptors and not other irrelevant receptors. 2-BFI and BU224 were discovered in an effort to chemically modify the structure of idazoxan to selectively increase the binding affinity to I_2 receptors and decrease the affinity to adrenergic α_2 receptors (Hudson et al., 1999; Hudson et al., 2003). Receptor binding studies have shown that 2-BFI demonstrates nearly 5000-fold selectivity at I_2 receptors over I_1 receptors and > 2500 -fold selectivity at I_2 receptors over adrenergic α_2 receptors (Hudson et al., 1997; Thorn et al., 2012), making it one of the most selective I_2 receptor ligand and an important research tool to understand the I_2 receptor pharmacology (Lione et al., 1996). BU224 is also a very selective I_2 receptor ligand (selectivity: $I_2/I_1 > 800$ -fold; $I_2/\alpha_2 > 1000$ -fold) and widely used (Hudson *et al.*, 1999). Despite these impressive selectivity data, it is unclear whether both compounds have meaningful affinities at other receptors.

Numerous *in vivo* functional studies have examined a wide range of 2-BFI and BU224 doses in rats (An et al., 2012; Hudson et al., 1997; Hudson et al., 2003; Li et al., 2011; Macinnes and Duty, 2004; MacInnes and Handley, 2002; 2003; Sampson et al., 2012; Thorn et al., 2012). However, none of these studies simultaneously examined the potential adverse effects of 2-BFI and BU224 and tests usually ended when the particular behavioral endpoints were reached. Similar situation also occurs in studies employing mice. An earlier study reported that 2-BFI but not BU224 (intracerebroventricularly, 10 μ g) enhanced morphine antinociception in mice (male ICR mice) (Sanchez-Blazquez et al., 2000). When given subcutaneously (s.c.), BU224 up to a dose of 10 mg/kg did not demonstrate

antidepressant-like activity in female BKTO mice in a forced swimming test (O'Neill et al., 2001). However, a recent study found that 2-BFI at doses of 19 and 56 mg/kg (s.c.) produces marked antidepressant-like and anxiolytic effects in male Swiss mice. Remarkably, these doses of 2-BFI do not alter the general motor activity in these mice (Tonello *et al.*, 2012). In the current study, we examined a whole range of doses of 2-BFI and BU224 in two different strains of mice in both males and females for inducing epileptic seizures. Both compounds at doses of 40 mg/kg (i.p.) consistently induced epileptic seizures both in male and in female C57/BL6 and in ICR mice, although female mice appear to be more sensitive than males. In addition, 40 mg/kg 2-BFI also evoked characteristic epileptic spikes in freely moving mice in electroencephalography measures (data not shown), in consistent with the observed seizure behaviors. The striking differences between the present study and the Tonello et al. (2012) study are intriguing, and several factors could be the potential causes, including strain differences and route of administration (i.p. in the current study vs. subcutaneous in Tonello et al. study). This study did not measure the reported beneficial effects (e.g., antinociception and antidepressant-like effects) in the same groups of mice. Regardless, the apparent neurotoxicity related to epileptogenesis of 2-BFI and BU224 markedly discounts their potential therapeutic values as the safety window (toxic dose/therapeutic dose) may be very narrow.

Importantly, the observed epileptogenic effects do not appear to be a general effect of imidazoline I₂ receptor ligands. In particular, CR4056 is a very selective I₂ receptor ligand and has no appreciable affinity to 30+ other receptors, enzymes and ion channels (Ferrari *et al.*, 2011). Up to a dose of 100-fold larger than the dose that produces marked analgesic activities, CR4056 does not show any noticeable effects in a battery of behavioral and cardiovascular assays, demonstrating an impressive safety profile (Ferrari *et al.*, 2011). Similar to 2-BFI and BU224, CR4056 produces a hypothermic effect in rats (Thorn *et al.*, 2012). Moreover, in a rat 2-BFI discrimination assay (5.6 mg/kg 2-BFI versus saline, i.p.), we found that CR4056 fully substituted for 2-BFI, clearly indicating an overlapped pharmacological mechanism between the two drugs (to be published elsewhere). Thus, the question maintains: since 2-BFI and BU224 apparently share certain (likely I₂ receptor) mechanism with CR4056, why are their safety profiles so different? One possible interpretation is that 2-BFI and BU224 produced epileptic seizures via non-I₂ or idazoxan-insensitive I₂ receptor mechanisms. It is conceivable that 2-BFI and BU224 bind to non-I₂ receptors at relatively large doses and activation of those receptors are responsible for their epileptogenic actions. This possibility is supported by the facts that a large dose of idazoxan (10 mg/kg) could not block the effects of 2-BFI and BU224 in the current study and that, at a much larger dose than analgesic doses, CR4056 does not produce epileptogenic effects (Ferrari *et al.*, 2011). We also tested the effects of 3.2 mg/kg idazoxan and, not surprisingly, this dose of idazoxan also did not block the epileptogenic actions of 2-BFI and BU224 (data not shown). Idazoxan is the only established I₂ receptor antagonist available and demonstrates I₂ receptor antagonist activity in various functional studies (Li et al., 2011; Sanchez-Blazquez et al., 2000; Tonello et al., 2012). The lack of antagonism by idazoxan in the present study was not due to inadequate dosing or quick metabolism, as we and others have shown that 3 mg/kg or even a much lower dose (0.1 mg/kg) of idazoxan are adequate to block I₂ receptor mediated effects both in rats and mice and the effect lasts for at least 2 hours (Li et al., 2011; Tonello et al., 2012).

In summary, the present study demonstrates that 2-BFI and BU224 dose-dependently produce epileptic seizures in two strains of mice with a marked gender difference. The fact that these effects could not be antagonized by the I₂ receptor antagonist idazoxan suggests a non-I₂ or idazoxan-insensitive I₂ receptor mechanism. These results discount the therapeutic values of 2-BFI and BU224 for monotherapy given their narrow therapeutic window, but do not undermine the validity of imidazoline I₂ receptors as a promising drug target for the

treatment of some neuropsychiatric disorders including depression and chronic pain. In addition, they may remain to be valuable as adjuvants in conjunction with other established medications for the treatment of pain and depression as synergistic interactions could greatly decrease the dose needed and increase the therapeutic index of these compounds (Ferrari et al., 2011; Li et al., 2011).

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Figure 1.

Effects of 2-BFI (left), BU224 (middle) and PTZ (right) in male (triangles) and female (squares) mice for seizure induction (top), inducing grade V seizure (center) and lethality (bottom). Filled symbols indicate data significantly different from control. * indicate data significantly different between male and female mice ($P < 0.05$). Each data point represents data from 10–11 mice. The latency of seizure onset data was expressed as mean $\pm$ S.E.M. In many cases the error bar cannot be seen because they are buried in the symbols. Note that the cutoff time of 600 sec does not mean that mice had seizures 600 sec later. This cutoff time is set arbitrarily and mice never had seizures within the 30-min observation period.

Table 1

Epileptogenic effects of the combination of 2-BFI or BU224 with idazoxan or PTZ in C57/BL6 mice.

Drug Doses (mg/kg)	Mice gender	Epileptogenic latency (sec)	% mice reaching grade V seizure	% Lethality
Control	M	600±0	0	0
	F	600±0	0	0
10 Idazoxan	M	600±0	0	0
	F	600±0	0	0
10 Idazoxan + 40 2-BFI	M	274.9±17.7	10	0
	F	300.6±13.8	0	0
10 Idazoxan + 40 BU224	M	191.4±11.3	40	40
	F	171.4±11.1 ^a	80	80
40 2-BFI + 15 PTZ	F	160.6±9.2 ^a	0	0
40 2-BFI + 30 PTZ	M	135.4±14.6 ^a	40 ^a	40 ^a
20 BU224 + 15 PTZ	F	241.7±21.4	0	0
30 BU224 + 30 PTZ	M	151.1±10.6 ^a	18.2	18.2

Note: n=9–11 per group.

^a indicates data significantly different from the same dose of 2-BFI or BU224 alone.

Table 2

Epileptogenic effects of BU224 in male and female ICR mice.

BU224 Doses (mg/kg)	Mice gender	Epileptogenic latency (sec)	% mice reaching grade V seizure	% Lethality
Control	M	600±0	0	0
	F	600±0	0	0
20	M	600±0	0	0
30	M	263.6±22.0	30	30
40	M	200.5±22.4	100	100
10	F	600±0	0	0
20	F	438.8±58.2	10	0
40	F	184.6±15.6	90	60

Note: n=10–11 per group.