British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2012.01894.x
www.brjpharmacol.org

RESEARCH PAPER

Characterization of the hypothermic effects of imidazoline I_2 receptor agonists in rats

David A Thorn[1], Xiao-Fei An[1,4], Yanan Zhang[2], Maria Pigini[3] and Jun-Xu Li[1]

[1]*Department of Pharmacology and Toxicology, School of Medicine and Biomedical Sciences, University at Buffalo, the State University of New York, Buffalo, NY, USA,* [2]*Research Triangle Institute, Research Triangle Park, NC, USA,* [3]*Scuola di Scienze del Farmaco e dei Prodotti della Salute, Universita di Camerino, via S. Agostino, Camerino, Italy, and* [4]*Department of Endocrinology, Jiangsu Province Hospital of Chinese Medicine, Nanjing, China*

Correspondence
Dr Jun-Xu Li, Department of Pharmacology and Toxicology, School of Medicine and Biomedical Sciences, University at Buffalo, the State University of New York, 102 Farber Hall, 3435 Main Street, Buffalo, NY 12214-3000, USA. E-mail: junxuli@buffalo.edu

Keywords
imidazoline I_2 receptor; hypothermia; locomotion; drug combination; rats

Received
7 October 2011
Revised
24 January 2012
Accepted
3 February 2012

BACKGROUND AND PURPOSE

Imidazoline I_2 receptors have been implicated in several CNS disorders. Although several I_2 receptor agonists have been described, no simple and sensitive *in vivo* bioassay is available for studying I_2 receptor ligands. This study examined I_2 receptor agonist-induced hypothermia as a functional *in vivo* assay of I_2 receptor agonism.

EXPERIMENTAL APPROACH

Different groups of rats were used to examine the effects of I_2 receptor agonists on the rectal temperature and locomotion. The pharmacological mechanisms were investigated by combining I_2 receptor ligands and different antagonists.

KEY RESULTS

All the selective I_2 receptor agonists examined (2-BFI, diphenyzoline, phenyzoline, CR4056, tracizoline, BU224 and S22687, 3.2–56 mg·kg^{-1}, i.p.) dose-dependently and markedly decreased the rectal temperature (hypothermia) in rats, with varied duration of action. Pharmacological mechanism of the observed hypothermia was studied by combining the I_2 receptor agonists (2-BFI, BU224, tracizoline and diphenyzoline) with imidazoline I_2 receptor/ α_2 adrenoceptor antagonist idazoxan, selective I_1 receptor antagonist efaroxan, α_2 adrenoceptor antagonist/5-HT_{1A} receptor agonist yohimbine. Idazoxan but not yohimbine or efaroxan attenuated the hypothermic effects of 2-BFI, BU224, tracizoline and diphenyzoline, supporting the I_2 receptor mechanism. In contrast, both idazoxan and yohimbine attenuated hypothermia induced by the α_2 adrenoceptor agonist clonidine. Among all the I_2 receptor agonists studied, only S22687 markedly increased the locomotor activity in rats.

CONCLUSIONS AND IMPLICATIONS

Imidazoline I_2 receptor agonists can produce hypothermic effects, which are primarily mediated by I_2 receptors. These data suggest that I_2 receptor agonist-induced hypothermia is a simple and sensitive *in vivo* assay for studying I_2 receptor ligands.

Abbreviations

2-BFI, 2-(2-benzofuranyl)-2-imidazoline; BU224, 2-(4, 5-dihydroimidazol-2-yl) quinolone; CR4056, 2-phenyl-6-(1H imidazol-1yl) quinazoline; diphenyzoline, 2-(2-[1,1′-biphenyl]-2ylethyl)- 4,5-dihidro-1H-imidazole; efaroxan hydrochloride, 2-ethyl-2-(imidazolin-2-yl)-2,3-dihydrobenzofuran hydrochloride; phenyzoline, 4,5-dihidro-2-(2-phenylethyl)-1H-imidazole; S22687, 5-[2-methyl phenoxy methyl] 1, 3-oxazolin-2-yl) amine; tracizoline, 2-styryl-4,5-dihydro-lH-imidazole; WAY100135 (*S*)-*N*-*tert*-butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide

Introduction

Imidazoline receptors are a group of heterogeneous receptors that are widely distributed and recognize prevalently imidazoline compounds (Regunathan and Reis, 1996; Head and Mayorov, 2006). Three different imidazoline receptors have been described: I_1 receptors are critically involved in central control of hypertension (Head and Mayorov, 2006; Nikolic and Agbaba, 2011); two I_1 receptor preferring agonists, moxonidine and rilmenidine, are clinically used to control hypertension (Sica, 2007; Edwards *et al.*, 2011); I_2 receptors are thought to be involved in neuroprotection, pain and several CNS disorders (Garcia-Sevilla *et al.*, 1999; Li and Zhang, 2011); I_3 receptors are involved in pancreatic insulin secretion (Eglen *et al.*, 1998).

Imidazoline I_2 receptors have been suggested as a potential therapeutic target for certain brain disorders. Autoradiographical studies reveal that I_2 receptors are widely distributed in the CNS, with high bindings to the area postrema, interpeduncular nucleus, arcuate nucleus, mammillary peduncle, ependyma and pineal gland (Lione *et al.*, 1998). The density of I_2 receptors in humans is dynamically altered under some disease conditions (Garcia-Sevilla *et al.*, 1999). For example, the I_2 receptor density is decreased in victims of suicide, heroin addicts and Huntington's disease patients, unaltered in Parkinson's disease patients, and markedly increased in Alzheimer's disease and glial tumour patients (Garcia-Sevilla *et al.*, 1999). In rats, chronic treatment with an antidepressant imipramine increases while treatment with heroin decreases the brain I_2 receptor density (Sastre *et al.*, 1996; Zhu *et al.*, 1997). In addition, a recently renewed interest is to target I_2 receptors for the treatment of pain conditions (Li and Zhang, 2011). For example, a selective I_2 receptor agonist, CR4056, shows promising antihyperalgesic activity for inflammatory and neuropathic pain in preclinical studies and is currently seeking phase I clinical trial (Ferrari *et al.*, 2011). This evidence points to the possibility that I_2 receptors may be functionally involved in these disorders and continued research efforts may eventually lead to novel treatment strategies.

Although I_2 receptors have not been cloned, recent studies suggest a link between I_2 receptors and AMP-activated protein kinase and PI3K-AKT signalling pathways (Lui *et al.*, 2010; Zhang *et al.*, 2012). These new developments may eventually facilitate the understanding of the I_2 receptor system. Nevertheless, currently, the identification of I_2 receptor ligands is still reliant primarily on receptor-binding studies, and receptor binding data cannot predict the *in vivo* activity of I_2 receptor ligands. Attempts have been made to develop *in vivo* bioassays for the study of I_2 receptor ligands. For example, it has been suggested that enhancement of morphine antinociception could be used to differentiate I_2 receptor agonists and antagonists (Sanchez-Blazquez *et al.*, 2000). However, given the relatively modest effects of I_2 receptor agonists on the action of morphine, it is difficult to interpret the effects of the I_2 receptor ligands in a quantitative manner with this assay. Moreover, this assay has limited sensitivity in capturing the I_2 receptor agonism activity, as only I_2 receptor agonists with high efficacy can be recognized and ligands with lower efficacy such as BU224 may be erroneously tagged as an 'antagonist' (Li and Zhang, 2011). A simple and sensitive *in vivo* assay for I_2 receptor ligands will help increase the understanding of the functional role of I_2 receptors and facilitate the rapid development of novel I_2 receptor ligands. This study reports that I_2 receptor agonists reliably decreased body temperature in a highly quantitative manner in rats, which can be used as a sensitive *in vivo* assay for studying I_2 receptor ligands.

Methods

Subjects

A total of 57 adult male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were used in this study. Rats were housed individually on a 12/12-h light/dark cycle (behavioural experiments were conducted during the light period) with free access to water and food except during experimental sessions. Animals were maintained and experiments were conducted in accordance with the Institutional Animal Care and Use Committee, University at Buffalo, the State University of New York, and with the *1996 Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, National Academy of Sciences, Washington DC).

Body temperature measurement

Body temperature was measured in a quiet procedure room maintained under identical environmental controls (temperature, humidity and lighting) with the animal colony room. Rats were habituated to the procedure room for at least 30 min before each test. Body temperature was measured by gently inserting a rectal probe (5.0 cm) and recording temperature from the digital thermometer (BAT7001H, Physitemp Instruments Inc., Clifton, NJ, USA) (Li *et al.*, 2009). Rats were handled for at least 3 days before testing drugs in order to habituate rats to the procedure.

Forty-six rats were used in the hypothermia studies. Rats were randomly assigned to eight groups with five to six rats in each. Each group of rats was generally only used for studying one agonist alone and/or in combination with antagonists, and testing was conducted no more than once per week. One group of rats was used to study the effects of phenyzoline and tracizoline, another group to study the effects of CR4056 and clonidine. During a test session, a baseline body temperature measurement was immediately followed by the injection of a dose of a drug, and the follow-up measurements were conducted every 15 min until the effect of the drug dissipated or until 3 h had passed by. A notable exception was testing the effect of high doses of tracizoline for which the measurement was continued for a total of 5 h. When a drug combination was studied, the first drug was administered 10 min before the first measurement, which was immediately followed by the administration of a second drug.

Locomotor activity

The locomotor activity of the rats was monitored by a video surveillance camera mounted on the ceiling and connected to the corresponding software (Smart Junior, Panlab SL, Barcelona, Spain). Four black acrylic boxes ($40 \times 40 \times 30$ cm,

L × W × H) were used as test arena throughout the study. Eleven rats were randomly assigned to two groups (five and six each, respectively) and were used for all the studies. Because it has been shown that a selective I_2 receptor ligand, S23229 and its stereoisomer S23230 both markedly increased the locomotor activity in rats, accompanied by the overshoot of dopamine release in the brain, it has been proposed that I_2 receptor activation stimulates locomotor activity in rats (Barrot *et al.*, 2000). Thus, this study was designed to examine the potential locomotor-stimulating effects of drugs. To fulfil this purpose, rats were habituated to the test environment for at least three sessions to minimize novelty-induced hyperlocomotion. One saline injection session was followed to allow rats to be familiar with the injection procedure and further confirm the low baseline activity. Rats were generally tested once per week. During a test session, the rats were allowed 20 min to explore the test arena, which was followed by the injection of a drug. The locomotor activity was then recorded for 2 h.

Data analyses

For the body temperature data, the relative body temperature changes (°C, mean ± SEM) were calculated by subtracting the baseline body temperature readings (first measurement of each test session) from all the subsequent measurements and plotted as a function of time or dose. The significance of the drug effects was compared with saline treatment sessions and analysed using two-way repeated measures ANOVA (time × treatment) followed by Bonferroni's *post hoc* test. The maximal changes in body temperature for each test session were also used to construct the dose–effect curves of the test drugs. The effects were analysed using one-way repeated measure ANOVA followed by Bonferroni's *post hoc* test where appropriate.

For the locomotor activity studies, the data (total locomotion counts within 2 h) were converted into percentage of saline control using the follow formula: control % = (locomotion after drug/locomotion after saline) × 100. The data were considered significantly different from saline control if the 95% confidence limits do not include 100 (Li *et al.*, 2011a).

Drugs

2-BFI hydrochloride, BU224 hydrochloride, S22687, diphenyzoline oxalate, phenyzoline oxalate, tracizoline oxalate and CR4056 were synthesized according to standard procedures (Jarry *et al.*, 1997; Pigini *et al.*, 1997; Gentili *et al.*, 2006; Ishihara and Togo, 2007; Giordani *et al.*, 2008). Clonidine hydrochloride, idazoxan hydrochloride, efaroxan hydrochloride and yohimbine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). WAY100135 hydrochloride was purchased from Tocris Bioscience (Ellisville, MO, USA). Unless otherwise noted, all drugs were dissolved in physiological saline and administered i.p. CR4056 was suspended in 5% Tween 80 and sonicated before use. It has been shown that up to 16% Tween 80 in saline does not alter the locomotor activity in rodents (Castro *et al.*, 1995). Doses are expressed as mg of the form indicated earlier kg^{-1} body weight. Injection volumes were 1 $mL \cdot kg^{-1}$.

Results

All the I_2 receptor agonists with a wide range of selectivity at I_2 receptors over I_1 receptors (8- to 4917-fold) and α_2 adrenoceptors (45- to 7431-fold, Table 1) dose-dependently and significantly decreased the body temperature (Figure 1). 2-BFI (Figure 1A), diphenyzoline (Figure 1B) and phenyzoline (Figure 1C) produced a similar hypothermic effect, with smaller doses showing little effect and larger doses (32 $mg \cdot kg^{-1}$ for 2-BFI and diphenyzoline, and 56 $mg \cdot kg^{-1}$ for phenyzoline) progressively reaching the nadir (–3.56 ± 0.17, –2.82 ± 0.31 and –3.08 ± 0.24°C for 2-BFI, diphenyzoline and phenyzoline, respectively), and the effect lasting for at least 180 min. Although tracizoline showed a similar pattern for the hypothermic effect (nadir at –2.72 ± 0.12°C;

Table 1

Binding affinities and selectivities of I_2 receptor agonists at I_1, I_2 receptors and α_2 adrenoceptors

Drug	I_1 (K_i or IC_{50}, nM)	I_2(K_i, nM)	α_2 (K_i, nM)	I_2/I_1	I_2/α_2	References
Tracizoline	19.1	1.9	14,118	10	7431	Polidori *et al.* (2000); Gentili *et al.* (2006)
2-BFI	6392*	1.3	3,736	4917	2874	Hudson *et al.* (1997)
BU224	1747	2.1	2,231	832	1062	Hudson *et al.* (1999)
Phenyzoline	3697	2.5	1,985	1479	794	Gentili *et al.* (2006; 2008)
S22687	370	45	11,000	8	244	Barrot *et al.* (2000)
Diphenyzoline	6340	158.5	7,079	40	45	Gentili *et al.* (2006; 2008)
CR4056	ND	596 (IC_{50})	N.D, but inactive at 10 μM		–	Ferrari *et al.* (2011)
Idazoxan	1259	10.6	55.4	119	5	Hudson *et al.* (1997); Eglen *et al.* (1998)
Efaroxan	52	>10,000	13	<0.005	<0.001	Eglen *et al.* (1998)

Selectivity (I_2/I_1 or I_2/α_2) was determined by comparing the K_i or IC_{50} values of the compounds on both receptors.
*Personal communication with Dr Alan L. Hudson, University of Alberta.
ND, not determined.

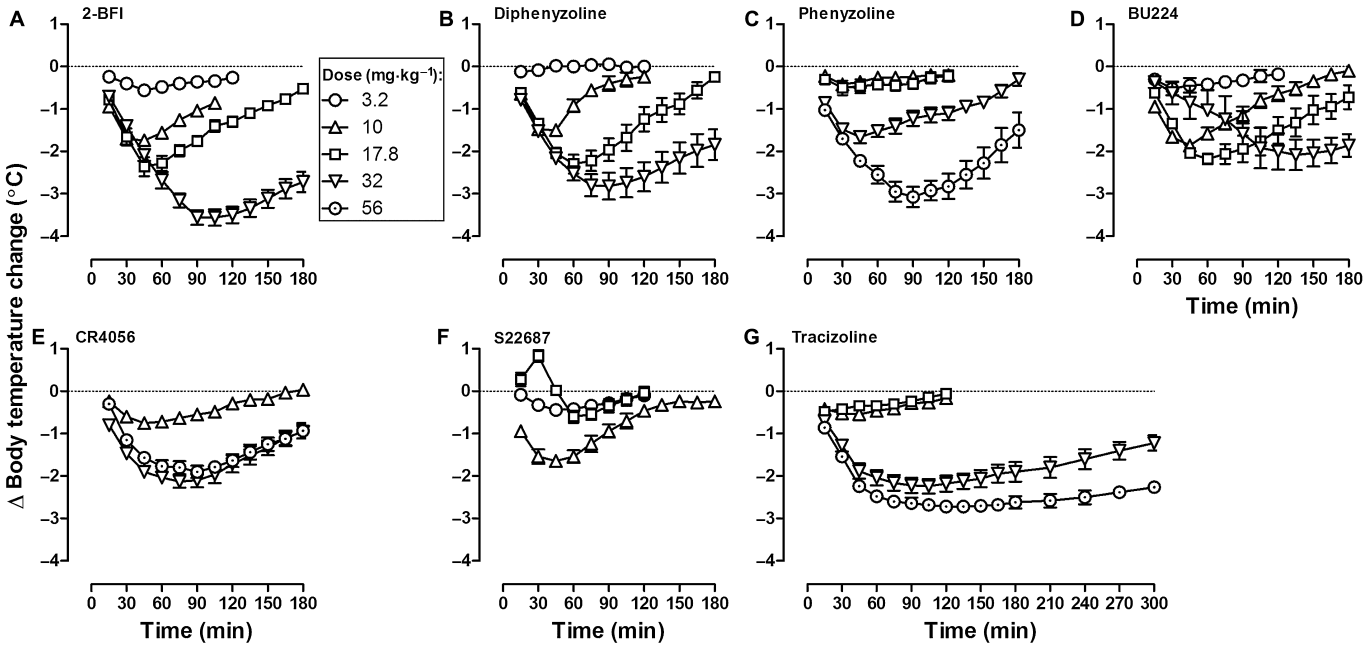

Figure 1

Effects of imidazoline I_2 receptor agonists on the body temperature in rats. Ordinates, body temperature changes (°C); Abscissa, time after drug administration (min). Each panel represents data from one compound with the drug name shown on top left of the panel.

Figure 1G), the duration of action was longer than 300 min. In contrast, BU224 showed an atypical dose–effect function (Figure 1D). At smaller doses (3.2–17.8 mg·kg^{-1}), the effect of BU224 was strikingly similar to that of 2-BFI. For example, at a dose of 17.8 mg·kg^{-1}, both 2-BFI and BU224 reached the nadir (–2.36 ± 0.22 and –2.18 ± 0.12°C for 2-BFI and BU224, respectively) 45–60 min after drug administration and the effect gradually dissipated 3 h later. However, unlike 2-BFI, 32 mg·kg^{-1} BU224 did not further decrease the body temperature but reached a similar nadir after a much longer period of time (nadir at –2.08 ± 0.36°C 135 min after drug administration). CR4056 also dose-dependently decreased the body temperature (Figure 1E); however, 32 mg·kg^{-1} CR4056 reached the nadir (–2.13 ± 0.14°C 90 min after drug administration) and increasing the dose did not further decrease the body temperature (–1.90 ± 0.14°C at a dose of 56 mg·kg^{-1}). S22687 demonstrated a different dose–effect function from any of the other I_2 receptor agonists (Figure 1F). At 10 mg·kg^{-1}, S22687 reached the hypothermic nadir (–1.64 ± 0.10°C) 45 min after drug administration and the effect lasted nearly 120 min (Figure 1F). However, at 17.8 mg·kg^{-1}, the body temperature was quickly increased (peak of 0.84 ± 0.14°C 30 min after drug injection) followed by a slight decrease (nadir of –0.60 ± 0.14°C 60 min after drug injection). The body temperature returned to pre-drug level 120 min after drug administration.

The maximal body temperature changes of the different doses were used to construct the dose–effect functions of the respective I_2 receptor agonists to facilitate visual inspection of the hypothermic effects (Figure 2). 2-BFI, diphenyzoline, phenyzoline and tracizoline produced a monotonic dose–effect function in decreasing the body temperature, with 2-BFI and diphenyzoline being somewhat more potent than phenyzoline and tracizoline. However, because the maximal effects of the drugs were unknown, the ED_{50} values could not be determined. Responses to BU224 and CR4056 reached the plateau at doses of 17.8 mg·kg^{-1} and 32 mg·kg^{-1}, respectively; while S22687 showed a clear bi-phasic dose–effect function. All the doses significantly decreased the body temperature except 3.2 mg·kg^{-1} diphenyzoline.

In order to understand the pharmacological mechanisms of the observed hypothermic effects induced by I_2 receptor agonists, the non-selective I_2 receptor/α_2 adrenoceptor antagonist idazoxan, the non-selective I_1 receptor/α_2 adrenoceptor antagonist efaroxan, and the selective 5-HT_{1A} receptor antagonist WAY100135 were combined with selected doses of I_2 receptor agonists. At a dose of 3 mg·kg^{-1}, idazoxan significantly attenuated the hypothermic effects induced by 10 mg·kg^{-1} 2-BFI (Figure 3A). Two-way ANOVA revealed significant main effects of time [F (6, 48) = 29.05, P < 0.0001] and idazoxan treatment [F (1, 48) = 46.68, P < 0.01]. In contrast, 2 mg·kg^{-1} yohimbine significantly potentiated the hypothermic effects of 2-BFI (Figure 3A). Two-way ANOVA revealed significant main effects of time [F (6, 54) = 34.35, P < 0.0001] and yohimbine treatment [F (1, 54) = 38.04, P < 0.0001]. Similar interactions were observed for BU224 (10 mg·kg^{-1}) and tracizoline (32 mg·kg^{-1}) in combination with 3 mg·kg^{-1} idazoxan or 2 mg·kg^{-1} yohimbine. For BU224, in combination with idazoxan, two-way ANOVA revealed significant main effects of time [F (7, 63) = 42.08, P < 0.0001] and idazoxan treatment [F (1, 63) = 34.60, P < 0.01]. For BU224, in combination with yohimbine, two-way ANOVA revealed significant main effects of time [F (11, 99) = 45.86, P < 0.0001] and yohimbine treatment [F (1, 99) = 38.05, P < 0.0001]. For tracizoline, in combination with idazoxan, two-way ANOVA revealed significant main effects of time [F (15, 135) = 13.77,

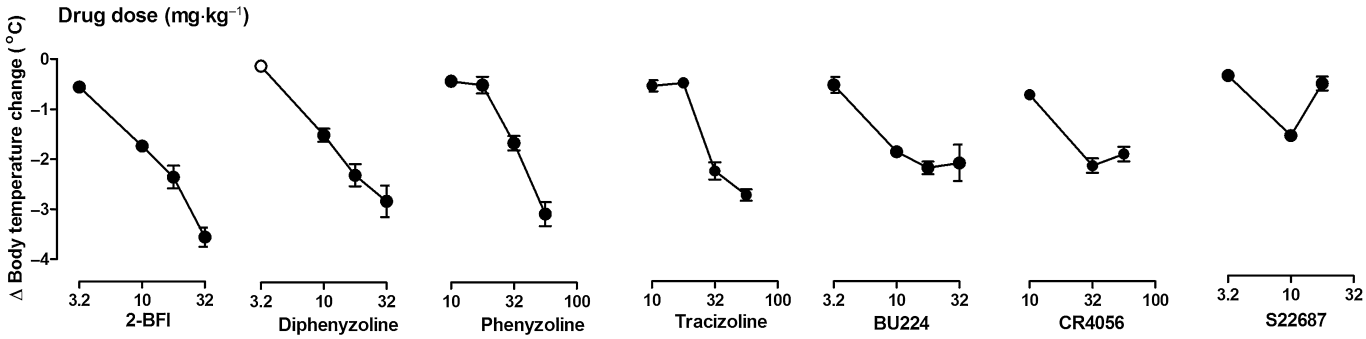

Figure 2

Dose–response functions for body temperature changes induced by I_2 receptor agonists. Each data point represents the maximal body temperature change from the tested dose of the drug. Filled symbols indicated significantly different from saline control. See Figure 1 for other details.

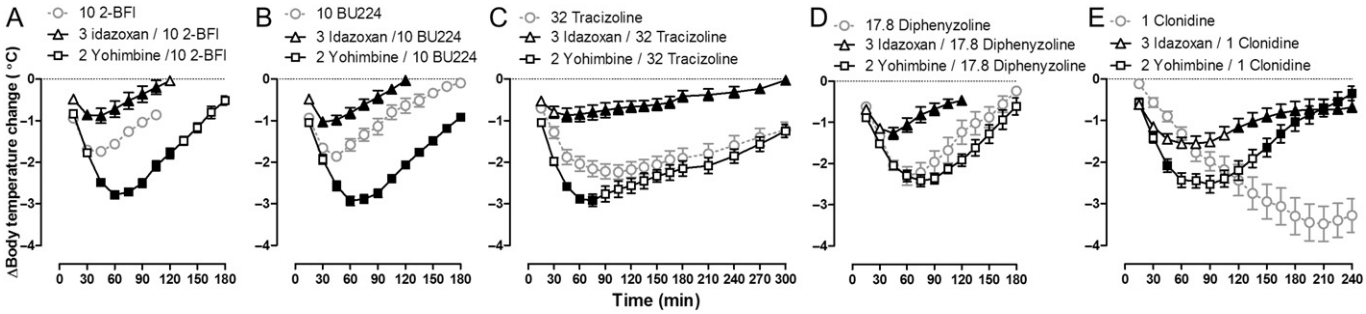

Figure 3

Effects of idazoxan (3 mg·kg^{-1}) and yohimbine (2 mg·kg^{-1}) on the hypothermic activities of 2-BFI (A), BU224 (B), tracizoline (C), diphenyzoline (D) and clonidine (E). Idazoxan and yohimbine were administered 10 min before each I_2 receptor agonist. Filled symbols indicated significantly different from the effect of I_2 receptor agonist alone. See Figure 1 for other details.

$P < 0.0001$], idazoxan treatment [F (1, 135) = 61.48, $P < 0.001$], and time × idazoxan treatment interaction [F (15, 135) = 5.72, $P < 0.0001$]. For tracizoline, in combination with yohimbine, two-way ANOVA revealed significant main effects of time [F (15, 135) = 53.30, $P < 0.0001$] and time × yohimbine treatment interaction [F (15, 135) = 2.99, $P < 0.0001$]. Idazoxan (3 mg·kg^{-1}) also significantly attenuated the hypothermic effects of diphenyzoline (17.8 mg·kg^{-1}) (Figure 3D, solid triangles); however, a combination of 2 mg·kg^{-1} yohimbine with diphenyzoline induced the hypothermia that was not different from that produced by diphenyzoline alone (Figure 3D). Two-way ANOVA revealed significant main effects of time [F ([7, 63) = 24.75, $P < 0.0001$], idazoxan treatment [F (1, 63) = 36.30, $P < 0.01$), and time × idazoxan treatment interaction [F (7, 63) = 12.84, $P < 0.0001$] for the combination of diphenyzoline and idazoxan. For the combination of diphenyzoline and yohimbine, two-way ANOVA only indicated a significant main effect of time [F (11, 99) = 67.46, $P < 0.0001$].

Clonidine (1 mg·kg^{-1}) significantly decreased the body temperature and this reached a nadir (–3.48 ± 0.42°C) 210 min after drug administration. Both 3 mg·kg^{-1} idazoxan and 2 mg·kg^{-1} yohimbine significantlty attenuated the hypothermic effect of clonidine (Figure 3E). For clonidine, in combination with idazoxan, two-way ANOVA revealed significant main effects of time [F (15, 15) = 16.22, $P < 0.0001$], idazoxan treatment [F (1, 150) = 28.36, $P < 0.01$], and time × idazoxan treatment interaction [F (15, 150) = 28.32, $P < 0.0001$]. For clonidine, in combination with yohimbine, two-way ANOVA revealed significant main effects of time [F (15, 150) = 20.39, $P < 0.0001$], yohimbine treatment [F (1, 150) = 10.98, $P < 0.05$], and time × yohimbine treatment interaction [F (15, 150) = 41.25, $P < 0.0001$].

2-BFI and BU224 were also studied in combination with non-selective I_1 receptor/α_2 adrenoceptor antagonist efaroxan or selective 5-HT_{1A} receptor antagonist WAY100135 (Figure 4). Efaroxan at a dose of 1 mg·kg^{-1} slightly but significantly potentiated the hypothermic effects of 10 mg·kg^{-1} 2-BFI (Figure 4A). Two-way ANOVA revealed significant main effect of time [F (6, 54) = 69.19, $P < 0.0001$] and time × efaroxan treatment interaction [F (6, 54) = 6.03, $P < 0.0001$], but the main effect of efaroxan treatment [F (1, 54) = 3.09, $P > 0.05$] did not reach statistical significance. However, for the WAY100135 (2 mg·kg^{-1}) + 2-BFI combination, two-way ANOVA only found statistical significance for time [F (6, 54) = 80.37, $P < 0.0001$]. Similarly, 1 mg·kg^{-1} efaroxan also significantly potentiated the hypothermic effects of 10 mg·kg^{-1} BU224 (Figure 4B). Two-way ANOVA

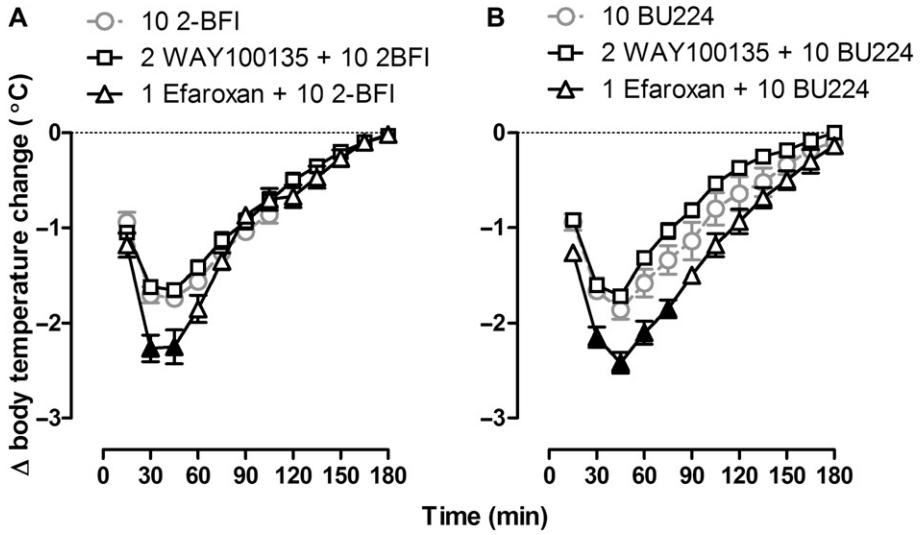

Figure 4

Effects of 1 mg·kg^{-1} efaroxan or 2 mg·kg^{-1} WAY100135 on the hypothermic activities of 2-BFI (A) and BU224 (B). See Figures 1 and 3 for other details.

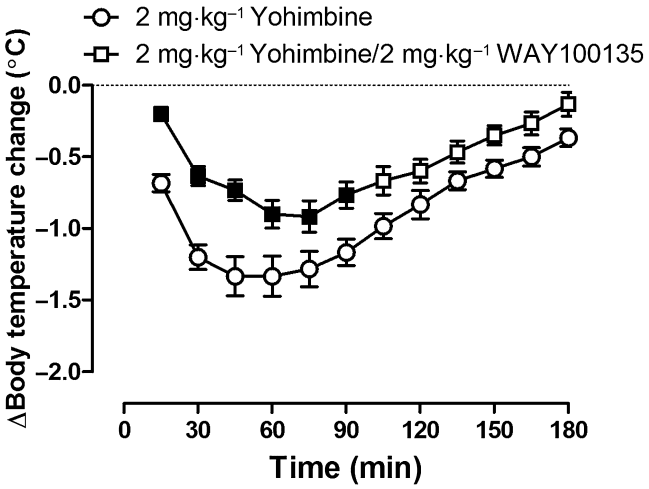

Figure 5

Effects of 2 mg·kg^{-1} yohimbine alone or in combination with 2 mg·kg^{-1} WAY100135 on the body temperature in rats. All data points for yohimbine alone were significantly different from saline control. Filled symbols indicated significantly different from the effect of yohimbine alone. See Figure 1 for other details.

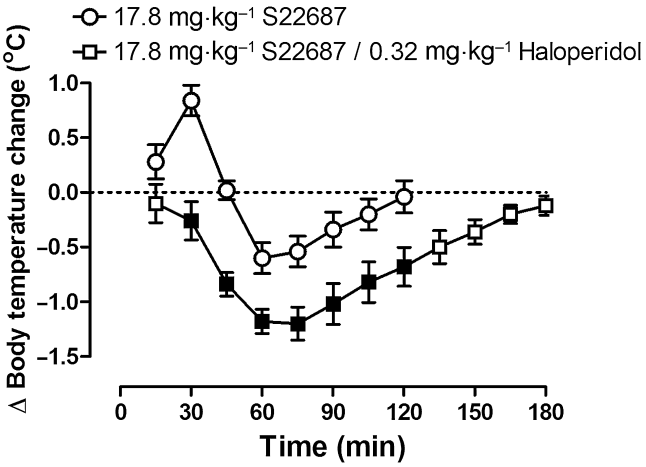

Figure 6

Effects of 17.8 mg·kg^{-1} S22687 alone or in combination with 0.32 mg·kg^{-1} haloperidol on the body temperature in rats. Filled symbols indicated significantly different from the effect of S22687 alone. See Figure 1 for other details.

revealed significant main effect of time [F (11, 99) = 79.78, P < 0.0001], eraroxan treatment [F (1, 99) = 5.01, P < 0.05], and time × efaroxan treatment interaction [F (1, 99) = 1.33, P < 0.01]. However, for the WAY100135 + BU224 combination, two-way ANOVA only found statistical significance for time [F (11, 99) = 85.93, P < 0.0001].

Yohimbine (2 mg·kg^{-1}) significantly decreased the body temperature in rats (Figure 5). This effect was significantly attenuated by 2 mg·kg^{-1} WAY100135. Two-way ANOVA revealed significant main effects of time [F (11, 110) = 52.66, P < 0.0001], WAY100135 treatment [F (1, 110) = 20.12, P < 0.01], and time × WAY100135 treatment interaction [F (11, 110) = 2.80, P < 0.001].

Although 0.32 mg·kg^{-1} haloperidol alone did not alter the body temperature (data not shown), it significantly decreased the body temperature changes induced by 17.8 mg·kg^{-1} S22687 (Figure 6, filled squares). Thus, in the presence of 0.32 mg·kg^{-1} haloperidol, the bi-phasic nature of 17.8 mg·kg^{-1} S22687-induced body temperature changes was no longer evident. In fact, S22687 produced a monotonic hypothermic effect, similar to that evoked by 10 mg·kg^{-1} S22687 (compare triangles in Figure 1F with squares in Figure 6). Two-way

Table 2

Effects of I_2 receptor agonists on the locomotor activity in rats

Drug dose (mg·kg^{-1})	Locomotion (% vehicle ± 95% confidence limit)
2-BFI	
10	86.2 (65.7, 106.7)
17.8	95.7 (50.2, 141.2)
Diphenyzoline	
10	111.1 (66.1, 156.1)
32	83.3 (76.6, 89.9)
Phenyzoline	
10	126.6 (76.2, 177.0)
32	92.7 (71.3, 114.1)
Tracizoline	
10	159.2 (125.7, 192.8)*
32	109.0 (85.4, 132.5)
BU224	
10	107.7 (80.9, 134.5)
17.8	73.4 (39.7, 107.1)
CR4056	
10	71.2 (57.3, 85.0)*
32	65.6 (44.8, 86.5)*
S22687	
10	269.4 (133.3, 405.5)*
17.8	755.4 (429.2, 1081.6)*
32	239.1 (152.7, 325.5)*

*Indicates data significantly different from saline control.

ANOVA revealed significant main effects of time [F (7, 56) = 41.76, $P < 0.0001$], haloperidol treatment [F (1, 56) = 31.39, $P < 0.01$], and time × haloperidol treatment interaction [F (7, 56) = 2.58, $P < 0.01$].

At the doses that significantly altered the body temperature (10 and 17.8 mg·kg^{-1}), S22687 markedly increased the locomotor activity (Table 2). Further increasing the dose of S22687 to 32 mg·kg^{-1} produced a hyperlocomotive effect that was lower than that of 17.8 mg·kg^{-1}, thus demonstrating a typical bell-shaped dose–effect curve. At the doses that significantly decreased the body temperature, 2-BFI, BU224, phenyzoline and diphenzyline did not significantly alter the locomotor activity in rats (Table 2). Although tracizoline slightly increased the locomotor activity at a dose of 10 mg·kg^{-1}, this effect was not dose-dependent as after a larger dose (32 mg·kg^{-1}) of tracizoline, the locomotor activity was not different from that of vehicle treatment. CR4056 significantly decreased the locomotor activity at doses that markedly decreased the body temperature.

Discussion

The primary findings of the current studies were that compounds selectively binding to imidazoline I_2 receptors consistently and dose-dependently decreased the body temperature in rats, and the effects were antagonized by the imidazoline I_2 receptor antagonist/α_2 adrenoceptor antagonist idazoxan, but not by the I_1 receptor antagonist/α_2 adrenoceptor antagonist efaroxan or 5-HT_{1A} receptor antagonist WAY100135. The hypothermic effects were further enhanced by the α_2 adrenoceptor antagonist/5-HT_{1A} receptor agonist yohimbine. The hypothermic effects of yohimbine were blocked by the selective 5-HT_{1A} receptor antagonist WAY100135, which most likely accounted for the observed enhancement of I_2 receptor ligands-induced hypothermia, therefore, the effects of idazoxan can only be interpreted as I_2 receptor antagonism. Collectively, these results suggest that activation of imidazoline I_2 receptors produces hypothermia, and consequently, this offers a validated and simple *in vivo* assay for understanding the neuropharmacology of the I_2 receptor system and facilitating the development of new I_2 receptor ligands.

The concept of imidazoline receptors has been proposed and studied for nearly two decades (Eglen *et al.*, 1998). Major progress has been made in understanding the I_1 receptor system and drugs that primarily act on I_1 receptors are clinically used for the treatment of hypertension and other chronic disorders (Nikolic and Agbaba, 2011). However, the understanding of I_2 receptors has long been hampered by the lack of valid functional assays and selective ligands. Over the years, several selective I_2 receptor ligands have been developed such as RS-45041-190, 2-BFI, BU224, tracizoline and LSL60101 (Alemany *et al.*, 1995; Brown *et al.*, 1995; Hudson *et al.*, 2003; Gentili *et al.*, 2006). These compounds have been valuable research tools to facilitate the better understanding of I_2 receptors. However, pharmacological selectivity of those compounds has only been demonstrated in *in vitro* receptor binding studies and has not been verified in *in vivo* assays, primarily because reliable *in vivo* functional bioassays related to I_2 receptor agonism are lacking. I_2 receptor activation has been suggested to produce hyperphagia in rats (Brown *et al.*, 1995; Polidori *et al.*, 2000). This effect waits to be elucidated as no pharmacological antagonism was attempted in previous studies and there are no data to confirm that the observed hyperphagic effects were truly mediated by I_2 receptors. Studies with selective I_2 receptor ligands on antidepressant-like effects have yielded mixed results (O'Neill *et al.*, 2001; Hudson *et al.*, 2003). It has been suggested that modulation of morphine analgesia may be used as an assay for the detection of ligands with I_2 receptor activity (Sanchez-Blazquez *et al.*, 2000). Although this assay is useful, the reading of any effect has to rely on the pharmacological effect of another drug, which complicates the interpretation of the results. Moreover, the modest interaction preludes generating orderly and highly quantitative data. Collectively, there is no *in vivo* functional assay that can easily capture compounds with imidazoline I_2 receptor activity.

In this study, several imidazoline I_2 receptor agonists with varied pharmacological selectivity for I_2 receptors over I_1 receptors (range of selectivity: 8- to 4917-fold) and α_2 adrenoceptors (range of selectivity: 45- to 7431-fold) were examined for their effects in body temperature (Table 1). Without exception, all the compounds showed robust and dose-dependent hypothermic effects, although the maximal effect and duration of action seemed to vary across the drugs. It was apparent that BU224 and CR4056 reached the plateau of the

hypothermic effect that was smaller than the effect produced by 2-BFI and phenyzoline. This could be due to either limited efficacy at I_2 receptors, or drug actions on another mechanism that counteract their effect on I_2 receptors (e.g. a second mechanism produces hyperthermic activity), or both. Although it was unclear which mechanisms accounted for the effects of BU224 and CR4056, the unexpected pattern of the duration of action of 32 mg·kg^{-1} BU224 (Figure 1D) suggested that at this high dose BU224 may act on another unidentified receptor that partially reversed its hypothermic effect. Another unexpected finding was that S22687 at a dose of 17.8 mg·kg^{-1} first increased the body temperature followed by a slight decrease of the body temperature. This dose of S22687 also markedly increased the locomotor activity in the rats (Table 2), an effect secondary to central dopamine release (Barrot *et al.*, 2000). It was postulated that the hyperlocomotion may increase the body temperature, which in turn counteracts S22687-induced hypothermia. A dose of 0.32 mg·kg^{-1} of the non-selective dopamine D_1/D_2 receptor antagonist haloperidol completely reversed the biphasic pattern of S22687-induced body temperature changes (Figure 5). This dose of haloperidol is sufficient to block a large population of dopamine D_1/D_2 subtype receptors and inhibits behavioural (e.g. hyperlocomotion, discriminative stimulus) effects of indirect-acting dopamine receptor agonists such as methamphetamine and cocaine (Costanza *et al.*, 2001; Steed *et al.*, 2011). This effect is unlikely to be a common mechanism of I_2 receptor drugs but rather mediated by non-imidazoline receptor mechanisms, as S22687 was the only I_2 receptor ligand that increased locomotor activity and other drugs with higher selectivity on I_2 receptors did not change the locomotor activity up to doses that markedly decreased the body temperature in rats (Table 2).

The I_2 receptor mechanism of the hypothermia induced by the compounds examined was confirmed by drug combination studies. There are currently no selective I_2 receptor antagonists available and idazoxan is frequently used as an I_2 receptor antagonist. Idazoxan non-selectively binds to both I_2 receptors and α_2 adrenoceptors (Table 1) and previous studies have demonstrated that idazoxan can block the antinociception induced by 2-BFI and BU224 (Li *et al.*, 2011b), attenuate the effects of 2-BFI, LSL60101 and phenyzoline for their potentiation of morphine-induced antinociception (Sanchez-Blazquez *et al.*, 2000; Gentili *et al.*, 2006), and inhibit CR4056-induced antinociception (Ferrari *et al.*, 2011). Consistent with previous studies, this study found that idazoxan significantly prevented the hypothermic effects of 2-BFI, BU224, tracizoline and diphenyzoline. However, because idazoxan also binds to α_2 adrenoceptors and is widely used as an α_2 adrenoceptor antagonist (Bill *et al.*, 1989; Dekeyne and Millan, 2006; Gamo *et al.*, 2010), the effects of a selective α_2 adrenoceptor antagonist yohimbine were also studied in combination with the I_2 receptor ligands to exclude the potential α_2 adrenoceptor mechanism. Surprisingly, yohimbine markedly potentiated the hypothermic effects of 2-BFI, BU224 and tracizoline (Figure 3). Yohimbine also binds to 5-HT_{1A} receptors and has been reported to produce hypothermia in rats (Dilsaver and Davidson, 1989; Winter and Rabin, 1992). Because activation of 5-HT_{1A} receptors produces hypothermia (Li *et al.*, 2009), we reasoned that yohimbine may produce hypothermia by activating 5-HT_{1A} receptors and the observed potentiation of the hypothermia induced by the I_2 receptor ligands might be due to the concurrent activation of I_2 receptors and 5-HT_{1A} receptors. Indeed, yohimbine alone markedly decreased body temperature, and the effect was antagonized by a selective 5-HT_{1A} receptor antagonist, WAY100135 (Przegalinski *et al.*, 1994). Interestingly, the hypothermic effect induced by yohimbine in combination with diphenyzoline was not different from that induced by diphenyzoline alone, which indicates that yohimbine partially blocked the hypothermic effects of diphenyzoline. Given that diphenyzoline only has 45-fold selectivity for I_2 receptors over α_2 adrenoceptors (Table 1), it is conceivable that the hypothermic effect of diphenyzoline was a congruent effect of activating both receptors. The α_2 adrenoceptor agonist clonidine was studied for comparison purposes. Many effects of clonidine, including its hypothermic effects, are blocked by α_2 adrenoceptor antagonists (Junnarkar and Singh, 1988; Halliday *et al.*, 1991). In contrast to the I_2 receptor ligands, the hypothermic effect of clonidine was markedly inhibited by both idazoxan and yohimbine, confirming the α_2 adrenoceptor mechamism.

Activation of I_1 receptors has also been shown to decrease body temperature (Cambridge and Robinson, 2005). However the hypothermic effects observed in this study are unlikely to be due to I_1 receptor agonism both because most I_2 receptor ligands have low affinity at I_1 receptors (nM vs. μM, Table 1) and because the I_1 receptor antagonist/α_2 adrenoceptor antagonist efaroxan, at a dose that significantly blocks the antinociceptive effects of a selective I_1 receptor agonist moxonidine (Shannon and Lutz, 2000), did not attenuate the hypothermic effects of I_2 receptor agonists (Figure 4). In fact, efaroxan slightly potentiated the hypothermic effects, which could be due to its 5-HT_{1A} receptor partial agonist property (Kleven *et al.*, 2005). Although it has been shown that I_2 receptor ligands also modulate brain 5-HT turnover (Hudson *et al.*, 1999) and 5-HT_{1A} receptor agonism decreases body temperature, the observed effects cannot be attributed to 5-HT_{1A} receptor activation, because the selective 5-HT_{1A} receptor antagonist WAY100135 did not attenuate 2-BFI- and BU224-induced hypothermia (Figure 4).

In the present study, BU224 decreased the body temperature to a maximum of –2.18°C, which was significantly lower than that produced by 2-BFI (–3.56°C). This is consistent with the literature suggesting the low-efficacy nature of BU224. Previous studies suggest that the effect of BU224 is assay-dependent. For example, BU224 similar to 2-BFI induces acute nociception in a writhing test and increases locomotion in nigrostriatal-lesioned rats (Macinnes and Duty, 2004; Li *et al.*, 2011b). However, BU224 prevents 2-BFI-induced enhancement of morphine antinociception in tail flick tests (Sanchez-Blazquez *et al.*, 2000; Thorn *et al.*, 2011), demonstrating I_2 receptor antagonist effects. This assay-dependency is in parallel with the profile of a partial agonist (or preferably low-efficacy agonist), and suggests that the efficacy demand of these assays is different. In this regard, body temperature change seems to have a moderate efficacy demand such that BU224 produces an effect that is smaller than a higher-efficacy agonist 2-BFI.

In summary, this study reported firstly that compounds selective for I_2 receptors produced hypothermic effects by activating I_2 receptors in rats. In combination with antago-

nism studies, this assay can be a useful and simple *in vivo* functional assay for studying I_2 receptor ligands and furthering the understanding of the functional significance of I_2 receptor systems. This study also demonstrated that activation of I_2 receptors does not consistently produce hyperlocomotion in rats and suggests that the previous assertion that I_2 receptor agonists may have abuse liability (Barrot *et al.*, 2000) requires further evaluation. This is particularly relevant as drugs acting on I_2 receptors may have important therapeutic potential for several neuropsychiatric disorders including pain and neuroprotection for ischaemia and brain injury (Qiu and Zheng, 2006; Li and Zhang, 2011).

Acknowledgements

The authors thank Dr Jerrold Winter, Department of Pharmacology and Toxicology, University at Buffalo, for the constructive discussions during the course of this study.

Conflict of interest

None.

References

Alemany R, Olmos G, Escriba PV, Menargues A, Obach R, Garcia-Sevilla JA (1995). LSL 60101, a selective ligand for imidazoline I2 receptors, on glial fibrillary acidic protein concentration. Eur J Pharmacol 280: 205–210.

Barrot M, Rettori MC, Guardiola-Lemaitre B, Jarry C, Le Moal M, Piazza PV (2000). Interactions between imidazoline binding sites and dopamine levels in the rat nucleus accumbens. Eur J Neurosci 12: 4547–4551.

Bill DJ, Hughes IE, Stephens RJ (1989). The thermogenic actions of alpha 2-adrenoceptor agonists in reserpinized mice are mediated via a central postsynaptic alpha 2-adrenoceptor mechanism. Br J Pharmacol 96: 133–143.

Brown CM, MacKinnon AC, Redfern WS, Williams A, Linton C, Stewart M *et al.* (1995). RS-45041-190: a selective, high-affinity ligand for I2 imidazoline receptors. Br J Pharmacol 116: 1737–1744.

Cambridge N, Robinson ES (2005). Effect of BU98008, an imidazoline1-binding site ligand, on body temperature in mice. Eur J Pharmacol 519: 86–90.

Castro CA, Hogan JB, Benson KA, Shehata CW, Landauer MR (1995). Behavioral effects of vehicles: DMSO, ethanol, Tween-20, Tween-80, and emulphor-620. Pharmacol Biochem Behav 50: 521–526.

Costanza RM, Barber DJ, Terry P (2001). Antagonism of the discriminative stimulus effects of cocaine at two training doses by dopamine D2-like receptor antagonists. Psychopharmacology (Berl) 158: 146–153.

Dekeyne A, Millan MJ (2006). Discriminative stimulus properties of the selective and highly potent alpha2-adrenoceptor agonist, S18616, in rats: mediation by the alpha2A subtype, and blockade by the atypical antidepressants, mirtazapine and mianserin. Neuropharmacology 51: 718–726.

Dilsaver SC, Davidson RK (1989). Chronic treatment with amitriptyline produces subsensitivity to the hypothermic effects of yohimbine. Prog Neuropsychopharmacol Biol Psychiatry 13: 211–215.

Edwards LP, Brown-Bryan TA, McLean L, Ernsberger P (2011). Pharmacological properties of the central antihypertensive agent, moxonidine. Cardiovasc Ther DOI: 10.1111/j.1755-5922.2011.00268.x. [Epub ahead of print].

Eglen RM, Hudson AL, Kendall DA, Nutt DJ, Morgan NG, Wilson VG *et al.* (1998). 'Seeing through a glass darkly': casting light on imidazoline 'I' sites. Trends Pharmacol Sci 19: 381–390.

Ferrari F, Fiorentino S, Mennuni L, Garofalo P, Letari O, Mandelli S *et al.* (2011). Analgesic efficacy of CR4056, a novel imidazoline-2 receptor ligand, in rat models of inflammatory and neuropathic pain. J Pain Res 4: 111–125.

Gamo NJ, Wang M, Arnsten AF (2010). Methylphenidate and atomoxetine enhance prefrontal function through alpha2-adrenergic and dopamine D1 receptors. J Am Acad Child Adolesc Psychiatry 49: 1011–1023.

Garcia-Sevilla JA, Escriba PV, Guimon J (1999). Imidazoline receptors and human brain disorders. Ann N Y Acad Sci 881: 392–409.

Gentili F, Cardinaletti C, Carrieri A, Ghelfi F, Mattioli L, Perfumi M *et al.* (2006). Involvement of I2-imidazoline binding sites in positive and negative morphine analgesia modulatory effects. Eur J Pharmacol 553: 73–81.

Gentili F, Cardinaletti C, Vesprini C, Ghelfi F, Farande A, Giannella M *et al.* (2008). Novel ligands rationally designed for characterizing I2-imidazoline binding sites nature and functions. J Med Chem 51: 5130–5134.

Giordani A, Lanza M, Caselli G, Mandelli S, Zanzola S, Makovec F *et al.* (2008). 6-1H-Imidazo-quinazoline and quinolines derivatives, new MAO inhibitors and imidazoline receptor ligands. *USA: Intl-Pat. WO 2009/152868A1, Rottapharm S.P.A.*

Halliday CA, Jones BJ, Skingle M, Walsh DM, Wise H, Tyers MB (1991). The pharmacology of fluparoxan: a selective alpha 2-adrenoceptor antagonist. Br J Pharmacol 102: 887–895.

Head GA, Mayorov DN (2006). Imidazoline receptors, novel agents and therapeutic potential. Cardiovasc Hematol Agents Med Chem 4: 17–32.

Hudson AL, Chapleo CB, Lewis JW, Husbands S, Grivas K, Mallard NJ *et al.* (1997). Identification of ligands selective for central I2-imidazoline binding sites. Neurochem Int 30: 47–53.

Hudson AL, Gough R, Tyacke R, Lione L, Lalies M, Lewis J *et al.* (1999). Novel selective compounds for the investigation of imidazoline receptors. Ann N Y Acad Sci 881: 81–91.

Hudson AL, Tyacke RJ, Lalies MD, Davies N, Finn DP, Marti O *et al.* (2003). Novel ligands for the investigation of imidazoline receptors and their binding proteins. Ann N Y Acad Sci 1009: 302–308.

Ishihara M, Togo H (2007). Direct oxidative conversion of aldehydes and alcohols to 2-imidazolines and 2-oxazolines using molecular iodine. Tetrahedron 63: 1474–1480.

Jarry C, Forfar I, Bosc J, Renard P, Scalbert E, Guardiola B (1997). 5-(arloxymethyl)oxazolines. *USA: US-Pat. 5,686,477, Adir et Compagnie.*

Junnarkar AY, Singh PP (1988). Antagonism of clonidine-induced hypothermia by alpha adrenoceptor antagonists in electrically stimulated mice. Pharmacol Res Commun 20: 451–463.

Kleven MS, Assie MB, Cosi C, Barret-Grevoz C, Newman-Tancredi A (2005). Anticataleptic properties of alpha2 adrenergic antagonists in the crossed leg position and bar tests: differential mediation by 5-HT1A receptor activation. Psychopharmacology (Berl) 177: 373–380.

Li JX, Zhang Y (2011). Imidazoline I2 receptors: target for new analgesics? Eur J Pharmacol 658: 49–56.

Li JX, Koek W, France CP (2009). Food restriction and streptozotocin differentially modify sensitivity to the hypothermic effects of direct- and indirect-acting serotonin receptor agonists in rats. Eur J Pharmacol 613: 60–63.

Li JX, Crocker C, Koek W, Rice KC, France CP (2011a). Effects of serotonin (5-HT)1A and 5-HT2A receptor agonists on schedule-controlled responding in rats: drug combination studies. Psychopharmacology (Berl) 213: 489–497.

Li JX, Zhang Y, Winter JC (2011b). Morphine-induced antinociception in the rat: supra-additive interactions with imidazoline I receptor ligands. Eur J Pharmacol 669: 59–65.

Lione LA, Nutt DJ, Hudson AL (1998). Characterisation and localisation of [3H]2-(2-benzofuranyl)-2-imidazoline binding in rat brain: a selective ligand for imidazoline I2 receptors. Eur J Pharmacol 353: 123–135.

Lui TN, Tsao CW, Huang SY, Chang CH, Cheng JT (2010). Activation of imidazoline I2B receptors is linked with AMP kinase pathway to increase glucose uptake in cultured C2C12 cells. Neurosci Lett 474: 144–147.

Macinnes N, Duty S (2004). Locomotor effects of imidazoline I2-site-specific ligands and monoamine oxidase inhibitors in rats with a unilateral 6-hydroxydopamine lesion of the nigrostriatal pathway. Br J Pharmacol 143: 952–959.

Nikolic K, Agbaba D (2011). Imidazoline antihypertensive drugs: selective I(1) -imidazoline receptors activation. Cardiovasc Ther DOI: 10.1111/j.1755-5922.2011.00269.x. [Epub ahead of print].

O'Neill MF, Osborne DJ, Woodhouse SM, Conway MW (2001). Selective imidazoline I2 ligands do not show antidepressant-like activity in the forced swim test in mice. J Psychopharmacol 15: 18–22.

Pigini M, Bousquet P, Carotti A, Dontenwill M, Giannella M, Moriconi R *et al*. (1997). Imidazoline receptors: qualitative structure-activity relationships and discovery of tracizoline and benazoline. Two ligands with high affinity and unprecedented selectivity. Bioorg Med Chem 5: 833–841.

Polidori C, Gentili F, Pigini M, Quaglia W, Panocka I, Massi M (2000). Hyperphagic effect of novel compounds with high affinity for imidazoline I(2) binding sites. Eur J Pharmacol 392: 41–49.

Przegalinski E, Filip M, Budziszewska B, Chojnacka-Wojcik E (1994). Antagonism of (+)WAY 100135 to behavioral, hypothermic and corticosterone effects induced by 8-OH-DPAT. Pol J Pharmacol 46: 21–27.

Qiu WW, Zheng RY (2006). Neuroprotective effects of receptor imidazoline 2 and its endogenous ligand agmatine. Neurosci Bull 22: 187–191.

Regunathan S, Reis DJ (1996). Imidazoline receptors and their endogenous ligands. Annu Rev Pharmacol Toxicol 36: 511–544.

Sanchez-Blazquez P, Boronat MA, Olmos G, Garcia-Sevilla JA, Garzon J (2000). Activation of I(2)-imidazoline receptors enhances supraspinal morphine analgesia in mice: a model to detect agonist and antagonist activities at these receptors. Br J Pharmacol 130: 146–152.

Sastre M, Ventayol P, Garcia-Sevilla JA (1996). Decreased density of I2-imidazoline receptors in the postmortem brain of heroin addicts. Neuroreport 7: 509–512.

Shannon HE, Lutz EA (2000). Effects of the I(1) imidazoline/alpha(2)-adrenergic receptor agonist moxonidine in comparison with clonidine in the formalin test in rats. Pain 85: 161–167.

Sica DA (2007). Centrally acting antihypertensive agents: an update. J Clin Hypertens (Greenwich) 9: 399–405.

Steed E, Jones CA, McCreary AC (2011). Serotonergic involvement in methamphetamine-induced locomotor activity: a detailed pharmacological study. Behav Brain Res 220: 9–19.

Thorn DA, Zhang Y, Peng BW, Winter JC, Li JX (2011). Effects of imidazoline I receptor ligands on morphine- and tramadol-induced antinociception in rats. Eur J Pharmacol 670: 435–440.

Winter JC, Rabin RA (1992). Yohimbine as a serotonergic agent: evidence from receptor binding and drug discrimination. J Pharmacol Exp Ther 263: 682–689.

Zhang F, Ding T, Yu L, Zhong Y, Dai H, Yan M (2012). Dexmedetomidine protects against oxygen-glucose deprivation-induced injury through the I2 imidazoline receptor-PI3K/AKT pathway in rat C6 glioma cells. J Pharm Pharmacol 64: 120–127.

Zhu H, Paul IA, McNamara M, Redmond A, Nowak G, Piletz JE (1997). Chronic imipramine treatment upregulates IR2-imidazoline receptive sites in rat brain. Neurochem Int 30: 101–107.