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Inhibition of brain [³H]cimetidine binding by improgan-like antinociceptive drugs

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Abstract

[³H]Cimetidine, a radiolabeled histamine H₂ receptor antagonist, binds with high affinity to an unknown hemoprotein in the brain which is not the histamine H₂ receptor. Improgan, a close chemical congener of cimetidine, is a highly effective pain-relieving drug following CNS administration, yet its mechanism of action remains unknown. To test the hypothesis that the [³H]cimetidine-binding site is the improgan antinociceptive target, improgan, cimetidine, and 8 other chemical congeners were studied as potential inhibitors of [³H]cimetidine binding in membrane fractions from the rat brain. All compounds produced a concentration-dependent inhibition of [³H]cimetidine binding over a 500-fold range of potencies (K_i values were 14.5 to >8,000 nM). However, antinociceptive potencies in rats did not significantly correlate with [³H]cimetidine-binding affinities (r = 0.018, p = 0.97, n = 10). These results suggest that the [³H]cimetidine-binding site is not the analgesic target for improgan-like drugs.

1. Introduction

The histamine H₂ receptor antagonist cimetidine exhibits high affinity, specific binding to a brain protein which is not the histamine H₂ receptor, but the identity and significance of this binding site have not been determined (Warrander et al., 1983; Smith et al., 1980; Burkard, 1978). The nature of the [³H]cimetidine-binding site is of considerable interest due to the non-histamine H₂ receptor-mediated actions of cimetidine, which include antinociception (Netti et al., 1984; Hough et al., 1997) and neurotoxicity (Shimokawa et al., 1996; Amabeoku and Chikuni, 1993; Edmonds et al., 1979). Recently, the [³H]cimetidine-binding site was pharmacologically characterized in detail as a heme-containing protein, possibly a member of the cytochrome P450 superfamily (Stadel et al., 2008).

Improgan (Table 1) is a chemical congener of cimetidine that shares cimetidine's antinociceptive properties following intracerebroventricular administration, but lacks affinity for the histamine H₂ receptor (Li et al., 1996). Improgan produces antinociception in several pain models, suggesting a favorable pre-clinical profile (Li et al., 1997; Bannoura et al., 1998). Evaluation of over 110 possible targets, including various ion channels and G protein-

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coupled receptors (Hough et al., 2000a and unpublished data), has not identified the site of improgan antinociceptive action. Improgan lacks affinity for many known antinociceptive receptors, including all known histamine (Mobarakeh et al., 2003), opioid (Hough et al., 2000b), and cannabinoid receptors (Hough et al., 2002). Improgan acts in the brain stem to stimulate descending pain-relieving mechanisms which may include supraspinal cannabinoid receptors and spinal α_2 adrenergic receptors, but the drug lacks affinity for these receptors as well (Hough et al., 2002; Hough et al., 2000a). Failure to identify the antinociceptive target for improgan has prevented further clinical development.

We recently found that improgan competes with [^3H]cimetidine binding in the rat brain (Hough et al., 2007). The same study reported discovery of CC12 (i.e. 4(5)-((4-iodobenzyl)thiomethyl)-1H-imidazole), a new cimetidine congener with nanomolar affinity ($K_i = 9.5 \text{ nM}$) for the [^3H]cimetidine-binding site. CC12 was also found to inhibit improgan antinociception, suggesting the possibility that the [^3H]cimetidine-binding site is the molecular target for improgan-like antinociceptive drugs. In order to characterize further the pharmacological properties and potential antinociceptive relevance of the [^3H]cimetidine-binding site, the effects of improgan, cimetidine and 8 additional antinociceptive congeners of improgan (Table 1) have been studied presently as inhibitors of [^3H]cimetidine binding.

2. Methods

2.1 Chemicals

Unless noted otherwise, the compounds in Table 1 were synthesized as described recently (Hough et al., 2006). Improgan was synthesized as described (Mobarakeh et al., 2003). Cimetidine and thioperamide maleate were purchased from Tocris Bioscience (Ellisville, MO). Burimamide was kindly provided by Dr. Mark Wentland (Rennselaer Polytechnic Institute, Troy, NY). CC10 was kindly provided by Dr. James Phillips (Curragh Chemistries, Cleveland, OH). Compounds in salt form were dissolved in saline. Free base forms were dissolved in dilute HCl, titrated to pH 5.5 – 6.0 and diluted with saline.

2.2 Synthesis of VUF6990

1-Cyano-3-[3-(1H-imidazol-4-yl)-propyl]-2-methyl-isothioureia (0.90 mmol, 200 mg, prepared according to Hough et al., 2006) was dissolved in cyclopropylmethylamine (3 ml) and heated under microwave conditions (30 min, 100 °C, 150 W). Co-evaporation with chloroform yielded the crude product which was purified over silica (acetone: methanol 9:1). The resulting colorless oil was crystallized with acetone/hexane, which gave the product (base) as a white foam (isolated yield 23%, purity 98% by NMR). δ_{H} (CD_3OD , 200 MHz) 7.57 (s, 1H), 6.82 (s, 1H), 3.24 (t, $J=9.0 \text{ Hz}$, 2H), 3.06 (d, $J=7.6 \text{ Hz}$, 2H), 2.62 (t, 7.1 Hz, 2H), 1.87 (m, 2H), 1.07 (m, 1H), 0.67-0.13 (m, 4H); δ_{C} (CD_3OD , 200 MHz) 3.9, 11.6, 24.9, 30.3, 42.3, 47.4, 117.3, 120.4, 135.9, 138.1, 161.2; m/z (ESI) calculated for $\text{C}_{12}\text{H}_{18}\text{N}_6$: 246.31, found: 247.1.

2.3 Animals

Male Sprague-Dawley rats (250–330 g, Taconic Farms, Germantown, NY) were used for all studies. They were housed in groups of 3–4 on a 12-h light/dark cycle (lights on from 0700 to 1900) with food and water ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of Albany Medical College.

2.4 Isolation of brain membrane fractions

Homogenates were prepared as recently described (Stadel et al., 2008). Rats were euthanized with an overdose of CO_2 or pentobarbital and brains were rapidly removed. In some cases

frozen brains were purchased (Taconic Farms, Germantown, NY). Brains were homogenized (polytron) in 10 volumes of homogenate buffer (100 mM Tris-HCl, 0.5 mM EDTA, pH 7.4), and centrifuged ($26,000 \times g$ for 15 min). Pellets were resuspended in buffer with a glass-teflon homogenizer, recentrifuged, and the resulting pellets stored at -80°C . On the day of assay, pellets were washed in assay buffer (100 mM Tris-HCl, pH 7.4), centrifuged ($26,000 \times g$ for 10 min), resuspended in a volume 5 times the wet weight of the original tissue, and analyzed for [^3H]cimetidine-binding activity.

2.5 Radioligand binding

[^3H]cimetidine binding experiments were performed following Smith et al. (1980) as recently described (Stadel et al., 2008). Resuspended crude membrane pellets (360-470 μg of rat brain protein) were incubated in a total volume of 0.1 ml containing 50 nM [^3H]cimetidine (20-25 Ci/mmol, G.E. Healthcare, Piscataway, NJ), various concentrations of competing ligand, and assay buffer for 60 min on ice. To evaluate non-specific binding, burimamide (30 μM) or cimetidine (10 μM) was added. Following incubation, samples were filtered through GF/B filters. Filters were rinsed three times with 1.5 ml of ice-cold assay buffer, placed in 5 ml of Ecoscint scintillation fluid, and counted in a scintillation counter. Protein content was determined using the bicinchoninic acid method (Pierce Chemical, Rockford, IL). For competition studies, percent specific binding was calculated using the following formula: $[(\text{drug} - \text{non-specific})/(\text{total} - \text{non-specific}) \times 100]$, where drug and total indicate the amount of binding in the presence and absence of competing ligand, respectively.

2.6 Intracerebral surgery

Surgeries, drug treatments, and antinociceptive testing of improgan and analogs were performed as recently described (Hough et al., 2006). Briefly, rats were anesthetized with pentobarbital (25 mg/kg, i.p.), supplemented with isofluorane. Guide cannulas were stereotactically implanted aimed toward the left lateral ventricle (-0.8 AP, 1.5 ML, and -3.3 DV, mm from bregma, Paxinos and Watson, 1986) and anchored to the skull with stainless steel screws and dental cement. Following surgery, animals were housed individually and allowed to recover for 5-7 days before testing. Each animal was used for a single experiment.

2.7 Injections and nociceptive testing

Subjects were tested on both the hot plate (Eddy and Leimbach, 1953) and tail flick tests (D'Amour and Smith, 1941) as recently described (Hough et al., 2006). For hot plate testing, animals were placed on a 52°C surface and the latency to lift or lick the hind paw was recorded. Baseline latencies were 10-15 s, with a cutoff time of 60 s. For the tail flick test, the ventral surface of the tail (2-5 cm from the tip) was exposed to radiant heat, and the latency for tail movement recorded. The heat source was set so that baseline latencies were generally 3-4 s with a 15-s maximal exposure. On the test day, animals underwent a single baseline hot plate test, followed by three tail flick tests performed at one min intervals. The animals were then gently secured with a laboratory pad and the injection cannula (which extended 1 mm beyond the guide to penetrate the ventricle) inserted. Animals received a single intracerebroventricular injection in a volume of 5 μl over delivered over a 5 min infusion. One min following the injection, the injection cannula was sealed approximately 2 mm above the juncture with the guide cannula with wire cutters. Subjects were re-tested at 5, 10 and 30 min following the end of drug infusion. Cannula placement was confirmed at the end of testing by an overdose of pentobarbital sodium (100 mg/kg, i.p.) followed by intracerebroventricular injection (5 μl) of India Ink, removal of the brain, and visual observation of the ink in the ventricular space. Data from animals with unsuccessful injections were excluded. Nociceptive assay results are expressed as latencies (s, mean $\pm$ SEM). The identity of all drug solutions was blinded to the investigator.

2.8. Data treatment and curve-fitting

Binding data and nociceptive latencies were fitted to sigmoidal dose-response curves with variable slopes by use of iterative non-linear regression (GraphPad Prism 4.0, San Diego, CA). Best fits yielded IC_{50} values for inhibitors of binding and antinociceptive ED_{50} values (Figs. 1 and 2). In the binding experiments, K_i values were calculated by use of the Cheng-Prusoff equation.

3. Results

Improgan, cimetidine, and seven other chemical congeners (Table 1) produced concentration-dependent inhibition of [^3H]cimetidine binding, yielding IC_{50} values between 25.4 (VUF6914) and 1,367.4 nM (improgan, Figs. 1A-C). CC10 (a conformationally-restricted congener of improgan, Table 1) displaced [^3H]cimetidine by only 18.4 % at the highest concentration studied (10 μ M, Fig. 1B). Extrapolation of these data suggest an IC_{50} value greater than 15 μ M for this drug. Except for CC10, Hill coefficients for the competition curves of all drugs in Fig. 1 were between -0.86 ± 0.12 and -1.28 ± 0.12 (mean $\pm$ SEM, for improgan and VUF6990, respectively).

VUF6990 produced antinociceptive activity on both the hot plate and tail flick tests at 5 and 10 min post-injection, with latencies returning toward baseline values at the 30 min time point (data not shown). VUF6990 antinociception was dose-dependent (Fig. 2A), with an estimated ED_{50} value of 86.6 nmol (Fig. 2A). The drug produced near-maximal effects following the largest dose tested (100 nmol, Fig. 2A). Vehicle treatment did not increase nociceptive latencies above baseline values. In contrast to the effects of all other drugs in Table 1, a wide range of doses of thioperamide produced no significant antinociceptive effects on either the hot plate (Fig. 2B) or tail flick tests (not shown) at any time. Except for VUF6990 and thioperamide, the antinociceptive potencies of all other compounds in Table 1 have been previously reported.

4. Discussion

The low affinity of cimetidine for the histamine H_2 receptor ($K_i = 0.8 \mu$ M, (Brimblecombe et al., 1975), and the low density of brain histamine H_2 receptors (Gajtkowski et al., 1983) prevent detection of the histamine H_2 receptor in brain with this radioligand. Nonetheless, [^3H]cimetidine binds with high affinity to an unidentified protein in the brain (Warrander et al., 1983; Smith et al., 1980). Recently, Stadel et al. (2008) reported that cyanide (a heme inhibitor) and metyrapone (a nonselective P450 inhibitor) competed with brain [^3H]cimetidine binding, suggesting that this site may be a P450 isoform. However, competition studies with 10 isoform-selective inhibitors, as well as direct binding studies with 8 human and 3 rat recombinant P450 isoforms, did not identify a [^3H]cimetidine-binding P450 (Stadel et al., 2008). Since approximately 89 rat and 57 human P450 isoforms are known (<http://drnelson.utmem.edu/CytochromeP450.html>), many additional isoforms require examination. Because cimetidine produces actions in animals and man that are not mediated by histamine H_2 receptors (Stadel et al., 2008; Cannon et al., 2004), it is important to identify this cimetidine-binding hemoprotein, and to assess its significance.

The molecular target for improgan remains unknown (Hough et al., 2007), despite extensive in vivo (Mobarakeh et al., 2003; Hough et al., 2000b) and in vitro studies (Hough et al., 2000a). In order to characterize further the improgan target, a series of seven chemical congeners of improgan were recently synthesized and studied in vivo for their antinociceptive actions (Hough et al., 2006, Table 1). As compared with improgan, drugs with longer and shorter alkyl chain lengths exhibited higher antinociceptive potencies, with the longest (8-carbon) chain (i.e. VUF6914) showing a 2.7-fold greater potency. Furthermore, conformational restriction of the side chain (as in the *trans*-cyclopropyl derivative CC10) had virtually no

effect on antinociceptive activity, as compared with the unconstrained analog VUF5733 (Hough et al., 2006, Table 1). Extending these earlier results, the present studies found that replacement of the N-methyl moiety in improgan (Z in Table 1), with the more lipophilic N-cyclopropylmethyl substituent in VUF6990 also increased antinociceptive potency (Table 1, Fig. 2A). These structure-activity relationships are useful tools in the search for improgan's molecular target.

Although thioperamide deviates significantly in chemical structure from the other drugs in Table 1 (e.g. it contains a thiourea [C=S] as opposed to a cyanoguanidine [C=NCN]) group, this drug was included in the present study because: 1) other thioureas structurally similar to improgan have antinociceptive properties (Hough et al., 1997), and 2) thioperamide has high affinity for the [³H] cimetidine binding site (Fig. 1, discussed further below). An earlier study reported that intracerebroventricular administration of 340 nmol of thioperamide produced no activity on hot plate or tail flick responses in rats (Hough et al., 1997). Assuming that the improgan-like thioureas (e.g. burimamide) produce antinociception by an improgan-like mechanism, it was suggested that incorporation of either the highly constrained piperidinyll chain or the N-cyclohexyl group (or both, see Table 1) into thioperamide might account for this drug's lack of antinociceptive activity (Hough et al., 1997). The present results with even larger doses of drug (Fig. 2B) seem to confirm that thioperamide does not have pronounced antinociceptive activity.

Several earlier studies have reported that the histamine H₃ receptor antagonist thioperamide has antinociceptive and/or anti-hyperalgesic properties (Oluyomi and Hart, 1991; Malmberg-Aiello et al., 1994; Farzin et al., 2002; Li et al., 1996; Huang et al., 2007). On the hot plate test, mild antinociceptive effects (up to 30 % of maximal) were observed after either peripheral (Malmberg-Aiello et al., 1994; Farzin et al., 2002) or CNS (Li et al., 1996) administration of low to moderate doses of thioperamide. Larger doses reversed these effects, yielding U-shaped dose-response curves (Malmberg-Aiello et al., 1994; Li et al., 1996), commensurate with the results of Fig. 2B. Since blockade of CNS histamine H₃ receptors releases histamine (Haas et al., 2008), and intracerebral histamine's dose-response curve on the hot plate test mimics the effects of thioperamide (Li et al., 1996), these mild, biphasic effects of thioperamide are likely to result from an action at the brain H₃ receptor. Because of the biphasic effects of thioperamide, the possibility that this drug's histamine H₃ receptor affinity might somehow interfere with the assessment of thioperamide's improgan-like activity should be considered. This seems not to be the case, however, since improgan lacks significant affinity for the histamine H₃ receptor, and improgan antinociception is readily detected in histamine H₃ receptor null mice (Mobarakeh et al., 2003). These results strongly argue that thioperamide has little or no affinity at the improgan antinociceptive target.

The present results show several notable features of the structure-activity relationship for the improgan congeners at the [³H]cimetidine-binding site. For instance, the 4-methyl-n-butyl (VUF5651) and the n-butyl (VUF5420) congeners have nearly identical binding affinities (K_i values = 122.4 and 154.2 nM, respectively, Table 1), suggesting a minimal impact of the 4-methyl group on binding. The 4-fold higher affinity of cimetidine (the thioether congener, K_i = 30.6 nM) relative to VUF5651 (the 4-methyl-n-butyl congener) may be due to the longer side chain introduced by sulfur. This conclusion seems consistent with the negligible impact of the 4-methyl substituent (above) and with the high binding potency of the n-pentyl-derivative, VUF6913. The most potent ligands for [³H]cimetidine binding identified presently are the longer-chained (6 to 8- carbon) congeners of improgan. It is not known if the high potency of these ligands is strictly due to increased lipophilicity or to more defined conditions. Lipophilicity may play some role, as replacement of the N-methyl moiety in improgan with the more lipid soluble N-cyclopropyl substituent (VUF6990) nearly doubled the binding affinity. Side chain lipophilicity alone does not seem to account for binding activity, since

VUF5733, the 2-carbon congener, is dramatically more potent than improgan, the 3-carbon congener ($K_i = 89$ vs 782 nM, respectively). CC10, the 2-carbon conformationally-restricted derivative, had unexpectedly low binding activity. The trans-geometry of this compound may suggest that access to the [^{3} H]cimetidine binding site requires a cis-geometry in the active congeners, but other interpretations are also possible.

Correlational analysis relating receptor-binding potencies to pharmacological responses is a classic approach for validating the biological relevance of a drug-binding site (Kenakin, 1997). For example, Compton et al. (1993) demonstrated significant correlations between the cannabinoid CB₁-receptor binding affinities and cannabinoid-like “tetrad” effects for 29 compounds. Similar studies have validated the clinical analgesic relevance of μ -opioid receptor binding assays (Wilson et al., 1975; Pert and Snyder, 1973). Presently, it was reasoned that if the [^{3} H]cimetidine-binding site represents the improgan antinociceptive receptor, then the [^{3} H]cimetidine-binding affinities for improgan-like congeners should correlate with their antinociceptive potencies.

Although nine of the ten compounds in Table 1 fully competed with [^{3} H]cimetidine-binding (Fig. 1), a significant correlation between binding affinities and antinociceptive potencies was not found (Fig. 3, Pearson correlation $r = 0.018$, $p = 0.97$, $n = 10$). For example, CC10 and VUF5733 have similar antinociceptive activities (106.1 vs. 137.2 nmol, respectively, Table 1), yet display highly disparate (>90 -fold) binding affinities ($>8,000$ vs. 89 nM, respectively, Table 1). Furthermore, as compared with improgan, more potent (VUF6914, $ED_{50} = 81.9$ nmol) and less potent (cimetidine, $ED_{50} = 417.3$ nmol) antinociceptive congeners maintain low nanomolar binding affinities ($K_i = 14.5$ and 30.6 nM, respectively, Table 1). Finally, the lack of antinociceptive activity of very high doses of thioperamide is in striking contrast with this drug's moderately high affinity ($IC_{50} = 175.3$ nM) for the [^{3} H] cimetidine binding site. Taken at face value, the absence of a significant correlation in Fig. 3 suggests that the [^{3} H]cimetidine-binding site does not mediate the antinociceptive effects of these improgan-like compounds.

Although the present results do not confirm an antinociceptive role for [^{3} H]cimetidine binding, neither do they completely exclude the analgesic relevance of this site. For example, if the drugs tested presently are behaving as agonists (improgan-like partial agonists may have been discovered, see Hough et al., 2006), then the absence of a correlation in Fig. 1 could be due to variations in agonist efficacy. Agonist ED_{50} values are known to depend upon both affinity and intrinsic efficacy (Kenakin, 1997), and notable differences in agonist intrinsic efficacies have been reported (Emmerson et al., 1996; Porreca et al., 1990; Quock et al., 1999). In order for the [^{3} H]cimetidine-binding site to mediate the presently-studied antinociceptive activities, wide variations in intrinsic efficacies would have to exist across the drug series. However, without an in vitro functional assay for the improgan receptor, the intrinsic efficacies of the drugs in Table 1 cannot be determined. Since competitive antagonists show significant correlations between binding activity and functional antagonism (without the additional complexities of agonist efficacy [Quock et al., 1999]), the best correlational study to assess the analgesic significance of the [^{3} H]cimetidine binding site would utilize improgan antagonists. Despite progress, an improgan receptor antagonist has not yet been definitely identified (Hough et al., 2007).

Pharmacokinetic factors such as drug metabolism and distribution can sometimes account for inconsistencies in correlations between ED_{50} values and binding affinities (e.g. see Pert and Snyder, 1973). However, the presently-studied compounds were administered directly into the brain lateral ventricle, circumventing both the liver and the blood brain barrier, and all were active within five minutes after administration, minimizing this potential concern.

The possible presence of multiple [³H]cimetidine-binding proteins in the brain could have prevented accurate determinations of affinity constants for impropen analogs, thereby potentially confusing correlational results. We have recently detected [³H]cimetidine binding in rodent liver with pharmacological properties distinct from the brain [³H]cimetidine-binding site (data not shown), suggesting the possible existence of more than one binding site. However, most saturation studies with [³H]cimetidine binding in rat brain homogenates found only one site (Kendall et al., 1980; Smith et al., 1980; Stadel et al., 2008). Furthermore, the present finding that all competing ligands of Table 1 exhibited Hill coefficients close to unity (Fig. 1) is commensurate with the existence of a single class of binding sites in the brain.

The present correlational analysis also makes the assumption that all of the compounds in Table 1 produce antinociception by the same mechanism. Although this is a reasonable assumption for such a series of close congeners, it may not pertain to all drugs in Table 1. For example, cimetidine may have an antinociceptive mechanism distinct from that of impropen (Hough et al., 2004). Further studies are required to determine the possible significance of cimetidine antinociception in the search for the impropen antinociceptive target.

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Figure 1.

Inhibition of [³H]cimetidine binding by impropion-like drugs. Whole brain crude membrane homogenates (360-470 µg) were incubated in triplicate, on ice, with 50 nM [³H]cimetidine for 60 minutes and filtered as described. Non-specific binding was evaluated with 10 µM cimetidine or 30 µM burimamide. Each data point represents the mean ± SEM of a total of 6-9 determinations, from 2-3 separate experiments. Except for CC10, competition curves for each drug show the best fit by non-linear regression. IC₅₀ values are given on the right. The dotted line connects CC10 data points. Examples of mean total and non-specific binding were 6352 cpms (0.29 pmol) and 1530 cpms (0.07 pmol), respectively. Impropion data were re-drawn from Hough, et. al. (2007).

Figure 2.

Effects of VUF6990 and thioperamide on hot plate nociceptive responses. Cannulated rats were tested for baseline responses, received intracerebroventricular injections (5 μ l) of either VUF6990 (A, top), thioperamide (B, bottom), or saline vehicle (Veh; A, B), and were re-tested. Nociceptive responses (sec, ordinate, mean $\pm$ SEM for the number of subjects in parentheses) are shown 5 min after drug administration at the doses specified (nmol, abscissa). The ED₅₀ value for VUF6990 (obtained by non-linear regression, dashed line) is given in A. The same vehicle data are shown in A and B.

Figure 3. Relationship between [^3H]cimetidine binding affinity and antinociceptive potency for a series of impropgan-like drugs. For each drug in Table 1 (identified by number on the right), the K_i value for [^3H]cimetidine binding (M, log scale, abscissa) is plotted against antinociceptive ED_{50} (mol, log scale, ordinate). Values taken from Table 1. No significant correlation was found between binding and antinociceptive potencies.

Drug	R	X	Z	3HCIM IC ₅₀ (nM) <i>a</i>	3HCIM K _i (nM) <i>b</i>	Antinociceptive ED ₅₀ (nmol) <i>c</i>
C10	H		-CH ₃	>15,000	>8,000	106.1

the (3HCIM)-binding IC₅₀ values from Fig. 1.

culated from respective IC₅₀ values (K_D for [³H]cimetidine =67 nM, [Stadel et al., 2008]).

otherwise, ED₅₀ values are from hot plate nociceptive data measured 5 min following intracerebroventricular administration in rats housed under a normal light-dark cycle (Hough et al.,

data taken 10 min after intracerebroventricular administration of cimetidine in rats housed under reverse (dark-cycled) conditions (Li et al., 1996).

Fig. 2.