Neuropharmacology and Analgesia

Pharmacological characterization of A-960656, a histamine H3 receptor antagonist with efficacy in animal models of osteoarthritis and neuropathic pain

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A R T I C L E   I N F O

Article history:
Received 13 December 2011
Received in revised form 28 March 2012
Accepted 29 March 2012
Available online 5 April 2012

Keywords:
Histamine
H3
A-960656
Neuropathic pain
Osteoarthritis

A B S T R A C T

Histamine H3 receptor antagonists have been widely reported to improve performance in preclinical models of cognition, but more recently efficacy in pain models has also been described. Here, A-960656 ((R)-2-((3-(piperidin-1-yl)pyrrolidin-1-yl)benzol[d]thiazol-6-yl)pyridazin-3(2H)-one) was profiled as a new structural chemotype. A-960656 was potent in vitro in histamine H3 receptor binding assays (rat \( K_i = 76 \) nM, human \( K_i = 21 \) nM), and exhibited functional antagonism in blocking agonist-induced \( [\gamma^S]GTP \gamma S \) binding (rat H3 \( K_i = 107 \) nM, human H3 \( K_i = 22 \) nM), and was highly specific for H3 receptors in broad screens for non-H3 sites. In a spinal nerve ligation model of neuropathic pain in rat, oral doses of 1 and 3 mg/kg were effective 60 min post dosing with an ED50 of 2.17 mg/kg and a blood EC50 of 639 ng/ml. In a model of osteoarthritis pain, oral doses of 0.1, 0.3, and 1 mg/kg were effective 1 h post dosing with an ED50 of 0.52 mg/kg and a blood EC50 of 233 ng/ml. The antinociceptive effect of A-960656 in both pain models was maintained after sub-chronic dosing up to 12 days. A-960656 had excellent rat pharmacokinetics (\( t_{1/2} = 1.9 \) h, 84% oral bioavailability) with rapid and efficient brain penetration, and was well tolerated in CNS behavioral safety screens. In summary, A-960656 has properties well suited to probe the pharmacology of histamine H3 receptors in pain. Its potency and efficacy in animal pain models provide support to the notion that histamine H3 receptor antagonists are effective in attenuating nociceptive processes.

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1. Introduction

The histamine H3 receptor is prominently expressed on neurons in the CNS, where it exerts negative modulation over the release of neurotransmitters, with the endogenous agonist histamine decreasing release, and synthetic antagonists shown to increase neurotransmitter release (Arrang et al., 1983; Blandina et al., 2010; Haas et al., 2008; Hill et al., 1997). Expressed on histaminergic neurons, the histamine H3 receptor is autoregulatory for control of histamine release, and is able to regulate levels of the cognate neurotransmitters when it is expressed on noradrenergic, cholinergic, or other neuronal types. A number of potent and selective histamine H3 receptor antagonists have been described, including agents that have advanced into clinical testing (Berlin and Boyce, 2007; Berlin et al., 2011; Brioni et al., 2011; Leurs et al., 2011).

The literature has extensive reports of the efficacy of antagonists in a broad range of preclinical models of cognition, schizophrenia, wake-promotion, site of action and mechanism of action investigations (Blandina et al., 2010; Brioni et al., 2011; Esbenshade et al., 2008). However, there are few publications on the potential of histamine H3 receptor antagonists in pain, indicating that the pharmacologic understanding of these agents in antinociception is poorly understood. Histamine (after intracerebroventricular administration) was long ago reported to reduce nociceptive responses to noxious mechanical, thermal, and chemical stimuli in rodents, as was systemic dosing of the histamine metabolic precursor \( L \)-histidine (Malmberg-Aiello et al., 1994). But most intriguing are recent reports of preclinical efficacy in pain models. Several structurally diverse histamine H3 receptor antagonists were reported effective in diabetic and surgical neuropathic pain models by Shirakura et al. (2004).

This was followed by reports of anti-nociceptive efficacy of three potent antagonists, with GSK-207040 and GSK-334329 reducing capsaicin-induced secondary hyperalgesia in rat (Medhurst et al., 2007a), and GSK-189254 and GSK-334329 effective after oral dosing in a chronic constriction injury-induced neuropathic pain and viral-induced alldynia models in rat (Medhurst et al., 2008).

We recently reported that histamine H3 receptor antagonists including GSK-189254 and ABT-239 are effective in rodents in a model of osteoarthritis pain as well as in a spinal nerve ligation model...
of neuropathic pain (Hsieh et al., 2010). A detailed investigation supported a CNS site of action in osteoarthritis pain, with lumbar intrathecal dosing induced efficacy that involved activation of adrenergic alpha-2 receptors.

This manuscript introduces A-960656 (Fig.1), a potent, selective and structurally novel histamine H3 receptor antagonist with excellent pharmacokinetic properties, CNS penetration, and efficacy in pain models.

2. Materials and methods

2.1. Preparation and properties of (R)-2-(2-(3-(piperidin-1-yl)pyrrolidin-1-yl)benzo[d]thiazol-6-yl)pyridazin-3(2H)-one hemihydrate (A-960656)

A-960656 was prepared in >95% purity as a white crystalline solid, melting point of 172–174 °C, as described in detail in the Supplemental Section S2.1

2.2. In vitro profile

2.2.1. In vitro activity at histamine H3 receptors in radioligand competition binding and functional assays

Assays for determination were carried using membranes expressing human H3 (in HEK) or rat H3 receptors (in C6 cells) as previously described (Cowart et al., 2007; Esbenshade et al., 2005). Membranes were prepared by homogenization in cold buffer (pH 7.4, 50 mM Tris–HCl, 5 mM EDTA, 10 mM MgCl₂, and protease inhibitors) followed by centrifugation twice at 40,000 g for 20 min at 4 °C, and the resulting pellet was resuspended in buffer containing 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, and 10 mM MgCl₂. Before freezing and storage, glycerol and BSA were added to give final concentration of 10% glycerol and 1% bovine serum albumin. The Kᵢ values were determined in competition binding assays using [³⁵S]H-alpha-methylhistamine at a concentration of 0.5–1.0 nM, in the presence of increasing test drug concentrations (11 concentrations spaced over a 5-log unit range), all for 30 min at 25 °C in a final volume of 0.5 ml of pH 7.4 buffer containing 50 mM Tris and 5 mM EDTA. Nonspecific binding was defined with 10 μM thipiperide. Binding reactions were terminated by suction under vacuum through polyethyleneimine (0.3%) presoaked Unifilter plates followed by 3 rapid washes with 2 ml of binding buffer. Bound radiolabel was quantitated by liquid scintillation counting, and IC₅₀ values and Hill slopes determined by Hill transformation. pKᵢ values were determined as pKᵢ = S.E.M. by the generalized Cheng–Prusoff equation using GraphPad Prism (GraphPad Software, San Diego).

Functional antagonism was determined in an in vitro assay of H3 agonist-induced increase in [³⁵S]GTPγS binding as previously described (Esbenshade et al., 2005). In brief, histamine H₃ receptor containing membranes (vide supra) were diluted in 25 mM HEPS, 2.5 mM MgCl₂, and 75 mM NaCl, pH 7.4, and wells containing 10 μg membrane protein were incubated with (R)-alpha-methylhistamine (30 nM for human and 300 nM for rat H₃ receptor), 5.0 μM unlabeled GDP, 0.5 nM [³⁵S]GTPγS, and various concentrations of A-960656. Samples were incubated at 37 °C for 5 min, then assays terminated with cold 50 mM Tris–HCl, 75 mM NaCl, and 2.5 mM MgCl₂, pH 7.6, then harvesting by vacuum filtration onto a Packard Unifilter 96-well GF/B plate (PerkinElmer Life and Analytical Sciences), followed by washing, drying of the plates, and the amount of bound [³⁵S]GTPγS determined scintillography after addition of Microscint 20. The percentage of [³⁵S]GTPγS bound in each sample was calculated as a percentage of that bound to control samples incubated in the absence of histamine H3 receptor ligands. Triplicate determinations were obtained at each concentration, and the data were analyzed using GraphPad Prism to obtain EC₅₀ or IC₅₀ values and Hill slopes. pKᵢ values were calculated using the generalized Cheng–Prusoff equation.

2.2.2. Assessment of potential for off-target interactions in binding screens

A-960656 was evaluated in competition binding assays for histamine H₁, H₂, and H₄ receptors as previously described (Esbenshade et al., 2005; Strakhova and Esbenshade, 2007). The compound was also screened in a commercial binding screen at 10 μM against a broad panel of 80 off-target receptors, enzymes, and ion channels (CEREP, www.CEREP.com, see Supplementary Section S2.3.1 for the tabulated list of targets screened).

2.2.3. Functional block of hERG channel current

The effect of A-960656 on hERG current was evaluated using HEK 293 cells stably expressing hERG. Drug effects were evaluated based on changes of tail currents measured during 4 second repolarizing pulses to −50 mV preceded by a 3 second depolarizing pulse to 0 mV (holding potential of −80 mV, pulses applied once every 15 s). Experiments were conducted at 36.5–37 °C with a 5 mM external K⁺ HEPES-buffered Tyrode’s solution. A-960656 was evaluated at target concentrations of 3, and 10 μM (1145 ng/ml and 3815 ng/ml), (n=2 per group). Measured bath concentrations of A-960656 were analyzed and found to be 2.45 μM and 10.52 μM (935 and 4013 ng/ml). These concentrations reduced hERG tail current by 33.9% and 61.0%, respectively, and were used to calculate the IC₅₀ value for hERG block of 5860 nM ± 60 nM.

2.3. Evaluation of A-960656 in in vivo pain models

2.3.1. Animals and drug dosing

All studies used male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 250–300 g at the time of testing. In every study group, two independent determinations of efficacy were made using groups of six animals (a total of 12 rats), with the results combined for analysis. Animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facilities at Abbott Laboratories in a temperature-regulated environment under a controlled 12 h light–dark cycle, with lights on at 6:00 a.m. Food and water were available ad libitum at all times except during testing. All testing was done following procedures outlined in protocols approved by Abbott Laboratories’ Institutional Animal Care and Use Committee and followed the Guidelines on Ethical Standards for Investigations of Experimental Pain in Conscious Animals laid down by the International Association for the Study of Pain (IASP) (Zimmermann, 1983). For use in vivo, A-960656 was dissolved in vehicle (10% DMSO and 90% hydroxy-propylcellulose solution (30% by weight in water)) and this solution dosed at 0.02 ml/kg body weight.

2.3.2. Rat spinal nerve ligation (SNL) model of neuropathic pain

As previously described in detail by Kim and Chung (1992), rats were placed under isoflurane anesthesia and a 1.5 cm incision was made dorsal to the lumbarosacral plexus. The paraspinous muscles (left side) were separated from the spinous processes, the L5 and L6 spinal nerves isolated, and tightly ligated with 5–0 silk suture distal to the dorsal root ganglion. Care was taken to avoid ligating the L4 spinal nerve. Following spinal nerve ligation, a minimum of 7 days of recovery and no more than 2 weeks was allowed prior to the behavioral testing (mechanical allodynia). Only rats with threshold scores ≤4.5 g were considered allodynic and utilized in pharmacological experiments.

Fig. 1. Chemical structure of A-960656.
Mechanical allodynia was measured using calibrated von Frey filaments (Stoelting, Wood Dale, IL). Paw withdrawal threshold (PWT) was determined by using the Dixon’s up-down method (Chaplan et al., 1994). Rats were placed into inverted individual plastic containers (20 × 12.5 × 20 cm) on top of a suspended wire mesh with a 1 cm² grid to provide access to the ventral side of the hind paws, and acclimated to the test chambers for 20 min. The von Frey filaments were presented perpendicularly to the plantar surface of the selected hind paw, and then held in this position for approximately 8 s with enough force to cause a slight bend in the filament. Positive responses included an abrupt withdrawal of the hind paw from the stimulus, or flinching behavior immediately following removal of the stimulus. A 50% withdrawal threshold was determined using an up-down procedure (Dixon, 1980). The strength of the maximum filament used for von Frey testing was 15.0 g. A group of SNL rats (one week post surgery) was dosed twice daily (p.o.) with either A-960656 (0.3, 1.0, and 3.0 mg/kg) or vehicle for 11 days. The behavioral measurements were conducted at 60 min post compound administration after the first dose on day 1 and day 11. Whole blood samples were collected immediately after the behavioral testing on day 11 for the exposure analysis by LCMS method according to the procedures described in rat PK study. A percent maximal possible effect (% MPE) of testing compound was calculated according to the formula: \( \frac{\log \text{[vehicle-treated threshold]} - \log \text{[vehicle-treatment threshold]}}{\log \text{[maximum threshold]}} \times 100 \), where the maximum threshold was equal to 15 g.

2.3.3. Rat knee joint osteoarthritis pain model

Unilateral knee joint osteoarthritis was induced in the rats by a single intra-articular (i.a.) injection of the reactive alkyllating agent sodium monooiodacetate (MIA, 3 mg in 0.05 ml sterile isotonic saline) into the right knee joint cavity under light isoflurane anesthesia using a 26 G needle, as previously described (Chandran et al., 2009; Hsieh et al., 2010). The dose of the MIA (3 mg intra-articular injection) was selected based on results obtained from preliminary studies wherein an optimal pain behavior was observed at this dose. Pain behavioral assessment of hind limb grip force was conducted by recording the maximum compressive force exerted on the hind limb strain gauge setup, using a commercially available grip force measurement system (Columbus Instruments, Columbus, OH). The grip force data was converted to a maximum hind limb cumulative compressive force (CFmax) (gram force)/kg body weight for each animal. The vehicle control group was assigned 0% whereas the age matched naive group was assigned as being 100% (normal). The antinociceptive effects of H3 antagonist A-960656 were then expressed as % return to normalcy compared to the naive group = \( \frac{\text{[Treatment CFmax} - \text{Vehicle CFmax}]}{\text{[Naive CFmax} - \text{Vehicle CFmax}]} \times 100 \).

A group of rats (20 days following the intra-articular injection of monooiodacetate) was dosed with A-960656 (0.1, 0.3, and 1.0 μmol/kg, p.o.) in a repeated dosing paradigm, in which the rats were dosed twice daily (p.o.) with either test compound or vehicle for 12 days. The behavioral measurements were conducted at 1 h post compound administration of the first dose on day 1 and day 12. Whole blood samples were collected immediately after the behavioral testing on day 12 for the exposure analysis by LCMS method as the procedure described in rat PK study.

2.3.4. Data analysis

Statistical analysis was carried out using GraphPad Prism (GraphPad Software, Inc., San Diego, CA), with values represented as mean ± S.E.M. All in vivo behavioral studies were conducted in a randomized, investigator-blinded fashion. Statistical significance on group means was measured by a one-way analysis of variance (ANOVA), followed by Bonferroni’s post-hoc analysis. In all cases P<0.05 was assumed as the level for statistical significance. EC50 values associated with 50% efficacy were calculated by linear regression analysis.

2.4. Pharmacokinetics of A-960656, plasma and brain tissue binding, and assessment of drug–drug interaction potential

Methods for 2.4.1 pharmacokinetic analysis of drug in blood, plasma, and brain samples are described in Supplemental Section S2.4.1. Methods for in vitro assays for binding to plasma protein and brain tissue are described in Supplemental Section 2.4.2. Assessment of membrane permeability and P-gp efflux liability in Caco-2 membranes was preformed as described in Supplemental Section S2.4.3.

2.5. In vivo tolerability in rat

2.5.1. Assessment of gross effects on CNS behaviors at high doses

A general observation test was carried out as previously described (Cowart et al., 2007; Fox et al., 2005). Adult male CD-1 mice (Charles River, 31–36 g) were separated into groups of 3, placed into observation cages (23 cm × 21 cm × 20 cm), and each group injected intraperitoneally with vehicle, or A-960656 at 3, 10, 30, 60 mg/kg. Animals were monitored for general effects on behaviors including tremor and seizure activity (including Straub tail, wild running, clonus, and tonus) and general changes in activity levels, noting results at time points 0, 0.5, 1, 2, 3, 6, and 24 h postdosing. All subjective observations in drug-treated mice were made with constant reference to a cage of vehicle-treated control mice.

2.5.2. Cardiovascular evaluation in rat

Male Sprague–Dawley rats (n = 3) were anesthetized with Inactin, instrumented, and allowed to recover and stabilize. Following a 30 min baseline period, A-960656 was infused intravenously at the rate of 3, 10, and 30 mg/kg/30 min in D5W vehicle (5% dextrose in water) at 1 ml/kg, with blood concentrations assessed at the end of each dosing and reaching 1840, 8490, and 18,380 ng/ml, respectively. Hemodynamic parameters assessed were heart rate, mean arterial pressure, vascular resistance, and left ventricular contractility.

2.5.3. Cardiovascular evaluation in anesthetized dog

A-960656 was administered by intravenous infusion at 3 escalating doses to anesthetized animals (n = 6). Hemodynamic and electrocardiographic observations were matched to blood concentrations, which achieved 545 ± 36, 2147 ± 297, and 6941 ± 780 ng/ml. The analysis of the QT interval used the Van de Water formula correction. All data were analyzed for statistical significance for drug-treated animals versus baseline and vehicle-treated animals.

3. Results

3.1. Synthesis and physical properties of A-960656

A-960656 was synthesized in >95% purity as a crystalline solid via a convergent six-step route on a multigram scale. Physicochemical properties were well within range of those reported to promote good CNS drug-likeness (Johnson et al., 2009; Wager et al., 2010a, 2010b), with a molecular weight of 381, log D7.4 of 1.0, polar surface area of 52 M, 32 mg/L, hydrogen bond donor-acceptor count of 6. The solubility of the free base crystalline solid was high (85 μM, 32 mg/L, pH 7.2 water), which facilitated oral and IV dosing studies in animals at doses ranging from 0.1 to 200 mg/kg.

3.2. In vitro profile

3.2.1. In vitro activity of A-960656 at histamine H3 receptors in radioligand competition binding and functional assays

The in vitro affinities at histamine H3 receptors was assessed in competition binding assays (Table 1), with A-960656 having a 76 nM Ki at rat, and 21 nM Ki at human histamine H3 receptors. The compound
exhibits functional antagonism by blockade of H₃ agonist-induced [³⁵S]GTPγS binding, with A-960656 having a Kᵦ of 107 nM in rat, and a Kᵦ of 22 nM at human histamine H₃ receptors. Thus the potency was nearly identical in binding and functional assays, nanomolar potency at human and rat receptors.

3.2.3. Assessment of A-960656 for block of hERG cardiac potassium channel

A-960656 was examined in a number of in vitro screens and found to be highly selective for the H₃ receptor. When tested at 10,000 nM versus a broad panel of receptors, enzymes, and ion channels in a commercial binding screen (CEREP, www.CEREP.com), A-960656 did not significantly interact with any of the 80 targets examined, so that no target was inhibited more than 23% at 10,000 nM (see Supplementary Section, S2.3.1). A-960656 was also examined in binding assays for human histamine H₁, H₂, and H₄ receptors and found highly selective with no significant binding at the highest concentrations examined: at histamine H₁, Kᵦ was >7100 nM; at histamine H₂, Kᵦ was >9300 nM; at histamine H₄, Kᵦ was >7200 nM. Thus, the selectivity of the compound for the human histamine H₃ receptor was >330-fold.

3.2.4. Efficacy of A-960656 in a model of neuropathic pain

Administration of A-960656 produced a significant reversal of nerve injury-induced tactile hypersensitivity in the rat SNL model of neuropathic pain (Fig. 2). On acute dosing (day 1), doses of 0.3, 1.0 and 3.0 mg/kg, p.o. A-960656 reversed tactile hypersensitivity by 17%, 32%, and 65%, respectively. To determine the analgesic efficacy following repeated administration, A-960656 was dosed twice daily for 11 days at 0.3, 1, and 3 mg/kg, p.o. which reversed hypersensitivity by 32%, 69%, and 81% respectively. Thus, A-960656 retained analgesic efficacy after sub-chronic dosing for 11 days. Acute administration of gabapentin (100 mg/kg, p.o.) was included as a positive control drug and it produced a significant response (76% on day 1 and 79% on day 11). Blood samples were collected immediately following behavioral testing (Table 2). Blood levels and brain levels increased proportionately with dose, and blood levels were nearly identical on acute dosing compared to 11 days of dosing, indicating no drug accumulation (brain exposures are summarized in Supplemental Section S2.3.2). The ED₅₀ was 2.17 mg/kg p.o. (day 1), with EC₅₀ blood level of 639 ng/ml. After sub-chronic daily dosing (11 days), the ED₅₀ was 0.78 mg/kg p.o. with an EC₅₀ blood level of 255 ng/ml.

3.2.5. Efficacy of A-960656 in a model of osteoarthritis knee joint pain

A-960656 exhibited analgesic efficacy in a rat model of osteoarthritis knee joint pain (Fig. 3). On acute dosing (day 1), A-960656 reversed monoiodoacetate-induced decreased grip force in a dose-dependent manner by 24%, 38%, and 64% respectively, at doses of 0.1, 0.3, and 1 mg/kg, p.o. A-960656 was administered twice daily for 12 days at doses of 0.1, 0.3, and 1 mg/kg, p.o., which significantly reversed monoiodoacetate-induced decreased grip force by 51%, 59%.
3.3. Pharmacokinetic profile of A-960656 in animals

A-960656 was well absorbed, with high oral bioavailability in all species examined (Table 4). In rat, dog, and monkey, the oral bioavailability was 84%, 59%, and 100%, respectively, with blood half-life of 1.9, 5.7, and 8.3 h, respectively. The compound was rapidly absorbed after oral dosing in rat as seen in Fig. 4, with blood concentrations reaching a maximum (Tmax) at 0.8 h, with the (Cmax) of 274 ng/ml at a dose of 1 mg/kg.

A-960656 rapidly achieved high brain exposures. When dosed at 1 mg/kg p.o. in rat, brain, blood, and plasma levels sampled at 1 h were 440, 283, and 206 ng/ml. Thus, the brain levels were similar to the systemic drug levels in circulation, and the calculated brain/blood ratio was 1.6 ×. In similar way, when drug exposures were examined at 5 h post dosing with 1 mg/kg p.o., the brain, blood, and plasma levels were 390, 110, and 87 ng/ml. The blood/plasma partition ratio was found to be 1.3 ×. Brain and blood levels from pain studies are shown in Supplemental Section Tables S2.3.2 and S2.3.3.

3.3.1. A-960656 in in vitro assays of membrane permeability, CYP inhibition, induction, and tissue binding

A-960656 is highly membrane permeable, with a very rapid permeation rate of 43 × 10^-6 cm/s in Caco-2 membranes. The compound showed no potential for efflux by drug transporters in the Caco-2 membranes, as the permeation rate was unchanged in the presence of P-gp (P-glycoprotein) inhibitors. Thus the in vitro data are consistent with the high oral bioavailability, rapid brain penetration, and high brain/blood ratios seen after dosing to rat. The binding of the drug to tissues was assessed, with A-960656 showing 84.7% rat plasma protein binding, and nearly identical binding to brain tissue homogenate, with 86.3% bound. A-960656 did not inhibit CYP enzymes with IC50 values >10 μM for CYP2C9, CYP2D6, and CYP3A4, and the compound showed no propensity to induce CYP (in vitro assessment with CYP-3A4). Taken together, the in vitro data indicated minimal or no pharmacokinetic propensity for drug-drug interactions.

3.4. Assessment of safety and tolerability

3.4.1. Assessment of gross effects on CNS behaviors at high doses

A-960656 was assessed at doses of 3, 10, 30, 60 mg/kg i.p. in mice, and behavioral responses carefully monitored for 24 h. The drug was well tolerated, with no observable adverse behavioral effects at multiples above the doses found effective in pain. Doses of 3 and 10 mg/kg produced no changes in behavior. Thus, at doses active in pain models (0.1–3 mg/kg), A-960656 did not show general behavior effects that would confound the interpretation that the compound exerts antinociceptive efficacy.

3.4.2. Cardiovascular evaluation in rat

A-960656 was assessed in anesthetized rat for cardiovascular safety margin after intravenous dose escalation at blood levels ranging from 25 to 18,380 ng/ml. Hemodynamic parameters (mean arterial pressure, heart rate, vascular resistance, and left ventricular contractility) were unaffected or only modestly affected (<12%) by A-960656 up to the highest exposure (18,380 ng/ml).

3.4.3. Cardiovascular evaluation in anesthetized dog

A-960656 was assessed in anesthetized dog with intravenous infusion at 3 escalating doses (n = 6) to achieve blood levels of 545 ± 36, 2147 ± 297, and 6941 ± 780 ng/ml. Hemodynamic parameters were unaffected by drug even at the highest exposure. However, the QT interval was prolonged (P < 0.05) by the drug even at the highest exposure. Thus, the in vivo electrocardiographic observation of QT prolongation is consistent with the earlier finding of hERG blockade in vitro. The potential for QT prolongation in human preclinical advancement of A-960656 into human clinical trials, but does not affect the use of or alter the finding of antinociceptive efficacy in animals.

Table 4

<table>
<thead>
<tr>
<th>Species</th>
<th>Cib (l/kg)</th>
<th>Vm (l/kg)</th>
<th>Oral t1/2 (h)</th>
<th>Tmax (h)</th>
<th>Cmax (ng/ml)</th>
<th>Oral bioavailability</th>
</tr>
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<tbody>
<tr>
<td>Rat</td>
<td>1.0</td>
<td>1.8</td>
<td>1.9</td>
<td>0.8</td>
<td>274</td>
<td>84%</td>
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<tr>
<td>Dog</td>
<td>0.3</td>
<td>1.5</td>
<td>5.7</td>
<td>0.5</td>
<td>420</td>
<td>59%</td>
</tr>
<tr>
<td>Monkey</td>
<td>0.5</td>
<td>3.7</td>
<td>8.3</td>
<td>2.2</td>
<td>261</td>
<td>100%</td>
</tr>
</tbody>
</table>

Cib = blood clearance after IV dosing; Vm = steady-state volume of distribution after IV dosing; t1/2 is the apparent oral elimination half-life; Tmax = maximum blood concentration after oral dosing; Cmax = peak blood level after oral dosing; oral bioavailability = oral AUC divided by the intravenous AUC (ratio of the area under the exposure-time curves); n = 3.

Fig. 4. Blood levels of A-960656 in rat after oral (open squares), and intravenous (solid squares) doses of 1 mg/kg. Results are shown as the average ± S.E.M. of n = 3 animals.

and 74%, respectively. Thus, A-960656 retained analgesic efficacy after sub-chronic dosing (12 days). In the study, diclofenac (30 mg/kg, p.o., administered acutely) was included as a positive control drug, and produced a statistically significant response (60% on day 1, and 73% on day 12). Blood samples were collected immediately following behavioral testing (Table 3). Blood levels and brain levels were increased proportionately with dose, and blood levels nearly identical on acute dosing compared to 12 days of sub-chronic dosing, indicating no drug accumulation over time (brain exposures are summarized in Supplemental Section S2.3.3). The ED50 was 0.52 mg/kg p.o. (day 1), with EC50 values>10 μg/ml. A-960656 did not inhibit CYP enzymes with IC50 values>10 μM for CYP2C9, CYP2D6, and CYP3A4, and the compound showed no propensity to induce CYP (in vitro assessment with CYP-3A4). Thus the in vitro data are consistent with the high oral bioavailability, rapid brain penetration, and high brain/blood ratio as seen in Fig. 4, with blood concentrations reaching a maximum (Tmax) at 0.8 h, with the (Cmax) of 274 ng/ml at a dose of 1 mg/kg.

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Table 3

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Blood exposure (ng/ml)</th>
<th>Blood exposure (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1 (acute dosing)</td>
<td>Day 12 (sub-chronic dosing)</td>
</tr>
<tr>
<td>0.1</td>
<td>61 ± 4</td>
<td>54 ± 4</td>
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<tr>
<td>0.3</td>
<td>125 ± 13</td>
<td>122 ± 7</td>
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<tr>
<td>1.0</td>
<td>493 ± 35</td>
<td>438 ± 28</td>
</tr>
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</table>

Fig. 4. Blood levels of A-960656 in rat after oral (open squares), and intravenous (solid squares) doses of 1 mg/kg. Results are shown as the average ± S.E.M. of n = 3 animals.
4. Discussion

A-960656 is a member a new structural class of histamine H3 receptor antagonists, as depicted in Fig. 1 (Black et al., 2009). The compound was designed to have physicochemical properties associated with good absorption, solubility, and CNS penetration (Johnson et al., 2009; Wager et al., 2010a, 2010b), with a low molecular weight of 381, log D<sub>7.4</sub> of 1.0, and low polar surface area (52 Å<sup>2</sup>). A-960656 is potent in vitro at human and rat histamine H3 receptors in radioligand competition binding assays, and demonstrates functional antagonism in blocking H3 agonist-induced increase of [3H]JcTPS binding (Table 1). It is highly selective (>330-fold) for histamine H3 receptors versus off-target sites screened in a commercial panel of >80 receptors, enzymes, and ion channels, and has no detectable interaction with histamine H1, H2, and H4 receptors. Because the compound is targeted for in vivo investigation, it was examined in vivo and found to be well tolerated in behavioral and cardiovascular assessments at doses at and above those found effective in the pain models. The finding of potential for QT prolongation at high blood exposures (relative to pain levels) does not affect the use of the agent to study pharmacology. We have previously described SAR and methodologies in other series able that were able to reduce hERG-based QT while preserving potent H3 receptor antagonism (Black et al., 2008; Cowart et al., 2007). Taken together, the in vitro selectivity and in vivo tolerability supports that anti-nociceptive efficacy of A-960656 in pain models is mediated specifically by histamine H3 receptors and is not confounded by non-specific behavioral effects.

The pharmacokinetic profile of the compound is well suited for in vivo use. In rat, high systemic levels are seen after oral or intravenous doses. A detailed examination in three species, rat, dog, and monkey, found good absorption (84–100% oral bioavailability), slow clearance, and long half-life (t<sub>1/2</sub> 1.9–8.3 h) as summarized in Table 4. It is rapidly absorbed in rat, with an oral t<sub>max</sub> of 0.8 h, and efficiently penetrates brain reaching high brain and blood levels at 1 h in the pharmacokinetic and pain studies. A-960656 has rapid membrane permeation in Caco-2 membranes with no propensity for P-gp efflux. This is important as P-gp (P-glycoprotein) is a major efflux transporter that could otherwise limit access of drugs to brain, and limit oral bioavailability. In the pain efficacy studies, comparable brain and blood levels are achieved at all doses, consistent with efficient brain penetration. Studies conducted to assess the non-specific partitioning of drug into tissues showed modest plasma protein binding (unbound drug fraction of 0.153) and a nearly identical degree of binding to brain tissue (unbound drug fraction of 0.137). Drug concentrations from the efficacy studies (Tables 3 and 4) show similar blood levels for similar doses (brain levels were also similar, see Supplemental Sections S2.3.2, S2.3.3). This supports that free concentrations of A-960656 should be similar in systemic circulation and in brain sites. Altogether, the compound’s potency, tolerability, and ability to sustain high drug levels after oral dosing met goals for an agent suited for evaluation in two models of pain.

A-960656 was evaluated in a spinal nerve ligation model of neuropathic pain. Onset of efficacy was rapid and significant at 60 min after oral doses of 1 and 3 mg/kg (Fig. 2). Efficacy was maintained on sub-chronic dosing for 11 days, even showing a slight improvement in efficacy on sub-chronic dosing, so that doses of 0.3–3 mg/kg were statistically effective (P < 0.01). Thus A-960656 is able to fully block mechanical allodynia, with doses of 1 and 3 mg/kg giving efficacy comparable to the clinically active drug gabapentin dosed at 100 mg/kg. Analysis of the dose response allowed calculation of the acute ED<sub>50</sub> as 2.17 mg/kg p.o. (day 1) and an EC<sub>50</sub> blood level of 639 ng/ml. Similarly, on the 11th day of sub-chronic dosed arm, the calculated ED<sub>50</sub> was 0.78 mg/kg p.o. and an EC<sub>50</sub> blood level of 255 ng/ml. A-960656 was effective in a model of osteoarthritis pain in rats. The onset of efficacy was rapid, as was previously seen in the neuropathic pain model. Efficacy was present at the 0.3 and 1 mg/kg doses after acute dosing, and was maintained on sub-chronic dosing for 12 days, even showing a slight improvement in efficacy on sub-chronic dosing, so that doses of 0.1, 0.3, and 1 mg/kg were statistically effective. Analysis of the dose response allowed calculation of the acute ED<sub>50</sub> as 0.52 mg/kg p.o. (day 1) and an EC<sub>50</sub> blood level of 233 ng/ml. Similarly, on the 12th day of the sub-chronic dosed arm, the calculated ED<sub>50</sub> was 0.11 mg/kg p.o. and an EC<sub>50</sub> blood level of 55 ng/ml.

Histamine H<sub>3</sub> receptors are found on presynaptic nerve terminals in neurons, prominently in the CNS, where they indirectly control the release of neurotransmitters (Arrang et al., 1983; Blandina et al., 2010; Cannon et al., 2007a; Haas et al., 2008; Hill et al., 1997). Histamine H3 receptors on histaminergic neurons are autoinhibitory controlling histamine release, and antagonists are known to release histamine in vitro and in vivo. Where localized on other neurons, histamine H3 receptor antagonists are able to induce release of acetylcholine, dopamine, and norepinephrine. We and others have studied a number of potent antagonists in vivo and found efficacy in diverse rodent models of cognition, and a number of reviews of efficacy in wide range of CNS behavioral models have appeared (Blandina et al., 2010; Brioni et al., 2011; Ebenshade et al., 2008).

Comparatively few studies have investigated the histamine system or histamine H3 receptor antagonists in pain sensation. Among these, histamine and its endogenous precursor L-histidine reduced pain in rodents after intracerebroventricular (i.c.v.) and systemic administration (Malmberg-Aiello et al., 1994). In the same report, the H<sub>3</sub> receptor agonist R-alpha-methylhistamine (dosed i.c.v.) was hyperalgesic, whereas the H<sub>3</sub> receptor thioperoamide blocked pain responses. Recent reports have appeared in the literature that some histamine H<sub>3</sub> receptor antagonists show efficacy in pathologically relevant preclinical pain models. Several structurally diverse histamine H<sub>3</sub> receptor antagonists were described as effective in diabetic and surgical neuropathic pain models (Shirakura et al., 2004). Later, two potent antagonists (GSK-207040 and GSK-334329) were reported to have anti-nociceptive efficacy in capsacin-induced secondary hyperalgesia (Medhurst et al., 2007a). GSK-189254 and GSK-334329 were effective in a chronic constriction injury-induced neuropathic pain model and in viral-induced allodynia in rat (Medhurst et al., 2008). We also recently reported that histamine H<sub>3</sub> receptor antagonists including GSK-189254 and ABT-239 are effective in rodents in a model of osteoarthritis pain, as well as in a spinal nerve ligation model of neuropathic pain (Hsieh et al., 2010).

A recent review of histamine H<sub>3</sub> receptor modulation of pain perception outlined uncertainties in the literature and highlighted the need for additional pharmacologic studies to clarify the pharmacology (Hough and Rice, 2011). The literature on histaminergic modulation of pain behaviors is complex, with different studies implicating activation or inhibition of H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub>, and H<sub>4</sub> receptors in inducing or inhibiting nociception. In addition to the reported efficacy of antagonism of the H<sub>3</sub> receptor in pain models, activation has also been reported to be antinociceptive (Cannon and Hough, 2005; Cannon et al., 2003; 2007b). Farzin and Nosrati (2007) concluded activation of histamine H<sub>1</sub> and H<sub>2</sub> receptors was pro-nociceptive in a formalin-induced pain model. In other mouse models, activation of histamine H1 and H3 receptors was pro-nociceptive, while antagonism of histamine H2 and H4 receptors was antinociceptive (Farzin et al., 2002). Khalilzadeh and others (2010) reported histamine microinjected into dentate gyrus in rats was antinociceptive in formalin-induced pain. Because histamine H<sub>3</sub> receptor antagonists have been shown able to induce release of numerous neurotransmitters, there is potential for multiple neurotransmitters to be involved modulating effects in pain models. We recognized the potential involvement of the adrenergic system in modulating the efficacy of histamine H<sub>3</sub> receptor antagonists, based on reports of norepinephrine release by histamine H<sub>3</sub> receptor antagonists and the extensive literature on the involvement of the adrenergic system in pain (reviewed by Pertovaara, 2006). We investigated GSK-189254, an H<sub>3</sub> receptor antagonist with antinociceptive efficacy (Medhurst et al., 2007b, 2008). Our studies in osteoarthritis pain...
supported a CNS site of action and involvement of adrenergic alpha-2 receptor activation, because efficacy after systemic and intrathecal dosing was blocked by co- dosing with intrathecal phenolamine (Hsieh et al., 2010). Taken as a whole, reports from a number of labs with different animal models implicate the histaminergic system in pain sensation. But the literature on the pharmacology of histaminergic systems in pain is complex, and as yet no simple clear picture has emerged unifying all the observed studies. There is thus a need for additional studies using pharmacologically selective and well behaved potent and selective compounds such as A-960656.

5. Conclusion

A-960656 is a potent and selective histamine H3 receptor antagonist with properties suited to in vivo investigation. The compound is highly soluble, CNS penetrant, and orally bioavailable in all species examined, and well tolerated in vivo. A-960656 is potent and efficacious in rat models of osteoarthritis and neuropathic pain that are relevant to the type of chronic pain observed in humans. Efficacy is achieved rapidly after acute oral administration, and sustained for several days of daily dosing. A-960656 belongs to a novel H3 antagonist pharmacophore that central histamine H3 receptor antagonism can modulate nociceptive processes. We believe further research with this molecule is justified to probe its pharmacology in more detail to advance the goal of discovering a new generation of pain medications based on histamine H3 receptor antagonism.

Acknowledgments

We thank Timothy B. Esbenshade, Thomas R. Miller, and Timothy Vorthers, for in vitro histamine assays, Scott Mittelstadt, Patricia Banfor, Brett Herzberg, Eugene Shek, and Jim Limberis for in vitro and in vivo cardiovascular profiling, and Kaitlin Browman for assessment of CNS effects in the Irwin test.

Appendix A. Supplementary data

Supplementary to this article can be found online at doi:10.1016/j.ejphar.2012.03.048.

References


