PXS-4681A, a Potent and Selective Mechanism-Based Inhibitor of SSAO/VAP-1 with Anti-Inflammatory Effects In Vivo

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ABSTRACT

Semicarbazide-sensitive amine oxidase (SSAO), also known as vascular adhesion protein-1 (VAP-1), is a member of the copper-dependent amine oxidase family that is associated with various forms of inflammation and fibrosis. To investigate the therapeutic potential of SSAO/VAP-1 inhibition, potent and selective inhibitors with drug-like properties are required. PXS-4681A [(Z)-4-(2-(aminomethyl)-3-fluoroallyloxy)benzenesulfonamide hydrochloride] is a mechanism-based inhibitor of enzyme function with a pharmacokinetic and pharmacodynamic profile that ensures complete, long-lasting inhibition of the enzyme after a single low dose in vivo. PXS-4681A irreversibly inhibits the enzyme with an apparent $K_i$ of 37 nM and a $k_{inact}$ of 0.26 min$^{-1}$ with no observed turnover in vitro. It is highly selective for SSAO/VAP-1 when profiled against related amine oxidases, ion channels, and seven-transmembrane domain receptors, and is superior to previously reported inhibitors. In mouse models of lung inflammation and localized inflammation, dosing of this molecule at 2 mg/kg attenuates neutrophil migration, tumor necrosis factor-$\alpha$, and interleukin-6 levels. These results demonstrate the drug-like properties of PXS-4681A and its potential use in the treatment of inflammatory disease.

Introduction

Amine oxidases are a family of enzymes that catalyze the oxidation of a wide variety of endogenous amines such as histamine or dopamine. There are two classes of amine oxidases that are characterized as being either flavin dependent, such as monoamine oxidase (MAO) A and B, or copper dependent, such as lysyl oxidase (LOX) and lysine demethylase. Semicarbazide-sensitive amine oxidase (SSAO) [also known as vascular adhesion protein-1 (VAP-1), primary amine oxidase or plasma amine oxidase, EC 1.4.3.21; gene name: amine oxidase copper containing-3 (AOC-3)] is a member of the copper-dependent family of amine oxidases (Salmi and Jalkanen, 1992). In humans, four AOC genes are known; three (AOC-1–3) translate into enzymatically active protein, and one pseudogene (AOC-4) into a truncated inactive form (Schwelberger, 2007). AOC-1 codes for a diamine oxidase (DAO) found in the kidney, gut, and lung and is involved in the metabolism of histamine (Green et al., 1987). AOC-2 encodes for an amine oxidase that has been characterized by its enzyme activity in the eye, which metabolizes larger monoamines than AOC-3 (Imamura et al., 1997; Kaitaniemi et al., 2009). AOC-3 is the best characterized AOC with many implications in human diseases.

SSAO/VAP-1 is a type 1 membrane-bound protein that has a distal adhesion domain and a catalytic amine oxidase site proximal to the membrane. Both sites have been shown to be critical for SSAO/VAP-1-mediated inhibition of leukocyte rolling, adhesion, and transmigration in response to inflammatory stimuli (OSullivan et al., 2004; Stolen et al., 2005; Lalor et al., 2007; Jalkanen et al., 2007). SSAO/VAP-1 catalyzes the oxidation of primary amines to aldehydes, releasing ammonia and hydrogen peroxide upon regeneration of the cofactor (Scheme 1), and is sensitive to carbonyl-reactive reagents (such as semicarbazide) but insensitive to selective flavin-dependent MAO-A and MAO-B enzyme inhibitors such as selegiline and clorgiline (Morin et al., 2001).

SSAO/VAP-1 is found in adipocytes, smooth muscle cells, and endothelial cells, and is highly expressed in the lung, aorta, liver, and ileum (Andrés et al., 2001). Membrane-bound SSAO/VAP-1 is proteolytically cleaved by a metalloprotease to release an active, soluble form of the protein (Stolen et al., 2004), which has been shown to be a biomarker of disease progression in patients with liver and kidney fibrosis (Kurkijärvi et al., 1998, 2000, 2001; Lin et al., 2008), cancer (Li et al., 2011), cardiovascular disease (Li et al., 2011; Aalto et al., 2012), and metabolic disorders (Boomsma et al., 1999; Li et al., 2009). Endogenous substrates for SSAO/VAP-1 include methylamine and aminoacetone (Precious et al., 1998; Lyles and Chalmers, 1992).

ABBREVIATIONS: AOC, amine oxidase copper containing; BTT-2052, (1S,2S)-2-(1-methylhydrazinyl)-2,3-dihydro-1H-inden-1-ol; DAO, diamine oxidase; HES, HEPES-EDTA mixed buffer; IL-6, interleukin 6; LJP 1207, N-((2-phenyl-allyl)-hydrazine hydrochloride; LJP 1586, Z-3-fluoro-2-(4-methoxybenzyl)allylamine hydrochloride; LOX, lysyl oxidase; LPS, lipopolysaccharide; MAO, monoamine oxidase; P450, cytochrome P450; PXS-4159A, (E)-4-(4-amino-2-fluorobut-2-enyloxy)-N-cyclohexylbenzamide hydrochloride; PXS-4681A, (Z)-4-(2-(aminomethyl)-3-fluoroallyloxy)benzenesulfonamide hydrochloride; rhMAO, recombinant human SSAO, semicarbazide-sensitive amine oxidase; TNF-$\alpha$, tumor necrosis factor-$\alpha$; VAP-1, vascular adhesion protein-1.
Small molecule inhibitors of SSAO/VAP-1 reduce inflammation in various in vivo models, including rheumatoid arthritis, lung inflammation and carrageen air pouch inflammation, and stroke (Xu et al., 2006; Yu et al., 2006; O’Rourke et al., 2008). Different types of small molecules have been developed and can be grouped into two main classes: irreversible, mechanism-based inhibitors based on either allylamines (compounds 1–3, Fig. 1) or hydrazines (compounds 4 and 5) and noncovalent/reversible binding inhibitors (compounds 6 and 7). These have been extensively reviewed elsewhere (McDonald et al., 2007; Dunkel et al., 2011).

Despite this wealth of potential new chemical entities, study of the in vivo effects of SSAO/VAP-1 enzyme inhibition has been hampered by lack of selectivity (compounds 1 and 2; O’Rourke et al., 2008), poor drug-like properties such as low solubility (compound 7) or insufficient pharmacokinetics, and class-dependent safety concerns such as the presence of hydrazine groups (compounds 4 and 5), or turnover of inhibitors by the enzyme (compound 3). Because of the considerable differences in pharmacology between species (Yu et al., 1994), it is also a challenge to design inhibitors of SSAO/VAP-1 with sufficient potencies across species (Inoue et al., 2013a,b) and thereby accurately determine their therapeutic potential and safety window. For example, PXS-4159A [(E)-4-(4-amino-2-fluorobut-2-enyloxy)-N-cyclohexylbenzamide hydrochloride; compound 3] has recently been reported as a potent, selective molecule with promising in vivo pharmacokinetic parameters that shows efficacy in a mouse model of lung inflammation when dosed orally at 100 mg/kg (Foot et al., 2012). However, despite these desirable properties, PXS-4159A was found to suffer from a narrow therapeutic window in rodents (due to poor efficacy against the rodent forms of the enzyme), limiting the study of the compound in in vivo models. It was also found to be an “imperfect” irreversible inhibitor in vitro with some degree of substrate turnover prior to inhibition. Herein we describe the in vitro profile and in vivo efficacy of a new allylamine-based
inhibitor, PXS-4681A ([Z]-4-(2-(aminomethyl)-3-fluoroallyloxy) benzenesulfonylamide hydrochloride), which possesses the ideal characteristics to fully study the therapeutic potential of SSAO/VAP-1 small molecule inhibitors.

Materials and Methods

Fluorometric Enzyme Activity Assays. All of the enzymatic activities were based on the production of H$_2$O$_2$ as described in Zhou et al. (1997) and used an Amplex Red amine oxidase kit (Invitrogen, Carlsbad, CA) with the appropriate substrates. Purified recombinant human SSAO/VAP1 (rhSSAO) was obtained from CSIRO (Melbourne, VIC, Australia), and was used with 0.6 mM benzylamine (Sigma-Aldrich, St. Louis, MO) as the substrate. Background readings per minute (low control) were obtained from assays run in the presence of 1 μM of the inhibitor mofegiline. Where present, PXS-4681A was preincubated for 30 minutes at 37°C before exposure to the substrate and IC$_{50}$ values were calculated from the concentration-response curves using Studies software (Dortmatics, Herts, UK).

Rat, mouse, rabbit, and dog abdominal fats were surgically removed and weighed right after being killed. All protocols were in compliance with local handling guidelines. Tissues were homogenized in ice-cold HES buffer (20 mM HEPES, 1 mM EDTA, sucrose 250 mM, 1 μM protease and phosphatase inhibitors, pH 7.4) prior to assay.

Inhibition of recombinant human diamine oxidase (rhDAO; kindly provided by Prof. Mitchell Guss, University of Sydney, Sydney, NSW, Australia) activity was measured as described for rhSSAO, with the exception that phenyl-ethylamine was used as substrate.

The AOC-2 gene product was produced from cDNA (kindly provided by Prof. Kati Klima, University of Turku, Turku, Finland) as described by Kaitaniemi et al. (2009). Inhibition of the enzyme activity was then measured as described for rhSSAO, with the exception that 200 μM putrescine and 10 μM of inhibitor aminoguanidine (both from Sigma-Aldrich) were used as the substrate and low control, respectively.

The AOC-2 gene product was produced from cDNA (kindly provided by Prof. Kati Klima, University of Turku, Turku, Finland) as described by Kaitaniemi et al. (2009). Inhibition of the enzyme activity was then measured as described for rhSSAO, with the exception that phenyl-ethylamine was used as substrate.

Bovine LOX was extracted by adapting the methodology from Rucker et al. (1996) from calf aorta (mixed sex); the homogenized tissue was washed extensively with phosphate-buffered saline to remove LOX inhibitors and readily soluble proteins, and then extracted in 4 M urea, 50 mM sodium borate buffer, pH 8.2. The supernatant was washed and concentrated by means of Amicon 10-kDa centrifuge filters (Millipore) in 1.2 M urea, 50 mM sodium borate buffer, pH 8.2, and in the presence of protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM N-ethylmaleimide, 0.5 mM p-aminobenzonic acid, 20 mg/ml soybean trypsin inhibitor, all from Sigma-Aldrich). Inhibition of LOX activity was tested under the same conditions as for rhSSAO with the exception that the LOX enzyme mixture was pretreated with 0.5 mM pargyline and 1 μM mofegiline, in 1.2 M urea buffer, 50 mM sodium borate, pH 8.2. The substrate used was 10 mM putrescine and 100 μM β-aminopropionitriile (Sigma-Aldrich) was used as low control.

The following substrates were used for recombinant human monoamine oxidases A and B (rhMAO-A and rhMAO-B, respectively; Sigma-Aldrich): 200 μM tyramine (Sigma-Aldrich) for rhMAO-A, and 100 μM benzylamine for rhMAO-B. The low controls for rhMAO-A and rhMAO-B assay were 1.5 μM clorgiline (Sigma-Aldrich) and 1 μM mofegiline, respectively.

Determination of Kinetic Parameters. The kinetic parameters for PXS-4681A were determined according to the Kitz–Wilson method (Kitz and Wilson, 1962; Holt et al., 2008).

Compound Oxidation. This assay determines the substrate propensities of a compound relative to background (dimethyl sulfoxide only). Compound oxidation by rhSSAO was measured by fluorometric assay (Holt and Palicic, 2006). Briefly, rhSSAO was incubated for 2 hours at 37°C in HEPES buffer before the addition of an equal volume of Amplex Red (20 μM), horseradish peroxidase (4 U/ml), and PXS-4681A (2.5 μM) in the same buffer. The kinetics of the formation of resorufin was measured immediately using Optima reader (BMG Labtech GmbH, Ortenburg, Germany), at 37°C.

Ion Channels and Off-Target Screening. Studies on hERG and Nav were carried out at Aptuit (Verona, Italy) and off-target screenings were performed by Ricerca Biosciences (Taipei, Taiwan), all according to standard conditions.

In Vitro Drug Metabolism and Pharmacokinetics Studies. Microsomal, hepatocyte, and whole blood stability studies, and the cytochrome P450 (CYP450) assays were performed by Ricerca Biosciences (Taipei, Taiwan), all according to standard conditions.

Pharmacokinetics and Pharmacodynamics. Studies were performed by ICP Firefly (Sydney, NSW, Australia), Microsomal, hepatocyte, and whole blood stability studies, and the cytochrome P450 (CYP450) assays were performed at the Centre for Drug Candidate Optimization, Melbourne, VIC, Australia.

Pharmacokinetics and Pharmacodynamics. Studies were performed by ICP Firefly (Sydney, NSW, Australia). Rats weighing approximately 280 g were administered PXS-4681A orally at 20 mg/kg or intravenously at 10 mg/kg. At various times between 15 minutes and 9 hours, blood samples were collected by tail vein bleed. Male BALB/c mice weighing approximately 20 g were administered 2 mg/kg test compound in a volume of 10 ml/kg for both intravenous and oral routes. Blood samples were collected from each animal at various times between 10 minutes and 3 hours by tail vein bleed. Plasma was prepared and kept frozen until analyzed for PXS-4681A by high-performance liquid chromatography–tandem mass spectrometry. Basic pharmacokinetic parameters were estimated by PK Solutions software (Summit Research Services).

To measure SAOS activity after one dose, mice were orally administered PXS-4681A at concentrations ranging from 0.06 to 10 mg/kg and weighed right after being killed. All protocols were in compliance with local handling guidelines.

Scheme 2. Synthesis and structure of PXS-4681A: i, (Boc)$_2$O; ii, tert-butyl dimethylsilyl chloride; iii, dimethyl sulfoxide, oxalyl chloride; iv, sodium hexamethyldisilazide, Ph$_3$PCH$_2$BF$_4$; v, separation of E/Z isomers via normal phase column chromatography; vi, Ph$_2$P, diisopropyl azodicarboxylate, 4-hydroxybenzenesulfonyl fluoride, and vii, TFA (trifluoroacetic acid) then HCl. MWT, molecular weight; Boc, tert-butyloxycarbonyl; TBDMBS, tert-butyldimethylsilyl.
2 mg/kg. Animals were killed after 3 h, and abdominal fat was collected. To measure the recovery profile of SSAO activity, mice were administered 2 mg/kg PO PX5-4681A and killed at various time points for abdominal fat collection. Tissue samples were homogenized as described previously and analyzed using a fluorometric method for rhSSAO activity.

Lipopolysaccharide Airway Inflammation. This study was performed by Washington Biotechnology (Baltimore, MD). One hour before disease induction, the different therapeutic groups were orally administered vehicle (phosphate-buffered saline), 0.2–2 mg/kg PX5-4681A, or 10 mg/kg dexamethasone. Inflammation was induced by endotracheal instillation of lipopolysaccharide (LPS) at 20 mg/kg in 40 μl vehicle. Six hours later, mice were killed, and bronchoalveolar lavage fluid was collected for recovery of airway luminal cells. This procedure was achieved by cannulating the trachea and lavaging the lungs with 0.5 ml of heparinized (10 U/ml) saline. An aliquot of the lavage was reserved for total and differential white cell counts. The remaining fluid was centrifuged and the supernatants used to measure cytokines.

Carrageenan Air Pouch Model. This study was performed by Washington Biotechnology. Mice were anesthetized, and 6 ml sterile air was injected subcutaneously as described elsewhere (Romano et al., 1997). After 3 days, the pouches were re-injected with 3 ml sterile air. On day 6, animals received 10 ml/kg vehicle or test materials; treated animals received either 10 mg/kg PO dexamethasone or 2 mg/kg PO PX5-4681A. At the end of the 1-hour pretreatment, the air pouch was injected with 1 ml carrageenan solution or 1 ml saline for the vehicle group. At 6 hours after carrageenan administration, the mice were killed, and the pouches were washed with saline. The exudates were used for volume, cell counts, and cytokine measurements.

Test Item Details. Mofegiline (compound 1) and LJP 1586 (compound 2; Z-3-fluo-ro-2-(4-methoxybenzyl)allylamine hydrochloride) were synthesized by SYNthesis (Shanghai, China) according to literature procedures (Evans et al., 1996; Wang et al., 2007). PX5-4681A (Scheme 2) was isolated as the hydrochloride salt from a seven-step synthetic sequence, starting from 3-aminopropane-1,2-diol. PX5-4681A has high solubility (>10 mg/ml) in aqueous media and is stable both as a dry powder and in solution for prolonged periods.

Results

Potency and Specificity of PX5-4681A. Inhibition of SSAO/VAP-1 was measured in a fluorescence-based in vitro assay using benzylamine as a substrate and either recombinant protein or homogenized tissue. PX5-4681A was found to be an inhibitor of SSAO/VAP-1 in human, rat, mouse, rabbit, and dog species with an IC50 of <10 nM in all cases (Table 1). This pan-species potency is a substantial improvement on the previously reported allylamine inhibitors (O’Rourke et al., 2008; Foot et al., 2012). Selectivity over the closely related DAO and LOX was shown to be excellent, at more than 250- and 4000-fold, respectively. The IC50 for the AOC-2 gene product was found to be 1.87 μM, which is consistent with the AOC-2 protein having a different substrate profile (Kaitaniemi et al., 2009). Inhibition of the FAD-dependent monoamine oxidases, MAO-A and MAO-B, was also found to be negligible (Table 1). The selectivity of LJP 1586 over MAO-B in the same assay was only 14-fold (Table 1), whereas PX5-4681A was found to be approximately 10,000-fold more selective for rhSSAO/VAP-1.

In Vitro Kinetics of PX5-4681A. To study the mechanism of action of PX5-4681A, the Kitz–Wilson method was then applied to measure the time-dependent inhibition of SSAO/VAP-1 (Kitz and Wilson, 1962). A double reciprocal plot was used to derive an apparent Ki of 37 nM and a kina of 0.26 min\(^{-1}\) (Fig. 2).

### Table 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>PX5-4681A (Compound 8)</th>
<th>Mofegiline (Compound 1)</th>
<th>LJP 1586 (Compound 2)</th>
<th>PX5-4159A (Compound 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhSSAO</td>
<td>0.003</td>
<td>0.018</td>
<td>0.038</td>
<td>0.011</td>
</tr>
<tr>
<td>Rat fat SSAO</td>
<td>0.003</td>
<td>0.006</td>
<td>0.042</td>
<td>0.77</td>
</tr>
<tr>
<td>Mouse fat SSAO</td>
<td>0.002</td>
<td>0.005</td>
<td>ND</td>
<td>0.15</td>
</tr>
<tr>
<td>Dog fat SSAO</td>
<td>0.003</td>
<td>0.004</td>
<td>ND</td>
<td>0.081</td>
</tr>
<tr>
<td>Rabbit fat SSAO</td>
<td>0.009</td>
<td></td>
<td>ND</td>
<td>&gt;2</td>
</tr>
<tr>
<td>rhDAO</td>
<td>0.80 (267×)</td>
<td>&gt;90 (1500×)</td>
<td>&gt;10 (263×)</td>
<td>26.13 (2300×)</td>
</tr>
<tr>
<td>Bovine aorta LOX</td>
<td>19.0 (&gt;4000×)</td>
<td>&gt;1 (&gt;55×)</td>
<td>&gt;30 (&gt;800×)</td>
<td>7.08 (640×)</td>
</tr>
<tr>
<td>rhMAO-A</td>
<td>17.11 (5700×)</td>
<td>1.78 (99×)</td>
<td>&gt;30 (&gt;800×)</td>
<td>&gt;30 (&gt;2700×)</td>
</tr>
<tr>
<td>rhMAO-B</td>
<td>29.84 (10,000×)</td>
<td>0.004 (0.2×)</td>
<td>0.53 (14×)</td>
<td>1.12 (100×)</td>
</tr>
</tbody>
</table>

ND, not determined.
Having established that PXS-4681A is a mechanism-based inhibitor, the potential for compound turnover by SSAO/VAP-1 was then assessed, using an assay to measure the amount of oxidation at a single concentration of 2.5 μM. As shown in Fig. 3A, the simultaneous application of enzyme and PXS-4681A did not cause any increase in fluorescence over background. PXS-4159A, which was previously shown to have a turnover number of approximately 10 (Foot et al., 2012), was used as a positive control. When the relative increase in fluorescence was measured after 30 minutes of incubation, no significant difference between dimethylsulfoxide background and PXS-4681A was observed, whereas PXS-4159A increased the number of counts (Fig. 3B).

**Off-Target Activity.** PXS-4681A was tested in vitro against a number of additional targets, at a single concentration of 10 μM (Table 2). This screen included some of the most relevant off targets (Bowes et al., 2012). PXS-4681A was found to have little activity against the targets tested, with the exception of carbonic anhydrase II, which was 80% inhibited at 10 μM (estimated IC₅₀ of 2.5 μM, >400-fold selective). Activity against this enzyme, and the structural similarity to dorzolamide, would suggest that there may also be a rapid uptake of PXS-4681A into the red blood cells (Biollaz et al., 1995). The distribution from plasma into red blood cells was confirmed because the blood/plasma ratio was high (Table 3). In addition to this panel, PXS-4681A showed no inhibition in patch-clamp recordings from hERG tail currents or hNav1.5 inward currents (Table 2).

**In Vitro and In Vivo Pharmacokinetic Properties.** In addition to an excellent potency and selectivity profile, PXS-4681A also has very promising pharmacokinetic properties. PXS-4681A was profiled for microsomal stability and activity against the most common P450 isozymes. In both human and rat microsomal preparations, PXS-4681A exhibited a very low rate of degradation and showed minimal inhibition of the P450s tested (Table 3). PXS-4681A was also tested for plasma and whole blood stability. As expected from the observed activity against carbonic anhydrase II, PXS-4681A was found to be stable in plasma but was rapidly taken up by red blood cells in whole blood.

The pharmacokinetic properties of PXS-4681A were investigated first in rats and then in mice (n = 3 in each group) and are reported in Table 4. In rats, PXS-4681A was well absorbed with good bioavailability and oral half-life at the 10 mg/kg i.v. dose and the 20 mg/kg PO dose. Because many models of inflammation are performed in mice, the pharmacokinetic profile of PXS-4681A was also evaluated in BALB/C mice (Table 4). Given the excellent exposure observed in the

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**TABLE 2**

<table>
<thead>
<tr>
<th>Receptor species</th>
<th>Species</th>
<th>Inhibition by 10 μM PXS-4681A %</th>
<th>Approximate IC₅₀ μM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbonic anhydrase II</strong></td>
<td>Human</td>
<td>80</td>
<td>2.5</td>
</tr>
<tr>
<td>Phosphodiesterase PDE3A</td>
<td>Human</td>
<td>−5</td>
<td>—</td>
</tr>
<tr>
<td>Adenosine A₁</td>
<td>Human</td>
<td>−4</td>
<td>—</td>
</tr>
<tr>
<td>Adrenergic α₂A</td>
<td>Human</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>Calcium channel L-type, benzothiazepine</td>
<td>Rat</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>Calcium channel L-type, phenylalkylamine</td>
<td>Rat</td>
<td>18</td>
<td>—</td>
</tr>
<tr>
<td>Dopamine D₂B</td>
<td>Human</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Histamine H₁</td>
<td>Human</td>
<td>9</td>
<td>—</td>
</tr>
<tr>
<td>Muscarinic M₂</td>
<td>Human</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>Serotonin (5-hydroxytryptamine) 5-HT₂C</td>
<td>Human</td>
<td>−2</td>
<td>—</td>
</tr>
<tr>
<td>Sodium channel, site 2</td>
<td>Rat</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>Transporter, dopamine (DAT)</td>
<td>Human</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>Transporter, serotonin (5-hydroxytryptamine) (SERT)</td>
<td>Human</td>
<td>12</td>
<td>—</td>
</tr>
<tr>
<td>hERG tail current</td>
<td>Human</td>
<td>&gt;100</td>
<td>—</td>
</tr>
<tr>
<td>hNav1.5</td>
<td>Human</td>
<td>&gt;100</td>
<td>—</td>
</tr>
</tbody>
</table>
been reported to induce neutrophil infiltration and inflammatory inflammation triggered by LPS application into the lungs has been established that 2 mg/kg was an adequate dose to achieve maximal inhibition of SSAO function after 6 hours in the rat study, dosing in the mice pharmacokinetic studies was lowered to 2 mg/kg for both intravenous and oral studies. Even at the lower (oral) dose, a maximal concentration of 0.6 μM was reached, with all derived pharmacokinetic values mirroring the rat data.

**Pharmacodynamic Profile In Vivo.** To determine the inhibition of SSAO related to the oral application of PXS-4681A, abdominal mouse fat was collected at various time points. Abdominal fat has high SSAO activity and provides a large assay window to detect SSAO inhibition. Mice orally administered a 0.2 mg/kg dose showed an 80% reduction in SSAO activity compared with the control group, whereas 0.6 and 2 mg/kg dosing resulted in near-total inhibition of the enzyme at 3 hours (Fig. 4A). The recovery of enzyme activity after a single dose of PXS-4681A was then studied. A 2 mg/kg dose resulted in virtually complete inhibition of SSAO activity up to 5 hours after administration, with only a 30% recovery in activity observed after 24 hours, consistent with de novo protein synthesis (Elliott et al., 1989). Inhibition of >50% was still apparent at 48 hours, which was the latest time examined (Fig. 4B).

From the pharmacokinetic/pharmacodynamic data, it was established that 2 mg/kg was an adequate dose to achieve maximal inhibition of SSAO function after 6 hours in the following mouse models of inflammation.

**Anti-Inflammatory Properties of PXS-4681A In Vivo.** Given the substantial literature evidence that inhibition of SSAO/VAP-1 reduces inflammation, the anti-inflammatory properties of PXS-4681A in vivo were next investigated. The inflammation triggered by LPS application into the lungs has been reported to induce neutrophil infiltration and inflammatory cytokines (Corteling et al., 2002; Wallin et al., 2004). The dose-response relationship for the effects of PXS-4681A on leukocyte influx induced by LPS lung injury was therefore first investigated. PXS-4681A at concentrations of 0.2 and 2 mg/kg had significant effects on leukocyte trafficking, with a neutrophil infiltration reduction of >33% in both dosing groups (Fig. 5A), down to the levels of the baseline and control (dexamethasone) groups. At the highest dose of 2 mg/kg, PXS-4681A reduced tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels by 54 and 27%, respectively, but showed no effect at 0.2 mg/kg (Fig. 5, B and C). This observation is consistent with the pharmacodynamic profile of PXS-4681A, and suggests that for maximal anti-inflammatory effect, complete inhibition of the enzyme activity is required.

The effect of PXS-4681A on carrageenan-induced skin inflammation was next studied at a single 2 mg/kg dose. PXS-4681A reduced local inflammation, causing a significant reduction in exudate volume by 25% (Fig. 6A). Concomitant with the diminished inflammation was a significant reduction in the neutrophil count in the exudate (Fig. 6B). Quantification of the cytokine levels in the exudate again showed a significant reduction in TNF-α and IL-6 (44 and 21%, respectively; Fig. 6, C and D). The reduction in inflammation seen in the PXS-4681A-treated group was comparable with that seen in the dexamethasone group.

### Discussion

PXS-4681A is a novel SSAO/VAP-1 inhibitor with single-digit nanomolar potency against human, mouse, rat, rabbit, and dog orthologs, and great selectivity over the related amine oxidases. It is a mechanism-based inhibitor with no substrate turnover in vitro and has a pharmacokinetic and pharmacodynamic profile that ensures long-lasting inhibition of the target enzyme in vivo after a single low dose. PXS-4681A has minimal off-target activity against a variety of enzymes and receptors.

When PXS-4681A was administered to mice prior to LPS-induced lung injury, animals were protected against inflammation, with reduced neutrophil infiltration and related cytokines. LPS is a bacterial surface antigen that initiates a plethora of inflammatory responses. It induces activation of macrophages and neutrophils, leading to the release of inflammatory cytokines, including TNF-α and IL-6 (Corteling et al., 2002; Wallin et al., 2004). These data suggest that inhibition of SSAO

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**TABLE 3**

<table>
<thead>
<tr>
<th>Species</th>
<th>Study</th>
<th>Result</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Microsomal stability half-life</td>
<td>223 min</td>
<td>CYP1A2, CYP2C9, CYP2D6, CYP3A4, CYP3A5</td>
</tr>
<tr>
<td></td>
<td>Intrinsic in vitro clearance</td>
<td>5 (μM/min/mg)</td>
<td>&gt;10</td>
</tr>
<tr>
<td></td>
<td>Plasma stability (2 h), fraction remaining</td>
<td>&gt;90%</td>
<td>6.7</td>
</tr>
<tr>
<td>Human</td>
<td>Microsomal stability half-life</td>
<td>150 min</td>
<td>CYP2C19</td>
</tr>
<tr>
<td></td>
<td>Intrinsic in vitro clearance</td>
<td>12 (μM/min/mg)</td>
<td>&gt;10</td>
</tr>
<tr>
<td></td>
<td>Plasma stability (2 h), fraction remaining</td>
<td>&gt;90%</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>Whole blood stability</td>
<td>Rapidly taken up into red blood cells</td>
<td></td>
</tr>
</tbody>
</table>
activity leads to decreased neutrophil rolling/extravasation, resulting in a reduction in inflammation.

This was further validated when PXS-4681A–treated animals underwent the carrageenan air pouch inflammation model. Injection of carrageenan prompts leukocyte infiltration that increases the local exudate and is suggestive of vascular permeability. In this setting, the air pocket serves as a reservoir of cytokines and cells that can be easily measured in the exudate. Leukocytes, especially neutrophils, are the main cellular components recruited to inflammation induced by carrageenan. These cells secrete TNF-α, which then induces adherence of neutrophils to endothelial cells and leads to a massive cellular infiltration into the air pouch space, further enhancing the inflammatory response (Romano et al., 1997; Ziaeea et al., 2012). In animals pretreated with PXS-4681A, the number of neutrophils that migrated into the air pouch was decreased by >50% and the observed levels of TNF-α and IL-6 were also diminished. In addition to this, the study showed that inhibition of SSAO/VAP-1 enzymatic activity reduced the exudate volume, suggesting a suppression of vascular leakage.

Taken together, the results from these in vivo models further validate the literature precedence for SSAO/VAP-1 as an anti-inflammatory target (O’Sullivan et al., 2004; Xu et al., 2006; Yu et al., 2006; Jalkanen et al., 2007; O’Rourke et al., 2008), and strengthen the evidence that the enzymatic activity of this protein plays a role in leukocyte recruitment, specifically neutrophils, in response to insult.

Despite the numerous SSAO/VAP-1 inhibitors present in the literature, none of the previously reported compounds has exhibited ideal drug-like characteristics required for full investigation of the in vivo effects of SSAO enzyme inhibition. Issues such as poor cross-species potency, unsuitable physicochemical properties, selectivity, and inherent class-based toxicity or off-target activity have proven to be a significant hurdle to drug development. For example, PRX.A, an inhibitor thought to be structurally related to compound 6, exhibited nanomolar potency against the mouse enzyme and was efficacious in a murine model of pulmonary metastasis (Ferjančič et al., 2013); however, this molecule also had undesirable off-target activity against human dopamine transporter and Na⁺ channel proteins at 1 μM. In addition,
Inoue et al. (2013a,b,c) documented the development of compound 7, which showed efficacy in a rat streptozotocin-induced ocular permeability model but suffered from poor pharmacodynamic coverage (see below).

Given that SSAO/VAP-1 is one of many proteins involved in a multifaceted immune response, it is likely that long-lasting inhibition of the enzyme will be required to have the most beneficial effect on disease state. This theory is supported by the data we observed in the mouse LPS model in which only the higher dose of 2 mg/kg, which completely abrogates enzyme activity, had an effect on TNF-α and IL-6 levels. A complete uninterrupted enzyme inhibition is likely to be a challenge for the noncovalent/reversible inhibitors going forward, and higher or more frequent dosing to achieve this may reduce the therapeutic window. For example, an in vivo study using compound 7 (dosing daily at 10 mg/kg PO over 2 weeks) indicated that this inhibitor was not able to completely inhibit plasma SSAO/VAP-1 levels 24 h after the final dose (Inoue et al., 2013c), further supporting this critical pharmacokinetic/pharmacodynamic relationship.

Therefore, it is likely that the most suitable candidate for clinical progression will be obtained from the mechanism-based inhibitors, in which irreversible, lasting inhibition of the enzyme can be achieved at much lower dosing. Given the inherent safety concerns with developing hydrazine-based inhibitors (such as compounds 4 and 5), the halo-allylamine–based inhibitors represent the most probable way forward if high selectivity over the related amine oxidases can be achieved. Mofegiline (compound 1), which has already been assessed in the clinic as an MAO-B inhibitor (and shown to be safe), has been an excellent tool compound for in vitro work; however, due to its dual action, it is difficult to further develop. Likewise, LJP 1586

**Fig. 6.** Inhibition of leukocyte trafficking and cytokine levels in a carrageenan-induced air pouch inflammation model showing total neutrophil counts (A) and concentrations of TNF-α (B) and IL-6 (C). Results are the mean ± S.E.M. of groups of eight mice. ***P < 0.001, by one-way analysis of variance followed by the Newman–Keuls multiple comparison test.


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