Anti-inflammatory effects of LJP 1586, an amine-based inhibitor of 

semicarbazide-sensitive amine oxidase activity.

Anne M. O’Rourke, Eric Y. Wang, Andrew Miller, Erika M. Podar, Kelly Scheyhing, Li 
Huang, Christina Kessler, Hongfeng Gao, Huong-Thu Ton-Nu, Mary T. MacDonald, 
David S. Jones and Matthew D. Linnik.

La Jolla Pharmaceutical Company, San Diego, CA
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Corresponding Author:
Anne M. O’Rourke, La Jolla Pharmaceutical Company, 6455 Nancy Ridge Drive, San Diego, CA 92121. Phone: 858-646-6617, FAX: 858-626-2845.
e-mail: anne.orourke@ljpc.com

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ABSTRACT

Semicarbazide-sensitive amine oxidase (SSAO, AOC3, vascular adhesion protein-1, VAP-1) is a copper-containing enzyme that catalyzes the oxidative deamination of primary amines to an aldehyde, ammonia and hydrogen peroxide. SSAO is also involved in leukocyte migration to sites of inflammation, and the enzymatic activity of SSAO is essential to this role. Thus, inhibition of SSAO enzyme activity represents a target for the development of small molecule anti-inflammatory compounds. Here, we have characterized the novel SSAO inhibitor Z-3-fluoro-2-(4-methoxybenzyl)allylamine hydrochloride (LJP 1586) and assessed it’s anti-inflammatory activity. LJP 1586 is a potent inhibitor of rodent and human SSAO activity, with IC50 values between 4 and 43 nM. The selectivity of LJP 1586 was confirmed with a broad panel of receptors and enzymes that included the monoamine oxidases-A and -B. Oral administration of LJP 1586 resulted in complete inhibition of rat lung SSAO with an ED50 between 0.1 and 1 mg/kg, and a pharmacodynamic half-life of greater than 24 h. In a mouse model of inflammatory leukocyte trafficking oral dosing with LJP 1586 resulted in significant, dose-dependent inhibition of neutrophil accumulation, with an affect comparable to that of anti-LFA-1 antibody. In a rat model of LPS-induced lung inflammation, administration of 10 mg/kg LJP 1586 resulted in a 55% significant reduction in transmigrated cells recovered by bronchoalveolar lavage. The results demonstrate that a selective, orally active small molecule inhibitor of SSAO is an effective anti-inflammatory compound in vivo, and provide further support for SSAO as a therapeutic anti-inflammatory target.
INTRODUCTION

Semicarbazide-sensitive amine oxidase (SSAO, AOC3, vascular adhesion protein-1 or VAP-1) is a copper- and topaquinone (TPQ) cofactor-containing enzyme that performs the oxidative deamination of primary amines. Endogenous substrates for SSAO include methylamine, formed from adrenaline and creatine metabolism, and aminoacetone, a product of amino-acid catabolism. The active enzyme is a dimer and exists as both a type II transmembrane protein and in soluble form in plasma (for reviews see Boomsma et al., 2003; Yu et al., 2003, Matyus et al., 2004; O’Sullivan et al., 2004). Experiments with genetically modified mice have demonstrated that the transmembrane form is the sole source of circulating plasma SSAO (Stolen et al., 2004). Cell surface SSAO is present at low levels on several tissues and is abundant on vascular smooth muscle cells and adipose tissue, where it is involved in regulating glucose uptake (Enrique-Tarancon et al., 1998; El Hadri et al., 2002).

SSAO plays a key role in inflammation by at least two mechanisms. First, the soluble products are highly reactive, and include hydrogen peroxide and reactive aldehydes, including formaldehyde when the substrate is methylamine. Plasma SSAO concentration is increased in several inflammatory diseases, including congestive heart failure, inflammatory liver disease and diabetes (O’Sullivan et al., 2004), where plasma concentrations of SSAO have been directly correlated with the degree of diabetic retinopathy (Gronvall-Nordquist et al., 2001). The vascular damage that accompanies these diseases is thought to result in part from protein cross-linking and oxidative stress mediated by the soluble SSAO reaction products (Yu et al., 2003).
SSAO also plays a role in the transmigration of leukocytes out of the bloodstream into sites of tissue inflammation. An understanding of this role began when the endothelial cell surface protein vascular adhesion protein-1 (VAP-1) was cloned and found to be identical to the transmembrane form of SSAO (Salmi and Jalkanen, 1992; Smith et al., 1998). SSAO is expressed at low levels on resting endothelium, but is upregulated in response to inflammatory stimuli (Salmi et al., 1993; Jaakkola et al., 2000). In vitro and in vivo studies have shown that endothelial SSAO participates at several points in the multi-step adhesion cascade that regulates transmigration (Koskinen et al., 2004; Stolen et al., 2005; Jalkanen et al. 2007). Anti-SSAO antibody-mediated inhibition of leukocyte transmigration has demonstrated beneficial effects in rodent models of liver allograft rejection (Martelius et al., 2004), diabetes and peritonitis (Merinen et al., 2005). In addition, mice in which the SSAO gene has been deleted display mild alterations in lymphocyte homing, but have a marked reduction in leukocyte migration in response to inflammatory challenge in induced models of peritonitis and autoimmune diabetes (Stolen et al., 2005).

Experiments with enzymatically inactive SSAO-transfected endothelial cells and with inhibitors of SSAO enzyme activity have shown that the adhesive function of SSAO is critically dependent on the amine oxidase activity (Koskinen et al., 2004). Thus, the interdependence of the adhesive and enzymatic functions of SSAO provides the opportunity to develop small molecule enzyme inhibitors where the anti-inflammatory effects potentially result both from blocking leukocyte migration, and by reducing the production of reactive soluble products.
Although small molecule SSAO enzyme inhibitors have shown clinical efficacy in several animal models of inflammation, most of the known SSAO enzyme inhibitors exhibit a lack of selectivity with respect to other copper-containing amine oxidases and the FAD-dependent monoamine oxidases, or they contain highly reactive structural elements (Matyus et al., 2004). Indeed, some of these molecules were initially developed as inhibitors of monoamine oxidases, and later were determined to additionally be potent inhibitors of SSAO activity (Lyles et al., 1987). In previous work we described a potent and selective inhibitor of SSAO, LJP 1207 [N′(2-phenyl-allyl)-hydrazine hydrochloride] that displayed anti-inflammatory activity in proof-of-concept rodent models of stroke, colitis, LPS-induced endotoxemia and multiple sclerosis (Salter-Cid et al., 2005; Xu et al., 2006; Wang et al., 2006; O’Rourke et al., 2007). However, LJP 1207 is a hydrazine derivative and this class of compounds has potential for toxicity upon prolonged administration.

We have now developed several series of orally active, mechanism-based SSAO inhibitors that are both potent and selective. We report here results for one of these compounds, Z-3-fluoro-2-(4-methoxybenzyl)allylamine hydrochloride (LJP 1586), which was evaluated for in vitro potency with tissue-derived and recombinant amine oxidases, and for specificity against a panel of enzymes and receptors. In vivo, LJP 1586 is shown to be orally active, and exhibits a sustained pharmacodynamic effect. In addition, the anti-inflammatory activity of LJP 1586 is confirmed in two rodent models of inflammation.
METHODS

Animals. Animals were purchased from the sources indicated below, and maintained at a contract vivarium facility (Perry Scientific, San Diego, CA). All protocols were approved by the Institutional Animal Care and Use Committee and conformed to United States Department of Agriculture requirements. Age-, weight- and sex-matched animals were used throughout, and were allowed food and water *ad libitum.*

Preparation of Tissue Homogenates. Lungs from 6 week old female Sprague Dawley rats (Harlan, Indianapolis, IN), and aortas from 6-8 week old Balb/c mice (Jackson Labs, Bar Harbor, ME) were surgically removed and weighed. Tissues were homogenized in ice cold 10 mM potassium phosphate buffer, pH 7.8 at 1: 20 (w/v), and the homogenate was then centrifuged at 10,000g for 10 minutes at 4°C. The supernatants were stored at -70°C until assayed. Frozen human umbilical cords (UC) were obtained from Lake Arrowhead Lab Consultants (Lake Arrowhead, CA). Cords were thawed in 10 mM potassium phosphate buffer, pH 7.8 and the umbilical arteries excised and weighed. The arteries were washed in buffer and then homogenized and stored as described above. Recombinant human SSAO expressed in Chinese hamster ovary (CHO) cells was assayed in clarified 1% NP-40-containing cell lysates.

Radiochemical Assay of SSAO Activity. SSAO activity in 100 µL aliquots of tissue homogenates was determined by a radiochemical method using 20 µM [14C] benzylamine as substrate, as described (Fowler and Tipton, 1981; Salter-Cid et al., 2005). In brief, the reaction was carried out for 30 minutes at 37°C in 100 mM potassium phosphate buffer, pH 7.2, and stopped with 2M citric acid. The [14C] benaldehyde product was extracted
into toluene/ethyl acetate (1:1 v/v) containing 0.6 % (w/v) 2,5-diphenyloxazole, and subjected to liquid scintillation counting. All assays were preincubated for 20 minutes at room temperature with 1 µM each of the MAO-A and MAO-B inhibitors clorgyline and pargyline. Where present, LJP 1586 was added for a further 30 minutes at room temperature. Background cpm were obtained from assays performed in the presence of 1 µM each pargyline and clorgyline and 1 mM of the SSAO inhibitor semicarbazide. IC50 values were calculated from multi-point dose-response curves using Prism software (GraphPad Software Inc, San Diego, CA).

**Cloning and Expression of Human SSAO and Diamine Oxidase.** Human SSAO expressed in CHO cells was produced as described previously (Salter-Cid et al., 2005). The DNA sequence for diamine oxidase (DAO, AOC1, E.C. 1.4.3.6; NM-001091) was synthetically produced by Genscript Corporation (Piscataway, NJ). A codon-optimized DAO sequence was subcloned into the mammalian expression vector pcDNA5/FRT. CHO cells with a stable genome integrated FLP recombination site allow Frt recombinase-mediated integration into a stable transcriptionally active site in the genome. The pcDNA5/FRT containing DAO was cotransfected using Fugene with pOG44, a vector that encodes the Frt recombinase. Cells were cultured in medium containing 0.5 mg/ml hygromycin, and samples of medium from transformed cells were screened for enzymatic activity using putrescine as the assay substrate as described below. Positive expression clones were expanded; the serum free media were collected and used directly as a source of diamine oxidase for enzyme analysis.
Fluorometric Assay of Diamine Oxidase. Expressed diamine oxidase activity was measured in transfected CHO media using an Amplex Red Monoamine Oxidase kit (Invitrogen, Carlsbad, CA) as described except putrescine was used as the substrate in place of benzylamine or tyramine. The DAO inhibitor aminoguanidine was used as a positive control inhibitor for these assays, and had an IC50 of 10 nM.

Colorimetric Assay of Monoamine Oxidases. Recombinant human monoamine oxidase-A and monoamine oxidase-B were obtained from BD Biosciences (San Jose, CA) and enzyme activity was measured using a coupled colorimetric method that detects released H$_2$O$_2$, as described previously (Salter-Cid et al., 2005).

Enzyme and Receptor Screen. Enzyme assays and ligand binding assays were performed using human recombinant proteins or isolated rodent proteins. Assays were performed by a contract research organization (Cerep, Redmond, WA).

Determination of Rat ED50. Groups of five Sprague Dawley rats were orally administered LJP 1586 at 0.1, 1 or 10 mg/kg, or an equal volume of PBS vehicle. At various times between 1 and 72 hours later the animals were sacrificed and the lungs excised. The lungs were weighed and then immediately processed for radiochemical SSAO assays, as described above. The results are expressed as the percent inhibition of enzyme activity in lung extracts from LJP 1586-dosed rats, compared to control, PBS-dosed animals.

Air-Pouch Leukocyte Trafficking. Female C57BL/6 mice were used at ages 10 to 12 weeks, and were obtained from Charles River Laboratories (Wilmington, MA). Mice
were injected with 2 ml of filtered air on the upper back. Twenty-four hours later the air pouches were injected with 0.5 ml of a solution of 1% carrageenan (Type IV Lambda; Sigma, St. Louis, MO) in RPMI medium (Invitrogen, Carlsbad, CA), or with medium alone. On day 2, cells were harvested from the pouch by two cycles of lavage with RPMI medium containing 10% FBS, 5 mM EDTA, and 5 U/ml heparin. Cells were then counted, and in some experiments samples were cytospun and stained with Wright-Giemsa to identify leukocyte subsets.

**LPS-Induced Lung Inflammation.** A solution of 100 µg/ml LPS (*E.Coli* 0111:B4; Sigma, St. Louis, MO) in PBS was prepared and loaded into an Omron Microair nebulizer (Omron Healthcare, Bannockburn, IL). The nebulizer was attached to an acrylic chamber, female Sprague Dawley rats aged 6-10 weeks were then placed in the chamber, and the nebulizer started. Rats were exposed to nebulized LPS for a total of 30 minutes, inclusive of chamber charge and evacuation periods. Experimental time zero was set as the time the animals were removed from the chamber. Animals were then returned to their cages for the duration of the experiment. At the appropriate time rats were sacrificed, the lungs excised and bronchoalveolar lavage (BAL) fluid collected after three cycles of lavage each with 3 ml of PBS. The BAL fluid from the 3 cycles was combined and total harvested cells enumerated. In some experiments, samples were cytospun and stained to identify leukocytes.

**Pharmacokinetics.** PK studies were performed by a contract research organization, Cerep (Redmond, WA). Three cannulated CRL:CD rats weighing 180-250 g were administered LJP 1586 orally with 5 mg/kg, or intravenously with 1 mg/kg. At various
times between 5 minutes and 24 hours, 200 – 300 µL blood samples were collected through a jugular catheter. Plasma was prepared and kept frozen until analyzed for detection of LJP 1586 by HPLC-MS/MS. Plots of plasma concentration of LJP 1586 versus time were constructed, and basic pharmacokinetic parameters were estimated by non-compartmental analysis using WinNonlin software (Pharsight, Mountain View, CA).
RESULTS

Potency and Specificity of LJP 1586. LJP 1586 was identified in an effort to develop mechanism-based small molecule inhibitors of human SSAO that were both potent and selective (Figure 1A). The TPQ-dependent reaction catalysed by SSAO is shown in Figure 1B, and involves reductive and oxidative half-reactions. During the reaction the primary amine interacts with the TPQ to produce a substrate Schiff base, which is converted to the product Schiff base followed by hydrolysis to the product aldehyde. Subsequently, the TPQ is re-oxidized by molecular oxygen in the presence of copper to produce hydrogen peroxide and ammonia (Klinman, 2003).

The inhibitory activity of LJP 1586 was determined in vitro using 
$[^{14}\text{C}]$ benzylamine as substrate and tissue or cell homogenates as sources of SSAO. LJP 1586 was shown to be a potent inhibitor of SSAO, with IC50 values ranging from 4 nM for enzyme from mouse aorta, to 43 nM for recombinant human SSAO stably expressed in CHO cells (Table 1).

LJP 1586 displayed good specificity for inhibition of SSAO over both copper-dependent DAO and the FAD-dependent monoamine oxidases, as well as a panel of additional enzymes (Tables 1 and 2). Using the IC50 calculated from human umbilical cord enzyme, the specificity of LJP 1586 relative to human recombinant MAO-A and DAO is greater than 3500-fold, while that for MAO-B is 82-fold.

To evaluate further the specificity of LJP 1586, the compound was tested in activity assays in vitro against 38 additional enzymes derived from several distinct classes. This
screen included multiple enzymes involved in inflammation as well as representatives from various enzyme families. In all cases inhibition of enzyme activity by 10 μM LJP 1586 was less than or equal to 20%. For the COX-1 and COX-2 enzymes inhibition was less than 10% (Table 2). In addition to the enzyme panel LJP 1586 was tested for its ability to inhibit specific ligand binding in vitro by a panel of 75 receptors. As shown in Table 2, this panel included non-peptide and peptide receptors, ion channels and amine transporters. LJP 1586 at 10 μM was found to inhibit less than 35% ligand binding to all receptors except the dopamine, norepinephrine and serotonin transporters, which were inhibited by 38, 68 and 47%, respectively. In these assays 50% inhibition at 10μM LJP 1586 corresponds to a specificity for inhibition of SSAO activity of > 370-fold over inhibition of amine transporter ligand binding. The inhibition observed with the transporters were not global effects on the dopaminergic, adrenergic or serotonergic families of receptors, as LJP 1586 at a concentration of 10 μM inhibited by less than 35% ligand binding by 4 adrenergic, 5 dopamine and 8 serotonin receptors.

**Pharmacokinetics and Pharmacodynamics of LJP 1586 in Rats.** Pharmacokinetic parameters for LJP 1586 were measured following administration via both oral and intravenous routes (Table 3). For both routes the elimination half-life of the compound was less than 2 hours, with a clearance after i.v. and oral dosing of 77 and 540 ml/min/kg, respectively. The maximum serum concentration of LJP 1586 after p.o. dosing at 5 mg/kg was 85 ng/ml, which corresponds to a concentration of approximately 400 nM. Linear extrapolation of the p.o. 5 mg/kg PK data to the range of doses used in efficacy studies predicts peak plasma levels of between ~ 7.5 nM at 0.1 mg/kg and 750 nM at 10 mg/kg p.o. These data indicate that the peak concentrations of LJP 1586 attained in the
circulation approximately 30 min after oral dosing would provide full inhibition of enzyme activity while minimizing off-target activity.

By comparison of the AUC after p.o. and i.v. dosing, the oral bioavailability of LJP 1586 in rats was estimated at 15%. Although low, the pharmacodynamic data discussed below suggest that low oral bioavailability does not represent a hurdle to effective target inhibition in vivo in the species tested.

To determine the extent to which orally dosed LJP 1586 inhibited the in vivo activity of SSAO, enzyme assays were performed on ex vivo rat lung tissue samples taken at various times after in vivo administration of LJP 1586. The choice of doses used for this study was guided by the estimated peak plasma concentrations extrapolated from the pharmacokinetic data outlined above. From the in vitro potency for rat enzyme, and the PK parameters discussed above, 0.1, 1 and 10 mg/kg doses were chosen for these experiments.

LJP 1586 and PBS vehicle were administered p.o. to rats, which were then sacrificed at various time points after dosing. Lungs were excised and SSAO activity determined in the lung homogenates. Figure 2 shows the results of an experiment in which SSAO activity was followed for up to 72 hr after administration of LJP 1586. SSAO activity in lungs of animals administered 0.1 mg/kg LJP 1586 was reduced at 3 and 6 hours by ~35%, compared with activity in lungs of animals administered PBS, and enzyme activity then returned over the following 64 hours. As expected from the potency of LJP 1586 in vitro, and from the in vivo PK data, single doses of 1 and 10 mg/kg LJP 1586 resulted in virtually complete inhibition of lung SSAO activity. This level of inhibition was
apparent at 3 hours after dosing, which was the earliest time examined, and then inhibition decreased until approximately 50% of enzyme activity was recovered at 72 hours (Figure 2). These data show that the inhibition of SSAO activity in rat lungs after a single oral dose of LJP 1586 was both rapid and sustained, and resulted in an ED50 for enzyme inhibition between 0.1 and 1 mg/kg.

The degree of inhibition of rat lung SSAO activity by LJP 1586 observed in these experiments is consistent with the extrapolated peak plasma concentrations estimated after oral administration of 0.1 and 1 mg/kg LJP 1586. Thus, following these doses the peak plasma concentration would span a concentration range that encompasses the in vitro IC50 for inhibition of rat lung SSAO by LJP 1586. Although mouse lung contains little SSAO activity compared with that detected in rat or human lungs (Andres et al., 2001), mouse aorta contains abundant SSAO activity. SSAO activity in mouse aorta homogenates was measured ex vivo after orally administering the same doses of LJP 1586 as used in the above experiments. Similar to the data reported above for rat lung, the ED50 for LJP 1586 inhibition of mouse aorta SSAO was between 0.1 and 1 mg/kg (data not shown).

In these in vivo experiments approximately 50% of lung SSAO activity was recovered 72 hours after a single administration of LJP 1586. This finding suggests that once inhibited, recovery of tissue enzyme activity requires either the release of bound compound from the enzyme, or expression of newly synthesized protein, or both. The results of preliminary experiments to address this question indicate that the reversibility of SSAO inhibition by LJP 1586 is low, suggesting that the observed recovery of SSAO
activity in vivo (Figure 2) primarily reflects a slow turnover of enzyme protein, rather than removal of bound inhibitor.

**LJP 1586 Inhibition of Acute Inflammation.** There is now considerable evidence that SSAO activity contributes to the recruitment of leukocytes to sites of inflammation in vivo. In order to determine whether leukocyte trafficking and inflammation in vivo could be inhibited by LJP 1586, we employed a mouse air pouch model that allows direct enumeration of cellular infiltrates (Sedgwick et al., 1983). In this model injection of carrageenan into subcutaneous air pouches elicits a localized inflammatory response that includes production of pro-inflammatory cytokines by resident cells and subsequent recruitment of leukocytes (Garcia-Ramallo et al., 2002). In the experiments described here infiltrating cells were harvested by lavage of air pouches 24 hours after injection of carrageenan. Air pouches that had been injected with medium in place of carrageenan were found to contain less than $3 \times 10^5$ cells in the lavage fluid. By contrast, injection of carrageenan induced an influx of cells that was detectable by four hours and reached a plateau between 16 and 24 hours. At the peak of response carrageenan stimulated a 10-30-fold increase in cells in the lavage fluid. Approximately 95% of infiltrating cells were identified as granulocytes by cytospin and staining (data not shown).

To determine the effect of SSAO inhibition on cell trafficking into the inflamed pouches, animals were dosed with 10 mg/kg LJP 1586 one hour prior to injection of carrageenan into the pouches. As the oral bioavailability of LJP 1586 may differ between mice and rats, we initially compared the effects of administering LJP 1586 either orally or intraperitoneally. A control group received an equivalent oral dose of PBS. LFA-1
(CD11a/CD18) plays a well-characterized role in the transmigration process by which leukocytes move from blood into tissue sites of inflammation (Luster et al., 2005). For this reason, we used anti-LFA-1 antibody as a positive control treatment with which to compare the effect of LJP 1586 on migration. Either anti-LFA-1 or an isotype control monoclonal antibody at 100 µg per animal were injected intravenously to additional groups of mice, also one hour prior to eliciting inflammation in the air pouch. Figure 3A shows the results of a representative experiment. Twenty-four hours after carrageenan injection animals that were dosed with the vehicle PBS had approximately 6 x 10^6 granulocytes in their pouches. By contrast, pouches from animals administered LJP 1586 had a marked reduction in cell accumulation, with 41 and 57% decrease after oral or i.p. administration respectively, thus demonstrating good oral bioavailability of LJP 1586 in mice. The number of cells harvested from pouches of animals treated with an isotype control antibody was not significantly different from that seen after PBS treatment. However, treatment of animals with the anti-LFA-1 antibody effectively reduced the cellular infiltrate by 62% (Figure 3A).

Based on the doses of LJP 1586 required to give 100% inhibition of SSAO in vivo, we expected doses between 1 to 10 mg/kg to yield a therapeutic effect on the inflammatory response. In an additional experiment we determined the dose-dependent effect of orally administered 1 or 10 mg/kg LJP 1586 on leukocyte trafficking when administration of LJP 1586 was delayed for 4 hours after carrageenan injection, rather than when administered prophylactically. As shown in Figure 3B, the inhibition of leukocyte accumulation by LJP 1586 was both statistically significant and dose-dependent, with 46% inhibition of cell trafficking by 1 mg/kg and 65% inhibition by 10
mg/kg LJP 1586. As had been seen previously, the inhibitory effect of 10 mg/kg orally administered LJP 1586 was in the same range as that observed in response to i.v. treatment with anti-LFA-1 antibody.

The data presented in Figure 3 thus demonstrate that orally administered LJP 1586 can effectively inhibit leukocyte trafficking in a mouse model of inflammation, and does so when dosing is delayed until after the inflammatory response is under way.

**LJP 1586 Effect on LPS-Induced Lung Inflammation.** A characteristic of several types of lung inflammation in humans is the accumulation of leukocytes in the airway, where they contribute to both acute and chronic lung injury in acute respiratory distress syndrome and chronic obstructive pulmonary disease (Barnes, 2004; Reutershan and Ley, 2004). Leukocyte transmigration into the bronchial airspace of the lung is complex and requires migration across the vasculature, interstitial tissue, and the airway epithelia (Reutershan and Ley, 2004). Given that LJP 1586 effectively inhibited recruitment of granulocytes to inflamed air pouches, we wished to assess further its anti-inflammatory activity in a clinically relevant rat model of acute inflammatory lung disease (van Helden et al., 1997).

Awake, freely moving rats were exposed to nebulized LPS. At various times after exposure, animals were sacrificed, lungs removed and transmigrated cells were collected by bronchoalveolar lavage. Lungs from animals exposed to the PBS vehicle contained less than $10^6$ cells, the majority of which were identified as alveolar macrophages by cytospin and staining (data not shown). By contrast, inhalation of a defined concentration of LPS resulted in the marked accumulation of leukocytes in BAL fluid
(Figure 4A). These cells consisted of approximately 90% granulocytes and the increase in cell number could first be detected within 1-2 hour of LPS inhalation, increasing over the following 5 hours. By 24 hours the number of cells present in BAL fluid returned to resting levels, and again predominantly consisted of macrophages.

To determine the effect of SSAO inhibition on cell recruitment to lungs, PBS or LJP 1586 was given orally to groups of rats 30 minutes prior to exposure of animals to LPS. In the experiment shown in Figure 4B animals dosed with PBS before exposure to LPS for three hours accumulated ~ 4 x 10^6 cells in their lungs, and this number was not significantly affected when animals were administered 0.1 mg/kg LJP 1586 (Figure 4B). However, LJP 1586 treatment at 1 and 10 mg/kg caused a dose-dependent decrease in the cellular infiltrate into LPS-stimulated lungs (Figure 4B). These doses resulted in a 37 and 55% reduction in the number of cells, respectively. The suppression of cell influx into lungs caused by 10 mg/kg LJP 1586 was statistically significant, as was the 72% reduction observed after administration of the positive control treatment of 3 mg/kg dexamethasone (Figure 4B). In another experiment we found that LJP 1586 inhibited by 41% ± 8% the accumulation of cells measured at 6 hours after inhalation of LPS, and inhalation of LPS did not affect the ED50 for LJP 1586 inhibition of lung SSAO activity (data not shown).

Collectively, the data discussed here demonstrate that the novel compound LJP 1586 is a potent, specific, orally available inhibitor of SSAO activity. Moreover, it effectively inhibits leukocyte transmigration and inflammation in vivo in two rodent models.
DISCUSSION

The movement of leukocytes out of the bloodstream into sites of inflammation is a coordinated multi-step process involving both soluble mediators and cell surface receptors (Luster et al., 2005). SSAO expressed on inflamed vascular endothelium has been shown in vitro and in vivo to play a key role in this process, and there is evidence suggesting that SSAO contributes to leukocyte-endothelium interactions at the rolling, firm adhesion and diapedesis steps (Koskinen et al., 2004; Stolen et al., 2005). The involvement of SSAO at multiple points in transmigration is one of several observations that support the notion that SSAO represents an important target for development of potential anti-inflammatory therapeutics.

Expression of vascular SSAO is normally low on endothelial cells, but is upregulated in response to inflammatory stimuli; however, SSAO is not expressed on leukocytes (Salmi et al., 1993 and our unpublished observations). These observations suggest that inhibition of SSAO should not interfere with normal immune surveillance, but instead have most impact on leukocyte recruitment during inflammation. Indeed, SSAO knockout mice show a mild defect in homing to mesenteric lymph nodes and spleen, but mount normal immune responses to both T-dependent and -independent antigens (Stolen et al., 2005). The ability of these animals to maintain normal immunity in the absence of detectable SSAO may have important implications for the safety of this specific anti-adhesion approach, as anti-adhesion therapies that target leukocytes have been implicated in rare cases of fatal progressive multifocal leukoencephalopathy thought to be a result of impaired immune surveillance (Langer-Gould et al., 2005).
While it is not yet known if there exists a specific leukocyte ligand for SSAO it is clear that the enzymatic activity is essential for SSAO-mediated leukocyte adhesion to endothelial cells (Koskinen et al., 2004). Antibodies to SSAO that sterically block adhesion but do not interfere with enzyme activity have been shown to be beneficial in animal models of acute and chronic inflammation (Merinen et al., 2005). However, there are clear advantages to the development of inhibitors that block SSAO enzyme activity. Small molecule therapeutics avoid the requirement for non-parenteral administration of antibodies, and reduce the potential for mounting a neutralizing immune response that may preclude treatment of chronic diseases. In addition, enzyme inhibitors prevent the formation of the soluble SSAO reaction products that themselves contribute to tissue damage. In this regard, hydrogen peroxide in particular has notable pro-inflammatory effects, and together with formaldehyde, contributes to the vasculopathy associated with a number of inflammatory diseases (Yu et al., 2003). Thus, inhibitors of SSAO enzyme activity have the potential to impact diseases in which tissue damage is mediated by soluble products of the reaction, in addition to via leukocyte infiltration.

Several compounds that inhibit SSAO activity have been previously described, but in general they were developed as MAO inhibitors and exhibit cross-reactivity on other amine oxidases, or are structurally unsuitable for use as human therapeutics (Matyus et al., 2004). In the present study we have characterized LJP 1586, a small molecule inhibitor of SSAO/VAP-1 that is potent, selective, and orally active and has demonstrated a good therapeutic window in ADME and toxicological studies performed to date.
The observed IC50 for in vitro inhibition of SSAO by LJP 1586 varied with the source of enzyme, with the IC50 for rodent enzymes generally being less than that for human tissues. This result has consistently been noted with our SSAO inhibitors from several distinct chemical series, and thus may reflect species-specific sequence differences at the enzyme active site, or cell and tissue-specific alterations in SSAO glycosylation patterns. Consistent with this possibility, modification of SSAO glycosylation has been shown to directly influence the enzymatic activity as well as the ability of SSAO to support leukocyte adhesion (Maula et al., 2005).

The specificity of LJP 1586 for inhibition of SSAO was demonstrated against a large panel of receptors and enzymes. Importantly, no significant inhibition of COX-1 and COX-2 activity was observed, demonstrating that inhibition of these enzymes is unlikely to contribute to the anti-inflammatory activity of LJP 1586. The selectivity for SSAO versus the related copper-containing enzyme DAO (AOC1), and the FAD-containing MAO-A was greater than 3500-fold. While the specificity against MAO-B and the amine transporters is lower, linear extrapolation of the pharmacokinetic data of LJP 1586 after a single oral dose suggests that doses greater than 30 mg/kg would be required to have a significant impact on MAO-B activity. Similar extrapolation indicates that doses greater than 135 mg/kg would be required to affect the amine transporters. These results suggest that complete inhibition of SSAO can be attained with LJP 1586 at doses that do not affect a substantial cross section of enzymes and receptors.

The pharmacodynamic effect of LJP 1586 was observed for longer periods of time than would be predicted from the serum half-life. The time to 50% recovery of inhibited
SSAO activity in vivo was approximately 72 hours. Such a protracted time to recovery of activity is consistent with low compound reversibility, and/or with slow turnover of enzyme at the cell surface. The latter possibility is supported by the finding that an anti-human VAP-1 monoclonal antibody remained bound to human dermal vascular endothelium in vivo for at least three days following a single infusion (Vainio et al., 2005).

The delayed recovery of SSAO activity after LJP 1586 administration compares favorably with the pharmacodynamic effects of antibodies that block other human leukocyte adhesion molecules in vivo, such as natalizumab, where single antibody doses are sufficient to saturate lymphocyte receptors for weeks after the initial dose (Miller et al., 2003). Moreover, SSAO gene deleted animals are no more susceptible to lethal bacterial infections than wild type animals, suggesting that a requirement for SSAO in protective inflammatory responses can be overcome during overwhelming conditions (Stolen et al., 2005).

LJP 1586 inhibited leukocyte trafficking to air pouches to a comparable extent to that observed with anti-LFA-1 antibody, and the reduction of infiltrating granulocytes to the lung compared favorably with dexamethasone, which has known pleiotropic effects (Rhen and Cidlowski, 2005). Movement of leukocytes to inflamed airways displays some differences to infiltration of other tissues. For example, exit from the vasculature takes place in capillaries rather than post-capillary venules, and thus, the requirement for rolling may thus be reduced. Furthermore, the process requires migration across vascular endothelium, interstitial tissue and the airway epithelial layer, and the involvement of
certain adhesion molecules in this process has been shown to be stimulus-dependent (Reutershans and Ley, 2005). The pulmonary site at which SSAO might act during inflammation-induced transmigration is not known. Lungs have relatively high SSAO expression, where it has been detected on pulmonary vessels but not airway epithelia (Singh et al., 2003); however, in another study expression on human alveolar pneumocytes has been reported (Andres et al., 2001).

Our results demonstrating a requirement for SSAO activity in leukocyte trafficking to lungs are consistent with a previous report showing that overexpression of SSAO in rodent lung increased recruitment of cells to the airways, both in the unchallenged state and after LPS treatment, and this was reduced by an amine oxidase inhibitor (Yu et al., 2006). In that study the reduced airway infiltration was accompanied by a decrease in the BAL fluid concentration of MIP-1α, TNF-α and IL-6. Our unpublished preliminary data suggests that the inhibition of lung SSAO by LJP 1586 is also accompanied by decreased cytokine and chemokine production, although whether this is due to an effect on cells in situ, or is secondary to the reduction in infiltrating cells, remains to be determined.

In addition to the effect of SSAO inhibition on granulocyte trafficking in the lung, the potential importance of blocking the production of reactive SSAO products at this site should be noted. In particular, methylamine is a major substrate for SSAO, and inhalation of this volatile amine leads to the localized production of formaldehyde, which has been shown to form protein adducts in the lung (Yu et al., 2006). Thus, inhibition of lung SSAO may have benefits in acute and chronic pulmonary disease above and beyond
its effects on leukocyte migration, and possibly include a favorable effect on fibrosis secondary to local formaldehyde production.

As yet, it is not known if SSAO plays a role in the preferential entry of leukocytes to a particular organ or tissue. The collective animal data thus far suggest that SSAO inhibition impacts a variety of inflammatory diseases where leukocyte infiltration mediates tissue destruction, and thus, the development of LJP 1586 as a safe and effective small molecule inhibitor of SSAO represents an important potential addition to the anti-inflammatory therapeutic repertoire.
REFERENCES


FOOTNOTES

Parts of this manuscript were previously presented in poster form at the annual meeting of the American College of Rheumatology in Washington, DC, November, 2006.

Reprint requests: A. M. O’Rourke, Ph.D., La Jolla Pharmaceutical Company, 6455 Nancy Ridge Drive, San Diego, CA 92121. e-mail: anne.orourke@ljpc.com
LEGENDS FOR FIGURES

Figure 1. The structure of LJP 1586 and SSAO Reaction Scheme

(A) The structure of LJP 1586. (B) The process catalyzed by SSAO occurs via two half-reactions involving successive reduction and oxidation steps.

Figure 2. Inhibition of Rat Lung SSAO Activity by LJP 1586

Groups of five rats were orally administered once either PBS, or the indicated doses of LJP 1586. At the indicated times thereafter animals were sacrificed and the lungs excised. SSAO activity in lung homogenates was then assayed. Results are presented as percent inhibition of SSAO activity, compared to activity in lungs of rats administered PBS vehicle. For this control group, activity of lung SSAO measured in the presence of MAO-A and MAO-B inhibitors and expressed as [14C] benzaldehyde formed, was 2798 ± 93 cpm. Results are mean ± SEM of 5 mice per group.

Figure 3. Inhibition of Leukocyte Trafficking by LJP 1586.

(A) Air pouches were introduced onto the backs of mice and 24 hr later animals were dosed either orally (p.o.), intraperitoneally (i.p.) or intravenously (i.v.) with the indicated treatment. One hr later 0.5 ml of 1% carrageenan was injected into the pouches and 24 hr later cells were collected from the pouch by lavage. Total cell counts are the mean ± SEM of 8 mice per group. ** P < 0.01 by one-way ANOVA followed by Dunnett’s test for multiple comparisons. For (B) air pouches were introduced and 24 hr later 0.5 ml of 1% carrageenan was injected into the pouches. Four hr later animals were dosed orally
with PBS or LJP 1586, or intravenously with mAb. Sixteen hr later cells were removed from pouches by lavage. The results are mean ± SEM of groups of 8 mice. ** P < 0.01 by one-way ANOVA followed by Dunnett’s test for multiple comparisons.

**Figure 4. Effect of LJP 1586 on Leukocyte Trafficking to LPS-Inflamed Lungs.**

(A) Groups of rats were exposed to nebulized LPS or PBS for 30 min. At the indicated times animals were sacrificed, lungs were excised, BAL fluid collected and cells enumerated. The results are the mean ± SEM of groups of 4 animals. (B) In a separate experiment animals were orally administered PBS, or the indicated doses of LJP 1586 or dexamethasone. They were then exposed to nebulized LPS for 30 min, and 3 hr later animals were sacrificed, lungs were excised, BAL fluid collected and cells counted. Results are mean ± SEM of groups of 4 rats. * P < 0.05, ** P < 0.01 by one-way ANOVA followed by Dunnett’s test for multiple comparisons.
Table 1. Potency and Specificity of LJP 1586 for SSAO.

<table>
<thead>
<tr>
<th></th>
<th>Hu UC SSAO&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hu CHO SSAO&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Rat Lung SSAO&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Mouse Aorta SSAO&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MAO-A</th>
<th>MAO-B</th>
<th>DAO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IC50 (µM)</strong></td>
<td>0.027 ± 7</td>
<td>0.043</td>
<td>0.009 ± 0.002</td>
<td>0.004</td>
<td>98 ± 6</td>
<td>2.2 ± 0.4</td>
<td>96</td>
</tr>
<tr>
<td><strong>Fold specificity for human UC SSAO</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;3600</td>
<td>82</td>
<td>&gt;3500</td>
</tr>
</tbody>
</table>

SSAO assays were performed as described in Methods using <sup>a</sup> human umbilical cord, <sup>b</sup> lysates of human SSAO-expressing CHO cells, or <sup>c</sup> rat lung or mouse aorta homogenates. MAO-A and –B, and DAO were recombinant human enzymes. IC50s are the average of duplicate determinations except UC SSAO, rat lung SSAO and MAO-A and -B, which are the mean ± SEM of 4 to 10 determinations.
Table 2. Activity of LJP 1586 on a Panel of Enzymes and Receptors.

<table>
<thead>
<tr>
<th>Class</th>
<th>Subclass</th>
<th>Percent Inhibition by 10 µM LJP 1586</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Peptide Receptors (38)</td>
<td>Adenosine, Adrenergic, Benzodiazepine, Cannabinoid, Dopamine, GABA, Glutamate, Histamine, Melatonin, Muscarinic, Prostanoid, Purinergic, Serotonin, Sigma</td>
<td>&lt; 35</td>
</tr>
<tr>
<td>Peptide Receptors (30)</td>
<td>Angiotensin, Bombesin, Bradykinin, Calcitonin-Related Peptide, Chemokine, Cholecystokinin, Cytokine, Endothelin, Galanin, Growth Factor, Melanocortin, Neurokinin, Neuropeptide Y, Opioid and Opioid-like, Somatostatin, Vasopressin</td>
<td>&lt; 25</td>
</tr>
<tr>
<td>Ion Channels (4)</td>
<td>Ca²⁺ (verapamil), K⁺, SK⁺Ca, Na⁺</td>
<td>≤ 15</td>
</tr>
<tr>
<td>Amine Transporters (3)</td>
<td>Dopamine, Norepinephrine, Serotonin</td>
<td>38, 68, 47</td>
</tr>
<tr>
<td>Enzymes (38)</td>
<td>Phospholipase A2; COX-1, COX-2; inducible nitric oxide synthase, constitutive NOS endothelial, cerebellar; 12-, 15-lipoxygenase; phosphodiesterase-3, -4; caspase-1, -3, -5; cathepsin-B, -H, -L, X; matrix metalloproteinase -1, -3, -7, -9; phosphatase-1B, -2B; EGFR, FGFR1, HER2/Erb2, IRK, p38α, p38β2, p38δ, p38γ kinases; acetylcholinesterase; catechol-O-methyl transferase; GABA transaminase; histamine N-methyltransferase; xanthine oxidase; myeloperoxidase; ATPase.</td>
<td>≤ 20</td>
</tr>
</tbody>
</table>
Table 3. Pharmacokinetics of Orally and Intravenously Administered LJP 1586 in Rats.

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Oral (5 mg/kg)</th>
<th>Intravenous (1 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{1/2}$ (min)$^a$</td>
<td>$86 \pm 6^b$</td>
<td>$105 \pm 15$</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (min)</td>
<td>$26 \pm 6$</td>
<td>$5^c$</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>$85 \pm 26$</td>
<td>$424 \pm 66^c$</td>
</tr>
<tr>
<td>CL (ml/min/kg)</td>
<td>$540 \pm 67^d$</td>
<td>$77 \pm 2$</td>
</tr>
<tr>
<td>$V_z$ (ml/kg)</td>
<td>ND</td>
<td>$11675 \pm 1997$</td>
</tr>
<tr>
<td>$V_{ss}$ (ml/kg)</td>
<td>ND</td>
<td>$5055 \pm 964$</td>
</tr>
<tr>
<td>$AUC_{\text{ALL}}$ (min x ng/ml)</td>
<td>$8903 \pm 1166$</td>
<td>$12404 \pm 546$</td>
</tr>
<tr>
<td>$AUC_{\text{INF}}$ (min x ng/ml)</td>
<td>$9556 \pm 1180$</td>
<td>$13101 \pm 407$</td>
</tr>
</tbody>
</table>

$^a$ Abbreviations: $T_{1/2}$, elimination half-life; $T_{\text{max}}$, time of maximum observed plasma concentration; $C_{\text{max}}$, maximal concentration; CL, clearance; $V_z$, volume of distribution; $V_{ss}$, volume of distribution at steady state; $AUC_{\text{ALL}}$, area under concentration-time curve from time of dosing to the time of last observation; $AUC_{\text{INF}}$, area under concentration-time curve from time of dosing, extrapolated to infinity.  
$^b$ Means ± SEM of 3 rats per group.  
$^c$ Earliest point examined.  
$^d$ Calculated from dose p.o. / $AUC_{\text{INF}}$ p.o.
A

Z-3-fluoro-2-(4-methoxybenzyl)allylamine
LJP 1586

B

\[
\begin{align*}
\text{HO} & \quad \text{RCH}_2\text{NH}_2 \\
\text{HO} & \quad \text{RH}_2\text{CHN} \quad \text{HO} \\
\text{HO} & \quad \text{RCHO} \\
\text{HO} & \quad \text{Asp/Glu}
\end{align*}
\]

\[
\begin{align*}
\text{HO} & \quad \text{RCH}_2\text{NH}_2 \\
\text{HO} & \quad \text{RH}_2\text{CHN} \quad \text{HO} \\
\text{HO} & \quad \text{RCHO} \\
\text{HO} & \quad \text{Asp/Glu}
\end{align*}
\]
Figure 2

Lung SSAO Activity (% Inhibition)

Time Post Oral Dose

- 3 hr
- 6 hr
- 24 hr
- 48 hr
- 72 hr

- 0.1 mg/kg
- 1 mg/kg
- 10 mg/kg
Figure 3
Figure 4