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The discovery and development of selective 3-fluoro-4-aryloxyallylamine inhibitors of the amine oxidase activity of semicarbazide-sensitive amine oxidase/vascular adhesion protein-1 (SSAO/VAP-1)

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ABSTRACT

A new class of 3-fluoroallyl amine-based SSAO/VAP-1 inhibitors is reported. These compounds have excellent selectivity over diamine oxidase, MAO-A and MAO-B. Synthesis and SAR studies leading to compound **28** (**PXS-4159A**) are reported. The pharmacokinetic profile of **28** in the rat, together with activity in a murine model of lung inflammation are also disclosed.

Semicarbazide-sensitive amine oxidase (AOC3, also known as vascular adhesion protein-1 [VAP-1], primary amine oxidase or plasma amine oxidase, EC 1.4.3.21) is a member of the copper-dependent family of amine oxidases (AOC).[1] SSAO/VAP-1 is a type 1 membrane-bound protein which 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.[2] SSAO/VAP-1 catalyzes the oxidation of primary amines to aldehydes with release of ammonia and hydrogen peroxide (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 like selegiline and clorgyline.

In humans four AOC genes are known; three (AOC-1–3) translate into enzymatically active protein and one (AOC-4) into a truncated inactive form.[3] AOC-1 codes for a diamine oxidase (DAO) found in the kidney, gut and lung and is involved in the metabolism of endogenous histamine. AOC-2 encodes for retinal amine oxidase which is found almost exclusively in the eye.[4] AOC-3 encodes for the primary monoamine oxidase SSAO/VAP-1 and is found in adipocytes, smooth muscle cells and endothelial cells, and is highly expressed in the lung, aorta, liver and ileum.[5] Membrane bound SSAO/VAP-1 is proteolytically cleaved by a metalloprotease to release an active, soluble form of the protein.[6] Endogenous substrates for SSAO/VAP-1 include methylamine and aminoacetone.[7] The enzyme also oxidizes other substrates, such as benzylamine, which is often used as the substrate to assay enzymatic activity. There are considerable substrate and inhibitor differences amongst species so it can be challenging to design potent inhibitors of human SSAO/VAP-1 with similar inhibitory potency against the rodent and canine enzymes.[8]

Different classes of SSAO/VAP-1 inhibitors have been reported in the literature and have been reviewed thoroughly elsewhere.[9] Inhibition of SSAO/VAP-1 has been shown to be efficacious in rodent models of eye inflammation, rheumatoid arthritis, carrageen air-pouch inflammation, liver fibrosis, and stroke, amongst other inflammatory models.[10] In addition, some inhibitors have proven efficacious in lung inflammatory responses.[10b] For example, mofegiline (**1**) attenuates the LPS-induced lung inflammatory response in mice over expressing human SSAO/VAP-1.[11] Therefore selective SSAO/VAP-1 inhibitors have the potential to treat lung inflammation in humans, including neutrophil-driven processes, such as severe asthma. The focus of this research project is to develop inhibitors for this indication.

Mofegiline (Fig. 1. **1**), which progressed to Phase 2 clinical trials as an adjunct to L-dopa for the treatment of Parkinson's disease, is a well known inhibitor of the amine oxidase activity of SSAO/VAP-1.

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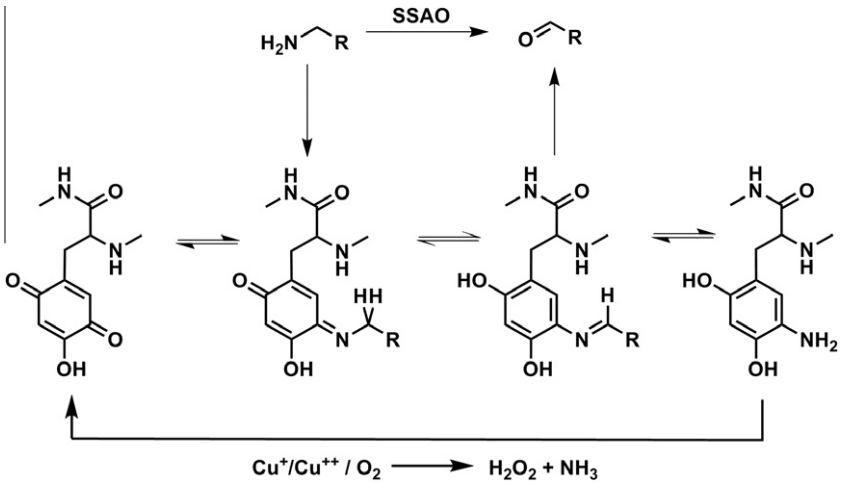
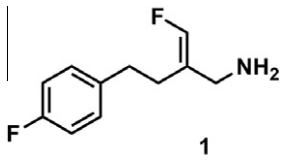


Scheme 1. SSAO/VAP-1 deaminates primary amines with re-oxidation of the reduced co-factor.



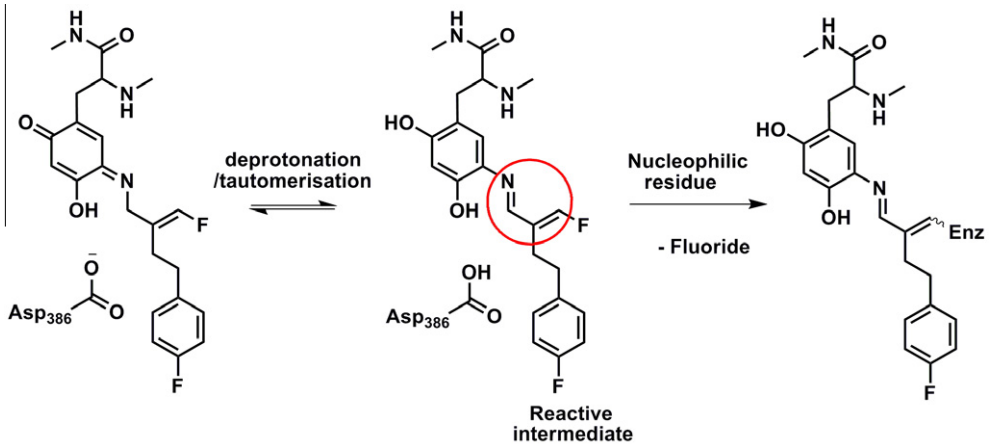
1

Figure 1. Structure of mofegiline (**1**).

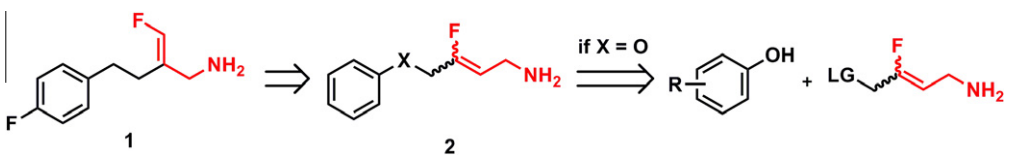
In a mechanism reminiscent of its mode of action to inhibit MAO-B,[12] mofegiline is believed to be processed as a substrate by SSAO/VAP-1 to generate a covalently attached, very reactive intermediate (highlighted in Scheme 2) which is alkylated by a neighboring nucleophilic residue. Loss of fluoride leads to irreversible inhibition and explains, in part, the very high potency that can be observed with this class of inhibitor. Inhibitors based on the haloallylamine scaffold have been designed for most amine oxidases; most recently LJP 1586, which was selected from a series of inhibitors which included mofegiline (**1**),[13] was reported to be an effective inhibitor of SSAO/VAP-1, with less activity as an MAO-B inhibitor.[10b]

This current Letter describes the design, synthesis and evaluation of new, potent fluoroallylamine inhibitors of SSAO/VAP-1 which show greater than 100-fold selectivity against the other amine oxidizing enzymes MAO-A, MAO-B and diamine oxidase.

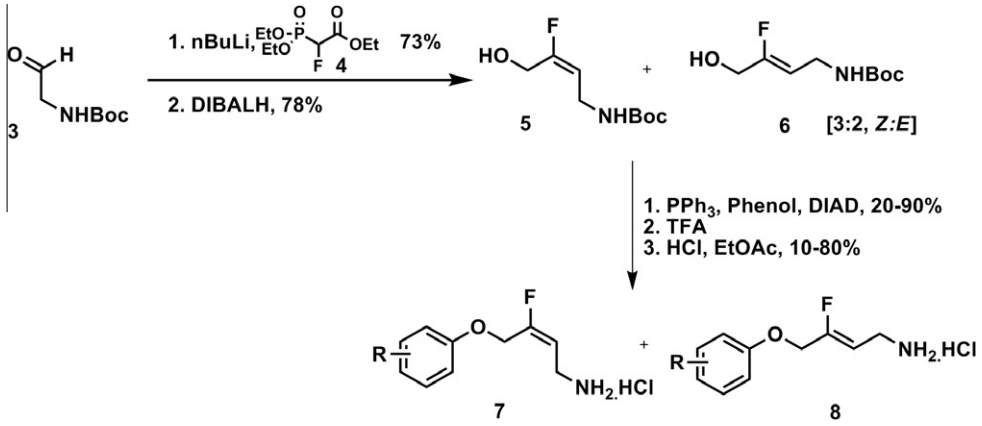
Several crystal structures of SSAO/VAP-1 have been published revealing an elongated binding pocket with the co-factor and copper sitting deep within the pocket.[14] Furthermore, modeling indicated that if **1** were re-designed with the phenylethyl moiety tethered to the 3-, rather than the 2-position of 3-fluoroallylamine, to give the more linear compound (**2**), this should be readily accepted by SSAO/VAP-1. These modeling studies did not



Scheme 2. Proposed mechanism-based inhibition of SSAO/VAP-1 by **1**.

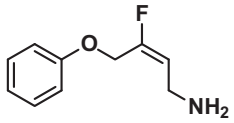
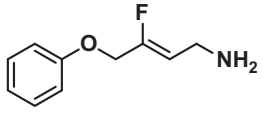
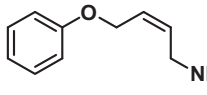
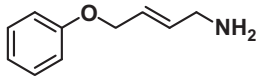


Scheme 3. Design of a lead with improved selectivity, rearrangement of the 2- to the 3-substituted fluoroallyamine scaffold.



Scheme 4. Preparation of *E* and *Z*-fluoroallylamines.

Table 1
Initial lead identification[16]

	Structure	SSAO IC_{50}[a,b] (μM)
9		0.29
10		20
11		>100
12		82

[a] Values are mean of three experiments.
[b] Resorufin/Amplex Red assay readout of H_2O_2 production.

conclusively determine whether **2** would be an effective mechanism-based inhibitor; however, the positioning of Asp386 suggested that turnover would occur. This deconvolution is shown in Scheme 3. To enable rapid SAR studies, an oxygen atom was incorporated into the side chain.

Synthetically, the first task was to prepare large amounts of both isomers of the key intermediate (**5** and **6**). This was accomplished by a Horner–Wadsworth–Emmons reaction of **4** with the *N*-Boc protected amino aldehyde (**3**), followed by DIBAL-H reduction to afford a separable mixture of **5** and **6** (Scheme 4).[15] Reaction of **5** and **6** with phenols under Mitsunobu conditions, followed by TFA deprotection and HCl salt formation, led to geometric isomers **7** and **8**, respectively. With unsubstituted phenol (R = H), compounds **9** and **10** were prepared (Table 1); the des-fluoro analogos **11** and **12** were also prepared by standard procedures. From this small series it can be deduced that the *E*-geometry is more favorable than the *Z*, and that the 3-fluoro substitution is essential. With this information in hand, a larger structure–activity relationship study was initiated to optimize potency and selectivity.

The most potent compound, **9** (IC_{50} = 290 nM) was found to have similar potency as an inhibitor of MAO-B (IC_{50} = 180 nM, Table 2), with virtually no activity against MAO-A (IC_{50} >30 μM). The challenge, therefore, was to improve inhibitory potency against SSAO/VAP-1 while reducing inhibition of MAO-B. The binding site of MAO-B is relatively narrow, such that large substituents on the aromatic ring would be expected to restrict binding considerably and thus lead to much weaker inhibition. In contrast the binding site of SSAO/VAP-1 is relatively large and can accommodate more bulky groups. With this understanding, substituents which break the planarity (with respect to the aromatic ring) were expected to be much less efficient inhibitors of MAO-B. Using the convergent synthetic route (Scheme 4), a range of phenols was coupled with intermediate **5** to afford the derivatives listed in Table 2.

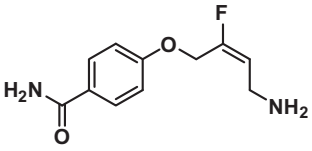
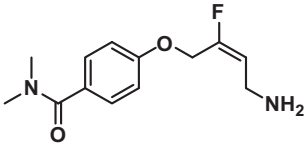
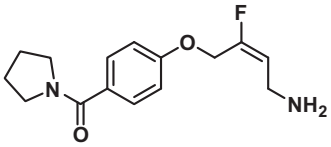
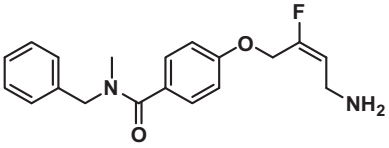
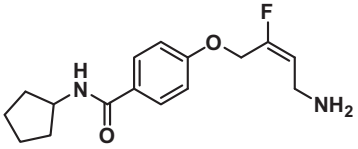
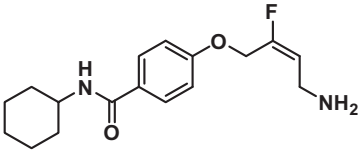
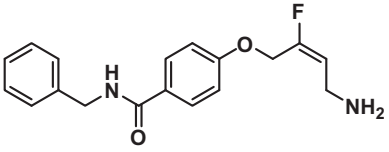
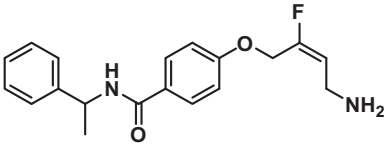
These data show that simple substitution at either the 3 or 4-positions with small electron-withdrawing and electron-donating

Table 2
Substituted aryl examples

	Aryl R-substitution	SSAO IC_{50}[a] (μM)	MAO-A IC_{50}[a] (μM)	MAO-B IC_{50}[a] (μM)
9	None	0.29	>30	0.18
13	4-OMe	0.16		0.31
14	4-OPh	0.24		0.45
15	3-Cl	0.25		0.03
16	4-SMe	0.02		0.16
17	4-^{t}Bu	0.21	>30	6.40
18	4-OCF_3	0.18		0.07
19	4-NO_2	0.07		0.01
20	3,4-Difluoro	0.19		0.09
21	4-CF_3	0.04		0.13
22	3-CF_3	0.15	>10	0.03
23	4-$CONH_2$	1.07	>30	>30

[a] Resorufin/Amplex Red assay readout of H_2O_2 production.

Table 3
Amide examples

	Structure	Human amine oxidases (IC_{50}; μM)[a]				Mouse adipocytes (IC_{50}; μM)[a]
		SSAO/VAP-1	MAO-A	MAO-B	DAO	SSAO/VAP-1
1		0.02	1.78	0.01	>10	0.01
9		0.29	>30	0.18		
23		1.07	>30	>30		
24		0.37		18.99		
25		0.14		10.96		
26		0.26		2.21		
27		0.02	>30	1.64	>30	0.53
28		0.01	>30	1.12	26.13	0.15
29		0.08		0.62		
30 (racemic)		0.04		2.98		
30a (*R*)		0.03	>30	1.61	2.46	
30b (*S*)		0.04	>30	6.43	74.07	0.13

[a] Resorufin/Amplex Red assay readout of H_2O_2 production.

groups did not influence inhibitory potency against SSAO/VAP-1 to any great extent. However, compound **17**, substituted with a bulky *tert*-butyl group was much less potent against MAO-B while maintaining potency against SSAO/VAP-1. It was also observed that the introduction of the hydrophilic, polar carboxamide (**23**) led to a considerable reduction in potency against SSAO/VAP-1 and especially MAO-B. Taking advantage of the observation that both bulky and polar groups impart selectivity over MAO-B, a series of amides were synthesized and evaluated. The tertiary amides **24**–**26** (Table 3) had the same profile as the unsubstituted amide **23**. However, the secondary amides (**27**–**29**) were more promising, especially those with larger lipophilic groups. The most potent and selective compounds in this series were **27** (20 nM [SSAO/VAP-1]; 32-fold selective vs MAO-B), **28** (10 nM; 112-fold) and racemic **30** (40 nM; 75-fold).[17]

When compound **28** is docked into the active site of human SSAO/VAP-1 prior to Schiff base formation with the TPQ co-factor, it becomes apparent that the lipophilic cyclohexyl fits snugly into

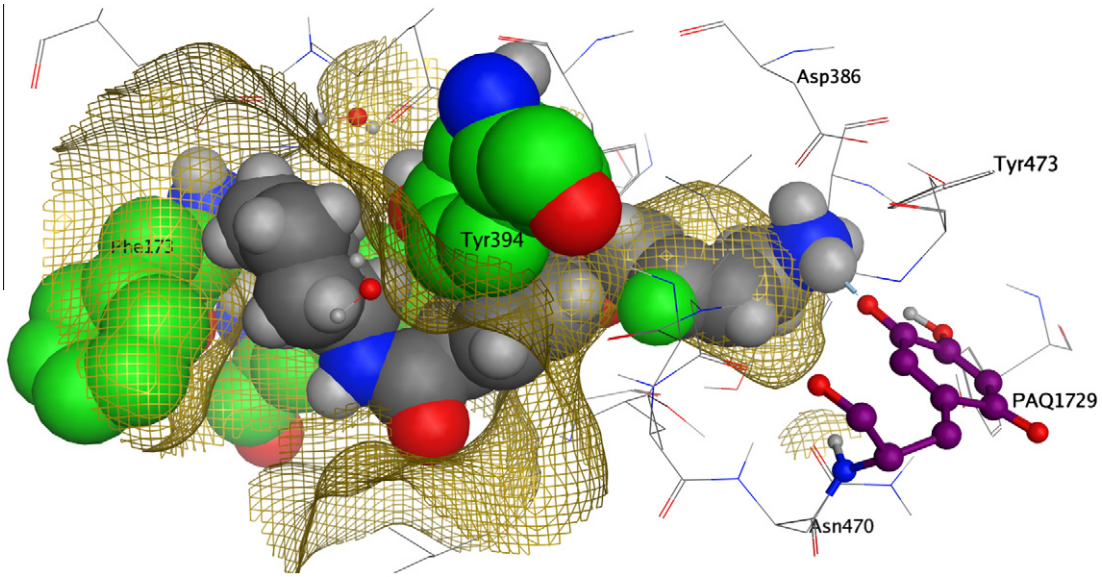


Figure 2. Docking of unbound **28** into active site prior to Schiff base formation. Solvent exposed entrance to the left, molecular surface is shown as contours. Topaquinone cofactor (off-copper) is shown in purple, lipophilic residues adjacent to cyclohexyl group in green.[18]

the binding pocket and makes positive interactions with the neighboring lipophilic phenylalanine and tyrosine resides (Fig. 2). This would lead to a lower binding constant (K_D), and equal or perhaps enhanced k_{inact}, and hence to an overall increase in inhibitory potency.

Compound **28** is a time-dependent, mechanism-based inhibitor of the amine oxidase activity SSAO/VAP-1 (Fig. 3). A Kitz and Wilson plot[19] determined the apparent K_I and k_{inact} values to be 1.64 μM and 0.46 min^{-1}, respectively, which are similar to those for mofegiline (1.61 μM and 1.03 min^{-1}). In vitro, the inhibition was shown to be irreversible after 24 h dialysis and the turnover number determined to be approximately 10 (data not shown). Further experiments to explore fluoride elimination were limited by the large quantity of enzyme required.[12]

Compound **28** was shown to be inactive (IC_{50} >10 μM) against a panel of traditional off-targets, inactive in the AMES test, did not inhibit CYP isozymes (data not shown) and had an acceptable pharmacokinetic profile (rat; bioavailability = 62%, half-life (po) = 97 min.). Compound **28** (**PXS-4159A)** was chosen for further evaluation.

Having established that **28** had an acceptable pharmacokinetic profile, the pharmacodynamic properties were evaluated in the mouse and rat. SSAO/VAP-1 amine oxidase activity was determined 6 h after an oral dose in gonadal adipose tissue, and compared against the more potent **1**. The EC_{50} values for **28** after a single oral dose were 10 mg/kg for both mouse and rat which is reflective of the weaker potency against the rodent enzyme.

Compound **28** was then tested in a mouse model of airway inflammation. Mice were challenged with LPS to induce a predominantly neutrophilic response.[20] Pre-treatment with **28** (100 mg/kg, ip) 3 h before LPS resulted in a marked decrease in cellular infiltration into the bronchoalveolar spaces (Fig. 4A). BAL fluid contained up to 80% fewer neutrophils compared to vehicle alone, indicating that in treated mice the recruitment of cells to the airways was diminished (Fig. 4B). This reduction followed the same trend as the positive treatment control, dexamethasone (Dexa). Cytokine levels were also measured. Whereas vehicle treated mice showed high levels of TNFα (Fig. 4C), significant decreases were observed with dexamethasone as well as with **28**, consistent with the diminished neutrophil influx into the airways.

In summary, a novel series of mechanism-based inhibitors of the amine oxidase activity of SSAO/VAP-1 in the allylamine class has been developed. These compounds were optimized to afford improved selectivity over MAO-B, achieved through modification

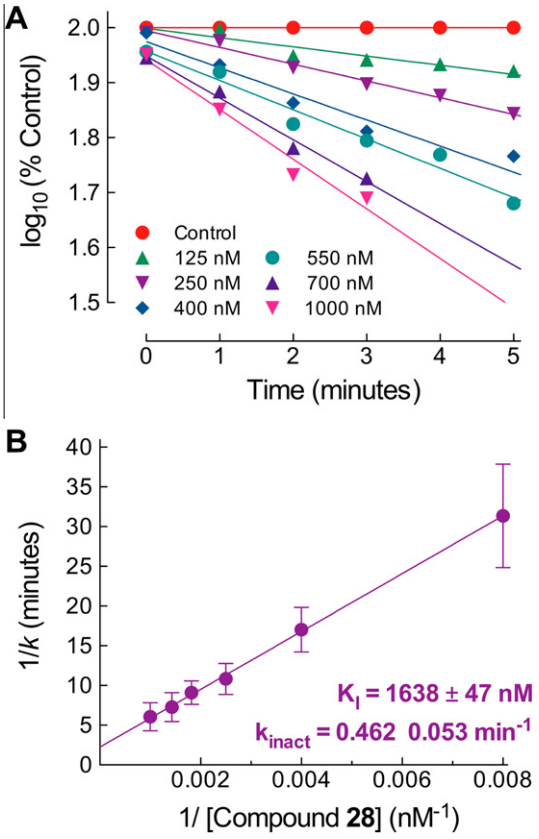


Figure 3. Kitz and Wilson analysis of **28**. (A) Representative plot of time-dependent inhibition of SSAO/VAP-1 by **28**. First order inhibition rate constants at each concentration of **28** are calculated as slope × 2.303. (B) Replot of inhibition rate constants from three experiments. Mean inhibition constants ± SEM shown in figure.

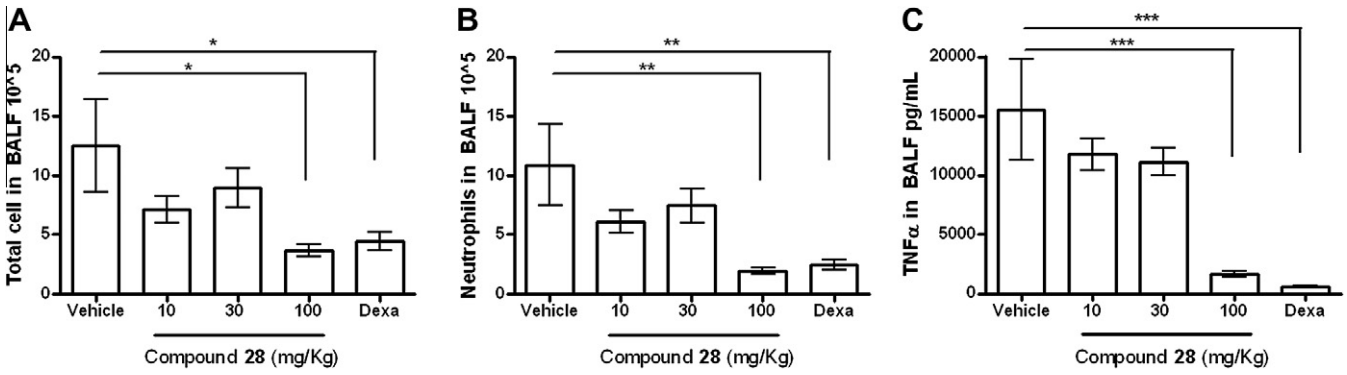


Figure 4. Evaluating compound **28** in a murine LPS model of lung inflammation. (A) Total cells in BALF; (B) neutrophil count; (C) TNFα levels. (n = 10 animals/group; P values; *0.05, **0.01, ***0.001 as analysed by one-way ANOVA).

of a distal amide group. Compound **28** exhibited excellent potency and selectivity in vitro, a promising pharmacokinetic profile and activity in an animal model of lung inflammation, and has been advanced for preclinical toxicology evaluation.

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15. Separated by normal phase chromatography; the lack of selectivity in the Horner–Wadworth–Emmons reaction is due to the inductive effect of the fluorine 'overriding' the electron-withdrawing power of the ester group.
16. Enzyme sources: recombinant human MAO-A and MAO-B were purchased from Sigma–Aldrich, recombinant human SSAO/VAP-1 (truncated; residues 34–763) was prepared by CSIRO, Parkville, Australia; full length human recombinant DAO was provided by Professor Mitchell Guss, University of Sydney.
17. Compound **28** was also evaluated against the AOC-2 gene product, (human transient) retinal amine oxidase and found to be inactive (IC_{50} >10 μM).
18. Figure generated using Molecular Operating Environment (MOE), 2011.10; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada H3A 2R7, 2011.
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