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6-Acylamino-2-[(ethylsulfonyl)oxy]-1*H*-isoindole-1,3-diones Mechanism-based Inhibitors of Human Leukocyte Elastase and Cathepsin G: Effect of Chirality in the 6-Acylamino Substituent on Inhibitory Potency and Selectivity

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Abstract—Inhibition of human leukocyte elastase(HLE) by a series of 6-acylamino-2-[(ethylsulfonyl)oxy)]-1*H*-isoindole-1,3-diones was determined and compared to their inhibition of ChT, PPE, and Cat G. The best inhibitor of the series was 6-((1′*S*)-camphanyl)amino-2-[(ethylsulfonyl) oxy]-1*H*-isoindole-1,3-dione **5b**, with a $k_{obs}/[I] = 11{,}000\ M^{-1}\ s^{-1}$. This study revealed that HLE shows a preference for the *S* stereochemistry and tolerates hydrophobic substituents in the S_n' binding sites. Molecular modeling of non-covalent HLE–inhibitor complexes was used as a tool to investigate our binding model. Buffer stability assays reveal that these compounds are susceptible to hydrolysis at physiological pH.

Introduction

Human leukocyte elastase (HLE) is a serine protease produced by polymorphonuclear leukocytes.[1] The primary role of HLE is the degradation of microorganisms ingested by leukocytes during phagocytosis. In the process, HLE degrades connective tissue proteins like elastin in pathological states.[2] Extensive elastolysis by HLE is primarily prevented by an endogenous inhibitor, α1-antitrypsin, also known as α1-PI. Depressed levels of active α1-PI, caused by a genetic deficiency or cigarette smoking, can lead to excessive proteolysis of elastin and result in the disease pulmonary emphysema.[3] It is believed that cigarette smoke causes the oxidation of the reactive methionine residue of α1-PI, thereby rendering it a less effective inhibitor of HLE.[4–6]

One of the current treatments for patients suffering from emphysema is high cost α1-PI replacement therapy ($20,000–$30,000 per patient per year).[7] Recently, much attention has focused on the synthesis of cost-effective, low molecular weight inhibitors of HLE that may incur therapeutic utility.[8–10] Mechanism-based inhibitors have been explored extensively as potent and specific inhibitors of HLE.[11] Enzyme catalyzed hydrolysis of the inhibitor exposes a reactive functionality which covalently and irreversibly binds to the enzyme. A multitude of diverse mechanism-based inhibitors have been reported in the literature. Among these are the 3-alkoxy-7-amino-4-chloroisocoumarins,[12,13] the saccharin derivatives,[14] the *N*-((alkylsulfonyl)oxy)succinimides,[15,16] and the β-lactams.[17] More recently, researchers have reported on the coumarinic derivatives,[18] thieno-oxazin-4-ones,[19] the 1,2,5-thiadiazolidin-3-one-1,1-dioxides,[20–22] and the *N*-((alkylsulfonyl)oxy) phthalimides.[23,24] It has been hypothesized by us and others that the mechanism of action of the *N*-sulfonyloxy phthalimides involves the formation of an imidazole-*N*-carboxamide covalent complex much like their *N*-sulfonyloxy succinimide counterparts.[23,25,26] This is achieved through the initial acylation of the catalytic serine residue followed by the release of a latent isocyanate. The isocyanate reacts with a catalytic histidine residue forming an imidazole-*N*-carboxamide.

Our proposed binding model is shown in Figure 1. The rationale for this assumption is based on the structural similarity observed between the 6-amino-2-[(ethylsulfonyl)oxy]isoindole-1,3-diones and 7-amino-4-chloro-3-ethoxyisocoumarins. The binding model of isocoumarin mechanism-based inhibitors of elastase has been established by X-ray crystallography of PPE-isocoumarin complexes[27–29] and further supported by molecular

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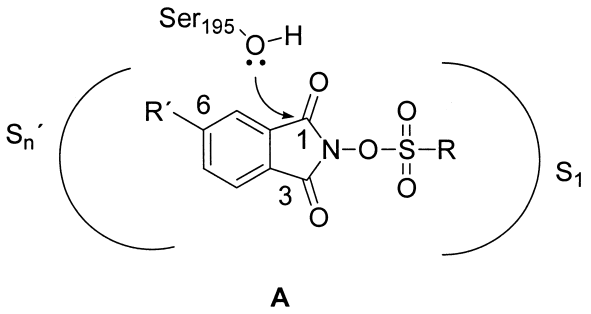


A

Figure 1. Proposed binding model.

modeling studies.[30,31] In Figure 2, flexible alignment of the two structures using the FlexAlign program in MOE® (Chemical Computing Group, Inc.) shows a remarkable similarity between the isocoumarins and the isoindole-1,3-diones. The proposed binding model in Figure 1 is based upon the isocoumarin model. Kerrigan et al. have demonstrated that flexible non-bulky hydrophobic substituents at the 6-position in tandem with small aliphatic (methyl or ethyl) groupings in the 2-(alkylsulfonyl)oxy substituent increase potency and selectivity of 6-(substituted)amino-2-[(alkylsulfonyl)oxy] isoindole-1,3-dione inhibitors of HLE over ChT.[24,25] In a similar fashion, hydrophobic groupings in the 7-position of isocoumarins increases potency and selectivity of these compounds for HLE.[13,28] It was observed that changing the *R* group from a methyl to an ethyl group increased selectivity up to 4-fold for inhibitors with non-bulky flexible chains in the 6-position. These studies support the possibility that the 6-substituent (R′) might be binding in the hydrophobic S_n' subsites of HLE. As an extension of earlier studies, exploration of the effect of bulky substituents possessing chirality on inhibitor potency and selectivity for HLE is addressed in this paper. Herein, we report the synthesis of a series of 6-acylamino-2-[(ethylsulfonyl)oxy)-1*H*-isoindole-1,3-diones with bulkier hydrophobic substituents at the 6-position and the in vitro activity of the derivatives towards HLE, bovine α-chymotrypsin (ChT), porcine pancreatic elastase (PPE), and human neutrophil cathepsin G (Cat G).

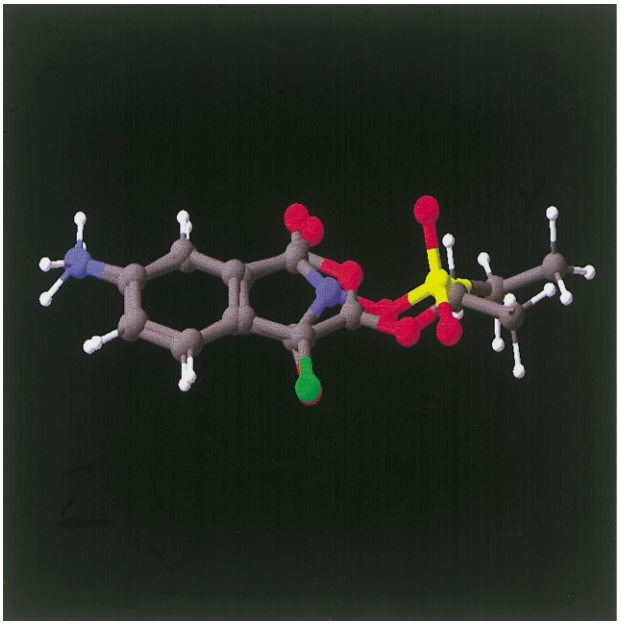


Figure 2. Overlay of aminoisocoumarin with aminoisoindole-1,3-dione.

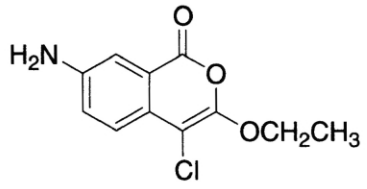
Results

Synthesis

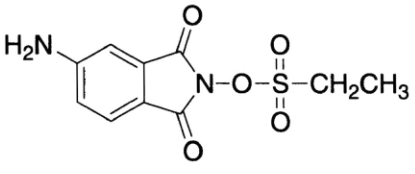
The sequence used for the preparation of the 6-acylamino-2-[(ethylsulfonyl)oxy)]-1*H*-isoindole-1,3-diones is outlined in Scheme 1. The commercially available 4-nitrophthalic anhydride was reacted with *O*-benzylhydroxylamine hydrochloride to form compound **2**, which was subsequently reduced via a transfer hydrogenation method to give intermediate **3**. Sulphonylation of **3** produced the starting material used for all subsequent reactions. Reaction of **4** with the appropriate acid chloride afforded the series of inhibitors discussed in this paper. Compounds, which were not available as acid chlorides, were refluxed with thionyl chloride in THF to form the intermediate acid chloride and immediately combined with **4** to form the corresponding inhibitor.

In vitro assays

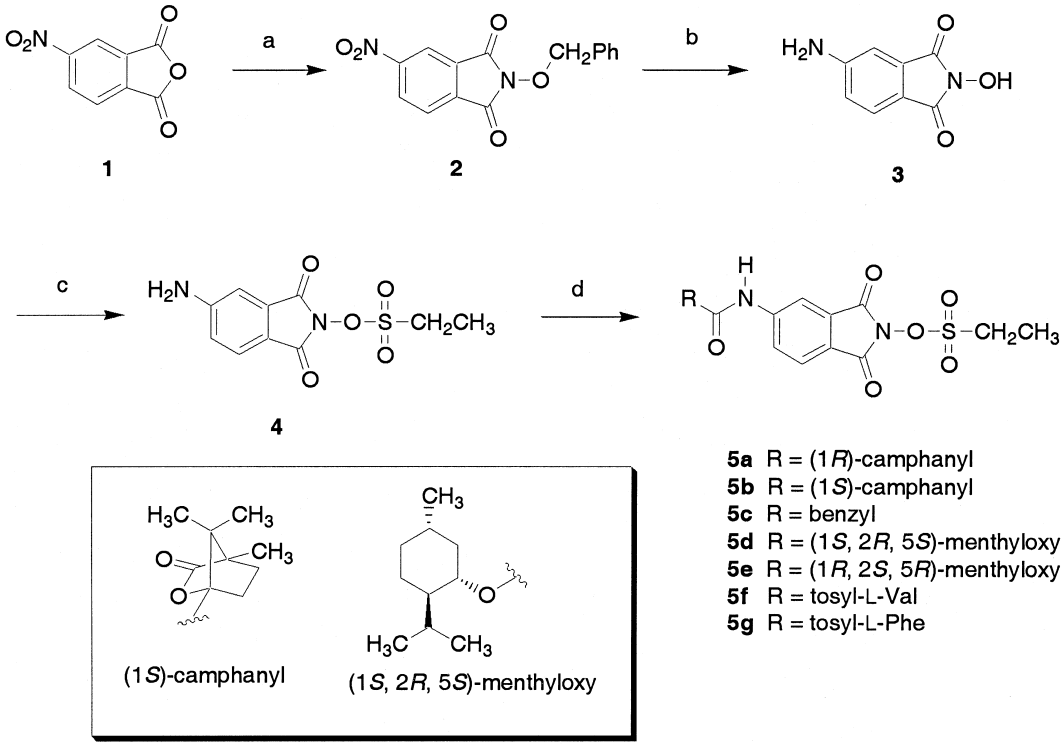
The reaction of an irreversible inhibitor with enzyme is generally accepted to proceed through a noncovalent complex (E−I*) followed by formation of the covalent complex (E−I in eq (1)). The inhibitory activity of compounds **5a**–**5g** toward HLE, ChT, PPE, and Cat G was determined using the progress curves method.[32] The *pseudo* first order rate constant (k_{obs}) was determined using eq (3).[33] The solver in Excel® (Microsoft Corp.) was used to simultaneously solve for two unknowns, A_∞ and $k_{obs,}$ by least squares fit to eq (3). Inhibition was measured by monitoring a decrease in absorbance at



7-amino-4-chloro-3-ethoxyisocoumarin



6-amino-2-[(ethylsulfonyl)oxy]-1*H*-isoindole-1,3-dione



Scheme 1. Reagents: (a) $PhCH_2ONH_2 \cdot HCl$, TEA, toluene, reflux; (b) 5% Pd/C, cyclohexene, EtOH/THF (1/1), reflux; (c) ethanesulphonyl chloride, $NaHCO_3$, H_2O, 0 °C; (d) RCOCl, TEA, THF -or- $NaHCO_3$, EtOAc.

410 nm due to the release of *p*-nitroaniline upon enzyme-catalyzed hydrolysis of the *p*-nitroanilide substrate. The results in the form of $k_{obs}/[I]$ values (see eq (2) are summarized in Table 1.

$$E + I \underset{K_i}{\rightleftharpoons} E - I^* \xrightarrow{k_3} E - I \quad (1)$$

$$k_{obs}/[I] = \frac{k_3}{K_i + [I]} \quad (2)$$

$$A_{calc} = A_\infty - A_\infty e^{-(k_{obs}t)} \quad (3)$$

A_∞ = final absorbance and A_{calc} = absorbance, calculated at time t.

Buffer stability studies

Compounds **4**, **5b** and **5f** were selected for buffer stability assays. All compounds were found to be stable under the buffer (pH 5.4) assay conditions used in the mobile phase of the HPLC assay. Each compound was incubated in either Tris or HEPES buffer, and their hydrolysis rates monitored via HPLC. Half-lives were calculated based on observations made at five different time intervals. Plots of $\ln[(A_t - A_f)/(A_o - A_f)]$ versus time, where A_t and A_f are the peak areas of the remaining non-hydrolyzed sulfonyloxy phthalimide at times t and the final reading, were used to determine the rate of hydrolysis. Half-life values were calculated using $t_{1/2} = 0.693/k$ where k (s^{-1}) is obtained from the slope (Table 2).

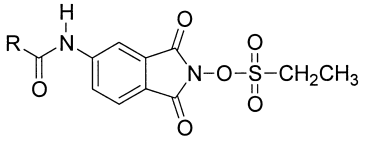
Discussion

Inhibition of elastase

Compound **5b** was the best inhibitor of HLE in this series. Compound **5b** was a 4-fold better inhibitor of HLE over ChT and 11-fold more selective for HLE over Cat G. Compound **5c** was the next best inhibitor, which was only slightly less potent than **5b** and very selective for HLE over the other three enzymes. For purposes of clarity, we will refer to the chiral center directly adjacent to the amide linkage of the side chain to the phthalimide ring when making reference to '*R*' and '*S*'. Assays involving those compounds possessing chirality in their side chains demonstrated that HLE has a preference for *S* stereochemistry over *R* for chiral centers adjacent to the amide linkage as can be seen from the increase in inhibition by **5b** over **5a** and **5d** over **5e**. This is consistent with other findings in the literature.[13] In stark contrast, chymotrypsin exhibits the opposite pattern in this series of compounds showing a preference for the *R* stereochemistry. Surprisingly, the tosyl-L-Val and tosyl-L-Phe substituted derivatives interacted poorly with HLE compared to their inhibition of ChT. In contrast, Kerrigan et al. found that the tosyl-L-Phe derivative of the methylsulfonyloxy phthalimides inhibited HLE very well ($k_{obs}/[I] = 160{,}000\ M^{-1}\ s^{-1}$).[24] This suggests that extension of the alkylsulfonyloxy chain strongly influences the S_n' binding interactions of the substituent at the 6-position.

Three representative compounds were selected for buffer stability study. Derivative **4** was the most stable of the three in both HEPES and Tris. Acylation of the

Table 1. Inhibitory activity of derivatives **5a–g** toward human leukocyte elastase, chymotrypsin, porcine pancreatic elastase and cathepsin G



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Compound	$k_{obs}/[I]$ (M^{-1} s^{-1})[a,b]			
	HLE[c]	ChT[d]	PPE[e]	Cat G[f]
5a	8100	17,000	270	1900
5b	11,000	2800	1100	950
5c	10,000	3500	3100	2000
5d	7500	3600	950	ND[g]
5e	3600	7800	2300	ND[g]
5f	6600	14,000	170	ND[g]
5g	9000	18,000	610	2200

[a]0.1 M HEPES, 0.5 M NaCl, pH 7.5 at 25 °C and < 6% DMSO.
[b]All data was collected in duplicate with an average relative standard deviation of ±14%.
[c]The substrate for HLE was MeO-Suc-Ala-Ala-Pro-Val-*p*NA ([E] = 31 nM, [S] = 95–100 mM).
[d]The substrate for ChT was Suc-Ala-Ala-Pro-Phe-*p*NA ([E] = 100–158 nM, [S] = 80 μM).
[e]The substrate for PPE was Suc-Ala-Ala-Ala-*p*NA ([E] = 200–400 nM, [S] = 225 μM).
[f]The substrate for Cat G was the same as that for ChT ([E] = 42.5 nM, [S] = 200 μM).
[g]ND, not determined.

Table 2. Buffer stability data for selected compounds[a]

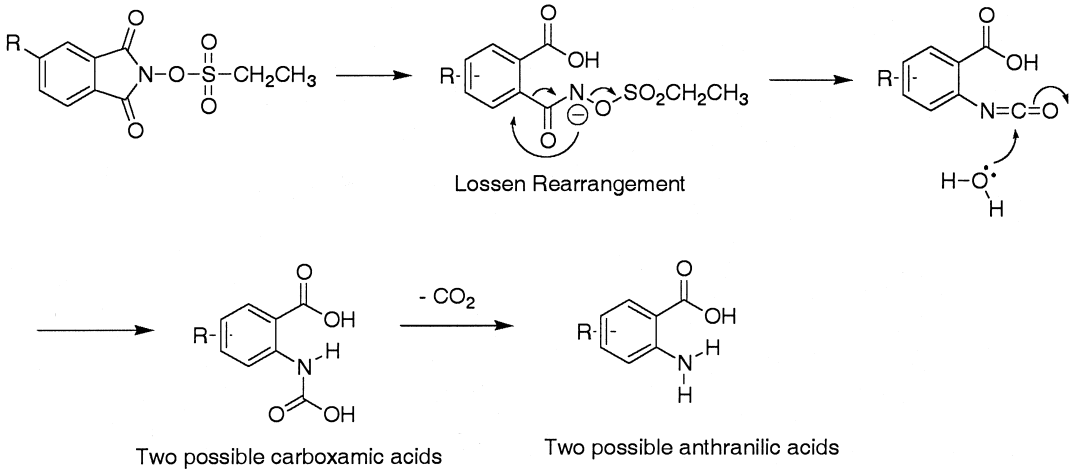
Compound	$t_{1/2}$ (min)	
	HEPES[b]	Tris[c]
4	8.9	6.1
5b	2.1	4.0
5f	6.1	6.8

[a]All log plots had $R^2 > 0.89$ with average $R^2 = 0.95$.
[b]0.1 M HEPES, 0.5 M NaCl, pH 7.5 at 25 °C and < 6% DMSO.
[c]0.05 M Tris, 0.15 M NaCl, pH 7.5 at 25 °C and < 6% DMSO.

6-amino function in the parent compound **4** appears to increase the susceptibility of compounds **5b** and **5f** to hydrolytic decomposition. These compounds have two potential sites for hydrolytic attack due to the asymmetric nature of the 6-substituted-isoindole-1,3-dione ring (Scheme 2). Opening of the ring exposes two possible intermediate isocyanates. Hydrolysis of the isocyanate gives two possible carboxamic acids, which upon loss of carbon dioxide will decompose to the anthranilic acids. An example of an HPLC trace (Fig. 3) of the progress of the hydrolysis reaction of compound **5f** in Tris buffer reveals the formation of two major product peaks, one at 6.8 min and one at 7.4 min. The peak at 10.5 min is the non-hydrolyzed starting material. This observation supports the suggested duality of binding modes. Additional work is under way to determine the exact nature of the structure of these two major products and their kinetics of formation and will be published elsewhere.

Modeling

The interaction energies of the noncovalent HLE-inhibitor complexes (E−I* in eq (1)) of the two sets of enantiomers are reported in Table 3. The interaction energy (E_{int}) is the sum of all nonbonding interactions in the complex (i.e., van der Waals, E_{vdw} and electrostatic, E_{elec} in eq. (4)). Interaction energies (E_{int}) are not binding free energies (i.e., ΔG_{bind}); however, they can be used to rank complexes and have been demonstrated to show moderate correlation with experiment.[34] We will refer to the chiral center directly adjacent to the amide linkage of the side chain to the phthalimide ring when making reference to '*R*' and '*S*'. In both sets of enantiomers, the *S* stereochemistry gave the lower interaction energy. In support of these results, the experimental results indicate that the *S* enantiomer is the better inhibitor (see Table 1). One reason for this preference for the *S* enantiomer is the nature of the binding of the chiral group in the S_n' sites. In Figure 4 is a picture of the AMBER89 energy minimized noncovalent complex of *S* enantiomer **5b** with HLE showing the relative position of key residues (PHE 41 and LEU 35 in S_n' and



Scheme 2. Possible buffer hydrolysis outcomes.

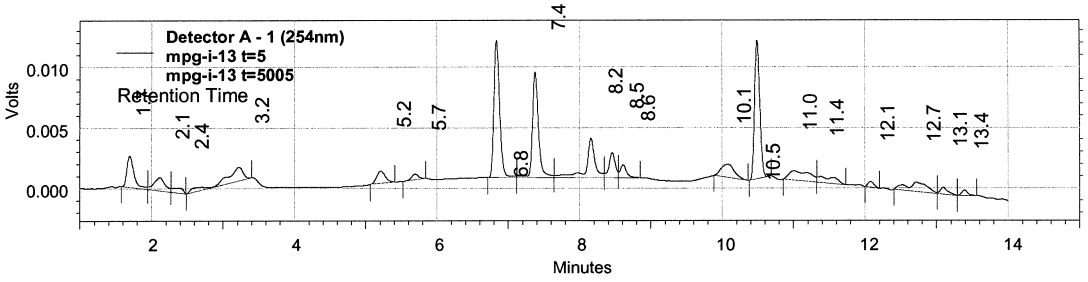


Figure 3. HPLC trace of **5f** in Tris buffer after 5 min of incubation.

Table 3. Interaction energies (E_{int}) of noncovalent HLE–inhibitor complexes

Compound	E_{int} (kcal/mol)	
	MMFF94[a]	AMBER89[b]
5a $R=(1R)$-camphanyl	−763	−5560
5b $R=(1S)$-camphanyl	−818	−5610
5d $R=(1S,2R,5S)$-menthyl	−927	
5e $R=(1R,2S,5R)$-menthyl	−857	
5d 'inverted'	−992	
5e 'inverted'	−1008	

[a]Gradient convergence at 0.05 kcal/(mol·Å).
[b]Gradient convergence at 0.001 kcal/(mol·Å).

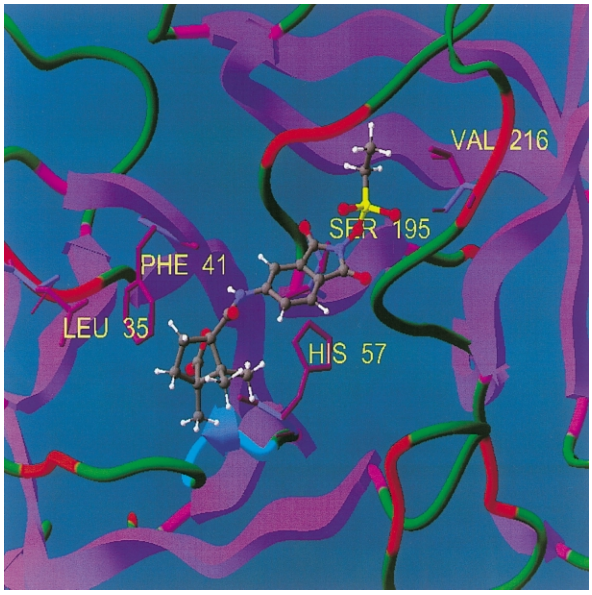


Figure 4. Model of compound **5b** noncovalent complex with HLE.

VAL 216 in S_1) relative to the inhibitor. The electrostatic potential surface map of the complex of '*S*' enantiomer **5b** with HLE in Figure 5 shows the lactone carbonyl oxygen of the camphanyl group making a favorable electrostatic interaction with the enzyme backbone along the peptide bond of CYS 58 and VAL 59. In comparison, the electrostatic surface potential map of the '*R*' enantiomer **5a** (not shown) has all the same features as the '*S*' enantiomer **5b** except for the favorable contact between the enzyme and the lactone. For the '*R*' enantiomer, the lactone ring oxygens are

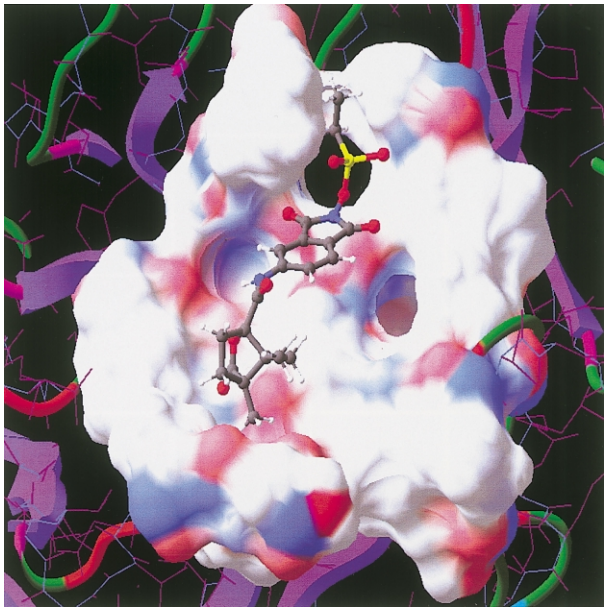


Figure 5. Electrostatic potential mapped to electron density in active site region of compound **5b** noncovalent complex with HLE.

pointing out and away from the S_n' pocket. In addition, some ring strain was noted in the '*R*' enantiomer complex possibly due to unfavorable steric interactions in S_1 and S_n'. We investigated the 'inverted' binding model (see Fig. 6) for the menthyl derivatives **5d** and **5e**. The *R* enantiomer is the lower energy complex in the 'inverted' model. The inverted model gave results that were in conflict with experimental observations. The energy gap (ΔE_{int}) between **5d** and **5e** was 16 kcal/mol for the inverted model compared to a ΔE_{int} of 70 kcal/mol for our proposed binding model (Fig. 1). These results do not necessarily rule out the inverted binding model; however, they do further support our binding hypothesis.

$$E_{int} = E_{vdw} + E_{elec} \quad (4)$$

Conclusion

Substitution in the 6-position of 6-acylamino-2-[(ethylsulfonyl)oxy]-1*H*-isoindole-1,3-diones is an effective method of improving selectivity of these inhibitors for HLE. Compounds **5b** and **5c** were both selective inhibitors of HLE over ChT, PPE, and Cat G. These data demonstrate

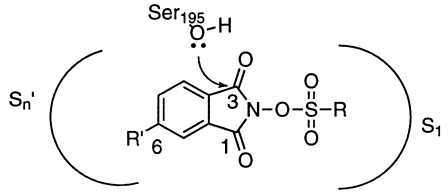


Figure 6. 'Inverted' binding model.

that, while hydrophobic groups may increase selectivity for HLE; the combination of groups in P1 and P2′ can have dire effects on potency. HLE does not tolerate the combination of bulky hydrophobic groups in both the P1 (ethylsulfonyloxy group) and P2′ regions of the inhibitor. The incorporation of an additional carbon in the alkylsulfonyloxy chain results in a loss of potency. Extension of this chain may lead to the formation of a higher energy complex in derivatives with bulky hydrophobic ring substituents perhaps through unfavorable steric interactions in both the S_1 and S_n' sites and hence less potent inhibition. The buffer stability assay indicates that these inhibitors will not be suitable for intravenous or oral administration. However, these compounds might be suitable for use in an inhaler.

Experimental

Chemistry

All reactions were carried out under an atmosphere of dry nitrogen. All melting points were recorded on a Mel-Temp II apparatus and are uncorrected. HPLC data was recorded on a Shimadzu Class VP HPLC using a YMC ODS-AQ (C-18) 120 Å 5 μm particle size 4.6 × 150 mm analytical column with the UV detector set at 254 nm. The solvent mixtures used were acetonitrile: 25 mM NH_4OAc pH 5.4 buffer (30:70) as Solvent A and acetonitrile (100%) as Solvent B. The gradient was ramped from 100% Solvent A to 100% Solvent B in a linear fashion over a 10-min time period followed by 4 min with 100% Solvent B with a flow rate of 1.00 mL/min. IR data was obtained on a Perkin–Elmer 1600 series FTIR spectrometer. Proton and ^{13}C spectra were recorded on a Varian Gemini 200 FTNMR spectrometer. Optical rotations were determined on a Perkin–Elmer 241 polarimeter using a micro-cell with a 1 decimeter path length. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Mass spectrometric analysis was performed by the Department of Chemistry, Washington University, St. Louis, MO. Flash chromatography[35] was carried out using 32–36 μm particle size silica gel (pore diameter, 60 Å). Thin-layer chromatography was performed using Allied-Signal silica plates and visualized by UV light, iodine vapor or phosphomolybdic acid spray. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl.

Tosyl-L-valine. Melting point (aq EtOH) 146.2–147.9 °C Lit.[36] 147 °C. ^1H NMR (DMSO) δ 0.78 (t, J = 6.6 Hz, 6H), 1.90 (m, 1H), 2.35 (s, 3H), 4.44 (m, 2H), 7.33 (d, J = 8.1 Hz, 2H), 7.65 (d, J = 8.2 Hz, 2H), 7.9 (d, J = 9.3 Hz, 1H).

2-*N*-(Benzyl)oxy-5-nitroisoindole-1,3-dione (2). Melting point (EtOH) 185–187.5 °C. Lit.[25] 188–190 °C. ^1H NMR (DMSO) δ 5.20 (s, 2H), 7.45 (m, 5H), 8.15 (d, J = 8.1 Hz, 1H), 8.48 (d, J = 1.8 Hz, 1H) 8.63 (dd, J = 8.2, 2.0 Hz, 1H).

6-Amino-2-*N*-hydroxy-1*H*-isoindole-1,3-dione (3). Melting point (EtOH) 270–273 °C Lit.[25] 268 °C. ^1H NMR (DMSO) δ 6.5 (s, 2H), 6.78 (d, J = 8.1 Hz, 1H), 6.90 (s, 1H), 7.45 (d, J = 8.3 Hz, 1H), 10.4 (s, 1 H).

6-Amino-2-[(ethylsulfonyl)oxy)]-1*H*-isoindole-1,3-dione (4). Compound **3** (1.96 g, 11.0 mmol) was taken up in H_2O (70 mL) and cooled to 0 °C. To this solution was added sodium bicarbonate (924 mg, 11.0 mmol) and ethanesulphonyl chloride (1.0 mL, 11.0 mmol). The reaction was allowed to stir overnight and the resulting precipitate was diluted in H_2O and collected. Recrystallization in EtOH afforded a yellow powder (1.83 g, 62%): mp 185–186 °C; ^1H NMR (DMSO) δ 1.49 (t, J = 7.2 Hz, 3H), 3.75 (q, J = 7.3 Hz, 2H), 6.80 (s, 2 H), 6.9 (d, J = 8.3 Hz, 1H), 7.0 (s, 1H), 7.6 (d, J = 8.3 Hz, 1H); ^{13}C NMR (DMSO) δ 8.3, 46.5, 107.9, 112.6, 118.0, 126.6, 131.3, 156.2, 162.2, 163.1; IR (KBr) 3476 (NH) 3389, 1733 (C=O) cm^{-1}; EIMS m/z 269.8 (M^+). Anal. calcd for $C_{10}H_{10}N_2O_5S$: C, 44.44; H, 3.73; N, 10.38. Found: C, 44.50; H, 3.78; N, 10.41.

6-((1′*R*)-Camphanyl)amino-2-[(ethylsulfonyl)oxy]-1*H*-isoindole-1,3-dione (5a). (1*R*)-(+)-Camphanic acid (500 mg, 2.3 mmol) in thionyl chloride (4 mL, 52 mmol) was stirred at reflux for 1.5 h. Thionyl chloride was removed in vacuo and the resulting acid chloride was immediately taken up in THF (5 mL) followed by addition of **4** (618 mg, 2.3 mmol) then triethylamine (0.4 mL, 2.8 mmol) at 0 °C. The mixture was gradually warmed to room temperature and allowed to stir overnight. The mixture was diluted in ethyl acetate (40 mL), washed with 10% HCl (16 mL), saturated sodium bicarbonate (2×8 mL), brine (16 mL), dried ($MgSO_4$) and concentrated. The resulting precipitate was recrystallized in methyl *tert*-butyl ether in cyclohexanes, yielding a beige powder (870 mg, 89%): mp 167–168 °C; ^1H NMR (DMSO) δ 0.90 (s, 3H), 1.10 (m, 7H), 1.49 (t, J = 7.3 Hz, 3H) 1.62 (m, 1H) 2.02 (m, 2H), 3.81 (q, J = 7.3 Hz, 2H), 7.93 (d, J = 8.4 Hz, 1H), 8.25 (dd, J = 8.2, 2.0 Hz, 1H), 8.41 (d, J = 2 Hz, 1H), 10.45 (s, 1H). IR (KBr) 3323 (NH), 2974, 1743 (C=O) cm^{-1}. EIMS m/z 450.2 (M^+). Anal. calcd for $C_{20}H_{22}N_2O_8S$: C, 53.33; H, 4.92; N, 6.22. Found: C, 53.57; H, 5.11; N, 6.20; $[\alpha]_D^{25}$ +18.8° (c 4, $CHCl_3$).

6-((1′*S*)-Camphanyl)amino-2-[(ethylsulfonyl)oxy]-1*H*-isoindole-1,3-dione (5b). This compound was prepared from (1*S*)-(−)-camphanic acid in the same manner as the procedure described for **5a**. The crude precipitate was recrystallized from methyl *tert*-butyl ether in cyclohexane giving a beige powder (200 mg, 48%): mp 168.9–169.0 °C; ^1H NMR (DMSO) δ 0.90 (s, 3H), 1.05 (m, 7H), 1.49 (t, J = 7.3 Hz, 3H), 1.60 (m, 1H), 2.00 (m, 2H), 3.75 (q, J = 7.3 Hz, 2H), 7.95 (d, J = 8.5 Hz, 1H), 8.25 (dd, J = 8.4, 1.5 Hz, 1H), 8.40 (s, 1H), 10.51 (s, 1H); ^{13}C NMR (DMSO) δ 8.3, 9.8, 16.5, 16.7, 28.6, 30.3, 40.4, 54.3, 54.9, 92.0, 115.7, 123.3, 125.5, 126.4, 129.9, 144.5, 161.8, 162.1, 166.8, 178.1. IR (KBr) 3324 (NH), 2965,

1749 (C=O) cm^{-1}. EIMS m/z 450 (M^+). Anal calcd for $C_{20}H_{22}N_2O_8S$: C, 53.33; H, 4.92; N, 6.22. Found: C, 53.57; H, 5.09; N, 6.16; $[\alpha]_D^{25}$ −19.5° (*c* 2, $CHCl_3$).

6-(Benzoyl)amino-2-[(ethylsulfonyl)oxy]-1*H*-isoindole-1, 3-dione (5c). Triethylamine (0.2 mL, 1.1 mmol) was added to a THF (6 mL) solution of compound **4** (250 mg, 0.9 mmol) and phenylacetyl chloride (0.1 mL, 1.0 mmol) at 0 °C. The mixture was allowed to stir at room temperature overnight. The work up employed was identical to that used for **5b**. Recrystallization in ethanol yielded a light-yellow powder (120 mg, 35%): mp 180.9–181.6 °C; ^{1}H NMR (DMSO) δ 1.45 (t, J = 7.3 Hz, 3H), 3.75 (m, 4H), 7.31 (m, 5H), 7.92 (m, 2H), 8.25 (s, 1H), 10.89 (s, 1H); ^{13}C NMR (DMSO) δ 8.2, 43.6, 46.4, 173.6, 122.2, 124.4, 125.8, 127.0, 128.7, 129.5, 130.3, 135.4, 145.8, 161.8, 162.1, 170.6. IR (KBr) 3372 (NH), 3058, 1739 (C=O) cm^{-1}. EIMS m/z 388 (M^+). Anal calcd for $C_{18}H_{16}N_2O_6S$: C, 55.66; H, 4.15; N, 7.21. Found: C, 55.63; H, 4.20; N, 7.12.

6-((1′*S*,2′*R*,5′*S*)-Menthyloxycarbonyl)amino-2-[(ethylsulfonyl)oxy]-1*H*-isoindole-1,3-dione (5d). Compound **4** (250 mg, 0.93 mmol) was combined with (+)-menthyl chloroformate (0.2 mL, 1.0 mmol) as described for the preparation of **5b**. This preparation afforded an oil, which was purified by column chromatography (ethyl acetate/hexanes, 1:1). The isolated oil was dried in vacuo giving a crystalline compound (220 mg, 53%): mp 242 °C dec. ^{1}H NMR (DMSO) δ 0.7–1.15 (m, 12H), 1.4 (m, 2H), 1.5 (t, J = 7.2 Hz, 3H), 1.65 (m, 2H), 1.95 (m, 2H) 3.85 (q, J = 7.2 Hz, 2H), 4.62 (dt, J = 6.6, 4.1 Hz, 1H), 7.88 (m, 2H), 8.09 (s, 1H), 10.5 (s, 1 H); ^{13}C NMR (DMSO) δ 8.3, 16.5, 20.8, 22.2, 23.2, 26.0, 31.2, 33.9, 46.5, 47.1, 74.8, 112.5, 112.2, 123.3, 125.9, 130.4, 146.4, 153.3, 162.3. IR (KBr) 3364 (NH), 2953, 1748 (C=O) cm^{-1}. HRFABMS calcd for $C_{21}H_{28}N_2O_7S$: 453.1696. Found: 453.1697. Anal calcd for $C_{21}H_{28}N_2O_7S$: C, 55.74; H, 6.24; N, 6.19. Found: C, 55.70; H, 6.29; N, 6.08; $[\alpha]_D^{25}$ +34.7° (*c* 2, $CHCl_3$).

6-((1′*R*,2′*S*,5′*R*)-Menthyloxycarbonyl)amino-2-[(ethylsulfonyl)oxy]-1*H*-isoindole-1,3-dione (5e). Compound **4** (250 mg, 0.9 mmol) was combined with (−)-menthyl chloroformate (0.2 mL, 1.0 mmol) as described for the preparation of **5d**. This preparation afforded the opposite enantiomer, which was purified by column chromatography (ethyl acetate/hexanes, 1:1). The oil obtained was dried in vacuo (160 mg, 38%). ^{1}H NMR (DMSO) δ 0.7–1.2 (m, 12H), 1.4 (m, 2H), 1.5 (t, J = 7.3 Hz, 3H); 1.65 (d, 2H), 1.98 (m, 2H), 3.75 (q, J = 7.3 Hz, 2H), 4.62 (dt, J = 6.5, 4.0 Hz, 1H), 7.88 (m, 2H), 8.10 (s, 1H), 10.5 (s, 1H); ^{13}C NMR (DMSO) δ 8.3, 16.4, 20.8, 22.2, 23.2, 26.0, 31.2, 33.9, 46.4, 47.1, 74.8, 107.4, 112.5, 121.2, 123.3, 125.9, 130.4, 146.3, 153.3, 161.9, 162.3. IR (KBr) 3364 (NH), 2953, 1748 (C=O) cm^{-1}. EIMS m/z (%) 188 (100), 163 (43), 315 (26). Anal. calcd for $C_{21}H_{28}N_2O_7S$: C, 55.74; H, 6.24; N, 6.19. Found: C, 55.84; H, 6.33; N, 6.11; $[\alpha]_D^{25}$ −45.5° (*c* 2, $CHCl_3$).

6-(Tosyl-L-valinyl)amino-2-[(ethylsulfonyl)oxy]-1*H*-isoindole-1,3-dione (5f). Compound **4** (250 mg, 0.9 mmol) was combined with tosyl-L-valine (1.0 g, 3.7 mmol) as described for the preparation of **5a**. This preparation afforded a crude oil. The crude oil was purified by column chromatography (ethyl acetate/hexanes, 1:1) giving a yellow powder after removal of solvent (140 mg, 31%): mp 177.6–180.1 °C dec. ^{1}H NMR (DMSO) δ 0.85 (dd, J = 6.6, 5.5 Hz, 6H), 1.55 (t, J = 7.3 Hz, 3H), 1.89 (m, 1H), 2.15 (s, 3H) 3.60 (t, J = 8.1 Hz, 1H), 3.78 (q, J = 7.2 Hz, 2H), 7.18 (d, J = 8.1 Hz, 2H), 7.60 (s, 1H), 7.62 (d, J = 8.1 Hz, 2H), 7.9 (m, 2H), 8.23 (d, J = 10.0 Hz, 1H), 10.55 (s, 1H); ^{13}C NMR (DMSO) δ 8.3, 18.8, 19.1, 20.0, 30.9, 46.5, 63.1, 113.9, 122.4, 124.7, 125.7, 126.9, 129.4, 129.9, 138.2, 142.8, 144.7, 162.1, 170.3. IR (KBr) 3251 (NH), 2963, 1748 (C=O) cm^{-1}. FABMS m/z 524.0 (M + H). Anal. calcd for $C_{22}H_{25}N_3O_8S_2$: C, 50.47; H, 4.81; N, 8.03. Found: C, 50.34; H, 4.74; N, 7.91.

6-(*N*-Tosyl-L-phenylalanyl)amino-2-[(ethylsulfonyl)oxy]-1*H*-isoindole-1,3-dione (5g). Triethylamine (0.1 mL, 0.9 mmol) was added to a THF (10 mL) solution of compound **4** (200 mg, 0.74 mmol) and *N*-*p*-toluenesulphonyl-L-phenylalanyl chloride (304 mg, 0.9 mmol) at 0 °C. The mixture was warmed to room temperature and stirred overnight. The work up employed was identical to that used for **5b**. The crude residue was recrystallized in EtOH affording a dark orange powder (148 mg, 35%); mp 189.9–191 °C dec. ^{1}H NMR ($CDCl_3$) δ 1.66 (t, J = 7.3 Hz, 3H), 2.43 (s, 3H), 3.06 (m, 2H), 3.65 (q, J = 7.3 Hz, 2H), 4.05 (m, 1H), 5.18 (d, J = 6.2 Hz, 1H), 6.95 (m, 2H), 7.2 (m, 5H), 7.55 (d, J = 8.1 Hz, 2H), 7.84 (m, 2H) 8.24 (s, 1H), 8.56 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 8.4, 21.7, 37.9, 48.2, 58.7, 74.2, 97.3, 115.4, 125.3, 125.7, 127.3, 127.7, 129.3, 121.3, 130.2, 134.7, 143.9, 144.7, 161.5, 169.3. FABMS m/z 572 (M + H). HRFABMS calcd for $C_{26}H_{26}N_3O_8S_2$: 572.1161. Found: 572.1137.

Biological assays

In vitro assays were carried out in plastic cuvettes on a Shimadzu UV–vis 2101PC scanning spectrophotometer equipped with a thermo jacketed cell holder. Substrate concentrations for enzyme assays were taken from Nakajima and Powers.[37] All inhibitor and substrate stock solutions were prepared in DMSO. Stock solutions of HLE and Cat G were prepared in 0.05 M NaOAc, 0.15 M NaCl buffer, pH 5.5; and stock solutions of PPE and ChT were prepared in 1 mM HCl. HEPES [4-(2-hydroxyethyl)-1-piperazineethane]sulfonic acid buffer (0.1 M HEPES, 0.5 M NaCl, pH 7.5) was used in all in vitro enzyme assays. Assays were run at 25 °C and initiated by addition of enzyme. The *pseudo* first order inhibition rates were monitored by following the decrease in absorbance at 410 nm of the *p*-nitroaniline group (ε_{410} = 8800 M^{-1} cm^{-1}) released from the substrate by enzymatic hydrolysis. Human Leukocyte Elastase and Cathepsin G were obtained from Athens Research and Technology, Inc., Athens, GA. Porcine Pancreatic Elastase and Chymotrypsin were obtained from Sigma-Aldrich, St. Louis, MO. All substrates were obtained from Bachem Bioscience, King of Prussia, PA.

Molecular modelling

All computational work was performed on an OCTANE® (Silicon Graphics, Inc.) system or a Pentium

III® (Intel, Inc.) 933 MHz personal computer. The small molecule inhibitors were built and geometry optimized using the Merck Molecular Force Field[38] (MMFF94) in PCSpartan Pro® (Wavefunction, Inc.). For those compounds used in the AMBER89 calculations, single-point ab initio calculations were performed on the resulting small molecule structures using the STO-3G*[39,40] basis set in Gaussian 98W.[41] The CHelpG electrostatic potential fitting procedure in Gaussian was used to compute charges on the individual atoms of each inhibitor after single point ab initio calculation.[42] For the MMFF94 calculations, MMFF94 charges were used on both the inhibitor and the protein. The ethylsulfonyl portion of the energy-minimized inhibitors was superimposed on the side chain of the valine residue of the MeOSuc-Ala-Ala-Pro-Val-chloromethyl ketone inhibitor in the crystal structure (PDB; 1PPG). Molecular mechanics calculations using the Merck Molecular Force Field (MMFF94) on the noncovalent enzyme-inhibitor complexes were performed in Sybyl® (Tripos, Inc.) on the OCTANE. Molecular mechanics calculations on enzyme-inhibitor complexes employing the Kollman all-atom implementation of AMBER89[43] was performed using MOE (Chemical Computing Group, Inc.) on the personal computer.

The refined 2.3 Å crystal structure of HLE (PDB; 1PPG) was used as a starting point for construction of the noncovalent enzyme inhibitor complexes.[44] All crystallographic water atoms and peripheral sugar residues were removed. The valine chloromethyl ketone inhibitor was extracted and used for initial positioning of the inhibitors. Hydrogen atoms were added and charges on the individual residues were loaded from the Kollman all-atom charge set[43] in Sybyl. The Kollman all-atom charge set in AMBER89 is derived from ab initio STO-3G calculations.[43] Inhibitors were pre-positioned in the active site of the enzyme followed by partial minimization of the entire complex. A 6 Å region about the inhibitor was defined as the cavity. The inhibitors/cavity region was minimized to the gradient convergence criteria specified in the text. All graphical displays were generated using Sculpt®, version 3.0 (MDL Information Systems, Inc.).

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