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Expedited Articles

Orally Bioavailable Benzisothiazolone Inhibitors of Human Leukocyte Elastase[1]

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Human leukocyte elastase (HLE) has been proposed as a primary mediator of pulmonary emphysema and other inflammatory airway diseases. HLE is capable of cleaving many proteins, including elastin, other components of connective tissue, certain complement proteins, and receptors. Under normal conditions an appropriate balance exists in the lung between HLE and endogenous inhibitors, which scavenge the released enzyme before it exerts deleterious effects in the lung. Emphysema is thought to result from an imbalance in the lung between HLE and endogenous inhibitor (elevated elastase or insufficient inhibitor) that leads to the destruction of alveoli. We have identified WIN 64733 (**2**) and WIN 63759 (**3**) as potent (K_i* = 14 and 13 pM, respectively), selective, mechanism-based inhibitors of HLE which are orally bioavailable in the dog (absolute bioavailability 46% and 21%, respectively). In this series the *in vitro* stabilities of the inhibitors in blood, jejunal homogenates, and liver S9 homogenates are useful predictors of oral bioavailability. After being administered orally (30 mg/kg) to dogs, compounds **2** and **3** are found in the lung, being detected in the epithelial lining fluid obtained by bronchoalveolar lavage (C_{max} of 2.5 and 0.47 μg/mL, respectively).

Human leukocyte elastase (HLE) has been proposed as a primary mediator of pulmonary emphysema and other inflammatory airway diseases. HLE is capable of cleaving many proteins, including elastin, other components of connective tissue, certain complement proteins, and receptors.[2] Under normal conditions an appropriate balance exists in the lung between HLE and endogenous inhibitors, which scavenge the released enzyme before it exerts deleterious effects in the lung. In response to inflammatory mediators, HLE and other proteases are released from the granules of neutrophils. An imbalance between HLE and endogenous inhibitory proteins (the so called protease–antiprotease theory) is postulated to exist in emphysema. Specifically, a deficiency of α_1-protease inhibitor leads to an excess of active HLE which causes elastin destruction in the lung.[3] In cystic fibrosis[4] and adult respiratory distress syndrome (ARDS)[5] patients, an excess of HLE in the lung is thought to exceed the protective barrier of the endogenous inhibitors and gives rise to significant

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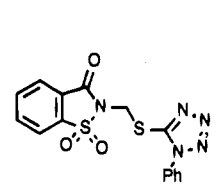
pathology in these diseases. HLE has been shown to be a potent secretogogue and may thereby contribute to the hypersecretion of mucous associated with cystic fibrosis and chronic bronchitis.[6] An inhibitor of HLE should prove useful in the treatment of these inflammatory airway diseases.

The protease–antiprotease theory for the role of HLE in emphysema, chronic bronchitis, or other pulmonary diseases has yet to be confirmed.[3,6] An active search to identify orally active or bioavailable HLE inhibitors has been underway for more than a decade.[7] Only one inhibitor has been in the clinic, ICI 200,880, delivered iv or by aerosol, and definitive efficacy data have not yet been reported. However, two small clinical studies of ICI 200,880 in ARDS patients supported the theory that HLE may play a role in ARDS.[8]

The culmination of many structure–activity relationship (SAR) studies[7,9] has led to the discovery of three series of orally active and bioavailable inhibitors of HLE. The β-lactam, mechanism-based inhibitor L-680,833 is potent, selective, and orally active in hamsters in the HLE-induced pulmonary hemorrhage model.[10] The initial lead β-lactam inhibitor L-658,758 had poor metabolic and hydrolytic stability and was not orally active in animals. When more stable inhibitors were prepared, they were found to be orally active.[11,12] A series of peptide-mimetic trifluoromethyl ketones[13] and a series of peptidyl pentafluoroethyl ketones[14] were reported to be reversible transition-state analog inhibitors of HLE which are orally active in hamsters. This paper describes a new class of orally bioavailable inhibitors of HLE that are well absorbed in dogs and distributed to the lung, the disease target organ.

Results and Discussion

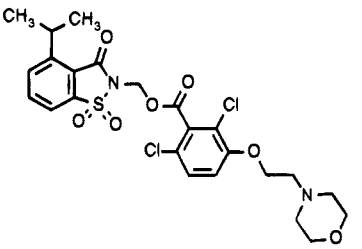
The lead compound in our benzisothiazolone class of HLE inhibitors was KAN 400473.[15,16] This compound was a 15 nM inhibitor of HLE but *in vitro* could not be detected in human blood even after very short incubation times (<1 min) and therefore would have poor oral bioavailability. Our approach to obtaining bioavailable inhibitors was to improve the *in vitro* metabolic stability of these reactive, mechanism-based inhibitors. SAR studies on this class of HLE inhibitors showed that at the 4-position an isopropyl substituent significantly improved both HLE inhibitory potency (apparent binding constant, $K_i^* = 0.3$ nM)[17] and human blood stability ($t_{1/2} = 45$ min). The introduction of a 6-methoxy group gave potent inhibitors with improved blood stability ($t_{1/2} = 260$ min). The enhanced stability was attributed to the attenuated reactivity of the benzisothiazolone carbonyl by the electron-donating 6-methoxy group.[16] The 2,6-dichlorobenzoate leaving group was optimum for potency, and the compounds retained stability in human blood.[16,18] However, these inhibitors are very lipophilic (clog $P > 4$) with poor aqueous solubility and poor *in vivo* activity, which we attribute to these physical properties. Therefore, a series of compounds with aqueous solubilizing substituents bearing amines and carboxylic acids were prepared. WIN 63294 (1) is a potent inhibitor of HLE both in *in vitro* and *in vivo* by the iv route of administration[18] ($K_i^* = 0.008$ nM and 87% inhibition at 1 mg/kg, iv in the HLE-induced hamster hemorrhage model[19]) but is not orally active in this hamster model. Inhibitor 1 was not stable in human blood nor in jejunum or liver S9 homogenates. We believe that the lack of oral activity in hamsters is due to poor metabolic stability. In dog blood and liver S9 homogenates, the stability is equally poor compared to human tissue.



KAN 400473

HLE, K_i^* = 15 nM

Human Blood $t_{1/2}$ = <5 min



WIN 63294 (1)

HLE, K_i^* = 0.008 nM

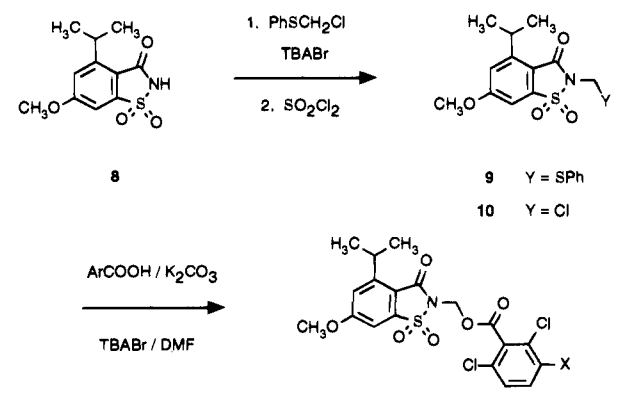
Human Tissue Stabilities ($t_{1/2}$):

blood	20.1 ± 3.9 min
jejunum	13 ± 1 min
liver	4.9 ± 0.8 min

On oral administration an inhibitor would be absorbed through the *intestine/jejunum*, transported by the portal vein to the *liver*, and then returned to the *blood* stream and circulated. We chose to examine inhibitor stability in these three tissues as the major barriers to absorption, metabolism, elimination, and achieving acceptable oral bioavailability. Since we had shown that the introduction of a 6-methoxy substituent improved human blood stability (in some examples by as much as 5-fold),[16] a series of inhibitors, **2**–**7**, with aqueous solubilizing groups and the 6-methoxybenzisothiazolone substituent were prepared.

The benzisothiazolones **2**–**5** were prepared by alkylation of substituted 2,6-dichlorobenzoic acids with 2-(chloromethyl)benzisothiazolone **10** (Scheme 1). Alkylation of the requisite benzoic acid with 2-(chloromethyl)-4-(1-methylethyl)-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide[17] or 2-(chloromethyl)-6-hydroxy-4-(1-methylethyl)-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide[20] in the presence of potassium carbonate and catalytic tetrabutylammonium bromide in DMF gave the inhibitor 1 or **6**, respectively. The synthesis of the carboxylic acid **7** is outlined in Scheme 2. The preparation of the substituted 2,6-dichlorobenzoic acids has been previously described.[20] The physical properties of new compounds are listed in Table 1.

Scheme 1



8

9 Y = SPh

10 Y = Cl

2-5

Scheme 2

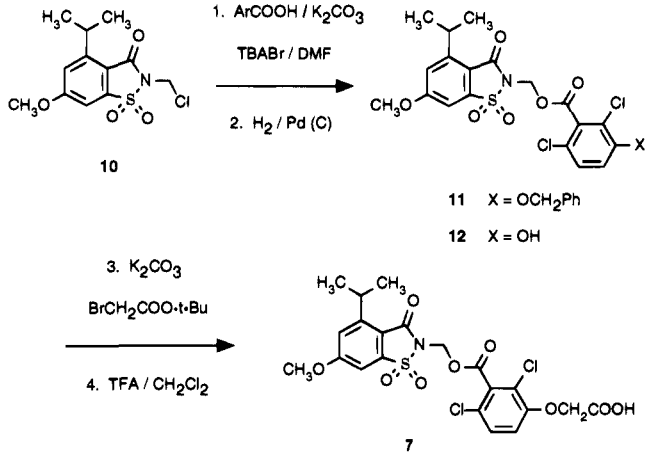


Table 1. Physical Properties

compd	% yield[a]	mp, °C	formula[b]
1	90	140–143	$C_{24}H_{26}Cl_2N_2O_7S$·HCl
2	36	102–104	$C_{25}H_{28}Cl_2N_2O_7S$·HCl·H_2O
3	61	225–226	$C_{25}H_{28}Cl_2N_2O_8S$·HCl
4	65	foam	$C_{24}H_{27}Cl_2N_3O_8S_2$·HCl·1.5$H_2O$
5	23	foam	$C_{24}H_{29}Cl_2N_3O_8S_2$·HCl·0.7$H_2O$
6	47	208–211	$C_{23}H_{25}Cl_2N_3O_8S$·HCl
7	*c*	206–208	$C_{21}H_{19}Cl_2NO_9S$
10	*c*	155–157	$C_{12}H_{14}ClNO_4S$
11	89	139–140	$C_{26}H_{23}Cl_2NO_7S$
12	*c*	152–154	$C_{19}H_{17}Cl_2NO_7S$

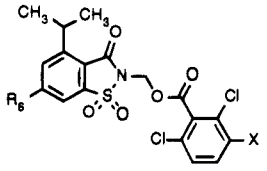
[a] Yield for the reaction of the 2-(chloromethyl)benzisothiazolone with ArCOOH. [b] Carbon, hydrogen, and nitrogen analyses were within ±0.4% of the theoretical values. [c] See the Experimental Section.

The benzisothiazolones **2**–**7** were potent inhibitors, and with the exception of the carboxylic acid **7**, the analogs were considerably more stable than 1. Inhibitor **3**, the 6-methoxy analog of 1, is equipotent as an HLE inhibitor *in vitro* and *in vivo* and is more stable in human tissues *in vitro* (Table 2). With improved liver stability, one would expect less first-pass metabolism and better oral bioavailability (BA). The most potent and tissue stable analog was inhibitor **2**. To test our hypothesis, the oral BA of the inhibitors **2**–**6** was determined in dogs.[21] The compounds are listed in Table 2 from the highest to lowest oral BA. A trend in the data shows that except for phenol **6** the jejunum and liver S9 homogenate stabilities correlate with oral BA and the elimination half-lives. We attribute the poor oral BA of **6** to rapid clearance by conjugation and elimination. Significant conjugate formation does not occur in the liver S9 homogenate incubations. Consistent with this hypothesis is the rapid elimination half-life and low iv AUC of the phenol **6** that results in low oral BA.

Since we are comparing human *in vitro* tissue stability with dog oral BA, *in vitro* stabilities in dog tissues were needed to confirm the dog as an appropriate species for preclinical studies. Also, inhibitors **2** and **3** are active in the hamster HLE-induced hemorrhage model[21] by the iv route of administration (ED_{50} of 1 and 3 mg/kg, respectively), but neither **2** nor **3** are orally active in hamsters. The data in Table 3 show that the inhibitor **3** on oral administration is likely to be rapidly degraded in the hamster intestine and liver, since the hamster jejunum and liver S9 half-lives were very short. However, the hamster blood half-life is greater than 4 h, and this result explains the iv activity and oral inactivity of **3** in hamsters. Monkeys and rats are predicted to be poor species for oral BA studies because **3** is not stable in monkey liver S9 homogenate or rat blood. Indeed, the compound **3** has very low oral BA in monkeys and rats. The inhibitor **3** is stable in dog blood and in jejunum and liver S9 homogenates and is orally bioavailable in dogs. Since **2** and **3** are orally bioavailable in dogs and the dog tissue stability mirrors the stability of **3** in human tissues, we predict that the inhibitor **3** and close analogs should be orally bioavailable in humans.

The inhibitors **2** (WIN 64733) and **3** (WIN 63759) have the best pharmacokinetic properties in dogs (Table 2) and are distributed to the lung when administered orally to dogs (Figure 1). After oral administration of **2** and **3** to dogs at 30 mg/kg, plasma samples were collected at selected intervals postdose and a bronchoalveolar lavage was performed to sample the epithelial lining fluid (ELF) of the lung. The ELF concentrations are corrected for dilution by using the urea concentrations in plasma and BAL fluid. The ELF levels of **3** are

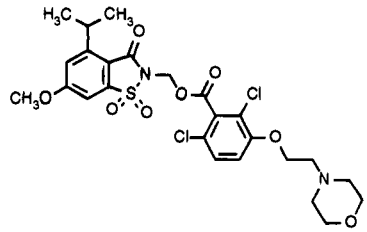
Table 2. Dichlorobenzoate Benzisothiazolones with Aqueous Solubilizing Groups



				human tissue stability,[b] $t_{1/2}$, min			dog[c]		
compd	R_6	X	HLE,[a] K_i*, nM	blood[d]	jejunum	liver	% BA	$t_{1/2elim}$, min	C_{max}, μg/mL
1	H	OCH_2CH_2-4-morpholinyl	0.008	20	13 ± 1	4.9 ± 0.8	NT[e]	NT	NT
2	OCH_3	OCH_2CH_2-1-pyrrolidinyl	0.014	110	218 ± 18	110 ± 18	46 ± 10	432 ± 36	0.325 ± 0.075
3	OCH_3	OCH_2CH_2-4-morpholinyl	0.013	36	170 ± 47	37.3 ± 2.60	21 ± 3	137 ± 25	1.2 ± 0.5
4	OCH_3	SO_2-1-piperazinyl-4-Me	0.011	90	99 ± 16	28.9 ± 1.60	21 ± 5	78 ± 6	0.39 ± 0.16
5	OCH_3	$SO_2N(Me)CH_2CH_2NMe_2$	0.013	130	80 ± 5	23.9 ± 0.82	14 ± 8	74 ± 10	0.30 ± 0.14
6	OH	SO_2-1-piperazinyl-4-Me	0.2	>1200	113 ± 4	40.2 ± 2.57	8 ± 3	23 ± 11	0.22 ± 0.12
7	OCH_3	OCH_2COOH	0.011	<15					

[a] The potency of inhibition is expressed as an apparent binding constant, defined as $K_i^* = k_{off}/k_{on}$. The binding constants are reproducible to within ±10%. Methods are described in ref 17. [b] Incubations were at 37 °C, and disappearance of parent was monitored by HPLC. [c] Bioavailability studies were conducted in beagle dogs with an oral dose of 20 mg/kg and an iv dose of 5 mg/kg, except for compound **2** which was administered orally at 10 mg/kg. Compounds **2**–**5** were formulated as a solution in 0.5% lactic acid, and compound **6** was formulated as a solution in 50% PEG 400 with 5% DMA in water. [d] The blood half-lives were reproducible to within ±25%. [e] NT = not tested.

Table 3. Interspecies Comparison of *in Vitro* Tissue Stability and Oral Bioavailability of Inhibitor **3**



	in vitro half-life (min)			*in vivo* bioavailability
	jejunum	liver S9	blood	(%)
hamster	1.3 ± 0.1	<5	259 ± 40.8	<3[a]
monkey	32.3 ± 16.8	2.9 ± 0.01	57.2 ± 6.3	<1
rat	60.8 ± 2.2	12.4 ± 0.08	6.0 ± 0.54	1
dog	394 ± 138	30.2 ± 2.6	90.0 ± 14.6	21[b]
human	170 ± 47	37.3 ± 2.6	35.5 ± 2.1	??

[a] Based on *in vivo* efficacy data. [b] Bioavailability was formulation dependent. When formulated in an oil-in-water emulsion, the absolute oral bioavailability was 67%.

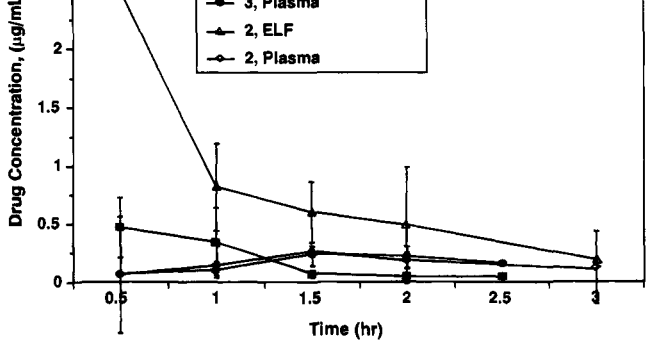


Figure 1. Plasma and bronchopulmonary epithelial lining fluid (ELF) concentrations of inhibitors **2** and **3** in dogs after oral administration of 30 mg/kg. Venous blood was withdrawn and bronchoalveolar lavage was performed on mongrel dogs at various times after oral administration of **2** and **3** (30 mg/kg). Each point represents the individual concentration (µg/mL) of inhibitor in the ELF and plasma for each dog (n = 3).

similar to the plasma levels, while the ELF levels of **2** are significantly higher than the plasma levels (Figure 1). Selective distribution of high pK_a amines to the lung has been reported for a series of drugs, and the lung had the highest tissue-to-plasma partition coefficients of all major organs.[22] The pK_a of **2** (7.7) is higher than the pK_a of **3** (5.3) by more than 2 log units and may account for the greater lung distribution of **2**.

The inhibitors **2** and **3** are specific inhibitors of HLE with *in vitro* specificities greater than 77 000 for HLE over other human proteases, including the serine proteases cathepsin G, thrombin, plasmin, and chymotrypsin and the matrix metalloproteases fibroblast collagenase and stromelysin. In summary, this study reports on a new series of selective, orally bioavailable inhibitors of HLE. Since *in vitro* metabolism studies (tissue stabilities) predicted *in vivo* activity in dogs, and since *in vitro* stability in humans is similar to dogs, inhibitors from this series may represent drug candidates for pulmonary diseases where HLE is involved.

Experimental Section

2-(Chloromethyl)-6-methoxy-4-(1-methylethyl)-1,2-benzisothiazol-3(2*H*)-one 1,1-Dioxide (10). A mixture of 51.0 g (0.20 mol) of 6-methoxy-4-(1-methylethyl)-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide[23] (**8**), 39.6 g (0.25 mol) of chloromethyl phenyl sulfide, and 6.4 g (0.02 mol) of tetrabutylammonium bromide in 280 mL of toluene was refluxed for 45 h, cooled, and concentrated. The residue was column chromatographed on silica gel (630 g) eluted with a gradient of hexanes to 15% hexanes in methylene chloride which gave 65.7 g (87%) of the phenyl sulfide **9** as a white solid: mp 76–78 °C. To a solution of 65.7 g (0.174 mol) of the phenyl sulfide **9** in 300 mL of methylene chloride was added dropwise 45 mL (0.56 mol) of sulfuryl chloride. The mixture was stirred at room temperature for 16 h and then concentrated. The residue was triturated with hexanes, and the solid was collected and recrystallized from benzene–cyclohexane to give 46.43 g (88%) of 2-(chloromethylbenzisothiazolone **10** as a white solid: mp 155–157 °C; ^{1}H NMR ($CDCl_3$) δ 1.38 (d, J = 7.0 Hz, 6H), 3.97 (s, 3H), 4.19 (m, 1H), 5.56 (s, 2H), 7.22 (m, 2H); MS 304 (MH^+), 268 (MH^+ – Cl). Anal. Calcd for $C_{12}H_{14}ClNO_4S$: C, 47.45; H, 4.65; N, 4.61. Found: C, 47.48; H, 4.55; N, 4.50.

[6-Methoxy-4-(1-methylethyl)-1-oxo-1,2-benzisothiazol-3(2*H*)-yl]methyl 2,6-Dichloro-3-[2-(4-morpholinyl)ethoxy]benzoate *S,S*-Dioxide Hydrochloride (3). A mixture of 2,6-dichloro-3-[2-(4-morpholinyl)ethoxy]benzoic acid[20] (3.12 g, 9.7 mmol), **10** (3.0 g, 9.9 mmol), milled potassium carbonate (1.93 g, 14 mmol), and tetrabutylammonium bromide (0.75 g) in 50 mL of DMF was heated at 70 °C for 1 h. After cooling to room temperature, the reaction mixture was concentrated *in vacuo*, diluted with ethyl acetate–ether (3:1), and washed with water and then brine. The organic extract was dried over $MgSO_4$, filtered, and concentrated and the residue crystallized from ethanol to give 3.5 g (61%) of **3** as an off-white solid: mp 146 °C. The hydrochloride salt was prepared with ethereal HCl, and crystallization from ethanol gave **3**·HCl: mp 225–226 °C; ^{1}H NMR (DMSO-d_6) δ 1.23 (d, J = 6.3 Hz, 6H), 3.40 (m, 4H), 3.50 (m, 2H), 3.95 (s, 3H), 4.15 (m, 5H), 4.65 (m, 2H), 6.05 (s, 2H), 7.00 (d, J = 9.2 Hz, 1H), 7.25 (m, 3H). Anal. Calcd for $C_{25}H_{28}Cl_2N_2O_8S$·HCl: C, 48.13; H, 4.68; N, 4.49. Found: C, 48.02; H, 4.64; N, 4.33.

[6-Methoxy-4-(1-methylethyl)-1-oxo-1,2-benzisothiazol-3(2*H*)-yl]methyl 2,6-Dichloro-3-(phenylmethoxy)benzoate *S,S*-Dioxide (11). Reaction of **10** with 2,6-dichloro-3-(phenylmethoxy)benzoic acid,[20] under the conditions described above for **3**, gave 11 in 89% yield: mp 139–140 °C; ^{1}H NMR ($CDCl_3$) δ 1.30 (d, J = 6.0 Hz, 6H), 3.90 (s, 3H), 4.20 (m, 1H), 5.15 (s, 2H), 6.05 (s, 2H), 6.90 (d, J = 9.0 Hz, 1H), 7.20 (m, 3H), 7.40 (m, 5H). Anal. Calcd for $C_{26}H_{23}Cl_2NO_7S$: C, 55.33; H, 4.11; N, 2.48. Found: C, 55.47; H, 4.11; N, 2.44.

[6-Methoxy-4-(1-methylethyl)-1-oxo-1,2-benzisothiazol-3(2*H*)-yl]methyl 2,6-Dichloro-3-hydroxybenzoate *S,S*-Dioxide (12). A mixture of 11 (2.5 g, 4.4 mmol), 10% Pd on carbon (0.7 g), and glacial acetic acid (1 mL) in 100 mL of ethyl acetate was hydrogenated on a Parr apparatus at 50 psi hydrogen for 1.5 h. The resulting mixture was filtered through a pad of florisil with ethyl acetate. The filtrate was washed with saturated $NaHCO_3$, water, and then brine. The organic layer was dried over $MgSO_4$, filtered and concentrated and the residue crystallized from ethyl acetate–hexanes to give 2.1 g (99%) of **12**: mp 152–154 °C; ^{1}H NMR ($CDCl_3$) δ 1.30 (d, J = 6.0 Hz, 6H), 3.90 (s, 3H), 4.18 (m, 1H), 6.10 (s, 2H), 7.00 (d, J = 9.0 Hz, 1H), 7.20 (m, 3H). Anal. Calcd for $C_{19}H_{17}Cl_2NO_7S$: C, 49.79; H, 3.74; N, 3.06. Found: C, 49.51; H, 3.60; N, 2.86.

[6-Methoxy-4-(1-methylethyl)-1-oxo-1,2-benzisothiazol-3(2*H*)-yl]methyl 2,6-Dichloro-3-(carboxymethoxy)benzoate *S,S*-Dioxide (7). A solution of **12** (0.78 g, 1.6 mmol), *tert*-butyl bromoacetate (0.38 g, 2.0 mmol), and milled potassium carbonate (0.30 g, 2.1 mmol) in 50 mL of acetone was refluxed for 16 h and then cooled to room temperature, filtered, and concentrated. Flash chromatography of the residue on silica gel eluted with ethyl acetate–hexanes (1:2) gave 0.65 g (67%) of the *tert*-butyl ester. The *tert*-butyl ester (0.55 g, 0.93 mmol) was dissolved in 15 mL of methylene chloride containing 5 mL of trifluoroacetic acid. The solution was stirred at room temperature under nitrogen for 2 h and concentrated and the residue triturated with hexanes/ether. The resulting solid was collected by filtration to give 0.40 (80%) of **7**: mp 206–208 °C; ^{1}H NMR (DMSO-d_6) δ 1.20 (d, J = 6.0 Hz, 6H), 3.90 (s,

3H), 4.15 (m, 1H), 4.50 (s, 2H), 6.00 (s, 2H), 6.78 (d, J = 9.0 Hz, 1H), 7.15 (m, 3H). Anal. Calcd for $C_{21}H_{19}Cl_2NO_9S$: C, 47.38; H, 3.60; N, 2.63. Found: C, 47.44; H, 3.57; N, 2.57.

***In Vitro* Stability Studies.** Blood from Sprague–Dawley rats, Syrian golden hamsters, beagle dogs, and cynomolgus monkeys was collected in heparinized vacutainer tubes and used within 4 h of collection. Animal liver and jejunum tissues were obtained fresh, immediately frozen in liquid nitrogen, and then stored at −80 °C until used. Frozen human liver and jejunum (unused transplant organs) were obtained from a research agency. Hepatic tissue was minced and homogenized with 3 volumes of 50 mM Na/K phosphate/KCl buffer, filtered, and then centrifuged at 9000g for 20 min. The upper fat layer was decanted after centrifugation and the remaining supernatant filtered giving the S9 fraction. Jejunal tissue was processed similarly in a pH 7.2 modified Ringer's buffer.

Liver S9 incubates (8 mg/mL protein) were fortified with an NADPH-generating system and preincubated for 5 min at 37 °C before the addition of the test compound. Test compounds were dissolved in DMSO and added to the blood, liver S9, and jejunum homogenates to give a starting concentration of 100 μM. The mixtures were incubated at 37 °C and sampled at various intervals. Reactions were quenched with acetonitrile (containing 0.1% trifluoroacetic acid and/or 4% DMSO), and the supernatant, following centrifugation, was assayed by reverse-phase HPLC for parent test compound. Control incubations were included with each assay.

***In Vivo* Bioavailability Studies.** Test compounds **2-5** were administered orally in 0.05% lactic acid in water at 20 mg/kg or intravenously in 5% dextrose/0.05% lactic acid in water at 5 mg/kg to fasted Sprague–Dawley rats, beagle dogs, and cynomolgus monkeys. Inhibitor **6** was administered orally in 50% PEG 400 and 5% DMA in water at 20 mg/kg or intravenously in PEG 400 at 5 mg/kg to fasted beagle dogs. Blood samples were drawn at various intervals postdose and assayed for concentrations of test compound by reverse-phase HPLC. For compounds **2**, **3**, and **5**, a 0.25 mL blood sample (rat or dog blood) was collected into a glass centrifuge tube containing an internal standard in 1 mL of a toluene/hexane (9:1) mixture. Following mixing on a reciprocal shaker for 10 min and centrifugation, the extract was evaporated to dryness and reconstituted with 50 μL of acetonitrile for HPLC analysis. For test compounds **4** and **6**, whole blood samples (1 mL) were precipitated by addition of 100 μL of 10% zinc sulfate in water and 1 mL of 0.1% trifluoroacetic acid in acetonitrile. Supernatants from the centrifuged samples were transferred to vials for assay or storage at −20 °C. Monkey blood samples of **3** were processed as described below. Freshly drawn blood was collected into a heparinized tube. A 0.5 mL aliquot was extracted with 0.8 mL of toluene/hexane (9:1) containing an internal standard. The organic layer from the centrifuged samples was evaporated to dryness at 40 °C under a stream of nitrogen. The sample was reconstituted in 250 μL of acetonitrile for analysis. Reverse-phase HPLC methods were optimized for the processed samples of each test compound, and internal standards were used.

Bronchoalveolar Lavage Study. Mongrel dogs weighing 10–15 kg were anesthetized with thiamylal (25 mg/kg, iv), and a cannula was inserted into the femoral vein for the collection of blood. A zero-time control blood and BAL sample was taken. After the dog recovered from anesthesia, **2** (solution in 28% hydroxypropyl β-cyclodextrin) or **3** (suspended in 0.75% methyl celluose) was administered 30 mg/kg via a stomach tube in a volume of 1 mL/kg. Twenty-five minutes later, the dog was again anesthetized. Bronchoalveolar lavage (BAL), as described below, was performed, and 3 mL blood samples were removed at various intervals after administration of the test compound. The BAL fluid was stored between 0 °C for analysis.

Each blood sample was transferred to a 3 mL vacutainer tube containing sodium heparin, gently mixed, and stored on ice. The samples were then centrifuged at 300g for 10 min. The plasma was decanted into aliquots and stored below 0 °C. Dog plasma samples (500 μL) were prepared by the addition of 50 μL of an internal standard (6 μg/mL) in acetonitrile. The sample was vortexed, precipitated by the addition of 50 μL of 10% zinc sulfate in water, and vortexed, and 500 μL of 0.1% trifluoroacetic acid in acetonitrile was added. Following vortexing and centrifugation, supernatants were transferred to autosampler vials for assay. Plasma samples were analyzed by reverse-phase HPLC with UV detection.

For BAL, a Tygon endotracheal catheter was inserted via the mouth and a rubber tube (interior catheter, 5 mm o.d.) threaded into the lung until it was unable to be advanced further. Twenty milliliters of sterile saline, followed by 10 of mL air, was slowly instilled into the lung via the interior catheter. The fluid was gently withdrawn from the lung over a period of 1–3 min. Typically, 60–80% of the instilled fluid was recovered. Immediately after each lavage procedure, the interior catheter was removed from the animal and later inserted into another region of the lung at the next time interval. BAL fluid samples were processed as follows. To a 1 mL aliquot of BAL fluid was added 50 μL of 0.2 M sodium phosphate buffer (pH 8.0), 50 μL of a solution containing 3 μg/mL of internal standard in acetonitrile, and 4 mL of ether. After thorough mixing (15 min on a rotary mixer) and phase separation, the ether layer was withdrawn and evaporated to dryness and the residue reconstituted in 200 μL of a 1:1 solution of acetonitrile/water containing 0.05% trifluoroacetic acid. This was subjected to reverse-phase HPLC analysis with UV detection. Urea concentrations in the processed BAL fluid and plasma samples were determined on a Cobas-Fara 2 analyzer (Roche Diagnostics). Concentrations of the test compound in ELF were calculated by multiplying the concentrations of test compound in BAL fluid by the ratio of the urea concentration in plasma over the urea concentration in BAL fluid.

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