Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 16 (2006) 5439–5444
Bioorganic & Medicinal Chemistry Letters

Peptidyl-urea based inhibitors of soluble epoxide hydrolases

Christophe Morisseau,[a] John W. Newman,[a] Hsing-Ju Tsai,[a] Preston A. Baecker[b] and Bruce D. Hammock[a,*]

[a]*Department of Entomology and UCD Cancer Center, University of California, Davis, CA 95616, USA*
[b]*Arête Therapeutics, 3912 Trust Way, Hayward, CA 94545, USA*

Received 15 June 2006; revised 14 July 2006; accepted 17 July 2006

Abstract—We prepared a series of amino acid derived cyclohexyl and adamantyl ureas and tested them as inhibitors of the human soluble epoxide hydrolase, and obtained very potent compounds (K_I = 15 nM) that are >10-fold more soluble than previously described sEH inhibitors. While our lead compound **2** showed low apparent bioavailability in dogs and rats, this series of compounds revealed that sEH inhibitor structures could accept large groups that could lead to better orally available drugs.

Soluble epoxide hydrolase (sEH; EC 3.3.3.2) catalyzes the addition of a water molecule to an epoxide resulting in the corresponding diol.[1] Endogenous substrates for sEH include epoxyeicosatrienoic acids (EETs), which are known for their vasodilatory effects as well as for their anti-inflammatory actions.[2] Hydrolysis of the epoxides by sEH diminished this activity.[3] The inhibition of sEH led to the accumulation of EETs and other lipid epoxides in the organism.[4] Furthermore, sEH inhibition in rodent models can successfully treat hypertension,[4b,5] and inflammatory diseases,[6] as well as protect against renal damage caused by hypertension.[7]

The development in our laboratory of novel, stable, highly potent, and selective inhibitors for sEH[4a] has allowed the elucidation of the biology associated with sEH.[1,2d] Crystal structure determinations show that the urea inhibitors establish hydrogen bonding and salt bridges between the urea function of the inhibitor and residues of the sEH active site.[8] However, these dialkyl-ureas have high crystal lattice energies as indicated by their high melting point, and also have limited solubility in water,[4] likely affecting their in vivo efficacy. While medicinal chemistry approaches were used to increase their inhibitory activity and solubility,[9] the latest compounds reported may not possess physical properties consistent with successful pharmaceutical candidates.[10] Therefore, there remains a need for novel sEH inhibitor structures with improved biological availability and stability for possible future orally administered in vivo drugs. There is also the need for synthetic methods amenable to high throughput combinatorial or parallel methods.

As shown previously, if placed at an appropriate distance from the urea moiety polar groups can be incorporated into one of the alkyl groups of the dialkyl-urea sEH inhibitors without loss of activity.[9] Such modifications give the new inhibitors better solubility and availability; however, only straight alkyl chain derivatives such as 12-(3-adamantyl-ureido)-dodecanoic acid (AUDA; see Fig. 1) were investigated. From the crystal structure of sEH,[8] we observed that while the catalytic tunnel is restricted around the catalytic residues it enlarges as one moves away from the site of reaction to yield relatively large cavities. These structural features suggest that the enzyme could accommodate branches on the main alkyl chain. To evaluate a wide variety of side chains, we used a semi-combinatorial approach. Because amino acids are simple bifunctional synthons with a wide variety of side chains, mono- and di-peptidic derivatives of urea based sEH inhibitors were synthesized (see Fig. 1) and their effects on sEH inhibition investigated.

We chose to modify (3-cycloalkyl-ureido)-alkanoic acids because these compounds are poor inhibitors of sEH when the alkyl chain is less than 6-carbons in length,[9]

Keywords: Cardiovascular diseases; Soluble epoxide hydrolase inhibitors.
* Corresponding author. Tel.: +1 530 752 7519; fax: +1 530 752 1537; e-mail: bdhammock@ucdavis.edu

0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.07.073

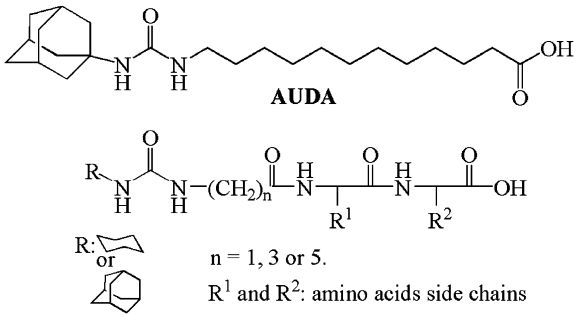
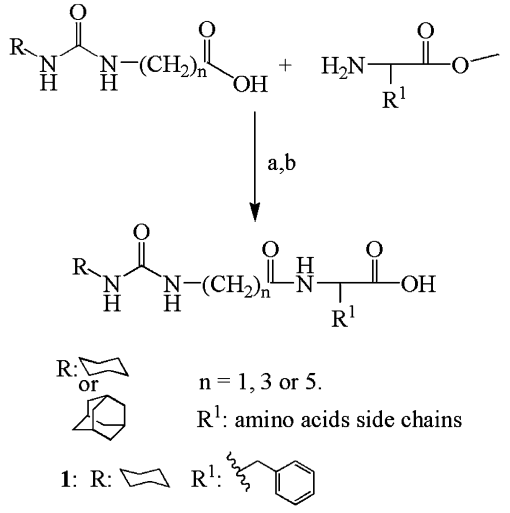


Figure 1. General structure of amino acid derived urea based inhibitors for sEH.

and because we previously showed that ester and amide derivatives of these compounds could yield potent sEH inhibitors. Furthermore, to make the peptidic bond, we select reactants, such as 1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide (EDCI) or *N*,*N*-dimethyl-4-aminopyridine (DMAP), that are not inhibitors of sEH themselves nor are their reaction products, such as 1-ethyl-3-(3-dimethylamino)-propyl urea. Therefore, any inhibition observed was derived from the targeted peptidic derivatives, allowing us to carry out reactions on an analytical scale (10 μmol), monitor the reactions with LC–MS, and test them for inhibition without the need to purify the products (Scheme 1). The presence of the desired products was confirmed by positive mode electrospray LC–MS in all cases reported. The yield of the reaction was estimated by quantifying the remaining (3-cycloalkyl-ureido)-alkanoic acid against a 4-point calibration curve. With the exception of cysteine and lysine, which formed secondary products and gave erratic yields and thus are not included herein, we found reaction yields of >95%. Dimethylformamide was added to the reaction mixtures to give target compound's concentration of 10 mM. These solutions were used to measure inhibitor potency directly on purified recombinant mouse and human sEH,[11] using a spectrophotometric substrate in a 96-well microplate assay.[12]

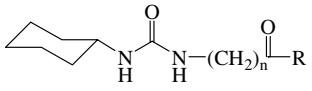


Scheme 1. Reagents and conditions: (a) EDCI, DMAP, CH_2Cl_2, 25 °C, 12 h, 95–99%; (b) KOH, DMF, H_2O, 25 °C, 12 h, 100%.

We first optimized the distance between the urea function and the first amino acid using a subset of amino acids and three (3-cyclohexyl-ureido)-alkanoic acids of different length (Table 1). For the mouse sEH, good inhibition is obtained for both inhibitor series with the 4- and 6-carbon carboxylate tails, suggesting that a 3-carbon spacer (i.e., $n = 3$) is sufficient for this enzyme. Compared to the free acid (R = OH), the histidyl derivative gives the best inhibition potency (smallest IC_{50}) for both chain length. Interestingly, overall for the human sEH the 4-carbon chain derivatives ($n = 3$) gave better inhibition results than shorter or longer chains. Similar results were observed for the methyl esters of the three (3-cyclohexyl-ureido)-alkanoic acids tested (R = OCH_3).[13] The crystal structure of the human sEH with these acids showed that the inhibitor with the 4-carbon carboxylate tail is oriented the opposite way in the active site, underlying that structure–activity relationships are more complex for the human sEH.[13] For example, increasing the chain length from 4 to 6 carbons resulted in higher IC_{50}s for all the amino acid derivatives, except for phenylalanine that is decreased by an order of magnitude. Furthermore, compared to the free acid (R = OH), only the phenylalanine derivative with $n = 5$ gave a significantly improved inhibition potency.

Therefore, in a second experiment to optimize the nature of the amino acid residues, we made amino acid

Table 1. Optimization of the alkyl chain length between the urea function and the first amino acid for optimal inhibition



n	R	Mouse sEH IC_{50}[a] (μM)	Human sEH IC_{50}[a] (μM)
1	OH	>100	>100
1	OCH_3	33	70
1	Arginine	>100	>100
1	Glutamate	>100	>100
1	Histidine	36	>100
1	Phenylalanine	18	>100
1	Valine	>100	>100
3	OH	1.9	36
3	OCH_3	0.33	6.2
3	Arginine	1.0	25
3	Glutamate	1.7	24
3	Histidine	0.05	30
3	Phenylalanine	1.6	21
3	Valine	1.4	17
5	OH	24	>100
5	OCH_3	0.11	3.2
5	Arginine	0.23	>100
5	Glutamate	51	>100
5	Histidine	0.06	>100
5	Phenylalanine	0.15	2.7
5	Valine	0.56	41
AUDA		0.05	0.1

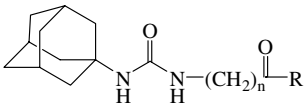
[a] IC_{50}s were determined as described using a spectrophotometric assay.[4a,12].

derivatives with $n = 3$ for the murine sEH and $n = 5$ for the human sEH. In addition, due to our previous discovery that for the human sEH the replacement of the cyclohexyl by 1-adamantyl resulted in $\geqslant$10-fold increase in inhibition, we also prepared derivatives of (3-adamantyl-ureido)-alkanoic acids (Table 2). Other than the catalytic residues, the active site of both the murine and human sEHs are quite hydrophobic.[1,8] Thus, one would expect that hydrophobic or aromatic amino acids would yield good inhibitors. While we observed such effects for the human sEH, the increase in inhibition for the murine sEH is not clearly linked to the hydrophobicity of the side chain. Furthermore, one would expect that polar side chains would yield poor sEH inhibitor as observed for the human sEH. However, for the murine enzyme, positively charged residues (i.e., the basic amino acids Arg and His) gave surprisingly good inhibition, with the best inhibition obtained with the histidine conjugate. Because the active site of sEH, besides being hydrophobic, is also relatively basic,[1] one could hypothesize that only uncharged residues would bind to the enzyme. This hypothesis is supported by the fact that histidine, which has a 3 pH unit lower pK_a than arginine, yields a far better inhibitor for the mouse sEH. Alternatively, one could envision π-cation interactions between the cationic side chain of the inhibitors and the aromatic residues that are lining the catalytic cavity of sEH.[1] This kind of interaction is very common in biological systems.[14]

Previously, while investigating largely hydrophobic inhibitors, we observed reasonable agreement between the two enzymes with regard to the structure–activity relationships among inhibitors.[4,9] As seen in Table 2, this is not the case here, indicating that for this structural series, it is important to optimize inhibitor structures with the targeted species (human) enzyme. Furthermore, it suggests that the series of inhibitors reported herein probably bind differently for each enzyme studied. While we obtained a very potent inhibitor for the murine sEH (the histidine derivative of the 4-carbon carboxylate), less than optimal inhibition was obtained for the human sEH. Toward improving inhibition potency for this latter enzyme, we tested the effect of adding a second amino acid on the phenylalanine derivatives. Therefore, we prepared and purified 2-[6-(3-cyclohexyl-ureido)-hexanoylamino]-3-phenyl-propionic acid **1**,[15] and following a procedure similar to the one described in Scheme 1, we prepared a series of amino acid derivatives of this compound on analytical scale. We obtained reaction yields >95% and the products were tested for their ability to inhibit the human sEH (Table 3). Compared to the free acid (R = OH), the presence of polar side chains did not enhance the potency of the inhibitor, while the presence of either aromatic or hydrophobic side chains reduced the IC_{50}s. These results agreed with the observed hydrophobicity of the human sEH catalytic cavity.[1,8] The best inhibition was obtained with isoleucine, phenylalanine, proline, tryptophan or valine derivatives, suggesting that the human sEH can fit compounds of various sizes as long as they are hydrophobic in nature.

Based on the results obtained at analytical scale (Tables 1–3), we prepared *N*-[6-(3-adamantyl-ureido)-hexanoyl]-phenylalanyl-tryptophan **2**.[16] We then determined the

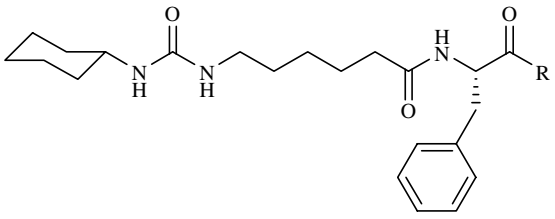
Table 2. Inhibition of mouse and human sEH by mono-amino acid derivatives of *n*-(3-adamantyl-ureido)-alkanoic acid



R	Mouse sEH $n = 3$ IC_{50} [a] (μM)	Human sEH $n = 5$ IC_{50} [a] (μM)
OH	>100	>100
OCH_3	0.10	1.8
Alanine	>100	>100
Arginine	16	>100
Aspartate	>100	>100
Glutamate	>100	>100
Glycine	>100	>100
Histidine	0.05	>100
Isoleucine	1.3	>100
Leucine	15	>100
Methionine	4.8	>100
Phenylalanine	0.8	2.7
Proline	82	>100
Serine	47	>100
Threonine	33	>100
Tryptophan	8.4	41
Tyrosine	18	80
Valine	30	55

[a] IC_{50}s were determined as described using a spectrophotometric assay. [4a,12].

Table 3. Inhibition of human sEH by mono-amino acid derivatives of **1**



R	IC_{50}[a] (μM)
OH (**1**)	1.0
Alanine	0.26
Arginine	2.1
Aspartate	>100
Glutamate	>100
Glycine	1.5
Histidine	5.5
Isoleucine	0.13
Leucine	0.88
Methionine	0.87
Phenylalanine	0.10
Proline	0.10
Serine	2.4
Threonine	1.3
Tryptophan	0.10
Tyrosine	0.23
Valine	0.10

[a] IC_{50}s were determined as described using a spectrophotometric assay.[4a,12]

inhibition constant of compound **2** for the human sEH (Fig. 2) and found a K_I of 15 ± 3 nM (n = 3). This is in the same order of magnitude as previously reported sEH inhibitors,[4,9] confirming that **2** is a very potent human sEH inhibitor in vitro, as suggested by analytical scale results. Our ultimate objective is to obtain novel sEH inhibitors with improved solubility, higher biological availability, and in vivo stability. Therefore, we next tested the peptidyl-urea sEH inhibitor solubility in sodium phosphate buffer (100 mM, pH 7.4) as previously described.[9a] All prepared amino acid derivatives had solubilities >500 μM. This is ⩾10-fold the solubility of the corresponding compounds with a straight carboxylate chain, such as AUDA,[9] suggesting that the peptidyl-urea based inhibitors could be given in drinking water, and should dissolve readily from tablets. Finally, we tested the oral bioavailability of **2** in a canine model (Table 4). The presence of amino acids in some compounds can greatly increase their bioavailability due to the presence of di/tri-peptide specific transporters in the gut.[18] Thus, one would expect that compounds like **2** might be readily bioavailable. Surprisingly, as shown in Table 4, small amounts of **2** were detected in dog plasma. Furthermore, expected metabolites for **2**, 6-(3-adamantyl-ureido)-hexanoic acid and its phenylalanine derivatives, were not detected, suggesting that **2** is poorly absorbed from the gut or rapidly metabolized by an

Table 4. Pharmacokinetic profile data for compounds **2** and AUDA as obtained via oral dosing in a canine model

	Time (min)	**2**	AUDA
Plasma concentration (nM)	0	0.00	0.00
	15	0.62	6.97
	30	0.82	20.43
	60	0.33	30.69
	120	0.00	26.31
	180	0.00	13.39
	240	0.00	6.12
	300	0.00	1.61
	360	0.00	1.17
	480	0.00	0.86
	1440	0.00	0.00
AUC[a] (×10^4 nM min)		<0.01	0.31

[a] Area under the curve, estimated from a plot of inhibitor plasma concentration (nM) versus time (minutes) following an oral dose of 0.3 mg/Kg of the indicated compounds in 6 mL of tristerate.[17]

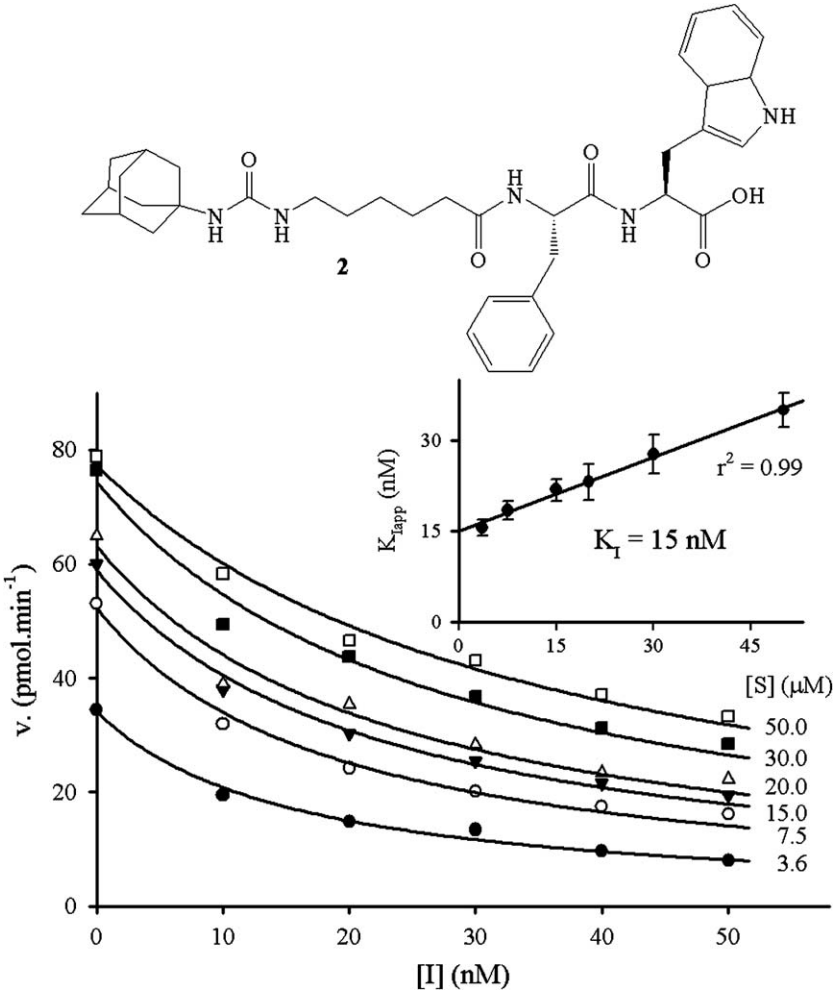


Figure 2. Determination of the K_I of **2** for the human sEH. The enzyme ($[E]_{final}$ = 3 nM) diluted in sodium phosphate buffer (100 mM, pH 7.4) was incubated for 5 min at 30 °C with the inhibitor ($[I]_{final}$ = 0–50 nM), before the addition of substrate (tDPPO; $[S]_{final}$ = 3.6–50 μM). The inhibition constant (K_I) was determined as described.[4a,9a]

alternate route, either within the gut or the body. Regardless, as measured by the AUCs of the parent molecules (Table 4), compound **2** appears far less bioavailable than AUDA, our reference inhibitor.[9a] Similar results were obtained in rats.

In conclusion, we have reported a new series of potent peptidyl-urea based sEH inhibitors which are substantially more water soluble than previously described sEH inhibitors. While our lead compound, **2**, showed low apparent bioavailability, this series of compounds revealed that sEH inhibitor structures could accept large groups if placed at an appropriate distance from the central pharmacophore. Further exploration within this area of the molecular structure could yield inhibitors with higher biological availability and stability, leading the way to orally available drugs. Furthermore, these findings suggest that refinements of the sEH inhibitor structure that depart from the simple, flexible aliphatic backbone characteristic of the AUDA-like compounds should be performed using human sEH. Moreover, it is clear that the procedure described here offers a rapid route to synthesize a variety of sEHI with both natural and non-natural amino acids by both liquid- and solid-phase procedures. One also can reverse the polar group on the urea side of the molecule using an amine linker to have the peptide chain serve as the N- rather than C-terminal equivalent. The structure and polarity of the peptide chain can be altered with amides and esters of free amino and carboxylic acid functionalities or by derivatives commonly used to alter the structure and polarity of the NH bond.

Acknowledgments

This study was supported in part by NIEHS Grant ES02710, NIEHS Superfund Grant P42 ES04699, NIEHS Center Grant P30 ES05707, and NHLBI STTR Grant R41 HL078016.

References and notes

1. Morisseau, C.; Hammock, B. D. *Annu. Rev. Pharmacol. Toxicol.* **2005**, *45*, 311.
2. (a) Harder, D. R.; Campbell, W. B.; Roman, R. J. *J. Vasc. Res.* **1995**, *32*, 79; (b) Campbell, W. B.; Gebremedhin, D.; Pratt, P. F.; Harder, D. R. *Circ. Res.* **1996**, *78*, 415; (c) Node, K.; Huo, Y.; Ruan, X.; Yang, B.; Spiecker, M.; Ley, K.; Zeldin, D. C.; Liao, J. K. *Science* **1999**, *285*, 1276; (d) Newman, J. W.; Morisseau, C.; Hammock, B. D. *Prog. Lipid Res.* **2005**, *44*, 1.
3. Spector, A. A.; Fang, X.; Snyder, G. D.; Weintraub, N. L. *Prog. Lipid Res.* **2004**, *43*, 55.
4. (a) Morisseau, C.; Goodrow, M. H.; Dowdy, D.; Zheng, J.; Greene, J. F.; Sanborn, J. R.; Hammock, B. D. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8849; (b) Yu, Z.; Xu, F.; Huse, L. M.; Morisseau, C.; Draper, A. J.; Newman, J. W.; Parker, C.; Graham, L.; Engler, M. M.; Hammock, B. D.; Zeldin, D. C.; Kroetz, D. L. *Circ. Res.* **2000**, *87*, 992.
5. (a) Imig, J. D.; Zhao, X.; Capdevila, J. H.; Morisseau, C.; Hammock, B. D. *Hypertension* **2002**, *39*, 690; (b) Jung, O.; Brandes, R. P.; Kim, I.; Schweda, F.; Schmidt, R.; Hammock, B. D.; Busse, R.; Fleming, I. *Hypertension* **2005**, *45*, 759; (c) Loch, D.; Hoey, A.; Morisseau, C.; Hammock, B. D.; Brown, L; *Cell. Biochem. Biophys.* in press.
6. (a) Schmelzer, K. R.; Kubala, L.; Newman, J. W.; Kim, I. H.; Eiserich, J. P.; Hammock, B. D. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 9772; (b) Smith, K. R.; Pinkerton, K. E.; Watanabe, T.; Pedersen, T. L.; Ma, S. J.; Hammock, B. D. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 2186.
7. (a) Zhao, X.; Yamamoto, T.; Newman, J. W.; Kim, I. H.; Watanabe, T.; Hammock, B. D.; Stewart, J.; Pollock, J. S.; Pollock, D. M.; Imig, J. D. *J. Am. Soc. Nephrol.* **2004**, *15*, 1244; (b) Imig, J. D.; Zhao, X.; Zaharis, C. Z.; Olearczyk, J. J.; Pollock, D. M.; Newman, J. W.; Kim, I. H.; Watanabe, T.; Hammock, B. D. *Hypertension* **2005**, *46*, 975.
8. (a) Argiriadi, M. A.; Morisseau, C.; Goodrow, M. H.; Dowdy, D. L.; Hammock, B. D.; Christianson, D. W. *J. Biol. Chem.* **2000**, *275*, 15265; (b) Gomez, G. A.; Morisseau, C.; Hammock, B. D.; Christianson, D. W. *Biochemistry* **2004**, *43*, 4716.
9. (a) Morisseau, C.; Goodrow, M. H.; Newman, J. W.; Wheelock, C. E.; Dowdy, D. L.; Hammock, B. D. *Biochem. Pharmacol.* **2002**, *63*, 1599; (b) Kim, I. H.; Morisseau, C.; Watanabe, T.; Hammock, B. D. *J. Med. Chem.* **2004**, *47*, 2110; (c) Kim, I. H.; Heirtzler, F. R.; Morisseau, C.; Nishi, K.; Tsai, H. J.; Hammock, B. D. *J. Med. Chem.* **2005**, *48*, 3621.
10. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Deliv. Rev.* **2001**, *46*, 3.
11. (a) Grant, D. E.; Storms, D. H.; Hammock, B. D. *J. Biol. Chem.* **1993**, *268*, 17628; (b) Beetham, J. K.; Tian, T.; Hammock, B. D. *Arch. Biochem. Biophys.* **1993**, *305*, 197; (c) Wixtrom, R. N.; Silva, M. H.; Hammock, B. D. *Anal. Biochem.* **1988**, *169*, 71.
12. Dietze, E. C.; Kuwano, E.; Hammock, B. D. *Anal. Biochem.* **1994**, *216*, 176.
13. Gomez, G. A.; Morisseau, C.; Hammock, B. D.; Christianson, D. W. *Protein Sci.* **2006**, *15*, 58.
14. Meyer, E. A.; Castellano, R. K.; Diederich, F. *Angew. Chem. Int. Ed.* **2003**, *42*, 1210.
15. 2-[6-(3-cyclohexyl-ureido)-Hexanoylamino]-3-phenyl-propionic acid **1** was prepared following the method described on Scheme 1, starting with 0.2 mmol of reagents. The target product was purified by reverse phase (C-18) chromatography with a 60:40 methanol/water solvent mixture. We obtained 70 mg (Yield: 93%) of **1** as a white solid; mp 141–142 °C. ESIMS $m/z = 404.26$ $(M+H)^+$; ^1H NMR (300 MHz, DMSO/TMS): δ 12.62 (s, 1H, COOH), 8.07 (d, $J = 8.4$ Hz, 1H, NH amide), 7.24–7.13 (br m, 5H, phenyl), 5.61 (m, 2H, NH urea), 4.38 (m, 1H, C_2) 3.30 (m, 1H, CH cyclohexyl), 3.04–2.75 (br m, 4H, C_3 and $C_{6'}$), 1.99 (t, $J = 7.4$ Hz, 2H, $C_{2'}$), 1.70–1.00 (br m, 16H, CH_2s from cyclohexyl and $C_{3'-5'}$) ppm.
16. *N*-[6-(3-adamantyl-ureido)-hexanoyl]-phenylalanyl-tryptophan **2** was obtained by reacting 6-(3-adamantyl-ureido)-hexanoic acid with the commercially available phenylalanyl-tryptophan dipeptide following the method described on Scheme 1, starting with 0.2 mmol of reagents. The target product was purified by reverse phase (C-18) chromatography with a 70:30 methanol/water solvent mixture. We obtained 45 mg (Yield: 35%) of **2** as a light tan colored solid; mp 115–120 °C (dec.); ESIMS $m/z = 642.60$ $(M+H)^+$; ^1H NMR (300 MHz, DMSO/TMS): δ 12.70 (br s, 1H, COOH), 10.86 (d, $J = 8.8$ Hz, 1H, NH indol), 8.25 (dd, $J = 7.6$ Hz, 1H, NH amide), 7.90 (dd, $J = 7.6$ Hz, 1H, NH amide), 7.55 (t, $J = 7.1$ Hz, 1H, CH indol), 7.34–6.92 (br m, 9H, phenyl and indol), 5.56 (m, 1H, NH urea), 5.42 (m, 1H, NH

urea), 4.62–4.38 (br m, 2H, C_2 and $C_{2'}$), 3.23–2.68 (br m, 6H, C_3, $C_{3'}$ and $C_{6''}$), 2.06–1.45 (br m, 17H, $C_{2''}$ and adamantyl), 1.40–0.93 (br m, 6H, $C_{3''-5''}$) ppm.

17. Selected soluble epoxide hydrolase inhibitors were prepared at 6 mg/mL in triglycerides. Each dog was dosed orally with three different inhibitors per experiment (dose = 0.3 mg/kg per inhibitor). Plasma samples were collected at 0, 15, 30, 60, 120, 180, 240, 300, 360, 480, and 1440 min, and analyzed by LC/MS/MS (Quattro Premier; Micromass, MA). The pharmacokinetic parameters were calculated with WinNonlin 5.0 (Pharsight, CA).
18. Oh, D. M.; Han, H. K.; Williamson, R. M.; Bigge, C. F.; Amidon, G. L.; Stewart, B. H.; Surendran, N. *J. Pharm. Sci.* **2002**, *12*, 2579.