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Synthesis and biological evaluation of sorafenib- and regorafenib-like sEH inhibitors

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ABSTRACT

To reduce the pro-angiogenic effects of sEH inhibition, a structure–activity relationship (SAR) study was performed by incorporating structural features of the anti-angiogenic multi-kinase inhibitor sorafenib into soluble epoxide hydrolase (sEH) inhibitors. The structural modifications of this series of molecules enabled the altering of selectivity towards the pro-angiogenic kinases C-RAF and vascular endothelial growth factor receptor-2 (VEGFR-2), while retaining their sEH inhibition. As a result, sEH inhibitors with greater potency against C-RAF and VEGFR-2 were obtained. Compound **4** (*t*-CUPM) possesses inhibition potency higher than sorafenib towards sEH but similar against C-RAF and VEGFR-2. Compound **7** (*t*-CUCB) selectively inhibits sEH, while inhibiting HUVEC cell proliferation, a potential anti-angiogenic property, without liver cancer cell cytotoxicity. The data presented suggest a potential rational approach to control the angiogenic responses stemming from sEH inhibition.

Soluble epoxide hydrolase (sEH, EC 3.3.2.10) is an enzyme that catalyzes the hydrolysis of epoxy fatty acids (EpFAs), including epoxyeicosatrienoic acids (EETs), to their less bioactive corresponding diols, such as dihydroxyeicosatrienoic acids (DHETs).[1] EETs possess anti-inflammatory,[2] anti-hypertensive[3] and analgesic properties.[4] Therefore, sEH has been a therapeutic target for numerous indications such as inflammation, pain, hypertension, atherosclerosis, pulmonary diseases, renal end-organ damage and diabetes.[2,5] EETs have also long been known as a pro-angiogenic factor particularly in the presence of vascular endothelial growth factor (VEGF).[6–9]While this is an attractive property during development and in certain cases such as wound healing,[10] studies suggested that EETs can promote cancer progression.[11] For example, Panigrahy et al. recently demonstrated their contribution to tumor growth and metastasis.[12]

Small-molecule kinase inhibitors[13] such as sorafenib and regorafenib, are generally flat, aromatic molecules which mimic the adenine group of ATP which binds to a highly conserved ATP-binding pocket to inhibit kinase function.[14] Sorafenib is a bi-aryl urea which was originally developed as a therapeutic agent targeting the pro-angiogenic kinase, C-RAF.[15] However, the structural features of sorafenib demonstrated multi-kinase inhibitory activities with potent anti-angiogenic properties via the inhibition of pro-angiogenic receptor tyrosine kinases (RTKs), such as the VEGFR-2.[16] As a result, sorafenib displays multi-inhibitory action in the RAF/MEK/ERK pathway and RTKs to combat tumor angiogenesis. It is currently used for the treatment of hepatocellular carcinoma (HCC)[17] and renal cell carcinoma (RCC).[18]

Based on the structural similarity between sorafenib and one class of sEH inhibitors (Fig. 1A), we tested and found that sorafenib (Nexavar®, BAY 43-9006), also displays potent inhibitory activity against sEH (human sEH IC_{50} = 12 ± 2 nM).[19] As expected, sorafenib exhibits similar anti-inflammatory responses as conventional sEH inhibitors in lipopolysaccharide-induced inflammation murine model.[19] In addition, we recently found that regorafenib (Stivarga™, BAY 73-4506), a second generation derivative of sorafenib for the treatment of colon or rectal cancer, is a more potent sEH inhibitor (human sEH IC_{50} = 0.5 ± 0.1 nM). Data on clinical blood levels from sorafenib-treated patients suggest that the sEH should be significantly inhibited, which may be beneficial during cancer treatment with sorafenib by reducing renal toxicity, hypertension and pain,[2] often associated with pan-kinase anti-angiogenic agents.[20]

Abbreviations: HCC, hepatocellular carcinoma; RCC, renal cell carcinoma; GI_{50}, concentration required to inhibit cell growth by 50%; EpFAs, epoxygenated fatty acids; *t*-CUPM, *trans*-4-{4-[3-(4-chloro-3-trifluoromethyl-phenyl)-ureido]-cyclohexyloxy}-pyridine-2-carboxylic acid methylamide; *t*-CUCB, *trans*-3-{4-[3-(4-chloro-3-trifluoromethyl-phenyl)-ureido]-cyclohexyloxy}-benzoic acid; *t*-AUCB, *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid; *t*-TUCB, *trans*-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid.

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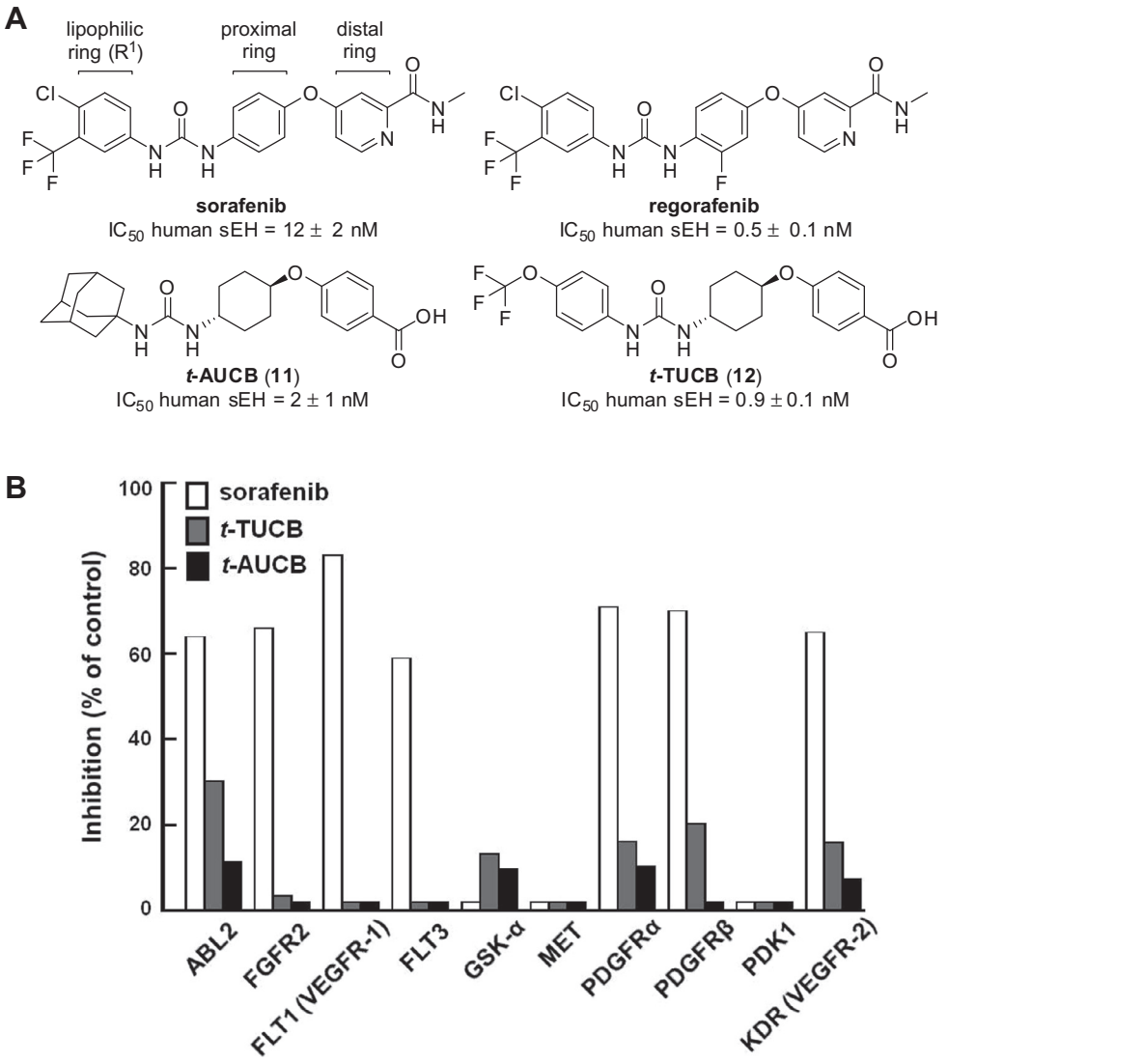


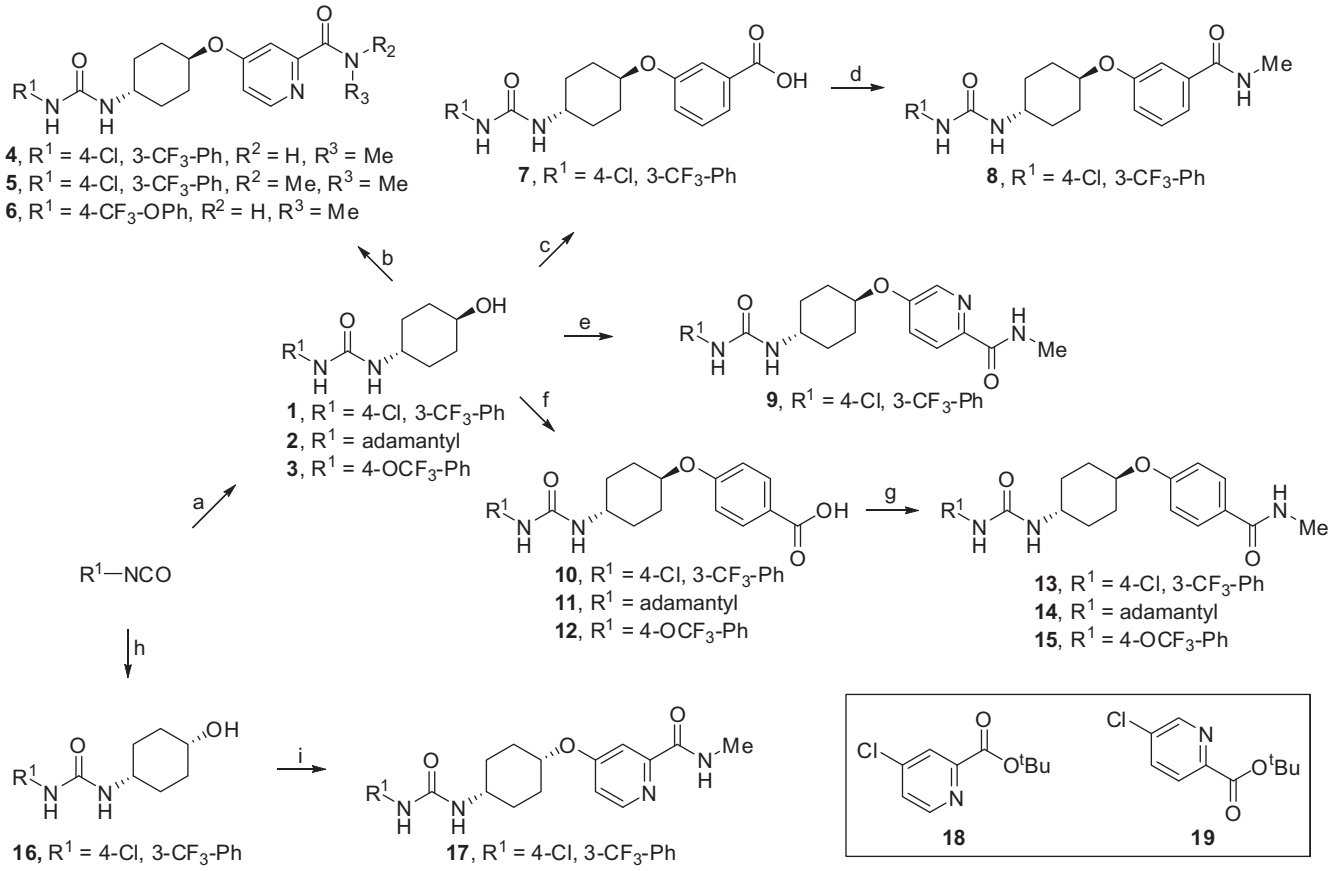
Figure 1. (A) Structures of sorafenib and common sEH inhibitors. (B) Selectivity of sorafenib, *t*-AUCB (**11**) and *t*-TUCB (**12**) at 10 μM concentration against 10 recombinant kinases.

On the other hand, urea-based sEH inhibitors {*t*-AUCB (**11**) and *t*-TUCB (**12**)} that are structurally related to sorafenib (Fig. 1A), did not display the cytotoxicity, growth inhibition, or apoptotic effects of sorafenib in RCC cell lines in our previous study.[19] The first question asked was whether lack of antiproliferative effect in RCC cells was reflected in their kinase inhibitory activities. We screened *t*-AUCB and *t*-TUCB against a panel of known sorafenib targets and found that these sEH inhibitors display no significant multi-kinase inhibition at 10 μM concentration (Fig. 1B). This confirmed that there is a distinct structure–activity relationship (SAR) between sorafenib and structurally related urea-based sEH inhibitors against kinase inhibition, and probably explains the lack of antiproliferative effects of *t*-AUCB and *t*-TUCB in RCC cells. Alternatively, it raises the question whether structural modifications of urea-based sEH inhibitors could yield altered kinase inhibition properties towards sorafenib's primary anti-angiogenic targets, C-RAF and VEGFR-2, in order to balance the potential adverse effect stemming from the angiogenic responses of EETs resulting from high doses of sEH inhibitors.[12]

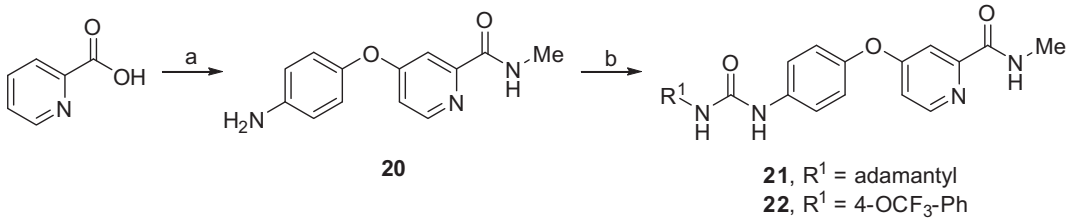
Herein, we report SAR study of hybrid compounds between sorafenib and conventional urea-based sEH inhibitors. To this end, we investigated whether these structural modifications could maintain sEH inhibition while altering kinase inhibitory activities (C-RAF and VEGFR-2, the two primary kinase targets of sorafenib believed to yield its anti-angiogenic properties) and cellular functions. The cellular responses of the compounds in this small library of sorafenib-like sEH inhibitors were determined in both endothelial HUVEC cells as an initial measurement of anti-angiogenesis, and two epithelial liver cell carcinoma cell lines (HepG2 and Huh-7) as an initial measurement of cytotoxicity.

The synthetic routes of urea-based sEH inhibitors containing the cyclohexyl group which are described herein have previously been disclosed.[21] The preparation of urea compounds **4**–**15**, **17**, **21** and **22** is depicted in Schemes 1 and 2. Briefly geometric isomers (*trans*- and *cis*-) were made starting from the corresponding alcohols, **1**–**3** and **16**, respectively, using Mitsunobu reaction we published previously.[22] Distal rings in compounds **4**–**15**, and **17** were then installed by nucleophilic aromatic substitution (S_NAr) reaction.[23] The compounds **21** and **22** were synthesized starting from picolinic acid according to the procedure used for the preparation of sorafenib.[24]

Our previous SAR study with sEH inhibitors such as *t*-AUCB reveal that replacement of the cyclohexyl group, by rigid groups such as a phenyl or an acetylenyl group, results in equal to or slightly



Scheme 1. Reagents and conditions: (a) *trans*-4-aminocyclohexanol, DMF, rt, 12 h; (b) (i) KO*t*-Bu, **18**, THF, 0 °C to rt, overnight, (ii) (a) 30% TFA in DCM, 6 h, (b) PyBOP, R_2R_3NH, DMF, rt, overnight; (c) (i) NaH, 3-fluorobenzonitrile, DMF, rt, 1 d, (ii) 6 N NaOH, EtOH, 90 °C, 1 d; (d) PyBOP, $MeNH_2$, DMF, rt, 12 h; (e) (i) KO*t*-Bu, **19**, THF, 0 °C to rt, overnight, (ii) (a) 30% TFA in DCM, 6 h, (b) PyBOP, $MeNH_2$, DMF, rt, 12 h; (f) (i) NaH, 4-fluorobenzonitrile, DMF, rt, 12 h, (ii) 6 N NaOH, EtOH, 90 °C, 1 d; (g) PyBOP, $MeNH_2$, DMF, rt, 12 h; (h) (i) *cis*-4-nitrobenzoic acid 4-aminocyclohexyl ester,[22] 4-chloro-3-(trifluoromethyl)phenyl isocyanate, Et_3N, DMF, rt, 12 h, (ii) 1 N NaOH, THF, rt, 12 h; (i) (a) KO*t*-Bu, **18**, THF, 0 °C to rt, overnight, (b) 50% TFA in DCM, 6 h, and (c) PyBOP, $MeNH_2$, DMF, rt, 12 h.



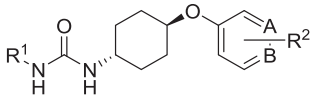
Scheme 2. Reagents and conditions: (a)[24] (i) $SOCl_2$, DMF, 80 °C, 16 h, (ii) $MeNH_2$, MeOH, THF, 0 °C, 4 h, (iii) *p*-hydroxyaniline, KO*t*-Bu, K_2CO_3, DMF, 80 °C, 6 h; (b) R^1-NCO, DMF, rt, 12 h.

less sEH inhibitory activity.[22,25] We hypothesized that the cyclohexyl group should be a bioisostere of the phenyl group due to its size and similar lipophilicity. Therefore we first modified the 'proximal group' in sorafenib (Fig. 1A). Two geometric isomers *t*-CUPM (**4**, *trans*) and **17** (*cis*), were initially obtained replacing the phenyl ring of sorafenib by the 1,4-cyclohexyl moiety as the proximal ring.

As seen in Table 1, the high potency of *t*-CUPM was surprising because kinase inhibitors utilize a phenyl or planar heterocyclic ring to maximize lipophilic interaction with residues at ATP-binding site conserved throughout the kinome. Although the phenyl group in sorafenib is proposed to interact with three aromatic residues—Trp530 of the hinge region, Phe582 at the end of the catalytic loop, and Phe594 of the DFG-out ATP-binding region found in the protein kinase C-RAF[26]—we observed similar activity towards C-RAF inhibition with the *trans*-isomer *t*-CUPM. Both *cis*- and *trans*-isomers improved sEH inhibition by about 20-fold, but the *cis*-isomer **17** had much poorer C-RAF kinase inhibition compared to the *trans*-isomer *t*-CUPM, directing our investigation towards only the *trans*-isomers.

As previously observed in the original SAR study with sorafenib,[27] conversion of a methylamide in *t*-CUPM to a dimethylamide **5** on the distal group (Fig. 1A) causes loss in C-RAF inhibition due to the loss of H-bonding. Interchange of a methylaminocarbonyl group and a nitrogen atom in pyridyl group of *t*-CUPM led only to 2.3-fold less activity against C-RAF, suggesting that the methyl amide group in the pyridyl group of the compound **9** still makes contact with residues that interact with sorafenib. Shifting the

Table 1
IC_{50} values of *N*-aryl, *N*′-cyclohexyl ureas **4**–**15**, and **17** for sEH and C-RAF



	R1	R^2		A	B	sEH	C-Raf
						IC_{50} (nm)	
Sorafenib[a]						12 ± 2	45 ± 5
Regorafenib[a]						0.5 ± 0.1	ND[b]
Sunitinib[19]						>10,000[19]	>10,000
t-CUPM (**4**)	4-Cl, 3-CF_3-Ph	3-C(=O)NHMe	*trans*	C	N	0.5 ± 0.1	75±5
5	4-Cl, 3-CF_3-Ph	3-C(=O)NMe_2	*trans*	C	N	0.5 ± 0.1	>10,000
6	4-OCF_3-Ph	3-C(=O)NHMe	*trans*	C	N	0.5 ± 0.1	>10,000
t-CUCB (**7**)	4-Cl, 3-CF_3-Ph	3-CO_2H	*trans*	C	C	8.6 ± 1.0	>10,000
8	4-Cl, 3-CF_3-Ph	3-C(=O)NHMe	*trans*	C	C	1.0 ± 0.1	293 ± 10
9	4-Cl, 3-CF_3-Ph	4-C(=O)NHMe	*trans*	N	C	0.5 ± 0.1	175 ± 20
10	4-Cl, 3-CF_3-Ph	4-CO_2H	*trans*	C	C	0.5 ± 0.1	4300 ± 400
t-AUCB (**11**)	Adamantyl	4-CO_2H	*trans*	C	C	1.5 ± 0.5	>10,000
t-TUCB (**12**)	4-OCF_3-Ph	4-CO_2H	*trans*	C	C	0.9 ± 0.1	>10,000
13	4-Cl, 3-CF_3-Ph	4-C(=O)NHMe	*trans*	C	C	0.5 ± 0.1	340±40
14	Adamantyl	4-C(=O)NHMe	*trans*	C	C	0.5 ± 0.1	>10,000
15	4-OCF_3-Ph	4-C(=O)NHMe	*trans*	C	C	0.5 ± 0.1	>10,000
17	4-Cl, 3-CF_3-Ph	3-C(=O)NHMe	*cis*	C	N	0.5 ± 0.1	1500 ± 200
TPPU (**1770**)[19]						3.7 ± 1.0[19]	>10,000
TUPS (**1709**)[19]						2.9 ± 1.0[19]	>10,000

[a] See the Supplementary data.
[b] ND = not determined.

position of the nitrogen atom in the pyridyl group or its removal has little effect on sEH activity. In contrast to C-RAF inhibition, the terminal carboxylic acid is not critical for potency on the distal ring of sEH inhibitors. When present the carboxylic acid is of similar activity on sEH to its esters and amides.[22,28] Thus avoiding the methylaminocarbonyl group provides another way to block kinase activity among sEH inhibitors structurally related to sorafenib.[29]

We then conversely left the proximal phenyl group intact—identical to that in sorafenib—and subsequently tested how replacement of the lipophilic group (R^1, (Fig. 1A) in sorafenib by groups found in *t*-AUCB and *t*-TUCB affected C-RAF kinase inhibition. While the adamantyl group (**21**, Table 2) is not compatible for C-RAF inhibition, surprisingly, *p*-trifluoromethoxyphenyl group (**22**) became slightly more potent than sorafenib itself, suggesting that the lipophilic group (R^1, Tables 1 and 2) is not restricted to 4-Cl, 3-CF_3-phenyl moiety for C-RAF inhibition. This indicates that the lack of inhibition of C-RAF in *t*-TUCB is more likely due to the lack of the amide group and the pyridine ring as the distal group than to the presence of the lipophilic group (R^1).

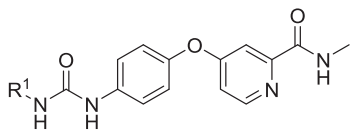
With these data in hand, we then converted the free carboxylic acid of sEH inhibitors *t*-CUCB (**7**), **10**, *t*-AUCB and *t*-TUCB to the corresponding methylamide group as in compounds **8**, and **13**–**15**, respectively. Except for compounds **8** and **13** which have the same lipophilic group (R^1) as seen in sorafenib, other compounds **14** and **15** lost C-RAF inhibitory activity.

However replacement of 4-chloro-3-trifluoromethoxy-phenyl group (R^1) in *t*-CUPM by 4-trifluoromethoxyphenyl group (R^1) with a *trans*-cyclohexyl proximal ring causes complete loss of C-RAF inhibition in compound **6**, suggesting the importance of the 3,4-substituents of the R^1 group when the proximal phenyl group is replaced by the cyclohexyl group. Most sEH inhibitors do not need large 3,4-substituted aryl group at the lipophilic group (R^1) for potent sEH inhibitory acitivity,[5] so lacking a single large 3, or 4-aryl substituent provides a simple way to reduce the potential of sEH inhibitors with a 1,4-cyclohexyl proximal ring to have inhibitory activity on the kinases studied here. Therefore we can conclude that unless the proximal ring is a complete planar group like the phenyl group as in sorafenib or compounds **21** and **22**, the lipophilic group (R^1) appears to require the 4-chloro,3-trifluoromethoxyphenyl moiety to inhibit C-RAF. Otherwise no C-RAF inhibition was observed in this series. Structurally diverse sEH inhibitors such as the ones with a piperidyl proximal ring[19] (TPPU and TUPS, Table 1) are not known to be kinase inhibitors.

We then evaluated all of our compounds against VEGFR-2 to determine which of these compounds have potentially anti-angiogenic property. As seen in Figure 2, only four compounds (*t*-CUPM, **9**, **21** and **22**) displayed potency towards VEGFR-2 at 10 μM concentration. These data yielded crucial evidence on the structural features which appear to be most import for VEGFR-2 inhibition. (1) There is some flexibility in the lipophilic group (R^1) and the proximal ring, and (2) compound **8** which is structurally similar to compound *t*-CUPM, shows little inhibitory activity suggesting the pyridine ring in R^2 is critical for VEGFR-2 inhibition.

To determine if potency against VEGFR-2 correlated with anti-angiogenic properties, we tested the ability of these compounds to halt the growth of HUVEC cells, which is used as preliminary model for angiogenesis.[30] We observed that compounds *t*-CUPM and **9** displayed similar effects on HUVEC cells compared to

Table 2
IC_{50} values of ureas **21** and **22** for sEH and C-RAF



	R^1	sEH	C-RAF
		IC_{50} (nM)	
Sorafenib	4-Cl, 3-CF_3-Ph	12 ± 2	45 ± 5
21	Adamantyl	0.5 ± 0.1	1000 ± 100
22	4-OCF_3-Ph	0.5 ± 0.1	30 ± 5

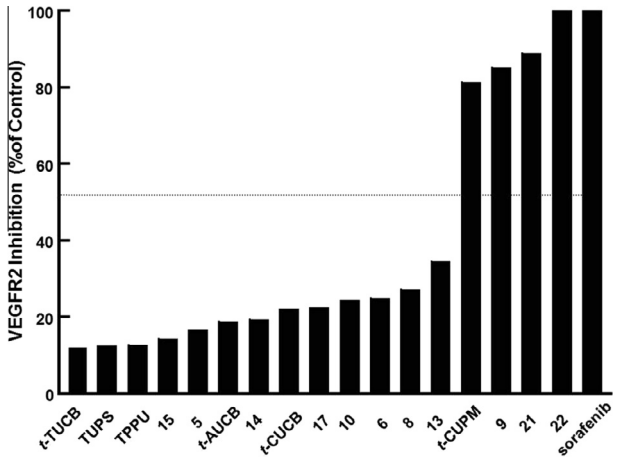


Figure 2. Percent inhibition against VEGFR-2 at 10 μM concentration of test compounds.

sorafenib, whereas **21** and **22** displayed over two-fold loss in growth inhibition (Table 3). Interestingly, the compounds *t*-CUCB, **8**, and **17** also inhibited HUVEC growth with similar to the compound *t*-CUPM and **9** despite their lack of VEGFR-2 inhibition. This suggested that some of these compounds may be cytotoxic rather than anti-angiogenic.

Finally, since C-RAF is overexpressed in a high number of HCC tumors,[31] and sEH is highly expressed in the liver,[32] we treated two epithelial liver carcinoma cells, HepG2 and Huh-7, to correlate sEH, C-RAF and VEGFR-2 inhibition to cytotoxicity and potential anti-tumorigenic properties. Consistent with our previous observations in RCC cell lines,[19] sEH inhibitors (TUPS and TPPU) that are structurally dissimilar to sorafenib displayed no observable effects on cell viability up to 100 μM (data only shown for 25 μM) (Table 3). Initial observation with compounds *t*-CUPM, **8**, **9**, and **13** suggested there may be a direct correlation between C-RAF inhibition and cytotoxicity; however, compounds **8** and **17** display similar ability to halt cellular proliferation despite significant differences in C-RAF inhibition. Moreover, among these compounds only *t*-CUPM and **9** inhibit both C-RAF and VEGFR-2. Lastly, **22** displayed similar cytotoxicity as *t*-CUPM, **9** and **22**, yet possessed significant differences in C-RAF inhibition. These data are consistent with previous work with sorafenib derivatives,[33] suggesting that C-RAF kinase inhibition may not be critical for the cytotoxic responses of sorafenib in HCC cells.

Table 3
GI_{50} concentrations of test compounds for HCC cells (HepG2 and Huh-7) and HUVEC

	GI_{50} (μM)		
	HepG2	Huh-7	HUVEC
Sorafenib	4.5	4.0	6
4	7.0	8.0	11
5	10	8	ND
6	7.0	7.0	>25
7	>25	>25	10.5
8	6.5	7.5	11
9	7.0	8.0	10.5
10	>25	>25	25
11	>25	>25	>25
12	15	20	>25
13	>25	16	>25
14	7.0	5.5	>25
15	7.0	5.5	>25
17	7.5	8.5	10
21	10	7.0	25
22	7.0	7.0	>25
TUPS	>25	>25	>25
TPPU	>25	>25	>25

Table 4
IC_{50} values of compounds *t*-CUPM, **17**, **21**, and **22** for B-RAF^{V600E}

	B-RAF^{V600E}
	IC_{50} (nM)
Sorafenib	13 ± 2 (38[18])
Regorafenib	19[37]
t-CUPM	570 ± 30
17	>5000
21	>5000
22	>5000

This study reveals that structural modification of sorafenib slightly alters sEH inhibitory activity, but affects C-RAF kinase and VEGFR-2 inhibition dramatically. Compounds possessing both a left side lipophilic group (R^1) and a right side distal group with a methylamide, or the identical right side groups as in sorafenib, retain C-RAF inhibition and/or VEGF receptor kinase inhibition. Such kinase inhibition is easy to remove from potent selective sEH inhibitors by altering the distal ring and thus avoid adverse effects associated with kinase inhibition. In addition, current study also demonstrated that a 1,4-cyclohexyl group, especially, as a *trans*-form as in the compound *t*-CUPM could be act as a bioisostere for the phenyl ring in sorafenib, which give an advantage over sorafenib by having narrower spectrum of kinase inhibition. This is illustrated by the selective inhibition of C-RAF over B-RAF^{V600E} (Table 4). This selectivity should reduce B-RAF driven side effects for example when treating tumors initiated by oncogenic Kras where B-RAF is not required but C-RAF is critical for downstream MEK signaling.[34] In addition, reducing the aromatic character of the sorafenib improved its physical characteristics.[35] Therefore the compound *t*-CUPM might have reduced side effects compared to sorafenib.

Lastly, while conventional sEH inhibitors *t*-AUCB and *t*-TUCB display little cytotoxicity, the corresponding methylamide derivatives (**14** and **15**, respectively) significantly affected HCC cell viabilities. These data reinforce the notion that inhibition of sEH does not contribute to cytotoxicity and suggest that the carboxylic acid derivative confers selectivity towards sEH. In addition, compound *t*-CUCB displays an ability to inhibit the growth of HUVEC stimulated by growth factors such as VEGF, but does not suppress cancer cell growth, suggesting that general cytotoxicity is not the mechanism for inhibition of HUVEC growth. Therefore, such compounds might possess a potential to reduce adverse effect associated with angiogenesis related to sEH inhibition.

In summary, we have performed a SAR study against sEH and kinases, such as C-RAF and VEGFR-2, with a series of compounds that resemble both sorafenib and conventional urea-based sEH inhibitors. Sorafenib is such a potent inhibitor of the sEH that this activity is thought to stabilize endogenous EpFAs including anti-hypertensive, anti-inflammatory, and analgesic EETs. Surprisingly regorafenib is 24-fold more potent than sorafenib as a sEH inhibitor. A recent study showed that epoxides of the ω-3 fatty acid DHA, when stabilized against hydrolysis, inhibit angiogenesis, tumor growth and metastasis by a non-VEGF dependent pathway.[36]

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.05.011.

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