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Thioether benzenesulfonamide inhibitors of carbonic anhydrases II and IV: Structure-based drug design, synthesis, and biological evaluation

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ABSTRACT

A novel series of potent thioether benzenesulfonamide inhibitors of carbonic anhydrases II and IV was discovered using structure-based drug design. Synthesis, structure–activity relationship, and optimization of physicochemical properties are described. Low nanomolar potency was achieved, and selected compounds with improved thermodynamic solubility showed promising in vitro inhibition of carbonic anhydrase activity in rabbit iris ciliary body homogenate.

1. Introduction

Glaucoma is a disease characterized by increased intraocular pressure (IOP). This increase in intraocular pressure leads to damage of the optic nerve and is an acknowledged risk factor for vision loss due to glaucoma.[1,2] Elevated IOP can result from either increased production or decreased drainage of aqueous humor, the fluid that provides structural support, oxygen, and nutrition to eye tissues.

Carbonic anhydrase (CA) inhibition has been demonstrated to reduce fluid flow into the eye and alleviate high IOP.[3–8] For over 40 years CA inhibitors (CAIs) were available only as pills, and these were intended for other therapies. These consisted of acetazolamide, methazolamide, and dichlorphenamide. Although well tolerated by many patients, they are also associated with serious side effects such as weight loss, leucopenia, and metabolic acidosis.[9–11] To minimize the incidence and severity of adverse events, topical ocular dosing of CAIs was explored as alternative treatment for glaucoma. In 1995 Trusopt, the first eye drop for topical dosing was introduced, using dorzolamide as the CA inhibitor (CAI). Another CAI, brinzolamide, followed in 1998. The incidence of side effects was lowered by local versus systemic dosing, but adverse events still occur in some patients.[12–15]

Another approach to reduce IOP is to increase the outflow of aqueous humor. There are two main known pathways for this alleviating drainage: through the trabecular meshwork and through the uveoscleral outflow. Prostaglandins, specifically PGF2α, have proven very effective in inducing the latter pathway[16] and a synthetic analog, latanoprost (Xalatan), is widely used for the treatment of glaucoma.

A combination therapy that operates through both mechanisms could provide greater IOP reduction for the treatment of glaucoma.[17–19] A CAI which is dosed once daily would be ideal for such combination therapy with other once-daily therapies such as latanoprost. This report discloses our initial effort toward the discovery of a novel inhibitor of carbonic anhydrase using structure-based drug design[20] and subsequent optimization through successive small compound arrays.

2. Chemistry

5-(Aminosulfonyl)-*N*-alkyl-2-substituted benzamides **3** were prepared as depicted in Scheme 1. 2-Fluorobenzoic acid (**1**) was converted to sulfonamide **2** with a slight modification of previously described conditions.[21] The acid underwent coupling to a variety of amide-building blocks using HATU.[22] The fluoride in the resulting intermediates was displaced with a variety of nucleophiles such as thiols, phenols, and alcohols using cesium carbonate in DMSO to generate analogs of interest **3**. In a selected case, thioether **3a** was converted to the corresponding sulfone using hydrogen peroxide in acetic acid.

In some cases the amides obtained from **2** were difficult to isolate using silica gel chromatography. To facilitate the isolation of these intermediates, the sulfonamide was protected as dimethylamino-methylidenes[31] as depicted in Scheme 1. The resulting

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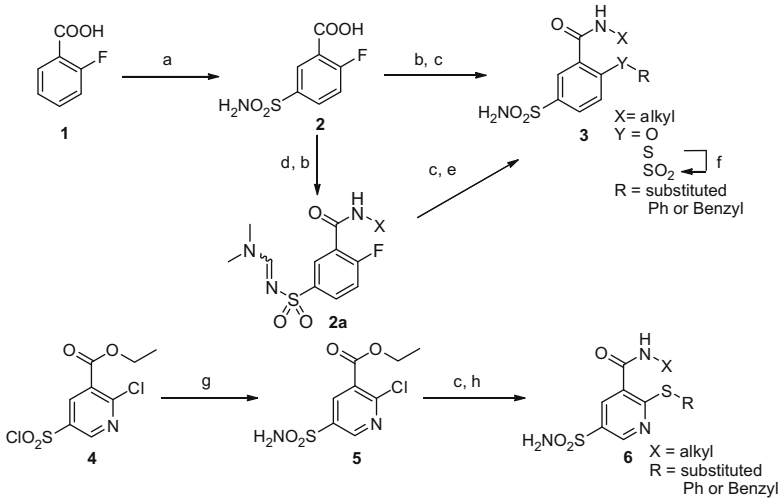


Scheme 1. Reagents and conditions: (a) $HOSO_2Cl$ then NH_3 (59%); (b) H_2NX, HATU (31–94%); (c) Cs_2CO_3, DMSO, HOR or HSR (7–100%); (d) $SOCl_2$, DMF (98%); (e) HCl, MeOH (45%); (f) H_2O_2, AcOH (63%); (g) NH_3(g), CH_2Cl_2 (91%); (h) Me_3Al, H_2NX, THF (55–84%).

methylidene was converted to amide **2a** as previously described. Aryl fluoride **2a** furnished analogs of interest **3** by introducing the thioether followed by removal of the sulfonamide-protecting group using methanolic HCl.

The synthesis of pyridine-containing sulfonamides started with ethyl 2-chloro-5-(chlorosulfonyl)nicotinate (**4**,[23] Scheme 1). Using ammonia in dichloromethane, the sulfonyl chloride **4** was converted to the sulfonamide **5**. This useful building block spawned small arrays of 6–12 analogs. The chloride was first displaced with thiols, and the resulting esters were then transformed to amides **6** using trimethyl aluminum under Weinreb's conditions.[24–26] When chloroester **5** was exposed to amines and trimethyl aluminum, the desired amides were formed as a mixture with the diamino adducts.

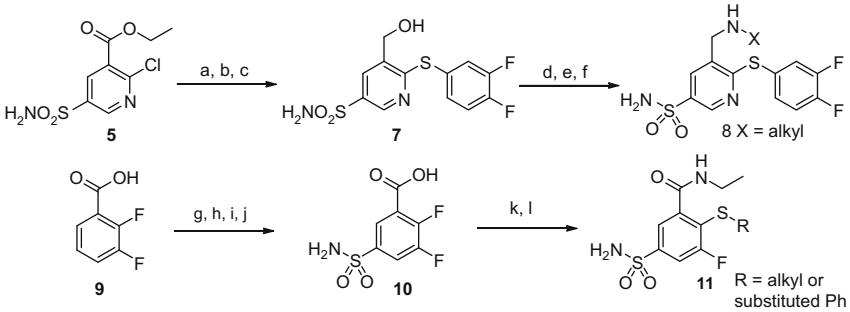
The synthesis of *meta*-aminomethyl derivatives **8** is depicted in Scheme 2. The Cl in **5** was displaced with 3,4-difluorothiophenol and the ester hydrolyzed to the corresponding acid. The resulting acid was reduced with borane–methyl sulfide. For the conversion of alcohol **7** to amines **8**, it was first necessary to protect the sulfonamide using trimethyl orthoacetate.[27,28] The resulting alcohol was then transformed to the tosylate, which was displaced with the desired amines. Finally, deprotection of the sulfonamide was achieved with TFA. Analog **14** (Table 2), without a *meta* substituent, was prepared following the chemistry described in Scheme 1, starting with 2-chloropyridine-5-sulfonyl chloride.[29]

meta-Fluoro analogs **11** (Scheme 2) were prepared by first converting 2,3-difluorobenzoic acid (**9**) to sulfonamide **10** using chlorosulfonic acid and ammonia. To facilitate the purification of the crude acid **10**, its corresponding methyl ester was made. The resulting ester was purified by normal phase chromatography, and pure acid **10** was regenerated by hydrolysis of the methyl ester in aqueous hydrochloric acid. Acid **10** underwent sequential conversion to the corresponding ethyl amide using HATU and fluoride displacement with thiols to give the desired *meta*-fluoro analogs **11**. The regiochemistry of sulfonylation and fluoride displacement were confirmed by careful analysis of the patterns for the aromatic protons for final product **11b** (Fig. 1). The coupling constants observed were consistent with the reported coupling constants for fluorobenzene:[30] *ortho* hydrogen–fluorine $J = 7.0$ Hz and *meta* hydrogen–hydrogen $J = 1.1$ Hz couplings. The reported *para* hydrogen–fluorine coupling (0.2 Hz in fluorobenzene)[30] was not observed.

Table 1
Summary of crystallographic data

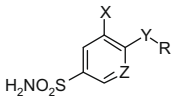
Compound	**6f**	**6d**	Dorzolamide
Resolution (Å)[a]	20–2.05 (2.12–2.05)	50–2.00 (2.07–2.00)	30–1.72 (1.78–1.72)
R_{cryst}[b] (%)	19.1	21.2	17.6
R_{free}[c] (%)	25.2	26.4	20.6

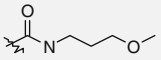
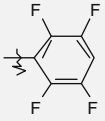
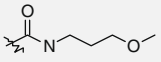
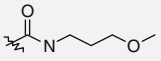
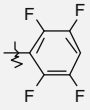
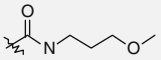
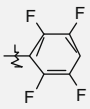
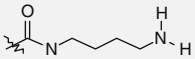
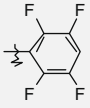
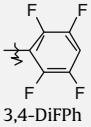
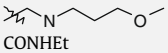
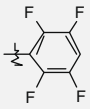
[a] Numbers in parentheses refer to the highest resolution shell.
[b] $R_{cryst} = (\sum_h |F_o - F_c| / \sum_h F_o) \times 100$, where F_o and F_c are the observed and calculated structure factors, respectively.
[c] R_{free} is computed as for R_{cryst} but with the test set (5%) of reflections only.



Scheme 2. Reagents and conditions: (a) Cs_2CO_3, DMSO, 3,4-difluorothiophenol (99%); (b) LiOH, THF, H_2O (100%); (c) BH_3–SMe_2, THF (28%); (d) trimethyl orthoacetate, CH_3CN; (e) TsCl, Et_3N, THF; (f) H_2NX then TFA (7–44% for three steps); (g) $HOSO_2Cl$; (h) NH_3, CH_2Cl_2; (i) $SOCl_2$, MeOH; (j) HCl (63% for four steps); (k) HATU, $EtNH_2$, DMF (27%); (l) Cs_2CO_3, DMSO, HSR (8–73%).

Table 2
CAII and CAIV inhibitory activity, kinetic solubility, and docking score in CAIV of analogs **3a–k, 6a–o,** 7, **8a–c, IIa–b,** and **14**



Analog	X	Y	R	Z	CAII IC_{50} (nM)	CAIV IC_{50} (nM)	Kinetic solubility (μM)	Docking score (kcal/mol)
3a	CONHBn	S	Ph	CH	12.3	241	<25.1	−7.9
3b	CONHEt	S	Ph	CH	21.3	575	205	−6.8
3c	CONHBn	O	Ph	CH	35.9	2040	28.5	−6.6
3d	CONHBn	S	Bn	CH	10.1	1480	<24.2	−7.3
3e	$CONHCH_2$-2-Pyr	S	2-(Hydroxymethyl)Ph	CH	106	952	>258	−6.5
3f	$CONHCH_2$-2-Pyr	S	2-Pyr	CH	84.1	>5000	>253	−7.2
3g	$CONHCH_2$-2-Pyr	S	Ph	CH	67.6	783	>275	−7.1
3h	$CONHCH_2CH_2OMe$	S	CH_2CH_2Ph	CH	20.2	1910	>304	−7.3
3i	CONH*i*Bu	S	*i*Pr	CH	21.5	>5000	689	−5.7
3j	CONHEt	S	cPent	CH	16.5	>5000	628	−6.4
3k	CONHBn	SO_2	Ph	CH	>5000	1450	55.9	−6.9
6a	CONHEt	S	Ph	N	20	283	118	−6.3
6b	$CONHCH_2CH_2NMe_2$	S	Ph	N	2710	>5000	>265	−6.3
6c	$CONHCH_2CH_2OMe$	S	Ph	N	57.1	361	35.6	−6.3
6d		S	Ph	N	48	246	>287	−6.6
6e	CONHPr	S	Ph	N	26.7	229	156	−6.4
6f	$CONHCH_2CH_2Ph$	S	Ph	N	26.6	64	<22.2	−7.7
6g	$CONHCH_2$-2-Pyr	S		N	23.2	23	<21.2	−7.2
6h	CONHEt	S	3-FPh	N	19.9	126	43.2	−6.2
6i	CONH-4-MeOBn	S	3,4-DiFPh	N	2.19	46.8	<19.9	−7
6j		S	3-FPh	N	25.8	118	89.6	−6.2
6k				N	10.6	23	214	−6.6
61		S	3,4-DiFPh	N	34.6	139	40.9	−6.4
6m	$CONHCH_2CH_2$-2-Pyr	S		N	18.4	20.9	211	−6.7
6n		S		N	45.9	33.1	>265	−4.7
6o	$CONHCH_2CH_2$-4OMeBn	S		N	11.5	23	<19	−6.7
7	CH_2OH	S	3,4-DiFPh	N	79.3	1230	>353	−5.4
8a	CH_2NH-4-MeOBn	S	3,4-DiFPh	N	52.8	402		−6.2
8b	CH_2NHEt	S	3,4-DiFPh	N	321	2660	449	−5.1
8c		S	3,4-DiFPh	N	386	2190	497	−4.3
11a	CONHEt	S	*i*Pr	CF	3.77	1890	498	−4.7
11b	CONHEt	S	*i*Bu	CF	2.79	1250	553	−5.1
14	H	S		N	8.29	188	558	−5.1

3. Results and discussion

The carbonic anhydrase program started in the legacy Pharmacia company with a screening effort of proprietary compounds that identified pyrazole benzenesulfonamides as inhibitors of CAII.[32] There was also a collaboration at Pharmacia with C. Supuran that identified celecoxib as an inhibitor of CAII.[33] For existing assays, esterase activity for the CAIV isozyme was much weaker than for the CAII isozyme.[34] To improve sensitivity as well as throughput, a new assay for CAIV was developed.[35,36] The best results were obtained using an acetazolamide-linked fluorescence tracer which

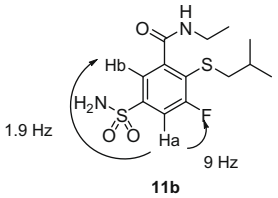


Figure 1. Coupling constants for *meta*-fluoro analog **11b**.

showed an improved fluorescence signal.[37] Pyrazole benzenesulfonamides such as **12** (Fig. 2) were found to have good potency for the CAII isozyme, but essentially no measurable activity for the CAIV isozyme in the new assay (CAII IC_{50} = 46.7 nM and CAIV IC_{50} >1000 nM). Since it was thought at the time of this work that inhibition of both isoforms II and IV of CA was necessary for the desired IOP-lowering effect, we initially focused on improving the CAIV potency of the lead matter.

Examination of an X-ray crystal structure of the complex of dorzolamide in engineered human CAIV (Fig. 3a) obtained in-house[38] identified a large lipophilic pocket formed by Lys 91, Glu 123, and Lys 206 in a rim with Val 121 and Ile 141 at the floor, that could be used to improve the CAIV potency and maintain CAII potency of the initial leads. This prompted the design of ligands, which were minimized and docked into CAIV and CAII (see Section 5 for computational methods employed). Modeling of thioethers of the general structure **13** predicted a good fit in the dorzolamide-CAIV structure with a good lipophilic interaction in the previously identified pocket. Chemistry toward this class of compounds was developed and the first two analogs made (**3a–b**, Table 2) showed an improvement in the CAIV potency. The crystal structure of **6d** and **6f** (Fig. 3b and c) later obtained in CAIV confirmed the initial docking experiments. Docking scores (predicted binding free energy, see Section 5) of the ligands in the CAIV structure associated with **6d** are shown in Table 2. The docking scores generally followed the CAIV potency trend (**3a–c** is a good example) but were not predictive for a number of analogs such as **3d**, **3f**, **3h, 3k, 6n**, and **14**. As a whole, the docking was useful for assessing new designs but not precise enough to rank order the ligands. The sulfonamide coordination to the zinc for all determined structures displayed identical geometries to that for dorzolamide with a N–Zn distance of 2.0 Å. To optimize this series further an empirical approach with small arrays of analogs (6–12 at a time) and quick assay turnaround was taken.

Intermediate fluoro-acid **2** was a key building block with two diversity points that was used for the rapid generation of small focused libraries as shown in Scheme 1 with data shown in Table 2. These libraries were devised to explore the solvent-exposed lipophilic pocket as well as the interactions on the other side of the active site, adjacent to Thr 200 and Asn 62. Initial structure–activity relationship (SAR) studies showed that a sulfur atom linkage was more potent than oxygen linkage and that *S*-aryl substituents were preferred over *S*-alkyls (**3a–d**, Table 2).

Solubility of the analogs made was assessed using a high throughput kinetic solubility assay (see Section 5). Most of the compounds initially made had low solubility. Since our goal was

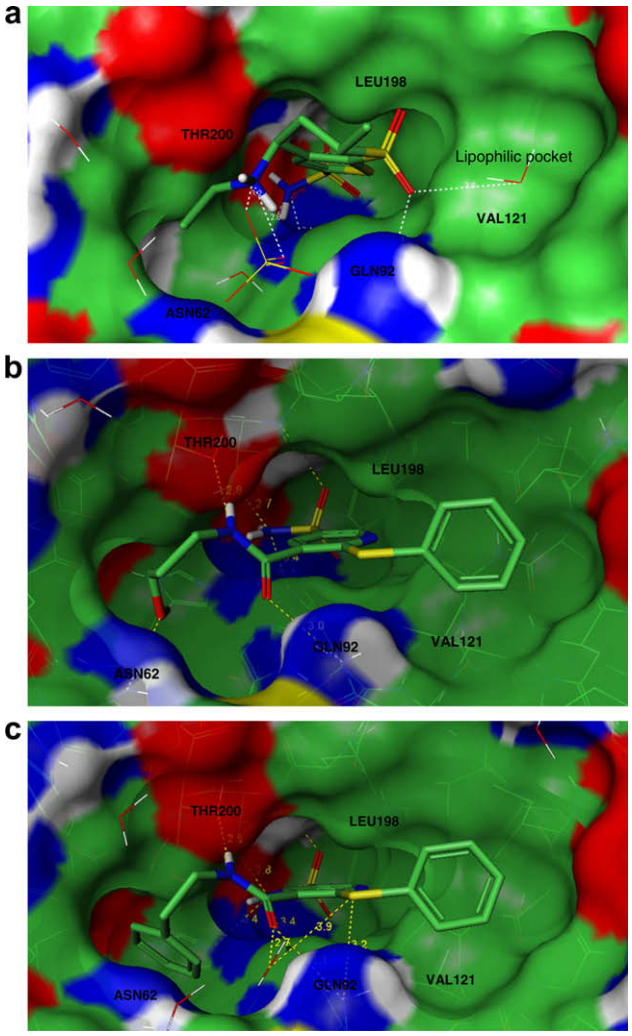


Figure 3. Connolly surface of the CAIV-binding pocket obtained from the crystal structures of CAIV with selected inhibitors. (a) Top view showing H-bonding network with dorzolamide. (b) Top view showing H-bonding network with **6d**. (c) Top view showing H-bonding network with **6f**.

a topical eye drop formulation which requires relatively high aqueous solubility, we next focused on improving solubility.

Our initial strategy to improve solubility was to introduce polarity on the thiophenyl and amide substituents. This initial effort lead to analogs with improved solubility but reduced CAIV and CAII potency (2-pyridylmethyls **3e–g** vs. **3a**, Table 2). This result is consistent with the X-ray data showing the thiophenyl moiety in the hydrophobic pocket of CAIV described above (Fig. 3b and c). Oxidizing the sulfur to the sulfone in order to reduce the lipophilicity of the compounds improved the solubility only slightly (**3k**, Table 2), but significantly reduced the CAIV potency. In another attempt to boost the solubility, the central phenyl core was replaced with a pyridine core. No improvement in solubility and a small increase in CAIV potency were observed

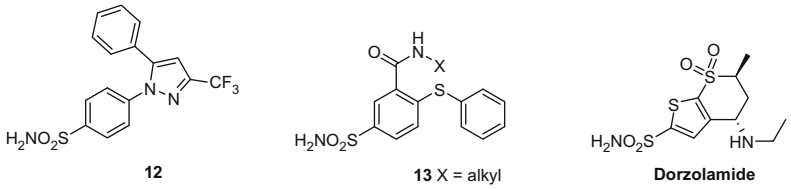


Figure 2. Pyrazole-sulfonamide **12**, thioether **13**, general structure, and dorzolamide.

(**6a** vs **3b**, Table 2). The lack of solubility improvement can be rationalized by the low basicity of the pyridine due to the electron-withdrawing effects of the sulfonamide and amide substituents. Introduction of polar substituents on the amide had mixed results: analogs with a polar group two carbons away from the amide had reduced CAII and CAIV potency (**3g**, **6b**, Table 2). However, with greater separation between the polar group and the amide, the CAII and CAIV potency was maintained (**6d**, **6m**, **6n**, Table 2). The methoxy propyl substituent of **6d** was particularly noteworthy since it maintained the CAII potency while improving the CAIV potency and the solubility. A crystal structure of 3-methoxypropylamide **6d** was obtained in CAIV (Fig. 3b) which revealed a potential hydrogen bond between the side chain amide oxygen and Asn 62 (2.7–3.7 Å distance) that could account for the small improvement in CAIV potency. In most cases, however, lipophilic amide substituents were required for greater CAII and CAIV potency (**6e**, **6f**, **6i**, Table 2) and these resulted in reduced solubility. Interestingly, X-ray analysis of **6f** in CAIV showed that the phenethyl substituent pointed inside a pocket of the protein formed by close interactions with Tyr 7, Asp 62, and His 94 side chains (Fig. 3c). This may explain the CAIV potency of this analog and rationalize the preference for lipophilic substituents in this region of the CAIV-binding pocket.

Further examination of the X-ray structure of dorzolamide and **6d** and **6f** in CAIV showed that the ethylamine group of dorzolamide occupied the same region of the protein as the amide substituent of the thioethers. Analogs with amine substituents at the *meta* position of the sulfonamide could potentially maintain potency while increasing the solubility. Unfortunately, these targeted analogs, while more soluble, had reduced potency for CAII and CAIV (**8a**–**c**, Table 2).

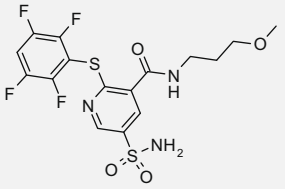
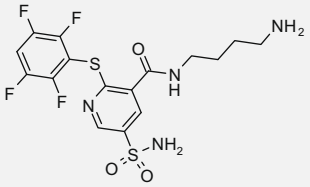
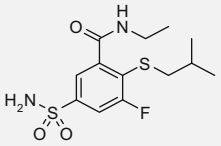
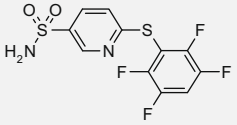
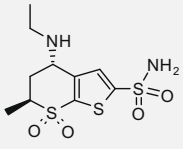
Introduction of fluorines on the *S*-aryl substituent resulted in improved CAIV potency (**6h**, **6k**, **6l**, Table 2). By examining the X-ray structure of **6d** in CAIV one can assume that fluorine substituents in the lipophilic pocket interact favorably with the lipophilic pocket of the protein. This trend is also observed for CAII and has also been noted by Supuran et al.[39,40] The tetrafluorophenyl substituent of **6k**, also identified by Supuran et al.,[40] was particularly intriguing since unlike the other arylfluorides it did not compromise solubility (214 μM kinetic solubility for **6k**), and greatly improved CAIV potency. The combination of this substituent with solubilizing groups on the amide led to analogs with good CAII and CAIV potency and solubility. For example, amine **6n** showed >265 μM kinetic solubility.

Our next strategy to improve solubility was to design targets with lower molecular weights.[41,42] When replacing the thiophenyl substituents with smaller thioalkyl substituents, solubility was greatly improved, but in most cases the CAIV potency was greatly reduced (**3i**–**j**, Table 2). Only branched alkyls such as isopropylthio **11a** and isobutylthio **11b** retained modest CAIV potency. In a similar fashion, the solubility was also improved with the smaller amide substituents (**3b** and **6a** vs **3a** and **6f**, Table 2) and the analogs with small non-amide substituents (**7, 14**, Table 2).

Shepard et al.[43] have shown that introducing a fluorine *meta* to the sulfonamide resulted in improved CAII potency in their series of analogs. Similarly, fluorobenzenesulfonamides **11a** and **11b** inhibited CAII with IC_{50}'s of 3.77 nM and 2.79 nM, respectively, and were some of the most potent CAII analogs in this series (Table 2).

A selected set of compounds, as shown in Table 3, was selected for further evaluation. The selection was made based on CAII potency, kinetic solubility, and CAIV potency. Since the kinetic solubility determination was done on a small scale from DMSO liquid

Table 3
Inhibition of carbonic anhydrase in iris ciliary body homogenate ('pH Stat Spike') of selected analogs

Analog	Structure	CAII IC_{50} (nM)	CAIV IC_{50} (nM)	Thermodynamic solubility (μM)	pH Stat Spike (% inh. 0.1 μM)	pH Stat Spike (% inh. 10 μM)
6k		10.60	23	124	47.5	80.9
6n		45.90	33.1	3090	65.4	84.1
11b		2.79	1250	395	67.6	85.5
14		8.29	188	231	74.2	99.3
Dorzolamide		<2	43	>61,600	68.0	86.0

stocks, more precise thermodynamic solubility[44] was determined with a larger sample in aqueous phosphate buffer. General ranking of solubility between kinetic and thermodynamic determinations was consistent, but the latter more relevant for future formulation. This set of analogs had moderate to good thermodynamic solubility (although much lower than that of dorzolamide). We next evaluated this set of compounds with an in vitro experiment where the inhibition of carbonic anhydrase was measured in rabbit iris ciliary body homogenate.[45,46] Good in vitro carbonic anhydrase inhibition was achieved at 10 μM and **6n**, **11b**, and **14** showed inhibition comparable to dorzolamide at 0.1 μM.

4. Conclusion

A novel series of potent inhibitors of CAII and CAIV has been discovered using a combination of small array synthesis and structure-based drug design. Each successive small array found more potent inhibitors and promising leads were co-crystallized to inform design of the next iteration. Different strategies to improve solubility while maintaining CAII and CAIV potency were explored. Several analogs with improved solubility have been identified and showed inhibition of CA activity in rabbit iris tissue homogenates similar to dorzolamide. Such promising activity warrants further examination, particularly in vivo IOP-lowering studies. Further studies on this series and related analogs are in progress and will be reported in due course.

5. Experimental section

5.1. General methods

All reagents were purchased from commercial sources and were used without further purification. Solvents were of analytical or anhydrous grade (Sigma–Aldrich). Reactions were monitored by HPLC. Analytical HPLC analysis was conducted using an Agilent 1100 HPLC with chromatography performed on a YMC CombiScreen ODS-A C_{18} column (4.6 × 50 mm, 5 μm) at 25 °C. The mobile phase was a 4-min binary gradient of acetonitrile (containing 0.1% TFA) and water (containing 0.1% TFA) (5–95%) with a flow rate of 3 mL/min. The retention times (t_R) are expressed in min with UV detection at 220 nM, 254 nM, and 280 nM. Reverse-phase preparative HPLC was performed using a Waters LC 4000 preparative HPLC system over a 250 × 50.8 mm Peeke C18 column (10 μm). A gradient of acetonitrile (containing 0.1% TFA or AcOH) and water (containing 0.1% TFA or AcOH) (5–95%) with a flow rate of 95 mL/min was used. Some of the partial TFA salt obtained from the RPHPLC purification was converted to an HCl salt using 3 N HCl in methanol and toluene. ^{1}H NMR spectra were recorded on an Avance DPX NMR Spectrometer (Bruker Biospin, Billerica, MA). Chemical shifts (δ) are reported in downfield from TMS. Mass spectra were obtained on an Agilent G1959A LC/MS with electrospray ionization (ESI) source mass spectrometer, and for high resolution, an Agilent G1969A LC/MSD TOF with electrospray ionization (ESI) source mass spectrometer. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA, and are within ±0.4% of theoretical values.

5.2. Chemistry

5.2.1. 5-(Aminosulfonyl)-2-fluorobenzoic acid (2)

2-Fluorobenzoic acid (320 g, 2.28 mol) was added to chlorosulfonic acid (750 mL) portionwise at ambient temperature. The resulting mixture was stirred at ambient temperature for 0.5 h and then heated at 130–140 °C overnight. The ambient temperature mixture was slowly poured onto crushed ice. After stirring for 0.5 h, the precipitate was filtered and washed with water thoroughly to afford a gray solid, which was added in portions to 28% aq ammonium hydroxide (700 mL) at 0 °C. The mixture was stirred at ambient temperature overnight and concentrated to a small volume under reduced pressure. 0.1 N aq H_2SO_4 was added slowly at 0 °C with vigorous stirring until pH 5. The precipitate was filtered, washed with water, and dried under vacuum at 50 °C to give 5-(aminosulfonyl)-2-fluorobenzoic acid (293 g, 58.7%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.39 (d, J = 1.1 Hz, 1H), 7.43 (br s, 3H), 7.83–7.91 (m, 1H), 8.22 (dd, J = 6.8, 2.5 Hz, 1H). LC/MS m/z 241.9 $[MNa]^+$. HRMS (TOF) calcd for $C_7H_7FNO_4S$ $[MH]^+$, 220.00743, found 220.00743.

5.3. Method A. General procedure for *meta* amide preparation

To a solution of **2** in DMF (0.23 M) was added HATU (1.5 equiv). The mixture was stirred at ambient temperature for 2 h and the amine (1.2 equiv) was added. The mixture was further stirred at ambient temperature for 18 h. The crude material was directly purified by RPHPLC using acetonitrile in water (5–95%, with 0.1% AcOH or TFA).

5.3.1. 5-(Aminosulfonyl)-*N*-benzyl-2-fluorobenzamide (15a)

To a stirred solution of **2** (63.0 g, 0.288 mol) in anhydrous DMF (1000 mL) at 0 °C were sequentially added HOBt hydrate (62.0 g, 0.461 mol), EDCI (88.0 g, 0.461 mol), *N*-methyl-morpholine (126.0 mL, 1.152 mol), and benzylamine (38.0 mL, 0.345 mol). The mixture was stirred overnight, poured into water, and filtered. The collected solid was washed with dilute aqueous HCl, dilute aqueous sodium hydroxide, and water, and dried in vacuo to yield the title compound (40.0 g, 45.1%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.51 (d, J = 5.8 Hz, 2H), 7.25–7.32 (m, 3H), 7.35–7.39 (m, 4H), 7.52 (t, J = 9.5 Hz, 1H), 7.91–7.99 (m, 1H), 8.08 (dd, J = 6.5, 2.4 Hz, 1H), 9.11 (s, 1H). LC/MS m/z 309.0 $[MH]^+$. HRMS (TOF) calcd for $C_{14}H_{14}FN_2O_3S$ $[MH]^+$, 331.05231; found, 331.05171. Anal. ($C_{14}H_{13}FN_2O_3S$·0.5HCl) C, H, N.

5.3.2. 5-(Aminosulfonyl)-*N*-ethyl-2-fluorobenzamide (15b)

Prepared following method A using 2 M ethylamine in THF. Yield: 87 mg, 31% as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.14 (t, J = 7.2 Hz, 3H), 3.22–3.34 (m, 2H), 7.43–7.59 (m, 3H), 7.91–7.98 (m, 1H), 8.06 (dd, J = 6.6, 2.5 Hz, 1H), 8.55 (s, 1H). HPLC t_R = 1.2 min (>95%). LC/MS (ESI) m/z 247.0 $[MH]^+$. HRMS (TOF) calcd for $C_9H_{12}FN_2O_3S$ $[MH]^+$: 247.05472, found: 247.05745. Anal. ($C_9H_{11}FN_2O_3S$·0.2AcOH·0.5H_2O) C, H, N.

5.3.3. 5-(Aminosulfonyl)-2-fluoro-*N*-(pyridin-2-ylmethyl)benzamide (15c)

Prepared following method A using 2-(aminomethyl)pyridine. Yield: 5.47 g, 94%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.02 (d, J = 5.6 Hz, 2H), 7.78 (s, 2H), 7.83 (dd, J = 10.2, 8.7 Hz, 1H), 8.00 (t, J = 6.8 Hz, 1H), 8.04 (d, J = 7.8 Hz, 1H), 8.23–8.28 (m, 1H), 8.43 (dd, J = 6.7, 2.4 Hz, 1H), 8.57 (t, J = 7.7 Hz, 1H), 9.02 (d, J = 4.6 Hz, 1H), 9.49–9.52 (m, 1H). LC/MS m/z 310.0 $[MH]^+$. HRMS (TOF) calcd for $C_{13}H_{13}FN_3O_3S$ $[MH]^+$, 310.0656; found, 310.0661.

5.3.4. 5-(Aminosulfonyl)-2-fluoro-*N*-(2-methoxyethyl)benzamide (15d)

Prepared following method A using 2-methoxyethylamine. Yield: 0.739 g, 58%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.30 (s, 3H), 3.41–3.53 (m, 4H), 7.55–7.57 (m, 2H), 7.95 (m, 1H), 8.06 (dd, J = 6.5, 2.4 Hz, 1H), 8.35 (dd, J = 6.8, 2.5 Hz, 1H). LC/MS m/z 277.1 $[MH]^+$.

5.4. Method B. General procedure for the aryl fluoride displacement

A solution of aryl fluoride, thiophenol (1.5 equiv), and cesium carbonate (2.5 equiv) in DMSO (0.25 M) was heated at 50–

100 °C for 2 h. The reaction mixture was treated with aq 5 N HCl (1 mL) and then directly purified by preparative reverse-phase HPLC using acetonitrile in water (5–95%, with 0.1% acetic acid or TFA).

5.4.1. 5-(Aminosulfonyl)-*N*-benzyl-2-(phenylthio)benzamide (3a)

Prepared following method B starting with **15a** and thiophenol. Yield: 52 mg (13%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.53 (d, J = 6.1 Hz, 2H), 7.09 (d, J = 8.3 Hz, 1H), 7.28–7.34 (m, 1H), 7.37–7.47 (m, 4H), 7.50–7.58 (m, 5H), 7.75 (dd, J = 8.5, 2.2 Hz, 1H), 7.94 (d, J = 2.0 Hz, 1H), 9.29 (t, J = 5.9 Hz, 1H). LC/MS m/z 399.0 $[MH]^+$. HRMS (TOF) calcd for $C_{20}H_{19}N_2O_3S_2$ $[MH]^+$: 399.0832, found: 399.0836. Anal. ($C_{20}H_{18}N_2O_3S_2\cdot0.4H_2O$) C, H, N.

5.4.2. 5-(Aminosulfonyl)-*N*-ethyl-2-(phenylthio)benzamide (3b)

Prepared following method B starting with **15b** and thiophenol. Yield: 24 mg (7%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.00 (t, J = 7.3 Hz, 3H), 3.09–3.18 (m, 2H), 6.88 (d, J = 8.6 Hz, 1H), 7.25 (s, 2H), 7.31–7.38 (m, 5H), 7.54 (dd, J = 8.5, 2.2 Hz, 1H), 7.69 (d, J = 2.0 Hz, 1H), 8.54 (t, J = 5.4 Hz, 1H). LC/MS m/z 337.0 $[MH]^+$. HRMS (TOF) calcd for $C_{15}H_{17}N_2O_3S_2$ $[MH]^+$: 337.0675, found: 337.0676. Anal. ($C_{15}H_{16}N_2O_3S_2\cdot0.4H_2O$) C, H, N.

5.4.3. 5-(Aminosulfonyl)-*N*-benzyl-2-phenoxybenzamide (3c)

Prepared following method B starting with **15a** and phenol. Yield: 405 mg (66%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.47 (d, J = 6.1 Hz, 2H), 6.97 (d, J = 8.8 Hz, 1H), 7.14 (d, J = 7.6 Hz, 2H), 7.21–7.30 (m, 6H), 7.41 (s, 2H), 7.46 (t, J = 8.0 Hz, 2H), 7.83 (dd, J = 8.8, 2.5 Hz, 1H), 8.09 (d, J = 2.3 Hz, 1H), 8.97 (t, J = 6.1 Hz, 1H). LC/MS m/z 383.0 $[MH]^+$. HRMS (TOF) calcd for $C_{20}H_{19}N_2O_4S$ $[MH]^+$: 383.1060, found: 383.1050. Anal. ($C_{20}H_{18}N_2O_4S$) C, H, N.

5.4.4. 5-(Aminosulfonyl)-*N*-benzyl-2-(benzylthio)benzamide (3d)

Prepared following method B starting with **15a** and benzyl mercaptan. Yield: 442 mg (67%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.40 (s, 2H), 4.54 (d, J = 6.1 Hz, 2H), 7.32–7.39 (m, 2H), 7.40–7.46 (m, 6H), 7.48–7.55 (m, 4H), 7.77 (d, J = 8.3 Hz, 1H), 7.84–7.91 (m, 2H), 9.22 (t, J = 5.9 Hz, 1H). LC/MS m/z 413.0 $[MH]^+$. HRMS (TOF) calcd for $C_{21}H_{21}N_2O_3S_2$ $[MH]^+$: 413.0988, found: 413.0996. Anal. ($C_{21}H_{20}N_2O_3S_2\cdot0.3H_2O$) C, H, N.

5.4.5. 5-(Aminosulfonyl)-2-{[2-(hydroxymethyl)phenyl]thio}-*N*-(pyridin-2-ylmethyl)benzamide hydrochloride (3e)

Prepared following method B starting with **15c** and 2-mercaptobenzyl alcohol. Yield: 155 mg (33%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.52 (s, 2H), 4.78 (d, J = 5.8 Hz, 2H), 6.82 (d, J = 8.3 Hz, 1H), 7.38 (t, J = 7.6 Hz, 1H), 7.44 (s, 2H), 7.48 (d, J = 7.8 Hz, 1H), 7.54 (td, J = 7.5, 1.3 Hz, 1H), 7.65–7.72 (m, 2H), 7.78 (t, J = 6.8 Hz, 1H), 7.88 (d, J = 8.1 Hz, 1H), 8.09 (d, J = 2.0 Hz, 1H), 8.36 (t, J = 7.8 Hz, 1H), 8.80 (d, J = 5.3 Hz, 1H), 9.60 (t, J = 5.7 Hz, 1H). LC/MS m/z 430.1 $[MH]^+$. HRMS (TOF) calcd for $C_{20}H_{20}N_3O_4S_2$ $[MH]^+$, 430.0890; found, 430.0891. Anal. ($C_{20}H_{19}N_3O_4S_2\cdot HCl\cdot H_2O$) C, H, N.

5.4.6. 5-(Aminosulfonyl)-*N*-(pyridin-2-ylmethyl)-2-(pyridin-2-ylthio)benzamide dihydrochloride (3f)

Prepared following method B starting with **15c** and 2-mercaptopyridine. Yield: 127 mg (54%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.83 (d, J = 5.6 Hz, 2H), 7.27–7.34 (m, 2H), 7.61 (s, 2H), 7.63 (d, J = 8.3 Hz, 1H), 7.78 (td, J = 7.8, 1.9 Hz, 1H), 7.88 (dd, J = 8.3, 2.0 Hz, 1H), 7.94 (t, J = 6.8 Hz, 1H), 7.96 (s, 1H), 8.17 (d, J = 2.0 Hz, 1H), 8.47 (d, J = 4.0 Hz, 1H), 8.51 (td, J = 8.0, 1.5 Hz, 1H), 8.86 (d, J = 5.1 Hz, 1H), 9.66 (t, J = 5.7 Hz, 1H). LC/MS m/z 401.0 $[MH]^+$. HRMS (TOF) calcd for $C_{18}H_{17}N_4O_3S_2$ $[MH]^+$, 401.0737; found, 401.0738. Anal. ($C_{18}H_{16}N_4O_3S_2\cdot2.0HCl\cdot2.1H_2O$) C, H, N.

5.4.7. 5-(Aminosulfonyl)-2-(phenylthio)-*N*-(pyridin-2-ylmethyl) benzamide hydrochloride (3g)

Prepared following method B starting with **15c** and thiophenol. Yield: 125 mg (57%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.59 (d, J = 5.6 Hz, 2H), 6.83 (d, J = 8.6 Hz, 1H), 7.23–7.33 (m, 7H), 7.53 (dd, J = 8.6, 2.0 Hz, 1H), 7.60 (t, J = 6.8 Hz, 1H), 7.70 (d, J = 8.1 Hz, 1H), 7.91 (d, J = 2.0 Hz, 1H), 8.19 (t, J = 7.5 Hz, 1H), 8.60 (d, J = 5.1 Hz, 1H), 9.41 (t, J = 5.7 Hz, 1H). LC/MS m/z 400.0 $[MH]^+$. HRMS (TOF) calcd for $C_{19}H_{18}N_3O_3S_2$ $[MH]^+$, 400.0784; found, 400.0785. Anal. ($C_{19}H_{17}N_3O_3S_2\cdot HCl\cdot H_2O$) C, H, N.

5.4.8. 5-(Aminosulfonyl)-*N*-(2-methoxyethyl)-2-[(2-phenylethyl) thio]benzamide (3h)

Prepared following method B starting **15d** and phenethyl mercaptan. Yield: 116 mg (100%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.89 (t, J = 7.5 Hz, 2H), 3.23–3.32 (m, 5H), 3.37–3.42 (m, 2H), 3.44–3.50 (m, 2H), 7.20–7.35 (m, 5H), 7.40 (s, 2H), 7.65 (d, J = 8.3 Hz, 1H), 7.75 (d, J = 2.1 Hz, 1H), 7.81 (dd, J = 8.3, 2.1 Hz, 1H), 8.62 (t, J = 5.4 Hz, 1H). LC/MS m/z 395.0 $[MH]^+$. HRMS (TOF) calcd for $C_{18}H_{23}N_2O_4S_2$ $[MH]^+$, 395.1094; found, 395.1100. Anal. ($C_{18}H_{22}N_2O_4S_2\cdot0.1H_2O$) C, H, N.

5.4.9. 5-({[(1*E*)-(Dimethylamino)methylene]amino}sulfonyl)-2-fluorobenzoic acid (16)

To a solution of **2** (1.0 g, 4.56 mmol) in toluene (10 mL) were slowly added thionyl chloride (1.66 mL, 22.81 mmol) and DMF (0.42 mL, 32.1 mmol). The reaction mixture was stirred at 70 °C for 48 h and then the reaction was carefully quenched with ice (10 g). The aqueous layer was extracted with ethyl acetate (3 × 25 mL) and the combined organic layer was dried ($MgSO_4$) and concentrated in vacuo to give the title compound (1.23 g, 98%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.93 (s, 3H), 3.17 (s, 3H), 7.51 (t, J = 9.5 Hz, 1H), 7.96–8.08 (m, 1H), 8.20–8.28 (m, 1H), 13.61 (br. s., 1H). LC/MS m/z 275.2 $[MH]^+$. HRMS (TOF): calcd for $C_{10}H_{12}FN_2O_4S$ $[MH]^+$: 275.04963, found: 275.05187. Anal. ($C_{10}H_{11}FN_2O_4S\cdot0.4HCl$) C, H, N.

5.4.10. Methyl 5-({[(1*E*)-(dimethylamino)methylene]amino}-sulfonyl) -2-fluorobenzoate (17)

A solution of **16** (160 mg, 0.58 mmol) in methanol was stirred at 40 °C for 4 h. The mixture was concentrated under reduced pressure and the residue was purified by column chromatography (silica gel, ethyl acetate–hexane, 50%) to yield the title product as a white solid (106 mg, 63%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.93 (s, 3H) 3.17 (s, 3H) 3.91 (s, 3H) 7.55 (dd, J = 10.6, 8.8 Hz, 1H) 8.01–8.12 (m, 1H) 8.22–8.31 (m, 2H). LC/MS m/z 289.1 $[MH]^+$. HRMS (TOF): calcd for $C_{11}H_{13}FN_2O_4S$ $[MH]^+$: 289.06528, found: 289.07424. Anal. ($C_{11}H_{13}FN_2O_4S\cdot0.2H_2O$) C, H, N.

5.5. Method C. Successive fluoride displacement, amide formation and sulfonamide deprotection

To a solution of methyl **17** and Cs_2CO_3 (1.1 equiv) in DMSO (0.35 M) was slowly added the thiol (1.1 equiv). The reaction mixture was stirred at 50 °C for 4 h, and diluted in ethyl acetate and water. The organic layer was washed with water, dried ($MgSO_4$), and concentrated under reduced pressure to yield the title product methyl ester intermediate which was used without further purification. This material was converted to the corresponding amide

using method D. The crude product was diluted in 3 N HCl in methanol (5 mL) and stirred at 60 °C for 3 h before reverse-phase purification.

5.5.1. 5-(Aminosulfonyl)-*N*-isobutyl-2-(isopropylthio)benzamide (3i)

Prepared via method C starting with 2-propanethiol and isobutylamine. Yield: 129 mg (58%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.00 (d, J = 6.8 Hz, 6H), 1.15 (d, J = 6.6 Hz, 6H), 1.72–1.86 (m, 1H), 2.88 (d, J = 6.8 Hz, 2H), 3.98–4.10 (m, 1H), 7.38 (s, 2H), 7.56 (d, J = 8.3 Hz, 1H), 7.66 (d, J = 2.3 Hz, 1H), 7.75 (dd, J = 8.3, 2.0 Hz, 1H), 8.39 (d, J = 7.8 Hz, 1H). LC/MS m/z 331.1 [MH]$^+$. HRMS (TOF): calcd for $C_{14}H_{23}N_2O_3S_2$ [MH]$^+$: 353.09663, found: 353.09641. Anal. ($C_{14}H_{22}N_2O_3S_2$+0.3H_2O) C, H, N.

5.5.2. 5-({[(1*E*)-(Dimethylamino)methylene]amino}sulfonyl)-*N*-ethyl-2-fluorobenzamide (2a)

To a solution of **16** (0.9 g, 3.28 mmol) in DMF (10 mL) were slowly added triethylamine (0.457 mL, 3.28 mmol) and HATU (1.25 g, 3.28 mmol). The mixture was stirred at ambient temperature for 1 h and ethylamine (1.64 mL, 2 M in THF, 3.28 mmol) was slowly added. The mixture was stirred at ambient temperature for 48 h and then diluted with methylene chloride and water. The organic layer was washed with water and concentrated in vacuo. The residue was purified by column chromatography eluting with ethyl acetate–hexane (30–100%) to give the title compound (455 mg, 51%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.14 (t, J = 7.3 Hz, 3H), 2.93 (s, 3H), 3.17 (s, 3H), 3.23–3.32 (m, 2H), 7.47 (dd, J = 9.9, 8.8 Hz, 1H), 7.87–7.98 (m, 2H), 8.26 (s, 1H), 8.49–8.59 (m, 1H). LC/MS m/z 302.0 [MH]$^+$. HRMS (TOF): calcd for $C_{12}H_{17}FN_3O_3S$ [MH]$^+$: 302.09692, found: 302.09980.

5.5.3. 5-(Aminosulfonyl)-2-(cyclopentylthio)-*N*-ethylbenzamide (3j)

Prepared following method B starting with **2a** and cyclopentyl mercaptan. The crude product was diluted in 3 N HCl in MeOH (3 mL), stirred at 60 °C for 3 h, concentrated, and purified by column chromatography eluting with ethyl acetate–hexane (10–100%). Yield: 49 mg (45%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.13 (t, J = 7.2 Hz, 3H), 1.42–1.77 (m, 6H), 2.04–2.20 (m, 2H), 3.17–3.30 (m, 2H), 3.73–3.86 (m, 1H), 7.38 (s, 2H), 7.62 (d, J = 8.5 Hz, 1H), 7.71 (d, J = 2.1 Hz, 1H), 7.78 (dd, J = 8.5, 2.1 Hz, 1H), 8.49 (t, J = 5.6 Hz, 1H). LC/MS m/z 329.0 [MH]$^+$. HRMS (TOF) calcd for $C_{14}H_{21}N_2O_3S_2$ [MH]$^+$, 329.09881; found, 329.09933. Anal. ($C_{14}H_{20}N_2O_3S_2$) C, H, N.

5.5.4. 5-(Aminosulfonyl)-*N*-benzyl-2-(phenylsulfonyl)benzamide (3k)

To a solution of **3a** (340 mg, 0.9 mmol) in acetic acid (4 mL) was added hydrogen peroxide (0.29 mL of 30 wt % in water, 2.56 mmol). The mixture was stirred at ambient temperature for 2 h, then at 75 °C for 2 h. The crude reaction mixture was directly purified by preparative reverse-phase HPLC using acetonitrile in water (5–95%, with 0.1% acetic acid or TFA) to give the title compound (272 mg, 63%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.51 (d, J = 5.8 Hz, 2H), 7.29 (t, J = 7.2 Hz, 1H), 7.38 (t, J = 7.6 Hz, 2H), 7.43–7.47 (m, 2H), 7.62 (t, J = 7.6 Hz, 2H), 7.67–7.72 (m, 3H), 7.83 (d, J = 1.8 Hz, 1H), 8.01–8.08 (m, 3H), 8.34 (d, J = 8.3 Hz, 1H), 9.23 (t, J = 5.9 Hz, 1H). LC/MS m/z 431.0 [MH]$^+$. HRMS (TOF) calcd for $C_{20}H_{19}N_2O_5S_2$ [MH]$^+$: 431.0730, found: 431.0719. Anal. ($C_{20}H_{18}N_2O_5S_2\cdot0.1H_2O$) C, H, N.

5.5.5. Ethyl 5-(aminosulfonyl)-2-chloronicotinate (5)

Ammonia was bubbled through a cooled −50 °C solution of **4** (170 g, 0.6 mol) in anhydrous CH_2Cl_2 (1500 mL) for 20–30 min. TLC (silica gel with EtOAc/petroleum ether = 1:2) showed that the reaction was complete. The reaction mixture was purged with N_2 to remove excess NH_3. The suspension was concentrated to give a light yellow solid, which was filtered, washed with water, and dried to give the title compound (144 g, 91%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.37 (t, J = 7.1 Hz, 3H), 4.42 (q, J = 7.1 Hz, 2H), 7.81 (s, 2H), 8.62 (d, J = 2.5 Hz, 1H), 8.96 (d, J = 2.5 Hz, 1H). HRMS (TOF) calcd for $C_8H_{10}ClN_2O_4S$ [MH]$^+$, 265.00443; found, 265.00534. Anal. ($C_8H_9ClN_2O_4S$) C, H, N.

5.6. Method D. General procedure for amide formation from esters

To a cooled 0 °C solution of the amine (3 equiv) in THF (0.2 M) was slowly added trimethylaluminum (2 M in toluene, 4 equiv). The mixture was stirred at ambient temperature for 1 h and the ester (1.0 equiv) was added. The mixture was stirred at 45 °C for 24 h and then the reaction was carefully quenched with methanol and 1 N HCl. The crude mixture was directly purified by preparative reverse-phase HPLC using acetonitrile in water (5–95%, with 0.1% acetic acid or TFA) to give the desired product.

5.6.1. 5-(Aminosulfonyl)-2-chloro-*N*-ethylnicotinamide hydrochloride (18a)

Prepared following method D starting with **5** and ethylamine. Yield: 580 mg (38%). This material was used without further purification. HPLC t_R = 1.4 min (>98%). LC/MS m/z 264.0 [MH]$^+$.

5-(Aminosulfonyl)-*N*-ethyl-2-(ethylamino)nicotinamide (365 mg, 30%) was isolated as a byproduct and the TFA salt resulting from the reverse-phase purification was converted to the HCl salt using 3 N HCl in MeOH. ^{1}H NMR (300 MHz, DMSO-d_6+D_2O) δ 1.05–1.21 (m, 6H), 3.25 (q, J = 7.3 Hz, 2H), 3.43 (q, J = 7.2 Hz, 2H), 8.20 (d, J = 2.3 Hz, 1H), 8.48 (d, J = 2.3 Hz, 1H). LC/MS m/z 273.1 [MH]$^+$. HRMS (TOF) calcd for $C_{10}H_{17}N_4O_3S$ [MH]$^+$, 273.1016; found, 273.1018.

5.6.2. 5-(Aminosulfonyl)-2-chloro-*N*-(pyridin-2-ylmethyl)-nicotin-amide (18b)

Prepared following method D starting with **5** and 2-(aminomethyl)pyridine. Yield: 1.52 g (68%) of an orange solid that contained some of the chloride displacement byproduct. The material was used without further purification. LC/MS m/z 327.0 [MH]$^+$.

5.7. Method E. General procedure for the 2-chloropyridine displacement

A solution of 2-chloropyridine, thiophenol (1.2 equiv), and cesium carbonate (2.5 equiv) in DMSO (0.15 M) was heated at 50–90 °C for 2–18 h. The reaction mixture was treated with aq 5 N HCl (1 mL), then directly purified by preparative reverse-phase HPLC using acetonitrile in water (5–95%, with 0.1% acetic acid or TFA).

5.7.1. Ethyl 5-(aminosulfonyl)-2-(phenylthio)nicotinate (19a)

Prepared following method E starting with **5** and thiophenol. The crude product (1.5 g, 100%) was used without further purification. LC/MS m/z 339.0 [MH]$^+$.

5.7.2. Ethyl 5-(aminosulfonyl)-2-[(2,3,5,6-tetrafluorophenyl)thio]nicotinate (19b)

Prepared following method E starting with **5** and 2,3,5,6-tetrafluorobenzenethiol. The crude product (8.58 g, 100%, white solid) was used without further purification. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.42 (t, J = 7.1 Hz, 3H), 4.48 (q, J = 7.2 Hz, 2H), 7.71 (s, 2H), 8.10–8.25 (m, 1H), 8.67 (d, J = 2.3 Hz, 1H), 8.81 (d, J = 2.5 Hz, 1H). LC/MS m/z 411.0 [MH]$^+$. HRMS (TOF): calcd for $C_{14}H_{11}F_4N_2O_4S_2$ [MH]$^+$: 411.00909, found: 411.01768.

5.7.3. Ethyl 5-(aminosulfonyl)-2-[(3,4-difluorophenyl)thio]nicotinate (19c)

Prepared following method E starting with **5** and 3,4-difluorothiophenol. The crude material was triturated twice with 50 mL of 10% ethyl acetate in hexane to give the title compound (14.5 g, 99%) as a white solid, which was used without further purification. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.40 (t, J = 7.1 Hz, 3H), 4.44 (q, J = 7.1 Hz, 2H), 7.39–7.48 (m, 1H), 7.51–7.63 (m, 1H), 7.69 (s, 2H), 7.71–7.77 (m, 1H), 8.59 (d, J = 2.3 Hz, 1H), 8.76 (d, J = 2.3 Hz, 1H). LC/MS m/z 375.0 $[MH]^+$. HRMS (TOF): calcd for $C_{14}H_{13}F_2N_2O_4S_2$ $[MH]^+$: 375.02793, found: 375.03571. Anal. ($C_{14}H_{12}F_2N_2O_4S_2$) C, H, N.

5.7.4. 5-(Aminosulfonyl)-*N*-ethyl-2-(phenylthio)nicotinamide (6a)

Prepared following method E starting with **18a** and thiophenol. Yield: 124 mg (100%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.18 (t, J = 7.3 Hz, 3H), 3.24–3.34 (m, 2H), 7.44–7.54 (m, 5H), 7.59 (s, 2H), 8.15 (d, J = 2.3 Hz, 1H), 8.64 (d, J = 2.3 Hz, 1H), 8.91 (t, J = 5.4 Hz, 1H). LC/MS m/z 338.1 $[MH]^+$. HRMS (TOF) calcd for $C_{14}H_{16}N_3O_3S_2$ $[MH]^+$, 338.0628; found, 338.0632. Anal. ($C_{14}H_{15}N_3O_3S_2$·0.1HCl) C, H, N.

5.7.5. 5-(Aminosulfonyl)-*N*-[2-(dimethylamino)ethyl]-2-(phenyl-thio)nicotinamide dihydrochloride (6b)

Prepared following method D starting with **19a** and *N,N′*-dimethylenediamine. Yield: 194 mg (73%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.94 (s, 6H), 3.92–4.02 (m, J = 4.0 Hz, 4H), 7.50–7.57 (m, 3H), 7.60–7.68 (m, 2H), 7.86 (s, 2H), 8.50 (d, J = 2.3 Hz, 1H), 8.99 (d, J = 2.5 Hz, 1H). LC/MS m/z 381.1 $[MH]^+$. HRMS (TOF) calcd for $C_{16}H_{21}N_4O_3S_2$ $[MH]^+$, 381.1050; found, 381.1061. Anal. ($C_{16}H_{20}N_4O_3S_2$·2HCl·0.7H_2O) C, H, N.

5.7.6. 5-(Aminosulfonyl)-*N*-(2-methoxyethyl)-2-(phenylthio)-nicotinamide (6c)

Prepared following method D starting with **19a** and 2-methoxyethylamine. Yield: 164 mg (69%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.21 (s, 3H), 3.33–3.38 (m, 2H), 3.39–3.43 (m, 2H), 7.34–7.38 (m, 3H), 7.39–7.43 (m, 2H), 7.49 (s, 2 H), 8.05 (d, J = 2.3 Hz, 1H), 8.54 (d, J = 2.3 Hz, 1H), 8.91 (t, J = 5.3 Hz, 1H). LC/MS m/z 368.0 $[MH]^+$. HRMS (TOF) calcd for $C_{15}H_{18}N_3O_4S_2$ $[MH]^+$, 368.0733; found, 368.0734. Anal. ($C_{15}H_{17}N_3O_4S_2$·0.3HCl) C, H, N.

5.7.7. 5-(Aminosulfonyl)-*N*-(3-methoxypropyl)-2-(phenylthio)-nicotinamide (6d)

Prepared following method D starting with **19a** and 3-methoxypropylamine. Yield: 103 mg (62%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.74–1.87 (m, 2H), 3.28 (s, 3H), 3.30–3.38 (m, 2H), 3.44 (t, J = 6.3 Hz, 2H), 7.45–7.54 (m, 5H), 7.59 (s, 2H), 8.15 (d, J = 2.3 Hz, 1H), 8.65 (d, J = 2.3 Hz, 1H), 8.91 (t, J = 5.6 Hz, 1H). LC/MS m/z 382.1 $[MH]^+$. HRMS (TOF) calcd for $C_{16}H_{20}N_3O_4S_2$ $[MH]^+$, 382.0890; found, 382.0894. Anal. ($C_{16}H_{19}N_3O_4S_2$·0.15HCl) C, H, N.

5.7.8. 5-(Aminosulfonyl)-2-(phenylthio)-*N*-propylnicotinamide (6e)

Prepared following method D starting with **19a** and propylamine. Yield: 86 mg (55%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.97 (t, J = 7.4 Hz, 3H), 1.54–1.63 (m, 2H), 3.21–3.31 (m, 2H), 7.45–7.54 (m, 5H), 7.59 (s, 2H), 8.13 (d, J = 2.3 Hz, 1H), 8.65 (d, J = 2.3 Hz, 1H), 8.90 (t, J = 5.6 Hz, 1H). LC/MS m/z 352.0 $[MH]^+$. HRMS (TOF) calcd for $C_{15}H_{18}N_3O_3S_2$ $[MH]^+$, 352.0784; found, 352.0786. Anal. ($C_{15}H_{17}N_3O_3S_2$·0.05HCl) C, H, N.

5.7.9. 5-(Aminosulfonyl)-*N*-(2-phenylethyl)-2-(phenylthio)-nicotinamide (6f)

Prepared following method D starting with **19a** and phenethylamine. Yield: 152 mg (84%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.90 (t, J = 7.4 Hz, 2H), 3.48–3.58 (m, 2H), 7.29–7.37 (m, 5H), 7.45–7.53 (m, 6H), 7.60 (s, 2H), 8.12 (d, J = 2.3 Hz, 1H), 8.65 (d, J = 2.3 Hz, 1H), 9.02 (t, J = 5.5 Hz, 1H). LC/MS m/z 414.0 $[MH]^+$. HRMS (TOF) calcd for $C_{20}H_{20}N_3O_3S_2$ $[MH]^+$, 414.0941; found, 414.0943. Anal. ($C_{20}H_{19}N_3O_3S_2$·0.05HCl) C, H, N.

5.7.10. 5-(Aminosulfonyl)-*N*-(pyridin-2-ylmethyl)-2-[(2,3,5,6-tetrafluorophenyl)thio] nicotinamide hydrochloride (6g)

Prepared following method E starting with **18b** and 2,3,5,6-tetrafluorobenzenethiol. Yield: 92 mg (42%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.87 (d, J = 5.3 Hz, 2H), 7.70 (s, 2H), 7.82 (t, J = 6.5 Hz, 1H), 7.93 (d, J = 8.1 Hz, 1H), 8.08–8.21 (m, 1H), 8.39 (td, J = 7.8, 1.1 Hz, 1H), 8.77 (d, J = 2.1 Hz, 1H), 8.80–8.84 (m, 3H), 10.08 (t, J = 5.5 Hz, 1H). LC/MS m/z 473.0 $[MH]^+$. HRMS (TOF) calcd for $C_{18}H_{13}F_4N_4O_3S_2$ $[MH]^+$, 473.0360; found, 473.0374. Anal. ($C_{18}H_{12}F_4N_4O_3S_2$·1.4HCl) C, H, N.

5.7.11. 5-(Aminosulfonyl)-*N*-ethyl-2-[(3-fluorophenyl)thio]nicotinamide (6h)

Prepared following method E starting with **18a** and 3-fluorothiophenol. Yield: 112 mg (92%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.18 (t, J = 7.3 Hz, 3H), 3.27–3.35 (m, 2H), 7.28–7.43 (m, 3H), 7.47–7.56 (m, 1H), 7.60 (s, 2H), 8.21 (d, J = 2.3 Hz, 1H), 8.69 (d, J = 2.3 Hz, 1H), 8.94 (t, J = 5.4 Hz, 1H). LC/MS m/z 356.0 $[MH]^+$. HRMS (TOF) calcd for $C_{14}H_{15}FN_3O_3S_2$ $[MH]^+$, 356.0534; found, 356.0538. Anal. ($C_{14}H_{14}FN_3O_3S_2$·0.1HCl) C, H, N.

5.7.12. 5-(Aminosulfonyl)-2-[(3,4-difluorophenyl)thio]-*N*-(4-methoxybenzyl)nicotinamide hydrochloride (6i)

Prepared following method D starting with **19c** and 4-methoxybenzylamine. Yield: 111 mg (69%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.76 (s, 3H), 4.44 (d, J = 5.7 Hz, 2H), 6.95 (d, J = 8.7 Hz, 2H), 7.23–7.30 (m, 1H), 7.33 (d, J = 8.7 Hz, 2H), 7.49–7.57 (m, 1H), 7.60 (s, 2H), 7.63–7.74 (m, 1H), 8.26 (d, J = 2.3 Hz, 1H), 8.69 (d, J = 2.3 Hz, 1H), 9.44 (t, J = 5.8 Hz, 1H), with rotamer peaks. LC/MS m/z 466.0 $[MH]^+$. HRMS (TOF) calcd for $C_{20}H_{18}F_2N_3O_4S_2$ $[MH]^+$, 466.0702; found, 466.0705. Anal. ($C_{20}H_{17}F_2N_3O_4S_2$) C, H, N.

5.7.13. 5-(Aminosulfonyl)-*N*-(3-methoxypropyl)-2-[(3-fluorophe-nyl)thio]nicotinamide (6j)

Prepared following methods E starting with **5** and 3-fluorobenzenethiol. The crude intermediate ethyl ester was converted to the title compound using method D and 3-methoxypropylamine. Yield: 136 mg (67%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.72–1.88 (m, 2H), 3.27 (s, 3H), 3.30–3.38 (m, 2H), 3.44 (t, J = 6.2 Hz, 2H), 7.27–7.44 (m, 3H), 7.46–7.56 (m, 1H), 7.61 (s, 2H), 8.20 (d, J = 2.3 Hz, 1H), 8.69 (d, J = 2.3 Hz, 1H), 8.95 (t, J = 5.5 Hz, 1H). LC/MS m/z 400.1 $[MH]^+$. HRMS (TOF) calcd for $C_{16}H_{19}FN_3O_4S_2$ $[MH]^+$, 400.0796; found, 400.0807. Anal. ($C_{16}H_{18}FN_3O_4S_2$·0.2HCl) C, H, N.

5.7.14. 5-(Aminosulfonyl)-*N*-(3-methoxypropyl)-2-[(2,3,5,6-tetrafluorophenyl) thio]nicotinamide (6k)

Prepared following method D starting with **19b** and 3-methoxypropylamine. Yield: 242 mg (65%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.76–1.88 (m, 2H), 3.28 (s, 3H), 3.36–3.38 (m, 2H), 3.44 (t, J = 6.3 Hz, 2H), 7.62 (s, 2H), 8.05–8.22 (m, 1H), 8.48 (d, J = 2.1 Hz, 1H), 8.72 (d, J = 2.1 Hz, 1H), 9.18 (t, J = 5.4 Hz, 1H). LC/MS m/z 454.0 $[MH]^+$. HRMS (TOF) calcd for $C_{16}H_{16}F_4N_3O_4S_2$ $[MH]^+$, 454.0513; found, 454.0513. Anal. ($C_{16}H_{15}F_4N_3O_4S_2$·0.05HCl) C, H, N.

5.7.15. 5-(Aminosulfonyl)-2-[(3,4-difluorophenyl)thio]-*N*-(3-methoxypropyl)nicotinamide (6l)

Prepared following method D starting with **19c** and 3-methoxypropylamine. Yield: 81 mg (60%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.73–1.87 (m, 2H), 3.27 (s, 3H), 3.30–3.35 (m, 2H), 3.43 (t, J = 6.2 Hz, 2H), 7.36–7.44 (m, 1H), 7.49–7.58 (m, 1H), 7.61 (s, 2H),

7.64–7.72 (m, 1H), 8.22 (d, J = 2.3 Hz, 1H), 8.69 (d, J = 2.3 Hz, 1H), 8.96 (t, J = 5.5 Hz, 1H). LC/MS m/z 418.2 $[MH]^+$. HRMS (TOF) calcd for $C_{16}H_{18}F_2N_3O_4S_2$ $[MH]^+$, 418.0702; found, 418.0709. Anal. ($C_{16}H_{17}F_2N_3O_4S_2$·0.1HCl) C, H, N.

5.7.16. 5-(Aminosulfonyl)-*N*-(2-pyridin-2-ylethyl)-2-[(2,3,5,6-tetrafluorophenyl)thio] nicotinamide hydrochloride (6m)

Prepared following method D starting with **19b** and 2-(2-aminoethyl)pyridine. Yield: 90 mg (68%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.32 (t, J = 6.1 Hz, 2H), 3.73–3.88 (m, 2H), 7.65 (s, 2H), 7.85 (t, J = 6.8 Hz, 1H), 7.94 (d, J = 7.9 Hz, 1H), 8.05–8.24 (m, 1H), 8.41 (t, J = 7.7 Hz, 1H), 8.56 (d, J = 1.7 Hz, 1H), 8.71 (d, J = 1.9 Hz, 1H), 8.81 (d, J = 5.1 Hz, 1H), 9.41 (t, J = 5.4 Hz, 1H). LC/MS m/z 487.0 $[MH]^+$. HRMS (TOF) calcd for $C_{19}H_{15}F_4N_4O_3S_2$ $[MH]^+$, 487.0516; found, 487.0502. Anal. ($C_{19}H_{14}F_4N_4O_3S_2$·2.3HCl·2H_2O·0.2toluene) C, H, N.

5.7.17. *N*-(3-Aminopropyl)-5-(aminosulfonyl)-2-[(2,3,5,6-tetrafluorophenyl)thio]nicotinamide hydrochloride (6n)

Prepared following method D starting with **19b** and 1,3-diaminopropane. Yield: 100 mg (83%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.83–1.94 (m, 2H), 2.86–2.96 (m, 2H), 3.40 (q, J = 6.6 Hz, 2H), 7.66 (s, 2H), 7.96 (br s, 3H), 8.07–8.20 (m, 1H), 8.64 (d, J = 2.0 Hz, 1H), 8.72 (d, J = 2.3 Hz, 1H), 9.36 (t, J = 5.6 Hz, 1H). LC/MS m/z 439.0 $[MH]^+$. HRMS (TOF) calcd for $C_{15}H_{15}F_4N_4O_3S_2$ $[MH]^+$, 439.0516; found, 439.0521. Anal. ($C_{15}H_{14}F_4N_4O_3S_2$·1.2HCl·0.5H_2O) C, H, N.

5.7.18. 5-(Aminosulfonyl)-*N*-[2-(4-methoxyphenyl)ethyl]-2-[(2,3,5,6-tetrafluorophenyl)thio]nicotinamide (6o)

Prepared following method D starting with **19b** and 4-methoxyphenethylamine. Yield: 107 mg (68%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.85 (t, J = 7.4 Hz, 2H), 3.45–3.57 (m, 2H), 3.74 (s, 3H), 6.89 (d, J = 8.7 Hz, 1H), 7.22 (d, J = 8.5 Hz, 1H), 7.69 (s, 2H), 8.03–8.22 (m, 1H), 8.55 (d, J = 2.1 Hz, 1H), 8.69 (d, J = 2.1 Hz, 1H), 9.45 (s, 1H). LC/MS m/z 516.0 $[MH]^+$. HRMS (TOF): calcd for $C_{21}H_{18}F_4N_3O_4S_2$ $[MH]^+$: 516.0670, found: 516.0670. Anal. ($C_{21}H_{17}F_4N_3O_4S_2$·0.2HCl) C, H, N.

5.7.19. 5-(Aminosulfonyl)-2-[(3,4-difluorophenyl)thio]nicotinic acid (20)

To a solution of **19c** (12.0 g, 32.1 mmol) in THF (60 mL) was added lithium hydroxide (2.30 g, 96.2 mmol) in water (60 mL). The reaction mixture was stirred at ambient temperature for 2 h. 5 N aq HCl (21 ml, 106 mmol) was slowly added and the THF was evaporated. The resulting solid was collected by filtration and dried overnight under vacuum to give the title compound (11.1 g, 99%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.42 (s, 1H), 7.50–7.59 (m, 1H), 7.63 (s, 2H), 7.67–7.74 (m, 1H), 8.58 (s, 1H), 8.72 (d, J = 2.3 Hz, 1H), 14.19 (br s, 1H). LC/MS m/z 345.0 $[M-H]^-$. HRMS (TOF) calcd for $C_{12}H_8F_2N_2O_4S_2$ $[MH]^+$, 346.9967; found, 346.9972. Anal. ($C_{12}H_8F_2N_2O_4S_2$·0.5H_2O) C, H, N.

5.7.20. 6-[(3,4-Difluorophenyl)thio]-5-(hydroxymethyl)-pyridine-3-sulfonamide hydrochloride (7)

To a cooled 0 °C solution of **20** (1.0 g, 2.9 mmol) in dry THF (5 mL) was slowly added borane–methyl sulfide complex 2 M in THF (2.9 mL, 5.8 mmol) over 5 min. The mixture was stirred at 0 °C to ambient temperature over 3 h and then the reaction was carefully quenched with methanol and water. The crude mixture was directly purified by RPHPLC using acetonitrile in water (5–95%, with 0.1% acetic acid or TFA). The resulting TFA salt was converted to the HCl salt using 3 N HCl in MeOH to give the title product (286 mg, 28%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.61 (s, 2H), 5.87 (br. s., 1H), 7.38–7.45 (m, 1H), 7.50–7.63 (m, 3H), 7.72 (td, J = 10.3, 8.0, 2.1 Hz, 1H), 8.20 (d, J = 2.3 Hz, 1H), 8.57 (d, J = 2.3 Hz, 1H). LC/MS m/z 333.0 $[MH]^+$. HRMS (TOF) calcd for $C_{12}H_{11}F_2N_2O_3S_2$ $[MH]^+$, 333.0174; found, 333.0166. Anal. ($C_{12}H_{10}F_2N_2O_3S_2$·0.6HCl) C, H, N.

5.7.21. Methyl (1*E*)-*N*-({6-[(3,4-difluorophenyl)thio]-5-(hydroxymethyl)pyridine-3-yl}sulfonyl)ethanimidoate (21)

To a solution of **7** (0.286 g, 0.810 mmol) in acetonitrile (4 mL) was added trimethyl orthoacetate (0.256 mL, 2.01 mmol). The reaction mixture was stirred at 85 °C for 18 h. The reaction mixture was concentrated under reduced pressure to give the title product (367 mg, 100%), which was used without further purification. LC/MS m/z 389.0 $[MH]^+$.

5.8. Method F. General procedure for the conversion of alcohol to amine

To a cooled 5 °C solution of **21** in THF (0.18 M) was added triethylamine (2.2 equiv) and *p*-toluenesulfonyl chloride (2.0 equiv). The mixture was stirred at 5 °C to ambient temperature over 3 h. The mixture was cooled back to 5 °C and the amine (30 equiv) was added. The mixture was stirred at ambient temperature overnight. The crude mixture was diluted with water (1 mL) and treated with TFA (0.65 mL) and then directly purified by RPHPLC using acetonitrile in water (5–95%, with TFA). The resulting TFA salt was converted to the HCl salt using 3 N HCl in MeOH to give the product.

5.8.1. 6-[(3,4-Difluorophenyl)thio]-5-{[(4-methoxybenzyl)-amino] methyl}pyridine-3-sulfonamide hydrochloride (8a)

Prepared following method F using 4-methoxybenzylamine. Yield: 12 mg (7%). ^{1}H NMR (300 MHz, MeOH) δ 3.86 (s, 3H), 4.41 (s, 2H), 4.45 (s, 2H), 7.02–7.11 (m, 2H), 7.37–7.43 (m, 2H), 7.50–7.59 (m, 3H), 8.31 (d, J = 2.3 Hz, 1H), 8.77 (d, J = 2.1 Hz, 1H). LC/MS m/z 452.1 $[MH]^+$. HRMS (TOF) calcd for $C_{20}H_{19}F_2N_3O_3S_2$ $[MH]^+$, 452.0909; found, 452.0915. HPLC t_R = 2.2 min (98%).

5.8.2. 6-[(3,4-Difluorophenyl)thio]-5-[(ethylamino)methyl]-pyridine-3-sulfonamide hydrochloride (8b)

Prepared following method F using ethylamine. Yield: 58 mg (44%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.32 (t, J = 7.2 Hz, 3H), 3.08–3.22 (m, 2H), 4.34 (t, J = 5.8 Hz, 2H), 7.44–7.52 (m, 1H), 7.54–7.65 (m, 1H), 7.70 (s, 2H), 7.75–7.83 (m, 1H), 8.43 (d, J = 2.3 Hz, 1H), 8.73 (d, J = 2.1 Hz, 1H), 9.58 (s, 2H). LC/MS m/z 360.0 $[MH]^+$. HRMS (TOF) calcd for $C_{14}H_{16}F_2N_3O_2S_2$ $[MH]^+$, 360.0647; found, 360.0646. Anal. ($C_{14}H_{15}F_2N_3O_2S_2$·1.5HCl·H_2O) C, H, N.

5.8.3. 6-[(3,4-Difluorophenyl)thio]-5-{[(3-methoxypropyl)-amino] methyl}pyridine-3-sulfonamide hydrochloride (8c)

Prepared following method F using 3-methoxypropylamine. Yield: 49 mg (42%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.92–2.06 (m, 2H), 3.14–3.17 (m, 2H), 3.28 (s, 3H), 3.46 (t, J = 5.9 Hz, 2H), 4.32–4.41 (m, 2H), 7.44–7.52 (m, 1H), 7.54–7.65 (m, 1H), 7.70 (s, 2H), 7.74–7.82 (m, 1H), 8.43 (d, J = 2.1 Hz, 1 H), 8.74 (d, J = 2.3 Hz, 1H), 9.53 (br s, 2H). LC/MS m/z 404.0 $[MH]^+$. HRMS (TOF) calcd for $C_{16}H_{20}F_2N_3O_3S_2$ $[MH]^+$, 404.0909; found, 404.0902. Anal. ($C_{16}H_{19}F_2N_3O_3S_2$·1.2HCl) C, H, N.

5.8.4. 5-(Aminosulfonyl)-2,3-difluorobenzoic acid (10)

2,3-Difluorobenzoic acid (**9;** 200 g, 1.26 mol) was added dropwise to chlorosulfonic acid (660 mL, 9.6 mol) in an ice-cold water bath. After the addition, the mixture was stirred at 115 °C for 48 h. The mixture was slowly poured into ice. The resulting mixture was stirred rapidly at ambient temperature for 2–3 h and filtered to give a yellow solid that was dried in vacuum to give 5-(chlorosulfonyl)-2,3-difluorobenzoic acid (215 g, 66.4%), which was used directly in the next step without purification. To a solution of CH_2Cl_2

(500 mL) saturated with NH_3 was added dropwise a solution of 5-(chlorosulfonyl)-2,3-difluorobenzoic acid (200 g, 0.78 mol) in CH_2Cl_2 (500 mL) at 0 °C. After the addition, the suspension was stirred at ambient temperature overnight. The mixture was filtered, and the solid was washed with water and dried under vacuum to give crude 5-(aminosulfonyl)-2,3-difluorobenzoic acid (210 g) as a yellow solid. To facilitate purification, the material was transiently converted to the methyl ester. Thionyl chloride (250 mL, 3.43 mol) was added to MeOH (500 mL). The solution was stirred at ambient temperature for 1 h, and the crude 5-(aminosulfonyl)-2,3-difluorobenzoic acid (150 g, 0.63 mol) was added portionwise at 0 °C. The reaction mixture was heated at 60 °C overnight. The mixture was concentrated under vacuum and the crude material was purified by column chromatography ($MeOH/CH_2Cl_2$; 1:20) to give the methyl ester of 5-(aminosulfonyl)-2,3-difluorobenzoic acid (100 g) as a light yellow solid. The solid was added to 3 N aq hydrochloric acid (1.5 L) portionwise. The mixture was heated at 70 °C overnight and then evaporated under vacuum to give the title compound (85 g, 63%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.67 (s, 2H) 8.03–8.11 (m, 1H) 8.13–8.19 (m, J = 5.6 Hz, 1H) 14.06 (br s, 1H). LC/MS m/z 235.9 $[M-H]^-$. HRMS (TOF) calcd for $C_7H_6F_2NO_4S$ $[MH]^+$, 237.9980; found, 237.9969. Anal. ($C_7H_5F_2NO_4S$) C, H, N.

5.8.5. 5-(Aminosulfonyl)-*N*-ethyl-2,3-difluorobenzamide (22)

Prepared following method A starting with **10** and ethylamine. Yield: 76 mg (27%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.14 (t, J = 7.2 Hz, 3H), 3.27–3.34 (m, 2H), 7.63 (s, 2H), 7.83–7.88 (m, 1H), 7.91–8.00 (m, 1H), 8.70 (s, 1H). HPLC t_R = 1.4 min (>95%). LC/MS (ESI) m/z 265.1 $[MH]^+$. HRMS (TOF): calcd for $C_9H_{11}F_2N_2O_3S$ $[MH]^+$: 265.0453, found: 265.0415. Anal. ($C_9H_{10}F_2N_2O_3S$) C, H, N.

5.8.6. 5-(Aminosulfonyl)-*N*-ethyl-3-fluoro-2-(isopropylthio)-benzamide (11a)

Prepared following method B starting with **22** and 2-propanethiol. Yield: 10 mg (8%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.09–1.22 (m, 9H), 3.20–3.31 (m, 2H), 3.44–3.58 (m, 1H), 7.58 (d, J = 1.5 Hz, 1H), 7.62 (s, 2H), 7.69 (dd, J = 8.9, 1.9 Hz, 1H), 8.54 (t, J = 5.5 Hz, 1H). LC/MS m/z 321.0 $[MH]^+$. HRMS (TOF) calcd for $C_{12}H_{17}FN_2O_3S_2$ $[MH]^+$, 321.0738; found, 321.0735. HPLC t_R = 2.1 min (92%).

5.8.7. 5-(Aminosulfonyl)-*N*-ethyl-3-fluoro-2-(isobutylthio)-benzamide (11b)

Prepared following method B starting with **22** and 2-methyl-1-propanethiol. Yield: 244 mg (73%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.94 (d, J = 6.6 Hz, 6H), 1.15 (t, J = 7.3 Hz, 3H), 1.53–1.71 (m, 1H), 2.81 (d, J = 7.0 Hz, 2H), 3.22–3.32 (m, 2H), 7.56 (d, J = 1.9 Hz, 1H), 7.60 (s, 2H), 7.67 (dd, J = 9.0, 1.9 Hz, 1H), 8.57 (t, J = 5.5 Hz, 1H). LC/MS m/z 335.0 $[MH]^+$. HRMS (TOF) calcd for $C_{13}H_{20}FN_2O_3S_2$ $[MH]^+$, 335.0894; found, 335.0889. Anal. ($C_{13}H_{19}FN_2O_3S_2$) C, H, N.

5.8.8. 6-[(2,3,5,6-Tetrafluorophenyl)thio]pyridine-3-sulfonamide (14)

Prepared following method E starting with 2-chloropyridine-5-sulfonamide and 2,3,5,6-tetrafluorobenzenethiol. Yield: 1625 mg (90%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.56–7.70 (m, 3H), 8.11 (dd, J = 8.5, 2.5 Hz, 1H), 8.15–8.29 (m, 1H), 8.74 (d, J = 1.9 Hz, 1H). LC/MS m/z 338.9 $[MH]^+$. HRMS (TOF): calcd for $C_{11}H_7F_4N_2O_2S_2$ $[MH]^+$: 338.9878, found: 338.9880. Anal. ($C_{11}H_6F_4N_2O_2S_2 \cdot 0.4HCl \cdot 0.7H_2O$) C, H, N.

5.9. Carbonic anhydrase-II (CA-II) fluorimetric assay—IC_{50} determination

Compounds were dissolved in DMSO at a concentration of 1 mM, then 50 μM, and transferred to a 96-well plate for further dilutions (1:3 dilutions, 11 points) in duplicate. Highest final concentration of compound in this CA-II Fluorimetric assay is 1 μM. Assays were conducted in a final volume of 100 μL in 50 mM Tris/HCl (pH 7.6), 100 mM Na_2SO_4, and 0.005% Tween-20 in a 96-well black assay plate. Fluorescein diacetate was used as the substrate. Enzyme inhibition was determined by pipetting 8 μL of human CA-II (5 nM, from Sigma–Aldrich, product # C6165) into assay plate that contained 2 μL of compound and 2 μL of substrate (10 μM) in 88 μL of assay buffer. The rate of the hydrolysis of fluorescein diacetate was measured spectrophometrically at 488 nm (excitation), 538 nm (emission), and 530 nm (cutoff) using a Molecular Devices SpectraMax M2 fluorescence reader at 25 °C. The IC_{50}, the inhibitor concentration resulting in 50% inhibition of the enzyme activity, was calculated using GraphPad Prism or similar in-house software with the IC_{50} curve fitting using the four parameter logistic equation.

5.10. Carbonic anhydrase-IV (CA-IV) fluorescence polarization assay—IC_{50} determination

Human CAIV was amplified from a human kidney cDNA library (Clonetech) using primers: 5′-ggaattccatatggcagagtcacactggtgctacgag and 5′-ccgctcgagttactaggactttatcaccgtgcgctgccc, with KOD Polymerase (Novagen). The PCR amplified product was cloned into a *Nde*I/ *Xho*I cut pET-43.1a(+) (Novagen) and transformed into *Escherichia coli* BL21 (DE3) (Invitrogen) cells. These cells were grown in Luria broth (LB) media (Biomyx) supplemented with 800 μM $ZnCl_2$ at 37 °C until an O.D.600 of 0.7, at which point the cells were induced with 100 μM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 20 h at 20 °C. The frozen pellet was resuspended in 50 mM 2-morpholinoethanesulfonic acid (MES) at pH 6.0, 100 mM NaCl, 800 μM $ZnCl_2$, and EDTA-Free protease inhibitors (Roche). The cells were lysed with a microfluidizer, the lysate was spun at 40,000 rpm for 45 min at 4 °C, and the soluble fraction was dialyzed overnight at 4 °C in 50 mM MES, pH 6.0, and 100 mM NaCl. The soluble fraction was then put over a 100 mL SP-Sepharose High Performance (GE Healthcare) column and eluted with a 50 mM MES, pH 6.0, 750 mM NaCl gradient. The peak fractions were then concentrated, via an Amicon Ultra-4 10,000 MWCO (Millipore) spin column to 2.0 mL and loaded onto a Sephacryl S-100 High Resolution (GE Healthcare) column in 25 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), pH 7.0, and 100 mM NaCl. The peak fractions were then concentrated, via an Amicon Ultra-4 10,000 MWCO (Millipore) spin column, to 7.0 mg/mL and left exposed at ambient temperature overnight. Fully oxidized protein was characterized by performing an Ellman's Assay, utilizing Pierce reagents, and non-reducing SDS–PAGE. The specific activity of the human CA-IV enzyme was confirmed with the literature inhibitors; IC_{50} for acetazolamide = 120 nM, ethoxzolamide = 88 nM, dorzolamide = 43 nM, and brinzolamide = 45 nM. These IC_{50} were generated using the FP assay described below and are comparable to the K_i/IC_{50} values in the literature.[47,48]

Compounds were dissolved in DMSO at the concentration of 1 mM, then to 250 μM in DMSO and transferred to a 96-well plate for further dilutions (1:3 dilutions, 11 points) in duplicate. Final compound highest concentration in CA-IV FP assay is 5 μM. Assays were conducted in a final volume of 100 μL in 50 mM Tris/HCl (pH 7.6), 100 mM Na_2SO_4, and 0.005% Tween-20 in a 96-well black assay plate. BODIPY®558/568-Acetazolamide (Cat # B-12270 from invitrogen-molecular, discontinued item) was used as the tracer. Binding inhibition was determined by pipetting 8 μL of human CA-IV (25 nM) into assay plate contained 2 μL of compound and 2 μL of tracer (2 nM) in 88 μL of assay buffer. The assay plate was incubated at ambient temperature for 30 min and read in the fluorescence polarization reader (Molecular Devices, Analyst) at 524/45 nm (excitation), 595/60 nm (emission), and 561 nm (beam splitter). IC_{50}, the inhibitor concentration resulting in 50%

inhibition of the enzyme activity, was calculated using GraphPad Prism or similar in-house software with the IC_{50} curve fitting using the four parameter logistic equation.

5.11. Kinetic solubility assay

The samples were predissolved as a 30 mM DMSO stock solution in VWR 96-well plates (200 μL, polypropylene PCR). The plates were allowed to thaw at room temperature and then placed in a 40 °C sonic water bath for 10 min to facilitate resuspension. An automated liquid handler was used to prepare 50-fold dilutions of stocks by combining 6 μL aliquots of stock solutions with 294 μL of 50 mM sodium phosphate buffer (pH 6.5) directly into a Millipore solubility filter plate. The final DMSO concentration was 2%, and the theoretical maximum concentration was 0.6 mM. The plates were heat sealed, and incubation was carried out at ambient room temperature (ca. 22–25 °C) for 24 h with shaking at 200 rpm. The plates were vacuum filtered and the filtrates were injected into the nitrogen detector using an automated liquid handler. The concentrations of filtrates were determined by chemiluminescent nitrogen detection.[49]

5.12. Thermodynamic solubility assay

A standard sample was prepared by dissolving 1–3 mg of sample in 5 mL of methanol. The test sample was prepared by mixing 2–5 mg of sample with 1.0 mL of 100 mM sodium phosphate buffer at pH 6.5. The mixture was stirred for 4 h at ambient temperature and centrifuged. The standard and saturated solutions were analyzed by HPLC. The solubility was calculated using the integrated areas for analyzed peaks.[44]

5.13. Computational methods

Reasonable conformers of computationally AMBER-derived candidate ligands were docked with the aid of AGDOCK according to a prior protocol[50] into the experimentally observed protein structures of CAII and CAIV described herein. The sulfonamide nitrogen-zinc distance was constrained to that experimentally observed and AGDOCK employed iteratively to a suitable ligand–protein complex geometry for further computational analysis. Subsequently, protein–ligand minimization for energetic analysis was performed using in-house software based on the MacroModel implementation of the AMBER force field (version 7),[51–53] with some added parameters to support specific chemical moieties found in Pfizer compounds and solved crystallographic structures (e.g., tetrazoles and some metal ions).

The minimization itself was done using a limited-memory Truncated-Newton minimizer[54,55] with partial preconditioning. Associated surface areas were computed using the LCPO (Linear Combination of Pairwise Overlaps) method, a fast method within the AMBER package that allows rapid and reasonably accurate surface area calculations with derivatives. Partial charges are assigned from the AMBER force field unless reasonable charges are provided in the input file, in which case the input charges are used. Bound ligand models were compared by calculated binding energies and visual inspection to try to predict favorable binding modes and attempt to prioritize which targets to synthesize earlier.

For the docking scores given in Table 2, the sulfonamide nitrogen of the ligands was constrained to that experimentally observed and the ligands docked using the docking program AGDOCK. This program uses an evolutionary algorithm (specifically, Evolutionary Programing) to explore the translational, rotational, and torsional spaces of the ligand within the binding site.[56,57] The docked structures were scored using the High Throughput Screening program developed at Agouron. This program uses an empirical energy function that has been parameterized using experimental data.[58]

5.14. Crystallographic methods

Co-crystals of the soluble portion of CA IV (see section on IC_{50} determination) with compounds (**6f**, **6d**, or dorzolamide) were obtained from 20% to 25% polyethylene glycol (PEG) 3350, 0.1 M sodium acetate (pH 4.5), 0.2–0.3 M ammonium sulfate, ±5 mM DTT, ±glucose (3%) by the method of vapor diffusion in hanging drops. Crystals grew at 13 °C over a period of 14 days. Data were collected at −180 °C after crystals were mounted in a loop (Hampton Research, Laguna Hills, CA) and dragged through a cryo-protecting solution of mother liquor containing 20% DMSO before flash cooling in liquid nitrogen. Data were collected either in-house or at the Advanced Light Source (ALS) at Berkeley and processed and scaled with HKL2000[59] (see Table 1 for data statistics). The structures were determined by molecular replacement using the protein chain only of the CA IV pdb structure 1ZNC[60] as the search model. Molecular replacement was carried out with MOLREP[61] through CCP4.[62] Refinement was carried out in REFMAC5,[63] with initial phase improvement using ARP/wARP.[64] Statistics for the final refined structures are presented in Table 1. Coordinates for the X-ray structure of Figures 3a–c have been deposited at the PDB under the file names 3FW3 (dorzolamide), 3F7U (**6d**), and 3F7B (**6f**).

5.15. Inhibition of carbonic anhydrase in iris ciliary body homogenate ('pH stat spike', similar to published procedure by Ponticello, et al)[46]

Dutch Belted rabbits ($N = 3$ per drug concentration) where euthanized with Beuthenasia and eyes where enucleated. Iris Ciliary Body (ICB) was collected via a wet dissection and snap frozen in liquid nitrogen. Samples were calibrated by using ICB wet weight. Compounds were formulated at specified concentrations (10 μM and 100 nM) in chilled 0.02 M Tris Buffer, pH 8.4, before homogenizing ICB samples (15 mg) in this buffer on the day of the titration assay. Each sample was assessed for carbonic anhydrase activity using a pH Stat titration machine (TitraLab). Samples containing 15 mg of homogenized ICB tissue were titrated with 0.025 N NaOH over 6 minutes while bubbling CO_2 (100 mls/min) into the ICB homogenate. The pH of the sample was held constant at pH 8.3 for at least 3 min. The titration assay was run $N = 3$ for each sample and the average was used to calculate the slope of the curve to determine enzymatic activity. Blank values were subtracted and the slope of the curve (mls/min NaOH added) was used to calculate percent inhibition of compound versus placebo-treated ICB.

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Supplementary data

Supplementary data (combustion analysis data, summary of crystallographic data and selected ^1H NMR spectra) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.014.

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