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Discovery of soluble epoxide hydrolase inhibitors through DNA-encoded library technology (ELT)

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ABSTRACT

Inhibition of soluble epoxide hydrolase (sEH) has recently emerged as a new approach to treat cardiovascular disease and respiratory disease. Inhibitors based on 1,3,5-triazine chemotype were discovered through affinity selection against two triazine-based DNA-encoded libraries. The structure and activity relationship study led to the expansion of the original 1,4-cycloalkyl series to related aniline, piperidine, quinoline, aryl-ether and benzylic series. The 1,3-cycloalkyl chemotype led to the discovery of a clinical candidate (GSK2256294) for COPD.

1. Introduction

DNA-encoded library technology (ELT) has developed over the 28 years since the concept was proposed in 1992.[1] Using a split-and-pool combinatorial synthesis strategy, ELT can provide greater coverage of chemical space that improves the chances of finding leads that may be considered "close to candidate".[2–4] By DNA-encoding each chemical warhead, this technology overcomes the problem of deconvolution, the historical challenge of other combinatorial chemistry methods. ELT has proven to be a powerful tool for ligand identification and target validation, attracting significant attention from both academia and industry. Numerous biologically active compounds have been discovered through affinity selection of DNA-encoded libraries against a broad range of targets.[5–11]

Epoxide hydrolases, found in both plants and animals, are enzymes that convert epoxides to diols by hydrolysis. In mammals, soluble epoxide hydrolase (sEH, EPHX2) is primarily responsible for the metabolism of arachidonic acid derivatives known as epoxyeicosatrienoic acids (EETs).[12] sEH converts EETs into dihydroxyeicosatrienoic acids (DHETs). Several publications[13,14] have described the beneficial vasodilatory, anti-inflammatory, and anti-thrombotic effects of EETs. Taking advantage of the beneficial effects of EETs, the inhibition of sEH can be applied to the treatment of various conditions mediated by the sEH enzyme through the prevention of EETs degradation. In addition, several studies have demonstrated a protective effect as a result of sEH inhibition in models involving cigarette smoke exposure.[15–17] Thus, the inhibitors of sEH can be applied to the treatment of chronic obstructive pulmonary disease (COPD), which accounted for 3 million deaths in 2019 and was the third leading cause of death worldwide according to the World Health Organization's report.[18]

Several classes of sEH inhibitors have been identified including urea,[19] amide,[20] hydrazone derivatives,[21] and others.[22] Our goal was to discover novel inhibitors of sEH through affinity selection against DNA-encoded libraries. In this paper, we will describe the discovery of unique

Abbreviations: ELT, encoded library technology; DEL, DNA-encoded library; sEH, soluble epoxide hydrolase; HP, DNA headpiece; SAR, structure activity relationship.

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triamino-triazine derivatives as potent inhibitors of sEH and the follow up SAR study that led to the expansion of hit series. One of the hit series led to a drug candidate, GSK2256294, for the treatment of COPD after minimal lead optimization.[17,23-24]

2. Results and discussions

2.1. Selection with DNA-encoded libraries

Two closely related libraries, A & B (Figure 1), based on the triazine chemotype were selected against sEH. Both libraries commenced with acylation of DNA headpiece (Supporting Information) using 192 Fmoc-amino acids. After Fmoc-deprotection, the triazine scaffold was installed using cyanuric chloride. Cycles 2 and 3 both employed S_NAr reactions to substitute the remained two chlorides, with 32 bifunctional acids installed at cycle 2 and 340 amines installed at cycle 3. The libraries diverged at the final cycle, in which amidation was performed using two sets of building blocks. DEL A was produced using 384 amines, giving a library containing 802,160,640 compounds and DEL B was produced using 48 amino esters. Final hydrolysis of DEL B gave a library containing 100,270,080 members. It is worth noting that as libraries progress beyond the first cycle, it becomes difficult to quantify conversions, yields and byproduct formation with each reaction. Thus nearly all building blocks used were tested in representative validation experiments using a DNA-linked substrate prior to synthesis of the libraries. Only those building blocks which gave high conversions (>70%) were used in the synthesis of DELs A and B.

We first performed the selections against DEL-A using the conventional affinity selection protocol as described previously.[5] The library was incubated with either 1 μM of protein alone, protein with a known sEH inhibitor (AUDA) (Figure 2a), or with buffer only (the no-target control). Then, streptavidin affinity matrix resin was added to capture the protein with bound library. The unbound molecules were washed away. The heat denaturation method was used to elute binders from the protein and the affinity resin. The heat eluted binders were then incubated with fresh aliquots of target protein and two more rounds of selection were performed. After the third round of selection, we quantified the number of recovered DEL molecules by qPCR. In comparison with the no-target control (NTC) condition, a very high number of DEL molecules were observed in the sEH alone selection, while the sEH with AUDA condition provided 15-times fewer potential ligands (Supporting Information). This result indicated that the sEH inhibitor AUDA can competitively bind over some of the sEH binders from the DEL-A library. After removing all sequences that occurred in the selection without the target (NTC), we still observed a large number of binders with most represented in fewer than 2 copies. This made it difficult to decide which species to follow up with off-DNA synthesis.

We next turned to the selection of DEL-B against sEH. We predicted that titrating down the protein concentration would help define the binders. The selection was performed using the same method as above with different concentrations of sEH, 1 μM, 100 nM and 10 nM, along with no target control. A visualization of the selection output in a cubic plot (Figure 2b) showed that related structures were selected after removing the molecules with only one copy number. More potential ligands were observed at the higher concentration of sEH. However, some compounds that were enriched in the higher concentrations of sEH were also present in the 10 nM sEH sample. To prioritize the off-DNA follow-up, we focused on the binding events from 10 nM of sEH. After removing all sequence reads that occurred only once, only 4-(aminomethyl) benzoic acid was selected at cycle 4, which means this building block outwins all the other 47 aminoacids and very likely is critical for the binding of the target. Building block preferences at other cycles are visualized in a 3D scatter plot (Figure 2c). There were 2 highly populated planes along the cycle 3 axis, which corresponded to *cis*- 4-aminocyclohexanecarboxylic acid and its mixture of *cis*- and *trans*- isomers. Within each plane, there were several lines corresponding to selected cycle 3 amines, some of which were structurally similar. The appearance of lines indicated that there was little preference for the cycle 1 building block, suggesting that cycle 1 chemistry may contribute very little to the binding.

2.2. Hit confirmation

To confirm the selected features from DEL-B, a representative set of the fully elaborated library molecules were synthesized (Table 1) using the protocol in Scheme 1. The building blocks at cycle 1 were the descarboxylic analogues of the cycle 1 amino acids. Biochemical assay showed these compounds had inhibitory activity ranging from 11 nM ~ 73 nM. The complete depletion of cycle 1 building block led to dramatic loss of activity (Table 1, 4). However, activity was retained with methylamine at cycle 1 (Table 1, 5). This confirmed the presumptive selection result that cycle 1 was not necessary for affinity. Since cycle 1 was the position linked to the DNA, we hypothesized that it might be exposed to the solvent and could also be used as a handle to improve the

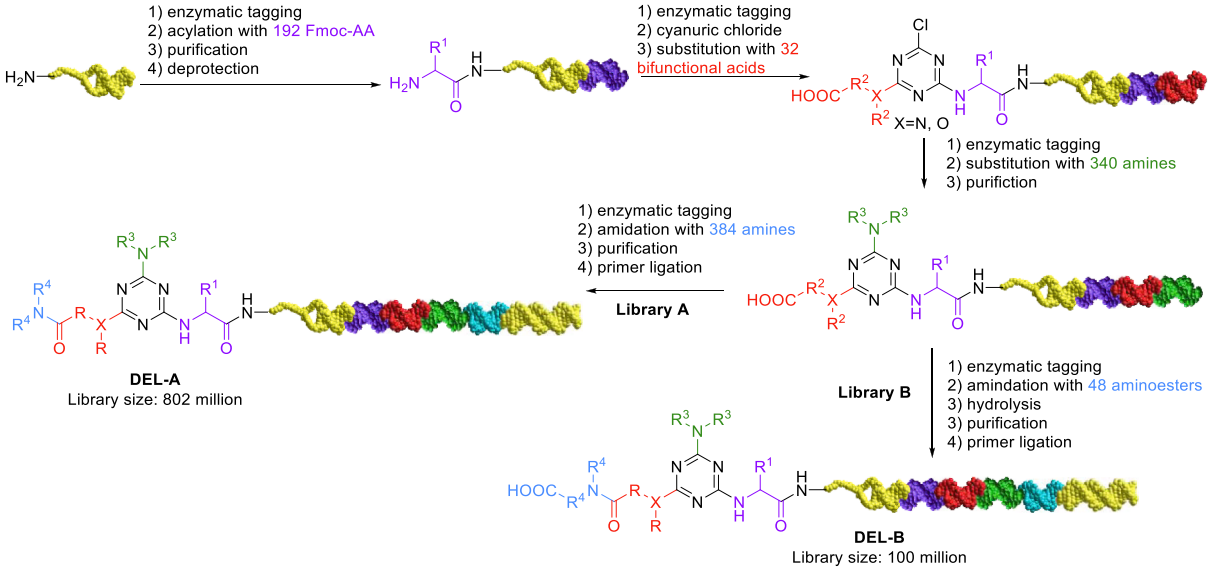


Figure 1. Synthesis of DEL-A & DEL-B.

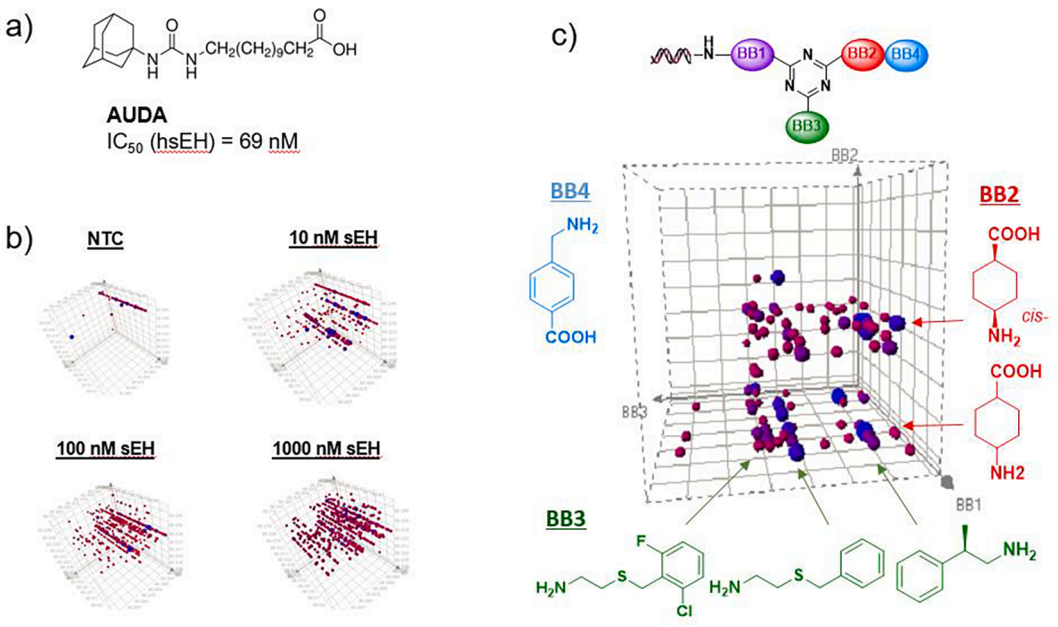
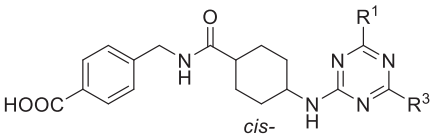


Figure 2. a) AUDA tool compound. b) Selection of library B against sEH. The selection output from NTC and under different concentrations of sEH. c) The cube corresponding to 4-(aminomethyl) benzoic acid as cycle 4 is shown in detail. The copy number of the selected species is indicated by the continuous color and size of the points (darker and bigger points indicate higher copy number). The structures shown represent the family of compounds defined by a line along the BB axis (arrow).

Table 1
Off-DNA hit confirmation.



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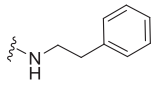
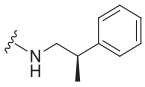
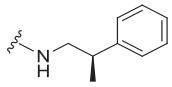
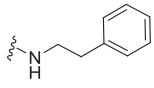
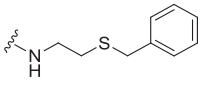
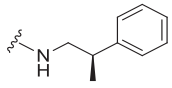
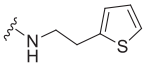
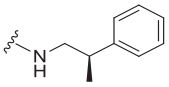
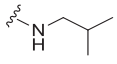
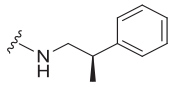
1			25	4	H		>5000
2			27	5	NHMe		24
3			39	6			38

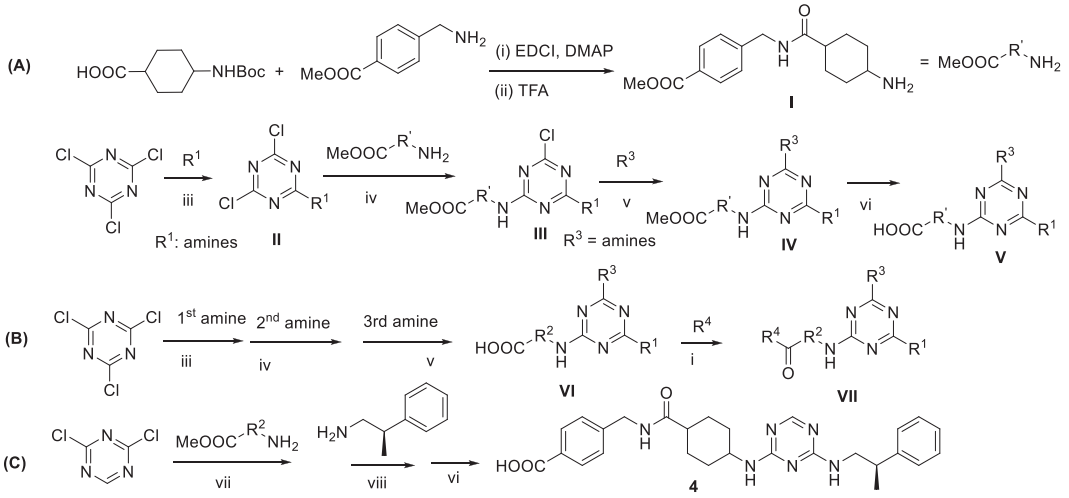
physicochemical properties of the inhibitors.

2.2.1. SAR at cycle 4 position

From the selection data of DEL-A, we did not observe obvious features with similar resolution as found in DEL-B. However, several cycle 4 amines were highly enriched, including (2-((trifluoromethyl)thiol) phenyl) methanamine and (2-(trifluoromethoxyl)phenyl) methanamine. A series of compounds with diversity at the cycle 4 position were synthesized following Scheme 1C. A substitution of 4-(aminomethyl) benzoic acid at cycle 4 with these two *ortho*-substituted benzylamines displayed similar activity (Table 2a, 7 & 8). With a trifluoromethyl group at the ortho position, compound **9** retained the activity. Trifluoromethyl substitution at the para position led to a 2-fold loss of activity (Table 2a, 10). With complete depletion of the substitution on the ring, compound **11** lost about 10-fold activity compared with the original acid synthon at the cycle 4 position. To understand why aromatic amines which were included in both libraries were not selected at the cycle 4 position, we synthesized the compound with 4-amino benzeneacetic acid (Table 2a, 12) at this position. The activity dropped >700-fold, which was consistent with the selection output.

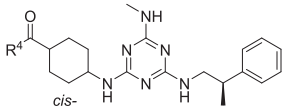
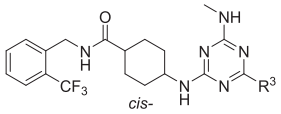
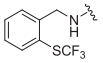
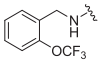
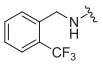
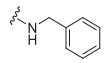
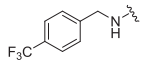
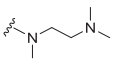
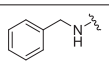
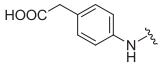
2.2.2. SAR at cycle 3 position

Considering the information inferred from the data cube and diversity of amines at cycle 3 being active, we further attenuated the cycle 3 structure. Substituting R^3 with piperidine, pyrrolidine and benzylamine yielded compounds that maintained similar excellent potency (Table 2b, 13, 14 & 15). This showed cycle 3, similar to cycle 1, was not critical for activity. These positions could then be modified to optimize the physical and pharmacokinetic properties of the chemotype. To increase the solubility of the compound, diamine derivatives,



Scheme 1. Synthesis of sEH off-DNA hits[a]. [a] Reagents and conditions: (i) amine (1 equiv), EDCI (1.25 equiv), DMAP (0.2 equiv), CH_2Cl_2, 0 °C – rt; (ii) TFA (50% in CH_2Cl_2), room temperature, 25 mins; (iii) amine (1 equiv), CH_3CN/H_2O (1/1), 1 N NaOH, pH 9 ~ 10, 0 °C; (iv) amine (1 equiv), CH_3CN/H_2O (1/1), 1 N NaOH, pH 9 ~ 10, room temperature 4 hrs-overnight; (v) amine (5 ~ 10 equiv), CH_3CN/H_2O (1/1), 80 °C; (vi) 1 N NaOH (50–100% v/v), RT 3 hrs - overnight; (vii) amine (1 equiv), DIEA (2.5 equiv), CH_3CN/H_2O (1/1), 0 °C ; (viii) amine (5 equiv), CH_3CN/H_2O (1/1), 80 °C for 2 hrs.

Table 2
SAR at a) cycle 4; b) cycle 3.

a)  *cis-*			b)  *cis-*		
Cmpd	R^4	IC_{50} (nM)	Cmpd	R^3	IC_{50} (nM)
7		38	13		31
8		29	14		19
9		39	15		36
10		63	16		16
11		273	17		9
12		19,000			

trimethylethane-1,2-diamine (Table 2b, 16) and 1-methylpiperazine (Table 2b, 17), were placed at the cycle 3 position. Both compounds showed an increase in the biochemical activity. As the piperazine derivative (Table 2b, 17) had the most potent activity (9 nM), we used that substitution at cycle 3 position for further SAR studies and hit-to-lead optimization.

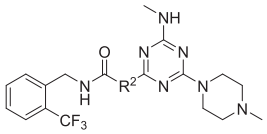
2.2.3. SAR at cycle 2 position

From the selection data, both the *cis-* and mixture of *cis-* and *trans-*stereoisomers of 4-aminocyclohexanecarboxylic acid were highly selected cycle 2 synthons. To determine the stereochemical preference at cycle 2, the *trans-*isomer of compound **5** was also prepared. Surprisingly, the *trans-*isomer was found to have the same potency as the *cis-*isomer. This suggested that the binding pocket for cycle 2 and cycle 4 was sufficient to tolerate further modification. To avoid the stereochemistry at the cycle 2 position, we modified the cycle 2 building block with aniline and piperidine analogs with 1-methylpiperazine at cycle 3. The potency of the aniline derivative (Table 3, 18) was approximately 4-fold lower, while the piperidine derivative (Table 3, 19) had comparable potency to the cyclohexyl analog (Table 3, 17). However, when azetidine was substituted at the cycle 2 position (Table 3, 20), the activity dropped dramatically. We further optimized the hits with diversity at the cycle 2 position (Table 3, 21-25). With different amine derivatives at cycle 2, such as benzylamine, 1,3 cyclohexyl amine, furanylmethanamine, tetrahydroquinoline and phenol, all compounds had single digit nanomolar activity.

2.2.4. CYP450 profile

CYP450 inhibition was also evaluated for compounds **16**–**19**. In general, the compounds showed a good CYP450 inhibition profile (Table 4). Activity was in the micromolar range across all targets tested, showing potency > 1000x lower for compounds **16**, **17**, and **19** and > 250x lower for compound **18** against CYP450s compared to sEH.

Table 3
SAR at cycle 2.



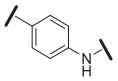
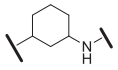
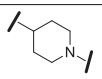
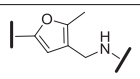
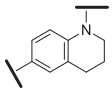
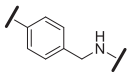
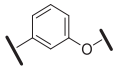
Cmpd	R²	IC_{50} (nM)	Cmpd	R²	IC_{50} (nM)
18		41	22		3
19		11	23		3
20		5,000	24		6
21		0.7	25		1

Table 4
P450 inhibition summary.

Cmpd	IC_{50} (μM)					
	1A2	2C9	2C19	2D6	3A4 (DEF)	3A4 (7BQ)
16	>33	32	>33	33	22	>33
17	>33	32	>33	>33	22	>33
18	>33	12	21	23	19	>33
19	>33	>33	>33	>33	>33	>33

3. Conclusion

In summary, employing DNA-encoded library technology, a series of potent sEH inhibitors were developed. Guided by selection output, extensive SAR studies and hit-to-lead optimization yielded a series of leads with diversity at cycle 2 and flexibility at both cycle 1 and cycle 3 positions. These positions were later utilized to improve oral bioavailability and physiochemical properties. Since few triazines have been developed as drugs,[25–26] and based on the unknown properties of this template, attempts to replace the triazine core with pyrimidine, pyridine, or phenyl were undertaken.[27] However, the triazine proved to be the optimal core from a potency and CYP450 standpoint. Among those triazine cores, 1,3-aminocycleohexanecarboxylate core **22** led to a drug candidate GSK2256294 as a novel anti-inflammatory for the treatment of COPD.[23] As a potent, selective, and orally available sEH inhibitor, GSK2256294 was well-tolerated with no serous adverse events attributable to the drug. These data support further investigation in patients with endothelial dysfunction or abnormal tissue repair.

4. Experimental section

4.1. Chemical synthesis

All reagents and solvents were of commercial quality and used without further purification unless indicated otherwise.

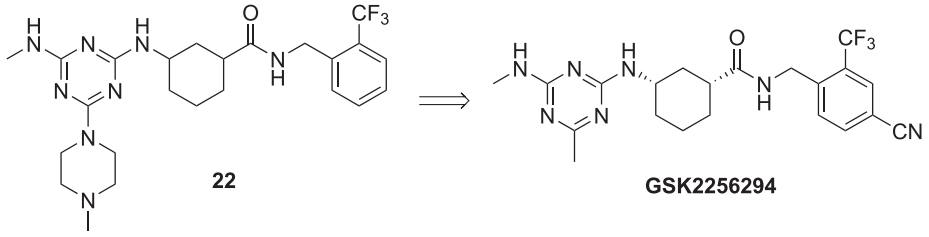
NMR spectra were recorded on a Varian Mercury 500. Chemical shifts are expressed in parts per million (ppm, δ units). Coupling constants (*J*) are in units of hertz (Hz). Splitting patterns describe apparent multiplicities and are designated as s (singlet), d (doublet), t (triplet), q (quartet), dd (double doublet), dt (double triplet), m (multiplet), br (broad). All mass spectra were performed under electrospray ionization (ESI) method. All the final compounds for biological assay were purified on Gilson system with a Phenomenex Luna 5 um C8(2), 100 mm × 21 mm 100A column with MeCN/H_2O (+0.1% TFA) solvents. High-resolution mass measurements were acquired on an Orbitrap Exactive Plus mass spectrometer (Thermo-Fisher) equipped with a heated electrospray source (HESI) and operated at 70 k resolution. HPLC utilized a Kinetex XB C18 column (Phenomenex, 1.7u, 2.1x50 mm at 30C), eluted with a 0.1% formic acid-acetonitrile gradient.

4.1.1. Synthesis of DNA-encoded libraries

The synthesis of library A has been reported previously.[5] Library B was synthesized similarly as library A from cycle 1–4. After pooling and precipitation with EtOH, the cycle 4 library material was further treated with 200 equivalents of NaOH to hydrolyze the ester prior to the final purification with reverse-phase HPLC.

4.1.2. Synthesis of compounds off-DNA.

General method A. A solution of 4-(Boc-amino)cyclohexanecarboxylic acid (2.0 g, 8.22 mmol, 1 equivalent), methyl 4-(aminomethyl)benzoate hydrochloride (1.89 g, 9.37 mmol, 1.14



equivalents) and *N*,*N*-dimethylamino pyridine (DMAP, 200.8 mg, 1.644 mmol, 0.2 equivalent) in methylene chloride (50 mL) was cooled with stirring in an ice bath. DIEA (1.79 mL, 10.275 mmol, 1.25 equivalents) was added, followed by addition of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI, 1.97 g, 10.275 mmol, 1.25 equivalents). The reaction mixture was stirred at 0 °C for 2 h and at room temperature for 3 h. The solution was diluted with methylene chloride (50 mL), which was further washed with saturated sodium bicarbonate, water, salt and dried over $MgSO_4$. The solvent was removed in vacuo to give the crude compound which was further purified by silica chromatography (60%-70% EtOAc in Hexane) to give acylation product (2.97 g, 92.5%). This compound (2.97 g) was treated with 50% TFA in dichloromethane (80 mL) at room temperature for 25 min. The reaction mixture was condensed to give a light-yellow oil which were the desired compound **I** as the TFA salt. MS: calcd for C16H22N2O3 + H^+ 291.16, found 291.11.

To a mixture of cyanuric chloride (1.22 mmol, 1 equivalent) in CH_3CN/H_2O (1/1, 2 mL) was cooled to 0 °C. A primary or secondary amine (1 equivalent) was added. The reaction mixture was adjusted to a pH of about 9–10 using 1 N NaOH. LC-MS was used to monitor the reaction. Without work-up and purification, the second amine (1 equivalent) was added to the reaction . The pH of the reaction solution was adjusted to 9 ~ 10 with 1 N NaOH and the reaction mixture was stirred at room temperature for 4 h. Then the third amine (10 equivalents) was added to the reaction mixture. The reaction was heated at 80 °C for 4 ~ 6 hrs monitored with LC-MS. Cooled to room temperature, the reaction solution was added 1 N NaOH (50% ~ 100% v/v) and stirred at room temperature for 3 h to overnight monitored with LC-MS to complete the hydrolysis of ester. The reaction mixture was concentrated in vacuo and the residue was purified with preparative HPLC to afford the final compound (**V**).

General method B. Following the general method A, 3 amines, one of which was free aminoacid, were added to cyanuric chloride subsequently. Then the acid was acylated with amine using EDCI/DMAP as described in general method A.

General method C. 2,4-Dichlorotriazine (96.6 mg, 0.612 mmol, 1 eq) was mixed with CH_3CN/H_2O (1/1, 6 mL) in an ice bath. Compound **I** as the TFA salt (272 mg, 0.673 mmol, 1.1 equivalents) was added. The pH of the reaction mixture was adjusted to about 9 by adding dropwise 1 N NaOH. After the addition, the reaction was determined to be complete based on LC-MS monitoring. Without work-up, the crude product was added second amine (5 equivalents). The reaction mixture was heated at 80 °C for 2–3 h. The reaction mixture was concentrated in vacuo and the residue was purified with preparative HPLC. To a solution of above purified compound (0.044 mmol, 1 equivalent) in MeOH (0.4 mL) was added 1 N NaOH (0.2 mL). The reaction mixture was stirred at room temperature for 2 to 3 h. The solution was neutralized with 1 N HCl and the crude product was purified with RP-HPLC.

4-((*cis*-4-((4-(phenethylamino)-6-(((R)-2-phenylpropyl)amino)-1,3,5-triazin-2-yl)amino)cyclohexane-1-carboxamido)methyl)benzoic acid (**1**). By use of (*R*)-(+)-beta-methylphenethylamine as R^1, compound **I** as R^2, and phenethylamine as R^3, the title compound was prepared by following general method A. The product was purified with RP-HPLC (Luna 5μ C8(2), 100x21mm, 25–80% CH_3CN/H_2O, 0.1% TFA, 20 min). HRMS [M + H]$^+$ calcd for C35H41N7O3 + H^+ 608.33436, found 608.33277. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ 1.26 (d, J = 7.02 Hz, 3H), 1.58–1.73 (m, 4H), 1.73–1.92 (m, 4H), 2.33–2.43 (m, 1H), 2.88 (t, J = 7.32 Hz, 2H), 3.10 (tq, J = 7.02 Hz, 1H), 3.42–3.49 (m, 1H), 3.52–3.60 (m, 3H), 4.01 (br. s, 1H), 4.37 (d, J = 5.80 Hz, 2H), 7.19–7.34 (m, 10*H*), 7.35–7.41 (m, 2H), 7.87–7.94 (m, 2H), 8.12 (br. t, J = 5.65 Hz, 1H).

4-((*cis*-4-((4-((2-(benzylthio)ethyl)amino)-6-(phenethylamino)-1,3,5-triazin-2-yl)amino)cyclohexane-1-carboxamido)methyl)benzoic acid (**2**). By use of S-benzylcysteamine hydrochloride as R^1, compound **I** as R^2, and phenethylamine as R^3, the title compound was prepared by following general method A. The product was purified with RP-HPLC (Luna 5μ C8(2), 100x21mm, 20–65% CH_3CN/H_2O, 0.1% TFA, 20 min). HRMS [M + H]$^+$ calcd for C35H41N7O3S + H^+ 640.30644, found 640.30497.

4-((*cis*-4-((4-((2-phenylpropyl)amino)-6-(((R)-2-(thiophen-2-yl)ethyl)amino)-1,3,5-triazin-2-yl)amino)cyclohexane-1-carboxamido)methyl)benzoic acid (**3**). By use of (*R*)-(+)-beta-methylphenethylamine as R^1, compound **I** as R^2, and 2-thiopheneethylamine as R^3, the title compound was prepared by following general method A. The product was purified with RP-HPLC (Luna 5μ C8(2), 100x21mm, 25–80% CH_3CN/H_2O, 0.1% TFA, 20 min). HRMS [M + H]$^+$ calcd for C33H39N7O3S + H^+ 614.29079, found 614.28956.

4-((*cis*-4-((4-(((R)-2-phenylpropyl)amino)-1,3,5-triazin-2-yl)amino)cyclohexane-1-carboxamido)methyl)benzoic acid (**4**). 2,4-Dichlorotriazine (96.6 mg, 0.612 mmol, 1 eq) was mixed with CH_3CN/H_2O (1/1, 6 mL) in an ice bath. Compound **I** as the TFA salt (272 mg, 0.673 mmol, 1.1 equivalents) was added. The pH of the reaction mixture was adjusted to about 9 by adding dropwise 1 N NaOH. After the addition, the reaction mixture determined to be complete based on LC-MS monitoring. Without work-up, the reaction mixture (1 mL, ~ 0.1 mmol) was added (*R*)-(+)-beta-methylphenethylamine (67.7 mg, 0.5 mmol, 5 equivalents). The reaction was heated at 80 °C for 2 hrs. The reaction mixture was concentrated in vacuo and the residue was purified with RP-HPLC (Luna 5μ C8(2), 100x21mm, 20–65% CH_3CN/H_2O, 0.1% TFA, 17 min) to give the desired ester product (2.2 mg). MS [M + H]$^+$ calcd for C28H34N6O3 + H^+ 503.27, found 503.39.

Methyl ester of compound 4 (2.2 mg) in MeOH (0.4 mL) was treated with 1 N NaOH (0.2 mL). The reaction mixture was stirred at room temperature for 2 to 3 hrs. The solution was neutralized with 1 N HCl and the crude product was purified with RP-HPLC (Luna 5μ C8(2), 100x21mm, 10–65% CH_3CN/H_2O, 0.1% TFA, 20 min). HRMS [M + H]$^+$ calcd for C27H32N6O3 + H^+ 489.26087, found 489.25929.

4-((*cis*-4-((4-(methylamino)-6-(((R)-2-phenylpropyl)amino)-1,3,5-triazin-2-yl)amino)cyclohexane-1-carboxamido)methyl)benzoic acid (**5**). By use of (*R*)-(+)-beta-methylphenethylamine as R^1, compound **I** as R^2, and methylamine as R^3, the title compound was prepared by following general method A. The product was purified with RP-HPLC (Luna 5μ C8(2), 100x21mm, 25–65% CH_3CN/H_2O, 0.1% TFA, 20 min). HRMS [M + H]$^+$ calcd for C28H35N7O3 + H^+ 518.28741, found 518.28618. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 1.26 (d, J = 7.02 Hz, 3H), 1.58–1.74 (m, 4H), 1.75–1.92 (m, 4H), 2.34–2.43 (m, 1H), 2.86 (br s, 3H), 3.06–3.15 (m, 1H), 3.40–3.50 (m, 1H), 3.51–3.62 (m, 1H), 4.02 (br s, 1H), 4.37 (d, J = 5.80 Hz, 2H), 7.22 (t, J = 6.80 Hz, 1H), 7.27 (d, J = 7.02 Hz, 1H), 7.32 (t, J = 7.63 Hz, 2H), 7.37 (d, J = 8.24 Hz, 2H), 7.90 (d, J = 8.24 Hz, H), 8.12 (br t, J = 5.65 Hz, 1H).

4-((*cis*-4-((4-(isobutylamino)-6-(((R)-2-phenylpropyl)amino)-1,3,5-triazin-2-yl)amino)cyclohexane-1-carboxamido)methyl)benzoic acid (**6**). By use of (*R*)-(+)-beta-methylphenethylamine as R^1, compound **I** as R^2, and ^{i}butylamine as R^3, the title compound was prepared by following general method A. The product was purified with RP-HPLC (Luna 5μ C8 (2), 100x21mm, 30–85% CH_3CN/H_2O, 0.1% TFA, 20 min). MS: calcd for C31H41N7O3 + H^+ 560.33, found 560.41.

cis-4-((4-(methylamino)-6-(((R)-2-phenylpropyl)amino)-1,3,5-triazin-2-yl)amino)-N-(2-((trifluoromethyl)thio)benzyl)cyclohexane-1-carboxamide (**7**). By use of (*R*)-(+)-beta-methylphenethylamine as first amine for chloro-substitution, *cis*-4-amino-cyclohexanecarboxylic acid as second amine for chloro-substitution, methylamine as third amine for substitution, and 2-(trifluoromethyl)thio-benzenemethanamine for acylation, the title compound was prepared by following general method B. HRMS [M + H]$^+$ calcd for C28H34F3N7OS + H^+ 574.25704, found 574.25602. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 1.26 (d, J = 7.02 Hz, 3H), 1.59–1.72 (m, 4H), 1.75–1.89 (m, 4H), 2.35–2.42 (m, 1H), 2.85 (br s, 3H), 3.39–3.61 (m, 2H), 4.02 (br. s, 1H), 4.55 (d, J = 5.80 Hz, 2H), 7.22 (t, J = 7.02 Hz, 1H), 7.27 (d, J = 7.32 Hz, 2H), 7.32 (t, J = 7.32 Hz, 2H), 7.43 (td, J = 7.63, 1.53 Hz, 1H), 7.49 (dd, J = 7.93, 0.92 Hz, 1H), 7.59 (td, J = 7.48, 1.22 Hz, 1H), 7.71 (d, J = 7.93 Hz, 1H), 8.06 (br t, J = 5.19 Hz, 1H).

cis-4-((4-(methylamino)-6-(((R)-2-phenylpropyl)amino)-1,3,5-triazin-2-yl)amino)-N-(2-(trifluoromethoxy)benzyl)cyclohexane-1-carboxamide (**8**). By use of (*R*)-(+)-beta-methylphenethylamine as first amine for chloro-substitution, *cis*-4-amino-cyclohexanecarboxylic acid as second amine for chloro-substitution, methylamine as third amine for substitution, and 2-(trifluoromethyl)-benzenemethanamine for acylation, the title compound was prepared by following general method B. HRMS $[M + H]^+$ calcd for $C28H34F3N7O2 + H^+$ 558.27988, found 558.27882. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 1.21–1.31 (m, 3H), 1.57–1.72 (m, 4H), 1.72–1.90 (m, 4H), 2.36–2.41 (m, 1H), 2.85 (br. s, 3H), 3.38–3.60 (m, 2H), 3.96–4.09 (m, 1H), 4.37 (d, $J = 5.80$ Hz, 2H), 7.22 (m, 1H), 7.27 (m, 2H), 7.30–7.36 (m, 3H), 7.36–7.44 (m, 3H), 8.00–8.07 (m, 1H). ^{1}H NMR (500 MHz, DMSO-d_6 & D_2O) δ ppm 1.19 (d, $J = 7.02$ Hz, 3H), 1.57–1.78 (m, 8H), 2.33 (m, 1H), 2.78 (br. s, 3H), 3.01 (m, 1H), 3.38 (m, 1H), 3.49 (m, 1H), 4.31 (br. s, 2H), 7.16 (m, 1H), 7.19 (m, 2H), 7.26 (m, 3H), 7.30–7.34 (m, 3H).

cis-4-((4-(methylamino)-6-(((R)-2-phenylpropyl)amino)-1,3,5-triazin-2-yl)amino)-N-(2-(trifluoromethyl)benzyl)cyclohexane-1-carboxamide (**9**). By use of (*R*)-(+)-beta-methylphenethylamine as first amine for chloro-substitution, *cis*-4-amino-cyclohexanecarboxylic acid as second amine for chloro-substitution, methylamine as third amine for substitution, and 2-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS $[M + H]^+$ calcd for $C28H34F3N7O + H^+$ 542.28497, found 542.28375.

cis-4-((4-(methylamino)-6-(((R)-2-phenylpropyl)amino)-1,3,5-triazin-2-yl)amino)-N-(4-(trifluoromethyl)benzyl)cyclohexane-1-carboxamide (**10**). By use of (*R*)-(+)-beta-methylphenethylamine as first amine for chloro-substitution, *cis*-4-amino-cyclohexanecarboxylic acid as second amine for chloro-substitution, methylamine as third amine for substitution, and 4-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS $[M + H]^+$ calcd for $C28H34F3N7O + H^+$ 542.28497, found 542.28412. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 1.26 (d, $J = 7.02$ Hz, 3H), 1.58–1.73 (m, 4H), 1.74–1.89 (m, 4H), 2.34–2.41 (m, 1H), 2.85 (s, 3H), 3.38–3.49 (m, 1H), 3.49–3.60 (m, 1H), 3.97–4.08 (m, 1H), 4.38 (d, $J = 5.80$ Hz, 2H), 7.22 (tt, $J = 7.02$, 1.53 Hz, 1H), 7.27 (d, $J = 7.02$ Hz, 2H), 7.32 (t, $J = 7.63$ Hz, 2H), 7.48 (d, $J = 7.93$ Hz, 2H), 7.67 (d, $J = 8.24$ Hz, 2H), 8.11–8.18 (m, 1H).

cis-N-benzyl-4-((4-(methylamino)-6-(((R)-2-phenylpropyl)amino)-1,3,5-triazin-2-yl)amino)cyclohexane-1-carboxamide (**11**). By use of (*R*)-(+)-beta-methylphenethylamine as first amine for chloro-substitution, *cis*-4-amino-cyclohexanecarboxylic acid as second amine for chloro-substitution, methylamine as third amine for substitution, and benzylamine for acylation, the title compound was prepared by following general method B. HRMS $[M + H]^+$ calcd for $C27H35N7O + H^+$ 474.29759, found 474.29698. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 1.26 (d, $J = 7.02$ Hz, 3H), 1.58–1.71 (m, 4H), 1.81 (br. d, $J = 5.49$ Hz, 4H), 2.32–2.39 (m, 1H), 2.85 (br s, 3H), 3.41–3.62 (m, 2H), 4.02 (m, 1H), 4.31 (d, $J = 6.10$ Hz, 2H), 7.19–7.35 (m, 10*H*), 7.96–8.05 (m, 1H).

cis-4-((4-(methylamino)-6-(piperidin-1-yl)-1,3,5-triazin-2-yl)amino)-N-(2-(trifluoromethyl)benzyl)cyclohexane-1-carboxamide (**13**). By use of *cis*-4-amino-cyclohexanecarboxylic acid as first amine for chloro-substitution, methylamine as second amine for chloro-substitution, piperidine as third amine for substitution, and 2-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS $[M + H]^+$ calcd for $C24H32F3N7O + H^+$ 492.26932, found 492.26826. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 1.47–1.55 (m, 4H), 1.59–1.66 (m, 6H), 1.75–1.91 (m, 4H), 2.33–2.42 (m, 1H), 2.79 (d, $J = 4.27$ Hz, 3H), 3.65–3.74 (m, 4H), 4.00 (br. s, 1H), 4.49 (d, $J = 5.80$ Hz, 2H), 7.47 (t, $J = 7.63$ Hz, 1H), 7.51 (d, $J = 7.63$ Hz, 1H), 7.65 (t, $J = 7.32$ Hz, 1H), 7.70 (d, $J = 7.93$ Hz, 1H), 7.99–8.08 (m, 1H).

cis-4-((4-(methylamino)-6-(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)amino)-N-(2-(trifluoromethyl)benzyl)cyclohexane-1-carboxamide (**14**). By use of *cis*-4-amino-cyclohexanecarboxylic acid as first amine for chloro-substitution, methylamine as second amine for chloro-substitution, pyrrolidine as third amine for substitution, and 2-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS $[M + H]^+$ calcd for $C23H30F3N7O + H^+$ 478.25367, found 478.25266. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 1.68 (m, 4H), 1.83 (m, 4H), 1.94 (m, 4H), 2.41 (m, 1H), 2.86 (br. s, 3H), 3.53 (m, 4H), 4.09 (br. s, 1H), 4.49 (d, $J = 5.80$ Hz, 2H), 7.48 (t, $J = 7.93$ Hz, 1H), 7.52 (d, $J = 8.24$ Hz, 1H), 7.65 (t, $J = 7.63$ Hz, 1H), 7.70 (d, $J = 7.93$ Hz, 1H), 8.06–8.15 (m, 1H).

cis-4-((4-(benzylamino)-6-(methylamino)-1,3,5-triazin-2-yl)amino)-N-(2-(trifluoromethyl)benzyl)cyclohexane-1-carboxamide (**15**). By use of *cis*-4-amino-cyclohexanecarboxylic acid as first amine for chloro-substitution, methylamine as second amine for chloro-substitution, benzylamine as third amine for substitution, and 2-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS $[M + H]^+$ calcd for $C26H30F3N7O + H^+$ 514.25367, found 514.25275.

cis-4-((4-((2-(dimethylamino)ethyl)amino)-6-(methylamino)-1,3,5-triazin-2-yl)amino)-N-(2-(trifluoromethyl)benzyl)cyclohexane-1-carboxamide (**16**). By use of *cis*-4-amino-cyclohexanecarboxylic acid as first amine for chloro-substitution, methylamine as second amine for chloro-substitution, N,N,N'-trimethylethylenediamine as third amine for substitution, and 2-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS $[M + H]^+$ calcd for $C24H35F3N8O + H^+$ 509.29587, found 509.29506. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 1.61–1.73 (m, 4H), 1.77–1.91 (m, 4H), 2.37–2.47 (m, 1H), 2.86 (s, 3H), 2.89 (s, 6H), 3.12 (s, 3H), 3.35 (t, $J = 6.41$ Hz, 2H), 3.89–3.96 (m, 2H), 4.06 (br. s, 1H), 4.49 (br. d, $J = 5.49$ Hz, 2H), 7.47 (t, $J = 7.63$ Hz, 1H), 7.52 (d, $J = 7.63$ Hz, 1H), 7.65 (t, $J = 7.32$ Hz, 1H), 7.70 (d, $J = 7.93$ Hz, 1H), 8.09 (br. t, $J = 5.04$ Hz, 1H).

cis-4-((4-(methylamino)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-yl)amino)-N-(2-(trifluoromethyl)benzyl)cyclohexane-1-carboxamide (**17**). By use of *cis*-4-amino-cyclohexanecarboxylic acid as first amine for chloro-substitution, methylamine as second amine for chloro-substitution, *N*-methylpiperazine as third amine for substitution, and 2-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS $[M + H]^+$ calcd for $C24H33F3N8O + H^+$ 507.28022, found 507.27936. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 1.66 (m, 4H), 1.84 (m, 4H), 2.41 (m, 1H), 2.80–2.91 (m, 6H), 3.27 (br, s, 4H), 3.70–4.29 (m, 5H), 4.49 (br. d, $J = 5.19$ Hz, 2H), 7.47 (t, $J = 7.63$ Hz, 1H), 7.52 (br. d, $J = 7.63$ Hz, 1H), 7.65 (t, $J = 7.32$ Hz, 1H), 7.70 (br. d, $J = 7.90$ Hz, 1H), 8.08 (br s, 1H).

4-((4-(methylamino)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-yl)amino)-N-(2-(trifluoromethyl)benzyl)benzamide (**18**). By use of methylamine as first amine for chloro-substitution, 4-amino benzoic acid as second amine for chloro-substitution, *N*-methylpiperazine as third amine for substitution, and 2-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS $[M + H]^+$ calcd for $C24H27F3N8O + H^+$ 501.23327, found 501.23255. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 2.87 (m, 6H), 3.15–3.42 (m, 4H), 4.70 (d, $J = 5.49$ Hz, 2H), 4.55–4.8 (m, 4H), 7.48 (t, $J = 7.70$ Hz, 1H), 7.58 (d, $J = 7.63$ Hz, 1H), 7.64 (t, $J = 7.48$ Hz, 1H), 7.72 (d, $J = 7.63$ Hz, 1H), 7.78–7.93 (m, 4H), 8.65 (br. t, $J = 5.65$ Hz, 1H).

1-(4-(methylamino)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-yl)-N-(2-(trifluoromethyl)benzyl)piperidine-4-carboxamide (**19**). By use of methylamine as first amine for chloro-substitution, methyl piperidine-4-carboxylate as second amine for chloro-substitution, *N*-methylpiperazine as third amine for substitution, and 2-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS $[M + H]^+$ calcd for $C23H31F3N8O + H^+$ 493.26457, found 493.2637. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 1.55 (qd, $J = 12.10$, 4.27 Hz, 2H), 1.81 (br. dd, $J = 12.97$, 2.90 Hz, 2H), 2.56 (tt, $J = 11.60$, 3.66 Hz, 1H), 2.81 (s, 3H), 2.85 (s, 3H), 2.92 (td, $J = 12.82$, 2.44 Hz, 2H), 3.10–3.42 (m, 4H), 3.75 – 4.20 (br, 4H), 4.48 (d, $J = 5.80$ Hz, 2H), 4.61 (br. d, $J = 13.12$ Hz, 2H), 7.47 (t, $J = 7.63$ Hz, 1H), 7.51 (d, $J = 7.63$ Hz, 1H), 7.65 (t, $J = 7.93$ Hz, 1H), 7.70 (d, $J = 7.93$ Hz, 1H),

8.16 (br. t, $J = 5.49$ Hz, 1H).

1-(4-(methylamino)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-yl)-N-(2-(trifluoromethyl)benzyl)azetidine-3-carboxamide (**20**). By use of methylamine as first amine for chloro-substitution, azetidine-3-carboxylic acid as second amine for chloro-substitution, *N*-methylpiperazine as third amine for substitution, and 2-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS $[M + H]^+$ calcd for C21H27F3N8O + H^+ 465.23327, found 465.23241.

4-(((4-(methylamino)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-yl) amino)methyl)-N-(2-(trifluoromethyl)benzyl)benzamide (**21**). By use of methylamine as first amine for chloro-substitution, 4-aminomethyl benzoic acid as second amine for chloro-substitution, *N*-methylpiperazine as third amine for substitution, and 2-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS: calcd for C25H29F3N8O + H^+ 515.24892, found 515.24837. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 2.80 (s, 3H), 2.83 (s, 3H), 3.10 – 3.30 (m, 8H), 4.54 (br. d, $J = 4.27$ Hz, 2H), 4.69 (d, $J = 5.49$ Hz, 2H), 7.43 (d, $J = 7.93$ Hz, 2H), 7.48 (t, $J = 7.63$ Hz, 1H), 7.56 (d, $J = 7.93$ Hz, 1H), 7.64 (t, $J = 7.32$ Hz, 1H), 7.73 (d, $J = 7.93$ Hz, 1H), 7.87 (d, $J = 8.24$ Hz, 2H), 8.76 (br. t, $J = 5.65$ Hz, 1H).

3-((4-(methylamino)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-yl) amino)-N-(2-(trifluoromethyl)benzyl)cyclohexane-1-carboxamide (**22**). A mixture of cyanuric chloride (3.86 g, 20.9 mmol, 1equivalent) in CH_3CN/H_2O (1/1, 200 mL) was cooled to 0 °C. Methylamine solution in water (40% w, 1.83 mL, 20.9 mmol, 1 equivalent) was added, followed by adding 1 N NaOH (20.9 mL, 20.9 mmol, 1 equivalent). The reaction mixture was stirred at cold for 15 min and LC-MS showed the reaction has completed. At cold, *N*-methylpiperazine (2.3 mL, 20.9 mmol, 1 equivalent) was added, followed by adding 1 N NaOH (4.2 mL, 0.2 equivalents). The solution turned to clear. The solvent was evaporated to give the crude product which was further purified with silica gel chromatography (eluant 70% B, solvent B is 10% MeOH in DCM with 1% Et3N) to give 4-Chloro-*N*-methyl-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-amine (1.8 g, 36% in 2 steps). ^{1}H NMR ($CDCl_3$, 400 MHz, mixture of rotamers): δ 2.33 (s, 3H), 2.44 (br, 4H), 2.95&2.96 (s, 3H), 3.86 (br, 4H), 5.1–5.7 (br, 1H).

To a solution of 3-(Boc-amino)cyclohexanecarboxylic acid (500 mg, 2.055 mmol, 1 equivalent) in dimethylformamide (10 mL) was added O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluoropho sphate (HATU, 781 mg, 2.055 mmol, 1 equivalent) and diisopropylethylamine (0.716 mL, 4.11 mmol, 2 equivalents). 2-trifluoromethyl benzylamine (360 mg, 2.055 mmol, 1 equivalent) was added and the reaction mixture was stirred at room temperature overnight. Additional diisopropylethylamine (0.358 mL, 2.055 mmol, 1 equivalent) was added and the reaction was stirred at room temperature for 2 h. The solvent was evaporated to give the crude product which was further purified with silica gel chromatography (eluant 40% Ethyl Acetate in Hexanes). The organic layer was dried over MgSO4, filtered and concentrated in vacuo. The resulting compound was re-purified with RP-HPLC. The purified compound was treated with 50% TFA in DCM (20 mL) at room temperature for 2 h, and then the solvents were removed in vacuo. The desired product was carried onto the next step without further purification. MS: calcd for C15H19F3N2O + H^+ 301.16, found 301.06.

The solution of 4-chloro-*N*-methyl-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-amine(30.0 mg, 0.124 mmol, 1 equivalent) and 3-amino-N-(2-(trifluoromethyl)benzyl)cyclohexanecarboxamide (103 mg, 0.248 mmol, 2 equivalents) in CH_3CN/H_2O (1/1, 2 mL) was adjusted to pH 9–10 with 1 N NaOH. The resulting solution was heated at 80 °C for 16 h. The solvent was removed under vacuo to give the crude which was purified with RP-HPLC to give the desired compound (0.94 mg, 1.5%). HRMS: calcd for C24H33F3N8O + H^+ 507.28022, found 507.27994. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 1.20–1.54 (m, 4H), 1.83 (m, 2H), 1.95 (m, 1H), 2.07 (br. d, $J = 12.21$ Hz, 1H), 2.46 (m, 1H), 2.77 (br. s, 3H), 2.91 (s, 3H), 3.05 – 3.65 (br, 8H), 3.82–3.90 (m, 1H), 4.47 (br. d, $J = 5.49$ Hz, 2H), 7.47 (t, $J = 7.32$ Hz, 1H), 7.51 (d, $J = 7.93$ Hz, 1H), 7.65 (t, $J = 7.63$ Hz, 1H), 7.69 (d, $J = 7.93$ Hz, 1H), 8.19 (br s, 1H).

5-methyl-4-(((4-(methylamino)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-yl)amino)methyl)-N-(2-(trifluoromethyl)benzyl)furan-2-carboxamide (**23**). By use of methylamine as first amine for chloro-substitution, 4-aminomethyl-5-methyl-furan-2-carboxylic acid as second amine for chloro-substitution, *N*-methylpiperazine as third amine for substitution, and 2-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS: calcd for C24H29F3N8O2 + H^+ 519.24383, found 519.24344. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 2.37 (s, 3H), 2.81 (s, 3H), 2.83 (s, 3H), 2.95–3.50 (m, 4H), 3.90–4.20 (m, 4H), 4.26–4.30 (m, 2H), 4.62 (d, $J = 5.80$ Hz, 2H), 7.06 (s, 1H), 7.47 (t, $J = 7.93$ Hz, 1H), 7.51 (d, $J = 7.93$ Hz, 1H), 7.63 (t, $J = 7.93$ Hz, 1H), 7.71 (d, $J = 7.93$ Hz, 1H), 8.48 (br. t, $J = 5.80$ Hz, 1H)

1-(4-(methylamino)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-yl)-N-(2-(trifluoromethyl)benzyl)-1,2,3,4-tetrahydroquinoline-6-carboxamide (**24**). By use of methylamine as first amine for chloro-substitution, 1,2,3,4-tetrahydroquinoline-6-carboxylic acid as second amine for chloro-substitution, *N*-methylpiperazine as third amine for substitution, and 2-trifluoromethyl benzylamine for acylation, the title compound was prepared by following general method B. HRMS: calcd for C27H31F3N8O + H^+ 541.26457, found 541.26408. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 1.95 (quin, $J = 6.33$ Hz, 2H), 2.79–2.90 (m, 8H), 3.11–3.46 (m, 4H), 4.00 (br. t, $J = 5.95$ Hz, 2H), 4.10 – 4.45 (br. s, 4H), 4.70 (d, $J = 5.49$ Hz, 2H), 7.48 (t, $J = 7.63$ Hz, 1H), 7.58 (d, $J = 7.63$ Hz, 1H), 7.65 (t, $J = 7.93$ Hz, 1H), 7.69 (dd, $J = 8.85$, 2.14 Hz, 1H), 7.71–7.76 (m, 2H), 7.96 (br. d, $J = 8.24$ Hz, 1H), 8.68 (t, $J = 5.80$ Hz, 1H).

3-((4-(methylamino)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-yl) oxy)-N-(2-(trifluoromethyl)benzyl)benzamide (**25**). To a solution of 3-hydroxybenzoic acid (1.0 g, 7.24 mmol, 1 equivalent) in 75 mL dimethylformamide was added *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU, 2.75 g, 7.24 mmol, 1 equivalent) and diisopropylethylamine (2.52 mL, 14.48 mmol, 2 equivalents). Immediately, (2-(trifluoromethyl)phenyl)methenamine was added (1.27 g, 7.24 mmol, 1 equivalent). The reaction mixture was stirred at room temperature for 1 h. The reaction solution was condensed and the residue was purified with silica chromatography (eluant 60% EtOAc), followed with RP-HPLC (Luna 5μ C8(2), 100x21mm, 20–60% CH_3CN/H_2O, 0.1% TFA, 20 min) to yield acylation product 3-hydroxy-N-(2-(trifluoromethyl)benzyl)benzamide (1.04 g, 49%). MS: calcd for C15H12F3NO2 + H^+ 296.09, found 295.94.

By use of methylamine for the first chloro-substitution, 3-hydroxy-N-(2-(trifluoromethyl)benzyl)benzamide for the second chloro-substitution, and *N*-methylpiperazine for the last chloro-substitution, the title compound was prepared by following general method A. HRMS $[M + H]^+$ calcd for C24H26F3N7O2 + H^+ 502.21728, found 502.21672. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ ppm 2.71–2.88 (m, 6H), 4.70 (br. d, $J = 5.80$ Hz, 2H), 7.21–7.30 (m, 1H), 7.35 (m, 1H), 7.46–7.55 (m, 2H), 7.58 (d, $J = 7.93$ Hz, 1H), 7.65 (t, $J = 7.93$ Hz, 1H), 7.73 (br. d, $J = 7.63$ Hz, 1H), 7.80 (d, $J = 7.93$ Hz, 1H), 8.87 (m, 1H).

4.2. Affinity selection.

The selection of sEH specific binders was done using conventional "in-solution" selection protocol as reported.[5] Different concentrations of purified dual-tagged sEH protein were incubated with ELT libraries in 1X Selection Buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1 mg/ml of salmon sperm DNA (Invitrogen, AM9680) for 1 h. The protein with bound ELT molecules was captured on streptavidin affinity matrix resin and washed several times with selection buffer. Bound library members were eluted from the protein by denaturation. The eluted molecules were subjected to a second and third rounds of selection with fresh protein at each round. The selections, described herein, were performed against 0.01 μM, 0.1 μM, 1 μM hsEH protein and no protein control (selection buffer only) in the presence and absence of 1 μM

AUDA. The outputs from the third selection round were PCR amplified, sequenced, translated into chemical structures and analyzed using TIBCO Spotfire software as described previously.[5]

4.3. Assay against human recombinant sEH

The biological activity of the compounds was measured using the nonfluorescent EnzChek epoxide hydrolase substrate (Molecular Probes, Catalog # E33956). Assay was performed in the buffer containing 50 mM HEPES (pH 8). The compounds were re-suspended in 100% DMSO and then serially diluted in the assay buffer (final concentration of DMSO 0.8%). 10 nM hsEH protein was pre-incubated with compounds for 5 min at room temperature, followed with addition of diluted EnzChek substrate (final concentration 10 μM). The reaction was run at room temperature, with fluorescence being measured every 30 s for 30 min at an excitation of 350 nm and an emission of 455 nm.

The activity of the compounds was calculated as the % of inhibition using the following equation: 100*(1-(slope of enzyme + inhibitor/slope of enzyme alone) and expressed as IC_{50} values (the concentration of inhibitor that blocks enzyme activity to 50%).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2021.116216.

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