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Application of encoded library technology (ELT) to a protein–protein interaction target: Discovery of a potent class of integrin lymphocyte function-associated antigen 1 (LFA-1) antagonists

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ABSTRACT

The inhibition of protein–protein interactions remains a challenge for traditional small molecule drug discovery. Here we describe the use of DNA-encoded library technology for the discovery of small molecules that are potent inhibitors of the interaction between lymphocyte function-associated antigen 1 and its ligand intercellular adhesion molecule 1. A DNA-encoded library with a potential complexity of 4.1 billion compounds was exposed to the I-domain of the target protein and the bound ligands were affinity selected, yielding an enriched small-molecule hit family. Compounds representing this family were synthesized without their DNA encoding moiety and found to inhibit the lymphocyte function-associated antigen 1/intercellular adhesion molecule-1 interaction with submicromolar potency in both ELISA and cell adhesion assays. Re-synthesized compounds conjugated to DNA or a fluorophore were demonstrated to bind to cells expressing the target protein.

1. Introduction

Targeting small molecule drugs to the interfaces between proteins has great therapeutic potential, but remains a challenge for traditional high-throughput screening drug discovery efforts. Three quarters of marketed pharmaceuticals target either enzymes or G-protein coupled receptors target classes known for containing classical ligand pockets that are amenable to traditional drug discovery efforts.[1] Most protein–protein interactions (PPI) occur over large, flat surface areas that have proved more difficult to target than classical ligand binding pockets.[2,3]

Integrin LFA-1 (lymphocyte function-associated antigen-1) is a major leukocyte cell adhesion molecule that binds to its major ligand ICAM-1 (intercellular adhesion molecule-1) on endothelial cells and dendritic cells.[4] LFA-1 plays a pivotal role in regulating leukocyte trafficking to sites of inflammation as well as in inducing immune responses, thereby representing an established therapeutic target for autoimmune and inflammatory diseases.[5] A therapeutic antibody to LFA-1, efalizumab (Raptiva™) has been demonstrated to be effective for the treatment of patients with psoriasis, a T-cell mediated autoimmune disease in the skin.[6] In addition, LFA-1 inhibitors are currently being investigated in clinical trials for the treatment of uveitis[7] and other ocular inflammation.[8]

The ligand binding domain of LFA-1 termed inserted (I) domain adopts a Rossmann fold that contains a metal ion-dependent adhesion site (MIDAS) located on the top, whereas its C- and N-terminal connections are located on the distal bottom face.[9] The ability of the I domain to bind ligand is regulated by conformational changes, as the affinity for its ligand is dramatically enhanced by a 'piston-like' downward axial displacement of its C-terminal helix.

Abbreviations: ELT, encoded library technology; LFA-1, lymphocyte function-associated antigen 1; ICAM-1, intercellular adhesion molecule 1; PPI, protein–protein interactions.

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0968-0896/

The C-terminal downward shift is conformationally linked to the conversion of the MIDAS to the high-affinity configuration that tightly binds to ligand. Since only the high-affinity form tightly binds to ICAM-1, perturbation of the activation-dependent conformational conversion of the LFA-1 I domain to the high-affinity form has been an important target for small-molecule drugs.[5] A class of potent small-molecule antagonists to LFA-1, termed alpha I allosteric antagonists, have been reported that bind to the cavity underneath the C-terminal helix of the I domain, thereby stabilizing the low-affinity LFA-1 conformation.[10–12] Thus far, the small-molecule antagonists that bind to the I domain were selected from a conventional random screening based on the results of functional assays that tested the ability of candidate compounds to block LFA-1-ICAM-1 interaction. Despite the critical role of the I domain in ligand binding, selection of antagonists by explicitly targeting the I domain has limited investigation.[13]

We have previously described encoded library technology (ELT) as a novel hit identification technology that includes the synthesis of combinatorial DNA-encoded libraries (DELs) containing 10^6–10^{10} members, the interrogation of those libraries for ligands by affinity selection, hit structure deconvolution through DNA sequencing and finally the resynthesis of small molecule hits off-DNA for activity confirmation.[14,15] We have also previously used ELT to identify inhibitors of Aurora A and p38 MAP kinases,[15] ADAMTS-5 metalloprotease[16] and sirtuins.[17] While encoded libraries have been reportedly used to discover inhibitors of protein–protein interactions, to our knowledge none of these inhibitors have demonstrated sub-micromolar potency.[18–20] With the intention of expanding the applications of ELT to PPIs, we launched a campaign to identify specific small molecule inhibitors of the LFA-1/ICAM-1 interaction by targeting the I domain, and we report here the first use of the technology to discover a potent class of small molecules that block the interaction of LFA-1 with its ligand ICAM-1.

2. Results and discussion

2.1. ELT selection and data analysis

Affinity selections were performed against soluble LFA-1 I-domain after chemical biotinylation. The target protein was immobilized on a streptavidin agarose resin packed column, and then exposed to the on-DNA library prior to extensive washing to remove non-binders. Heat denaturation of the protein was used to elute bound library molecules and the eluant was incubated with fresh protein to start a new round of selection. A parallel selection against a column lacking protein was done to allow for the identification of molecules enriched by non-target affinity. After three rounds of selection the eluted population was amplified and sequenced, and the sequences translated to identify small molecule binders to the LFA-1 I-domain. Only sequences that were unique and constituted a complete translatable ELT encoding were used for the analysis. The no target sample sequencing yielded 46,476 such sequences and the target sample gave 268,586. Data analysis yielded a few attractive features (chemically related enriched compounds that share one or more building blocks) across a number of ELT libraries. Most of the selected features were confirmed to be active inhibitors after off-DNA synthesis. In this paper, we report on one feature identified from a triazine library. Successful ELT campaigns using DNA-encoded libraries (DELs) incorporating 1,3,5-triazine as a core structure have been disclosed previously.[15,16] A schematic drawing of the desired triazine library is shown as DEL-A in Figure 1. DEL-A utilizes 192 Fmoc protected amino acids coupled with the amine moiety of the desired ELT headpiece as cycle 1, followed by Fmoc deprotection and functionalization of the amine group of the cycle 1 with cyanuric chloride. The triazine core is decorated with 479 amines as cycle 2. Further elaboration of the triazine moiety with 96 diamines as cycle 3 followed by further decoration of the cycle 3 with 459 amine-capping building blocks (carboxylic acids, aldehydes, sulfonyl chlorides and isocyanates) as cycle 4 generated a library with a theoretical complexity of 4.1 billion compounds. The synthesis details of this library have been previously disclosed.[15]

As previously described, the output of an ELT selection is conveniently visualized by a cubic scatter plot representation in which each axis represents building blocks used in a individual cycle in the library and the size of the data point represents the number of times each unique DNA sequence was observed.[15] Thus, after filtering out members of the library that are lowly represented or considered background because of presence in a no target selection, enriched chemotypes will appear as planes (one building block in common) or lines (two building in common). For a four cycle library such as DEL-A, two plots are required to visualize the complete selection. Representative plots that were used to analyze and visualize the selection outcome and the highly selected enriched families/features are shown in Figure 2. The selected feature is demonstrated by a plane in the cube analysis. This feature's enrichment is driven specifically by a single building block from cycle 1 (BB1), 2-amino-2,3-dihydro-1*H*-indene-2-carboxylic acid (**1**), which was attached to the DNA through its carboxylic acid functional group. The combination of the amino acid **1** and the triazine core represent the selected pharmacophore **6**. Within the selected plane, there are four prominent horizontal lines; an indication of preference for four different BB2s. There is a great similarity within these selected BB2s (compounds **2** through **5**) a good indication of some preliminary internal SAR for selection at cycle 2. Cycles 3 and 4 appear to be variable based on the lack of selection for specific building blocks as indicated by the lack of preference for spots within each line.

2.2. Chemistry

In preparation for off-DNA synthesis and activity confirmation a few spots within the selected lines were chosen for follow up. The synthetic strategy was designed to generate the pharmacophore earlier in the synthesis and add on the variable components later (Scheme 1). This allowed for testing the intermediate in the activity assay and therefore additional SAR exploration by testing the possible fragments and intermediates. Off-DNA synthesis initiated with ethylamine coupling with Boc-protected acid **7** and Boc removal of the desired product to afford amine **8**. We chose the most

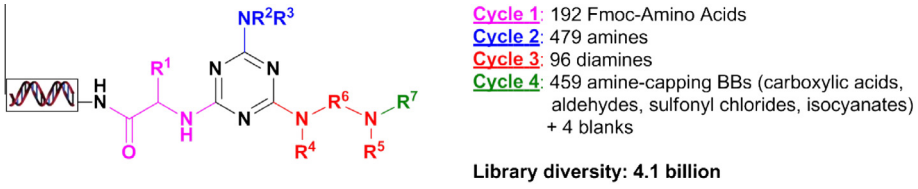


Figure 1. Design of DEL-A.

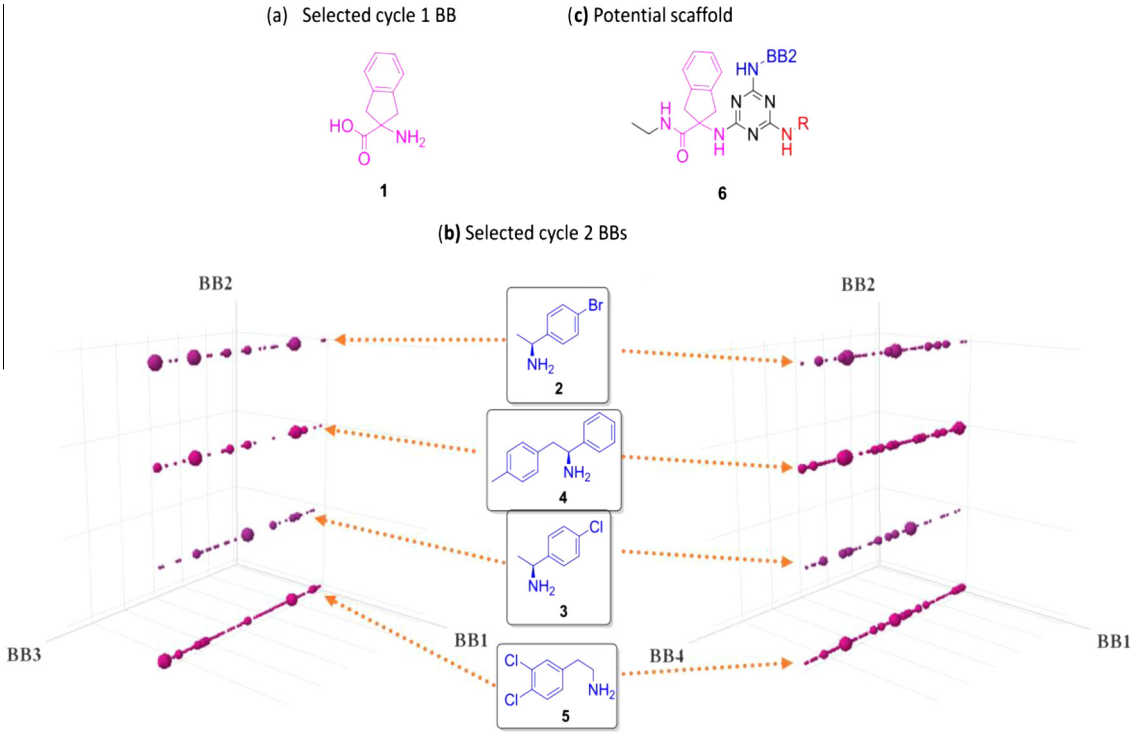
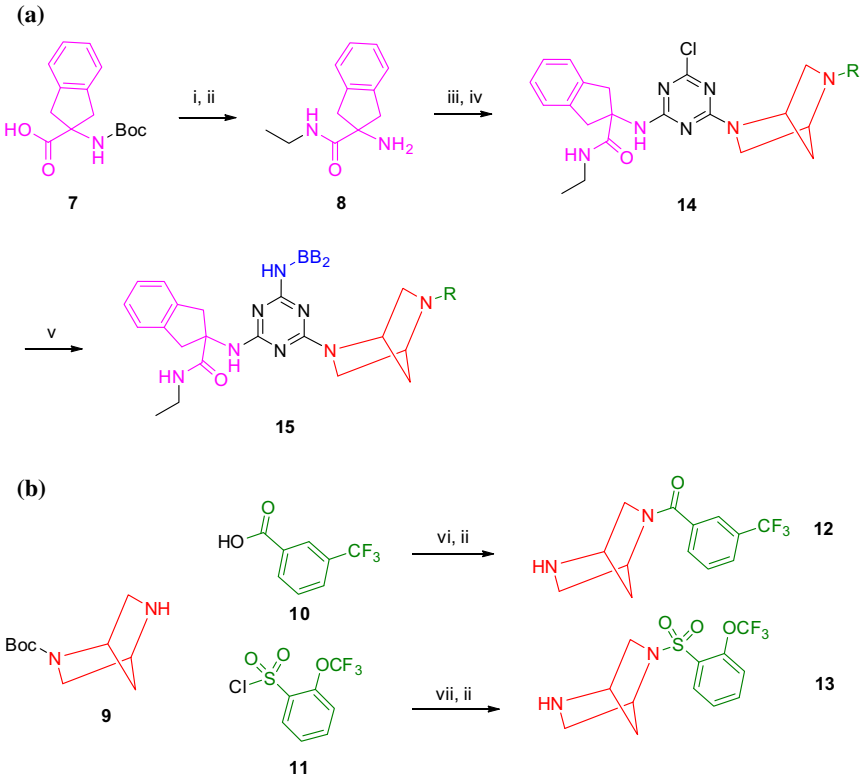


Figure 2. (a) Selected cycle 1 building block (BB1); (b) Spotfire™ cube data views of selected LFA-1 features with selected cycle 2 building blocks (BB2s); (c) potential chemotype pharmacophore analyzed from the feature analysis.



Scheme 1. Off-DNA synthesis of LFA-1 selected hits. Reagents and conditions: (i) ethylamine hydrochloride (1.0 equiv), 2-(1*H*-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU, 1.2 equiv), *N*,*N*-diisopropylethylamine (DIPEA, 3.0 equiv), *N*,*N*-dimethylformamide (DMF); (ii) 20% trifluoroacetic acid (TFA) in dichloromethane (DCM); (iii) cyanuric chloride (1.0 equiv), DIPEA (3.0 equiv), MeCN; (iv) desired amine, DIPEA (3.0 equiv), MeCN, room temperature; (v) desired amine, DIPEA (3.0 equiv), MeCN; 80 °C; (vi) HATU (1.2 equiv), DIPEA (3.0 equiv), DMF; (vii) DIPEA (2.0 equiv), tetrahydrofuran (THF), rt.

prominently selected cycle 3 BB, 2,5-diazabicyclo[2.2.1]heptane **9**, and a couple of cycle 4 variable region BBs, compounds **10** and **11**, and pre-assembled the desired cycle 3 and cycle 4 precursors **12** and **13** for off-DNA synthesis. A one pot reaction of the amine **8** with cyanuric chloride followed by addition of the precursors **12** and **13** gave aryl halide **14**. Displacement of the chloride on compound **14** under heating conditions provided final compound **15**.

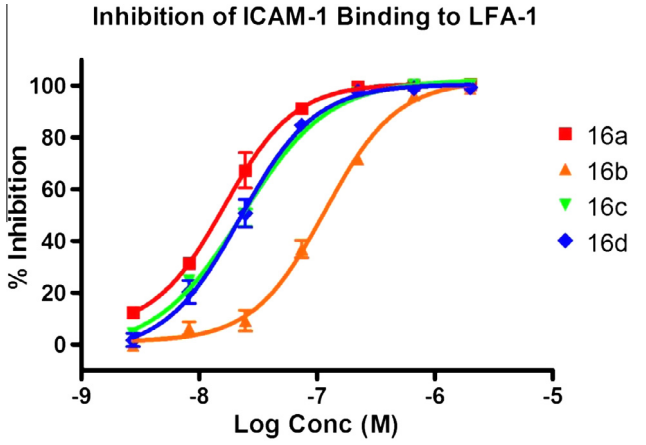
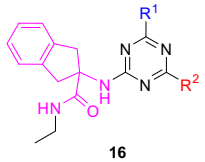


Figure 3. Dose dependent inhibition of ICAM-1 binding to LFA-1 by resynthesized compounds **16a–d** as measured by ELISA. Data points are the mean of two replicates and error bars represent SEM.

2.3. Activity assay and SAR establishment

The synthesized compounds were tested for inhibition of the binding of ICAM-1 to LFA-1 by an ELISA-type ligand binding assay. Representative results from the dose dependent titration of the first four synthesized compounds are shown in Figure 3. Given the precursors **12** and **13** as two combinations of cycles 3 and 4, we chose only two cycle 2 BBs in our explorations. The four potential combination compounds (Table 1, entries 1–4) plus a truncated cycle three analog (entry 5) were the first compounds synthesized off-DNA and tested for feature activity confirmation. Choosing the cycle 2 BB as (*S*)-1-(4-bromophenyl)ethanamine (**2**), the corresponding amide compounds **16a** gave an IC_{50} potency of 16 nM, 7-fold more potent than the corresponding sulfonamide analog **16b**. Replacing the (*S*)-1-(4-bromophenyl)ethanamine (**2**) with the achiral 2-(3,4-dichlorophenyl)ethanamine (**5**), produce amide **16c** with an IC_{50} potency of 23 nM while the corresponding sulfonamide **16d** gave analogous activity of 22 nM as amide **16a**. This demonstrated the importance of both the cycle 2 groups and the presence of an amide/sulfonamide moiety in the inhibitor

Table 1
Activity of the first set of compounds made off-DNA



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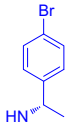
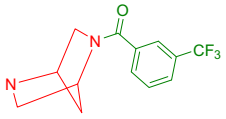
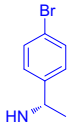
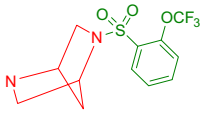
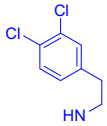
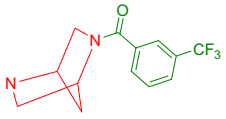
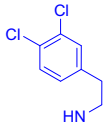
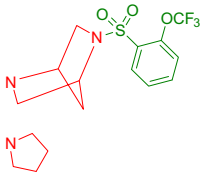
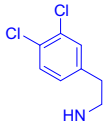
Entry	Compounds	R^1	R^2	IC_{50}[a]
1	**16a**			16 nM
2	**16b**			117 nM
3	**16c**			23 nM
4	**16d**			22 nM
5	**16e**			12,900 nM

[a] Inhibition of ICAM-1 binding to LFA-1 was measured by ELISA.

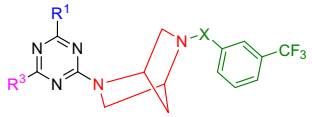
structure. Truncation of cycle3–cycle4 group to pyrrolidine diminished the inhibitor activity to double digit μM, clear indication of the major role played by the cycle3–cycle4 bridged diamine–amide/sulfonamide moieties in the target inhibition.

Exploration of additional SAR around cycle 2 (R1) and cycle 1 (R3) was fruitful in defining the necessary moieties responsible for the chemotype affinity. In this investigation we chose cycle3–cycle-4 region constant as the corresponding amide. Maintaining the cycle 1 as 2-amino-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (**8**), changing the cycle 2 residue from (*S*)-1-(4-bromophenyl)ethanamine to the des-bromo analog reduced the activity by 10-fold. Inverting the α-methyl group chirality from *S* to *R* was detrimental to the compound activity, while elimination of the α-methyl group all together produce weak μM activity. Further SAR exploration by removal of the two chloro groups from compound **16c** gave compound **17d** with a 20-fold diminished potency. Further truncation of **17d** by removal of the phenyl group abolished the compounds activity (Table 2, entry 5). Replacement of cycle3–cycle4 group to pyrrolidine diminished the inhibitor activity to double digit μM a clear indication of the major role played by the cycle3–cycle4 bridged diamine–amide/sulfonamide moieties target inhibition. Additional SAR exploration around cycle 1 was observed to be rewarding. Replacement of the ethylamide group with a carboxylate maintained compound potency at 37 nM while the des-carboxylate analog was observed to be 10-fold less potent. Further truncation of the cycle group by removal of the phenyl group abolished the compound activity (**17h**, entry 8). Given the excellent potency of the carboxylate **17f**, the corresponding sulfonamide was synthesized and was confirmed to have a potency of 65 nM (**17i**, entry 9).

2.4. Cell adhesion activity

Given the great potency of the chemotype we examined a number of compounds in a cell adhesion assay. A human lymphocyte Jurkat cell-line that expresses native wild-type (WT) LFA-1 was used to test for compound inhibition of cell adhesion to ICAM-1. Jurkat cells were allowed to interact with ICAM-1 substrates. Upon PMA stimulation, Jurkat cells showed a good level of cell adhesion to ICAM-1. Both the competitive antagonist antibody TS1/22[21] used as a control and a number of our compounds potently inhibited Jurkat cell adhesion to ICAM-1 (Fig. 4A). To study the mode of action of the LFA-1 antagonists that we discovered, we employed K562 cells stably transfected to express wild type LFA-1 (WT, Fig. 4B–D) or mutant LFA-1 (HA, Fig. 4B–D) that contains an engineered disulfide locked high-affinity I domain.[22] The mutant LFA-1 is blocked only by competitive but not allosteric inhibitors. However, upon DTT treatment that disrupts the engineered disulfide, the mutant LFA-1 is reverted to a functionally wild-type LFA-1, thereby becoming inhibitable by either competitive or allosteric antagonists In this way, this mutant LFA-1 allows for discrimination between competitive and allosteric modes of LFA-1 inhibition.[23] TS1/22 inhibited not only wild-type LFA-1 (Fig. 4B), but also mutant LFA-1 both in the absence (Fig. 4C) and presence (Fig. 4D) of DTT treatment, thereby being confirmed to be a competitive inhibitor. By contrast, an established allosteric antagonist LFA703[24] as well as our compounds were able to inhibit WT LFA-1 (Fig. 4B) but unable to inhibit the mutant LFA-1 in the absence of DTT treatment (Fig. 4C). However, they were able to inhibit the mutant HA LFA-1 in the presence of DTT treatment (Fig. 4D). These results suggest that our compounds function in the same mode as LFA703; that is, in allosteric inhibition. As our compounds bind to the I domain, their binding sites are likely to locate to the allosteric pocket underneath the C-terminal helix, to which established alpha I allosteric antagonists including LFA703 were found to bind.

Table 2
Structure–activity study (SAR) exploration around the BB1 and BB2 component of the scaffold



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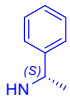
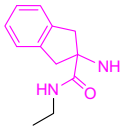
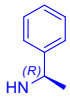
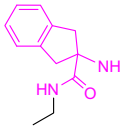
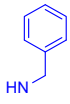
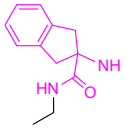
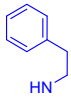
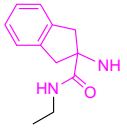
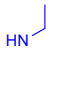
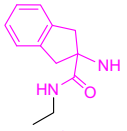
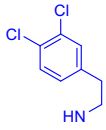
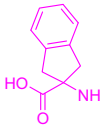
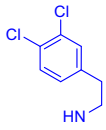
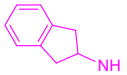
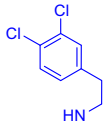
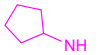
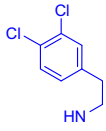
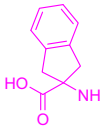
Entry	Compounds	R^1	R^3	X	IC_{50}[a]
1	**17a**			–CO–	180 nM
2	**17b**			–CO–	>10,000 nM
3	**17c**			–CO–	7900 nM
4	**17d**			–CO–	488 nM
5	**17e**			–CO–	>10,000 nM
6	**17f**			–CO–	37 nM
7	**17g**			–CO–	358 nM
8	**17h**			–CO–	>10,000
9	**17i**			$–SO_2–$	65 nM

[a] Inhibition of ICAM-1 binding to LFA-1 was measured by ELISA.

2.5. Synthesis and affinity of fluorescently labeled compounds

Compounds **16c** and **16d** were chosen as representative for further investigation. We generated fluorescent compounds by directly conjugating the Cy3 dye to the DNA attachment point (Scheme 2). Also, we synthesized compounds **16c** and **16d** with DNA-headpiece attached to generate compounds **20a** and **20b** then ligated them to biotinylated-DNA to generate compounds **21a** and **21b** (Scheme 3). The addition of fluorescently labeled streptavidin phycoerythrin (PE) allowed visualization of binding by a warhead

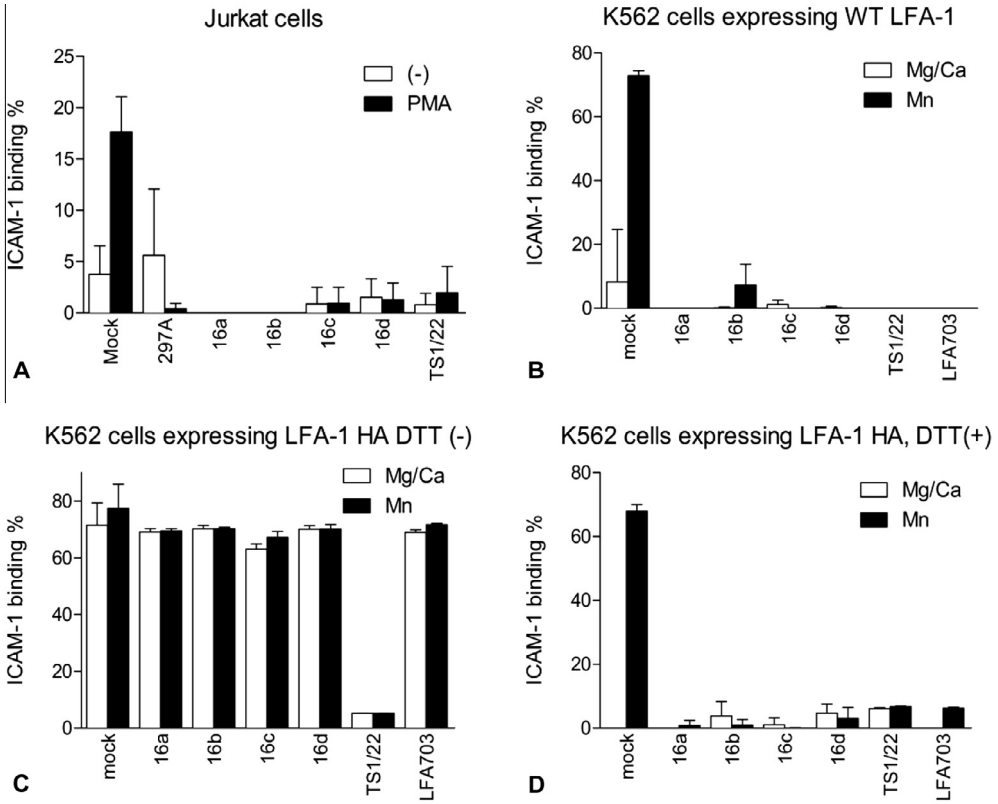
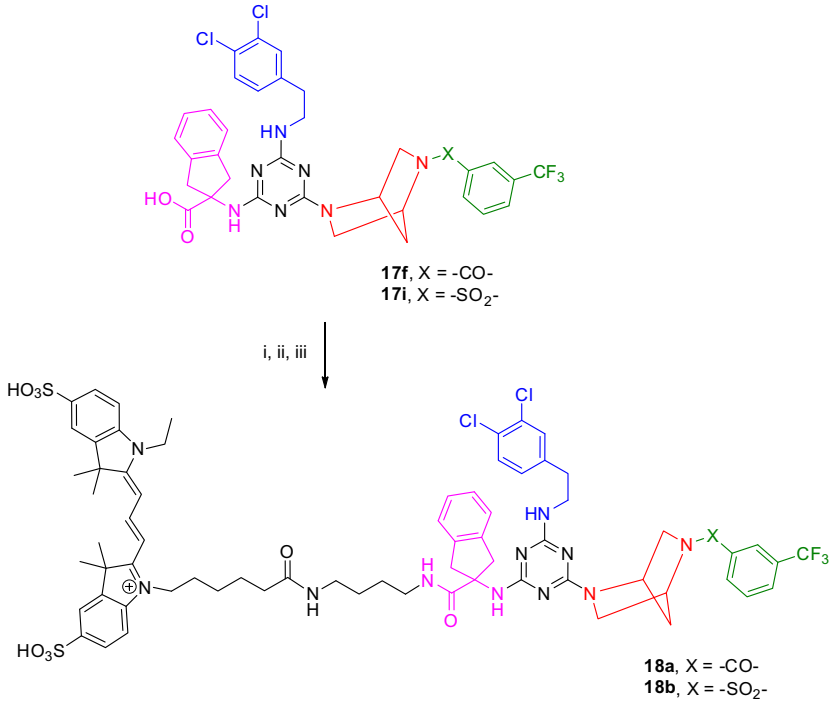
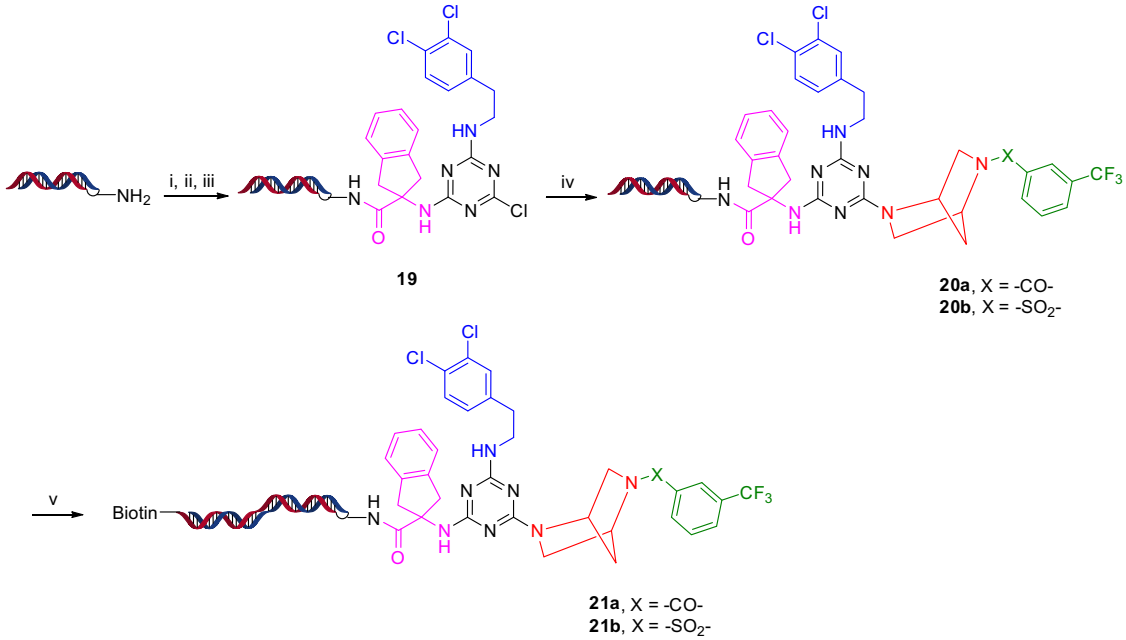


Figure 4. Inhibition of LFA-1-mediated cell adhesion to ICAM-1. (A) Adhesion to ICAM-1 of Jurkat cells expressing native WT LFA-1 in the presence or absence of PMA stimulation. (B and C) Adhesion to ICAM-1 of K562 transfectants expressing WT (B) or mutant high-affinity (C and D) LFA-1 in resting (Mg/Ca) or stimulating (Mn) conditions. (A–D) Data are one of two representative experiments with mean ± S.D. of quadruplicates. Compounds **16a–d** at 50 μM, a reference allosteric antagonist LFA703 at 50 μM, and a reference competitive antagonist TS1/22 at 10 μg/mL were used.



Scheme 2. Fluorophore labeling of selected LFA-1 hits. Reagents and conditions: (i) *tert*-butyl (4-aminobutyl)carbamate (1.5 equiv), HATU (1.5 equiv), DIPEA (5.0 equiv), DMF; (ii) 20% TFA in DCM; (iii) Cy3-NHS ester (1.0 equiv), DIPEA (3.0 equiv), DCM/DMSO (3:1).



Scheme 3. On-DNA synthesis of biotinylated selected LFA-1 hits. Reagents and conditions: (i) 2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-2,3-dihydro-1*H*-indene-2-carboxylic acid (40.0 equiv), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, 40.0 equiv), sodium borate buffer (pH 9.4), MeCN/H_2O (1:1), 0 °C, 2 h; (ii) 10% piperidine in sodium borate buffer (pH 9.4); (iii) cyanuric chloride (30 equiv), MeCN/H_2O (1:1), 0 °C, 2 h then added 2-(3,4-dichlorophenyl)ethanamine (50 equiv) in DMA, 0 °C, 12 h; (iv) Compound **12** or **13** (50.0 equiv), sodium borate buffer (pH 9.4), MeCN, 80 °C, 1 h; (v) enzymatic ligation of a biotinylated-DNA.

attached to DNA as in the library. The Cy3 labeling initiated with a peptide coupling of compounds **17f** and **17i** to mono-Boc decorated 1,4-diamino butane. After removal of the Boc protecting group the corresponding amine was coupled with Cy3-NHS ester to afford final compounds **18a** and **18b**. The corresponding on-DNA biotinylated approach was initiated in a library mimetic fashion. The synthesis proceeded analogously to our previous reports using ELT headpiece (HP).[15] Coupling of the free amine functional group of the HP with Fmoc-protected cycle 1 amino acid was followed by the Fmoc removal to produce cycle1 as free amine. Reaction of the cycle amine with cyanuric chloride followed by addition of the second amine afforded on-DNA precursor **19**. Reaction of precursor **19** with amine **12** and **13** provided final compounds **20a** and **20b** which were further ligated with a biotinylated piece of DNA to produce **21a** and **21b**. We consider the immediate knowledge of a usable attachment point to be a major benefit of the ligands discovered through ELT, as tool compounds can be rapidly synthesized. By making the DNA conjugated molecules we were able to examine the affinity of the small molecule warhead as in the original library. In addition to determining the potency of the conjugated compounds in the LFA-1 ELISA, we also investigated the compound's binding to LFA-1 in its natural state on the cell surface using fluorescent flow cytometry.

The Cy3 and DNA conjugated compounds were both inhibitors of the LFA-1/ICAM-1 interaction (Table 3). These data were compared to one another as well as the corresponding ethylamide **16c** (23 nM) and **16d** (22 nM). The Cy3 conjugated compounds produce comparable data to the ethylamine **16c** and **16b** while the on-DNA compounds gave 10-fold lower potency but retained sufficient activity for further cell surface LFA-1 investigation.

Table 3
LFA-1 activity of modified inhibitors

Entry	Compounds	Modification	IC_{50}[a]
1	**18a**	Cy3	100 nM
2	**18b**	Cy3	28 nM
3	**20a**	DNA	330 nM
4	**20b**	DNA	295 nM

[a] Inhibition of ICAM-1 binding to LFA-1 was measured by ELISA.

We then used the fluorescently labeled compounds **18a** and **18b** and the on-DNA compounds (**20a** and **20b**) to examine the binding of these compounds to native LFA-1. Flow cytometry was used to measure the fluorescence increase due to compound binding to either parent K562 cells (naïve) that lack LFA-1 expression or 19385WT (K562 cells stably transfected to express LFA-1[25]). After incubation of the cells with biotinylated on-DNA compounds (**21a** and **21b**) streptavidin PE was added to detect binding. Fluorescence increase due to compound binding was compared to phycoerythrin labeled mouse anti-human CD11a antibody binding as a positive control for LFA-1 expression (Fig. 5). We observed a higher background fluorescence signal from the Cy3 labeled compounds on the naïve cells, but fluorescence signal increased 2–3 fold when LFA-1 expressing cells were used (Table 4). The on-DNA compounds had lower levels of background signal and an LFA-1 dependent fluorescent signal increase of 45 and 13 fold (Table 4). This data indicates the requirement of LFA-1 expression for binding and shows that the original on-DNA library member as selected by affinity to soluble LFA-1 I-domain retains affinity for the native protein as expressed on a cell membrane.

3. Conclusion

Our strategy of selecting for binders to a soluble recombinant protein domain from a complex chemical library was validated by our discovery of potent small molecules with novel SAR that inhibit the LFA-1/ICAM-1 PPI in both ELISA and cell adhesion assays. This potency was retained after modification for use as tool compounds and the compounds proved to retain affinity for native protein. The latter phenomena provided an opportunity for ELT selections against a desired target in its natural state on cell surface. We are currently exploring this technology development opportunity.

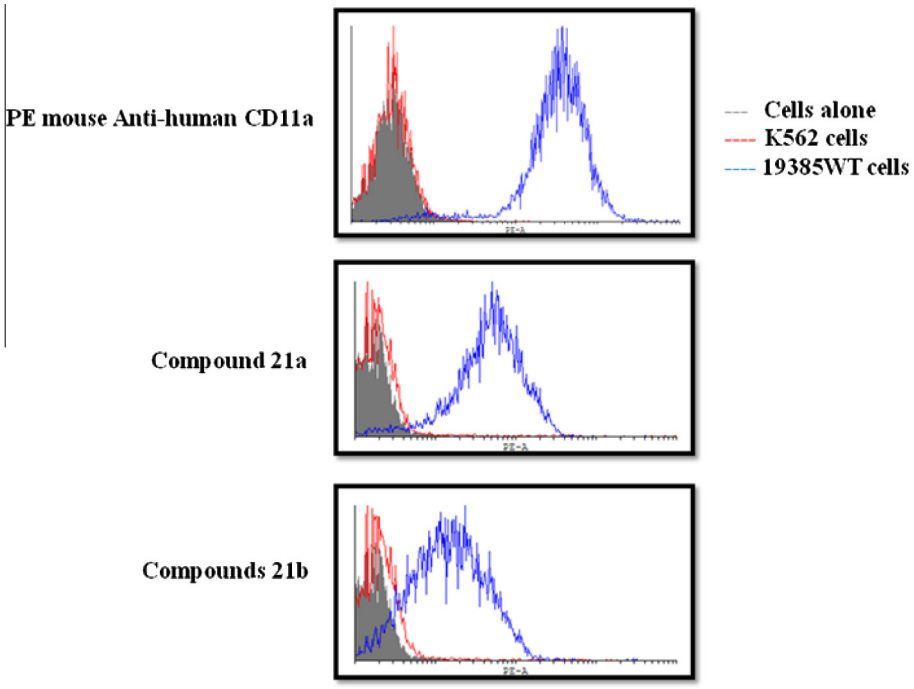


Figure 5. Fluorescent cell cytometry of the binding of on-DNA, fluorescently labeled compounds to LFA-1 expressing cell. The *x* and *y* axes show number of cells per channel and fluorescence intensity in each channel, with intensity on a log scale.

Table 4
Fluorophore signal increase due to inhibitor binding to LFA-1

	Mean signal	K562 naïve cells	K562 LFA-1 expressing cells	Fold signal increase
1	PE αCD11a	93	12,072	129
2	**18a**	215	699	3
3	**18b**	610	1360	2
4	**21a**	41	1833	45
5	**21b**	51	652	13

4. Experimental procedures

4.1. Protein expression

The LFA-1 I domain was expressed in *Escherichia coli* as an inclusion body, solubilized in guanidine, refolded by dilution, and purified to homogeneity by a gel filtration as previously described.[26] Soluble extracellular portion of LFA-1 (αLβ2 heterodimer) containing the C-terminal coiled-coil artificial transmembrane domains and Hexahistidine and Strep-II tags was expressed in CHO Lec 3.2.8.1 cells and purified to homogeneity by a sequential affinity chromatography as previously described.[27]

4.2. Cell adhesion assays

Cell adhesion assays using a V-bottom plate were performed as previously described[28] with minor modifications.[29] Briefly, Jurkat cells were cultured in RPMI1640/10% FBS at 37 °C. V-bottom 96 wells (Corning) were coated with ICAM-1-Fc (1 μg/mL) (R&D Systems) or BSA (1 μg/mL) overnight. Following wash with Tris-buffered saline/0.05% Tween (TBST), wells were blocked with HEPES-buffered saline (HBS)/2% BSA for 2 h at room temperature. After wash, wells were given 50 μL each of HBS/2 mM $MgCl_2/CaCl_2$ or HBS/2 mM $MgCl_2/CaCl_2$ + phorbol myristate acetate (PMA) 200 ng/mL. Cells were labeled with calcein acetoxymethyl ester (calcein-AM) (Invitrogen). 50 μL of cells containing compounds, mock (DMSO), LFA-1 competitive antagonist TS1/22 (final concentration 10 μg/mL) were given to wells. DMSO concentration was held constant at 0.5%. Cells were incubated at 37 °C for 45 min in the dark, and centrifuged at 200*g* for 5 min. After centrifugation, nonadherent cells that accumulated in the center of the V bottom wells were quantified using the Fluoroskan Ascent microplate fluorometer (Thermo Scientific) with the filter sets of excitation at 485 nm and emission at 535 nm. ICAM-1 binding % was defined in the following formula as follows.

$$[1 - (FI_{ICAM-1}/FI_{BSA})] \times 100 = \% \text{ of adhesive cells}$$

FI_{ICAM-1}: the fluorescence signal when cells bind to ICAM-1; FI_{BSA}: the fluorescence signal in absence of ICAM-1 and presence of BSA.

In some experiments, K562 transfectants expressing wild-type or mutant disulfide-locked high-affinity LFA-1 K287C/K294C (Ref. 22) were used. K562 transfectants were cultured in RPMI1640/10% FBS, 4 μg/mL puromycin at 37 °C. V-bottom 96 wells were coated with ICAM-1-Fc (1 μg/mL) or BSA (1 μg/mL) overnight. Following wash with TBST, wells were blocked with HBS/2% BSA for 2 h at room temperature. After wash, wells were given 50 μL each of HBS/2 mM $MgCl_2/CaCl_2$ or HBS/2 mM $MnCl_2$. Cells were labeled with 2′,7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM)(Invitrogen). An aliquot (50 μL) of cells containing compounds, mock (DMSO)(final concentration 10 μM), LFA-1 allosteric antagonist LFA703 (final concentration 50 μM), or competitive antagonist TS1/22 (final concentration 10 μg/mL) were given to wells. Cells were incubated at room temperature for 30 min in the dark, and centrifuged at 400*g* for 5 min. In some experiments, K562 cells expressing high-affinity LFA-1 (K287C/K294C) were pretreated with 20 mM dithothreitol (DTT) for 10 min and then underwent continuous treatment with 5 mM DTT during a 30-min incubation with ICAM-1. After that, the procedures were same as those with Jurkat cells.

4.3. Affinity screening of encoded library

Sulfo-NHS-LC-biotin (Pierce Chemical, USA) was used to biotinylate LFA-1 I-domain protein by incubating 150 μg of protein with a 10:1 biotin/protein ratio for 30 min at room temperature. Biotinylated protein was then desalted into phosphate buffered saline and used for selection. For affinity selection, 100 μL of 0.1 mg/mL protein solution was pipeted up and down in a 200 μL Phynexus column packed with 5 μL agarose streptavidin resin for 20 min to immobilize 10 μg of protein. 5 nmol of library in selection buffer (25 mM Hepes, pH 7.4; 150 mM NaCl; 1 mM CHAPS; 1 mM $MgCl_2$; 1 mg/mL sheared salmon sperm DNA (Ambion)) was incubated on the column for 1 h, the column was then washed 10 times with 100 μL of selection buffer to remove non-binders. Bound molecules were eluted by heat denaturing the protein at 80 °C for 10 min. Eluant was then incubated with fresh immobilized protein to start a second round of affinity selection. Three rounds of selection were performed. To exclude molecules bound to the resin matrix a parallel selection was done in identical fashion with no protein target as a no target control (NTC). For DNA sequencing, the final eluant containing 5e7 molecules was amplified by PCR for 25 cycles, then sequenced using Illumina high throughput sequencing technology.

4.4. ELISA testing for LFA-1 antagonism

An ELISA-type assay to study LFA-1-ICAM-1 interaction was performed as previously described with minor modification. Briefly, LFA-1 protein was diluted to 2.5 μg/mL in Tris buffered saline (TBS), pH 8.0 plus 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Protein was directly immobilized by adding 50 μL/well to an ELISA plate and incubated overnight at 4 °C. All subsequent steps were carried out at room temperature. Wells were washed 3 times with 300 μL/well with TBS plus 0.05% Tween-20 (TBST), then blocked with 150 μL/well TBS+0.1% gelatin for 1 h. Compounds were serially diluted in 100% DMSO at 200× the final assay concentration prior to being diluted 1:100 to 2× final concentration in Hepes buffered saline (HBS). Wells were again washed 3 times with 300 μL/well TBST prior to 25 μL of serially diluted compounds being added. Compounds were incubated with LFA-1 for 30 min, then 25 μL/well of 20 μg/mL ICAM-1-Fc in HBS+2 mM $MnCl_2$ was added and plates were incubated for 60 min. Wells were washed 5 times with 300 μL/well TBST+1 mM $MnCl_2$. Horseradish peroxidase conjugated goat anti-human IgG (Pierce) was added to allow the detection of bound ICAM-1-Fc. Antibody was diluted 1:5000 into HBS+1 mM $MnCl_2$ and 50 μL/well was added prior to incubation for 30 min. Wells were washed 10 times with 300 μL/well TBST+1 mM $MnCl_2$. 100 μL/well ABTS substrate (Invitrogen) was added and reaction allowed to proceed for 20 min before being stopped by the addition of 100 μL/well 0.01% sodium azide in citric acid. Absorbance at 415nM was then measured. Wells containing no LFA-1 were used for background signal subtraction and percent inhibition was calculated from wells containing no compound.

4.5. DNA ligation

Compounds **20a** and **20b** with library DNA headpiece attached were ligated to biotinylated double stranded DNA by incubating with 20 μM T4 DNA ligase (NEB) in ligation buffer at 16 °C for 18 h. Ligation reactions were then ethanol precipitated prior to further experiments.

4.6. Cell-binding analysis via FACS

K562 cells 19385 cells were cultured in RPMI media, supplied with 10% FBS in a 5% CO_2 incubator with shaking. Cells were collected by centrifugation, counted, and washed twice with PBS. 1 μM compound concentration in PBS buffer was incubated with 2×10^5 cells in the presence of 0.5 mg/mL of sheared salmon sperm DNA to block non-specific DNA interaction at 4 degrees for 1 h. Cells were then washed with 100 μL of PBS buffer 3 times. For biotinylated on-DNA compounds, cells were then incubated with Streptavidin-PE (Invitrogen) at 1:100 ratio in 50 μL of PBS at 4 degrees for 30 min. After staining, cells were washed and fluorescence was measured by FACS analysis on a LSR II flow cytometer.

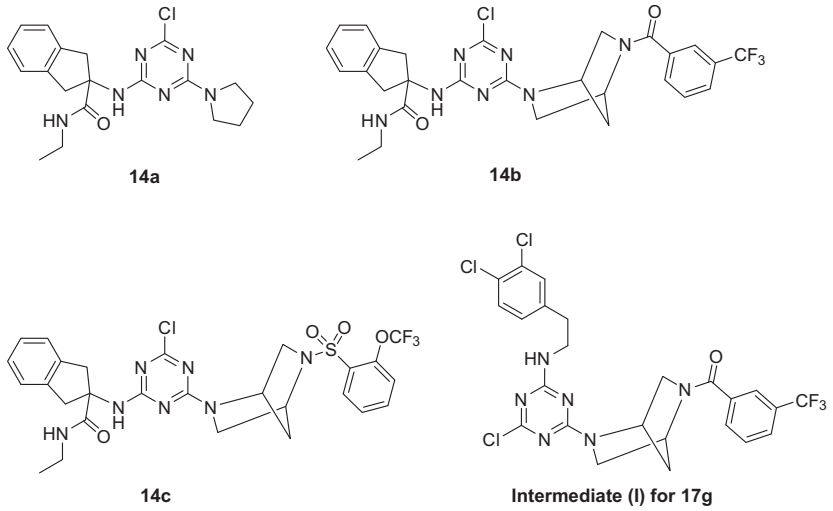
4.7. Compound synthesis

4.7.1. General method

^{1}H NMR spectra were recorded on a Varian Mercury 400 plus. 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). Purification of final compounds for biological testing was performed on a Gilson GX-281 system with a Phenomenex Luna 5μ C8(2) 100 × 21.20 mm 100A column running gradient of 10–80% MeCN/H_2O (+0.1% TFA) over 20 min with flow rate of 22 mL/min. The purity of final compounds was checked using an Agilent 1100 HPLC system coupled with a Thermo Finnigan LCQ Mass Spectrometer–Phenomenex Luna 3μ C8(2) 100A 50 × 3.00 mm column running gradient of 10–95% MeCN/H_2O (+0.1% formic acid) over 15 min with flow rate 0.5 mL/min. High-resolution mass measurement was performed on Bruker MicroTOF electrospray mass spectrometer coupled with an Agilent 1100 HPLC system. All mass spectra were performed by electrospray ionization (ESI) positive mode (Scheme 4).

4.7.1.1. 2-Amino-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (8). To a mixture of 2-((*tert*-butoxycarbonyl)amino)-2,3-dihydro-1*H*-indene-2-carboxylic acid (**7**) (500 mg, 1.803 mmol) and HATU (823 mg, 2.164 mmol) in 10 mL DMF was added DIPEA (0.945 mL, 5.41 mmol) and ethylamine hydrochloride (221 mg, 2.70 mmol), and the reaction was allowed to stir at rt overnight. The reaction mixture was diluted with EtOAc (100 mL), washed with 10% NH_4Cl (2 × 100 mL), saturated $NaHCO_3$ (1 × 100 mL), saturated NaCl (1 × 100 mL), dried with $MgSO_4$, and evaporated to dryness. 500 mg (1.643 mmol) of the residue was dissolved in 6 mL DCM and added TFA (3.16 mL, 41.1 mmol). The reaction mixture was stirred at room temp for 2 h, then azeotroped with DCM (2 × 20 mL). The crude TFA salt was put on vacuum pump overnight, and the residue was re-dissolved in EtOAc (50 mL) and washed with 1 N NaOH (1 × 50 mL), and saturated NaCl (1 × 50 mL), dried with $MgSO_4$, filtered, and evaporated to dryness to obtain the desired free amine 2-amino-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (**8**) (350 mg, 1.628 mmol, 99% yield). MS (ESI) m/z $[M+1]^+$ = 205.04.

4.7.1.2. 2,5-Diazabicyclo[2.2.1]heptan-2-yl(3-(trifluoromethyl) phenyl)methanone (12). To a mixture of 3-(trifluoromethyl) benzoic acid (**10**) (450 mg, 2.37 mmol) and HATU (912 mg, 2.4 mmol) in DMF (6 mL) was added DIPEA (2 mL, 11.5 mmol). To the reaction mixture was then added *tert*-butyl 2,5-diazabicyclo[2.2.1]heptane-2-carboxylate (**9**) (469 mg,2.37 mmol) and the reaction was allowed to stir at rt for 1 h. The reaction mixture was diluted with EtOAc (100 mL), washed with 10% NH_4Cl (2 × 100 mL), saturated $NaHCO_3$ (1 × 100 mL), saturated NaCl (1 × 100 mL), dried with $MgSO_4$, and evaporated to dryness to give the Boc-protected material. The Boc-protected material was re-dissolved in 6 mL DCM and to the solution was added TFA (3 mL, 39 mmol). The reaction was stirred at rt for 2 h. The solvent was



Scheme 4. Structures of intermediates.

evaporated to dryness. The residue was azeotroped with DCM (2 × 20 mL) to yield 2,5-diazabicyclo[2.2.1]heptan-2-yl(3-(trifluoromethyl)phenyl)methanone (**12**) as a TFA salt (911 mg, >99% yield). MS (ESI) *m*/*z* $[M+1]^+$ = 271.14.

4.7.1.3. 2-((2-(Trifluoromethoxy)phenyl)sulfonyl)-2,5-diazabicyclo[2.2.1]heptane (13). To a solution of *tert*-butyl 2,5-diazabicyclo[2.2.1]heptane-2-carboxylate (**9**) (469 mg, 2.37 mmol) and DIPEA (2 mL, 11.5 mmol) in DMF (6 mL) was added 2-[(trifluoromethyl)oxy]benzenesulfonyl chloride (617 mg, 2.37 mmol) slowly. The reaction mixture was allowed to stir at rt for 2 h. The reaction mixture was diluted with EtOAc (100 mL), washed with saturated $NaHCO_3$ (1 × 100 mL), saturated NaCl (1 × 100 mL), dried with $MgSO_4$, and evaporated to dryness to give the Boc-protected material. The Boc-protected material was dissolved in DCM (6 mL) and to the solution was added TFA (3 mL, 39 mmol). The reaction was stirred at rt for 2 h. The solvent was evaporated to dryness and the residue was azeotroped with DCM (2 × 5 mL) to yield 2-((2-(trifluoromethoxy)phenyl)sulfonyl)-2,5-diazabicyclo [2.2.1]heptane **(13)** as a TFA salt (1.0 g, >99% yield). MS (ESI) *m*/*z* $[M+1]^+$ = 323.17.

4.7.1.4. 2-((4-Chloro-6-(pyrrolidin-1-yl)-1,3,5-triazin-2-yl) amino)-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (14a); 2-((4-chloro-6-(5-(3-(trifluoromethyl)benzoyl)-2,5-diazabicyclo [2.2.1]heptan-2-yl)-1,3,5-triazin-2-yl)amino)-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (14b); 2-((4-chloro-6-(5-((2-(trifluoromethoxy)phenyl)sulfonyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-yl)amino)-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (14c). In three separate vials, to a suspension of 2,4,6-trichloro-1,3,5-triazine (50 mg, 0.271 mmol) in MeCN (3 mL) at 0 °C was added a solution of the 2-amino-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (**8**, 55.4 mg, 0.271 mmol) in MeCN (1 mL) dropwise. To the reaction mixture was then added DIPEA (0.142 mL, 0.813 mmol) dropwise. The reaction mixtures were stirred at 0 °C for 15 min. To each vial was added the desired secondary amine (pyrrolidine for **14a**, 2,5-diazabicyclo[2.2.1] heptan-2-yl(3-(trifluoromethyl)phenyl) methanone (**12**) for **14b**, and 2-((2-(trifluoromethoxy)phenyl) sulfonyl)-2,5-diazabicyclo [2.2.1]heptane (**13**) for **14c**) in MeCN (1 mL) dropwise at 0 °C. The reaction mixtures were allowed to warm up to room temp. The reactions' completion was confirmed by LCMS after 2 h of stirring. The mixtures were then purified with prep Gilson HPLC. **14a** (81 mg, 0.222 mmol, 81% yield), MS (ESI) *m*/*z* $[M+1]^+$ = 387.13; **14b** (84 mg, 0.135 mmol, 51 % yield). MS (ESI) *m*/*z* $[M+1]^+$ = 586.22; and **14c** (75 mg, 0.081 mmol, 30% yield), MS (ESI) *m*/*z* $[M+1]^+$ = 638.08.

4.7.2. General method for synthesis of 16 and 17(a–e)

To a suspension of one of compound **14** (0.090 mmol) in NMP (2 mL) at rt was added the desired amine (0.135 mmol). To the reaction mixture was then added DIPEA (0.047 mL, 0.270 mmol). The reaction mixture was then heated at 100 °C overnight. The desired product was purified by prep Gilson HPLC.

4.7.2.1. 2-((4-(((*S*)-1-(4-Bromophenyl)ethyl)amino)-6-(5-(3-(trifluoromethyl)benzoyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-yl)amino)-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (16a). Compound **14b** and (*S*)-1-(4-bromophenyl)ethanamine were used for the reaction to give **16a** (40 mg, 0.048 mmol, 53% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.66–8.26 (m, 2H), 7.80–7.39 (m, 6H), 7.27–7.06 (m, 6H), 6.00 (s, 1H), 5.07–4.38 (m, 3H), 3.79–3.15 (m, 10H), 2.06–1.87 (m, 2H), 1.53 and 1.51 (d, *J* = 8 Hz, 3H), 1.11–0.92 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.1, 168.6, 167.8, 160.3, 155.0, 154.4, 142.2, 144.8, 139.6, 131.8, 131.7, 131.7, 129.3, 128.2, 128.0, 127.3, 127.2, 127.1, 124.5, 124.4, 120.6, 117.7, 114.8, 59.4, 56.7, 56.6, 50.6, 43.3, 35.0, 34.9, 34.7, 22.3, 19.0, 14.7, 14.6. HRMS $(M+H)^+$ calcd for $[C_{36}H_{36}BrF_3N_8O_2 + H]$ 749.2170; found 749.2139.

4.7.2.2. 2-((4-(((*S*)-1-(4-Bromophenyl)ethyl)amino)-6-(5-((2-trifluoromethoxy)phenyl)sulfonyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-yl)amino)-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (16b). Compound **14c** and (*S*)-1-(4-bromophenyl)ethanamine were used for the reaction to give **16b** (25 mg, 0.028 mmol, 31% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48–8.38 (broad s, 1H), 8.26–8.01 (m, 2H), 7.68–7.64 (t, 1H), 7.47–7.4 (m, 4H), 7.20–7.06 (m, 6H) 6.01–5.87 (d, 1H), 5.04–4.97 (m, 1H), 4.73–4.59 (m, 2H), 3.72–3.3 (m, 10H), 1.86–1.67 (m, 2H), 1.53–1.47 (m, 3H), 1.08–1.02(m, 3H). HRMS $(M+H)^+$ calcd for $[C_{35}H_{36}BrF_3N_8O_4S + H]$ 801.1789; found 801.1769.

4.7.2.3. 2-((4-((3,4-Dichlorophenethyl)amino)-6-(5-(3-(trifluoromethyl)benzoyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-yl)amino)-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (16c). Compound **14b** and 2-(3,4-dichlorophenyl)ethanamine were used for the reaction to give **16c** (20 mg, 0.024 mmol, 27%

yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.47–8.00 (m, 2H), 7.61–7.56 (m, 4H), 7.36–7.02 (m, 7H), 6.12–6.02 (d, 1H), 5.09–4.95 (m, 1H), 4.76–4.43 (dd, 1H), 3.82–3.13 (m, 12H), 2.87–2.76 (m, 2H), 2.09–1.89 (m, 2H), 1.12–0.91 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.1, 160.3, 154.3, 139.7, 138.5, 138.4, 135.8, 132.4, 130.9, 130.7, 130.5, 129.2, 128.4, 127.5, 127.1, 124.5, 124.5, 124.4, 120.6, 117.7, 115.8, 67.3, 59.6, 57.7, 56.7, 54.2, 43.7, 43.1, 36.2, 34.8, 34.5, 14.7, 14.6. HRMS $(M+H)^+$ calcd for $[C_{36}H_{35}Cl_2F_3N_8O_2$ + -H] 739.2285; found 739.2276.

4.7.2.4. 2-((4-((3,4-Dichlorophenethyl)amino)-6-(5-((2-(trifluoromethoxy)phenyl)sulfonyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-yl)amino)-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (16d). Compound **14c** and 2-(3,4-dichlorophenyl)ethanamine were used for the reaction to give **16d** (20 mg, 0.023 mmol, 25% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41–8.29 (d, 1H), 8.05–7.91 (m, 2H), 7.67–7.63 (t, 1H), 7.45–7.01 (m, 9H), 6.02–5.90 (d, 1H), 4.72–4.63(m, 2H), 3.82–3.15(m, 12H), 2.86–2.82 (t, 2H), 1.91–1.69 (m, 2H), 1.10–1.02 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.2, 154.3, 146.0, 139.6, 138.4, 134.8, 132.5, 131.7, 130.8, 130.7, 130.6, 128.3, 127.3, 127.2, 127.1, 126.7, 124.5, 124.4, 120.4, 67.3, 60.2, 59.8, 57.9, 55.5, 54.3, 54.0, 43.5, 43.4, 41.8, 37.2, 34.9, 34.4, 14.7. HRMS $(M+H)^+$ calcd for $[C_{35}H_{35}Cl_2F_3N_8O_4S$ + H] 791.1904; found 791.1891.

4.7.2.5. 2-((4-((3,4-Dichlorophenethyl)amino)-6-(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)amino)-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (16e). Compound **14a** and 2-(3,4-dichlorophenyl) ethanamine were used for the reaction to give **16e** (15 mg, 0.025 mmol, 28% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (s, 1H), 7.63 (s, 1H), 7.36–7.02 (m, 7H), 6.10 (s, 1H), 3.75–3.25 (m, 12H), 2.87–2.83 (t, 2H), 2.00–1.94 (m, 4H), 1.08–1.05 (t, 3H). HRMS $(M+H)^+$ calcd for $[C_{27}H_{31}Cl_2N_7O$+ H] 540.2040; found 540.2020.

4.7.2.6. *N*-Ethyl-2-(4-((*S*)-1-phenylethylamino)-6-(5-(3-(trifluoromethyl)benzoyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-ylamino)-2,3-dihydro-1*H*-indene-2-carboxamide (17a). Compound **14b** and (*S*)-1-phenylethanamine were used for the reaction to give **17a** (34.5 mg, 0.046 mmol, 51% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41–8.05 (m, 2H), 7.81–7.54 (m, 4H), 7.37–7.10 (m, 9H), 6.00 (s, 1H), 5.11–4.39 (m, 2H), 3.77–2.91 (m, 11H), 2.04–1.88 (m, 2H), 1.56 (d, 3H), 1.12–1.05 (m, 2H), 0.94–0.92 (t, 1H). HRMS $(M+H)^+$ calcd for $[C_{36}H_{37}F_3N_8O_2$ + H] 671.3065; found 671.3059.

4.7.2.7. *N*-Ethyl-2-(4-((*R*)-1-phenylethylamino)-6-(5-(3-(trifluoromethyl)benzoyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-ylamino)-2,3-dihydro-1*H*-indene-2-carboxamide (17b). Compound **14b** and (*R*)-1-phenylethanamine were used for the reaction to give **17b** (37 mg, 0.05 mmol, 55% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21–8.07 (m, 2H), 7.83–7.59 (m, 4H), 7.32–7.05 (m, 9H), 6.03 (s, 1H), 5.10–4.39 (m, 2H), 3.86–2.77 (m, 11H), 2.06–1.86 (m, 2H), 1.57 (d, 3H), 1.12–1.04 (m, 2H), 0.93–0.90 (t, 1H). HRMS $(M+H)^+$ calcd for $[C_{36}H_{37}F_3N_8O_2$ + H] 671.3065; found 671.3049.

4.7.2.8. 2-((4-(Benzylamino)-6-(5-(3-(trifluoromethyl)benzoyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-yl)amino)-*N*-ethyl-2,3-dihydro-1*H*-indene-2-carboxamide (17c). Compound **14b** and benzylamine were used for the reaction to give **17c** (38.7 mg, 0.045 mmol, 51% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.76–8.70 (m, 1H), 8.14–7.57 (m, 5H), 7.33–7.14 (m, 9H), 6.05 (broad s, 1H), 5.08–4.44 (m, 4H), 3.84–3.12 (m, 10H), 2.09–1.90 (m, 2H), 1.13–0.90 (m, 3H). HRMS $(M+H)^+$ calcd for $[C_{35}H_{35}F_3N_8O_2$ + H] 657.2908; found 657.2922.

4.7.2.9. *N*-Ethyl-2-(4-(phenethylamino)-6-(5-(3-(trifluoromethyl)benzoyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-ylamino)-2,3-dihydro-1*H*-indene-2-carboxamide (17d). Compound **14b** and phenylethylamine were used for the reaction to give **17d** (54.5 mg, 0.07 mmol, 81% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60–8.54 (m, 1H), 7.82–7.52 (m, 5H), 7.31–7.14 (m, 9H), 6.06 (br s, 1H), 5.10–4.89 (d, 1H), 4.82–4.46 (m, 1H), 3.84–3.15 (m, 12H), 2.89 (br s, 2H), 2.10–1.89 (m, 2H), 1.13–1.04 (dt, 2H), 0.94–0.91 (t, 1H). HRMS $(M+H)^+$ calcd for $[C_{36}H_{37}F_3N_8O_2$ + H] 671.3065; found 671.3067.

4.7.2.10. *N*-Ethyl-2-((4-(ethylamino)-6-(5-(3-(trifluoromethyl)benzoyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-yl)amino)-2,3-dihydro-1*H*-indene-2-carboxamide (17e). Compound **14b** and ethylamine hydrochloride were used for the reaction to give **17e** (43.9 mg, 0.056 mmol, 63% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.69–8.60 (m, 1H), 7.82–7.56 (m, 4H), 7.32–7.14 (m, 4H), 6.11 (s, 1H), 5.13–5.05 (t, 1H), 4.84 and 4.46 (d, 1H), 3.86–2.89 (m, 13H), 2.10–1.94 (m, 2H), 1.26–0.90 (m, 6H). HRMS $(M+H)^+$ calcd for $[C_{30}H_{33}F_3N_8O_2$ + H] 595.2752; found 595.2748.

4.7.2.11. 2-((4-((3,4-Dichlorophenethyl)amino)-6-(5-(3-(trifluoromethyl)benzoyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-yl)amino)-2,3-dihydro-1*H*-indene-2-carboxylic acid (17f). To a suspension of 2,4,6-trichloro-1,3,5-triazine (200 mg, 1.085 mmol) in MeCN (3 mL) at 0 °C was added a solution of the 2-amino-2,3-dihydro-1*H*-indene-2-carboxylic acid (316 mg, 1.085 mmol) in MeCN (2 mL) dropwise. To the reaction mixture was then added DIPEA (0.568 mL, 3.25 mmol) dropwise. The reaction mixture was stirred at 0 °C for 30 min. To the reaction vial was then added amine **12** (292 mg, 1.085 mmol) in MeCN (2 mL) dropwise at 0 °C. The reaction mixture was allowed to warm up to room temp. The reaction's completion was confirmed by LCMS after stirring for 2 h. To the vial was then added 2-(3,4-dichlorophenyl)ethanamine (412 mg, 2.169 mmol). The reaction mixture was then heated at 100 °C overnight and purified by prep Gilson HPLC to yield **17f** (120 mg, 0.16 mmol, 14.8% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50–8.32 (m, 1H), 8.11 (br s, 1H), 7.80–7.53 (m, 4H), 7.32–6.97 (m, 7H), 5.05–4.35 (m, 2H), 3.91–3.24 (m, 11H), 2.85–2.75 (m, 2H), 2.06–1.92 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{34}H_{30}Cl_2F_3N_7O_3$ + H] 712.1812; found 712.1782.

4.7.2.12. (5-(4-((3,4-Dichlorophenethyl)amino)-6-((2,3-dihydro-1*H*-inden-2-yl)amino)-1,3,5-triazin-2-yl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)(3-(trifluoromethyl)phenyl)methanone (17g). To a suspension of 2,4,6-trichloro-1,3,5-triazine (191 mg, 1.036 mmol) in MeCN (5 mL) at 0 °C was added a solution of the 3,4-dichlorophenylehthylamine (196 mg, 1.036 mmol) in MeCN (2 mL) dropwise. To the reaction mixture was then added DIPEA (1.086 mL, 6.22 mmol) dropwise and then stirred at 0 °C for 15 min. To the vial was added the secondary amine **12** (279 mg, 1.036 mmol) in MeCN (2 mL) dropwise at 0 °C. The reaction mixture was allowed to warm up to room temperature and allowed to stir for additional 1 h, then diluted with EtOAc (100 mL), washed with 10% NH_4Cl (2×100 mL), saturated $NaHCO_3$ (1×100 mL), saturated NaCl (1×100 mL), dried with $MgSO_4$, and evaporated to dryness. The residue was purified with ISCO flash chromatography (40 g silica gel column, 10–80% EA in Hexanes in 40 min with retention time at 23 min) to give the intermediate (5-(4-chloro-6-((3,4-dichlorophenethyl)amino)-1,3,5-triazin-2-yl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)(3-(trifluoromethyl)phenyl)methanone (**I**): (510 mg, 0.89 mmol, 86% yield). MS (ESI) m/z $[M+1]^+$ = 571.07. To a suspension of the intermediate **I** (100 mg, 0.175 mmol) in NMP (1 mL) at rt was added a

solution of 2,3-dihydro-1*H*-inden-2-amine (46.5 mg, 0.35 mmol) and DIPEA (0.122 mL, 0.7 mmol). The reaction mixture was then heated at 90 °C overnight and purified by prep Gilson HPLC to give **17g** (40 mg, 0.046 mmol, 26.3% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96–7.55 (m, 6H), 7.37–6.99 (m, 7H), 5.12–4.48 (m, 3H), 3.83–3.54 (6H), 3.38–3.22 (m, 2H), 3.04–2.81 (m, 4H), 2.12–2.00 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{33}H_{30}Cl_2F_3N_7O + H]$ 668.1914; found 668.1892.

4.7.2.13. (5-(4-(Cyclopentylamino)-6-((3,4-dichlorophenethyl) amino)-1,3,5-triazin-2-yl)-2,5-diazabicyclo[2.2.1]heptan-2-yl) (3-(trifluoromethyl)phenyl)methanone (17h). The procedure for the synthesis of **17g** was followed with the use of cyclopentanamine (30 mg, 0.35 mmol) to yield **17h** (39 mg, 0.048 mmol, 27.3% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89–7.56 (m, 5H), 7.44–7.26 (m, 3H), 7.07–6.99 (m, 1H), 5.17–4.50 (m, 2H), 4.27–4.16 (m, 1H), 3.83–3.36 (m, 6H), 2.87–2.80 (m, 2H), 2.14–1.98 (m, 4H), 1.76–1.57 (m, 6H). HRMS $(M+H)^+$ calcd for $[C_{29}H_3\,{}^{\circ}Cl_2F_3N_7O + H]$ 620.1914; found 620.1909.

4.7.2.14. 2-((4-((3,4-Dichlorophenethyl)amino)-6-(5-((2-(trifluoromethoxy)phenyl)sulfonyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-yl)amino)-2,3-dihydro-1*H*-indene-2-carboxylic acid (17i). The procedure for the synthesis of **17f** was followed with the use of amine **13** to yield **17i** (120 mg, 0.15 mmol, 13.8% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46–7.98 (m, 3H), 7.68–7.60 (m, 1H), 7.43–6.97 (m, 9H), 4.87–4.23 (m, 2H), 3.77–3.09 (m, 11H), 2.79–2.76 (t, 2H), 1.88–1.57 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{33}H_{30}Cl_2F_3N_7O_5S + H]$ 764.1431; found 764.1411.

4.7.3. Synthesis of fluorescence polarization (FP) ligands

4.7.3.1. Synthesis of compound 18a. To a mixture of **17f** (50 mg, 0.07 mmol) and HATU (40 mg, 0.105 mmol) in DMF (3 mL) was added DIPEA (0.061 mL, 0.351 mmol) and 1,1-dimethylethyl (4-aminobutyl)carbamate (19.8 mg, 0.105 mmol), and the reaction was allowed to stir at rt overnight. LCMS analysis showed complete conversion. The reaction mixture was diluted with EtOAc (100 mL), washed with 10% NH_4Cl (2 × 100 mL), saturated $NaHCO_3$ (1 × 100 mL), saturated NaCl (1 × 100 mL), dried with $MgSO_4$, and evaporated to dryness. The residue was re-dissolved in DCM (3 mL) and added TFA (0.25 mL, 3.23 mmol) and stirred at rt for 2 h. The solvent was removed under vacuum and azeotroped with DCM (2 × 20 mL) to yield the free amine intermediate (54.7 mg, 0.07 mmol). A fraction of the free amine intermediate (2.4 mg, 0.003 mmol) was then re-dissolved in DCM (3 mL) and added DIPEA (1.6 μL, 0.009 mmol) and then Cy3-NHS (3.5 mg, 0.003 mmol, in 1 mL DMSO) and the reaction mixture was stirred at rt for 2 h. The mixture was purified with Gilson HPLC to yield **18a** as a TFA salt (0.43 mg, 0.24 μmol, 8% yield). MS (ESI) m/z $[M+1]^+$ = 1394.35.

4.7.3.2. Synthesis of compound 18b. The procedure for the synthesis of **18a** was used with **17i** as starting material to yield **18b** as a TFA salt (0.69 mg, 0.37 μmol, 12% yield). MS (ESI) m/z $[M+1]^+$ = 1446.40.

4.7.3.3. Synthesis of compound 19. DNA headpiece (HP) (0.5 μmol, 500 μL, 1 mM) in sodium borate buffer (pH = 9.4) was coupled with 2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-2,3-dihydro-1*H*-indene-2-carboxylic acid (40 equiv, 20 μmol, 100 μL, 200 mM) in MeCN using DMT-MM protocols reported previously[13] and then treated to with piperidine (10% volume) to yield free amino group. Then the freshly prepared DNA HP conjugate with free amine group (0.4 μmol, 400 μL, 1 mM) in sodium borate buffer (pH = 9.4) was cooled to 4 °C and added a freshly prepared cyanuric chloride in MeCN (30 equiv, 12 μmol, 60 μL, 200 mM) in portions, and the mixture was shaken gently at 4 °C overnight. LCMS showed almost complete conversion. To the solution was added a solution of 2-(3,4-dichlorophenyl)ethanamine in DMA (50 equiv, 20 μmol, 100 μL, 200 mM), and the mixture was shaken gently at 4 °C overnight. LCMS showed complete conversion to **19**. To the solution was then added 10% volume of 5 M NaCl solution and 3X volume of cold anhydrous ethanol, cooled at −78 °C for 1 h and then centrifuged at 3000r/min for 10 min. The supernatant was discarded and the residue was re-dissolved in 400 μL of sodium borate buffer (pH = 9.4) as product **19** for the next step. MS (ESI) m/z $[M-3]/3^-$ = 1880.03 (calcd 1880.0).

4.7.3.4. Synthesis of compound 20a. To a solution of **19** (0.25 μmol, 250 μL, 1 mM) in sodium borate buffer (pH = 9.4) was added a solution of **12** in MeCN (50 equiv, 12.5 μmol, 62.5 μL, 200 mM) and the mixture was shaken gently at 80 °C for 1 h. LCMS showed complete conversion to **20a**. To the solution was then added 10% volume of 5 M NaCl solution and 3× volume of cold anhydrous ethanol, cooled at −78 °C for 1 h and then centrifuged at 3000r/min for 10 min. The supernatant was discarded and the residue was re-dissolved in 400 μL of water and purified with Prep HPLC to yield **20a** (0.06 μmol, 24% yield). MS (ESI) m/z $[M-3]/3^-$ = 1957.96 (calcd 1958.7).

4.7.3.5. Synthesis of compound 20b. The procedure for the synthesis of **20a** was used with **19** (0.18 μmol) and **13** (9.0 μmol) as starting materials to yield **20b** (0.09 μmol, 52% yield). MS (ESI) m/z $[M-3]/3^-$ = 1975.38 (calcd 1976.0).

Conflict of interest

The authors have declared no conflict of interest.

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