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.

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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. Most protein–protein interactions (PPI) occur over large, flat surface areas that have proved more difficult to target than classical ligand binding pockets.

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. 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. 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. In addition, LFA-1 inhibitors are currently being investigated in clinical trials for the treatment of uveitis and other ocular inflammation.

The ligand binding domain of LFA-1 termed inserted (I) domain adopts a Rossman 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. 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.
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. 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. 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.

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 10^8–10^10 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. We have also previously used ELT to identify inhibitors of Aurora A and p38 MAP kinases; ADAMTS-5 metalloprotease and sirtuins. 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. 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 ethylamine coupling with Boc-protected acid. This allowed for testing the intermediate in the activation assay and therefore additional SAR exploration by testing the possible fragments and intermediates. Off-DNA synthesis initiated earlier in the synthesis and add on the variable components later.

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. 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 and Boc removal of the desired product to afford amine. We chose the most
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-(1H-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.

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

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compounds</th>
<th>R^{1}</th>
<th>R^{2}</th>
<th>IC_{50}^{a}</th>
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<tbody>
<tr>
<td>1</td>
<td>16a</td>
<td></td>
<td></td>
<td>16 nM</td>
</tr>
<tr>
<td>2</td>
<td>16b</td>
<td></td>
<td></td>
<td>117 nM</td>
</tr>
<tr>
<td>3</td>
<td>16c</td>
<td></td>
<td></td>
<td>23 nM</td>
</tr>
<tr>
<td>4</td>
<td>16d</td>
<td></td>
<td></td>
<td>22 nM</td>
</tr>
<tr>
<td>5</td>
<td>16e</td>
<td></td>
<td></td>
<td>12,900 nM</td>
</tr>
</tbody>
</table>

^{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-1H-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 altogether 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 (17f, 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 TS1/2221 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 LFA70324 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.

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

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Table 2

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compounds</th>
<th>R1</th>
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<tr>
<td>1</td>
<td>17a</td>
<td>HN</td>
<td>CO−</td>
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<td>180 nM</td>
</tr>
<tr>
<td>2</td>
<td>17b</td>
<td>HN</td>
<td>CO−</td>
<td></td>
<td>&gt;10,000 nM</td>
</tr>
<tr>
<td>3</td>
<td>17c</td>
<td>HN</td>
<td>CO−</td>
<td></td>
<td>7900 nM</td>
</tr>
<tr>
<td>4</td>
<td>17d</td>
<td>HN</td>
<td>CO−</td>
<td></td>
<td>488 nM</td>
</tr>
<tr>
<td>5</td>
<td>17e</td>
<td>HN</td>
<td>CO−</td>
<td></td>
<td>&gt;10,000 nM</td>
</tr>
<tr>
<td>6</td>
<td>17f</td>
<td>HN</td>
<td>CO−</td>
<td></td>
<td>37 nM</td>
</tr>
<tr>
<td>7</td>
<td>17g</td>
<td>HN</td>
<td>CO−</td>
<td></td>
<td>358 nM</td>
</tr>
<tr>
<td>8</td>
<td>17h</td>
<td>HN</td>
<td>CO−</td>
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<td>&gt;10,000 nM</td>
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<tr>
<td>9</td>
<td>17i</td>
<td>HN</td>
<td>SO2−</td>
<td></td>
<td>65 nM</td>
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</table>

* Inhibition of ICAM-1 binding to LFA-1 was measured by ELISA.

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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).
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 rated 1,4-diamino butane. After removal of the Boc protecting group the corresponding amine was coupled with Cy3-NHS ester.

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 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 l-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.

Table 3

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compounds</th>
<th>Modification</th>
<th>IC50/μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18a</td>
<td>Cy3</td>
<td>100 nM</td>
</tr>
<tr>
<td>2</td>
<td>18b</td>
<td>Cy3</td>
<td>28 nM</td>
</tr>
<tr>
<td>3</td>
<td>20a</td>
<td>DNA</td>
<td>330 nM</td>
</tr>
<tr>
<td>4</td>
<td>20b</td>
<td>DNA</td>
<td>295 nM</td>
</tr>
</tbody>
</table>

* Inhibition of ICAM-1 binding to LFA-1 was measured by ELISA.
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. Soluble extracellular portion of LFA-1 (αβ 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.

4.2. Cell adhesion assays

Cell adhesion assays using a V-bottom plate were performed as previously described with minor modifications. 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 200g 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.

$$\frac{1}{2} \left( \frac{\text{FI}_{\text{ICAM-1}}}{\text{FI}_{\text{BSA}}} \right) \times 100 = \% \text{ of adhesive cells}$$

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 400g 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.

![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.](image)

**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

<table>
<thead>
<tr>
<th>Mean signal</th>
<th>K562 naïve cells</th>
<th>K562 LFA-1 expressing cells</th>
<th>Fold signal increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PE αCD11a</td>
<td>93</td>
<td>12,072</td>
</tr>
<tr>
<td>2</td>
<td>18a</td>
<td>215</td>
<td>699</td>
</tr>
<tr>
<td>3</td>
<td>18b</td>
<td>610</td>
<td>1360</td>
</tr>
<tr>
<td>4</td>
<td>21a</td>
<td>41</td>
<td>1833</td>
</tr>
<tr>
<td>5</td>
<td>21b</td>
<td>51</td>
<td>652</td>
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</tbody>
</table>

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 400g 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 pipetted 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 MgCl2; 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 MgCl2 and 1 mM CaCl2. 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 to 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 MnCl2 was added and plates were incubated for 60 min. Wells were washed 5 times with 300 μl/well TBST+1 mM MnCl2. 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 MnCl2 and 50 μl/well was added prior to incubation for 30 min. Wells were washed 10 times with 300 μl/well TBST+1 mM MnCl2. 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 415 nm 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% CO2 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 × 10³ 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

1H 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 5 μm C8(2) 100 × 21.2 mm 100A column running gradient of 10–80% MeCN/H2O (+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 3 μm C8(2) 100A 5 × 3.0 mm column running gradient of 10–95% MeCN/H2O (+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-1H-indene-2-carboxamide (8). To a mixture of 2-[(tert-butoxycarbonyl)amino]-2,3-dihydro-1H-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% NH4Cl (2 × 100 mL), saturated NaHCO3 (1 × 100 mL), saturated NaCl (1 × 100 mL), dried with MgSO4, 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 MgSO4, filtered, and evaporated to dryness to obtain the desired free amine 2-amino-N-ethyl-2,3-dihydro-1H-indene-2-carboxamide (8) (350 mg, 1.628 mmol, 99% yield). MS (ESI) m/z [M+H]+ = 205.04.

4.7.1.2. 2,5-Diazabicyclo[2.2.1]heptan-2-yl[3-(trifluoromethyl)phenyl]methane (12). To a mixture of 3-(trifluoromethyl) benzoic acid (10) (450 mg, 2.37 mmol) and HATU (912 mg, 2.44 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% NH4Cl (2 × 100 mL), saturated NaHCO3 (1 × 100 mL), saturated NaCl (1 × 100 mL), dried with MgSO4, 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
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]heptan-2-yl)amine (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-((trifluoromethoxy)oxy)benzenesulfonic chloride (617 mg, 2.37 mmol) slowly. The reaction mixture was allowed to stir at rt for 2 h. The solvent was evaporated to dryness. The residue was azeotroped with DCM (2 mL, 11.5 mmol) in DMF (6 mL) to yield 2-((2-(trifluoromethoxy)phenyl)sulfonyl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)amine (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-1H-indene-2-carboxamide (14a); 2-((4-chloro-6-(pyrrolidin-1-yl)-N-ethyl-2,3-dihydro-1H-indene-2-carboxamide (14b); 2-((4-chloro-6-(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)amino)-N-ethyl-2,3-dihydro-1H-indene-2-carboxamide (14c).

In three separate vials, to a suspension of tert-butyl 2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-yl)amino)-N-ethyl-2,3-dihydro-1H-indene-2-carboxamide (14d).

To a solution of tert-butyl 2,5-diazabicyclo[2.2.1]heptan-2-yl)-1,3,5-triazin-2-yl)amino)-N-ethyl-2,3-dihydro-1H-indene-2-carboxamide (14e).

Scheme 4. Structures of intermediates.

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)amino)-N-ethyl-2,3-dihydro-1H-indene-2-carboxamide (16a). Compound 14b and (S)-1-(4-bromophenyl)ethanamine were used for the reaction to give 16a (40 mg, 0.044 mmol, 53% yield). 1H NMR (400 MHz, CDCl3) 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). 13C NMR (100 MHz, CDCl3) 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)+ calc'd for [C16H13BrF3N2O2S + H] 749.1270; found 749.1239.

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)amino)-N-ethyl-2,3-dihydro-1H-indene-2-carboxamide (16c). Compound 14c and (S)-1-(4-bromophenyl)ethanamine were used for the reaction to give 16c (25 mg, 0.028 mmol, 31% yield). 1H NMR (400 MHz, CDCl3) 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)+ calc'd for [C15H12BrF3N2O2S + 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)amino)-N-ethyl-2,3-dihydro-1H-indene-2-carboxamide (16c).

Compound 14b and (3,4-dichlorophenethyl)ethanamine were used for the reaction to give 16c (20 mg, 0.024 mmol, 27%

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yield.\textsuperscript{1} 1H NMR (400 MHz, CDCl\textsubscript{3}) \( \delta \) 8.47–8.40 (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). \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) \( \delta \) 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\textsuperscript{+}) \textsuperscript{1}c\textsuperscript{2} calculated for [C\textsubscript{30}H\textsubscript{32}F\textsubscript{12}N\textsubscript{3}O\textsubscript{2}H] \(+\) 739.2285; found 739.2276.

4.7.2.4. \( 2\text{-}((4\text{-}(3\text{-}4\text{-}Dichlorophenethyl)amino)-6\text{-}(5\text{-}(2\text{-}((4\text{-}((3,4\text{-}Dichlorophenethyl)amino)-6\text{-}(pyrrolidin-1\text{-}yl)-7\text{-}ethyl-2,3\text{-}dihydro-1\text{-}H\text{-}indene-2\text{-}carboxamido)}(17d). Compound 14b and phenethylamine were used for the reaction to give 17d (54.5 mg, 0.07 mmol, 81\% yield). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \( \delta \) 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\textsuperscript{+}) \textsuperscript{1}c\textsuperscript{2} calculated for [C\textsubscript{30}H\textsubscript{32}F\textsubscript{12}N\textsubscript{3}O\textsubscript{2}H] \(+\) 739.2285; found 739.2276.

4.7.2.5. \( 2\text{-}((4\text{-}(3\text{-}4\text{-}Dichlorophenethyl)amino)-6\text{-}(pyrrolidin-1\text{-}yl)-1\text{-}3\text{-}triazin-2-ylamino)-2\text{-}3\text{-}dihydro-1\text{-}H\text{-}indene-2\text{-}carboxamide)\) (18a). Compound 14a and \( 2\text{-}((4\text{-}(3\text{-}4\text{-}Dichlorophenethyl)amino)-ethanamine were used for the reaction to give 16d (20 mg, 0.023 mmol, 25\% yield). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \( \delta \) 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, 2H), 4.72–4.63 (m, 2H), 3.82–3.15 (m, 12H), 2.86–2.82 (t, 2H), 2.19–1.69 (m, 2H), 1.10–1.02 (m, 3H). \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) \( \delta \) 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, 124.0, 67.3, 60.2, 59.8, 57.9, 55.5, 54.0, 43.4, 41.8, 37.2, 34.9, 34.4, 14.7. HRMS (M+H\textsuperscript{+}) \textsuperscript{1}c\textsuperscript{2} calculated for [C\textsubscript{32}H\textsubscript{30}F\textsubscript{12}N\textsubscript{3}O\textsubscript{3}S\textsubscript{+}H\textsuperscript{+}] 791.1904; found 791.1891.

4.7.2.6. \( 2\text{-}((4\text{-}(5\text{-}(3\text{-}fluoromethylbenzoyl)-2\text{-}5\text{-}diazacyclob[2.2.2]heptan-2-yl)-1\text{-}3\text{-}triazin-2-ylamino)-2\text{-}3\text{-}dihydro-1\text{-}H\text{-}indene-2\text{-}carboxamide)\) (17a). Compound 14b and \( 5\text{-}(3\text{-}fluoromethylbenzoyl)-2\text{-}5\text{-}diazacyclob[2.2.2]heptan-2-yl)\)-1,3,5-triazin-2-ylamino)-2,3-dihydro-1H-indene-2-carboxamide (17a). Compound 14a and \( 5\text{-}(3\text{-}fluoromethylbenzoyl)-2\text{-}5\text{-}diazacyclob[2.2.2]heptan-2-yl)\)-1,3,5-triazin-2-ylamino)-2,3-dihydro-1H-indene-2-carboxamide (17b). Compound 14b and \( 5\text{-}(3\text{-}fluoromethylbenzoyl)-2\text{-}5\text{-}diazacyclob[2.2.2]heptan-2-yl)\)-1,3,5-triazin-2-ylamino)-2,3-dihydro-1H-indene-2-carboxamide (17c). Compound 14b and benzylamine were used for the reaction to give 17c (38.7 mg, 0.045 mmol, 51\% yield). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \( \delta \) 8.76–8.70 (m, 1H), 8.14–7.57 (m, 5H), 7.33–7.14 (m, 9H), 6.05 (65 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\textsuperscript{+}) \textsuperscript{1}c\textsuperscript{2} calculated for [C\textsubscript{35}H\textsubscript{33}F\textsubscript{12}N\textsubscript{3}O\textsubscript{2}H] \(+\) 657.2908; found 657.2922.

4.7.2.9. \( N\text{-}Ethyl-2\text{-}((4\text{-}(phenethylamino)-6\text{-}(5\text{-}(3\text{-}(trifluromethyl)benzoyl)-2\text{-}5\text{-}diazacyclob[2.2.2]heptan-2-yl)-1\text{-}3\text{-}triazin-2-ylamino)-2,3-dihydro-1H-indene-2\text{-}carboxamide)\) (17d). Compound 14b and phenethylamine were used for the reaction to give 17d. Compound 14b and phenethylamine were used for the reaction to give 17d. Compound 14b and phenethylamine were used for the reaction to give 17d. Compound 14b and phenethylamine were used for the reaction to give 17d.
solution of 2,3-dihydro-1H-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).1H NMR (400 MHz, CDCl3) 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 [C29H30ClF3N2O+]+ = 481.1892; found 481.1891.

4.7.3. Synthesis of fluorescent 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 mixture was stirred at rt for 2 h. The solvent was removed under vacuum and azeotroped with DCM (3 mL) and then centrifuged at 3000 r/min for 10 min. The supernatant was discarded and the residue was re-dissolved in 400 mL of water and purified with Prep HPLC to yield 20a (0.06 mmol, 24% yield). MS (ESI) m/z [M+1]/3 = 1975.38 (calcld 1975.60).

4.7.3.5. Synthesis of compound 20b. The procedure for the synthesis of 20a was used with 19 (0.18 mmol) and 13 (9.0 mmol) as starting materials to yield 20b (0.09 mmol, 52% yield). MS (ESI) m/z [M+1]/3 = 1957.96 (calcld 1958.7).

Conflict of interest

The authors have declared no conflict of interest.

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