Published in final edited form as:

J Med Chem. 2015 February 12; 58(3): 1466–1478. doi:10.1021/jm5018187.

Discovery of Small Molecule Inhibitors to Krüppel-Like Factor 10 (KLF10): Implications for Modulation of T Regulatory Cell Differentiation

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Abstract

The Krüppel-like family of transcription factors (KLFs) constitute a subfamily of C₂H₂-type zinc finger proteins with distinct cell-type expression patterns and regulate functional aspects of cell growth and differentiation, activation, or development. KLF10 has been previously shown to critically regulate the acquisition of CD4+CD25+ T regulatory cell differentiation and function, an effect important to the maintenance of self-tolerance, immune suppression, and tumor immunosurveillance. To date, there are no selective pharmacological inhibitors to KLF10. Herein, we report on the discovery of first-in-class small molecule compounds that inhibit the KLF10-DNA interaction interface using computer-aided drug design (CADD) screens of chemical libraries. Interrogation of a 'druggable' pocket in the 2nd zinc-finger of KLF10 revealed three small molecules, #48, #48-15, and #15-09, with similar scaffolds and binding patterns. Each of these small molecules inhibited KLF10-DNA binding and transcriptional activity, conversion of CD4+CD25[−] T cells to CD4+CD25⁺ T regulatory cells, and KLF10 target gene expression. Taken together, these findings support the feasibility of using CADD with functional assays to identify small molecules that target members of the KLF subfamily of transcription factors to regulate biological functions in health and disease. We hope these novel compounds will serve as useful mechanistic probes for KLF10-mediated effects and T regulatory cell biology.

Zinc fingers (ZFs) are the most ubiquitous family of transcription factors (TFs) with more than 1000 members, comprising ≈1% of the human genome.¹ The Krüppel-like family of

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Supporting Information Available: Attached document contain Supplemental Figures 1 – 3 and Supplemental Tables 1–3 that include the raw data (flow cytometry and RT-PCR) used to generate Figure 4 and 5 (Inhibition of conversion of CD4+CD25[−] T cells to CD4+CD25⁺ T regs by small molecule compounds), structures of previously reported inhibitors of another transcription factor GLI, the analytical data that support purity of compounds #48, #48-15, and #15-09, and the list of molecules from the SBVS-150 set. This material is available free of charge via the Internet at <http://pubs.acs.org>.

transcription factors (KLFs), so named for their homology to the *Drosophila melanogaster* Krüppel protein, is a C₂H₂-type zinc finger (containing 2 cysteine and 2 histidine residues) with a simple structure consisting of 25–30 amino acid residues that includes two β -pleated sheets in the amino terminal half and an α -helix in the carboxyl terminal half, held together at the base by a zinc (Zn⁺²) ion.^{2–4} The ZFTFs are responsible for interacting with either a CACCC-element or GC-rich sites in the promoter region of target genes, thereby regulating transcriptional activity and gene expression.⁵ These DNA binding proteins are requisite components of the transcriptional machinery involved in mediating cellular phenotypic responses to extracellular signals. The KLF family currently includes 18 members, namely KLF 1–18 with distinct cell-type expression patterns.⁶ Furthermore, KLFs participate in various functional aspects of cell growth and differentiation, activation, or development. As such, therapeutic targeting of select KLF family members may be desirable for achieving distinct biological effects.

CD4+CD25+ T regulatory cells (T regs) actively participate in the maintenance of self-tolerance and immune suppression and constitute a significant obstacle for effective tumor immunosurveillance, vaccine-induced anti-tumor immune responses, and clearance of bacterial pathogens.^{7–11} We and others have previously demonstrated that KLF10, a transforming growth factor beta 1 (TGF- β 1)-responsive transcription factor, is a critical regulator of T reg cell differentiation and function *in vitro* and *in vivo*.^{12, 13} KLF10^{–/–} mice exhibit reduced peripheral T reg cells with reduced expression of Foxp3, a hallmark of T reg cell differentiation. Indeed, KLF10 mediates this effect, in part, by binding to the proximal CACCC sites of the Foxp3 and TGF- β 1 promoters. These observations raise the possibility that KLF10 may be an excellent therapeutic target for a range of disease states in which reduced T reg cell numbers or function would be advantageous such as enhanced anti-tumor immunity.^{7–11}

Currently, there is no clinically available treatment that specifically targets T reg cells using a small drug-like molecule. Herein, we report computational discovery of small molecule inhibitors of the KLF10-DNA interaction interface, an effect that reduced KLF10-DNA binding and transcriptional activity. Furthermore, we also report an investigation into the effects of our inhibitor compounds on the conversion of CD4+CD25[–] T cells to CD4+CD25+ T regs and FoxP3 expression. Overall, our findings support the feasibility of using computer-aided drug design (CADD) with functional assays to identify small molecules that target members of the KLF subfamily of transcription factors to regulate biological functions.

Results

Homology model of KLF10 reveals a large binding pocket within the DNA-binding region

A homology model of KLF10 protein was generated using template coordinates of a DNA-binding protein having three consensus-sequence-based zinc finger domains. The overall topology of the KLF10 structure matches with the expected fold of classical C₂H₂ zinc finger domains in which two short strands of antiparallel β strands linked by a rubredoxin-like turn are followed by an α -helix that contains two Zn⁺²-coordinating histidine residues. In our current efforts to identify modulators of TF-dependent transcription, we hypothesized

that small organic molecules capable of binding to the KLF10 zinc finger region that interfaces the DNA, would be able to inhibit the binding of KLF10 with DNA and, subsequently, reduce transcriptional activity. An analysis of the zinc finger-DNA binding region using multiple binding pocket identification programs such as SiteMap^{14, 15} (Schrodinger), MolCad¹⁶ (Tripos) and SiteFinder¹⁷ (Chemical Computing Group), uniquely identified a relatively large pocket in the middle zinc finger which has maximum surface contacts with the DNA (Fig. 1A–B). We subclassified this binding pocket into α - and β -pockets depending on the proximity with α -helix or β -sheet residues encompassing it.

***In silico* screens identified small molecule inhibitors of KLF10**

High-throughput docking of chemical libraries using Glide docking^{18, 19} with extra precision (XP)²⁰ scoring into grids encompassing the α - and β -pockets resulted in ~1400 unique hits. This subset was further evaluated with another two independently developed dockers: Surflex²¹ and FlexX.²² The highest scored 700 molecules were then subjected to consensus scoring and ADME/Tox profiling analysis to select 40 molecules, which were subsequently tested in KLF10 reporter assays as detailed below.

Identified compounds inhibit KLF10 transcriptional activity

We developed a cell-based reporter assay to test the transcriptional activity of *in silico* identified compounds. A KLF10 expression plasmid and luciferase reporter driven by a CACCC-responsive promoter were transfected into HeLa cells. Exogenous KLF10-induced luciferase activity was approximately 2.5-fold compared with transfection of empty vector (Figure 1C–E). *In vitro* screening using this luciferase reporter assay resulted in the discovery of 11 inhibitors of KLF10 reporter activity (Table 1). Several structural analogs of two scaffolds, #48 and #15 (Table 2 and 3) were computationally investigated to explore the chemical space governing the activity; many of these analogs showed equal or better potency than the parent compound in luciferase reporter assays. For example, the induction of luciferase activity by exogenous KLF10 expression was almost completely blocked by 100 μ M of compound #48 (96.6%, Figure 1C). In the presence of 100 μ M of compound #48-15, reporter activity was reduced to even lower than basal conditions (–20% vs. control, Figure 1D). Similarly, compound #15-09 also potently inhibited KLF10 induced luciferase activity to –31% of basal conditions (Figure 1E). In contrast, small molecule compounds #3, #23, #37, #40, #65, #79, #83, #96, #104, #106, #114, #118, #121, #126, and #146 had no effect on KLF10-induced luciferase activity (Figure 1F). We also examined dose-dependent responses of compounds #48, #48-15, and #15-09 on KLF10 transcriptional activity. IC₅₀s, calculated based on non-linear regression analysis, for compounds #48, #48-15, #15-09 were 112 μ M, 40 μ M, and 43 μ M, respectively (Figure 2A, 2B, 2C). Collectively, these findings highlight the identification of three small molecule compounds, #48, #48-15, and #15-09, which could effectively inhibit KLF10 transcriptional activity.

Lead compounds inhibit binding of KLF10 to DNA

To determine whether identified compounds inhibit the binding of KLF10 to DNA, KLF10 was incubated with double-stranded DNA containing a KLF10 consensus binding site in the presence of identified compounds #48, #48-15, or #15-09, or DMSO control. We first

validated that purified KLF10 protein bound to the KLF10 consensus DNA-binding site in a specific manner as the binding was completely blocked by unlabeled oligonucleotide DNA probe, and there was no binding using KLF10 protein lacking the DNA-binding domain (GST-KLF10 amino acid 111–210); in addition, the KLF10 DNA-protein complex was super-shifted by a KLF10 antibody (Figure 3A). The identified small molecule compounds were next examined for effects on KLF10 DNA-protein binding. As shown in Figure 3B, compound #48 inhibited KLF10 DNA-binding activity by 25% and 11% at 40 μ M and 8 μ M, respectively. Compound #48-15 inhibited KLF10 DNA-binding activity by 45% and 12% at 40 μ M and 8 μ M, respectively (Figure 3C). In addition, compound #15-09 inhibited KLF10 DNA-binding activity by 83% and 25% at 50 μ M and 8 μ M concentrations, respectively (Figure 3D). In contrast, no dose-dependent inhibition on DNA-binding was observed for another member of the KLF family, KLF1 (Figure 3E–F), showing specificity. Taken together, these data indicate that small molecule compounds #48, #48-15, and #15-09 could each inhibit binding of KLF10 protein to DNA.

Lead compounds inhibit conversion of CD4+CD25– T cells to CD4+CD25+ T regulatory cells

Our previous work and that of others have identified a critical role for KLF10 in its ability to convert CD4+CD25– T cells into CD4+CD25+ T regs.^{12, 13} To test if these lead compounds had any effect on T reg cell differentiation, CD4+CD25– T cells were cultured for the conversion of CD4+CD25– T cells to CD4+CD25+ T regulatory cells in the presence of the indicated small molecule compounds #48, #48-15, and #15-09. As shown in Figure 4, Supplemental Figure 1, and Supplemental Table 1, compound #48, #48-15, and #15-09 inhibited the percentage of CD4+CD25+ T regs by 57.4%, 59.1%, and 29.2%, respectively. Collectively, these findings suggest that small molecule compounds #48, #48-15, and #15-09 have the ability to alter T reg cell differentiation.

Identified small molecule compounds inhibited FoxP3 expression during the conversion of CD4+CD25– T cells to CD4+CD25+ T regulatory cells

Foxp3, a forkhead transcription factor, is a critical protein involved in the differentiation of T regs.^{23–27} Indeed, our previous report demonstrated that KLF10 can potentially induce Foxp3 expression by binding to an evolutionarily conserved CACCC DNA-binding site in the proximal Foxp3 promoter.¹² To examine the effects of the identified tool compounds #48, #48-15, and #15-09 on Foxp3 expression, CD4+CD25– T cells were cultured for the conversion to CD4+CD25+ T regs in the presence of each of the identified compounds. Compound #48, #48-15, and #15-09 inhibited Foxp3 expression by 84.7%, 82.5%, and 90.8%, respectively, at 50 μ M (Figure 5 and Supplemental Table 2). Taken together, these observations indicate that these compounds effectively suppress the KLF10 target gene Foxp3, a critical regulator of T reg cell differentiation.²⁸

Discussion

In recent years, *in silico* CADD and lead optimization strategies including structure-based and ligand-based virtual screens have become popular and, successfully complement or, in certain cases, replace HTS approaches for early stage identification of potential lead

compounds with improved chemical diversity and enhanced pharmacodynamic and pharmacokinetic properties. KLFs constitute an important class of transcriptional regulators in diverse aspects of cell growth and differentiation. Furthermore, specific KLFs have been implicated in disease states ranging from tumorigenesis, inflammation, and cardiovascular disease, among others. However, given the lack of enzymatic activity, transcription factors in general have largely been considered 'non-druggable' to small molecule inhibition.^{29–31} This provides an exciting and challenging opportunity for those in the field of drug discovery. To date, examples of small molecules targeting transcription factors have largely focused on protein-protein interactions of 'dimeric' transcription factors, so-called due to their need to form heterodimers or homodimers for efficient transcriptional activation and DNA binding including c-Myc/Max, HIF1- α /HIF-1 β , and STAT3 dimers.^{29–31}

There are few reported attempts of targeting other ZFTFs by small drug-like molecules. Lin et al.³² screened a 250-membered heterocycle library containing diverse scaffolds for activation of the reporter gene after transfection with the zinc finger C7 protein, a structural variant of the murine zinc finger zif268. As a result, a potent 2-(4'-quinoline)benzimidazole compound was identified which induced luciferase activity by about 18-fold at 100 μ M concentration (EC_{50} = 35 μ M); however, at concentrations above 100 μ M it exhibited significant cytotoxicity. Lauth et al.³³ used a cell-based screen to identify small-molecule antagonists of GLI-mediated transcription, thereby, inhibiting Hedgehog signaling implicated in several malignancies. They discovered two small molecules, GANT61 and GANT58 (NSC 136476 and NSC 75503, respectively; where GANT stands for *Gli*-ANTagonist), which inhibited the GLI-induced transcription in transfected HEK293 cells at 30 μ M concentration (Supplemental Figure 2).

We report the first-in class discovery of inhibitors of the KLF10-DNA interaction interface using small 'drug-like' molecules. For structure-based screening, we generated a working homology model of KLF10 in association with DNA with attention to modeling of side chains of amino acids at the DNA interface (Figure 1A). In the absence of prior knowledge of binding pocket or point-mutation data critical in DNA binding, we carefully studied the protein-DNA interface of KLF10 using independently developed pocket identification algorithms to identify, with consensus, a potentially druggable pocket for high throughput docking (HTD) of small molecule databases. A large, shallow pocket was observed within the middle zinc finger region and we sub-classified this binding pocket as α - and β -pocket depending on the proximity with α -helix or β -sheet residues surrounding it (Figure 1B). We hypothesized that tighter binding of small molecules in this pocket would provide an opportunity to interfere with KLF10-DNA binding and subsequent inhibition of transcriptional activity.

A fast *in silico* HTD screen of publically available databases using the α - and β -pockets separately was performed with constrained Glide docking in SP settings. To offer both pockets an equal opportunity for binding, we selected ~1800 molecules that exhibited binding in both pockets. In the next level of filtering we provided every molecule in this set to dock without constraints and used a more accurate scoring function available through Glide XP docking. The outcome of this analysis showed that a good portion of these

molecules (~400 molecules) were thrown away from this interface and ~1400 molecules (*GlideXP-1400*) were able to virtually bind in one of the proposed pockets.

While the current state of the art in docking simulations and scoring technologies are very good at generating the correct binding pose of a given ligand, they are less facile in identifying the bioactive conformation as a top scoring pose (scoring issue) or rank-ordering the bioactive poses of different ligands (rank-ordering issue), effects that determine the success of a docker in high throughput virtual screening. Because none of the presently available dockers are capable of performing well in overcoming the scoring and rank-ordering issues, researchers apply consensus docking and scoring strategies to improve the enrichment of *in silico* screens³⁴. In the present study, we performed docking of the *GlideXP-1400* molecule set using two additional well-known dockers commercially available to the scientific community: Surflex and FlexX. We selected 50% of the molecules top-ranked by all three dockers (Glide XP, Surflex and FlexX) and chose a common set of 150 molecules (*SBVS-150*) after performing ADME/Tox filtering analysis (Supplemental Table 3). The KLF10 luciferase reporter screen of 40 molecules from the SBVS-150 set validated the activity of 11 structurally different inhibitor scaffolds (Table 1). We selected compounds #48 and #15 for further chemical space expansion leading to more than 30 additional inhibitors. Each of the three compounds #48, #48-15 and #15-09 inhibited KLF10 transcriptional activity (Figure 1–2), KLF10-DNA binding (gel mobility shift assay) (Figure 3), conversion of CD4+CD25– T cells to CD4+CD25+ T regs (Figure 4), and Foxp3 gene expression (Figure 5).

Binding Mode and Molecular Interactions Analysis of compounds #48, #48-15 and #15-09

Owing to the shallow pocket, there was variation in top binding poses from different docking runs. In order to explore this variability problem in multiple scales of speed and accuracy, we performed two types of optimizations on the top 50 poses of #15 and #48 series of inhibitors obtained from the Glide XP docking run as below: (1) Glide ‘refine’ment, in which only the ligand was allowed to relax in the protein binding pocket followed by re-scoring of each pose with Glide XP function; and (2) eMBRACe minimization, which allowed both ligand and its surrounding 5 Å residues to relax, followed by in-place scoring and ranking of eMBRACe minimized complexes using Glide. An analysis of output of these two optimization procedures revealed that rank ordering or enrichment of eMBRACe-optimized complexes correlated better to luciferase activity of these hits, justifying the need for protein and ligand relaxation to obtain a more stable binding pose (Figure 6). However, none of these attempts categorically separated actives from false positives (decoys).

Docking with eMBRACe minimization of compound #48 within the KLF10 pocket shows the possibility of various binding modes without substantial loss of binding energy (Docking Score, DS: –5.4 to –4.5), with its resorcinol ring occupying either the α - or β -pocket. The top pose of compound #48 stably docked in α -pocket (Figures 6A), however other poses can also fit into β -pocket. In the α -pocket, the resorcinol ring of compound #48 participates in π -interactions with the phenyl ring of Phe410 and two hydroxyl groups form hydrogen bond interactions with side chains of His419 (through *p*-OH) and Glu415 (through *o*-OH). Among

the Glide 'refine' poses (Figures 6A; green sticks), the top pose occupied only the α -pocket with two -OH forming hydrogen bonds with His419 (through *o*-OH) and Arg418 (through *p*-OH) and toluene ring resting in the extreme of the α -pocket, whereas the rest of the poses occupied both pockets with either of two possible sets of hydrogen bonds between hydroxyl groups of compound #48 and His419 and Glu415 or Glu415 and Lys397 (C=O). Compound #48-15 represents a binding mode with two rings occupying two subpockets and a hydroxyl group forming a hydrogen bond either with His419 or Glu415, an effect that may be due to its symmetrical linker (-CO-CH₂-CO-) connecting two rings (Figures 6B). An analysis of Glide 'refine' poses (Figures 6B, green sticks) also revealed a unique binding mode occupying both pockets, with a hydroxyl group making hydrogen bonding interactions with the side chain carboxyl of Glu415 and the backbone amine of Ala411 (bifurcated H-bond), whereas the carbonyl oxygen of ester in compound #48-15 forms a hydrogen bond with Lys397. In the representative binding modes of the eMBRACE minimized poses (Figures 6B, plum sticks), the phenolic ring occupied β -pocket (in some cases it forms H-bond with carboxylic oxygen of Glu415), while oxygen of methyl ether in the α -pocket forms a hydrogen bond with side chain nitrogen of His419 and carbonyl oxygen of linker ester forms a hydrogen bond with Lys397.

The binding mode analysis of #15-09 revealed that it occupies both pockets (Figures 6C; gray, green and plum sticks), with the pyrimidothiophene ring residing in the β -pocket and *m*-OH group of the other ring in the α -pocket, forming hydrogen bonds with the guanidinium side chain of Arg418, backbone oxygen of Glu415, and/or the side chain imidazole of His419. The Glide 'refine' as well as eMBRACE minimized poses also revealed the similar pattern of binding for compound #15-09 but more equivocally and with least variation in poses due to relaxation of ligand and/or receptor pocket after optimization.

Thus, the general binding patterns of compound #15 series scaffolds has an overall similarity with the binding pattern of #48 compound series scaffold such that two aromatic rings occupy the two pockets. This may be, in part, due to the similarity in the linker [-X-CH₂-C(=O)-], separating the aromatic rings placed in two respective pockets and forming a bridge to connect them. This similarity, in part, may also underlie the mechanism of binding of these compounds, as has been reflected in their luciferase potencies (IC₅₀ values of #48-15 and #15-09 are ~40 μ M), ability to inhibit DNA binding, and T reg cell differentiation. The length and nature of the linker [X=O versus X = OC(=O)] in these series (Tables 2 and 3) may play a serious role in proper placement of aromatic rings in respective pockets which may afford further stability through aromatic and hydrophobic interactions in addition to physical anchoring for tighter binding. As a result, modifying the linker could provide future scope for additional series of inhibitors, which may be even more potent than the ones reported here.

Conclusion

In summary, we demonstrate proof-of-principle for the discovery of small molecule inhibitors of the KLF10-DNA interaction interface using *in silico* screens of chemical libraries. Interrogation of a 'druggable' pocket in the 2nd zinc-finger of KLF10 revealed three molecules, #48, #48-15, and #15-09, with similar scaffolds and binding patterns.

Indeed, each of these small molecules inhibited KLF10-DNA binding and transcriptional activity, conversion of CD4+CD25⁻ T cells to CD4+CD25⁺ T regs, and KLF10 target gene expression. Taken together, these findings support the feasibility of using CADD with functional assays to identify small molecules that target members of the KLF subfamily of transcription factors to regulate biological functions in health and disease.

Materials and Methods

Computational Methods

All molecular modeling operations were carried out in the Maestro (version 9.0 Schrödinger Inc., USA), Sybyl (version 7.2; Certara Inc. USA) and Molecular Operating Environment (version 2009.10; Chemical Computing Group Inc., Canada) modeling packages running on Dell Precision 690 workstation with 8 CPUs, 10 GB memory and Red Hat Enterprise 5 Linux Operating System.

Homology Modeling of KLF10

A truncated model of three zinc-fingers of KLF10 (amino acids 361–459) was built using F-chain of X-ray crystal structure² (PDB code: 1MEY) of a designed zinc finger protein in complex with DNA. Sequences of KLF10 and template proteins exhibited 51% identity, leaving weaker coordinate information for two small loops: amino acids 431–436 and 449–451. The two loops were refined using *Extended High* (accuracy) loop sampling method in Prime (version 2.1).^{35, 36} The coordinates for Zn⁺² ions were extracted from the template structure and verified for coordination distances with corresponding cystines and histidines. The (coordinate bonded) Cys – Zn⁺² – His pattern was preserved from the X-ray structure positions during side chain refinements by applying distance constraints. This was followed by a protein structure analysis and refinement of disallowed backbone (θ , ψ , and Ω) and side chain dihedral angles, peptide planarity, bond length and angle deviations and steric clashes between side chains. Finally, DNA-bound KLF10 structure was built by extracting DNA coordinates from X-ray crystal structure and resolving steric clashes by rotamer search of side chains of interface residues having bumps with DNA nucleotides, followed by optimization of overall interaction energy of complex using OPLS-2005 (Optimized Potential for Liquid Simulations)³⁷ molecular mechanics energy minimizations (Figure 1). The optimized structure (without DNA coordinates) was then subjected to binding pocket analysis using pocket identification algorithms employing different methods. For example, SiteMap^{14, 15} which is a combination of geometry-based and energy-based methods³⁸ along with molecular surface calculations in Maestro was applied in addition to MolCad¹⁶ (Sybyl/Tripes), which is a grid-based flood-filled solvation technique³⁹. Another tool that was used in pocket identification, SiteFinder¹⁷ is based on the calculation of appropriately exposed alpha spheres which are clustered to produce a collection of sites. Each site consists of several alpha spheres, at least one of which is hydrophobic. The sites are then ranked according to the number of hydrophobic contacts made with the receptor, i.e. the number of hydrophobic atoms within a contact distance of any of the retained alpha spheres to uniquely identify a ‘druggable’ pocket at the interface.

These programs identified, with consensus, a relatively big pocket in the second ZF (aa 385–425) which spans from β -sheet to α -helix (near Zn^{+2}), subdivided with a small cleft into, correspondingly termed, α - and β -subpockets, which were subsequently used for high throughput docking (HTD) of chemical libraries (Figure 1B).

Structure-based Virtual Screening (SBVS)

The chemical libraries used in the present screening included structures from Chembridge (<http://www.chembridge.com>; ~400,000 molecules), Asinex (<http://www.asinex.com>; ~200,000 molecules), National Cancer Institute (http://dtp.nci.nih.gov/docs/3d_database/dis3d.html; ~250,000 molecules), and ZINC⁴⁰ databases (downloaded as of June 2008 and accessed online later for similarity and substructure searches). The structures were prepared using Ligprep (version 2.2), which performed hydrogen addition and 3D structure optimization. Epik (through Ligprep) was used to identify possible ionization and tautomer states at physiological pH (7.0 ± 2.0), along with generating ring conformers and stereoisomers using default settings. The ionization state penalties for each protomer state were also calculated and stored. Two separate receptor grids for docking calculations were prepared, one for each of α - and β -subpocket, by assigning individual residues forming the respective pocket, with hydrophobic and H-bond constraints. A high-throughput docking (HTD) was performed to filter out weak binders in α - and β -subpockets separately using Glide (version 5.0)^{18, 19} in standard precision (SP) scoring mode. An analysis of hits that satisfied the hydrogen bond and hydrophobic constraints revealed that approximately four-times more hits preferred α -pocket over β -pocket, and ~1800 hits were common to both pockets. A third receptor grid encompassing residues from both α - and β -subpockets was used for Glide (version 5.5) docking without constraints, and using more accurate extra precision (XP) scoring.²⁰ Post-docking analysis identified that 1400 hits occupied either α - and/or β -subpocket, leaving out remaining molecules docked off-site of the TF-DNA interface. This *GlideXP-1400* set was then further docked using additional two independently developed dockers, Surflex²¹ and FlexX²² in Sybyl (version 7.2). The hits common in top 700 rank-order of each docking algorithm were compared to shortlist 150 molecules (*SBVS-150* set) after ADMET evaluation using QikProp (version 3.1; Schrodinger) and Sarchitect (Strand Life Science). A subset of 40 compounds was evaluated in luciferase reporter assays. Compounds were acquired from different vendors and dissolved in DMSO. The 2D structures of active compounds are shown in Table 1–3. The purity of compounds #48, #48-15, and #15-09 was verified to be 95% using HPLC equipped with (a) Column: Onyx C18 50×4.6mm (flow rate = 3.75ml/min), (b) UV detector operating at dual wavelengths, and (c) evaporative light-scattering detector (Supplemental Figure 3). Additionally, small molecules were also screened for substructural features that appear in frequent hitters (promiscuous compounds) in several biochemical HTS. Such compounds are also known as Pan Assay Interference compounds (PAINS) and researchers have created systematic filters to remove such compounds from virtual screens⁴¹. We employed PAINS1, PAINS2, and PAINS3 filter definitions as implemented in Schrodinger's Canvas program that correspond to filter C, filter B, and filter A, respectively, of previous study⁴¹. None of our screened compounds (Table 1–3) showed PAINS attributes as per the structure definitions of PAINS1 (filter C) and PAINS3 (filter A). However, compounds #57 (Table 1), #15-07 and #15-09 (Table 3) were marked as possible PAINS

according to PAINS2 (filter B) substructure definitions. Since these compounds were passed in other two PAINS (1 and 3) filters, we assume that the promiscuous liability is relatively less, making worth investigating their effects in this novel (non-enzymatic) and relatively undruggable target space.

Chemical Structure Exploration of Lead Compound Scaffolds

Potent and promising hits were subjected to chemical space exploration by performing substructure search of their scaffolds to find their close analogs (second-generation compounds) in databases. The structural information of inhibitors identified in SBVS screens was employed to identify a working pharmacophore query using Phase^{42, 43} (version 3.1; Schrodinger, Inc). This query was used to bias the selection of second-generation compounds towards chemical features of known inhibitors to improve enrichment. The structural analogs were subsequently evaluated using Glide XP docking to rank-order a small subset for activity testing (Table 2 and 3).

Constructs, cell culture and transfection, and luciferase reporter assays

CACC-TK-luc plasmid contains three tandem copies of CACCC sites upstream to a luciferase reporter in pGL3-basic as previously described.^{12, 44} Empty vector pCMV-SPORT6 and human KLF10 expressing plasmid pCMV-SPORT6-KLF10 are from Open Biosystems (Huntsville, AL). HeLa cells from ATCC were cultured in DMEM medium with 10% Fetal Bovine Serum, 1% antibiotics. Cells were transfected by using Fugene® 6 transfection reagent from Roche. In brief, cells were plated (40 000/well) in triplicate in a 12-well plate. After growing to 70–80% confluency, cells were transfected with 500 ng of CACC-TK-luc reporter construct, 200 ng β -galactosidase expression plasmids, and 100 ng pcDNA3.1 empty vector or pcDNA3.1-KLF10. After 24 h incubation, cells were treated with 100 μ M or indicated concentration of small molecule compounds for 24 h. Treated cells were collected in 200 μ l 1 $\times$ Reporter Lysis Buffer (Promega). The activity of luciferase were measured and normalized to total protein read for the same lysate performed in triplicate replicates.

Real time qPCR

T cells were suspended in TRIzol® reagent (Invitrogen), and total RNA was isolated according to the manufacturer's instructions. Reverse transcriptions were performed by using the Reverse Transcription Kit and QuantiTect SYBR Green reverse transcription-PCR Kit from Qiagen for quantitative real-time quantitative PCR analysis on a M $\times$ 3000P Real-time PCR system (Stratagene) following the manufacturer's instructions. Primers were used to detect mouse FoxP3 as previously described.¹²

Gel mobility shift assays

KLF10 recombinant protein was obtained from Novus Biologicals. Oligonucleotides 5'-GCAGTGAAAAGGGGTGTGTCAGGATGC and 5'-GCATCCTGACACACCCCCTTTTCACTGC were generated as previously described⁴⁵ and labeled with Biotin using Biotin 3' End DNA Labeling Kit (Pierce). End-labeled oligos (1 μ mol/L) were annealed in a buffer system with 10 mM Tris, 50 mM NaCl, pH8.0 by

heating at 95 °C for 5 min, then slowly cooled to 65 °C for 30 min incubation, and cooled to room temperature. Lightshift chemiluminescent EMSA kit (20148) was from Thermo Scientific. Binding reactions contain 1× binding buffer, 2.5% glycerol, 5mM MgCl₂, 50 ng/μl poly (dI-dC), 0.05% NP-40, and 5 fmol Biotin-labeled probes in the presence of different concentrations of compounds, and 100 ng purified recombinant KLF10. Compounds were incubated with KLF10 in a reaction system for 4 hrs at room temperature, then Biotin-labeled probes was added for 1 hr incubation. Binding reactions were electrophoresed for 80 min at 100 volts on 6% polyacrylamide gel in 0.5 × TBE. The binding reactions were transferred to nylon membranes at 100 volts for 45 min, and transferred DNA was cross-linked to membrane using an HL-2000 Hybrilinker instrument. Biotin-labeled DNAs were detected by chemiluminescence.

T regulatory cell differentiation assay and flow cytometry

Mouse spleens were meshed through a cell strainer into Phosphate Buffered Saline (PBS) with 2% Fetal Bovine Serum and 1% Penicillin/Streptomycin, and cells were collected by spinning at 1000 rpm for 10 min. CD4+CD25- T cells were purified by using CD4+CD25+ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, cells were incubated with Biotin-conjugated antibodies, followed by anti-biotin microbeads and PE-conjugated anti-CD25 antibody for 30 min on ice with gentle shaking. Mixtures were loaded into pre-washed LD columns in the Magnetic field, and CD4+ cells were collected and then incubated with anti-PE microbeads. Mixtures were loaded into prewashed MS columns in the magnetic field to collect CD4+CD25- T cells (non-bound cell fraction). Cells were plated into 12-well plates pre-coated with 1μg/ml anti-CD3e (eBioscience) overnight in RPMI1640 Medium with 10% FBS, 1% Penicillin/Streptomycin, 1% non-essential amino acids, and 2-mercaptoethanol, 1μg/ml anti-CD28 (BD Biosciences), and 1ng/ml TGF-β1 (Peprotech). Cells were cultured at 37°C up to five days for the conversion of CD4+CD25- T cells to CD4+CD25+ T regs. For flow cytometry, cells were stained with anti-mouse CD25 Alexa Fluor®488 (eBioscience) and analyzed by using multicolor FACS (ARIA; BD Biosciences) as previously described.¹²

Supplementary Material

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Abbreviations

CD4	cluster of differentiation 4
CD25	cluster of differentiation 25
eMBrAcE	Multi-Ligand Bimolecular Association with Energetics
Foxp3	forkhead box P3
HTD	high throughput docking
KLF10	krüppel-Like Factor 10
PAINS	Pan Assay Interference compounds

TFs	transcription factors
T regs	T regulatory cells
TGF-β1	transforming growth factor beta-1
SP	standard precision
SD	standard deviation
XP	extra precision
ZFs	Zinc fingers

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Figure 1.

Homology model of KLF10 protein revealing the proposed pocket of small molecule binding and its relative location in the DNA-bound complex (A–B): Molecular surface of DNA-binding domain of KLF10 protein (blue) along with DNA strands (plum) shown to represent the relative orientation/location of proposed binding pockets along with compound #48-15 (A; green, mesh representation). Proposed binding pocket within second zinc finger of KLF10 (electrostatic surface; blue color for positive potential and red color for negative potential), with alpha and beta subpockets separated by Glu415 side chain (B). Inhibition of

KLF10 transcriptional activity by small molecule compounds #48, #48-15, and #15-09 (C–E): HeLa cells were transiently transfected with human KLF10 and a CACCC-luciferase reporter. After transfection, cells were allowed to grow for 24 hrs and subsequently treated with compounds #48 (C), #48-15 (D), and #15-09 (E) for another 24 hrs at which time they were harvested for luciferase activity assays. In (F), HeLa cells were transfected in a similar manner as above, except they were treated with small molecule compounds #3, #23, #37, #40, #65, #79, #83, #96, #104, #106, #114, #118, #121, #126, and #146. Data show mean $\pm$ SD, n=3. *, $P < 0.01$. Con, empty-vector control.

Figure 2.

Dose-response curves of KLF10 transcriptional activity of HeLa cells treated with small molecule compounds #48, #48-15, and #15-09. HeLa cells were transiently transfected with human KLF10 and a CACCC-luciferase reporter. After transfection, cells were allowed to grow for 24 hrs and subsequently treated with different amounts of #48 (A), #48-15 (B), and #15-09 (C) for another 24 hrs at which time they were harvested for luciferase activity assays.

Figure 3.

Effect of identified small molecule compounds on KLF10 DNA-binding activity by electromobility gel shift assays. (A) GST-KLF10 specifically bound to a KLF10 consensus DNA-binding sequence. Small molecule compounds #48 (B), #48-15 (C), and #15-09 (D) inhibited KLF10 DNA-binding activity. (E–F) In contrast, there was no inhibition of DNA-protein binding of the related KLF, KLF1, by small molecules.

Figure 4.

Inhibition of conversion of CD4+CD25⁻ T cells to CD4+CD25⁺ T regs by small molecule compounds #48, #48-15, and #15-09. CD4+CD25⁻ T cells were isolated and cultured for the conversion to CD4+CD25⁺ T regs in the presence or absence of small molecule compounds #48 (A), #48-15 (B), and #15-09 (C) for 3 days and harvested for analysis by flow cytometry. Data show mean $\pm$ SD, n=3. *, $P < 0.05$.

Figure 5.

Inhibition of FoxP3 expression by small molecule compounds #48, #48-15, and #15-09. CD4+CD25- T cells were isolated and cultured for the conversion to CD4+CD25+ Tregs in the presence or absence of small molecule compounds #48 (50 μ M), #48-15 (50 μ M), and #15-09 (100 μ M) and harvested for analysis by qPCR for Foxp3 expression. Data show mean $\pm$ SD, n=3. *, $P < 0.01$.

(A) #48 **(B) #48-15**

(C) #15-09

Figure 6.

Binding modes of compounds #48 (A), #48-15 (B) and #15-09 (C) as predicted by Glide docking simulation. Protein side chains in green refer to the original position of receptor coordinates used in docking. The compound in gray stick refers to the rescored pose; the compound in torquise (sky blue) stick refers to the refined ligand pose; the compound in plum/pink stick refers to the eMBRACe minimized (and xGlide rescored top) pose. Yellow dotted lines indicate H-bonds between ligand and receptor. The eMBRACe minimization considers flexibility of both ligand and residues within 5 Å pocket surrounding the ligand and is shown superimposed (plum colored side chain) to outline the changes after minimization.

Table 1

The first-generation inhibitors of KLF10 reporter activity.

Compound ID	% Inhibition at 100 μM ($\pm$ sd) [§]	IC ₅₀ (μM) [¶]	Glide Rank	Surflex Rank	FlexX Rank	Chemical Structure
#15	45.5 $\pm$ 4.1	n.d.	34	79	76	
#48	54.5 $\pm$ 8.0	112	59	54	122	
#57	33.1 $\pm$ 5.6	n.d.	6	94	128	
#71	41.4 $\pm$ 4.2	100	84	138	140	
#71-S	46.7 $\pm$ 3.0	n.d.	N.A.	N.A.	N.A.	
#77	61.7 $\pm$ 2.6	10	135	104	16	
#82	31.9 $\pm$ 0.4	n.d.	78	141	66	

Compound ID	% Inhibition at 100 μM ($\pm$ sd) [§]	IC ₅₀ (μM) [¶]	Glide Rank	Surflex Rank	FlexX Rank	Chemical Structure
#86	57.6 $\pm$ 2.3	n.d.	133	115	30
#117	37.6 $\pm$ 7.1	200	80	140	105
Tolcapone [€]	71.8 $\pm$ 0.9	50	N.A.	N.A.	N.A.
Atomoxetine [€]	47.1 $\pm$ 4.6	100	N.A.	N.A.	N.A.

[§]Percent inhibition at 100 μM (sd, standard deviation);

[¶]Compound concentration in μM that inhibited 50% of KLF 10 reporter activity; n.d., not determined.

[€]Tolcapone (Tasmar[™], Catechol-O-methyl transferase inhibitor) and Atomoxetine (Strattera[™], Norepinephrine reuptake inhibitor) are approved drugs.

Represents respective rank order within SBVS-150 set by Glide XP, Surflex, and FlexX dockers' scores. N.A. indicate hits which were not part of the original consensus docking protocol, but were included later to test similar molecule (#71-S) or repurposing of approved drugs[€]

Table 2

Chemical expansion (structure-activity relationship) of hit compound **#48** scaffold.

<div> </div>											
Compound ID	% Inhibition at 100 μ M ($\pm$ sd) [§]	IC50 (μ M) [¶]	R ₂	R ₄	R ₅	X	R ₂ '	R ₃ '	R ₄ '	R ₅ '	
#48	54.5 $\pm$ 8.0	112	OH	OH	CH ₃	O	H	H	CH ₃	H	
#48-02	62.1 $\pm$ 0.5	n.d.	OH	OH	C ₂ H ₅	O	H	H	C2H5	H	
#48-03	41.1 $\pm$ 2.6	n.d.	OH	OH	C ₂ H ₅	O	H	H	H	H	
#48-04	75.65 $\pm$ 1.9	n.d.	OH	OH	C ₂ H ₅	O	H	CH=CH-CH=CH		H	
#48-05	91.4 $\pm$ 0.6	n.d.	OH	OH	C ₂ H ₅	O	H	H	CH(CH ₃) ₂	H	
#48-06	91.6 $\pm$ 4.6	n.d.	OH	OH	C ₂ H ₅	O	H	H	Ph	H	
#48-07	49.3 $\pm$ 1.0	n.d.	OH	OH	C ₂ H ₅	O	H	H	F	H	
#48-08	39.0 $\pm$ 3.5	n.d.	OCH ₃	H	H	OCO	OH	H	H	CH ₃	
#48-09	43.5 $\pm$ 0.7	n.d.	OCH ₃	OCH ₃	H	OCO	OH	H	H	CH ₃	
#48-10	39.6 $\pm$ 2.6	n.d.	OCH ₃	OCH ₃	H	OCO	OH	H	H	OCH ₃	
#48-11	81.5 $\pm$ 3.3	n.d.	OH	OH	C ₂ H ₅	H	H	H	OCH ₃	H	
#48-12	83.5 $\pm$ 0.4	n.d.	OH	OH	H	O	H	H	C ₂ H ₅	H	
#48-13	41.4 $\pm$ 5.0	n.d.	OH	OH	CH ₃	O	H	H	F	H	
#48-14	79.1 $\pm$ 1.4	n.d.	OH	OH	C ₂ H ₅	O	H	H	I	H	
#48-15	78.0 $\pm$ 1.3	40	H	OCH ₃	H	OCO	OH	H	H	CH ₃	
#48-20	35.49 $\pm$ 3.4	n.d.	OH	OH	H	O	H	OCH ₃	H	H	
#48-19	22.3 $\pm$ 2.4	n.d.	CH ₃	H	CH ₃	OCO	OH	H	CH=CH-CH=CH		

[§]Percent inhibition at 100 μ M (sd, standard deviation)

Compound concentration in μM that inhibited 50% of KLF10 reporter activity; n.d., not determined.

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Table 3

Chemical expansion (structure-activity relationship) of hit compound #15 scaffold.

Compound ID	% Inhibition at 100 μ M ($\pm$ sd) [§]	IC50 (μ M)	R ₂	R ₃	R ₄	R _{3'}	R _{4'}	R _{5'}
#15	45.5 $\pm$ 4.1	n.d.	H	OH	OH	H	H	H
#15-07	39.1 $\pm$ 0.6	n.d.	H	OH	OH	CH ₃	H	CH ₃
#15-09	85.9 $\pm$ 1.3	43	H	OH	OH	S-C(-CH ₃)=C(-CH ₃)		
#15-11	39.3 $\pm$ 1.6	n.d.	OH	H	OH	CH ₃	H	CH ₃
#15-13	32.6 $\pm$ 4.1	n.d.	OCH ₃	OCH ₃	H	CH=CH-CH=CH	OH	H
#15-17	32.3 $\pm$ 5.8	n.d.	H	COOCH ₃	-OH	CH ₃	H	CH ₃
#15-21	37.4 $\pm$ 2.8	n.d.	H	CH ₃	-CH ₃	CH ₃	H	CH ₃

[§] Percent inhibition at 100 μ M (sd, standard deviation)[¶] Compound concentration in μ M that inhibited 50% of KLF10 reporter activity; n.d., not determined.