

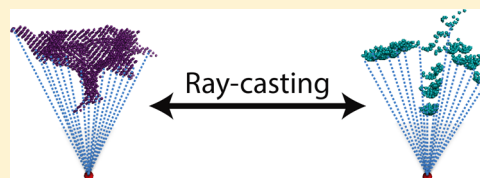
DARC: Mapping Surface Topography by Ray-Casting for Effective Virtual Screening at Protein Interaction Sites

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Supporting Information

ABSTRACT: Protein–protein interactions represent an exciting and challenging target class for therapeutic intervention using small molecules. Protein interaction sites are often devoid of the deep surface pockets presented by “traditional” drug targets, and crystal structures reveal that inhibitors typically engage these sites using very shallow binding modes. As a consequence, modern virtual screening tools developed to identify inhibitors of traditional drug targets do not perform as well when they are instead deployed at protein interaction sites. To address the need for novel inhibitors of important protein interactions, here we introduce an alternate docking strategy specifically designed for this regime. Our method, termed DARC (Docking Approach using Ray-Casting), matches the topography of a surface pocket “observed” from within the protein to the topography “observed” when viewing a potential ligand from the same vantage point. We applied DARC to carry out a virtual screen against the protein interaction site of human antiapoptotic protein Mcl-1 and found that four of the top-scoring 21 compounds showed clear inhibition in a biochemical assay. The K_i values for these compounds ranged from 1.2 to 21 μM , and each had ligand efficiency comparable to promising small-molecule inhibitors of other protein–protein interactions. These hit compounds do not resemble the natural (protein) binding partner of Mcl-1, nor do they resemble any known inhibitors of Mcl-1. Our results thus demonstrate the utility of DARC for identifying novel inhibitors of protein–protein interactions.



■ INTRODUCTION

Protein–protein interactions comprise the underlying mechanisms for cell proliferation, differentiation, and survival; manipulation of these interactions thus represents a promising avenue for therapeutic intervention in a variety of settings. Despite the urgent need for small-molecule modulators of protein interactions, however, as recently as five years ago the dearth of success stories meant that even the druggability of this target class was very much in question.^{1,2} At the same time, the challenges associated with these important targets help spur refinement of innovative new techniques, including fragment-based methods,³ peptidomimetics,⁴ and nonpeptidic mimetics of protein secondary structural elements.^{5,6} By now, these advances have enabled discovery of inhibitors against many different protein–protein interactions and have led to an impressive array of compounds in various stages of clinical trials and preclinical optimization.^{7,8}

Modern methods for structure-based virtual screening have proven effective for a variety of applications, providing novel hits to address a very wide range of “traditional” targets such as enzymes and G protein-coupled receptors.^{9,10} Unfortunately, structure-based virtual screening tools for identifying small-molecule inhibitors of protein–protein interfaces have lagged behind; due to their relatively exposed binding modes, the same

docking methods that perform well for traditional drug targets struggle to correctly identify compounds that disrupt protein interactions.¹¹ This problem is particularly acute because the natural partners of these targets, proteins, cannot easily be used for inspiration in designing small-molecule inhibitors or as templates for ligand-based virtual screening, as is possible for many enzymes^{12,13} and G protein-coupled receptors.¹⁴

In a number of cases, protein structures have been solved both in complex with a biological protein partner and also in complex with a small-molecule inhibitor. Comparison of the unbound protein structure to the equivalent structure in complex with a small molecule shows that binding is not associated with a large conformational change, and yet, the concave pocket on the protein surface in which the small molecule binds is often absent in the unbound structure so that the flat surface of the unbound structure must often undergo local rearrangement to reveal the small-molecule binding site.¹⁵ This has confounded structure-based methods for identifying

Special Issue: Computational Methods for Medicinal Chemistry

Received: January 27, 2015

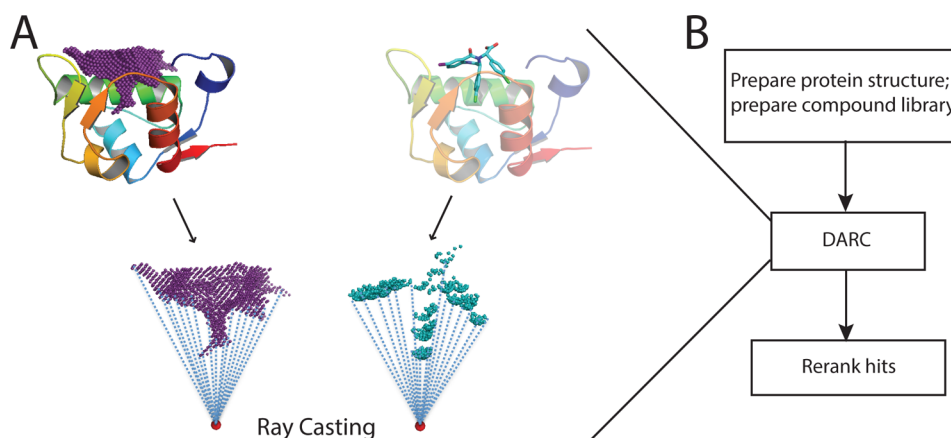


Figure 1. Docking approach using ray-casting. (A) DARC first casts a set of rays emanating from an origin within the protein (red dot), and maps the topography of the surface pocket by monitoring intersection of these rays with the pocket (left). To evaluate the shape complementarity of a given ligand for this pocket, DARC casts the same rays (from the same origin) and monitors their intersection with the ligand (right). If ligand (in its current position and orientation) is perfectly shape-complementary to the pocket, each ray will intersect the ligand at the same distance from the origin as it intersected the protein surface pocket. By moving the ligand to maximize this shape complementarity, a ligand can be docked into a protein surface pocket. (B) A schematic diagram of the complete workflow split into three stages: pre-DARC preparation, DARC, and post-DARC reranking/filtering of the docked models.

inhibitors: how can the protein structure be used to identify an inhibitor, if the protein structure will change upon binding?

We recently described a method for identifying protein surface pockets suitable for small-molecule binding, from either cocrystal structures or from simulations starting from the unbound protein structure.¹⁶ Here, we introduce a method that we call DARC (Docking Approach using Ray-Casting) for matching these surface pockets to shape-complementary small molecules. Because many inhibitors of protein interactions are found to bind in shallow flat pockets on the protein surface,^{11,15} this method is specifically designed for such protein–ligand interactions. Further, our method is intrinsically low-resolution to allow for minor conformational variation of the protein upon ligand binding. We anticipate this method will provide suitable starting points for cases in which the protein structure is not perfectly optimal for the ligand and cases in which further small conformational changes of the protein are expected to accompany ligand binding.

COMPUTATIONAL APPROACH

Computational methods are implemented in the Rosetta software suite;¹⁷ Rosetta is freely available for academic use (www.rosettacommons.org), with the new features described here included in the 3.6 release.

Scoring with DARC. To map the pocket shape on the protein surface, we use a variation of the LIGSITE¹⁸ algorithm described fully in our earlier work.¹⁶ In its most basic form, this method involves building a three-dimensional grid around a protein (or select region of a protein) then tagging the grid points that are covered by the protein. Next, each grid point not covered by the protein is examined to determine whether the point is bounded on two opposing sides by the protein along any direction of the grid, including diagonals. A continuous group of such special points are labeled a “pocket.” We then restrict this collection of “pocket” points to those “surface pocket” points that immediately contact the protein: these points together define the concave surface with which a ligand may interact.

From an “origin” point within the protein (determination of this point is described in Supporting Information), we then cast a series of rays toward the surface pocket; each ray connects the origin point to one “surface pocket” point (Figure 1a). For each ray, we express the position of the “surface pocket” point relative to the origin using spherical coordinates ($\theta, \phi, \rho_{\text{pocket}}$); collectively, these spherical

coordinates represent a map of the binding pocket’s topography when viewed from the protein interior.

When viewed from the same vantage point (the origin), the topography of a suitably bound ligand is expected to be highly complementary to that of the protein’s binding pocket. To evaluate the degree of complementary, we therefore cast the same set of rays (defined by their angles θ and ϕ) and determine the distance at which each ray first intersects the ligand (Figure 1a). If the ligand is indeed complementary to the pocket, the distance to the ligand (ρ_{ligand}) for a given ray will closely match the distance to the pocket (ρ_{pocket}) for the corresponding ray.

We define the DARC “overlap score” as the degree to which the pocket and ligand topographies match, by summing over all rays as follows:

DARC overlap score

$$= \sum_{\text{rays}} \left\{ \begin{array}{ll} c1 \times (\rho_{\text{ligand}} - \rho_{\text{pocket}}) & \text{if } \rho_{\text{pocket}} < \rho_{\text{ligand}} \\ c2 \times (\rho_{\text{pocket}} - \rho_{\text{ligand}}) & \text{if } \rho_{\text{ligand}} < \rho_{\text{pocket}} \\ c3 & \text{if ray does not intersect ligand} \\ c4 & \text{if ray does not intersect pocket} \end{array} \right\} \quad (1)$$

From a physical perspective, rays that reach the pocket before the ligand indicate underpacking between protein and ligand (condition no. 1); alternatively, rays that reach the ligand before the pocket indicate a steric clash (condition no. 2). Rays that do not intersect the small molecule at all are penalized as well (condition no. 3); from a physical perspective, this corresponds to the ligand not completely filling the pocket. Finally, we include a series of rays that do not intersect the pocket; intersection of these rays with the ligand indicates that the ligand is too large for the pocket (condition no. 4).

For a given set of rays, evaluating the intersection distance of each ray with the ligand is a task that is naturally very amenable to parallel computing; indeed, the need to efficiently carry out the related “ray tracing” operation provided motivation for development of graphics processing units (GPUs). We have recently demonstrated that the ray-casting approach we describe here can also be carried out on GPUs, leading to dramatic speed enhancement relative to the analogous calculation on a CPU alone.¹⁹

Determination of the values for parameters $c_1/c_2/c_3/c_4$ used in these studies is described in Supporting Information.

Docking with DARC. Determining the optimal bound pose of a small molecule within a pocket (i.e., “docking”) entails varying the ligand position and orientation to minimize the DARC score eq 1. Although the first derivative of the DARC score can be calculated with respect to the ligand position and orientation, we instead carry out a derivative-free minimization using the particle-swarm optimizer implemented within the Rosetta software suite.¹⁷ The ligand internal degrees of freedom are held fixed during this search; to account for ligand flexibility, we prebuild ligand “conformers” using OMEGA^{20–22} and independently dock each of these. The final DARC score for a given ligand is taken to be the minimum DARC score from among each of its docked conformers.

Application of DARC for virtual screening is summarized through the workflow in Figure 1b. The starting pocket shape derives from a protein crystal structure and can be an unbound structure, a bound structure in which the ligand has been discarded, or a structure generated from simulation (e.g., the “pocket optimization” simulations we have described for identifying low-energy pocket-containing conformations¹⁶).

RESULTS

Building a Virtual Screening Benchmark. Comparing DARC to other methods for virtual screening requires development of a benchmark to evaluate the performance of each method for correctly identifying known inhibitors from among “decoy” compounds. Assembling a large benchmark set is complicated by the relatively small number of available crystal structures of small-molecule inhibitors of protein interactions that have been solved in complex with their protein targets. Rather than assemble many tests with a very small number of active compounds (known inhibitors), we instead built two separate benchmark tests around protein interaction sites for which numerous chemically diverse inhibitors have been reported: Bcl-xL’s interaction with Bak peptides and XIAP’s interaction with Smac/Diablo peptides. These two proteins (and their homologues) were excluded from parametrization of DARC (see Table S1 in Supporting Information) to ensure that DARC would not inadvertently be trained to perform well against these two protein targets.

When generating a set of “decoy” compounds for this screening experiment, it is important that these compounds be suitably matched to the “active” compounds. If the decoy compounds are systematically different in some physicochemical property, for example, a docking method may successfully pick out active compounds simply due to some implicit bias (e.g., by simply picking the largest compounds, or the most hydrophobic, etc.). On the other hand, it is important to consider that decoy compounds are not necessarily inactive but rather “presumed inactive”; typically, they have not been explicitly tested experimentally against the target proteins in the benchmark to ensure they are not active. If decoy compounds are too similar to active compounds in chemical structure, certain decoys may themselves be active, which will confound analysis of the results (because the “right answer” in the benchmark is to label decoy compounds as inactive).

To generate well-matched decoy compounds for these benchmarks, we drew from either (i) a set of 85 drug-like compounds that comprise the Astex Diverse Set,²³ or (ii) a set of 6912 compounds reported to inhibit a different protein–protein interaction than our protein targets, compiled from the TIMBAL database.^{24,25}

To reduce the likelihood that “decoy” compounds might be active, we removed any decoy compounds that exhibited strong

chemical similarity to a compound in the “active” set for this target (2D Fingerprint Tanimoto score²⁶ > 0.7). To ensure nonredundancy of the active set and nonredundancy of the decoy set, we also removed any compound with strong chemical similarity to another compound already present in the same set (2D Fingerprint Tanimoto score > 0.7).

For Bcl-xL, this approach yielded 27 (diverse) active compounds, with 33 decoy compounds from the Astex set or 328 decoy compounds from the TIMBAL set. For XIAP, this yielded 14 (diverse) active compounds, with 45 decoy compounds from the Astex set or 425 decoy compounds from the TIMBAL set. Although smaller than the TIMBAL decoy sets, the Astex decoys have the advantage that these compounds have typically been advanced through further optimization for their respective targets; this makes the Astex decoys less likely to exhibit (undesired) activity against Bcl-xL or XIAP.

In addition, we also generated a third set of decoy compounds using the DUD-E server.²⁷ In this case, we identified 50 “custom” property-matched decoys from each active compound. Thus, the 27 compounds active against Bcl-xL led to a set of 1350 DUD-E decoys, and the 14 compounds active against XIAP led to a set of 700 DUD-E decoys. The careful matching of the decoys’ physicochemical properties to those of specific active compounds makes this a much more challenging benchmark; at the same time, however, there is an increased likelihood that some decoy compounds may themselves be active against the target protein.

Screening against a Ligand-Bound Protein Structure.

The challenge in virtual screening entails identifying as many active compounds as possible from a library (true positives) while minimizing the number of inactive compounds incorrectly assigned as active (false positives). A very stringent score cutoff for any method will result in few compounds being assigned as active (such that some active compounds will be missed), whereas relaxing this cutoff will lead to many more compounds assigned as active (such that more false positives will be included). Because only a small fraction of the total compound library is expected to be further evaluated or validated experimentally, a typical goal for virtual screening is to rank active compounds at the very top of the sorted list (good “early” behavior), corresponding to the ability to build a small subset of the original library that is strongly enriched in active compounds.²⁸ The “late” behavior (the fraction of the library that must be screened to ensure no active compounds are missed) is irrelevant for virtual screening applications because a screening tool that offers to eliminate only a small fraction of (inactive) compounds from a large library is not particularly useful.

We started from the crystal structures of Bcl-xL and XIAP, each solved in complex with an inhibitor that had been excluded from our library of active compounds. In addition to DARC, we used five other methods to score and rank each compound in this benchmark experiment. Four of these (DOCK,^{29,30} AutoDock,³¹ rDock,³² and PLANTS³³) are receptor-based screening tools that we used to dock each compound in the protein interaction site of each protein. The fifth (ROCS^{34–36}) is a ligand-based screening tool, which ranks compounds on the basis of how well their three-dimensional structure mimics the volume and chemical features of a template: in this case, the structure of the inhibitor solved in complex with the target protein. Parameters used for each of

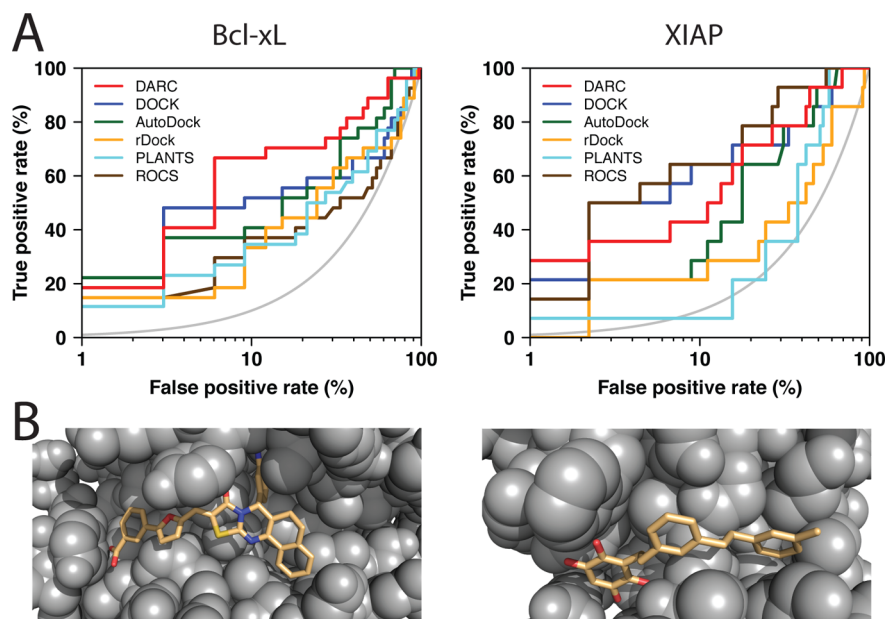


Figure 2. Virtual screening benchmark experiment using an inhibitor-bound protein structure. (A) This receiver operating characteristic (ROC) plot compares the performance of various methods (DARC, DOCK,^{29,30} AutoDock,³¹ rDock,³² PLANTS,³³ and ROCS^{34–36}) for predicting whether a given compound is active against a particular protein interaction site. Drug-like “decoy” compounds were drawn from the Astex diverse set,²³ then further filtered to remove any compounds that are similar in chemical structure to an active compound or any other decoy compound. The results are presented on a semilog plot to highlight the “early” performance of the methods; the gray curve indicates the random retrieval of compounds (i.e., a random predictor). Left: discriminating 27 diverse compounds active against Bcl-xL from among 33 matched decoy compounds. Right: discriminating 14 diverse compounds active against XIAP from among 45 matched decoy compounds. (B) DARC-docked models of representative active compounds against Bcl-xL (left) and XIAP (right). These compounds were not chemically similar to those used in solving these crystal structures (such compounds were excluded from this benchmark).

these other packages are included in the Supporting Information.

Given the rank order of each compound using each of the three methods, we prepared separate receiver operating characteristic (ROC) curves for both Bcl-xL and XIAP, corresponding to an experiment in which we screen a library comprised of active compounds and Astex decoys (Figure 2a). Each point on such a plot corresponds to a given score cutoff, and each point indicates the fraction of active compounds collected at this cutoff as a function of the inactive compounds incorrectly assigned as active at this cutoff. On this plot, the critical “early” behavior described earlier corresponds to a steep rise at the leftmost part of the plot: a useful method for screening must pick out as many active compounds as possible while accumulating a very small fraction of the inactive compounds. All three methods outperform the behavior that would result from randomly ranking the compounds in the library (gray curve), especially in this “early” region. We also observe similar results when carrying out the same screening experiment using decoy compounds drawn from the TIMBAL set rather than from the Astex set (Figure S1 in Supporting Information). With the exception of ROCS applied to XIAP, meanwhile, none of the methods perform at a comparable level when used to distinguish known active compounds from their closely matched decoys in the DUD-E set (Figure S2 in Supporting Information).

As seen in representative examples of docked active compounds (Figure 2b), the DARC-generated models exhibit clear shape complementarity that allowed these compounds to be (correctly) identified as true ligands; experimentally derived structures of these compounds in complex with their protein partners have not been reported to date. While low-resolution

considerations of shape complementarity alone cannot be sufficient to reliably predict binding affinity or details of the interactions, it is striking that for these two benchmark tests the simple approach employed by DARC successfully distinguishes active from inactive compounds with similar accuracy as more sophisticated methods. We further note that the performance of DARC in this benchmark is remarkably robust to the location of the “origin” point from which rays emanate and provided a reasonable approach is used to define this point (Figure S3 in Supporting Information). Thus, these observations support the hypothesis that the crude pocket shape on the protein surface provides a strong restriction on the chemical “shape space” of inhibitory compounds.

Screening against a “Pocket Optimized” Protein Structure. The fact that a protein interaction site is likely to undergo a conformational change upon inhibitor binding¹⁵ makes the choice of receptor conformation of particular interest for this target class, especially given that the performance of most approaches decreases considerably when an unbound starting structure is used in place of a ligand-bound starting structure.³⁷ A variety of methods exist for incorporating receptor flexibility, typically by docking against multiple receptor conformations from either crystal structures^{38–42} or simulation.^{43–45}

Rather than start from unbound crystal structures of Bcl-xL and XIAP, which lack the surface pocket required for inhibitor binding, we instead started from conformations generated using the “pocket optimization” approach we have recently described.¹⁶ Briefly, we developed a biasing potential that drives conformational sampling in molecular mechanics simulations toward conformations containing surface pockets suitable for small-molecule binding. This biasing potential

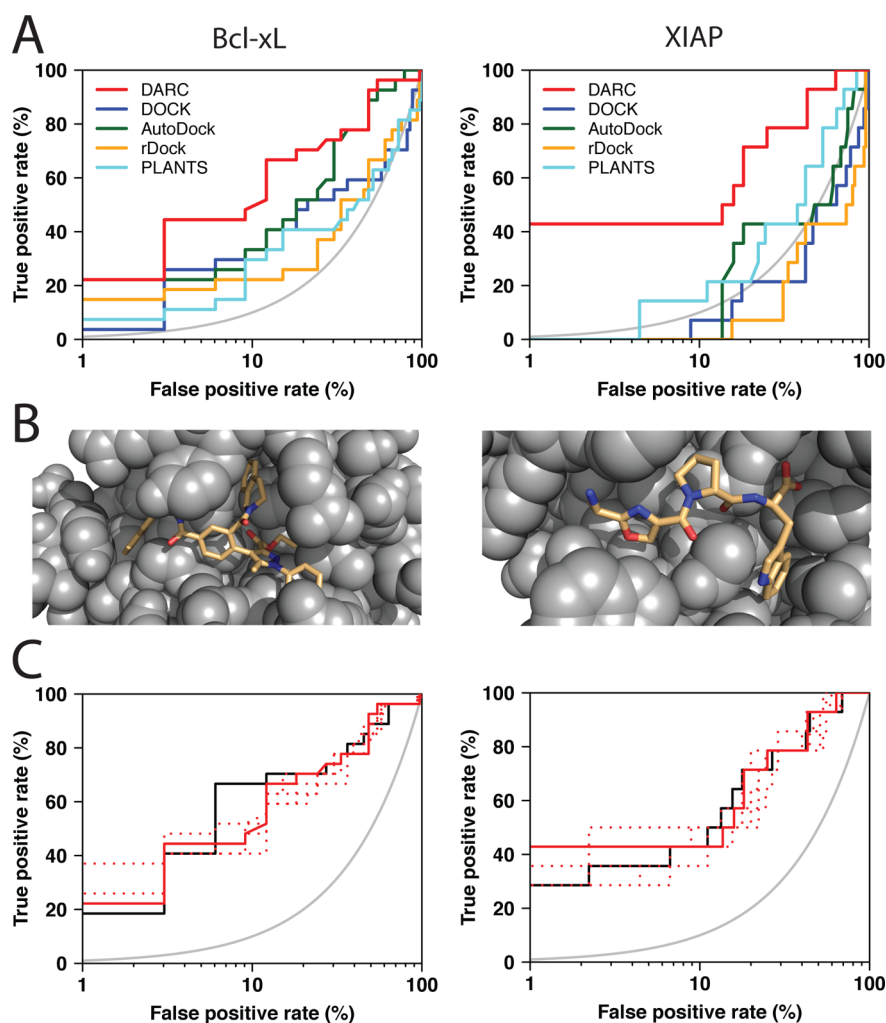


Figure 3. Virtual screening benchmark experiment using protein structures from “pocket optimization” simulations. (A) This benchmark experiment involves discrimination of the same active versus “decoy” compounds as in Figure 2, however, this time compounds were screened against a protein conformation generated via “pocket optimization” simulations initiated from an unbound crystal structure (instead of protein conformations from an inhibitor-bound crystal structure).¹⁶ The results are again presented on a semilog plot to highlight the “early” performance of the methods; the gray curve indicates the random retrieval of compounds (i.e., a random predictor). (B) Models of representative active compounds docked by DARC to the “pocket optimized” conformation of Bcl-xL (left) or XIAP (right). (C) The performance of DARC in this benchmark is essentially equivalent when screening against a known ligand-bound structure (solid black line), the lowest-energy “pocket optimized” conformation (i.e., as described in the previous panels) (solid red line), or any of five other low-energy “pocket optimized” conformations (dashed red lines); this observation demonstrates the insensitivity of the method to details of the protein structure.

operates solely on simple geometric considerations so that the shapes of the resulting surface pockets are influenced solely by properties of the protein surface and *not* knowledge of any particular ligand. Starting from unbound crystal structures, we carried out simulations to generate “pocket optimized” conformations of Bcl-xL and XIAP: conformations containing a surface pocket, but without the precise details that would be encoded in a ligand-bound structure (our application of this approach to generate these protein conformations is described in Supporting Information).

We rescreened the same compound library described earlier, this time using a “pocket optimized” conformation of Bcl-xL or XIAP (Figure 3a). We did not include ROCS in this stage of the benchmark because there is no structure of a cognate ligand available for this protein conformation that could be used as a template. Unsurprisingly, the performance of DOCK, AutoDock, rDock, and PLANTS all suffer dramatically in this more challenging regime. In contrast, the performance of DARC is

remarkably similar to the earlier experiment, demonstrating that the low-resolution nature of the underlying calculations makes DARC relatively robust to slight mismatches between protein and ligand. Indeed, representative examples of active compounds docked to these alternate conformations demonstrate that the overall pocket shapes remain complementary to these ligands (Figure 3b). We note that these observations also hold when carrying out the same experiment using decoy compounds drawn from the TIMBAL set rather than the Astex set (Figure S4 in Supporting Information), but that again all of these methods exhibit diminished performance against the DUD-E set (Figure S5 in Supporting Information).

In summary, slight differences in these protein conformations lead to diminished performance from other receptor-based docking methods in this regime, but DARC performs equally well in this experiment as in the previous benchmark that used protein conformations from ligand-bound crystal structures. This finding is confirmed by extending this experiment to

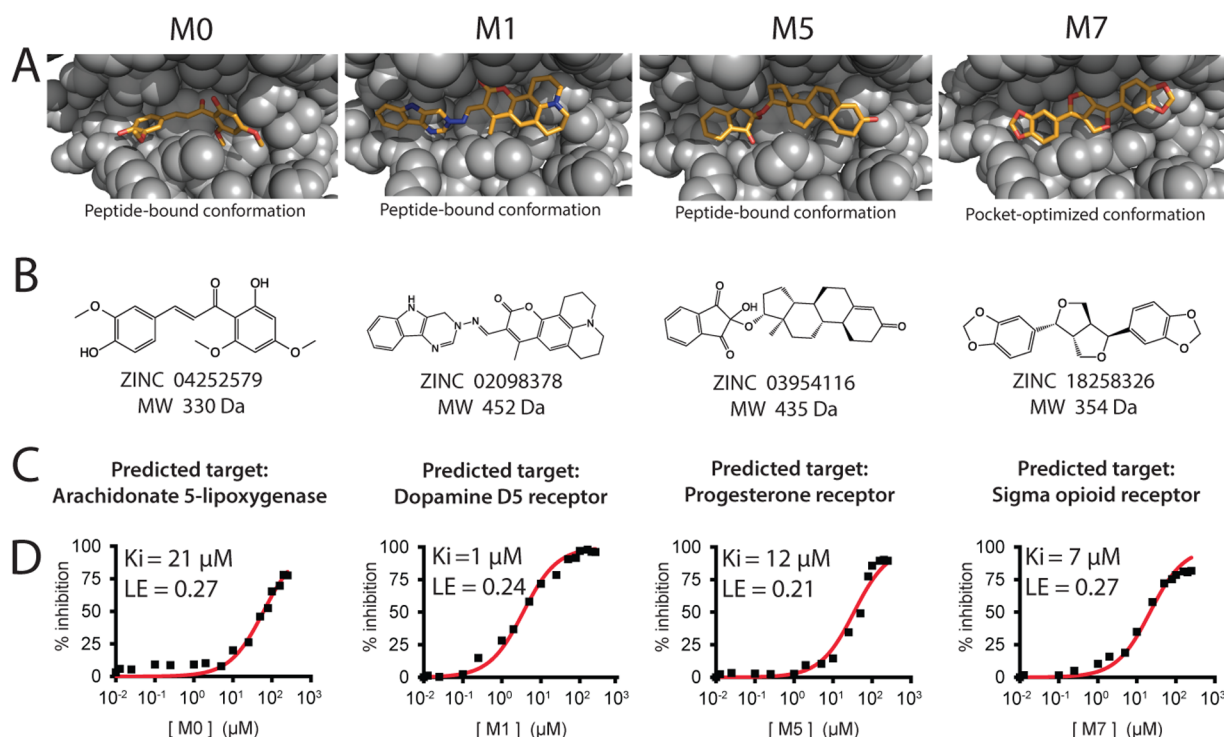


Figure 4. DARC can be used to identify novel inhibitors of human Mcl-1. Screening was carried out against the protein interaction site of Mcl-1, and 21 of the top-scoring compounds were tested in a biochemical assay. (A) Docked models generated by DARC for four compounds emerging from our screen. Compounds were screened either against an experimentally derived peptide-bound structure of Mcl-1 or against an Mcl-1 conformation generated via “pocket optimization” simulations.¹⁶ (B) The chemical structures of the compounds in each model are shown. We note that some of these have reactive functional groups: such compounds could be removed from the library prior to screening in applications where such moieties are undesirable. (C) The most likely activity of each compound was predicted using the similarity ensemble approach (SEA).⁷⁵ None of these compounds are assigned as likely inhibitors of Bcl-2-family proteins, underscoring their lack of similarity to known inhibitors of this family. (D) Each of these four compounds inhibit Mcl-1’s interaction with a FITC-labeled cognate peptide, as determined via fluorescence polarization. CHAPS detergent (0.1%) was included in this assay, to ensure that these results were not due to compound aggregation; we also observe equivalent inhibition in the absence of CHAPS. Each curve is fit to a one-site inhibition model, with a single free parameter in the fitting. Direct binding of each of these compounds to Mcl-1 was subsequently confirmed via biolayer interferometry (see Supporting Information).

consider a series of five more distinct low-energy conformations for each protein, as generated by the “pocket optimization” approach; we find that the performance of DARC in this benchmark is notably insensitive to the precise details of the protein conformation (Figure 3c).

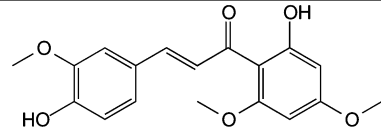
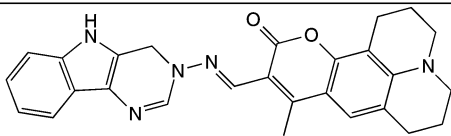
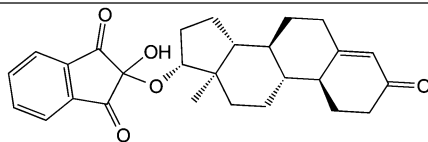
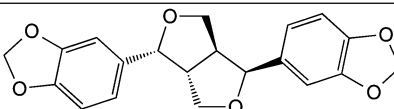
Collectively, these experiments point to different regimes in which each class of method is expected to prove superior. Detailed docking methods, such as DOCK, AutoDock, rDock, and PLANTS, are expected to out-perform DARC if the protein structure is solved in complex with a ligand of similar chemotype to the desired compounds. By contrast, DARC allows greater discrimination if the protein conformation is not quite optimal for the ligand; this can occur when one wishes to discover inhibitors with a radically different chemotype than known ligands or when the protein conformation derives from simulation.

Identifying Novel Mcl-1 Inhibitors Using DARC. To evaluate the performance of DARC in a realistic application, we next applied this approach to screen for novel classes of inhibitors for Mcl-1, a member of the Bcl-2 family of proteins. Individual members of this protein family can serve either a pro- or antiapoptotic role and interact with one another through a structurally conserved binding motif. Small-molecule inhibitors of Bcl-2/Bcl-xL have shown promising efficacy in overcoming chemo/radioreistance in various tumor models including prostate cancer.^{46–50} One such compound is ABT-

737, a potent Bcl-2/Bcl-xL inhibitor;⁵⁰ a more recent Bcl-2-selective derivative of this compound is currently in clinical trials.⁵¹ Recent studies have shown that cancer cells resistant to ABT-737 have high levels of Mcl-1 and that knockdown of Mcl-1 promotes ABT-737-induced apoptosis.^{35,48,49,52,53} Taken together, these observations motivate the pressing need for development of potent and selective inhibitors of Mcl-1 in treating a variety of cancers to be used either as a single agent or in combination with inhibitors of Bcl-2/Bcl-xL.⁵⁴

The strong evidence supporting the potential impact of effective Mcl-1 inhibitors has spurred intense efforts using a variety of complementary approaches,^{54,55} including high-throughput screening of standard libraries using a fluorescence polarization competition assay^{56,57} and of an sp³-rich library using differential scanning fluorimetry.⁵⁸ Aiming to build upon the success of the ABT-737 series for Bcl-2, several groups have since applied similar NMR-based fragment screening to Mcl-1; fragment hits were prioritized on the basis of ligand efficiency (binding free energy per heavyatom), first yielding weakly binding compounds that were subsequently merged or elaborated to give inhibitors with improved potency at the expense of some ligand efficiency.^{59–62} Inspired by the helical conformation adopted by Mcl-1’s interaction partners, a number of groups have grafted the interaction partners’ side chains onto small-molecule scaffolds that allow them to be presented in a similar geometry^{63–65} or have used “stapled”

Table 1. DARC Screening Hits Confirmed Experimentally As Inhibitors of Mcl-1^a

Compound	Chemical structure	K _i (μM)	L.E. (kcal/mol•heavyatom)
M0		21	0.27
M1		1.2	0.24
M5		12	0.21
M7		7	0.27

^aThe complete set of DARC hits (including those that did not inhibit Mcl-1) are included as Table S2 in Supporting Information. K_i values were determined via a fluorescence polarization competition assay (see Supporting Information), and then bio-layer interferometry was used to directly confirm binding to Mcl-1 (see Supporting Information).

derivatives of the helical peptides.^{66–68} Such approaches predicated on mimicry of a known helical binding partner can rapidly lead to potent inhibitors, but the large chemical scaffolds required to recapitulate the helical geometry diminish from their ligand efficiency.

Like other members of the Bcl-2 family, Mcl-1's interaction partners bind to an exposed hydrophobic groove on the protein surface.⁶⁹ We recently applied our “pocket optimization” approach to this protein surface and generated ensembles of low-energy Mcl-1 conformations that present surface pockets suitable for small-molecule binding.⁷⁰ Among these ensembles, we find conformations similar to those observed in crystal structures of Mcl-1 bound to diverse small-molecule ligands, and we also observe “distinct” conformations that have not yet been observed in any experimentally derived structures.⁷⁰ We elected to use DARC to screen chemical libraries for compounds that would complement one of two Mcl-1 conformations: either an experimentally derived conformation or a conformation derived from “pocket optimization” simulations. At the time we initiated these studies, no unbound structure was available,⁷¹ so for the former we used a peptide-bound crystal structure (PDB ID 2pqk).⁶⁹ For the latter, we used the lowest-energy pocket-containing conformation generated from 1000 independent trajectories.

Drawing from the ZINC database,⁷² we compiled a small virtual library corresponding to 62442 highly diverse compounds with drug-like properties (MW ≤ 500 Da, xlogP ≤ 5, etc., see Supporting Information) available for immediate purchase from a commercial vendor. We then applied DARC to separately dock and rank each compound using the peptide-

bound structure of Mcl-1 and also separately using the “pocket optimized” conformation of Mcl-1.

The docked models produced by DARC are intrinsically low resolution because they rely only on matching the protein–ligand topography. To achieve further high-resolution discrimination, we therefore included an additional final step (Figure 1b): for each of the top ranked 10% of the complexes produced by DARC we carried out fullatom gradient-based minimization in Rosetta, using the standard Rosetta fullatom energy function¹⁷ and ligand parameter definitions.⁷³ All internal dihedral angles of the protein were included as degrees of freedom, along with the ligand position and orientation relative to the protein. We then filtered the resulting models to remove those with no intermolecular hydrogen bonds or an abundance of buried unsatisfied polar groups. From the remaining models, we then purchased the top-scoring 21 compounds on the basis of DARC score (Table S2 in Supporting Information). Of these 21 compounds, 11 were identified by screening against the (peptide-bound) crystal structure of Mcl-1, and the other 10 were from screening against the “pocket optimized” structure.

The structural basis for the favorable scores in our DARC screen is evident from the models of these compounds in complex with Mcl-1 (Figure 4a). In each case the ligand, in its modeled conformation, exhibits exquisite shape complementarity for the protein surface; this is unsurprising given that the DARC score is expressly built to identify the ligand position, orientation, and conformation that will maximize similarity to the topography of the protein surface. Nonetheless, there is also clear diversity among the models; the ligand shapes are different from one another, and they fill the protein surface

pocket in distinctly different ways. In the case of M0, for example, a hydrophobic groove on the Mcl-1 surface is neatly complemented by the spatial arrangement of the two aromatic rings in the selected ligand conformation.

The selected compounds also represent a diverse array of different chemical scaffolds (Figure 4b), with no evident similarity to one another. It must be noted that of the compounds shown, only M7 (a natural product) is devoid of potentially reactive functional groups. This is, of course, a reflection of the screening library: in a typical drug discovery application, such compounds should be removed from the library prior to screening because their potential for advancement is likely to be extremely limited.⁷⁴ For the purpose of evaluating the performance of DARC in this virtual screening experiment, however, we did not exclude such compounds.

By visual inspection, the structures of these compounds bear no obvious resemblance to any known inhibitors of Mcl-1 or to any known inhibitors of any other Bcl-2 family members. To systematically identify biologically active compounds most resembling these compounds, we applied the similarity ensemble approach (SEA); this method uses ligand similarity to identify likely receptors for a query ligand.⁷⁵ In most cases, SEA identifies chemical scaffolds with clear resemblance to our queries, but these related compounds have each been described in the context of very different activities (Figure 4c); even delving further into each list, we did not find similarity to other inhibitors of Bcl-2 family members. This observation highlights the fact that these compounds would have been very difficult to pick out using ligand-based screening due to their lack of similarity to any known inhibitors of this protein family.

We next examined the ability of each compound to inhibit the interaction of Mcl-1 with one of its binding partners. We used a fluorescein-labeled peptide derived from the Noxa protein which exhibits an increase in polarization upon Mcl-1 binding (Figure S6a in Supporting Information). Upon addition of a known Mcl-1 inhibitor, AT-101 (i.e., R-(−)-gossypol), we observe a dose-dependent decrease in polarization that confirms this compound competes with Noxa for Mcl-1 binding and is consistent with previously reported data⁷⁶ (Figure S6b in Supporting Information). Using this assay we found that six of the 21 compounds from our computational screen appeared to inhibit the interaction between Mcl-1 and its cognate peptide with K_i values ranging from 1.2 to 21 μ M (Figure 4d). These results were not affected by the presence or absence of detergent (CHAPS), suggesting that compound aggregation was not contributing to the observed inhibition. We then used an orthogonal label-free assay based on biolayer interferometry (BLI) to test for direct binding of these compounds to Mcl-1 and through this assay confirmed the activity for four of these six compounds (see Figure S7 in Supporting Information): M0, M1, M5, and M7 (Table 1). In all four cases we found that inhibition did not increase over time in the competition assay and that the BLI signal showed a dissociation phase (decreased amplitude) upon transfer into buffer lacking the compound of interest; together, these imply that all four compounds bind in a reversible manner.

Given the chemical structures of these four most potent compounds, these observed K_i values correspond to ligand efficiency (binding free energy per heavyatom) of 0.21 to 0.27 kcal/mol-heavyatom, which is typical of other promising small-molecule inhibitors of protein–protein interactions reported in the literature.⁸ With respect to other Mcl-1 inhibitors in

particular, these four initial hits are less active than the most potent compounds arising from screens of libraries designed to mimic the side chain interactions of helical cognate peptides;^{63–65} however, the DARC hits exhibit greater ligand efficiency due to the extensive size of the compounds designed for mimicry of the helix.

Superposition of the modeled complexes for each of the DARC hits with the structure of Mcl-1 bound to a cognate peptide reveals that these compounds are decisively *not* recapitulating the interactions of the helical peptide (Figure 5a). While the cognate peptide uses a cluster of aliphatic residues at its N-terminus and aromatic residues at its C-terminus, compound M0 (as a representative example) occupies only the regions of the Mcl-1 surface engaged by the N-terminus of the peptide. Given that it is not constrained to fit onto the backbone of the helix, this compound also fits more deeply into the binding pocket on the Mcl-1 surface. In contrast, the most potent of the helix mimetics was designed to recapitulate the complete set of interactions provided by the helical template.⁶³ The side chains that comprise these interactions are separated by a span of 15 residues in sequence, and while this led to an inhibitor with K_i of 0.2 μ M, the large chemical scaffold needed to present these interactions reduced the ligand efficiency of this compound to 0.21 kcal/mol-heavyatom.

While the underlying methodology used in our DARC screen did not explicitly select for compounds that bind in place of the N-terminus of the cognate peptide, each of the resulting hits complemented this region. This is in stark contrast to the binding modes observed in crystal structures of Bcl-xL solved in complex with potent inhibitors such as ABT-737 and WEHI-539; each of these bind to the region of the protein surface used by the C-terminus of the cognate peptide, and thus have relatively little overlap with the models of the DARC hits (Figure 5b). In the time since we carried out our computational screen, crystal structures of Mcl-1 solved in complex with four distinct classes of inhibitor have been reported^{58–60,77} and in each case the ligand occupies the region of the protein surface corresponding to the N-terminus of the cognate peptide. Thus, the lack of DARC hits complementing the protein surface used by inhibitors of Bcl-xL may indicate a lack of druggability for the analogous surface of Mcl-1.

In contrast to Mcl-1 inhibitors derived by mimicry of the cognate helix, the most promising hits emerging from fragment screens initially proved less potent than those identified by DARC but had superior ligand efficiency.^{59–62} One representative class of fragments inhibited Mcl-1 with K_i of only \sim 100 μ M, albeit with a promising ligand efficiency of about 0.3 kcal/mol-heavyatom.⁵⁹ NMR-derived models of the binding mode for this class of compound suggested that they induce a conformational change in Mcl-1, which in turn produces a very deep pocket required for binding; upon merging with another fragment class, crystallography confirmed that the larger compound also induced this conformational change.⁵⁹ In retrospect, each of the Mcl-1 crystal structures in complex with an inhibitor (solved after completion of our screen) revealed a conformational change that allowed the ligand to access a deep pocket that was not evident from the unbound or peptide-bound structures^{58–60,77} (Figure 5c). The observation that extensive ligand burial is related to high ligand efficiency (across many small-molecule inhibitors of protein–protein interactions)¹¹ is thus consistent with the impressive ligand efficiency of these (deeply buried) Mcl-1 fragment hits.

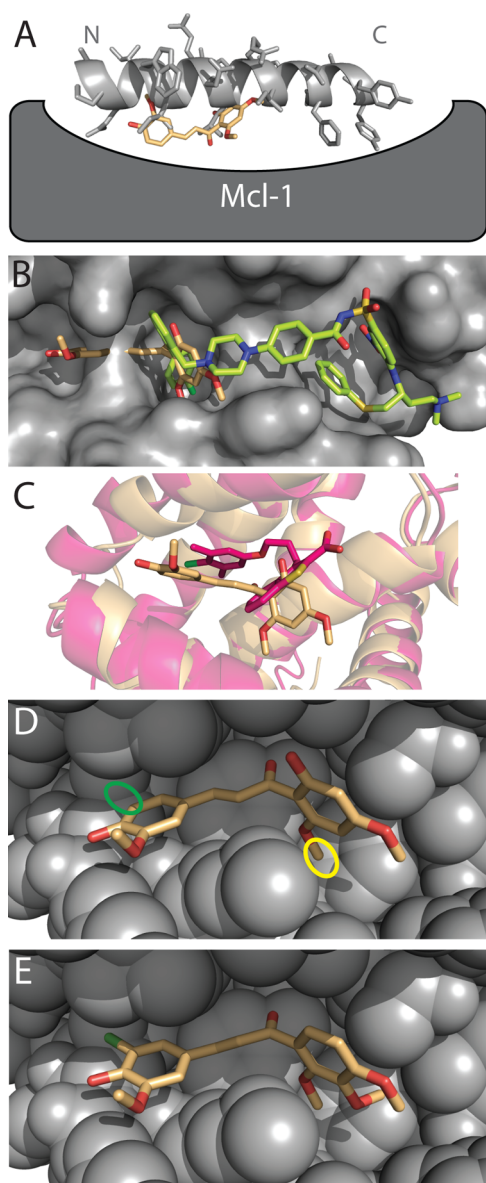


Figure 5. Evaluating DARC's model of the M0/Mcl-1 complex. (A) Though M0 was identified in a screen against a peptide-bound conformation of Mcl-1, M0 (peach) is distinctly *not* mimicking the interactions of the cognate peptide (light gray). In the DARC-generated model, M0 competes with the N-terminus of the peptide for Mcl-1 binding but fits more deeply into the Mcl-1 surface pocket than the peptide side chains. (B) This M0 binding mode is distinctly different from that of known Bcl-xL inhibitors such as ABT-737 (green), which instead overlap with the C-terminus of the peptide-binding site. The surface of the Bcl-xL protein is shown (gray), solved in complex with ABT-737. (C) Other recently described inhibitors of Mcl-1, including the representative example shown here (dark pink),⁵⁹ bind at a similar location to M0; however, each distinct series of inhibitors induce their own unique Mcl-1 conformational change to allow deeper ligand burial. (D) The locations of two M0 positions that play key roles in our model of the complex: the 6'-methoxy group (yellow) and the unsubstituted 5-position (green). (E) A model of the D1, the most potent M0 derivative, in complex with Mcl-1. This compound preserves the crucial 6'-methoxy group, and embellishes the 5-position with a chlorine substitution to improve packing. Closely analogous compounds to D1 that lack the methoxy group at the 6'-position (D3) or that lack the chlorine at the 5-position (D5) exhibit reduced activity.

However, such conformations were not among those included in our DARC screen, and accordingly the DARC hits could not have taken advantage of the deep pockets presented upon conformational rearrangement of the protein.

Evaluating the DARC Model of M0 Binding. An important benefit that can arise from the use of structure-based modeling (such as DARC) is the prospect of immediately using the model of the complex to guide design of derivatives once an initial hit compound is validated experimentally. To test whether a DARC model can indeed serve this purpose, we next sought to test whether the tighter-binding derivatives could be designed from the DARC model. Despite the fact that M0 (2',4-dihydroxy-3,4',6'-trimethoxychalcone) was the least potent of the four hits in our original screen and concerns of promiscuity because similar chalcones have been found to exhibit other diverse activities in biological assays, the straightforward synthetic accessibility of the chalcone series prompted us to select this scaffold for further investigation. While this compound would not necessarily be an optimal starting point for advancement into a drug or biological probe, then, selecting of this compound as a starting point allowed us to make and test more derivatives than would have been possible with the other three validated DARC hits.

Starting from compound M0, we therefore we sought to ask whether such a model could be used to explain the structure–activity relationship (SAR) observed across a series of related compounds. Because our goal was to gauge the veracity of the modeled complexes that led to selection of these compounds, and not simply to identify the most potent derivatives, we further restricted our study to compounds very close to the parent compound, M0; we reasoned that these would best allow us to test our model of the structure, reducing the likelihood of conformational changes leading to drastic and unexpected SAR.

Before designing a series of analogues, however, we noted that other chalcones are known to form covalent adducts to proteins (the chalcone α,β -unsaturated carbonyl system is a Michael acceptor and thus may react with unpaired cysteine side chains).^{78,79} We therefore carried out analysis via surface plasmon resonance (Figure S8 in Supporting Information) and HSQC chemical shift mapping (Figure S9 in Supporting Information) to further characterize this interaction (see Supporting Information). Taken together, the stoichiometric Hill coefficient⁸⁰ and insensitivity to the presence of detergent⁸¹ in the fluorescence polarization assay, the fact that the SPR response did not exhibit superstoichiometric behavior,⁸² and the observation of a small number of distinct chemical shift differences in the HSQC spectrum⁸³ all provide evidence that the observed activity of M0 is not due to compound aggregation that deactivates Mcl-1 but results from specific binding. The NMR experiment also supports two features of our model for M0 binding: first that the M0 binding mode is not completely overlapping with that of the Bcl-xL inhibitors (Figure 5b) and second that M0 binding does not induce extensive protein conformational changes as seen in other Mcl-1 inhibitors (Figure 5c).

We then proceeded to produce a variety of M0 derivatives using a simple aldol condensation reaction (Figure S10 in Supporting Information). We were concerned that designing these analogues from our model of the M0/Mcl-1 complex might bias the resulting series toward certain regions of chemical space and thus not provide a fair assessment of our model. To evaluate the structure–activity relationship more

Table 2. Chemical Analogues of M0^a

Compound	Chemical structure	Ki (μM)	L.E. (kcal/mol•heavyatom)
M0		21	0.27
D1		2	0.29
D2		3	0.30
D3		7	0.33
D4		10	0.31
D5		28	0.33
D6		20	0.32
D7		40	0.25
D8		>50	N.D.

Table 2. continued

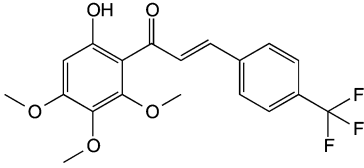
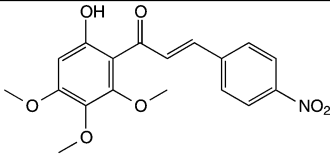
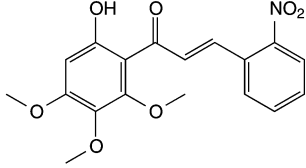
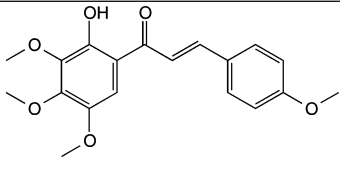
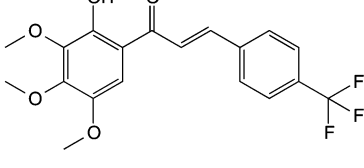
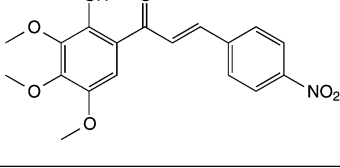
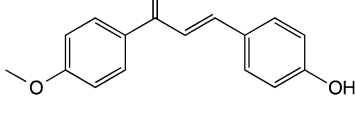
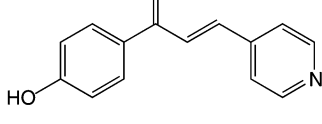
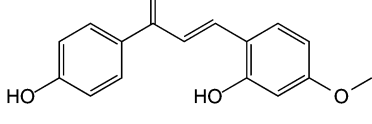
Compound	Chemical structure	Ki (μM)	L.E. (kcal/mol•heavyatom)
D9		>50	N.D.
D10		>50	N.D.
D11		>50	N.D.
D12		>50	N.D.
D13		>50	N.D.
D14		>50	N.D.
D15		>50	N.D.
D16		>50	N.D.
D17		>50	N.D.

Table 2. continued

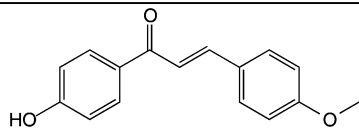
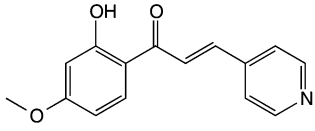
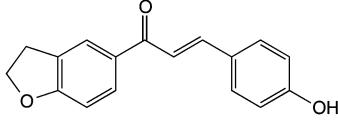
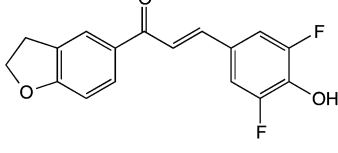
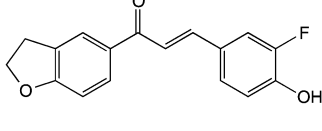
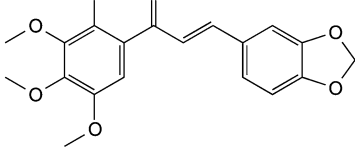
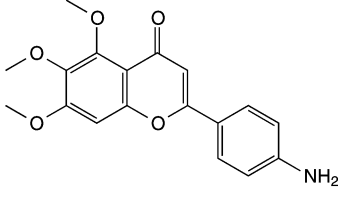
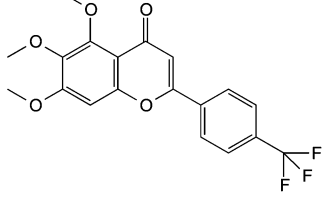
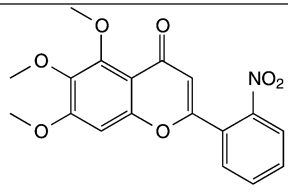
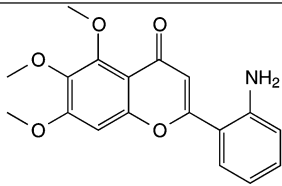
Compound	Chemical structure	Ki (μM)	L.E. (kcal/mol•heavyatom)
D18		>50	N.D.
D19		>50	N.D.
D20		>50	N.D.
D21		>50	N.D.
D22		>50	N.D.
D23		>50	N.D.
D24		>50	N.D.
D25		>50	N.D.
D26		>50	N.D.

Table 2. continued

Compound	Chemical structure	Ki (μM)	L.E. (kcal/mol•heavyatom)
D27		>50	N.D.

^aWe designed and synthesized a series of 27 M0 derivatives and tested each of these for inhibition of human Mcl-1 using a fluorescence polarization competition assay (see Supporting Information). This series of analogues was designed without consideration of DARC's model of the M0/Mcl-1 complex to allow unbiased evaluation of the model.

objectively, we therefore designed a set of compounds by selecting the most readily available starting materials based only upon the chemical structure of M0 and without consideration of our model of the complex. Using the same fluorescence polarization competition assay described earlier, we characterized a total of 27 analogues of M0 (Table 2, Figure S11 in Supporting Information). As described below, the SAR deduced from this series presents a compelling narrative when interpreted using our model of M0 binding (Figure 5d).

The most potent of these M0 analogues are D1 and D2: they each inhibit Mcl-1 about 7-fold more potently than M0. Compounds D1 and D2 differ only by a single methoxy versus fluorine substitution at the 3-position; M0 harbors a methoxy here. This methoxy group is exposed in our model of the M0/Mcl-1 complex, consistent with the observation from D1 versus D2 that its replacement with fluorine does not affect activity. Compounds D1 and D2 together share three differences relative to M0: both have a chlorine at the 5-position, both have a methoxy group at the 5'-position, and both lack the 2'-hydroxyl of M0. The latter two positions are exposed in our model of M0 binding and are thus assumed not to strongly affect activity. In contrast, the 5-position (unsubstituted in M0) faces toward the protein and points toward a small cavity (Figure 5d), thus the improved packing resulting from this substitution may explain the slightly enhanced potency of D1/D2 relative to M0.

The only other two compounds in this series with potency exceeding that of M0 are D3 and D4; this equipotent pair again differs only by the same fluorine versus methoxy substitution that distinguished D1 and D2. Relative to D1/D2, compounds D3/D4 lack the 5'- and 6'-methoxy groups. While the 5'-position is unsubstituted and exposed in our model of the M0/Mcl-1 complex, the 6'-methoxy group is deeply buried in a hydrophobic cavity (Figure 5d); loss of this interaction may explain the reduced potency of D3/D4 relative to D1/D2.

Across this series of 27 M0 analogues, only compounds D1–D4 proved more potent than M0. While the SAR described above is consistent with our model of M0 binding, these results are also notably *inconsistent* with the behavior expected if these compounds were simply carrying out thia-Michael additions. Kinetic studies using a model thiol (cysteamine) demonstrate that the presence of the 6'-methoxy group decreases reactivity, and the 2'-hydroxy group increases reactivity.⁸⁴ Through the comparisons above, we find that the most potent compounds for inhibition of Mcl-1 correspond to those expected to be *least* reactive, further implying that the inhibition we observe is not

due to reactivity of these compounds with Mcl-1's unpaired cysteine side chain.

Through the results presented in Table 2 we demonstrate that chalcones are not “privileged scaffolds”^{85,86} for inhibition of Mcl-1, an assertion that is best supported by specific examples. Compounds D7/D12/D13/D14 lack the key 6'-methoxy group described earlier (Figure 5d) and accordingly are less potent than M0. Compound D5 preserves each of the M0 functional groups that interact with Mcl-1 in our model (i.e., the 6'-methoxy group and the 4-hydroxyl group) and accordingly exhibits a very similar inhibition constant as M0. Compound D8 maintains the substituents of D2 on one ring but lacks the substituents on the other ring (including the 6'-methoxy group) and is thus less potent. Compounds D9/D10/D11 maintain the 6'-methoxy group but harbor a variety of alternate substituents on the other ring, making them less potent as well. Compounds harboring extra fused rings (D20/D21/D22/D23) are inevitably less potent, as are compounds in which the chalcone linkage has been replaced with a flavone (D24/D25/D26/D27). Overall, the assertion that chalcones are not privileged scaffolds for inhibition of Mcl-1 or generic helix mimetics is supported by the observation that most analogues presented here, designed without consideration of our model of binding, inhibit Mcl-1 less potently than the parent compound M0. In summary then, it is the precise complementarity of select compounds for the surface of Mcl-1 that dictate their activity and not some property of this chemical scaffold.

Ultimately, direct structural evidence will be required to confirm that these compounds are indeed binding via the designed pose. Unfortunately, our efforts to crystallize Mcl-1 in complex with a member of this chemical series have not yet proven successful. Validating the models that lead to selection of active compounds through structural biology will be important not only for retrospective evaluation of the DARC models but also as a starting point for inspiring design of subsequent analogues. Accordingly, we used DARC to compare our model of the M0/Mcl-1 complex to a model in which M0 was replaced with D1 (Figure 5e). We find that DARC scores D1 even more favorably than M0 due to the improved packing resulting from substitution of chlorine at the 5-position. Thus, we expect that compounds with even better shape complementarity for Mcl-1 than those selected in our initial screen, compounds such as D1, can be identified by screening larger chemical libraries with DARC. By further analogy to D1, we anticipate that such compounds will also exhibit superior

potency relative to the initial hits described in this first screen. Despite the improved potency relative to M0, we nonetheless note that compound D2 does not exert the desired biological effect using an *in vitro* cellular assay (see Supporting Information, Figure S12). This underscores the need to test candidate inhibitors in cellular assays as well because biochemical activity may not necessarily translate to cellular activity. Indeed, careful analysis has shown that a number of other inhibitors, each with comparable binding affinity for Mcl-1 as that of compound D2, also appear not to act on Mcl-1 in cells.^{57,87,88}

DISCUSSION AND CONCLUSION

Screening on the Basis of Surface Topography.

Because the protein surfaces of “traditional” drug targets have evolved to bind some cognate small-molecule ligand, they typically include a deep pocket or groove that can be targeted by inhibitors. In contrast, protein interaction sites typically do not include such surface features and accordingly small-molecule inhibitors acting at these sites rely on shallower bound poses.¹¹ Because these shallow binding modes prove challenging to predict by conventional docking approaches, modern virtual screening tools exhibit diminished performance when used to predict inhibitors of protein interactions (relative to their performance when applied to “traditional” drug targets).¹¹ Here we present DARC, an alternate approach to docking and virtual screening. By matching the topography of the protein surface to the buried face of the ligand, we find that DARC outperforms popular canonical tools (DOCK, AutoDock, rDock, and PLANTS) when screening for inhibitors of protein–protein interactions.

Given the importance of shape complementation for binding, several other fast approaches have been described to rapidly evaluate poses and enable large-scale virtual screening. These include methods based on Fourier correlation theory,^{89,90} spherical harmonics,^{91,92} geometric hashing,⁹³ and negative images.^{94–96} Most recently, others have used variations of ray-casting to compare internal pockets in proteins to one another.^{97,98} In addition to their intended usage, a subtle, but very important, difference between the latter work and DARC is the origin from which rays emanate. This other study casts rays from within the pocket (at the center of mass), resulting in a shape description that is most useful when the pocket is mostly (or completely) enclosed by the protein.^{97,98} In contrast, DARC casts rays that originate from “behind” the pocket (i.e., inside the protein); this instead emphasizes shape complementarity at the deepest regions of the binding groove and is thus better suited for describing the shallow bound poses typical of small-molecule inhibitors acting at protein interaction sites.

While shape complementarity is clearly a necessary feature of ligands that will bind to a protein surface, this alone obviously cannot be sufficient. In the present study, we used DARC to optimize and evaluate surface shape complementarity without consideration of electrostatic complementarity or solvation effects; for this reason, we included a “re-ranking” step in our virtual screen against Mcl-1 to filter out models with no intermolecular hydrogen bonds or an abundance of unsatisfied buried polar groups. In retrospect, the hydrophobicity of the Mcl-1 surface pocket provided a convenient testing ground for the ability of DARC to identify shape-complementary ligands without the complication of polar groups on the protein surface. In the future, however, we expect that incorporation of

electrostatic complementarity into DARC lead to improved performance in virtual screens against protein surfaces that display surface polar groups.⁹⁹

Approaches for Identifying Inhibitors of Protein–protein Interactions. In many cases, artificial ligands that bind to a specific protein surface bear some resemblance to an endogenous ligand that also binds to that surface; analysis of precedent drug targets shows that knowledge of a substrate, product, or effector with “drug-like” physicochemical properties is a good predictor of druggability for a given protein surface.¹⁰⁰ Analogues of endogenous ligands can also provide a starting point for designing inhibitors.¹⁰¹ In the case of protein interaction sites, however, the natural ligand is not a small molecule and thus does not provide an obvious template from which to start. This has driven advances in new methodologies for identifying inhibitors of these “nontraditional” targets, most notably including mimicry of key interacting groups of the protein partner^{63–65,102–104} and fragment-based approaches.^{59,105–107}

In addition to chemical scaffolds that mimic secondary structural elements,^{63–65} exciting new computational approaches have facilitated identification of compounds that instead mimic the geometric orientation of key “hotspot” or “anchor” side chains in a protein–protein interface.^{102–104} While either mimicry-based approach may provide a robust starting point for recapitulating the interactions of a protein partner using a small-molecule scaffold, both are likely to encounter the same intrinsic limitation: ligand efficiency is unlikely to exceed that of the interacting groups from the protein partner, which in turn is usually less than that those achieved by small-molecule inhibitors that do not explicitly mimic interactions of the protein partner.¹⁵ Accordingly, an important advantage of *de novo* structure-based screening methods that do not rely on mimicry, such as DARC, is the potential to identify inhibitors that achieve superior ligand efficiency through deeply buried interactions not available to a protein-based ligand.

In contrast, fragment-based approaches often prioritize small compounds with very high ligand efficiency from the outset: this allows exploration of deep crevices that would not necessarily be evident from the structure of the protein–protein complex. Here, the challenge often lies in elaborating initial fragment hits (by growing, merging, or linking them) into larger compounds that maintain these highly productive interactions.^{108,109} In our DARC screen against Mcl-1, we focused on compounds larger than traditional fragments; this led to initial hits with easily detectable activity, albeit at some expense of ligand efficiency. Much as the “multiple solvent crystal structures” method¹¹⁰ uses very small fragments to probe the protein surface for productive interactions with isolated functional groups, screening a commercially available library of “prototype” compounds, as we have done here, allows rapid evaluation of specific protein–ligand interactions predicted *in silico* and can provide potential chemical scaffolds for further optimization.

Screening against Multiple Protein Conformations.

The compounds identified as “hits” by DARC, and by structure-based screening methods in general, naturally depend on the conformation of the protein target. With respect to inhibitors of protein–protein interactions in particular, a number of examples have been shown to bind concave surface pockets that are absent in the corresponding unbound protein structures; these “cryptic” pockets are revealed by local

conformational changes associated with inhibitor binding.¹⁵ In the case of Bcl-2 family members, the protein surface can also adopt a number of different conformations to accommodate ligands with radically different shapes and chemical properties.⁷⁰

Variation in the protein structure is most commonly included in virtual screening by first generating an ensemble of relevant conformations from crystal structures^{38–42} or simulation^{43–45} and then separately screening against each of these conformations. Nonetheless, identifying the optimal conformations to include in these ensembles is still a challenging task.^{111–113} By preferentially exploring conformations that contain a surface pocket suitable for binding some (unspecified) ligand,¹⁶ the “pocket optimization” approach provides a natural complement to DARC screening. In principle, each of these low-energy pocket-containing conformations represents a starting point for screening, and capturing the potential diversity of pockets on the protein surface will be essential for fully realizing the available diversity of potential inhibitors.

In our Mcl-1 screen, DARC identified inhibitors both when screening against the peptide-bound conformation and when screening against the pocket-optimized conformation. Due to the conformations selected for these screens, however, our models did not take advantage of deep pockets that have been observed in recent inhibitor-bound crystal structures of Mcl-1^{58–60,77} (Figure 5c); the particular pocket-optimized conformation used in our screen was much more similar to the peptide-bound conformation, making these deep pockets unavailable to DARC. Careful analysis showed that similar conformations to those observed in the inhibitor-bound crystal structures were indeed represented in the ensemble produced by pocket optimization⁷⁰ but were not included here because we only screened against a single pocket-optimized conformation. In the future, then, inclusion of additional pocket-containing protein conformations may allow identification of inhibitors that bind more deeply and exhibit improved ligand efficiency.

Advantages to Structure-Based Virtual Screening.

Enhanced and reliable tools for structure-based virtual screening will enable development of new tool compounds, particularly in academic settings where the costs associated with large-scale biochemical (or phenotypic) screening can be prohibitive. However, the ability to precisely complement a specific surface pocket also makes structure-based virtual screening particularly attractive in a variety of other contexts that may prove challenging for traditional biochemical screening. These include building “conformation-selective” inhibitors (such as compounds that are sensitive to the phosphorylation state of a kinase activation loop¹¹⁴), targeting sites that enable unique binding kinetics (such as a newly discovered pocket on ERK1/2¹¹⁵), and building allosteric inhibitors that address both the wild-type and drug-resistant isoforms of a target (such as BCR-ABL¹¹⁶ or HIV-1 protease¹¹⁷).

In light of the plasticity of many protein interaction sites,¹⁵ these tools may also prove particularly useful for designing inhibitors that exhibit specific selectivity profiles. We have shown that the activity of a given compound against various members of the Bcl-2 family can be predicted from whether or not each protein samples a complementary surface pocket.⁷⁰ In other words, a given compound will inhibit those Bcl-2 family members that include conformations to suitably accommodate

this compound but will not inhibit other family members that cannot adopt such conformations. By identifying “common” pockets harbored on the surfaces of multiple family members, it may be possible to identify inhibitors designed to act against multiple family members (pan-inhibitors). “Pocket optimization” simulations also reveal highly unique pockets for each family member that are sampled by one family member but are not accessible to any other family member.⁷⁰ Thus, DARC’s ability to identify ligands that precisely address a given family member’s unique “signature” pockets may also provide a means to identify highly selective compounds at a very early stage of development.

METHODS

Detailed descriptions of computational and experimental methods are provided in the Supporting Information, along with sample command-lines and instructions for using DARC.

ASSOCIATED CONTENT

Supporting Information

Detailed descriptions of computational and experimental methods; additional supporting experimental results; sample command-lines and instructions for using DARC (PDF). Molecular formula strings (CSV). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00150.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Amy Keating for providing the plasmid encoding Mcl-1, and we thank Anne Cooper and Philip Gao for assistance with protein production. We are grateful to OpenEye Scientific Software (Santa Fe, NM) for providing an academic license for the use of OMEGA and QuacPac. We are grateful to the developers of the DOCK, AutoDock, rDock, and PLANTS software for providing academic licenses for the use of these programs. This work was supported by grants from the National Institute of General Medical Sciences (1R01GM099959, 5P50GM069663, 8P30GM103495, and 8P20GM103420) and the National Center for Research Resources (5P30RR030926 and 5P20RR017708). This work was also supported by the National Science Foundation through XSEDE allocation MCB130049, the University of Kansas Undergraduate Research Awards (S.M.), and the Alfred P. Sloan Fellowship (J.K.).

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