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Discovery of a Potent Small Molecule IL-2 Inhibitor through Fragment Assembly

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Protein−protein binding interfaces are considered to be improbable sites for high-affinity small molecule ligands; yet this target class represents the majority of therapeutically important targets.[1] These binding surfaces are typically large and featureless, and they lack the well-defined pockets or mechanism-based contacts that confer binding energy to enzyme inhibitors. While leads against an enzyme target can be improved by incorporating functionality known to be important on the basis of substrate preferences, protein−protein targets do not offer such opportunities. Consequently, it is difficult to envision how a small ligand can bind this target class with high affinity.

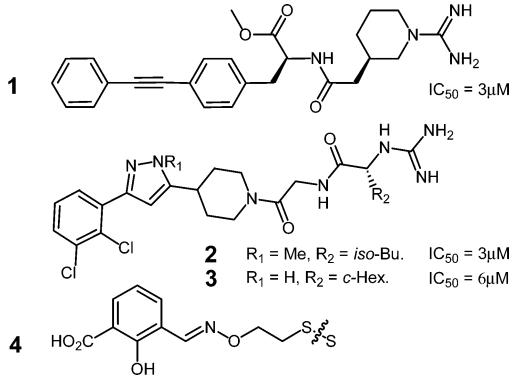
Fragment assembly methods hold great promise because they access very large numbers of potential fragment combinations without requiring the synthesis of each individual compound. Ideally, a set of small fragments (<200 MW) is screened, and only those fragments with affinity for the target are combined, thereby increasing the efficiency of the search process. Two significant challenges encountered in fragment assembly are the identification of low-affinity fragments and the determination of how to link different fragments productively. A number of fragment assembly methods have been described including SAR by NMR, dynamic libraries, and virtual screening.[2] We have developed an approach called tethering[3] that can rapidly identify low-affinity fragments that bind to specific sites on a target protein. Herein we describe the first application of this strategy to generate a high-affinity inhibitor against a protein−protein target.

Binding of the cytokine interleukin-2 to its receptor induces T-cell proliferation and is an important therapeutic target for immune disorders.[4] Our interest in this target was stimulated by a report of a small molecule (Ro26-4550, **1**, Chart 1) that binds to IL-2 and is a low micromolar antagonist of IL-2Rα binding ($IC_{50} = 3\ \mu M$).[5] We initiated a medicinal chemistry program using structure-based design and parallel synthesis that generated a novel lead series (**2**, **3**). However, this series also reached a low micromolar affinity plateau, and efforts to identify new binding interactions were unsuccessful.[6]

Analysis of the X-ray structure of **1** binding at the "hot spot" of IL-2 revealed that the protein is adaptive and able to undergo significant rearrangements which create the small molecule binding site.[7] This observation refutes the perception that protein−protein interactions are flat and featureless and suggests that the surface of IL-2 could present additional nonobvious binding sites capable of binding a small molecule with high affinity. The adaptive nature of the site creates an additional challenge though, because accurate structure-based predictions are more difficult.

We applied tethering with the goal of improving the affinity of the lead compound **2**. Ten individual cysteine mutations were designed to search the perimeter of the IL-2 "hot-spot" based on alanine scanning data and a crystal structure of **1** bound to IL-2. The mutants were then screened against a library of 7000 disulfide-containing fragments. Analysis of the screening results showed that, while most regions selected few if any fragments, one region, accessible by two different cysteine mutants (Y31C and L72C, cf. Figure 1), selected a number of structurally related fragments. Intriguingly, this is the same region that was found to be structurally adaptive, undergoing rearrangements upon binding of different ligands.[7]

Chart 1. IL-2/IL-2Rα Antagonists: **1** (Ro26-4550), **2**, and **3**[a]



[a] Fragment **4** is one of the hits selected by the IL-2 L72C mutant.

Statistical analysis of the fragments identified in this region indicated that the mutants Y31C and L72C preferentially selected small aromatic carboxylic acids (see Supporting Information).

Molecular modeling suggested that the fragments could occupy a deep hydrophobic cavity within the adaptive region. The fact that tethering requires covalent attachment of the fragments greatly facilitates computational prediction of the fragments' binding sites, even within a region of the protein that can adopt multiple low-energy conformations. An overlay of the modeled tethering hits with a crystal structure of analogue **3** bound to IL-2[6] (Figure 1) suggested that productive merging of fragments onto **2** might be achieved by linking these fragments to the dichloro-phenyl ring of **2**. On the basis of these observations, we designed a focused set of 20 compounds (Chart 2), incorporating the chemical functionalities (specifically aromatic carboxylic acids) that had been selected by tethering. Eight of the 20 compounds inhibited IL-2/IL-2Rα binding at submicromolar concentrations, demonstrating a 5−50-fold improvement in potency over the starting scaffold. All of the tight-binding inhibitors contained a carboxylic acid. Thus, the acidic functionality identified by tethering was clearly required for the improved binding. For example, the benzoic acid derivative **7** displays an IC_{50} of 0.20 μM, representing a 15-fold improvement over **2**. In contrast, control compounds **6** and **8** are at least 10-fold *less* potent than **2** and 200-fold less potent than **7**.

Analogue **13** was the most active compound with an IC_{50} of 60 nM. Characterization by surface plasmon resonance showed that this compound binds to IL-2 with a 1:1 stoichiometry and a K_d = 100 nM. Compound **13** was also found to have an $EC_{50} = 3\ \mu M$

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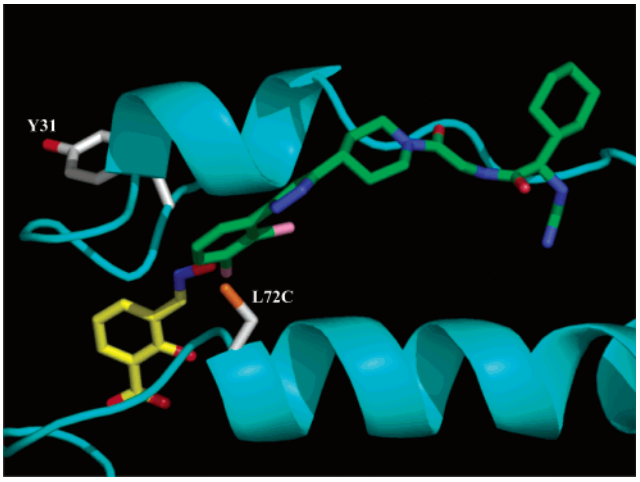
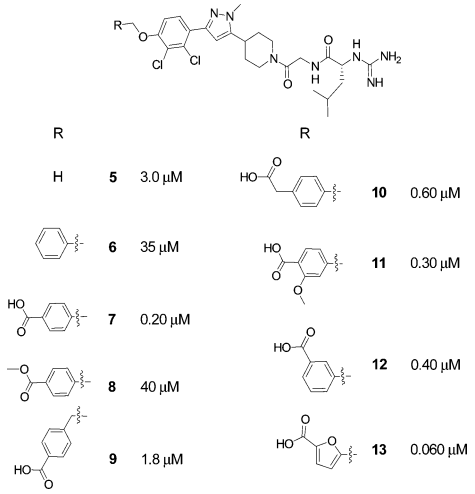


Figure 1. Crystal structure of **3** (green) complexed with IL-2 (cyan) shown as an overlay with a model of where **4** (yellow) is proposed to bind when tethered to L72C (white). IL-2 residues 1−29, 48−61, 81−133, and the disulfide tether linkage of **4** are omitted for clarity.

Chart 2. Representative Examples from the 20 Member Library Prepared To Merge Tethering Hits onto Compound **2**



in a short-term cell-based assay when cells were stimulated with IL-2, but it had no activity when the same cells were stimulated with the homologous cytokine IL-15 (see Supporting Information for details). These compounds represent the first examples of a high-affinity ligand for a cytokine target and demonstrate that protein−protein interactions are potential small molecule targets. Exploiting flexibility and adaptivity at an otherwise flat and featureless surface could be a general strategy for this target class.

Fragment assembly is a conceptually powerful strategy that requires experimentally challenging steps, including fragment identification and productive fragment linking. Tethering is an efficient method for interrogating protein surfaces and identifying fragments: the site-specific nature of tethering reveals binding sites as well as the preferred chemical properties of fragments that bind these sites. In this example, tethering identified a new site and a strategy for merging the selected class of fragments onto an existing low micromolar hit. A 50-fold affinity improvement was found in the adaptive region, suggesting that for targets of this nature the most productive binding sites might also be the most difficult regions for applying structure-based design approaches. These observations underscore the value and potential of an empirical process like tethering to probe specific regions on a target. The selected fragments indicated distinct chemical features that were important for binding; only compounds containing aromatic carboxylic acids showed affinity improvements. Attempts to identify additional binding contacts with conservative substitutions significantly *reduced* the affinity of the ligand. The efficiency with which affinity was improved is remarkable and demonstrates a novel method for designing focused libraries to rapidly improve leads that have resisted chemical optimization.

Supporting Information Available: Statistical analysis of the tethering hits, computational analysis of potential binding modes, syntheses of the compounds described, binding analysis of compound **13**, and cell-based assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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