Use of "Tethering" for the Identification of a Small Molecule that Binds to a Dynamic Hot Spot on the Interleukin-2 Surface

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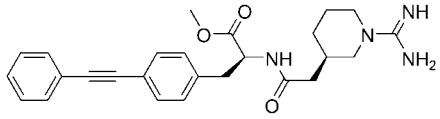
The modulation of protein–protein interactions by small organic molecules represents one of the most rewarding yet challenging topics of current research at the interface of organic chemistry and biochemistry.[1] Since the biological function of most proteins depends on their interactions with other macromolecules, disruption or enhancement of these interactions by cell-permeable molecules provides a means of influencing protein function. Cell-permeable molecules that allow a given protein to be turned on or off with high temporal and spatial control are therefore desirable tools for the analysis of complex biological systems in basic research.[2] However, the following difficulties need to be overcome: 1) protein–protein interfaces are significantly larger than the surface areas of small molecules, 2) many protein–protein interfaces lack obvious binding pockets for small molecules, and 3) mechanism-based or natural product-based lead structures rarely exist.[1]

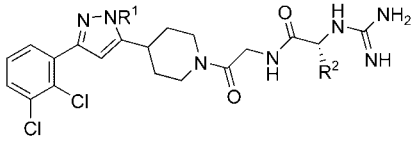
A solution for the problem of size difference between small molecules and protein–protein interfaces was offered in 1995 by the group of J. Wells, who proposed the presence of "hot spots" in protein–protein interfaces.[3] Hot spots are subregions of protein–protein interfaces that contribute significantly to the overall free energy of binding between the proteins, and whose size is comparable to the surface area of drug-like molecules. Further research by Wells[4] and other scientists[5] recently provided additional evidence that the problem originating from the frequent absence of obvious binding pockets for small molecules in flat protein surfaces can be overcome.

The articles by Wells, Braisted, and Oslob highlighted here[4, 5a] point to an aspect of hot spots that encourages the initial screening of diverse chemical libraries: flexible protein surfaces. These articles describe the discovery of small-molecule inhibitors of the interactions between interleukin-2 (IL-2) and its receptor IL-2Rα, and elucidate the inhibitors' mechanisms of action. Compound **1**, a micromolar inhibitor of the IL-2/IL-2Rα interaction, acts by binding to the IL-2Rα-binding region of IL-2.[6] This region of IL-2 had previously been defined by mutational studies, which analyzed the importance of individual amino acids for binding to IL-2Rα,[7] and consists of a rigid and a flexible region. Efforts to optimize **1** by structure-based design and parallel synthesis led to novel lead structures **2** and **3** (Scheme 1), whose potency did not exceed the potency of the original inhibitor **1**. X-ray analysis revealed that inhibitor **3** binds to the hot spot of the IL-2/IL-2Rα interaction in a similar manner to the parent compound **1**.

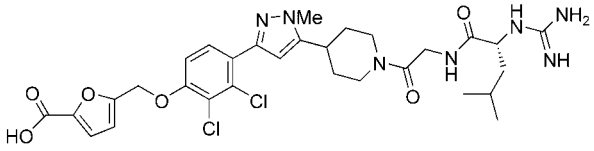
In order to identify more active inhibitors, a fragment-assembly method referred to as "tethering" was applied.[8] Tethering can identify low-affinity fragments that bind to a specific site of a protein. It involves generating protein mutants in which cysteine mutations are introduced at the perimeter of the protein region of interest. Subsequently, the mutant proteins are probed with disulfide-containing chemical libraries under conditions that facilitate thiol–disulfide exchange, and molecules that bind to the site near the cysteine mutation (even if the affinity is low) are captured by disulfide bonds. The identity of the small molecules covalently attached to the protein is then analyzed by mass spectrometry (Scheme 2).



1 IC_{50} = 3 μM M_W = 447 g mol^{-1}



2 R^1 = Me, R^2 = *i*Bu IC_{50} = 3 μM M_W = 522 g mol^{-1}
3 R^1 = H, R^2 = *c*Hex IC_{50} = 6 μM M_W = 534 g mol^{-1}



4 IC_{50} = 0.06 μM M_W = 663 g mol^{-1}

Scheme 1. *IL2/IL-2Rα inhibitors **1–4**.*

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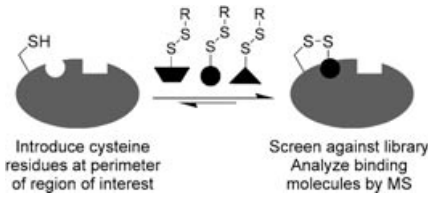

Scheme 2. *Principle of tethering (adapted from ref. [5b]).*

The application of tethering to the perimeter of the inhibitor binding site on IL-2, which coincides with the IL-2Rα binding region, revealed that two cysteine mutants selected small aromatic carboxylic acids. The cysteine residues in these mutants were located in the structurally adaptive part of the IL-2Rα binding region. A combination of molecular modeling studies and X-ray analysis of compound **3** bound to IL-2 suggested that the selected fragments could be merged onto the core structure of compound **2** by linking the fragments onto the dichlorophenyl ring of **2**. This led to the identification of compound **4** ($M_W =$ 663 g mol^{-1}, Scheme 1), which was found to be 50 times more active than the compound **2** and which inhibited the IL-2/IL-2Rα interaction with an IC_{50} value of 60 nM.

The X-ray structures of IL-2 without ligand as well as in complex with compounds **3** and **4** (Figure 1) reveal how the surface of IL-2 adapts to accommodate these inhibitors. In the absence of a ligand, no obvious binding pocket for a small molecule is observed (Figure 1 A). Both inhibitors anchor with their hydrophilic guanido moiety to the carboxylate side chain of a glutamic acid residue located in the rigid part of the IL-2Rα binding hot spot. To accommodate the hydrophobic dichlorophenyl moiety of **3**, two amino acids (F42 and L72) in the adaptive part of the hot spot shift to create a hydrophobic binding pocket that is not present in the absence of the inhibitor (Figure 1 B). Further conformational changes of the IL-2 hot spot are observed in the complex with inhibitor **4** and allow binding of the additional furanoic acid moiety of **4** in a hydrophobic yet polar binding pocket between P34, K35, and R38 (Figure 1 C). Binding of **4** to IL-2 creates an elongated groove reaching from P34 to E62. Thus, the conformation of IL-2 changes as necessary to adapt to the newly added functionalities in compound **4**.

While the induction of a binding pocket on the IL-2 surface in the presence of a small molecule may initially come as a surprise, one needs to bear in mind that the structure of a protein binding site cannot be visualized in the absence of the binding partner.[9] The traditional "induced fit" model[10] would explain the generation of the protein binding site by conformational changes induced during the process of binding between IL-2 and the inhibitors. An alternative model regards proteins as statistical ensembles of conformational states.[11] The latter model hypothesizes that the conformer containing the binding site for the inhibitor pre-exists even in the absence of the inhibitor, albeit to an extent too small to be detected in the X-ray structure. In the presence of the inhibitor, this conformer is postulated to become stabilized and thereby to become predominant. Regardless of the mechanism by which the binding pocket on IL-2 is generated, it is important to realize that the absence of a binding pocket for a small molecule in the unbound structure of a protein does not mean that a protein conformation displaying such a binding pocket could not be induced or stabilized. However, binding of a small molecule to an induced or stabilized binding pocket presumably has an entropic disadvantage compared to its binding to a deep binding pocket that already exists in the absence of the small molecule: in the latter case, substitution of water molecules upon binding of the inhibitor may compensate for the entropy reduction accompanying the binding event.

Tethering adds to the repertoire of fragment-assembly methods,[12] such as SAR by NMR[13] or dynamic combinatorial libraries,[14] and has proved to be a useful tool for the empirical optimization of an existing lead compound binding to a flexible protein site. Because of protein flexibility, it appears unlikely that compound **4** would have been identified by rational design. A drawback of tethering when compared to NMR-based screening[15] is the requirement for disulfide-containing small-molecule fragments, which are not readily commercially available as this point. Furthermore, despite several reports that describe the application of tethering for the identification of bioactive small molecules,[8,16] the general scope of the method is still unclear. A major challenge related to both SAR by NMR and tethering appears to be the productive merging of the existing lead structure with the newly discovered fragments.

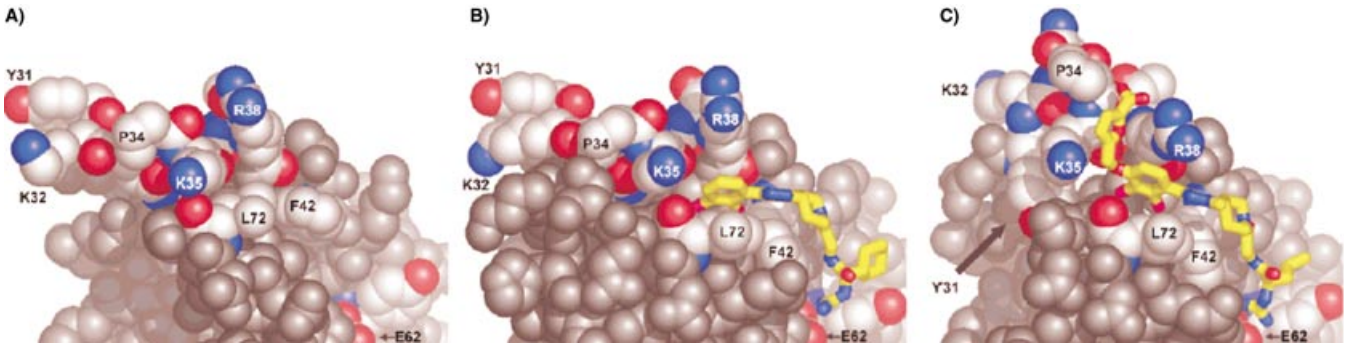

Figure 1. *X-ray structures of A) unligated IL-2, as well as in complex with inhibitors B) **3** and C) **4**. Reprinted with permission from ref. [4]. Copyright 2003 American Chemical Society.*

The example of the IL2/IL-2Rα inhibitors adds to the list of small-molecule inhibitors of protein–protein interactions that have been discovered despite the difficulties related to this topic (see introduction).[1,17] However, as the current level of understanding of the mechanisms of action of small-molecule inhibitors of protein–protein interactions is still very limited, a significant amount of further research will be necessary to allow for more efficient and systematic discovery of these valuable tools for the analysis of protein functions in basic research.

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Keywords: bioorganic chemistry · inhibitors · molecular biology · proteins · X-ray spectroscopy

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