Identification of a Small Molecule Inhibitor of the IL-2/IL-2Rα Receptor Interaction Which Binds to IL-2

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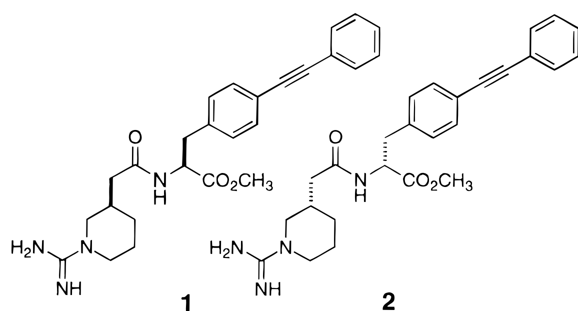
Interleukin-2 (IL-2) is a 15.5 kDa cytokine that has a predominant role in the growth of activated T cells. IL-2 stimulates T-cell proliferation by binding on the T-cell surface with picomolar affinity to a heterotrimeric receptor complex consisting of α, β, and γ chains.[1] Antibodies that recognize the α receptor subunit (IL-2Rα) and block IL-2 binding have proven clinically effective as immunosuppressive agents,[1,2] and thus, we have sought small molecules capable of blocking the IL-2/IL-2Rα interaction as potential orally active successors to the antibody drugs. The design of such agents is based on a combination of structural information obtained by X-ray crystallographic[3,4] and NMR studies[5] of IL-2 which reveal the backbone to include a bundle of four α-helixes (A, B, C, and D) with an up−up−down−down arrangement and site-directed mutagenesis of human IL-2 which identified residues in the AB loop (K35, R38, T41, F42, K43, Y45) and the B helix (E62, L72) as being critical for the binding of IL-2 to IL-2Rα.[6,7]

One member of a series of acylphenylalanine derivatives intended to mimic the R38−F42 region of IL-2 was found to be a competitive inhibitor of IL-2/IL-2Rα binding with a mid-micromolar IC_{50}. Structure−activity studies led to the synthesis of **1** with an IC_{50} of 3 μM. Although the acylphenylalanine



derivatives were designed to complex with IL-2Rα by emulating residues R38 and F42 of the IL-2 ligand, we considered the possibility that they might instead interact with the ligand. NMR studies using uniformly ^{15}N-labeled IL-2 indicate that the resonances of residues in the vicinity of the binding epitope previously identified by site-directed mutagenesis are selectively perturbed in a concentration-dependent manner by **1**, but not by its inactive enantiomer **2,** *indicating that **1** inhibits IL-2/IL-2Rα binding by associating with IL-2*. To our knowledge, this represents the first well-characterized example of a small molecule, nonpeptide inhibitor of a cytokine/cytokine receptor interaction.

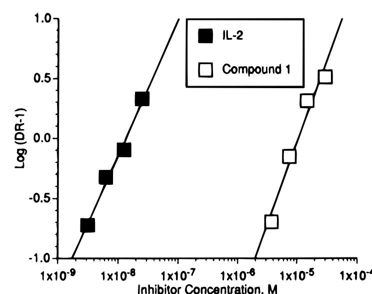


Figure 1. Schild regressions of the effects of unlabeled IL-2 and compound **1** on [^{125}I]IL-2 binding to soluble IL-2Rα. Plots are derived from Scatchard analyses of [^{125}I]IL-2 binding to immobilized receptor in the presence of the plotted concentrations of unlabeled IL-2 or compound. DR represents the dose ratio and is defined as the K_d of [^{125}I]IL-2 binding in the presence of inhibitor divided by the K_d in the absence of inhibitor.

Compounds were evaluated for the ability to inhibit IL-2/IL-2Rα interactions using a scintilation proximity competitive binding assay.[8] The assay basically measures binding of ^{125}I-labeled IL-2 to immobilized soluble IL-2Rα in the presence of various concentrations of inhibitor compound. As determined from Hill plots of the inhibition curves, compound **1** inhibited binding of IL-2 to IL-2Rα with an IC_{50} of 3 μM at pH 7.4. In contrast, it's enantiomer, **2,** gave only 14% inhibition of binding at the highest concentration tested (500 μM), while IL-2 had an IC_{50} of 13 nM. To characterize the inhibitory activity of **1,** binding assays were carried out with varying concentrations of ^{125}I-labeled IL-2 in the presence of varying concentrations of **1** or unlabeled IL-2. Scatchard analyses were performed and the K_d values for [^{125}I]IL-2 binding at each inhibitor concentration were plotted versus inhibitor concentration in a Schild plot[9] (Figure 1), which indicates that the observed inhibitory activity of **1** is consistent with a competitive reversible inhibition.

To assess the mechanism of inhibition, NMR experiments were undertaken with uniformly ^{15}N-enriched IL-2.[10] The $^1H-^{15}N$ NMR assignments were obtained by analysis of 3D $^1H-^1H-^{15}N$-TOCSY- and NOESY-HSQC spectra.[11] These resonance assignments were, in most cases, determined to be similar to those previously published.[12] Perturbations of the $^1H-^{15}N$ chemical shifts were measured in 2D $^1H-^{15}N$-HSQC spectra[13] which were acquired in the presence of 0.0, 0.5, 1.0, and 2.0

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(10) ^{15}N-labeled IL-2 was produced by metabolic labeling of IL-2 produced in the yeast *Pichia pastoris*. Briefly, Pichia cells were transformed with an expression vector that directed the synthesis and secretion of human IL-2 under the control of a methanol-inducible promoter. The cells were grown in media containing [^{15}N]ammonium sulfate for 2 days at 30 °C. The cells were then concentrated, and IL-2 expression was induced by addition of methanol to the culture. The culture medium containing secreted IL-2 was harvested. IL-2 was purified by affinity chromatography over an IL-2Rα column as described previously: Bailon, P.; Weber, D. V.; Kenney, R. F.; Fredericks, J. E.; Smith, C.; Familletti, P. C.; Smart, J. E. *Bio/Technology* **1987**, *5*, 1195−1198.
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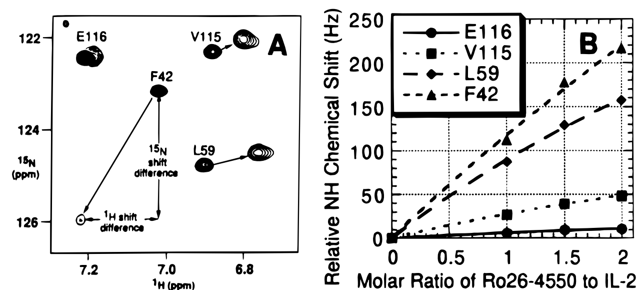

Figure 2. (A) Expansion of $^{15}N-^{1}H$-HSQC spectra of [^{15}N]IL-2. The arrows indicate the chemical shifts observed for four backbone NH resonances in the absence (dark signals) and presence (light signals) of 2 equiv of **1**. The absolute values of ^{1}H and ^{15}N shift changes are illustrated for F42. (B) Vector magnitude chemical shift changes $(((\Delta\delta^{1}H)^2 + (\Delta\delta^{15}N)^2)^{1/2})$ for the same NH signals shown in part A. Here the chemical shift changes for 0.59 mM [^{15}N]IL-2 NH signals are calculated upon addition of 1.0, 1.5, and 2.0 equiv of **1**.

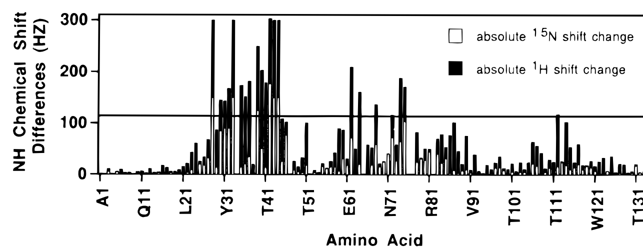

Figure 3. Sum of the absolute value of $\Delta\delta^{1}H$ and $\Delta\delta^{15}N$ chemical shift differences for [^{15}N]IL-2 resonances in the presence and absence of 2.0 equiv of **1**. ^{1}H shift differences are in black and ^{15}N shift differences are in white. Shift differences are calculated as $\Delta\delta = |\delta_{2eq} - \delta_{0eq}|$, where δ_{2eq} represents the chemical shift (in hertz) for the [^{15}N]-IL-2 signal the presence of 2.0 equiv of **1**, and δ_{0eq} represents the chemical shift for [^{15}N]IL-2 alone. Chemical shifts were measured in 2D $^{1}H-^{15}N$-HSQC spectra[15] at 40 °C and pH 4.6, in a buffer containing 10 mM sodium acetate, 0.02% sodium azide, 10% D_2O, and 90% H_2O, and using a Varian Unity*plus* 600 spectrometer. The largest combined shift difference ($\Delta\delta^{1}H + \Delta\delta^{15}N$) was measured to be 300 Hz for F42. The shift differences for the residues I28, N33, K43, and F44 have an estimated lower limit of 300 Hz for their combined ($\Delta\delta^{1}H + \Delta\delta^{15}N$) shift. Their shifts could not be accurately quantitated due to exchange broadening of the HSQC signal in the presence of **1**. The horizontal line at 115 Hz is the arbitrary threshold above which $^{1}H-^{15}N$ resonances are defined as "highly perturbed" by the addition of 2 equiv of **1** to [^{15}N]IL-2.

equiv of **1** to [^{15}N]IL-2 as indicated in Figure 2. While titration curves shown in Figure 2B correspond to dissociation constants of 2−6 mM for the IL-2 complex with **1**, the drop in the binding constant is primarily due to the difference in pH between the NMR solution conditions (pH = 4.6) and the binding assay[14] (pH = 7.4). Accurate determination of K_d at pH = 7.4 by NMR is difficult because of limited solubility of the protein; however, approximate K_d measurement by NMR at pH = 7.5 clearly illustrates a stronger complex (lower K_d) than that observed at pH = 4.6 (data not shown). Figure 3 illustrates the sum of absolute chemical shift changes for both backbone and side-chain $^{1}H-^{15}N$ resonances of [^{15}N]IL-2. The horizontal line at 115 Hz represents an arbitrary threshold above which $^{1}H-^{15}N$ resonances have been defined as "highly perturbed" by addition of 2 equiv of **1**. No effect on the [^{15}N]IL-2 NMR resonances was observed upon addition of up to 2 equiv of the enantiomer **2**, indicating that the chemical shift changes observed in the presence of **1** are the result of a specific interaction between **1** and IL-2.

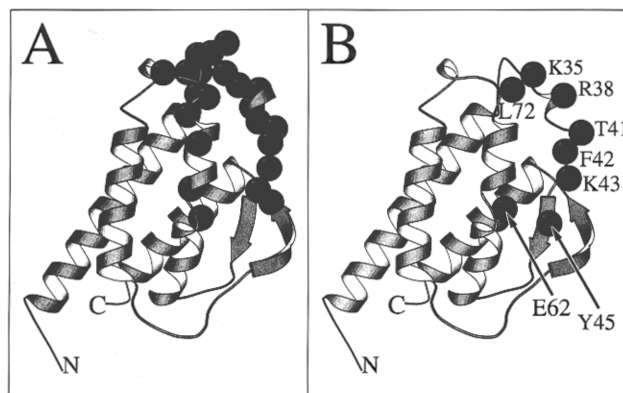

Figure 4. Molscript[16] rendering of the X-ray crystal structure of human IL-2.[4] (A) Nitrogen atom positions are represented as dark spheres for the highly perturbed $^{1}H-^{15}N$ resonances defined in Figure 2. $^{1}H-^{15}N$ resonances which are highly perturbed upon addition of **1** include I28, N30, Y31, K32, N33, K35, L36, T37, M39, L40, T41, F42, K43, F44, E62, K64, E68, L72, Q74, S75, and A112 backbone NHs and the side-chain $N\delta_2H$ of N33. (B) α-Carbon atom (Cα) positions where mutagenesis studies have identified IL-2 side-chain interactions important in the binding of Hu-IL-2 to IL-2Rα.[6,7]

The N atoms corresponding to the residues with highly perturbed resonances are shown as dark spheres on the ribbon diagram shown in Figure 4A. It is clear from this figure that these residues form a contiguous patch which primarily involves the N-terminal half of the AB loop and the carboxy-terminal half of the B helix. The same orientation of IL-2 is shown in Figure 4B, in which the dark spheres represent the Cα positions of residues identified as important for the binding of human IL-2[6,7] to IL-2Rα by site-directed mutagensis. The evident correspondence between the region of IL-2 perturbed by association with **1** (Figure 4A) and that shown to be involved in the binding to IL-2Rα (Figure 4B) suggests that **1** interferes with IL-2/IL-2Rα binding by competing with IL-2Rα for its binding site on IL-2. Further work including attempts to solve the X-ray and NMR structures of the IL-2/**1** complex are currently underway in our laboratories.

Supporting Information Available: Text describing the synthesis of **1** and **2** and a supplement to Figure 2 showing the complete $^{15}N-^{1}H$-HSQC spectra of [^{15}N]IL-2 in the presence of 0.0, 1.0, 1.5, and 2.0 equiv of **1** (7 pages). See any masthead page for ordering and Internet access instructions.

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