Small molecule cytokine mimetics

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A number of reports describe small peptides, and even *bona fide* small organic molecules, that activate homodimeric cytokine receptors and show cytokine-like activity *in vitro* and *in vivo*. These cases can be examined in light of the mechanistic and thermodynamic principles that govern cytokine-receptor activation.

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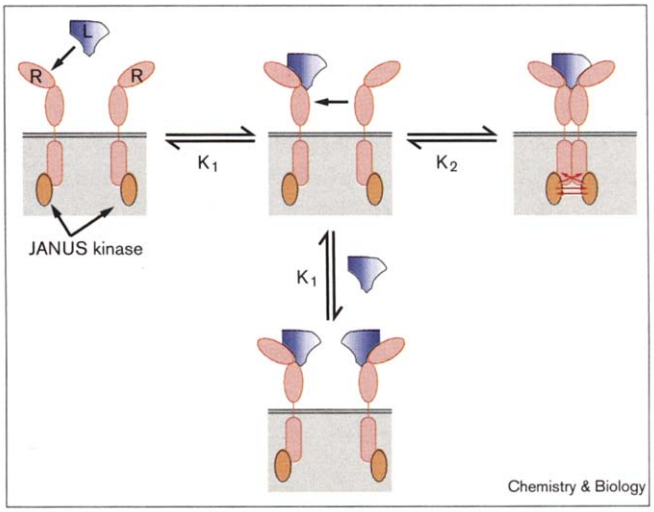
Introduction

Cytokines are extracellular signaling molecules that mediate communication between cells and their environment through interaction with cell-surface receptors [1,2]. Members of this extensive family of proteins play key roles in many critical biological processes including regulation of the cellular and humoral immune responses, hematopoiesis, inflammation, wound healing and apoptosis. Given the varied and important biological activities of cytokines, it is not surprising that many successful protein drugs are either cytokines themselves, or antibodies or soluble receptor mimics that antagonize cytokine function. Examples of cytokines that are marketed as drugs include human growth hormone (hGH), erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor, interferon-α, interferon-β, interleukin (IL)-2 and IL-11. Numerous other cytokines or cytokine-receptor antagonists are in advanced stages of clinical development. All of these protein drugs share the key drawback that they must be delivered to the patient perenterally, for example by subcutaneous, intramuscular or intravenous injection. There is therefore great interest in developing cytokine agonists or antagonists that are orally available. One approach to achieving this aim involves developing small-molecule agonists that can activate cytokine receptors, or small-molecule antagonists that block the response to a cytokine by interacting with its receptor or with downstream components of its signaling pathways. Until recently, there was reason to believe that the task of finding a small-molecule agonist that could mimic the action of a protein the size of a typical cytokine (~15–50 kDa) would be extremely difficult, if not impossible. Recent reports have shown, however, that relatively small peptides [3–5], and even *bona fide* small organic molecules [6,7], can bind to and activate certain homodimeric cytokine receptors, and can, to some degree, mimic the effects of these cytokines *in vitro* and *in vivo* in experimental systems. Here we examine these cases in light of the mechanistic and thermodynamic principles that govern cytokine-receptor activation. In so doing, we discuss the essential properties of small molecule cytokine-receptor agonists, and we examine various strategies for identifying and optimizing lead molecules. Although examples from the literature include only agonists of homodimeric cytokine receptors (i.e. those receptors comprising two identical receptor chains), we include a brief discussion of how the lessons learned from these published examples might apply to receptors of more complex stoichiometry, and also to receptor antagonists.

Receptor activation by ligand-induced receptor chain dimerization

For a number of cytokines, receptor activation has been shown to occur by a mechanism of ligand-induced receptor chain oligomerization [8–10]. This mechanism can be exemplified by the human growth hormone receptor (GH-R) [11], a class I cytokine receptor [1]. GH-R comprises two identical subunits, each of which is a single-pass transmembrane protein containing an extracellular portion comprising two fibronectin type-III repeats, a membrane-spanning region and a cytoplasmic portion of unknown structure. The receptor is activated via a two-step mechanism, as shown in Figure 1 [12]. In the first step, hGH (L) binds to the extracellular portion of one receptor chain (R) to form a binary complex, R•L. Subsequently, a second GH-R molecule binds to form a ternary complex, R•L•R. Recruitment of the second GH-R chain is brought about by the combination of existing weak binding sites in GH-R and hGH into a single, extended binding site in R•L that confers significantly increased affinity for binding the second GH-R chain [11]. The net effect of hGH binding to the cell is therefore to bring two molecules of GH-R together into a single complex, such that signaling kinases that are noncovalently associated with the cytoplasmic portions of the receptor chains can interact with each other and with additional downstream signaling molecules, thus transmitting the signal to the interior of the cell. A significant body of data supports the notion that, for GH-R [11,12], and several other homodimeric class I cytokine receptors such as the receptors for EPO (EPO-R) [13,14], G-CSF (G-CSF-R) [12,15] and thrombopoietin (TPO-R) [16–18], the bringing together of two receptor chains on the cell surface is necessary to trigger signaling (see the note at end of the text for a recently proposed alternative mechanism for EPO-R activation). Important details such as the role of conformational changes in the receptor [19,20], the precise orientation between receptor chains required for signaling [21,22], the involvement of higher-order receptor aggregation events subsequent to formation of the initial ternary complex [23,24], and the importance of on- and off-rate kinetics [25] are still being determined, however. In addition to the homodimeric class I receptors mentioned above, a mechanism of ligand-induced receptor dimerization has been established or is strongly indicated for a number of other oligomeric cytokine receptors, including the heterodimeric receptor for IL-4 comprising the IL-4Rα and common gamma (γ_c) chains [26,27], the heterotrimeric receptor for IL-2 [28], and the tetrameric ($\alpha_2\beta_2$) receptor for IFN-γ [29]. Moreover, analogous activation mechanisms have also been established for a number of oligomeric receptors of other structural classes such as TNF family members [9], and tyrosine kinase and serine/threonine kinase receptors for growth factors such as EGF and TGF-β [8,30]. As a result, it has become widely assumed that most or all of the receptors that fall into any of the structural classes represented in the examples above

Figure 1



The ligand-induced receptor dimerization mechanism as it applies to a homodimeric class I cytokine receptor such as GH-R (adapted from [9]). Receptor activation occurs in two steps. The cytokine or agonist ligand (L) binds to one receptor chain (R), with dissociation constant K_1, to form binary complexes, R•L. In a subsequent step, R•L recruits a second receptor chain to form a ternary complex, R•L•R. The affinity of this second step, which is a bimolecular association event constrained to the two dimensions of the cell membrane, is given by the dissociation constant K_2, which has units of molecules/μm^2 [27]. The bringing together of the receptor cytoplasmic domains, together with their associated JANUS signaling kinases, allows them to interact with each other and with additional downstream signaling molecules, thereby transmitting the signal to the interior of the cell. At very high concentrations of L, the system is forced into a state in which all R molecules are occupied by an equivalent number of L molecules, therefore antagonizing the formation of receptor dimers, as illustrated by the lower pathway. Note that the extent of direct contact between the receptor chains in R•L•R varies for different receptors, and that the agonist ligand can have either identical or nonidentical binding sites for the receptor.

are activated by ligand-induced oligomerization [10,30], although some exceptions or significant mechanistic variations might exist even among closely related members of the cytokine receptor superfamily [31,32].

The task confronting small-molecule agonists of cytokine receptors

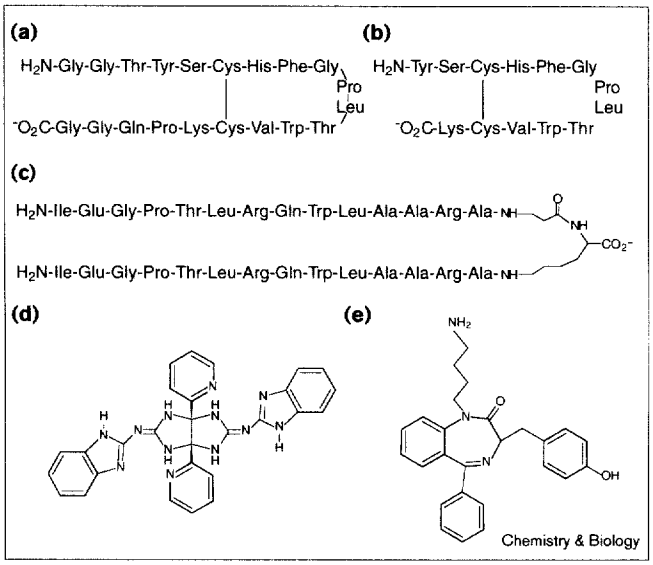
To trigger the activation of a receptor such as GH-R by directly cross-linking two receptor chains, as illustrated in Figure 1, an agonist ligand must achieve the following: it must bind to one of the receptor chains with reasonable affinity; it must do so in a position such that additional unsatisfied binding functionality projects in the direction required for contact with a second receptor chain; it must have a sufficient affinity for the second receptor chain to shift the equilibrium for receptor dimerization to significantly favor the dimeric state of the receptor; the resulting receptor dimers must have a relative orientation between receptor chains that leads to productive interaction

between the receptor cytoplasmic domains and their associated signaling molecules; and it might also be important that the activating ligand has a sufficiently slow dissociation rate such that the lifetime of the activated receptor complexes exceeds the threshold required for effective signaling to occur. Examination of published cases of peptide or small molecule cytokine-receptor agonists allows us to assess the degree to which each of these requirements was successfully met in different cases, and therefore to draw inferences about the relative importance of these different properties for the function of a small-molecule receptor agonist.

Published examples of peptide and small molecule cytokine-receptor agonists

Peptide or small-molecule agonists have been reported for three different cytokine receptors: EPO-R, TPO-R and G-CSF-R. All three are homodimeric receptors from the class I subfamily of cytokine receptors [1]. In 1996, Wrighton *et al.* [3] reported the discovery of a 20 residue cyclic peptide, EMP-1 (Figure 2a), derived from phage selection, that competes with EPO for binding to a soluble form of the EPO-R extracellular domain and, at micromolar concentrations, stimulated cell proliferation in an EPO-dependent functional assay. Phosphotyrosine analysis of the lysates of stimulated cells suggested that EMP-1 and EPO induced the phosphorylation of a similar set of intracellular proteins, and that they had similar effects on cell-cycle kinetics. EMP-1 also showed activity *in vivo* in two murine models of erythropoiesis. A crystal structure of the complex of EMP-1 with recombinant soluble EPO-R showed that the complex contains two EMP-1 molecules in contact with two receptor molecules [33], indicating that EMP-1 functions as a homodimer. This conclusion was substantiated by reports that covalently coupling EMP-1 molecules to enforce a dimeric structure enhanced agonist activity by greater than tenfold [34]. Structure–activity studies of EMP-1 have led to the construction of a yet smaller derivative peptide, a 13-mer called EMP-20 (Figure 2b), that in cell proliferation assays *in vitro* has agonist activity roughly comparable to EMP-1 [35]. The same group responsible for EMP-1 has also reported a covalently dimerized 14-residue peptide, AF13948 (Figure 2c), again derived from a phage-selection approach, that was able to activate a different homodimeric cytokine receptor, TPO-R, in assays measuring TPO-R-dependent cell proliferation, megakaryocyte colony formation and magakaryocyte maturation *in vitro* [4]. Remarkably, this peptide appeared virtually equipotent with recombinant human TPO in these assays. The authors also report that AF13948 was active in a mouse model of thrombopoiesis. A second group [5] has subsequently reported the independent discovery of phage-derived peptide TPO-R agonists of related sequence to those of Cwirla *et al.* [4], and have demonstrated that one of them activates JAK/STAT signaling in a TPO-responsive cell line [36].

Figure 2



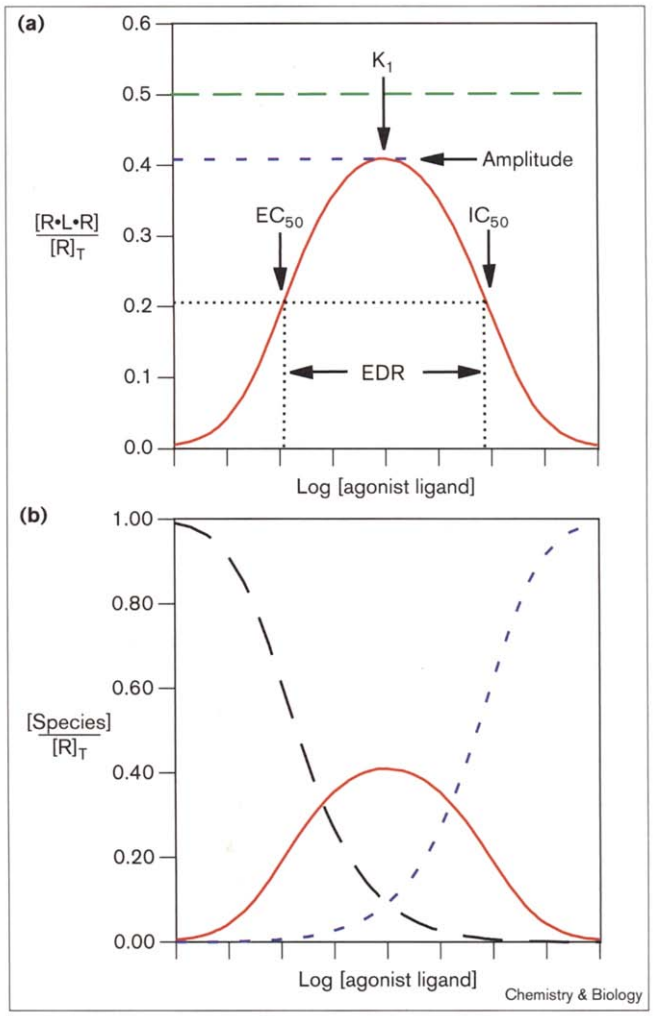
Structures of some reported examples of peptide and small molecule cytokine-receptor agonists. **(a)** EMP-1 [3]; **(b)** EMP-20 [35]; **(c)** AF13948 [4]; **(d)** SB247464 [6]; **(e)** TM41 [7]. (a) and (b) bind to EPO-R as noncovalent dimers; (c) comprises two identical 14-mer peptides covalently dimerized by means of a lysine-β-alanine linker.

Reports of small organic molecules that possess cytokine receptor agonist activity also exist. Tian *et al.* [6] described a small organic molecule, SB247464 (Figure 2d), that at micromolar concentrations showed agonist activity towards the murine G-CSF-R. This molecule (molecular weight ~500 Da), was derived by screening a compound library directly for agonist activity in a cell-based assay using a muG-CSF-dependent reporter gene. SB247464 also gave responses in several other muG-CSF-dependent colony-forming and signaling assays—including demonstrating an ability to induce G-CSF-dependent signaling events such as the phosphorylation of G-CSF-R itself, JAK1, JAK3, STAT3 and STAT5—and showed activity *in vivo* in a murine model of granulopoiesis [6,37]. SB247464 appeared highly specific: it did not elicit a response in assays dependent on activation of the receptors for EPO, interferon-α or interferon-γ and, strikingly, was also unable to activate the human G-CSF-R in similar types of *in vitro* assays. More recently, Kimura *et al.* [7] have reported a small organic molecule, TM41 (Figure 2e), that competes against TPO in a TPO-R-binding ELISA, and, at a concentration of ~100 μM, was shown to stimulate cell proliferation and to activate STAT5 in a human TPO-R-dependent cell line [7].

Agonist potency and efficacy and bell-shaped dose–response curves

A striking characteristic of the small-molecule G-CSF-R agonist SB247464 was that it gave bell-shaped dose-response curves in a variety of cell-based functional assays

Figure 3



Dependence of the concentration of the activated receptor complex R•L•R (expressed as a fraction of total receptor, $[R]_T$) on the concentration of cytokine or agonist ligand, L. The curves shown were calculated from the equations of Perelson and DeLisi [38] for a value of $[R]_T/K_2 = 100$. **(a)** The agonist concentration that produces the maximal level of R•L•R and therefore defines the mid-point of the bell-shaped curve is equivalent to K_1, the dissociation constant for the initially formed binary complex R•L (see Figure 1). The ligand concentration resulting in half-maximal R•L•R is designated EC_{50} and the ligand concentration resulting in 50% self-inhibition of R•L•R formation is similarly designated IC_{50}. The width of the curve at half-maximal height is given by IC_{50}/EC_{50}, and defines the effective dose range (EDR) over which the agonist ligand will produce at least half-maximal R•L•R formation. The EDR is a function of the effective affinity for the binding of R•L to R to form R•L•R, which depends on K_2 (Figure 1) and on $[R]_T$, the total concentration of receptor present locally on the cell surface. The amplitude of the curve at $[L] = K_1$ is shown by the blue line (short dashes). The theoretical maximum amplitude of $[R•L•R] = 1/2[R]_T$ is shown by the green line (long dashes). **(b)** Identical simulation to that shown in (a), but showing how each of the three forms of the receptor, [R•L•R] (solid line), [R•L] (short dashes) and [R] (long dashes), varies with the concentration of agonist ligand.

[6,37]. Peak response was observed at ~1 μM SB247464, independent of the assay used. The concentration of SB247464 required for peak activity was several logs higher than that required for the natural murine cytokine, which, in the same assays, achieved a maximal response at ~10–100 pM. Moreover, in some assays the amplitude of the signaling response seen at peak doses of SB247464 was several-fold lower than the response seen with muG-CSF itself. Efficient optimization of the properties of a molecule such as SB247464 clearly requires an understanding of how the dose-response curve observed in functional assays can be quantitatively related to the interactions between the molecule and the target receptor.

Bell-shaped dose–response curves in cell-based functional assays are a characteristic feature of homodimeric receptors that are activated by ligand-induced dimerization [12,14]. Because the two receptor chains involved in the signaling complex are identical, at very high concentrations of ligand the system becomes forced into a state in which each receptor molecule is occupied by a separate molecule of ligand (Figure 1), such that, at equilibrium, virtually all receptor molecules are present as R•L complexes. There is, therefore, little formation of the ternary R•L•R complex — and hence little receptor activation — at either very low or very high concentrations of agonist ligand (Figure 3), and functional assays that depend upon the formation of R•L•R complexes show self-antagonism by the ligand at high concentrations. Such behavior is expected in homodimeric receptors systems, whether the receptor is activated by a small molecule such as SB247464, by monoclonal antibodies targeted to the receptor extracellular domains [12,14,17] or by the natural cytokine itself [12,14,25], when dimerization is induced by the binding of a single agonist molecule. Other, related activation mechanisms can also give bell-shaped dose–response curves (see below, and note at end of text). Experimental observation of a full bell-shaped dose–response curve is sometimes difficult, however, if self-antagonism occurs only at very high concentrations of the agonist ligand, as is typically true for highly potent agonists [14].

Importantly, a mathematical treatment applicable to the ligand-induced dimerization mechanism has been described by Perelson and DeLisi [38], resulting in equations that can be used to calculate how the concentration of R•L•R varies as a function of the concentration of L. The published equations can be applied directly to symmetrical agonists, and the discussion that follows is framed in these terms. Application of the equations to asymmetrical agonist such as hGH itself strictly speaking requires statistical correction by a factor of two, but the effect of this correction on the behavior of the systems we discuss here is negligible. Receptor activation and signaling is a direct consequence of the formation of R•L•R complexes, so this treatment provides a basis for interpreting experimental dose–response data from functional assays in terms of the separate interaction affinities of the agonist ligand

with each of the two receptor chains (i.e. in terms of K_1 and K_2 from Figure 1). In cell-based experimental systems, the application of these mathematical treatments is complicated by the fact that the dependence of the functional response on R•L•R formation might not be linear. For example, in the case of so-called 'spare receptors' [12,39] the receptor is present in excess with respect to one or more limiting downstream signaling components, and therefore a maximal response is achieved with activation of only a fraction of the available receptors. Fortunately, such nonlinearity does not preclude the application of this model to functional data to allow meaningful and quantitative conclusions to be drawn about the binding properties of the agonist ligand. A detailed discussion of the mathematical treatments themselves is beyond the scope of this article, but conclusions derived from their application to agonists of homodimeric cytokine receptors [14,39] are summarized below.

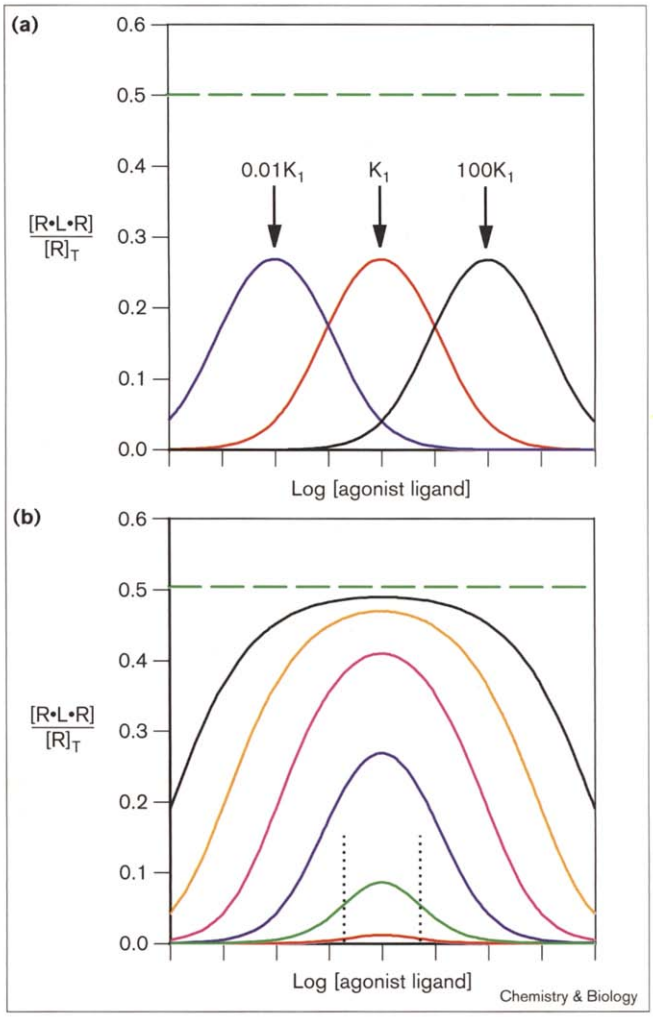
Significance of the mid-point of the dose–response curve

Assuming cytotoxicity effects and other assay artifacts can be excluded, the equations of Perelson and DeLisi [38] predict that the concentration of agonist ligand that corresponds to the peak or mid-point of the bell-shaped dose–response curve should equal K_1, the affinity of the ligand for binding to the first receptor chain (Figure 1). Figure 1 shows that the initial binding of L to R and the self-antagonism that occurs at very high concentrations of L are both governed by K_1 (Figure 1), so at $[L] = K_1$ these effects are equally balanced and the concentration of R•L•R is maximal (Figure 3) [38]. A nonlinear relationship between the concentration of R•L•R and the size of the functional response, such as would be seen with spare receptors, would be expected to have an equal effect on the agonist and antagonist limbs of the dose–response curve, and would therefore be expected to preserve symmetry about K_1 [39]. In a cell-based functional assay a bell-shaped dose–response curve that is centered around a ligand concentration of 1 μM therefore implies that L binds to the first receptor chain with $K_D \approx 1\ \mu M$ (Figures 3,4a). This interpretation can potentially be confirmed by independent experiments because direct determination of the affinity of this interaction is sometimes possible [14].

Significance of the width of the dose–response curve

In a functional assay in which a given agonist shows a bell-shaped dose–response, the ratio of the IC_{50} for self-antagonism to the EC_{50} for the agonist response represents the width of the dose–response curve at half its maximum height (Figure 3). This ratio can be thought of as a measure of the effective dose range (EDR) for the agonist in the assay concerned, because it equals the range of concentrations over which the agonist gives a half-maximal or greater response. It can be inferred from application of Perelson and DeLisi's equations [38] that the magnitude of the EDR will be governed primarily by the effective affinity of the second step in receptor activation, that is the local or effective affinity of R•L for binding to R on the cell surface. This effective affinity is governed by K_2, the intrinsic affinity for the binding of R to R•L (Figure 1), but

Figure 4



How the bell-shaped dose–response curve for R•L•R formation as a function of agonist ligand concentration varies with changing values for K_1 or K_2. The curves shown were calculated using the mathematical treatment of Perelson and DeLisi [38]. The theoretical maximum amplitude of $[R•L•R] = 1/2[R]_T$ is shown by the green line (long dashes). **(a)** For fixed values of K_2 and $[R]_T$, increasing or decreasing K_1 by a factor of 100 causes the mid-point of the dose–response curve to shift to correspondingly higher or lower agonist concentrations. The width and amplitude of the curve are unaffected. **(b)** For fixed values of K_1 and $[R]_T$, decreasing K_2 by incremental factors of 10 over the range of values $[R]_T/K_2 = 0.1–10{,}000$ increases the width and amplitude of the dose–response curve without altering its mid-point. A decrease in the dissociation constant K_2 corresponds to an increase in the affinity of R•L for binding to R on the cell surface (Figure 1). An identical result would be obtained if $[R]_T$ were increased at constant K_2. The theoretical minimum width of $IC_{50}/EC_{50} \approx 30$ is shown by the vertical dotted lines.

depends also on the local density of receptor chains present on the cell membrane [27]. A high intrinsic affinity (i.e. a low K_2) and a high local concentration of receptor chains on the cell surface both favor the observation of a wide dose–response curve (Figure 4b). This is because the more thermodynamically stable is R•L•R, the lower is the concentration of L required to form R•L•R, and the higher — by an identical factor — the concentration of L required to disrupt R•L•R formation through the self-antagonism illustrated in Figure 1. The experimental observation of a very broad dose–response curve would therefore indicate that the total concentration of receptor on the cell surface ($[R]_T$) is much larger than K_2, and therefore the effective affinity for the binding of R•L to R on the cell surface is high. (For a discussion of the dimensionality issues inherent in this comparison, arising from the need to consider receptor concentration in the two dimensions of the cell membrane, see [27].) The relationship between [R•L•R] and $[R]_T/K_2$ that underlies this expected effect on the width of an experimental dose–response curve is illustrated in Figure 4b. The sensitivity of EDR to variations in K_2 has been shown experimentally by means of mutations in the region of the cytokine involved in binding the second receptor chain [12]. Published data showing bell-shaped dose–response curves for cytokines such as EPO [14] and hGH [12,25,39] in cell-based assays show EDR values of up to 6 logs, suggesting that there is a high effective affinity of R•L for R on the cell surface in these cases [27]. In addition to the above-mentioned parameters, the width of the dose–response curve in a functional assay is also influenced by factors that affect the quantitative relationship between the extent of receptor activation (i.e. the level of R•L•R formation) and the functional response. The existence of spare receptors, for example, would in some circumstances be expected to somewhat broaden the dose–response curve [12,39].

Significance of the amplitude of the dose–response curve

The level of functional response achieved at the peak of a bell-shaped dose–response curve, as compared with the maximal response that would be expected for 100% receptor activation, is another measure of how effective the agonist is at inducing the formation of R•L•R complexes. Applying the equations of Perelson and DeLisi [38] leads to the prediction that this measure is also primarily governed by the total receptor concentration on the cell surface and by K_2. Only if $[R]_T$ is high relative to K_2 will the effective affinity for the binding of R•L to R be sufficient to induce the majority of receptors to become dimerized, even at the peak of the dose–response curve. For example, to bring about dimerization of ~80% of receptor molecules at $[L] = K_1$, $[R]_T/K_2$ must be = 80 [38]. Even in the case of a very strong association between R•L and R, a low response or no response might be observed if the geometry of the resulting R•L•R complex does not allow the receptor chains to achieve a relative orientation that is suitable for efficient signaling [21,22]. A clue to diagnosing this situation can be gained from the shape of the dose–response curve. For example, if EDR is large (i.e. greater than ~3 logs), a maximal signal that is significantly lower than that seen for the natural cytokine in the same assay is unlikely to be due to a low effective affinity of R•L for R, because the width of the dose–response curve implies that at $[L] = K_1$ the majority of R chains exist as R•L•R complexes. Such an observation would instead tend to suggest that the agonist ligand is effective at promoting dimerization of the receptor, but that the resulting complex has a geometry that is not optimal for signaling.

A recent report by Pearce *et al.* [25] suggests that predictions such as those made above, derived from an equilibrium model, might break down for highly potent agonists for which kinetic effects have become dominant. Whether such observations will prove to be a general characteristic of receptor systems of this kind represents an exciting new line of inquiry. Their conclusions support the notion that the equilibrium model is applicable to agonist ligands that have only weak or moderate potency, however. It therefore seems likely that the conclusions drawn from analysis of the equilibrium model can fruitfully be applied to the discovery and characterization of small-molecule lead compounds that have agonist activity, and to the optimization of these properties at least up to the point where they begin to approach the activity seen for the natural cytokines.

Analysis of reported peptide and small molecule cytokine-receptor agonists

The fact that the small-molecule muG-CSF-R agonist SB247464 gave bell-shaped dose–response curves in several cell-based functional assays, taken together with the molecule's twofold rotational symmetry (Figure 2d) and the known ability of dimerized G-CSF-R to signal [12], strongly suggests that SB247464 brings about activation of the receptor by forming a 1:2 complex with G-CSF-R (although this might not be true for G-CSF itself, for which various stoichiometries in addition to 1:2 have been proposed for the complex [40]). Evaluating the published dose–response curves for SB247464 in the light of the above analysis allows us to draw a number of inferences about the interactions of the agonist with the two G-CSF-R molecules presumed to exist in the activated receptor complex. The fact that the peak response is consistently seen at an agonist concentration of ~ 1 μM SB247464 across several different functional assays [6,37] implies that the molecule binds to the first receptor chain with an affinity of $K_1 \approx 1\,\mu M$. The amplitude of the dose–response curve obtained with SB247464 in a muG-CSF-R-dependent luciferase promoter assay is relatively low; the peak response for the small molecule is only ~30% of the maximal response seen with muG-CSF in the same assay. The dose–response curve was also shown in several assays to be narrow, with $IC_{50}/EC_{50} \approx 30$, which is close to the theoretical lower limit (Figure 4b). These two features

imply that the binding of SB247464 to one molecule of G-CSF-R results in the induction of only a relatively low effective affinity for the binding of a second G-CSF-R molecule at the receptor expression level that exists on the cells used. The relatively low peak signaling amplitude implies that, even at [SB247464] = K_1, only a fraction (albeit a substantial one) of receptors become dimerized on these cells. The fact that the amplitude of the dose–response curve appears approximately commensurate with its narrow width implies, however, that the R•L•R complexes that form bring the receptor chains into a mutual orientation that leads to relatively efficient signaling. Although increasing the efficacy and potency of SB247464 presumably would require modification of its structure to increase its affinity for G-CSF-R and thereby lower K_1 and K_2, it appears that this molecule provides a good framework for structural embellishment and optimization to achieve this result.

The EPO-R agonist EMP-1 (Figure 2a) forms a 2:2 complex with the receptor, and therefore does not conform to the mechanistic paradigm illustrated in Figure 1. The two molecules of EMP-1 make direct contact with each other in the receptor complex, and the binding energy derived from this interaction is presumably almost wholly responsible for stabilizing the dimerized form of the receptor as the area of direct contact between receptor chains is relatively small [33]. Functional assays that require formation of a 2:2 complex and involve significant productive contact between ligand molecules would also be expected to give rise to a bell-shaped dose–response curve, because a high enough concentration of EMP-1 would be expected to force the system into a state in which all receptor chains are individually occupied by noncovalent EMP-1 dimers, resulting in the predominance of a nondimerized R•L•L state by a pathway analogous to the lower pathway in Figure 1. The experimental data reported for EMP-1 [3] do not extend to high enough concentrations of EMP-1 to unambiguously establish this expected self-antagonist behavior. In contrast, covalently dimerized derivatives of EMP-1 presumably form a 1:2 complex with EPO-R, and therefore would be expected to follow the mechanism shown in Figure 1 and to manifest the behavior described in Figures 3 and 4. Indeed, a biotinylated form of EMP-1, when cross-linked through streptavidin, does appear to give a bell-shaped dose–response in proliferation assays and, additionally, displays enhanced potency [34]. Other covalently dimerized forms of EMP-1 show EC_{50} values that are more than tenfold lower than that found for EMP-1 itself [34]. The dose–response data reported for these covalently dimerized forms is not sufficient to establish definitively the expected bell-shaped dose–response, but the data shown suggest that EDR is ≥ 3 logs in a proliferation assay based on cells made EPO-dependent by transfection with EPO-R. Even this lower limit suggests that the binding of dimeric forms of EMP-1 to EPO-R induces a substantial effective affinity for EPO-R dimerization at the level of receptor expression present on the cells used. It is difficult to draw any conclusions about how either the width or the amplitude of the dose–response curve for EMP-1 and derivatives compares with that seen for EPO itself because the reports do not show a side-by-side comparison of these agonists in the same experiment, but in a different assay EPO has been shown to have an EDR of ~6 logs [14]. (See note at end of text.)

The TPO-R agonist peptide of Cwirla *et al.* [4], AF13948, is also a covalent dimer of identical peptide sequences (Figure 2c). On the basis of the >1000-fold enhanced potency of AF13948 compared with the monomeric peptide from which it was derived [4] and the known ability of dimerized TPO-R to signal [17,18], AF13948 can also be presumed to form a 1:2 complex with its receptor. As was the case for EMP-1, published dose–response data for AF13948 also do not extend to high enough agonist concentrations to fully define the expected self-antagonistic portion of the dose–response curve, although it is possible to set a lower limit of EDR of ≥ 2 logs for the peptide (and for TPO itself) in a proliferation assay based on TPO-R-transfected Ba/F3 cells. A direct comparison with the natural cytokine [4] indicates that AF13948 achieves a functional response with an amplitude and an EC_{50} that were indistinguishable from that of TPO. This result does not necessarily imply that AF13948 dimerizes TPO-R as effectively as does TPO. In the absence of fully defined dose–response curves we cannot be sure, for example, that the peptide does not induce dimerization with a higher K_2 that is being offset by a lower value for K_1. Nevertheless, the dimerization achieved by AF13948 is clearly sufficient to achieve a level of R•L•R complexes that can effectively match the response seen with the cytokine in this assay. Finally, data for the small-molecule TPO-R agonist of Kimura *et al.* [7], TM41 (Figure 2e), show hints of a bell-shaped dose–response, but the data are not sufficient to clearly define the mid-point or the width of the curve beyond the lower limits of $K_1 \geq 100\ \mu M$ and EDR ≥ 30.

One conclusion that can be drawn from examination of the EPO-R and TPO-R studies cited above is that, despite the extensive and highly informative characterization to which the agonist molecules were subjected, detailed analysis of the binding energetics that underlie their ability to bring about receptor activation is, in most cases, hampered by a failure to fully define the expected self-antagonistic limb of the functional dose–response curves. Although fully defining the predicted bell-shaped dose–response curve might be superfluous to the characterization of a particular agonist when considered in isolation, the unique information that such data can provide could be critical for any meaningful comparison of the properties of different agonists for the same receptor, and as such might play an important role in optimizing the properties of such molecules.

Lessons for screening and lead optimization

The reports described above exemplify several different approaches to identifying lead molecules with agonist properties towards homodimeric cytokine receptors. The G-CSF-R agonist SB247464 was discovered by directly screening a compound library for agonist activity, using a G-CSF-R-dependent reporter gene assay [6]. In contrast, Wrighton *et al.* [3] working with EPO-R, and Cwirla *et al.* [4] and Kimura *et al.* [5] working with TPO-R used phage display technology to screen for peptide sequences that bound to the extracellular region of the receptor, and then tested for agonist activity in a secondary assay. Wrighton *et al.* [3] benefited from the unexpected ability of a peptide to bind to the receptor as a dimer, whereas Cwirla *et al.* [4] covalently dimerized their molecule as a deliberate strategy. The success of dimerizing a molecule identified through its ability to bind to a single receptor chain, to convert it into a molecule that can induce the dimerization of two receptor chains, might be highly sensitive to the shape, size and conformational preferences of the linker structure [21,22]. It is likely, therefore, that even the early stages of agonist lead discovery will benefit from testing a range or library of dimer constructs in which the linker is varied to allow a range of distances and orientations between the binding moieties to be tested.

Some specific suggestions regarding lead discovery can be drawn from consideration of the published cases cited above.

Choice of compound dilutions for use in agonist screening assays

Even in the best cases, initial lead molecules are likely to be relatively weak agonists with low effective dose ranges. Screening compounds directly in an agonist-activity assay at a single high concentration might therefore result in active compounds being overlooked, because the potential for self-antagonism could cause a compound that is active at lower concentrations to appear inactive at higher ones. The theoretical minimum value for the effective dose range can be calculated from the mathematical model of Perelson and DeLisi [38], and has a value of $IC_{50}/EC_{50} \sim 30$ (Figure 4b). Testing compounds over as wide a concentration range as possible using 30-fold serial dilutions should therefore ensure that at least one test concentration falls between the putative agonist's EC_{50} and its IC_{50}, giving a response that is at least half of the peak response. Screening test compounds using fivefold serial dilutions would be expected to give at least two data points with >50% maximal agonist response, which might help identify both false positive and false negative results.

Assays based on high-expressing cell lines

As mentioned above, the effective dose range for an agonist is predicted to be a function of K_2 and of the concentration of receptor chains expressed on the cell surface (Figures 3 and 4b), and can also be influenced by the level of spare receptors. The relationship illustrated in Figure 4b suggests that cell-based assays used to screen directly for agonist activity are likely to be more sensitive in their detection of weak agonists if the target receptor is expressed at high levels, provided that ligand-independent signaling remains negligible. The relative sensitivities of different candidate cell lines can be assessed by comparing the effective dose ranges found using the natural cytokine or, if the full bell-shaped dose–response cannot practically be determined with the cytokine itself, by choosing cell lines that give the lowest EC_{50} for stimulation with the cytokine (Figure 4b). An additional consequence of this sensitivity to receptor expression levels is that, because both the extent of any spare receptors and the density of receptor expression on the cell surface might vary from day to day, the same agonist might give dose–response curves of varying widths in different assays. Unless such variations can be excluded or controlled, it is prudent to ensure that quantitative comparisons of the properties of different agonist molecules are performed only with data obtained in the same experiment.

Lead optimization: deconvoluting agonist structure–activity data

The information provided by a quantitative analysis of dose–response data can be extremely valuable to the lead optimization process, whether using traditional methods, sophisticated structure-based strategies or a combinatorial chemistry approach. This is because only such an analysis can provide a quantitative picture of how the previous round of structural changes affected the affinity of the compounds for binding to each of the two receptor chains in the R•L•R complex. A key question, therefore, is how this information can be extracted from the kind of bell-shaped dose–response curves seen for lead molecules such as SB247464. The quantitative analysis of bell-shaped dose–response curves described above suggests that changes in the EC_{50} values observed for the agonist responses of a set of molecules does not necessarily correlate with changes in their binding affinity to one or the other receptor chain. For example, a structural modification to a lead molecule might increase the molecule's affinity for binding to the first receptor chain (i.e. decrease K_1), but if the modification resulted in a poorer geometry for bridging across to the second receptor chain, it might significantly decrease dimerization efficiency by raising K_2. The net effect of these changes would be to shift the dose–response curve to the left but also to narrow it and, depending on the balance between these effects, the EC_{50} value might increase, decrease or stay the same. An experimental example of such a case is referred to by Dower [41]. Clearly, in order to understand the consequences for activity of structural changes in the agonist, it is necessary to consider their effects on K_1 and K_2 separately. For a system exemplifying the behavior described above, this aim can be

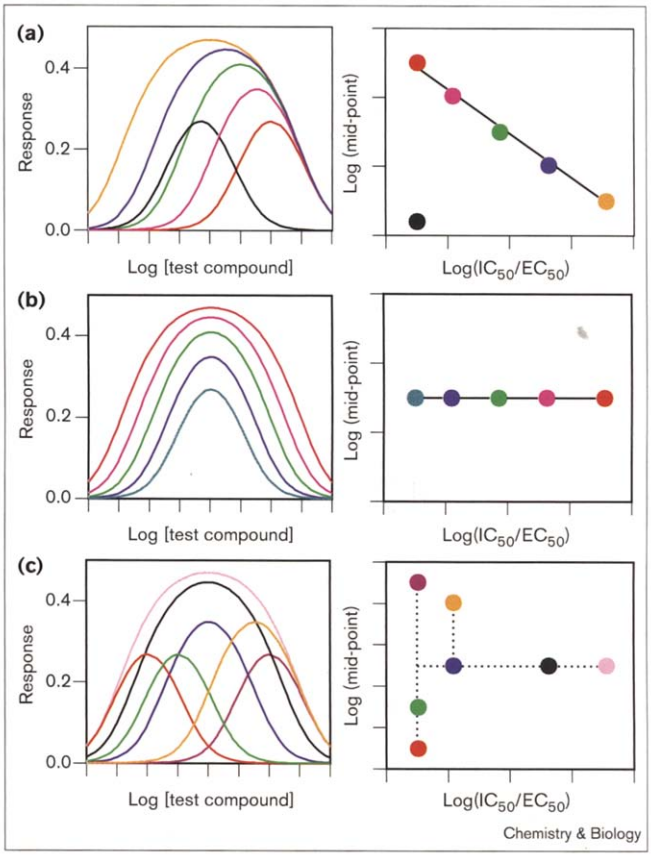


Figure 5

How the effects of changes in the properties of the agonist ligand can potentially be deconvoluted, thereby allowing a quantitative analysis of the changes in terms of their effects on K_1 and K_2, by considering separately variations in the mid-point of the curve and in IC_{50}/EC_{50}. The left-hand column shows simulated dose–response data, generated using the equation of Perelson and DeLisi [38] assuming that response is proportional to [R•L•R]. The right-hand column shows the result of plotting the mid-point versus IC_{50}/EC_{50} for each compound, illustrating how the effect of any particular structural change can potentially be quantified. The color of each data point in the right-hand plots matches that of the corresponding simulated dose–response curve shown in the left-hand column. **(a)** The predicted effects for a series of compounds in which the affinity for binding to each of the two receptor chains is changing proportionately, such as might be seen as a consequence of symmetrical modifications being made to both of the identical receptor-binding regions of a covalently dimerized or symmetrical agonist. A set of compounds of this type would be expected to result in dose–response curves that show correlated changes in width and mid-point, as illustrated by the simulated data shown. The plot of mid-point versus IC_{50}/EC_{50} illustrates the deconvolution of the dose–response curves to highlight and quantify the underlying proportionate changes in K_1 and K_2. The black curve, and its corresponding data point that falls below the line in the plot of mid-point versus IC_{50}/EC_{50}, represents a compound with a K_1 value that is disproportionately lower than K_2, such as might result from a structural change that reduced the ability of a relatively tight binding agonist to bridge between the receptor chains. **(b)** Predicted effects for a series of compounds with identical affinities for binding to the first receptor chain, but varying abilities to promote dimerization of the receptor (i.e. compounds that show variations in K_2 but not K_1). A pattern such as this might be seen for a set of covalently dimerized agonist compounds containing identical ligand-binding regions connected through linker structures of varying geometry. A set of compounds of this type would be expected to cause the dose–response curve to change in width and amplitude but to cause little or no change in the mid-point, as illustrated by the simulated data shown. The plot of mid-point versus IC_{50}/EC_{50} illustrates how the underlying effects on K_2 can potentially be quantified. **(c)** Predicted effects for a series of compounds with varied properties, as shown by the simulated data. The plot of mid-point versus IC_{50}/EC_{50} illustrates how the effect of any specific structural change can be determined in terms of whether, and by how much, it affected K_1, K_2 or both. The dotted lines illustrate how relationships between the activities of subsets of compounds that share certain binding properties can be identified and quantified in this way.

achieved by considering separately the effects of structural changes on the mid-point of the dose–response curve (i.e. effects on K_1), and on the effective dose range (i.e. on IC_{50}/EC_{50}, which is a measure of K_2), and using these effects as separate indices of binding to the first and second receptor chains, respectively. This suggestion presupposes that a full bell-shaped dose–response curve (i.e. data sufficient to define both EC_{50} and IC_{50}) can be obtained for each compound included in the analysis, as was done for SB247464 [6]. An illustration of how such an analysis might be utilized is shown in Figure 5, which shows three sets of simulated dose–response curves, such as might be expected to arise from assaying sets of test compounds designed using three different lead optimization strategies, and attempts to illustrate how effects on K_1 and K_2 can be deconvoluted and quantitatively analyzed. A limitation to this approach is that, for very weak agonists (e.g. those with $IC_{50}/EC_{50} < 100$), the width of the dose–response curve is predicted to become relatively insensitive to changes in K_2 [38]. Nevertheless, the simulations in Figure 5 suggest that analyzing dose–response data in terms of the changes in mid-point and in IC_{50}/EC_{50} might constitute a useful approach to deconvoluting and interpreting agonist dose–response data.

Agonists versus antagonists

In addition to the utility of orally available cytokine-receptor agonists, there is also considerable scope for the therapeutic application of receptor antagonists. Many of the lessons inferred from the quantitative analysis of receptor activation, described above, can be equally well applied to developing molecules designed to prevent activation from occurring. For example, it was stated above that molecules that bind to the receptor but confer insufficient affinity for binding a second receptor chain make poor agonists. Such molecules are promising starting points for antagonist development, however, because they bind to and occupy the receptor but do not induce dimerization and activation efficiently. How such a lead molecule might behave is illustrated by the black curve in Figure 5a. One target profile for an antagonist would therefore be to aim for a molecule that has a low K_1 value and a high K_2 value; that is a molecule with a high affinity for binding to the first receptor chain but negligible propensity to cause receptor dimerization. If such a molecule binds in a site that blocks

binding of the natural cytokine, it will act as an antagonist. Proteinaceous molecules with these properties have been obtained by site-directed mutagenesis of cytokines [12]. It must be noted, however, that a number of cytokine receptors have been extensively screened for inhibitors using a variety of conventional approaches, and this activity has not generally led to the discovery of potent small-molecule antagonists. This anecdotal evidence implies that, even for cytokine–receptor pairs that derive most of their interaction affinity from localized binding 'hotspots' [11], it is not a trivial undertaking to identify a small molecule that binds to one receptor chain with sufficient affinity to directly block cytokine binding.

Two alternative approaches to targeting such receptors can therefore be considered. The first involves setting up the screening assay to specifically target the second step in receptor activation, the binding of R•L to R (Figure 1). In certain cases, this second step in receptor activation might be of lower affinity — and therefore easier to block — than the initial binding of the cytokine to R [27]. The analysis presented above suggests that screening assay formats and conditions can potentially be chosen that, in contrast to a typical competitive binding ELISA, allow or even favor the discovery of molecules that block the dimerization step. For example, by screening in a functional assay for molecules that can inhibit the activity of a very high concentration of cytokine, the assay might thereby be biased against the detection of molecules that block the initial binding of cytokine to R — a step in direct competition with cytokine binding — and biased towards the detection of molecules that inhibit by alternative means, such as by blocking dimerization. Alternatively, antagonists that block dimerization could, potentially, be detected using a cell-based receptor-binding competition assay in which the concentration of labeled cytokine competitor is fixed at around EC_{50} and is well below K_1. Under these conditions, significant binding of the labeled cytokine will be seen only if the second receptor chain is able to participate in the formation of a ternary complex, so molecules that block receptor dimerization will be detected as inhibitors of cytokine binding in this assay. A second alternative approach to developing antagonists involves aiming for molecules that, like agonists, can bind two receptor chains simultaneously. Instead of optimizing the linker element of a dimeric lead molecule to aim for a productive orientation of receptor chains, however, such a molecule might be optimized for a nonproductive alignment that does not result in signaling. The potential advantage of this approach is that the inhibitor gains the avidity advantage of binding to two receptors simultaneously, and therefore enjoys the enhanced binding affinity that can be achieved by combining two moderate-affinity interactions to give high-affinity binding. This approach is likely to be restricted to receptors that have fairly stringent requirements for receptor chain orientation, however.

Cytokine receptors of more complex stoichiometry

Homodimeric receptors, such as GH-R, EPO-R, G-CSF-R and TPO-R, comprise only a small subset of the broad family of cytokine receptors [1,30]. It is therefore useful to consider how the ideas discussed above might apply to cytokine receptors of more complex stoichiometry or mechanism. A group of receptors that are closely related in structure and mechanism to the homodimeric receptors discussed above are class I cytokine receptors of heterodimeric composition [1]. Viewed in functional terms, this group can be considered to include any class I cytokine receptor that is activated by the ligand-induced association of two dissimilar receptor subunits. These can be formally heterodimeric receptors, such as the receptor for IL-4 comprising the IL-4Rα and γ_c chains [26,27], but can also include certain receptors of higher formal stoichiometry, such as the IL-2 receptor that comprises the IL-2Rα, IL-2Rβ and γ_c receptor chains [28]. The IL-2Rα and IL-2Rβ chains are pre-associated on the cell surface [42], and the key step in the activation of this receptor is therefore the bimolecular association of the preformed IL-2α/β complex with γ_c; this receptor, and perhaps other formally heterotrimeric receptors such as IL-15R, can therefore be considered as functionally heterodimeric. The development of small-molecule agonists or antagonists of heterodimeric receptors that are activated by ligand-induced dimerization is highly analogous to the homodimeric cases discussed above, except for considerations of symmetry. Instead of identifying a molecule that can bind to one R chain and then duplicating it as a covalent dimer, an analogous approach for heterodimeric receptor targets would be to screen or select for molecules that bind to each of the receptor chains separately, and then link the selected and presumably dissimilar lead fragments together via an appropriate linker or scaffold. An alternative approach would involve screening for molecules that bind tightly to one receptor chain, and then using the resulting binding fragments as the basis for a more open-ended combinatorial library that can be screened in a functional assay to identify derivatives able to engage in simultaneous binding to the second receptor chain. The development of antagonists of heterodimeric cytokine receptors, and particularly the potential for achieving inhibitors that act noncompetitively with respect to the cytokine, has been discussed elsewhere [27]. In addition to these heterodimeric systems, lessons learned from peptide and small-molecule agonists of homodimeric class I cytokine receptors are likely to apply equally to other classes of receptor that are activated by a mechanism of ligand-induced receptor-chain oligomerization [30].

Conclusions

A number of peptides and small molecules have been described that have activity as agonists of homodimeric cytokine receptors. We have discussed these cases in terms

of the thermodynamic and mechanistic principles underlying the receptor-activation mechanism of ligand-induced dimerization. Moreover, we have attempted to show that this kind of analysis can allow the properties of these molecules to be understood in terms of the requirements for binding to and appropriately orienting two receptor chains to bring about activation and signaling. The results suggest a number of ways in which strategies for lead discovery and lead optimization can be evaluated. In particular, we promote the value of obtaining experimental dose–response data that fully define both limbs of the bell-shaped curve expected for agonists of homodimeric receptors in cell-based functional assays. We also advocate approaching the analysis of structure–activity data from such assays by considering the effects on the mid-point and on the width of the dose–response curves as separate indices of the affinity of the agonist molecule for binding to the first and second receptor chains, respectively.

Preconceptions about whether or not a certain goal can be achieved can be pivotal in determining whether or not it is in fact accomplished; such beliefs have an even greater impact on defining what is attempted. Thanks to the exciting recent reports on peptide and small-molecule agonists of cytokine receptors reviewed here, practitioners of small-molecule drug discovery have now been given reason to believe that small molecules might be developed that have the essential functional properties of much larger proteinaceous cytokines. In so doing, these paradigm-shifting results might have brought the possibility of orally available drugs with cytokine-like activity a large step closer to reality.

Note added in proof

Since this article was submitted, two reports have been published which suggest that EPO-R might exist as preassociated, inactive dimers in the absence of EPO (Livnah, O., Stura, E.A., Middleton, S.A., Johnson, D.L., Joliffe, L.K. & Wilson, I.A. (1999). *Science* **283**, 987-990; Remy, Y., Wilson, I.A. & Michnick, S.W. (1999). *Science* **283**, 990-993). The authors of these studies propose that binding of EPO activates the receptor by bringing about a particular orientation between receptor chains within the pre-existing dimer. If this proposal is correct, other published data concerning activation of EPO-R place significant restrictions on how such a mechanism might function. For example, the observation of self-antagonism by EPO [14] implies that, at very high concentrations of EPO, the preassociated receptor dimers can be converted to an inactive $(EPO)_2(EPO\text{-}R)_2$ complex analogous to the lower pathway in Figure 1. Derivation of the appropriate binding equations (C.W.B. & A.W., unpublished observations) demonstrates that, in the simplest case consistent with these restrictions, a preassociated receptor mechanism of this kind might result in behavior essentially identical to that described in Figures 3 and 4, with the exception that the width of the bell-shaped dose–response curve would be expected to be insensitive to the receptor expression level.

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