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REVIEW ARTICLE

Cytokine Receptor Dimerization and Activation: Prospects for Small Molecule Agonists

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Abstract—Ligand-induced dimerization of cell surface receptors has emerged as a general mechanism for the initiation of signal transduction. A number of therapeutically important receptor families are believed to be activated by this process. Recently available structural information, particularly for the erythropoietin receptor, has provided insight into the mechanism of receptor activation. These findings have also revealed important constraints on the nature of receptor–agonist complexes. The prospects of discovering small-molecule mimetics of such receptor agonists are discussed, including strategies which have led to the identification of a small number of peptide and non-peptide cytokine mimetics.

Contents

Cytokine Receptor Dimerization and Activation of Signal Transduction

Receptor dimerization has been established as a general mechanism for the initiation of signal transduction, and many cell-surface receptors are believed to be activated by such a process.[1] These include protein–tyrosine kinase receptors (Table 1),[2] antigen receptors,[3] the tumor necrosis factor receptor family,[4] protein-serine/threonine kinase receptors,[5] and members of the cytokine receptor superfamily (Table 2).[6] Important therapeutic applications may emerge from either the development of agonists or antagonists of such receptor or protein dimerization, and representative examples are provided in Table 3 for the cytokine receptor superfamily. Class I cytokine receptors, including erythropoietin,[7] thrombopoietin,[8] growth hormone,[9] granulocyte colony-stimulating factor,[10] and prolactin receptors[11] share many key structural motifs. Their make-up consists of an extracellular ligand-binding domain containing two fibronectin type III motifs, a single transmembrane domain and an intracellular domain which is non-covalently associated with members of a family of tyrosines kinases known as Janus kinases or just another kinase (JAKs).[12] Activation of the cytokine receptor, by ligand induced homodimerization, results in JAK autophosphorylation, phosphorylation of substrate binding sites, and subsequent binding and phosphorylation of several substrates including signal transducer and activator of transcription (STAT) proteins. These transcription factors then form homo- or heterodimeric complexes which translocate to the nucleus and bind to specific enhancer sequences (STAT binding elements (SBEs)) and promote gene transcription (Fig. 1).[13,14]

Erythropoietin Receptor

Erythropoietin (EPO), one of the most extensively studied cytokines, is a 34 kDa glycoprotein hormone which regulates erythropoiesis, the process of growth and differentiation of red blood cell progenitors.[15] Recombinant EPO is currently one of the most successful biotechnology products, (annual world-wide sales: $4 billion)[16] making it a target of significant medical and commercial importance.

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Table 1. Protein–tyrosine kinase receptors activated by dimerization or oligomerization[a]

Family	Examples
PDGF receptor	PDGFR-α, PDGFR-β, SCFR, CSF-R, Fik-2
EGF receptor	EGFR (erbB), erbB-2 (Neu), erbB-3, erbB-4
FGF receptor	FGFR-1, FGFR-2, FGFR-3. FGFR-4
IGF receptor	Insulin R, IGF-1R
HGF receptor	HGFR (Met), MSPR (Ron)
VEGF receptor	Flt-1, Flt-2 (KDR)
Neurotrophin receptor	Trk, TrkB, TrkC
Eph receptor	Eph, Elk, Eck, Cck5, Sek, Eck, Erk

[a]Abbreviations: R, receptor; PDGF, platelet-derived growth factor; SCF, stem cell factor; CSF, colony-stimulating factor; EGF, epidermal growth factor; FGF, fibroblast growth factor, IGF, insulin-like growth factor; HGF, hepatocyte growth factor; MSP, macrophage-stimulating protein; VEGF, vascular endothelial growth factor; FN, fibronectin.

Table 2. Class I cytokine receptors[a]

Family	Examples	Activation Characteristics
GH receptor	GHR, EPOR, PRLR, G-CSFR	Homodimers
IL-3 receptor	IL-3R, GM-CSFR, IL-5R	Heterodimeration with β_C
IL-6 receptor	IL-6R, LIFR, CNTFR, IL-11R	Heterodimerization with gp130
IL-2 receptor	IL-2R, IL-2Rβ, IL-4R, IL-7R	Heterodimerization with IL-2R γ chain

[a]Abbreviations: R, receptor; GH, growth hormone; EPO, erythropoietin; PRL, prolactin; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; LIF, leukemia inhibitory factor; CNTF, ciliary neurotrophic factor.

Table 3. Approved or potential therapeutic applications of cytokine agonists and antagonists

Cytokine	Agonist	Antagonist
EPO	Anemias, selective blood donation	Cancer, leukemia
TPO	Thrombocytopenia	
IL-2	Cancer	Histoincompatability
IL-3	Leukopenia, myeloid reconstitution	Leukemia
IL-4	Inflammation, cancer	Allergy
IL-6	Thrombocytopenia	Cancer, osteoporosis, inflammation
IL-11	Thrombocytopenia	
IL-12	Cancer, infections	Histoincompatability, autoimmunity
G-CSF	Neutropenia, myeloid reconstitution	Leukemia
GM-CSF	Leukopenia, myeloid reconstitution	Leukemia
IFN α/β	Cancer, viral infections, autoimmunity	Inflammation
IFN γ	Chronic granulonatous disease, infections	Inflammation, autoimmunity

EPO's high affinity receptor (EPOR) is displayed on the surface of immature erythroid cells in the bone marrow. Considerable progress in recent years has led to a better understanding of the mechanism of EPOR activation, and the prospects of discovering small molecule agonists.

The key step in EPOR activation is the binding of EPO through the hormone's high affinity (~1 nM) and low affinity (~1 μM) binding sites,[17] which results in a 2:1 receptor:ligand complex. A number of dimeric EPOR complexes have also been reported in which the receptor is activated by a ligand other than EPO (Fig. 2). Bivalent antibodies, based on monovalent Fabs which recognize only one receptor chain, were found to dimerize and activate EPOR.[18] Single-chain EPO dimers were synthesized in which one EPOR binding site was mutated and inactive on each monomer and found to induce EPOR signal transduction.[19] Small EPO-mimetic peptides (EMPs), discovered from phage-display libraries, were reported which dimerize themselves and form an active 2:2 peptide:receptor complex (Figs 3 and 6(a)).[20,21] A non-peptide agonist of EPOR has also been described in which copies of a small molecule antagonist were linked by a dendrimer core forming an active octamer (Fig. 6(e)).[22] In addition, EPOR mutants (Arg130→Cys130) which form disulfide dimers, were found to be constitutively active.[23] The nature of these complexes is undoubtedly different, depending on the size and structure of the bridging ligand. Based on these findings, constraints on the organization of the dimeric complex seem quite flexible, and one may think many modes of dimerization are acceptable for EPOR activation.

However, results from recent studies suggest that dimerization alone is not sufficient for EPOR activation. The X-ray structure has been solved for a dimeric EPOR-peptide complex which does induce receptor activation.[24] EMP33, a derivative of agonist peptide EMP1 containing 3,5-dibromotyrosine in place of Tyr^4, binds and dimerizes EPOR, but does not induce signal transduction. The major distinguishing feature in the X-ray structures of the EMP1–EPOR and EMP33–EPOR complexes is the angle between the D1 domains of the two receptor chains (Figs 3 and 4). In the perfectly symmetrical EMP1 dimer, these domains are 180° to each other, whereas with EMP33 an angle of 165° is observed. The crystal structure of the EPO–EPOR complex is also now available,[25] and here the angle of the two receptor domains is 120°. The EPO–EPOR dimer is considerably more active than the EMP1–EPOR complex, but this can not be simply explained by the efficiency with which the ligands bind the receptor. Covalently-linked EMPs displayed EPOR-binding activity comparable to that of EPO, but remained much less effective in functional assays.[26] The activity of these complexes may instead be explained by the differences in the relative orientation of the two receptor chains in the dimerized structure.[27] There are limitations on the structure of active EPOR dimers, and effective agonists must not only bind two receptor molecules, but also do so in a specific manner.

Analysis of the X-ray structure of dimerized EPOR *in the absence of ligand*,[28] along with evidence for the existence of such complexes in vivo,[29] provide more insight into the nature of EPOR activation and the role of the agonist ligand in the dimerization mechanism. In the interface of the unliganded EPOR dimer, the two receptor chains are directly associated via their two ligand-binding sites. In this conformation, the receptor D2 domains are twisted at a 135° to one another which

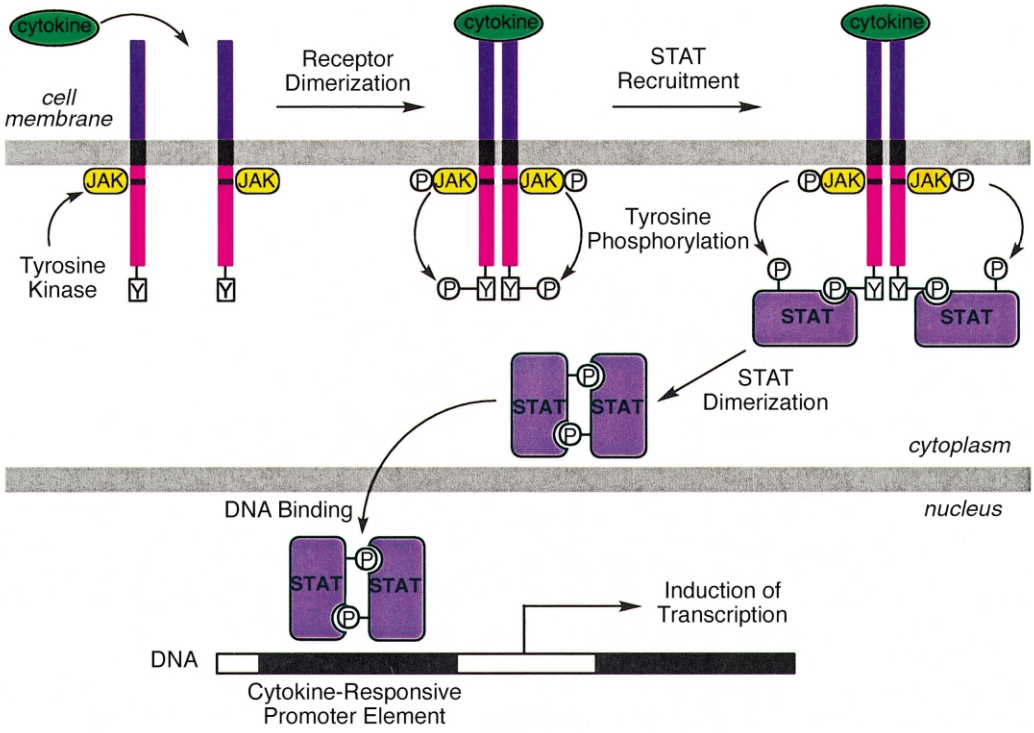

Figure 1. General mechanism of cytokine activation of the JAK/STAT signal transduction pathway. Cytokine binding induces dimerization and activation of its cell surface receptor. This activates associated JAK kinases and results in the phosphorylation of several intracellular targets including the receptor itself and recruited STAT transcription factors. These proteins then form homo- or heterodimeric complexes which ultimately bind DNA and promote gene transcription. Based on a figure by Lamb et al.[14]

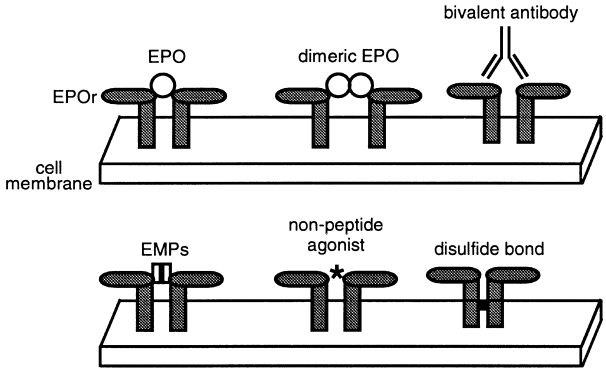

Figure 2. EPOR can be activated by dimerization through complexes with different types of agonist ligands, including EPO, dimeric EPO,[19] bivalent antibodies,[18] EMP peptides,[20,21] and non-peptide agonists[22] or as a constitutively active mutant.[23] Based on a figure by Qiu et al.[19]

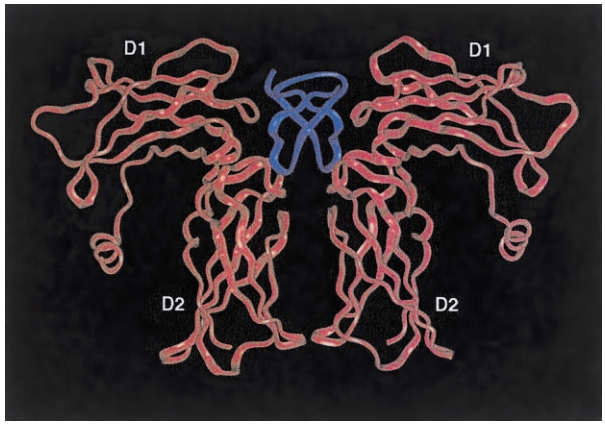

Figure 3. X-ray structure of the extracellular ligand-binding fragment of EPOR (red) dimerized by EMP1 (blue).[21] The two receptor fibronectin type III domains are labeled D1 and D2.

separates the transmembrane regions by a distance of 73 Å, considerably more than the 39 Å (45° orientation of the D2 domains) and 30 Å (coplanar D2 domains) separation observed for the EMP1 and EPO dimers, respectively. This distance translates into the intracellular receptor domains and the associated JAK2 kinases, which must presumably be brought significantly close together to allow for their transactivation and the propagation of the intracellular signal (Fig. 5). The binding of other proteins, such as STAT5, may also be influenced by the positioning of the receptor's intracellular domains.

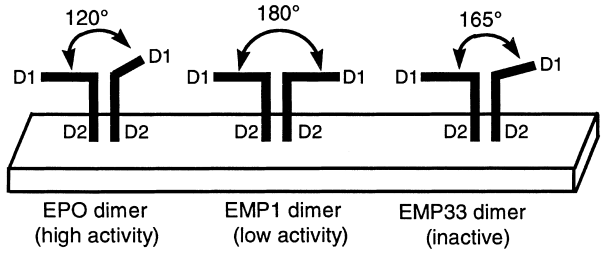

Figure 4. The relative orientation of the two receptor chains in the dimerized complex is important for EPOr activation. The angles outlined by the D1 domains of the two EPOR molecules are indicated for the dimeric receptor complexes with EPO,[25] EMP1,[21] and EMP33.[24]

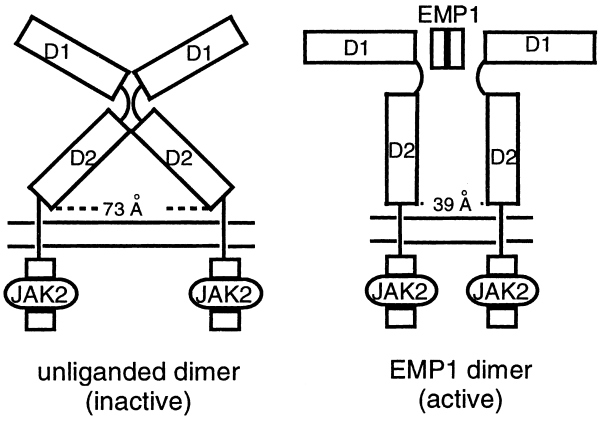

Figure 5. Schematic comparison of the structures of EPOR dimers in the absence of ligand (*left*) and in the presence of EMP1 (*right*). The mode of dimerization affects the distance separating the intracellular receptor components. Based on a figure by Livnah et al.[28]

Thus, the 'on' or 'off' states of EPOR may be determined by the positioning and orientation of the intracellular catalytic regions of the complex mediated by the binding of ligands to the receptor's extracellular domain. Since different dimer orientations are possible, receptor agonists must not only bind both EPOR chains, but also do so in a manner which properly positions their intracellular domains and allows for the activation and function of associated factors. Thus, the prospects of designing or discovering functional EPO mimetics is more challenging than simply promoting dimerization, as receptor binding must induce a specific conformational change. This may well be extended to other cytokine receptors which share similar structural and functional characteristics.[30] If an approach is utilized in which molecules are first screened for receptor binding and then dimerized to allow for interaction with two receptor chains,[22] the choice and nature of the linking strategy may prove critical in identifying functional agonists.

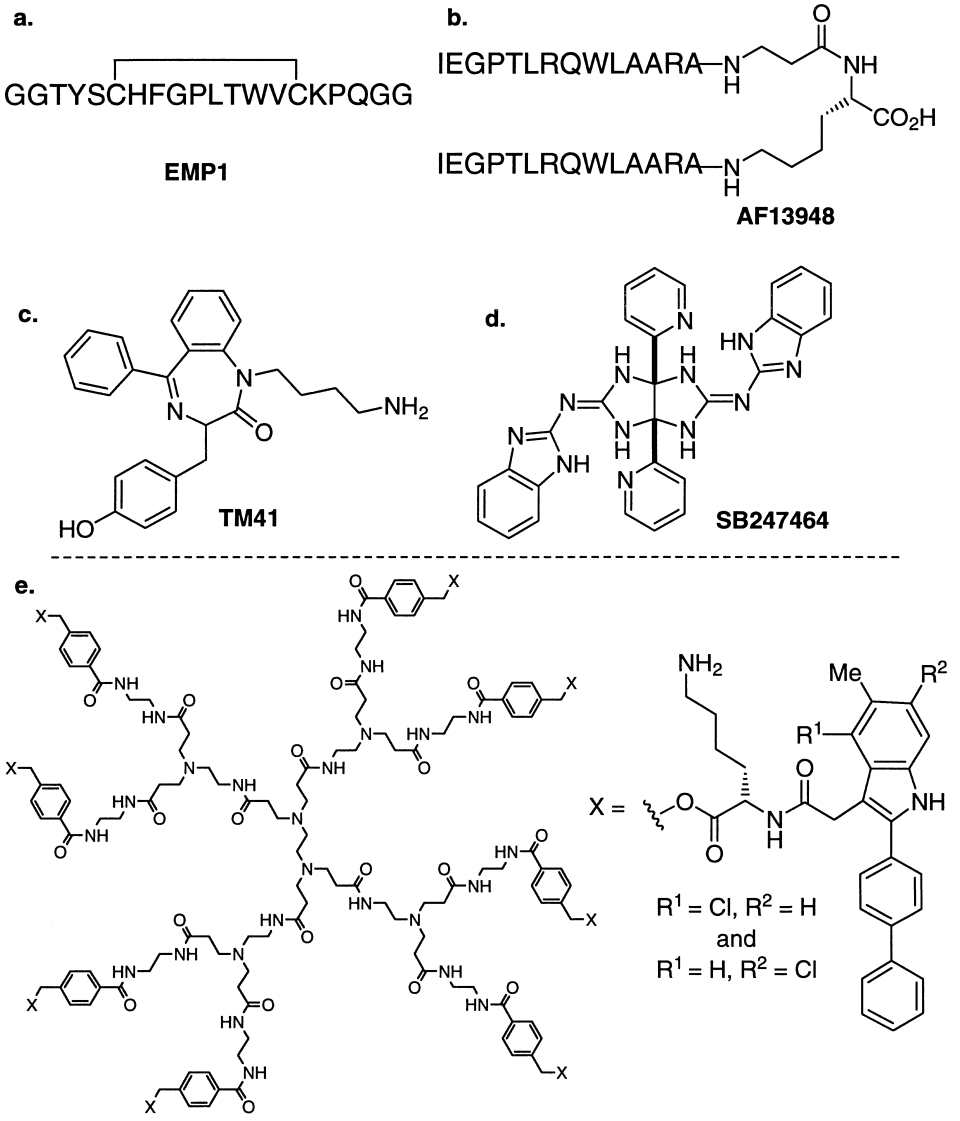

Figure 6. Structures of some reported peptide and non-peptide small molecule agonists of cytokine receptors.

Small Molecule Cytokine Receptor Agonists

The search for small molecule cytokine mimetics has seen promising success, despite the stringent requirements for agonist activity outlined with EPOR. In addition to EMP1 and related peptides (Fig. 6(a)) and the Merck non-peptide agonist (Fig. 6(e)), there are other reports of small molecule cytokine mimetics. A 14-amino acid peptide, derived from screening phage display libraries, was found to bind and activate the thrombopoietin receptor (TPOR). When dimerized through a short β-alanine-lysine linker, a more active variant (AF13498, Fig. 6(b)) was obtained which was found to activate TPOR with an EC_{50} equipotent with the natural cytokine.[31] Another group has also described the discovery of peptide TPO mimetics,[32] and have recently identified a non-peptide agonist of the same receptor. Benzodiazapinone TM41 (Fig. 6(c)),[33] which competes with TPO for binding to the receptor's extracellular region, stimulated the activation of STAT5 and the proliferation of a TPO-dependent cell line. At approximately the same time, a second example of a non-peptide cytokine mimetic was disclosed in which the granulocyte colony-stimulating factor receptor (GCSFR) was activated by SB247464 (Fig. 6(d)).[34] This symmetrical molecule, discovered by the direct screening of compounds for agonist activity, is remarkably small ($M_r = 527$ Da) compared to the natural cytokine protein ($M_r \sim 120$ kDa) and presumably activates the receptor through binding to a region other than the GCSF binding site. Interestingly, SB 247464 has murine granulopoiesis activity, but does not activate human GCSFR.

Despite these successes, the discovery of small molecules with agonist activity is clearly a challenging endeavor. There are few reports of small compounds capable of disrupting specific protein–protein interactions (antagonists),[35] and the additional requirement of binding and dimerizing two receptor molecules in a specific manner makes the process even more difficult. Two general approaches to the identification of small molecule receptor agonists have been pursued, and each has been successful. Compounds that bind one receptor molecule can first be identified from competitive binding assays (antagonists), and then dimerized or oligomerized through various linking strategies to provide potential agonists.[22,36] Alternatively, compound libraries can be directly screened for agonist activity in functional assays.[34] The optimal approach will likely depend on the specific receptor involved and the capabilities of available assays, and will be facilitated by further knowledge of receptor dimerization constraints on functional activity. In all cases, functional small molecule cytokine mimetics, such as those presented in Figure 6, have been discovered from the screening of large and diverse chemical libraries.

Many receptors, such as EPOR,[21] are believed to recognize their ligands utilizing small clusters of residues, as was first noted with the growth hormone receptor in which a functional 'hot spot' dominated by two tryptophan residues was shown to be responsible for three-quarters of the receptor–ligand binding energy.[37] Therefore, it is reasonable to expect that the screening of small molecules could provide a general solution for identifying novel receptor antagonists and agonists. As more detailed structural information of receptor-agonist complexes becomes available, the prospects of discovering or designing such functional mimetics will certainly improve.

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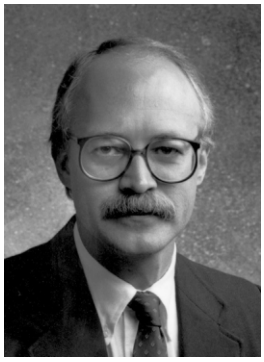
Biographies



Born 22 August, 1953, Dale L. Boger received his B.Sc. in chemistry from the University of Kansas (1975) and Ph.D. in chemistry from Harvard University (1980). Immediately following graduate school, he returned to the University of Kansas as a member of the faculty in the Department of Medicinal Chemistry (1979–1985), moved to the Department of Chemistry at Purdue University (1985–1991), and joined the faculty at The Scripps Research Institute (1991 to present) as the Richard and Alice Cramer Professor of Chemistry. His research interests span the fields of organic and bioorganic chemistry and include the development of synthetic methodology, the total synthesis of natural products, heterocyclic chemistry, bioorganic chemistry, medicinal chemistry, the study of DNA–agent and protein–ligand interactions, and antitumor agents.

Joel Goldberg was born 17 June, 1972 in Boston, Massachusetts and graduated from Tufts University (B.A. 1994). He later joined Professor Boger's research group at The Scripps Research Institute where he received a National Defense Science and Engineering Graduate Fellowship. In 2000, he completed his Ph.D. studies and moved to the Swiss Federal Institute of Technology (ETH Zürich) for postdoctoral work as a National Science Foundation International Research Award Fellow. His research interests include combinatorial chemistry, protein–ligand interactions, and directed molecular evolution.