Discovery of cytokine mimics using cell-based systems

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The successful cloning and subsequent clinical application of recombinant cytokines and/or growth factors has generated a number of important therapeutics. In contrast to the G-protein-coupled receptors, identification of small-molecule agonists of the cytokine and/or growth factor receptor family has proved difficult. The first small peptides and non-peptidic small-molecule agonists for several receptors have recently been reported. The initial identification and/or crucial characterization of these molecules as true mimics was dependent on the use of cell-based functional assays. This article will review recent cell-based assay technologies that are suitable for HTS and that are being applied to the discovery of novel cytokine and growth factor mimics.

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▼ Several recombinant cytokines and growth factors including insulin, human growth hormone (hGH), interferon-α (IFN-α), IFN-β, erythropoietin (Epo) and granulocyte-colony stimulating factor (G-CSF) have proved to be safe and therapeutically effective[1,2]. However, proteins have several potential and often limiting characteristics as therapeutic agents including: a non-oral route of administration; the potential for generation of neutralizing antibodies[3,4]; injection site complications[5,6]; poor pharmacokinetics/pharmacodynamics[7,8]; and cost. The pharmaceutical industry has long been interested in the identification of non-peptide small molecules that mimic the activities of the natural ligands of cell surface receptors. Small molecules that act as agonists at these receptors have the potential to provide advantages such as oral bioavailability, lack of immunogenicity and improved pharmacokinetics. Significant advances have been made in developing small-molecule agonists and antagonists for a variety of G-protein-coupled receptors (GPCRs)[9]. However, the single transmembrane domain cytokine and/or growth factor receptors have, until recently, proved to be exceptionally difficult targets.

In the past few years, reports of peptides, peptidomimics and organic small molecules capable of mimicking some or all of the activities of several cytokines and/or growth factors have emerged. Peptides that mimic the activity of Epo (Refs 10–12) and thrombopoietin (Tpo)[13,14] were identified using either directed approaches or selection from random phage-display libraries. Maliartchouk *et al.*[15] synthesized a focussed library of peptidomimics designed as agonists of the tropomyosin receptor kinase A (TrkA) receptor and identified a small molecule that selectively binds to and activates this receptor. Peptides and peptidomimics used as therapeutic agents are predicted to share some of the same limitations as the native cytokines and/or growth factors that they are designed to replace[16,17]. These peptides are best considered as a starting point for the rational design of non-peptidic small molecules[18] rather than as potential therapeutics in their own right.

In addition to peptide-based mimics, small molecules that mimic the activities of G-CSF (Ref. 19), Tpo (Ref. 20), Epo (Ref. 21), and insulin[22] have also been described. Each of these compounds exhibits agonist activity in *in vitro* assays measuring early signaling events following receptor activation. Both G-CSF and Epo mimics are capable of supporting the appropriate differentiation of hematopoietic precursor cells when tested in standard colony-forming assays[19,21]. Remarkably, the small-molecule G-CSF and insulin agonists also exhibit activity in *in vivo* models[19,22]. Subcutaneous administration of the G-CSF agonist in mice elicits an increase in peripheral blood neutrophil counts with an efficacy equivalent to that obtained with G-CSF (Ref. 19) and oral administration of the insulin mimic decreases plasma glucose in two mouse models of diabetes[22].

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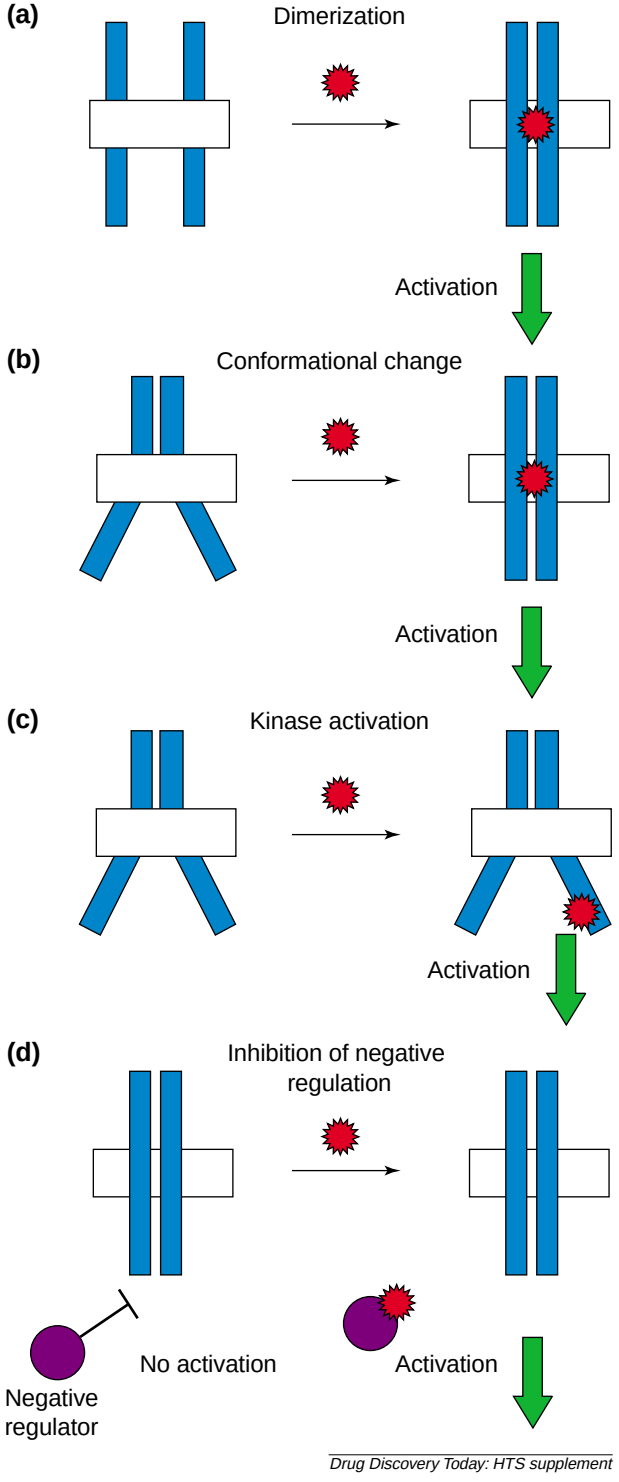

Figure 1. Potential mechanism of action of a small-molecule cytokine and/or growth factor mimic. **(a)** Receptor dimerization induced by small-molecule binding; **(b)** conformational change in a pre-existing receptor dimer leading to receptor activation; **(c)** conformational change leading to activation of tyrosine kinase activity of the receptor or an associated kinase; and **(d)** inhibition of a negative regulatory process (such as a phosphatase).

Several mechanisms by which a small molecule could mimic the activity of a cytokine and/or growth factor can be envisioned (Fig. 1). Dimerization of this class of receptors is a crucial step in activation[23] although at least some receptors could exist as preformed dimers[24–26]. Binding of a small molecule could lead to receptor dimerization (Fig. 1a) or a conformational change in a preformed dimer (Fig. 1b), both resulting in receptor activation. Alternatively, a small molecule could lead to a conformational change in the intracellular domain of the receptor, leading to activation of intrinsic or associated tyrosine kinase activity (Fig. 1c). A third mechanism would be a small molecule that inhibited negative regulation of receptor activation such as a selective tyrosine phosphatase inhibitor[27] (Fig. 1d).

It has been suggested that the peptide agonists (as previously described) act by dimerizing the receptor through interactions with the extracellular domain – a mechanism that has been demonstrated for an Epo mimic peptide[28]. In this case, the peptide mimic was co-crystallized with the extracellular domain of the Epo receptor, and a structure at 2.8 Å resolution was determined. The structure revealed that a peptide-dimer induced the formation of an Epo receptor dimer, resulting in a 2:2 complex with nearly perfect twofold symmetry. The small-molecule G-CSF mimic has twofold rotational symmetry and is proposed to function by inducing dimerization of the G-CSF receptor subunits[19] (Fig. 2). Similarly, the small-molecule Epo mimic induces dimerization of the extracellular domain of the Epo receptor[21]. By contrast, the insulin mimic is suggested to function by binding to the intracellular domain of the insulin receptor, altering the conformation of the tyrosine kinase domain and leading to receptor activation[22]. The small-molecule TrkA agonist binds to the extracellular domain of the receptor but the molecule is not symmetric and Maliartchouk *et al.*[15] suggested that it had no propensity to dimerize in solution. It was also suggested that the molecule functions by stabilizing a signaling conformation of pre-formed TrkA dimers rather than inducing dimerization, a model supported by the ability of low concentrations of nerve growth factor (NGF) to enhance activity of the molecule.

The discovery of the small-molecule mimics of cytokines and/or growth factors already described has generated significant excitement, and pharmaceutical companies are interested in extending the approach to identify additional agonists for these as well as other receptors. As the probability of identifying such molecules in random screening appears to be much lower than for other molecular targets, the development of sensitive and robust assays compatible with HTS is essential. Several approaches can be taken to develop high-throughput screens including both biochemical and cell-based assays. The peptide mimics previously described in this article were initially selected on the basis of binding of phage-display libraries

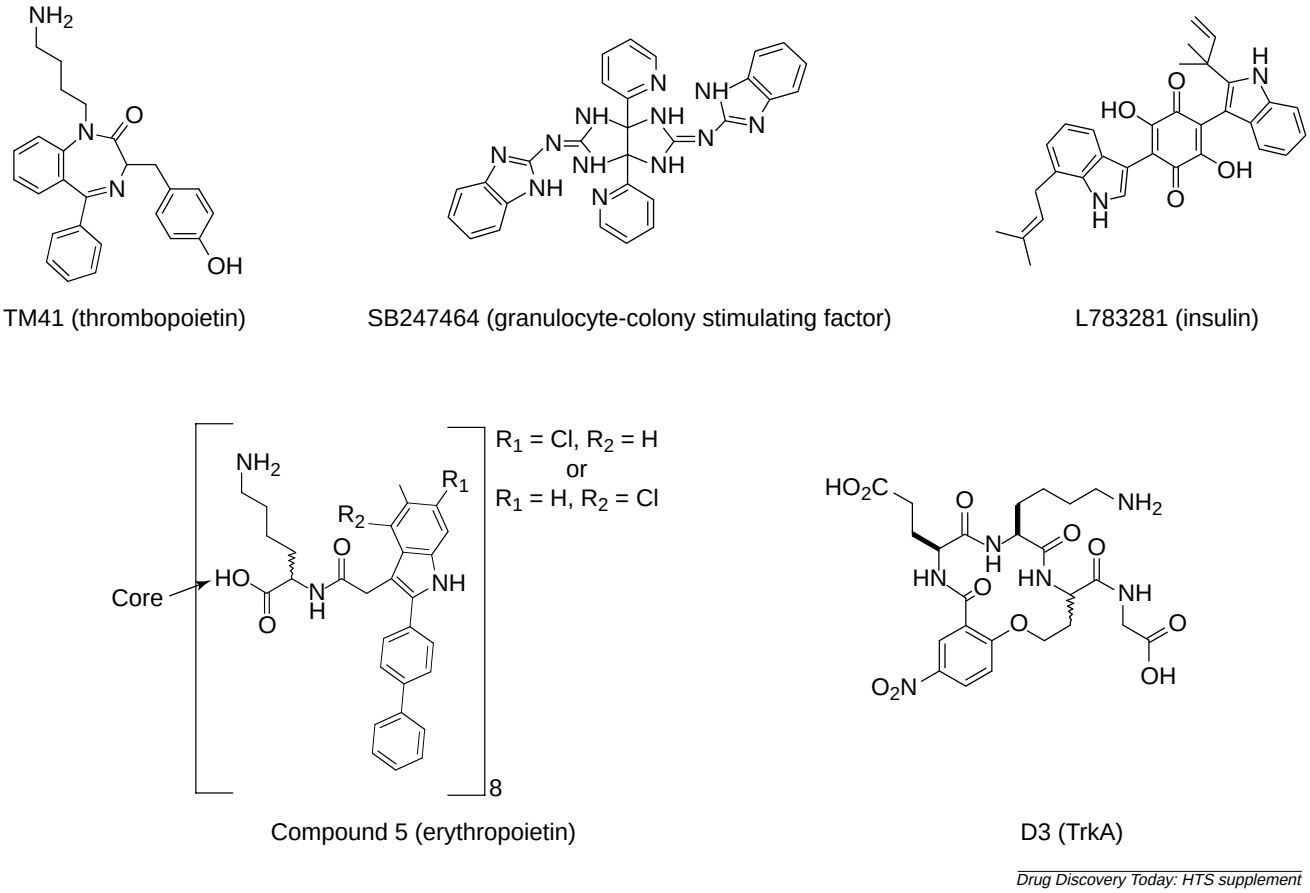

Figure 2. Structures of published small-molecule cytokine and/or growth factor agonists. Compound 5 consists of eight copies of the indicated structure joined by a central dendrimer core[21].

to the extracellular domain of the receptor, an approach that is not directly applicable to traditional small-molecule libraries. The small-molecule mimics of Tpo (Ref. 20) and Epo (Ref. 21) were identified based on their ability to inhibit cytokine binding to its cognate receptor. In both of these approaches, the vast majority of peptides or small molecules identified did not exhibit agonist activity. By contrast, the G-CSF and insulin mimics were identified using cell-based assays that measure the ability of compounds to activate receptor signaling directly. The cell-based assays that have been utilized or proposed as primary high-throughput screens capable of identifying small-molecule receptor agonists will now be described.

Cell-based approaches

Transcriptional reporter-gene assays

Cell-based transcriptional reporter assays[29] have been utilized in HTS to identify agonists for intracellular receptors[30] and GPCRs (Refs 31,32). In the past few years, this approach has been adapted to develop assays for cytokine and/or growth factor receptors. One approach has been to take advantage of the direct activation of the signal transducers and activators of transcription (STAT) family of transcription factors following activation of this class of receptors[33]. The STAT proteins are substrates for receptor or receptor-associated tyrosine kinases and, subsequent to phosphorylation, act directly as DNA-binding transcriptional activators. Thus, transcription of a reporter gene regulated by a STAT-responsive promoter is proportional to receptor activation. Although the pathway from receptor activation to reporter-gene transcription is relatively direct, the amplification at each step (STAT-phosphorylation, transcription/translation and reporter enzymatic activity) make this an extremely sensitive technique. This approach has been reported with cell lines expressing endogenous[19] or recombinant receptors[34], and cell lines expressing chimeric receptors consisting of a fusion of the extracellular domain of the target receptor of interest with the intracellular domain of a second receptor[35]. The reporter constructs and recombinant receptors can be introduced transiently into cells, or stable cell lines can be isolated.

Using this approach, Tian *et al.*[19] identified a small-molecule G-CSF mimic by screening a diverse compound library. The assay utilized a luciferase reporter consisting of four copies of a synthetic STAT-binding element linked to a minimal promoter. This reporter construct was stably incorporated into a G-CSF-receptor-expressing cell line. Storz *et al.*[34] developed a similar high-throughput assay for insulin receptor activation using a STAT5-based transcriptional reporter. The reporter construct was again composed of a multimerized STAT-binding element linked to a minimal promoter upstream of the luciferase gene. Secreted placental alkaline phosphatase (SEAP) under the control of an IFN inducible gene was utilized as a reporter for chimeric receptors consisting of the extracellular domain of either the interleukin-5 receptor or the Epo receptor fused to the transmembrane and intracellular domains of the IFN type I receptor[35]. The promoter of the IFN-responsive 6-16 gene was used to drive transcription of the SEAP reporter gene.

Examples of transcriptional reporter assays being implemented as primary screens have been reported[19]. Transcriptional reporter assays have been adapted to high-density plate formats (384-well and 1536-well) and, depending on the cell line, can be amenable to full automation[36]. Cell lines expressing the receptor of interest and containing intact signaling pathways can be used for the identification of compounds that act at the receptor as well as intracellular targets. The chimeric receptor approach, utilizing only the extracellular domain of the target receptor, will not identify compounds whose function requires interaction with the intracellular domain of the receptor (such as the insulin mimic already described[22]).

Assays measuring transcription of endogenous genes

In principle, transcription of 'endogenous' cytokine-/growth factor-regulated genes can be monitored rather than introducing an artificial reporter-gene construct. In practice, reporter genes often provide a more sensitive readout, exhibit a higher dynamic range and utilize simple readouts compared with the measurement of transcription of endogenous genes. A high-throughput assay for modulators of the insulin receptor based on detection of ligand-induced *c-fos* mRNA expression has recently been reported[37]. The assay utilized a branched DNA (bDNA) assay to provide a sensitive, high-throughput quantitation of *c-fos* mRNA in a microtiter plate format. The application of this approach as a high-throughput screen is limited by the complexity of carrying out a cell-based activation step followed by cell lysis and multistep mRNA detection.

A generalized method has been described that incorporates a gene-trapping approach, utilizing the endogenous promoter of a gene, regulated by a signal-transduction pathway of interest, to drive expression of a reporter gene[38]. In this approach, a promoterless reporter gene (β-lactamase) with an upstream splice acceptor and downstream polyadenylation sequence is introduced into target cells expressing the cytokine and/or growth factor receptor of interest. Random integration of the promoterless construct produces transcriptional reporter 'tags'. A non-toxic cell-permeant fluorogenic substrate for β-lactamase is used to identify the expression level of the integrated β-lactamase reporter. A combination of fluorescence-activated cell sorting (FACS) and stimulation with the cytokine and/or growth factor, can be used to achieve isolation of clonal cell lines in which the reporter has integrated into a cytokine and/or growth factor regulated gene. These clones can be used to identify the target gene, or characterized further and used directly for high-throughput cell-based screening.

Rapid technological progress is being made in the measurement of endogenous gene expression. The bDNA assay described by Shyamala *et al.*[37] requires multiple incubation and wash steps but should be suitable for HTS. The cell lines identified by the gene-trapping approach can theoretically be incorporated directly into a high-throughput screen. The primary limitation in the use of endogenous gene expression is the significant time and effort involved in identifying relevant target genes and, in the case of the gene-trapping approach, validating the specificity and physiological relevance of the regulation of the gene.

Cell-based activation with subsequent biochemical assay for receptor activation or phosphorylation state

Zhang *et al.*[22] identified an insulin mimic using a two-step assay consisting of a cell-based activation step followed by a biochemical assay measuring insulin receptor kinase activity. Chinese hamster ovary cells that over-express the insulin receptor were incubated with test compounds or insulin controls; the cells were then lysed, the insulin receptor immunoprecipitated and insulin receptor tyrosine kinase activity was assayed using [γ-^{32}P]-ATP and a tyrosine-containing peptide substrate. Several groups have assessed the phosphorylation state of the receptor after treatment of intact cells with test compounds as an alternative to measuring receptor kinase activity as a readout of receptor activation. Such assays have been used for the oncogenic protein ErbB2 (Ref. 39), receptors for insulin-like growth factor I (IGF-I)[40], epidermal growth factor (EGF)[41] and the neurotrophins[40,42,43]. These assays involve incubation of intact cells with test compounds, followed by lysis and capture of the receptor on an appropriate antibody immobilized on a microtiter plate. The extent of receptor phosphorylation is then assessed with a generic anti-phosphotyrosine antibody using either a colorimetric sandwich ELISA or time-resolved fluorescence. The quantity of receptor can be determined simultaneously using a dual ELISA format and the ratio of activated to total receptor measured. Although the growth factor receptors

in the examples already described are in the receptor tyrosine kinase class, the approach could be extended to the class I and class II cytokine receptors.

These two-step approaches incorporate the ability to measure receptor activation in intact cells and the use of standard biochemical assay methodologies for readouts. The primary limitation of these assay formats is the requirement for multiple liquid handling, incubation and wash steps.

Cell-based assays based on protein-fragment complementation by receptor dimerization

Dimerization (or higher-order oligomerization) of cytokine and/or growth factor receptors by ligand binding is generally thought to be a key step required for activation of downstream signaling events[26,44]. Receptor homo-dimerization for the growth hormone (GH) receptor has been studied in the greatest detail[44,45]. In addition to these and other studies on growth factor–receptor complexes, a number of studies suggest that dimerization/oligomerization by other means can be sufficient for receptor activation and downstream signaling[28,46]. Mutation of a single amino acid in the membrane-proximal extracellular domain of the Epo receptor from arginine to cysteine leads to formation of constitutively activated disulfide-linked homodimers of the receptor[47]. Antibodies directed against the extracellular domains of the GH (Ref. 48), prolactin[49] and Epo (Ref. 46) receptors have been described that exhibit agonist activities in their bivalent form but are not agonists when used as monovalent Fab fragments. These antibodies have been proposed to activate receptor signaling by creating receptor homodimers. The crystal structure of a peptide mimic of Epo, bound to the Epo-receptor extracellular domain, revealed a complex of one peptide dimer binding to two molecules of the Epo receptor[28]. It was proposed that a small-molecule G-CSF mimic activates the G-CSF receptor by effecting dimerization[19].

Several cell-based assays that specifically measure receptor dimerization have been proposed based on the data demonstrating that receptor dimerization induced by multiple methods can lead to receptor activation. Remy and Michnick[50] utilized complementation of two fragments of dihydrofolate reductase (DHFR) to monitor dimerization of the Epo receptor. The extracellular and transmembrane domains of the Epo receptor were fused to one of the two DHFR fragments, and the two chimeric constructs were cotransfected into cells. Complementation of the DHFR fragments was monitored by incubation with fluorescein–methotrexate, which binds stoichiometrically to the reconstituted DHFR. Cells incubated with the fluorescent methotrexate exhibited high levels of fluorescence when incubated with either Epo or an Epo-mimic peptide. Fluorescence was saturable and exhibited single site binding isotherms that were consistent with previous studies for the two Epo-receptor ligands. Blakely *et al.*[51] adopted a similar approach using complementary fragments of the enzyme β-galactosidase to measure EGF receptor dimerization. Alternative methods that could be employed to detect receptor dimerization on intact cells include adaptation of fluorescence resonance energy transfer (FRET)[52,53] or similar methods, such as proximity imaging (PRIM)[54].

In vitro biochemical assays that measure receptor dimerization, such as those recently described for the Epo receptor[55], or those using proximity-based methods such as FRET, can be readily configured. Although the cell-based dimerization assays described here might have a lower throughput than such biochemical assays, they offer the advantage of measuring dimerization in the native membrane. The cell-based complementation assays might also enable detection of primary screening hits with low affinity that might be missed by a biochemical assay involving separation steps.

Approaches based on receptor dimerization could have several limitations in comparison with assays measuring receptor activation. One limitation is that although receptor dimerization/oligomerization is required, it is not always sufficient for receptor activation. For example, a peptide antagonist of the Epo receptor is capable of inducing receptor dimerization but the orientation of receptor subunits is altered relative to agonist peptide–receptor complexes[56]. Peptide agonists that dimerize the Epo receptor are less efficient at activating the receptor, possibly because of subtle differences in the orientation of the receptor subunits within the dimer[57]. In addition, there is evidence for the existence of receptors as preformed dimers on the cell surface[25,53,58] – a ligand-induced conformational change being the key event in receptor activation[24]. Thus, a molecule that dimerizes the receptor could be inactive or even antagonize receptor activation by the native cytokine and/or growth factor. A second limitation is that approaches that primarily target receptor dimerization would fail to identify lead molecules such as the small insulin mimic described by Zhang *et al.*[22] This molecule was proposed to activate directly the tyrosine kinase activity of the intracellular domain of the insulin receptor β subunit.

Melanophore assays

An amphibian melanophore assay, originally described as a functional assay for GPCRs (Ref. 59), has been adapted to identify modulators of the platelet-derived growth factor β-receptor (PDGF-β)[60] and, more recently, for the Epo receptor[61]. The assay monitors receptor-mediated pigment granule aggregation/dispersion in a *Xenopus laevis* melanophore cell line expressing the recombinant receptor of interest. Receptors signaling through activation of adenylate cyclase or protein kinase C (PKC) stimulate pigment dispersion and lead to cell darkening

and receptors coupled to inhibition of adenylate cyclase cause cell lightening. The readout for the assay is thus phototransmission and can be carried out using a simple plate reader. PDGF signaling is coupled to phospholipase C and subsequent activation of PKC, and is directly amenable to this approach. An assay to measure Epo-receptor dimerization was developed by using a chimeric fusion protein consisting of the extracellular domain of the Epo receptor fused to the cytoplasmic domain of the EGF receptor. This fusion results in activation of PKC subsequent to Epo binding. Alternative high-throughput assays that measure cAMP (Ref. 62) and calcium mobilization[63] have been described that measure the activation of GPCRs, and could be adapted for use in screening for cytokine and/or growth factor mimics. These approaches could utilize either native receptors or chimeric receptors as already described for the melanophore assay.

Conclusions and perspectives

The ability of small (300–600 Da) organic molecules to mimic the function of proteins as large as G-CSF and insulin *in vitro* and *in vivo* is remarkable. Both of these non-peptidic small molecules were discovered using HTS with cell-based assays that measure receptor activation. Primary screening of small-molecule libraries using methods such as those described here will undoubtedly yield additional compounds that act as cytokine and/or growth factor mimics. As more examples of such molecules are published, it is possible that general principles will emerge that will enable the development of targeted combinatorial libraries for this class of molecular target. The challenge will be to identify molecules that, through traditional medicinal chemistry efforts, can be optimized to generate viable clinical candidates.

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