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Erythropoietin Mimetics Derived from Solution Phase Combinatorial Libraries

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Abstract: The erythropoietin receptor (EPOr) is activated by ligand-induced homodimerization, which leads to the proliferation and differentiation of erythroid progenitors. Through the screening of combinatorial libraries of dimeric iminodiacetic acid diamides, novel small molecule binders of EPOr were identified in a protein binding assay. Evaluation of a series of analogues led to optimization of binding subunits, and these were utilized in the synthesis of higher order dimer, trimer, and tetramer libraries. Several of the most active EPOr binders were found to be partial agonists and induced concentration-dependent proliferation of an EPO-dependent cell line (UT-7/EPO) while having no effect on a cell line lacking the EPOr (FDC-P1). An additional compound library, based on a symmetrical isoindoline-5,6-dicarboxylic acid template and including the optimized binding subunits, was synthesized and screened leading to the identification of additional EPO mimetics.

Introduction

Ligand-induced receptor dimerization or oligomerization has emerged as a general mechanism for signal transduction,[1] and important members of several receptor superfamilies are activated by this process (Figure 1).[2−5] Many of these receptors appear to bind their ligands using only small clusters of residues for a majority of the binding interaction,[6−8] which has led to the expectation that smaller molecules may be capable of inducing their activation. Unlike the discovery of small molecule antagonists of biological activity functioning through the disruption of protein−protein interactions, such agonists must function by promoting a productive protein−protein interaction. Recently, the first peptide,[7−9] as well as nonpeptide,[10−12] agonists promoting receptor homodimerization have been identified through phage display or the random screening of compound libraries. Though successful, the limited number of examples, the size of the agonists,[7−9,12] or questionable nature of the receptor activation[10,11] have not yet provided a generalizable approach to the discovery or development of such small molecule receptor agonists.

The erythropoietin receptor (EPOr)[13] is a member of the cytokine receptor superfamily, whose members share similar structural motifs[14] and mechanisms of signal transduction, in which activation is believed to be achieved through an appropriately oriented ligand-induced homodimerization.[15−17] EPOr's endogenous high affinity ligand, erythropoietin (EPO), is a 34 kDa glycoprotein hormone that controls red blood cell production by promoting the proliferation and differentiation of erythroid progenitors.[18] Recombinant EPO is one of the most successful biotechnology products[19] and is currently used to treat anemias stemming from kidney failure, cancer chemotherapy, and AIDS.[20] Moreover, clinical observations on patients receiv-

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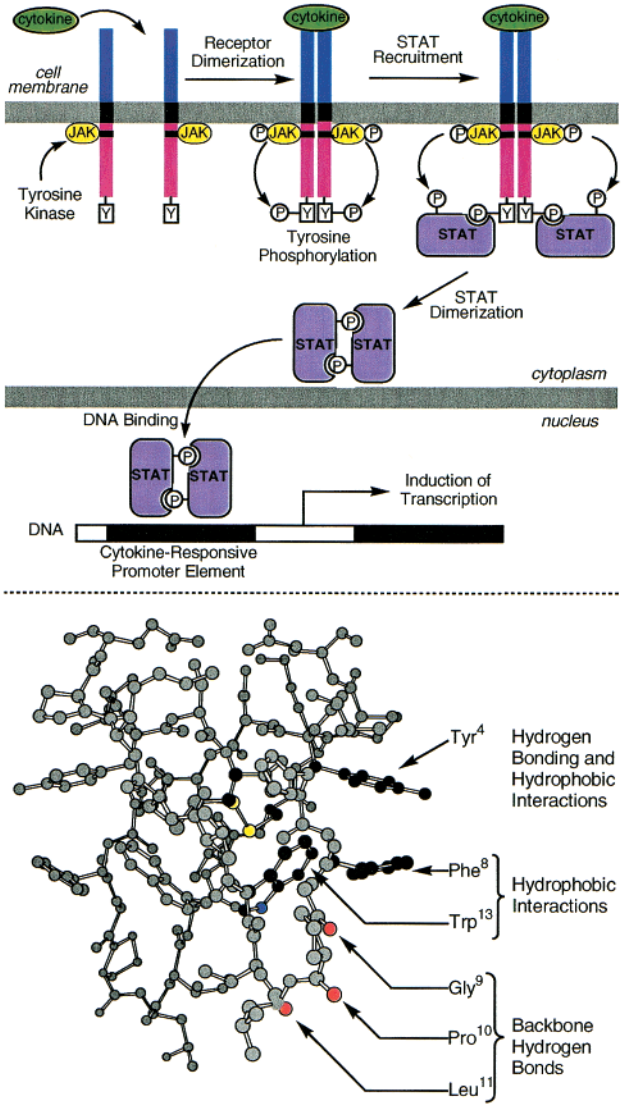

Figure 1. Top: General mechanism of cytokine activation of the JAK/STAT signal transduction pathway. Bottom: Key residues of EMP1 involved in the agonist peptide−EPOr interaction, taken from the X-ray structure of EMP1 bound to EBP.[8] Two major sites of interaction are evident: a hydrophobic pocket, outlined by Tyr^4 (which also hydrogen bonds to EPOr), Phe,[8] and Trp^{13} of EMP1, and hydrogen bonds from three contiguous residues (Gly,[9] Pro,[10] and Leu^{11}) of the peptide backbone.

ing recombinant human EPO and subsequent in vivo testing where it was found to induce complete myeloma tumor regression in 30−60% of the treated mice have shown that it may act as an antitumor therapeutic agent in its own right.[21] Thus, the discovery of effective small molecule EPO mimetics that might replace the use of the injectable recombinant protein would be of significant scientific, medical, and commercial importance.

In the absence of candidate lead structures, we turned to combinatorial chemistry[22] to identify potential EPO mimetics. Moreover, we adapted an approach whereby candidate ligands were initially screened for EPOr binding, then dimerized in efforts to convert EPOr antagonists into EPOr agonists. We enlisted a technically nondemanding solution phase strategy which dependably delivers pure individual compounds or small to large combinatorial mixtures. Symmetrical cyclic anhydride templates, such as *N*-BOC iminodiacetic acid anhydride (**1**), which are readily substituted at up to three positions, can later be combined through a variety of linking strategies to provide symmetrical and unsymmetrical dimer and higher-order libraries.[23,24] In addition to dimerization through amide bond forming reactions, recent efforts have expanded the scope of this approach by introducing dimerization and trimerization through the formation of carbon−carbon bonds via olefin metathesis,[25] Stille,[26] and biaryl[27] coupling reactions. In all cases, reaction and workup conditions were designed to permit the isolation and purification of products through simple liquid−liquid or liquid−solid extractions. Notably, solution phase, but not solid phase, combinatorial chemistry uniquely permits the convergent[25] dimerization of such functionalized subunits to provide candidate leads for promoting receptor activation, and this has been the basis behind much of our prior work. We previously described the synthesis of diverse libraries derived from **1** that were suited for the study of such protein−protein interactions.[24] Herein, we report the identification of novel erythropoietin mimetics through the screening of these and related combinatorial libraries based on iminodiacetic acid and isoindoline-5,6-dicarboxylic acid templates that was conducted over a period of several years.

Results and Discussion

Iminodiacetic Acid Libraries: EPOr Binding Activity. In recent studies, the identification of cyclic peptide dimers with the ability to mimic EPO,[7,8] together with structural details of the intricate ligand−receptor and receptor−receptor interactions, have been described.[8,28] In the X-ray structure of the complex between EMP1 (erythropoietin mimetic peptide 1) and the extracellular binding fragment of EPOr (EBP), the peptide ligand and receptor were observed as a symmetrical 2:2 dimer.[8] Although each EMP1−EBP interface was relatively large (420 $Å^2$), key binding sites were identified that could potentially be exploited by smaller molecules. Two main sites of interaction were observed between the cyclic peptide ligand and the receptor (Figure 1). A hydrophobic core comprised of two phenylalanine side chains and a methionine residue of EBP binds the tyrosine, phenylalanine, tryptophan, and cystine residues of EMP1. The hydroxy group on the peptide tyrosine (Tyr^4) also serves as a hydrogen bond acceptor in this pocket. The second key

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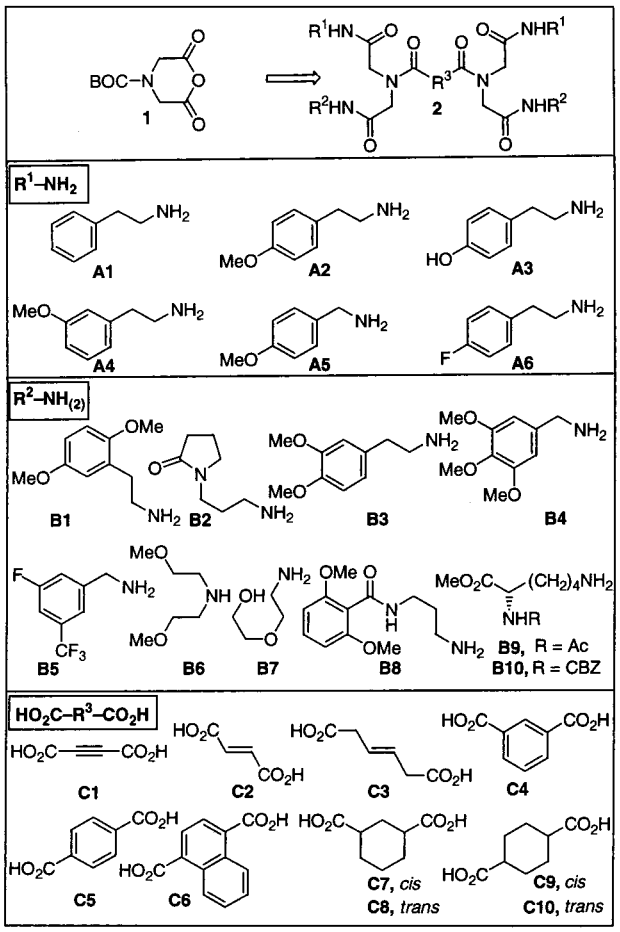

Figure 2. The structure and building blocks of library **2**. The library was synthesized and screened as mixtures containing single R^1 (**A1**−**A6**) and R^2 (**B1**−**B10**) substitutions, linked by an equimolar mixture of subunit R^3 (**C1**−**C10**), 60 mixtures of 10 compounds. For the synthesis and characterization of this library, see ref 24.

interaction entails three closely situated hydrogen bonds between the peptide backbone of EMP1 and EBP.

Solution phase combinatorial synthesis was employed to generate an initial library of 600 C_2-symmetrical compounds (60 mixtures of 10 compounds, library **2**, Figure 2) which were designed to bind EPOr in the same manner as EMP1. The library members contained functionality appropriately spaced to loosely mimic the hydrophobic and hydrogen bonding interactions revealed in the EMP1−EBP X-ray structure. The R^1-NH_2 (group A) amines were included to mimic Tyr^4 of EMP1, whereas the R^2-NH_2 (group B) amines included functionality capable of emulating the hydrogen bonding peptide backbone of EMP1. The approach constituted the dimerization linkage of iminodiacetic acid diamides with a mixture of rigid dicarboxylic acids. The entire synthesis sequence to assemble the library required three steps, and purification at each step was achieved by the removal of excess reactants and reagents by liquid−liquid extraction protocols.[29]

The library was screened (50 μM mixture concentration, 1% DMSO) for binding in an assay that measured the ability of the compounds to displace ^{125}I-EPO from immobilized EPOr.[30] Several of the compound mixtures displayed EPO−EPOr

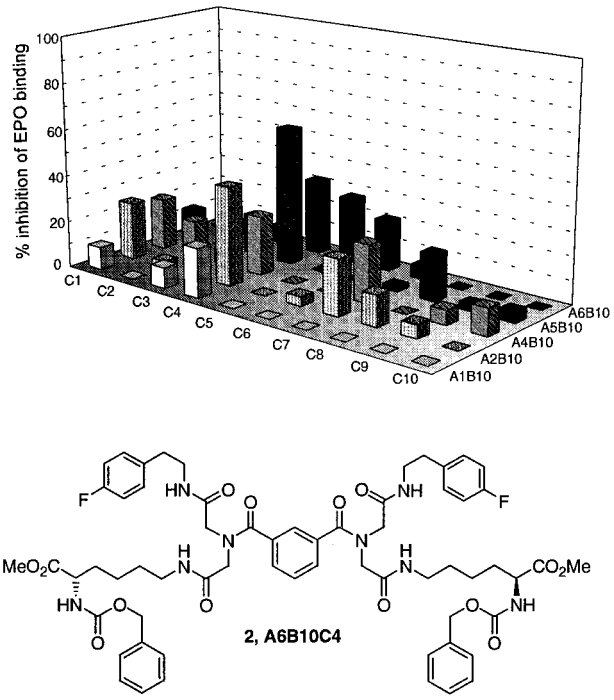

Figure 3. Competitive binding activity (% inhibition of EPO binding) of the individual compounds from the deconvolution of the most active sublibraries of mixture library **2**. ^{125}I-EPO, in the presence of the library members (50 μM), was incubated with immobilized EPOr, as described in ref 27. Compounds were tested in triplicate, and the bars represent the average % inhibition of ^{125}I-EPO binding to the receptor. **2, A6B10C4** was selected as a lead for further derivitizations.

competitive binding activity, the most active containing *N*-α-CBZ-lysine methyl ester (**B10**) in the R^2 position. Mixture sublibraries were deconvoluted by synthesizing the individual components of the mixtures that displayed the highest competitive binding activity. Thus, the 50 compounds that comprised the five most active mixtures were prepared to identify the optimal linking dicarboxylic acid, R^3.

These deconvoluted compounds were tested in the competitive binding assay, and the results are shown in Figure 3. Throughout the five series, the **C4** linking dicarboxylic acid (R^3) exhibited the best binding activity. Thus, with R^2 fixed as **B10** and R^3 fixed as **C4**, a range of R^1 derivatives exhibited good competitive binding activity. Compound **2, A6B10C4** (Figure 3), which displayed 32% inhibition at 50 μM, was selected as a lead for subsequent modifications. Its activity was respectable when compared to that of EMP1 which has an IC_{50} of 5 μM in this binding assay.[28,31] Compounds **2, A2B10C4** (42% inhibition at 50 μM) and **2, A5B10C4** (59% inhibition at 50 μM) were also identified as possessing significant activity.

Two series of follow-up libraries were prepared to optimize the amine binding groups. At this stage, the *N*-α-CBZ-lysine methyl ester (**B10**) group was identified as being critical for the observed activity, which was modulated by the R^1-NH_2 (group A) substitution. Thus, a series of structurally diverse primary and secondary amines (**A7**−**A115**, Figure 4) was utilized, in conjunction with the *N*-α-CBZ-lysine methyl ester (**B10**) and isophthalic acid (**C4**) linker, to prepare 109 new derivatives. Screening results from the competitive binding assay

(29) For a detailed description of the synthesis of this library, see ref 24.
(30) The protein binding assay was conducted by The R. W. Johnson Pharmaceutical Research Institute, as described in ref 28.
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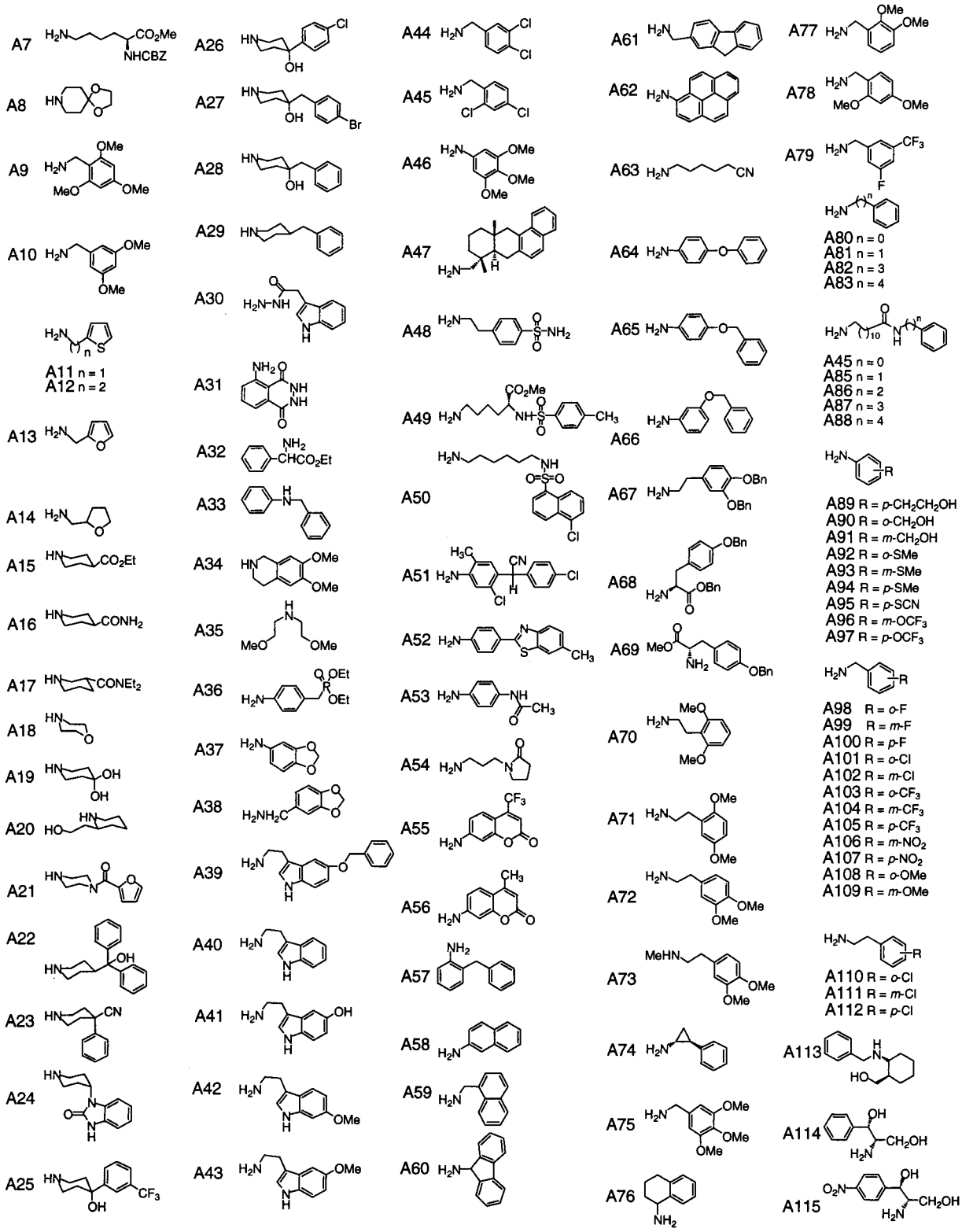


Figure 4. Structures of the 109 amine subunits (**A7**−**A115**) used in combination with the **B10** and **C4** subunits to explore the binding activity of lead compound **2, A6B10C4**. For the synthesis of compounds **2, A7B10C4**−**A115B10C4**, see ref 24.

(Figure 5) revealed that most of the amine subunits did not improve the activity and, in many cases, resulted in a loss in affinity for the receptor, indicating the effectiveness of the initially chosen R^1-NH_2 amines (**A1**−**A6**). In some cases, interesting activity was observed, most notably with cyclic acetal **A8** (79% inhibition at 50 μM). The most active of these R^1-NH_2 substitutions were included in the synthesis of subsequent higher order libraries (vide infra).

The second series of analogues of **2, A6B10C4** contained modifications to the *N*-α-CBZ-lysine methyl ester (**B10**) (Figure 6). These compounds maintained the 4-fluorophenethylamine (**A6**) group and the isophthalic acid (**C4**) linker identified from

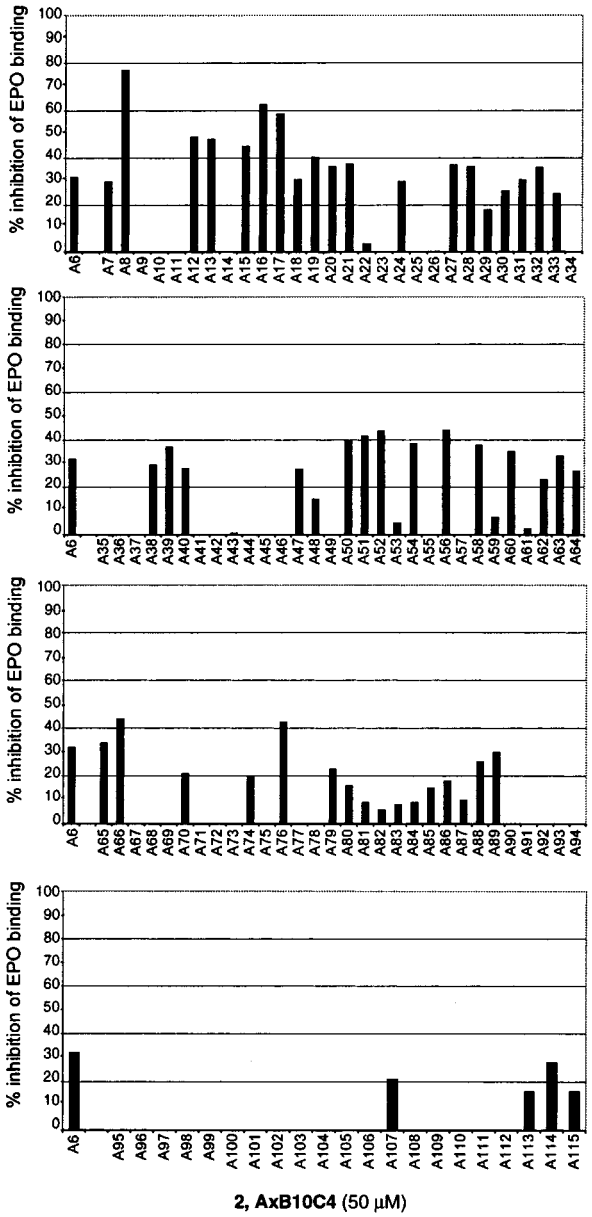


Figure 5. Competitive binding activity of compounds **2, A7B10C4**−**A115B10C4**. Each compound (50 μM) was incubated with ^{125}I-EPO and immobilized EPOr, as described in ref 27. Compounds were tested in triplicate, and the bars represent the average % inhibition of ^{125}I-EPO binding to the receptor.

the initial deconvoluted library. These agents included commercially available amines (**B11**−**B26** and **B85**−**B87**) possessing some of the structural features of *N*-α-CBZ-lysine methyl ester, as well as synthesized amines which contained systematic changes to the **B10** subunit, especially the ester and carbamate functionalities. Each of these compounds (**2, A6B10C4**−**A6B87C4**) was screened in the EPO competitive binding assay (50 μM concentration, Figure 7). Most of the analogues displayed very little activity in comparison to lead compound **2, A6B10C4**. The most active were **2, A6B17C4** and **2, A6B18C4** which inhibited EPO binding by 32 and 36%, respectively. The decreased activity of compounds containing **B37**−**B43** indicates that the ester functionality has importance for binding, although lysinol derivative **2, A6B45C4** maintains modest activity. A variety of sizes are tolerated among the ester substitutions (**B46**−**B65**), although most larger substitutions were less effective than the methyl ester, and none provided an increase in potency. The carbamate functionality also surfaced as necessary for activity, and none of the derivatives in which it was removed (**B23**, **B26**−**B35**) were generally active. Many substitutions on the aromatic ring are permitted, although none of the new carbamates (**B66**−**B84**) led to activity that exceeded that of the original benzyl carbamate, **B10**. Compound **2, A6B36C4**, which has the same general substitution as the *N*-α-CBZ-lysine methyl ester but with the positions of the carbamate oxygen and nitrogen atoms reversed, maintains some activity (15% inhibition). The stereochemistry at the α-position is important as the D-lysine derivative **2, A6B44C4** (5%) is significantly weaker than the lead compound with the L-stereochemistry. Replacement of the lysine α-amino by a hydroxy group (included in **B32**−**B36**) also diminishes activity.

Recognizing the significance of the *N*-α-CBZ-lysine methyl ester, this group was introduced in both positions of the iminodiacetic acid diamide template (**A7** = **B10**) and was tested in combination with all 10 linking dicarboxylic acids (compounds **2, A7B10C1**−**10**). Here, the acetylene dicarboxylic acid linker (**C1**) provided the highest activity (45% inhibition at 50 μM), followed by the isophthalic acid (**C4**) linked agent (22% inhibition at 50 μM).

Higher Order Libraries. Subsequent libraries were prepared which expressed the iminodiacetic acid diamides as trimers and tetramers, either through a tricarboxylic acid linkage or through sequential dimerization couplings with dicarboxylic acids. The structures in these libraries contained the key amine binding groups identified in the initial EPOr binding screen. In the trimer series, a total of 560 compounds were prepared (80 mixtures of 7), and the five most active mixtures in the competitive binding assay were deconvoluted by resynthesis of the 35 individual compounds.[32] Several of these agents displayed activity on the same level as the iminodiacetic acid dimers, but none surpassed the initial series. As expected, compounds containing the *N*-α-CBZ-lysine methyl ester (**B10**) exhibited significant inhibition; however, the most potent agent in the series was **3, A2B6C11**, which at 50 μM resulted in a 38% inhibition of EPO binding (Figure 8a). The tetramer compounds were synthesized on a broader scale where a total of 1596 compounds were screened in mixtures of 8−10.[32] Deconvolution of the most active mixtures and screening 176 individual compounds provided agents that were of similar activity to the corresponding iminodiacetic acid diamide dimers discussed above. The two most active tetramers in the binding assay contained the **A8** and **A13** binding groups, combined with the *N*-α-CBZ-lysine methyl ester (**B10**), which displayed 52% (**4, A8B10C12**) and 47% (**4, A13B10C12**) inhibition of EPO

(32) In the trimer series (library **3**), eight group A amines (R^1-NH_2) and 10 group B amines (R^2-NH_2) identified as the most active subunits from the dimer library screening were utilized, each combination linked through a mixture of seven tricarboxylic acid linkers, resulting in 560 homotrimers as 80 mixtures, seven compounds per mixture. The general library structure and the subunits of the most active compound, **3, A2B6C11**, are shown in Figure 8a. For the tetramer series (library **4**), three of the most active group A subunits (R^1-NH_2) and 15 of the most active group B subunits (R^2-NH_2) were utilized, each combination linked through sequential dimerization reactions with mixtures of 8−10 dicarboxylic acids used in the final couplings, resulting in 1596 compounds as 168 mixtures of 8−10. The general library structure and the subunits of the two most active agents identified from the binding assay (**4, A8B10C12** and **4, A13B10C12**) are shown in Figure 8b. Structures of the complete set of subunits used in libraries **3** and **4** may be found in Supporting Information. For details of their synthesis and characterization, see ref 24.

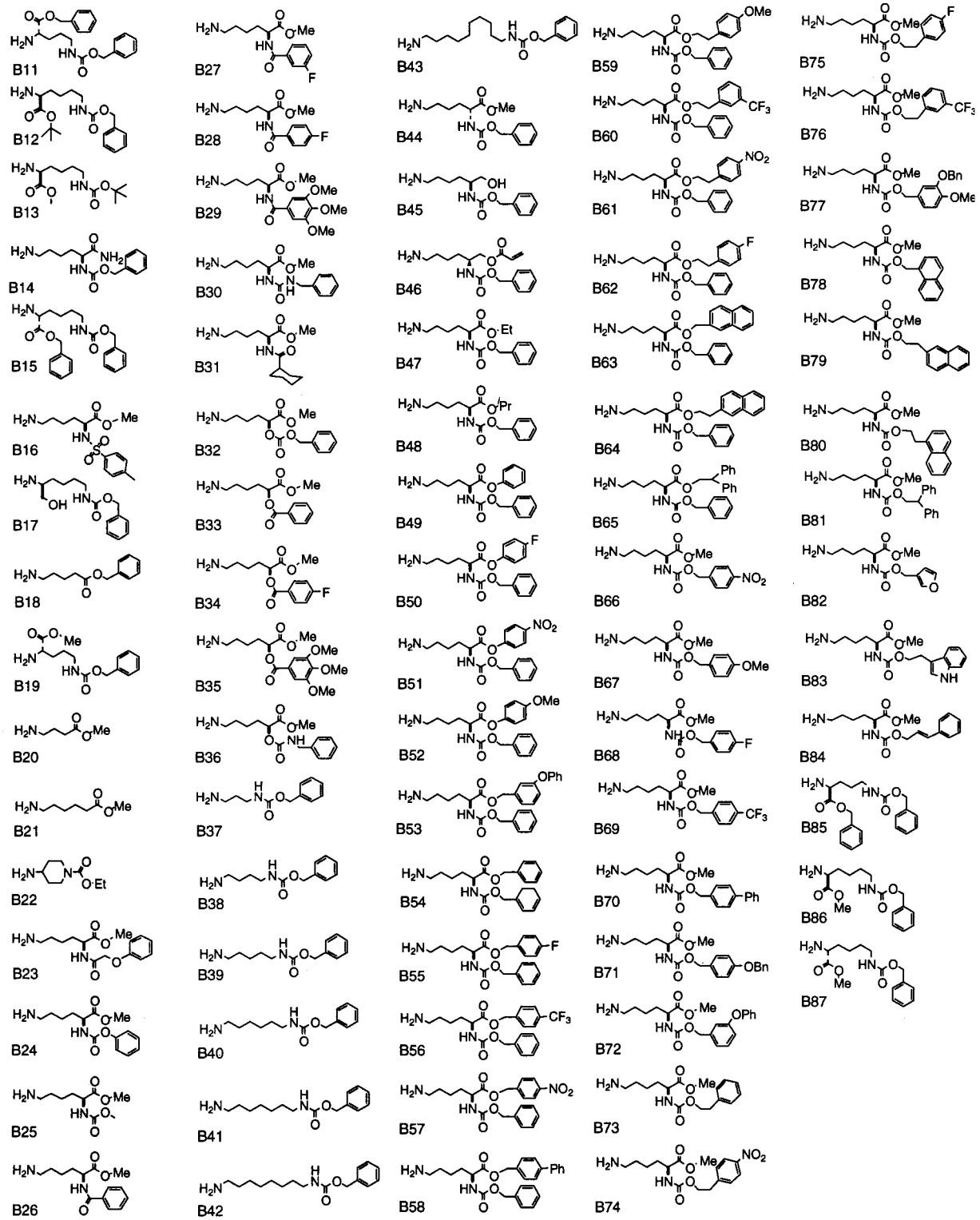


Figure 6. Structures of the 77 amine subunits (**B11**−**B87**) containing modifications to the *N*-α-CBZ-lysine methyl ester (**B10**), present in the most active library compounds. Each of these amines was combined with the **A6** and **C4** subunits and screened in the competitive binding assay. For details of the synthesis of compounds **2, A6B11C4**−**A65B87C4**, see ref 24.

binding at 50 μM concentration (Figure 8b). The choice of the linking dicarboxylic acid was less important for the observed activity, but the highest inhibition was detected with octadecanedioic acid (**C12**). Thus, the trimer and tetramer agents, in which three or four copies of the same functionalized iminodiacetic acid binding subunits are present, bind the target receptor, and because of their higher valency have a greater chance of interacting with a second receptor molecule. The screening of such higher order libraries, in which multiple copies of identical binding subunits are present, has been successfully adopted by others for the discovery of EPOr agonists.[12]

A second series of tetramer agents was prepared in which 10 dicarboxylic acids were used to link each of the eight most active compounds of the optimized iminodiacetic acid diamide dimers

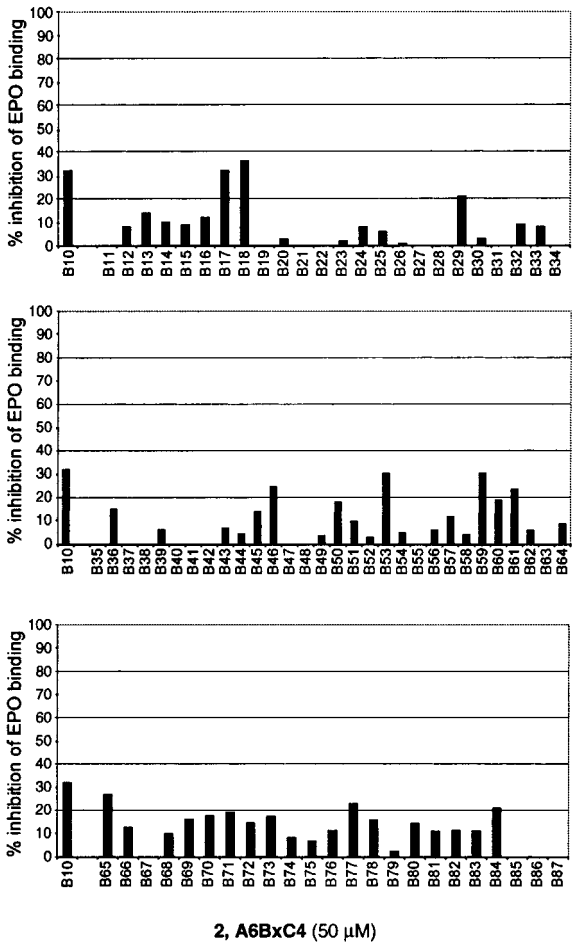


Figure 7. Competitive binding activity of compounds **2, A6B11C4**−**A65B87C4**. Each compound (50 μM) was incubated with ^{125}I-EPO and immobilized EPOr, as described in ref 28. Compounds were tested in triplicate, and the bars represent the average % inhibition of ^{125}I-EPO binding to the receptor.

(library **2**). These agents (library **5**, Figure 9)[29] were prepared for testing in a functional activity assay (vide infra) where interaction with two receptor molecules is necessary.

Isoindoline Libraries. A second dimeric library was also developed to provide an alternative structural framework for the incorporation of the protein-binding subunits. Here, the cyclic anhydride chemistry was extended to the preparation of a prototypical 1000-member library of dimeric isoindoline-5,6-diamides. The key amine binding groups identified from the initial iminodiacetic acid libraries were incorporated into the new library. Thus, an alternative rigid cyclic anhydride template (**6**, Figure 10) was utilized, from which the pendant binding groups are displayed. The use of such rigid templates may be especially important for receptor dimerization agonist libraries considering recent evidence that suggests activation is achievable only by a precise (re)orientation of the target receptor chains.[15,17]

The *N*-BOC-isoindoline template **6** was generated in situ by treatment of dicarboxylic acid **7** with EDCI[33] and was reacted in parallel with amines **A1′**−**A10′** (Figure 11), providing 10 individual monoamides **8, A1′**−**A10′** in good to excellent yields (average = 88%, Scheme 1). The products were purified by liquid−liquid extractions and characterized by ^{1}H NMR, IR, and HRMS. In each case, the desired product was isolated in ≥95% purity. Each monoamide was then divided into 10 portions and coupled (EDCI) to amines **B1′**−**B10′** providing 100 individual diamides **9, A1′B1′**−**A10′B10′** in 87% average yield after acid/base extractive workup. Representative samples were analyzed (matrix characterization) by ^{1}H NMR, IR, and HRMS, indicating the presence of the desired diamide products in excellent purity regardless of the reaction efficiencies.

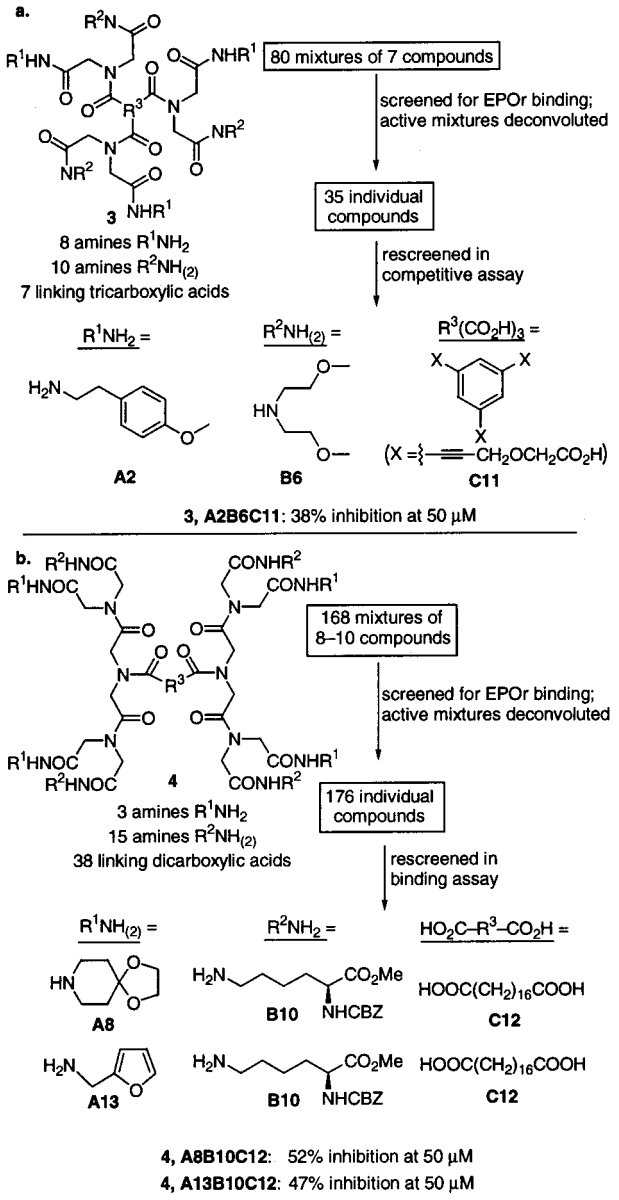


Figure 8. Structures and binding activity (% inhibition of EPO binding) for the most active trimer and tetramer iminodiacetic acid diamide libraries. (a) A total of 560 trimers were prepared as 80 mixtures of seven compounds, utilizing the key R^1-NH_2 and R^2-NH_2 amine binding subunits identified from the dimer library screening. The most active mixtures were deconvoluted, and compound **3, A2B6C11** displayed the highest activity, inhibiting EPO binding by 38% at 50 μM. (b) A total of 1596 tetramers were prepared as 168 mixtures of 8−10 compounds, utilizing the key R^1-NH_2 and R^2-NH_2 amine binding subunits identified from the dimer library screening. The most active mixtures were deconvoluted, and compounds **4, A8B10C12** and **4, A13B10C12** displayed the highest activity, inhibiting EPO binding by 52 and 47%, respectively, at 50 μM. Structures of the full set of subunits used in libraries **3** and **4** may be found in Supporting Information.

(33) Boger, D. L.; Lee, J. K.; Goldberg, J.; Qing, J. *J. Org. Chem.* **2000**, *65*, 1467.

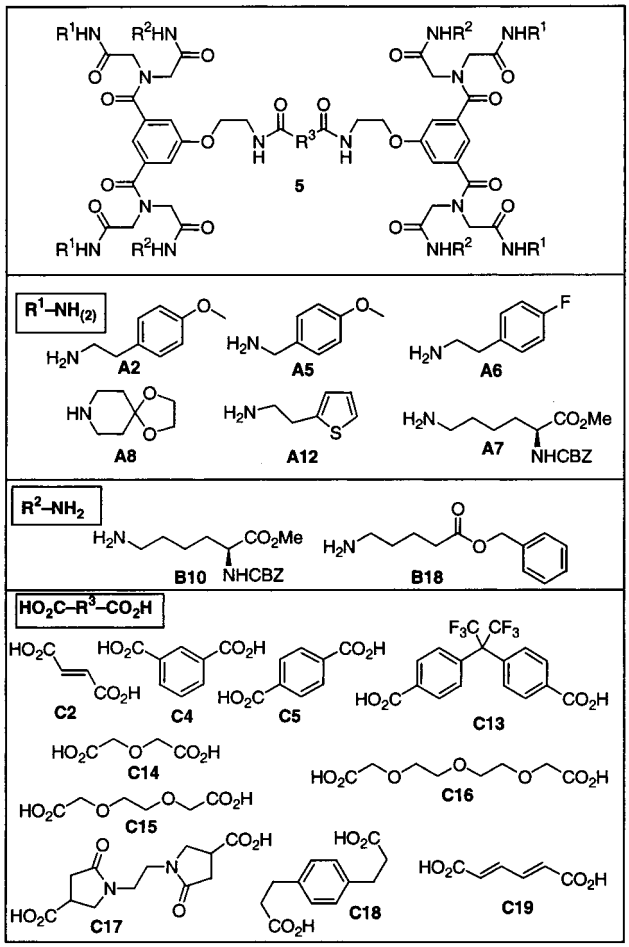


Figure 9. Structures of tetramer library **5**. The most active dimeric iminodiacetic acid diamide compounds (**2**), including the linking isophthalic moiety, were dimerized through dicarboxylic acid couplings. This series was prepared for testing in functional activity screens, where an increased number of protein-binding subunits would be available for interaction with two receptor molecules. For the synthesis and characterization of library **5**, see ref 24.

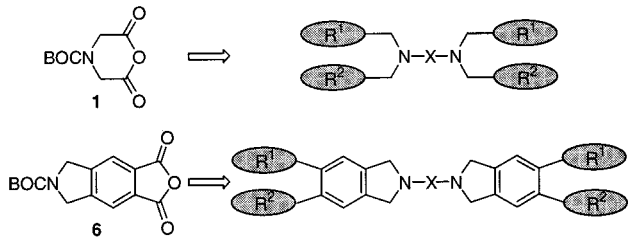


Figure 10. Comparison of cyclic anhydride templates **1** and **6**.

The dimerization of the *N*-BOC-protected diamides **9, A1′B1′**−**A10′B10′** was then investigated to provide the C_2-symmetrical ligands. Following protocols developed for the iminodiacetic acid diamide libraries,[24] samples of **9** were treated with 4 N HCl-dioxane to remove the BOC protecting group and provide the secondary amine. However, preliminary analysis indicated an impure mixture of reaction products after the HCl treatment. For example, treatment of **10** with 4 N HCl-dioxane (25 °C, 1 h), followed by evaporation of the solvent, provided a residue consisting of the desired deprotected diamide **11** (50%) together with a 1:1 mixture of phthalimide **12** and 4-fluorophenethylamine hydrochloride, indicating that acid-catalyzed cyclization of the isoindoline-5,6-dicarboxamide competes with

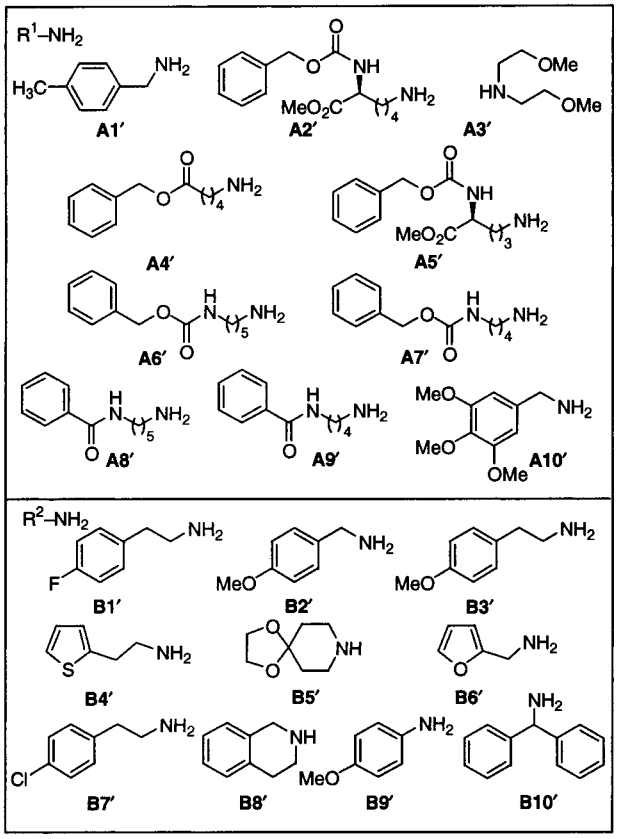
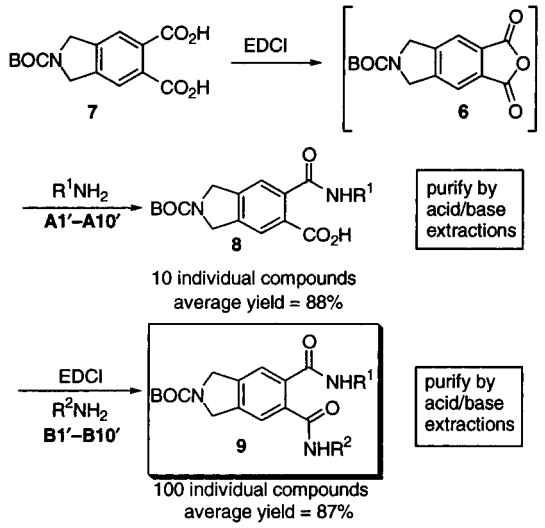


Figure 11. The structures of the R^1-NH_2 (**A1′**−**A10′**) and R^2-NH_2 (**B1′**−**B10′**) amines used to functionalize template **6**.

Scheme 1

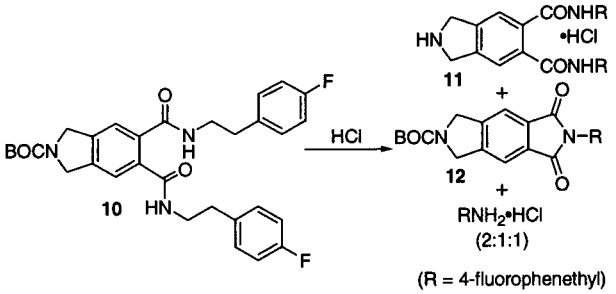


N-BOC deprotection (Scheme 2).[34] The use of alternative conditions (solvents and temperatures) for the HCl deprotection reaction resulted in similar product mixtures.

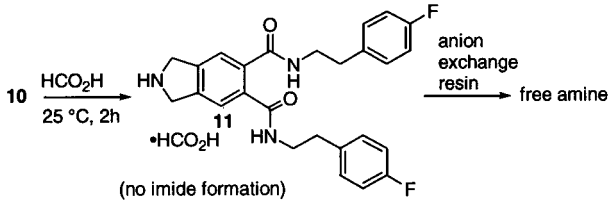
Consequently, milder reagents were investigated for the removal of the BOC protecting group. Treatment of **10** with formic acid (25 °C, 2 h) followed by evaporation of the reaction solvent was found to cleanly provide **11**, without any detectable competitive imide formation. Effective, yet technically nondemanding conditions, which are practical for a large library

(34) Diamide **10** was prepared from dicarboxylic acid **7** by successive couplings (EDCI) with 4-fluorophenethylamine.

Scheme 2



Scheme 3



synthesis, were developed to free base the resulting formic acid salts. A solution of the evaporated crude reaction product in 1:1 MeOH−CH_2Cl_2 was treated with the OH^- form of Amberlite IRA-400 ion-exchange resin.[35] Subsequent filtration and evaporation were found to remove all traces of formic acid, providing the free secondary amine (Scheme 3).

The dimerization of the isoindoline diamides was first investigated using **9, A1′B1′** as a representative substrate. The *N*-BOC protecting group was removed by treatment with formic acid and subjected to dimerization coupling (EDCI) with each of the 10 dicarboxylic acids **C1′**−**C10′** (Figure 12), providing the dimerized isoindoline products **13, A1′B1′C1′**−**C10′**. The reactions were worked-up by dilution of the reaction solvent (DMF) with 1:1 MeOH−CH_2Cl_2 and treatment with a mixture of cation (Dowex 50WX8-200) and anion (Amberlite IRA-400, OH^- form) exchange resins. This procedure was found to effectively remove not only unreacted starting materials, but also EDCI and its reaction byproducts. Thus, simple filtration and evaporation of solvent provided the 10 individual products **13, A1′B1′C1′**−**A1′B1′C10′**, free of any impurities. The yields of the isolated products were good to excellent (79−94%), and each was characterized (^{1}H NMR, IR, HRMS) establishing the identity and purity (≥95%) of the products.

Mixture couplings were investigated by subjecting **9, A1′B1′** to the same deprotection and coupling conditions utilizing an equimolar mixture of dicarboxylic acids **C1′**−**C10′**. A reaction stoichiometry in which an excess of amine (2.5 equiv) relative to dicarboxylic acid (0.1 equiv of each dicarboxylic acid, 1.0 equiv total) was utilized to drive the reactions to completion and ensure that differences in the individual coupling rates would not affect the integrity of the product mixture. After the ion-exchange resin workup, a 79% yield (based on the average product MW) of **13, A1′B1′C1′**−**C10′** was obtained. This mixture was analyzed by MS, and the parent ion of each expected product was observed.

By utilizing the optimized reaction and workup conditions outlined above, the full set of 100 isoindoline-5,6-diamides **9, A1′B1′**−**A10′B10′** was dimerized through dicarboxylic acid linkers **C1′**−**C10′** (Figures 11 and 12, Scheme 4). The reactions

(35) This was prepared by treating Amberlite IRA-400 resin with 1 N aqueous NaOH and washing with H_2O, MeOH, and CH_2Cl_2.

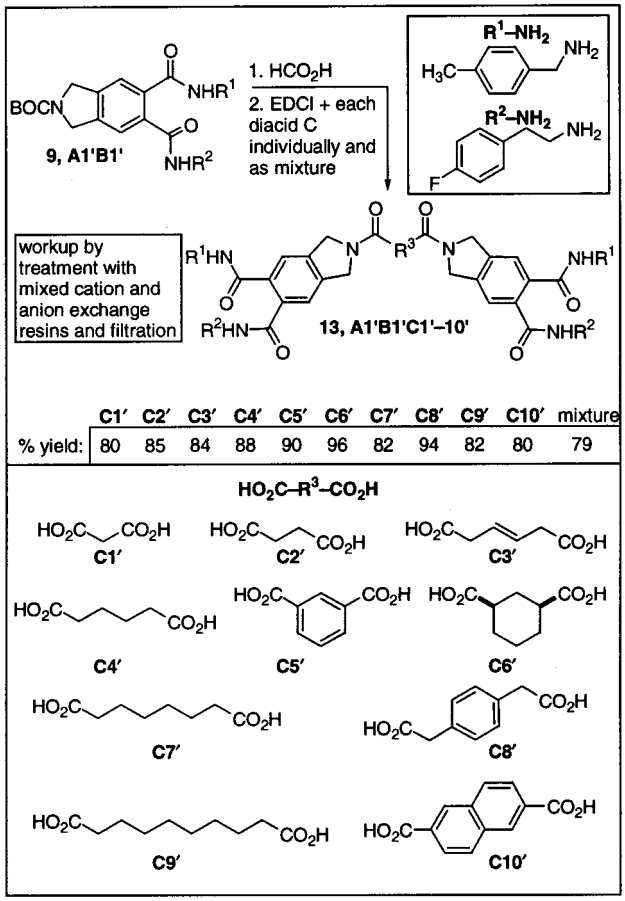
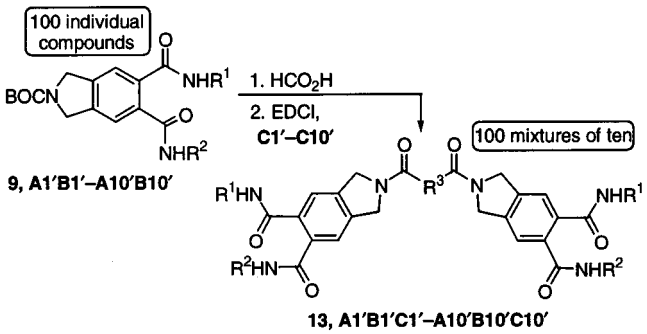


Figure 12. Dimerization of the functionalized isoindoline template through dicarboxylic acid couplings.

Scheme 4



provided library **13, A1′B1′C1′**−**A10′B10′C10′** as 100 mixtures of 10 compounds in an average of 72% yield (Table 1).

EPO-Dependent Cell Proliferation. The most active iminodiacetic acid-derived agents in the binding assay (**2**−**5**), as well as the entire isoindoline library (**13**, as mixtures of 10 compounds), were screened for functional activity by measuring the ability to stimulate the growth of an EPO-dependent cell line (UT-7/EPO)[36] through EPOr binding and dimerization. Mitogenic activity was determined by measuring cellular radioactivity (CPM) due to incorporation of ^{3}H-thymidine in the cell line (UT-7) developed to grow only in the presence of EPO. Primary screening of **2**−**5** was conducted at a single compound concentration (10 μM). Many of the compounds were

(36) Komatsu, N.; Yamamoto, M.; Fujita, H.; Miwa, A.; Hatake, K.; Endo, T.; Okano, H.; Katsube, T.; Fukumaki, Y.; Sassa, S.; Miura, Y. *Blood* **1993**, *82*, 456.

Table 1. % Yields (Based on the Average Product Molecular Weight) for the Final Coupling Step for Mixture Library **13**

13	B1′	B2′	B3′	B4′	B5′	B6′	B7′	B8′	B9′	B10′
A1′	100	86	84	98	70	99	93	94	81	100
A2′	79	79	81	84	65	72	88	53	88	81
A3′	69	75	64	73	56	40	65	70	76	79
A4′	71	51	89	70	45	56	54	26	82	66
A5′	65	83	61	72	53	35	58	42	61	79
A6′	82	74	76	77	53	64	80	54	76	86
A7′	82	74	82	64	48	74	84	64	95	74
A8′	86	80	80	68	60	65	86	57	82	98
A9′	99	100	84	86	57	60	79	76	86	86
A10′	59	77	75	76	57	39	72	42	45	79

average yield = 72.2%

Table 2. Mitogenic Activity of Iminodiacetic Acid Library Compounds (10 μM) in an EPO-Dependent Cell Line (UT-7)[a]

compound no.	% increase in proliferation	compound no.	% increase in proliferation
2, A1B10C1	*88*	**5, A5B10C4**	69
2, A2B10C1	*54*	**5, A5B10C5**	65
2, A5B10C4	*61*	**5, A5B10C14**	64
2, A5B10C8	*33*	**5, A5B10C16**	74
2, A6B10C4	*39*	**5, A5B10C18**	44
2, A6B17C4	*37*	**5, A6B10C2**	35
2, A7B10C1	*95*	**5, A6B10C19**	35
2, A7B10C4	*33*	**5, A6B10C4**	28
2, A12B10C4	*34*	**5, A6B10C16**	23
2, A13B10C4	*42*	**5, A6B10C18**	38
2, A17B10C4	42	**5, A6B18C2**	46
4, A8B10C12	34	**5, A6B18C19**	26
4, A13B10C12	40	**5, A6B18C4**	41
5, A1B10C2	88	**5, A6B18C5**	−3
5, A12B10C2	24	**5, A6B18C13**	−5
5, A12B10C4	16	**5, A6B18C15**	29
5, A12B10C14	24	**5, A6B18C17**	19
5, A12B10C16	6	**5, A6B18C18**	6
5, A12B10C18	29	**5, A7B10C4**	33
5, A2B10C2	35	**5, A8B10C2**	29
5, A2B10C4	−14	**5, A8B10C19**	11
5, A2B10C18	18	**5, A8B10C4**	30
5, A5B10C19	40	**5, A8B10C16**	33

[a] Data reported are the average % increases in proliferation, relative to DMSO control, as measured by ^{3}H-thymidine incorporation. Selected compounds (italic) were retested over a concentration range; see Figure 13 and Table 5.

able to induce cell proliferation at this concentration, and representative results are presented in Table 2. A standard curve was also established for EPO, and a maximum response of 812% was observed. The greatest increase in proliferation was observed with compound **2, A7B10C1**, which contains the key *N*-α-CBZ-lysine methyl ester (**B10**) binding group in both the R^1-NH_2 and R^2-NH_2 positions. Compounds from the tetramer library **5**, which contain four copies of the same functionalized iminodiacetic acid diamides present in the dimer series (**2**), induced a similar increase in proliferation, indicating that the additional binding group copies did not further increase the observed stimulated cell growth. Thus, the dimeric compounds, which contain two sets of binding groups, were found to be sufficient for functional activity.

Screening results from mixture library **13** (10 μM mixture concentration, 1% DMSO) are presented in Table 3. Because the R^1-NH_2 and R^2-NH_2 binding groups previously identified from the screening of the iminodiacetic acid libraries were incorporated into **13**, the new library members were expected to similarly bind EPOr. The extended, more rigid, nature of the isoindoline template was included to probe the effects of linker

Table 3. Mitogenic Activity of Dimeric Isoindoline Library, **13** (10 μM Mixture Concentration) in an EPO-Dependent Cell Line (UT-7)[a]

13	B1′	B2′	B3′	B4′	B5′	B6′	B7′	B8′	B9′	B10′
A1′	−4	−23	−20	−26	−16	−44	−34	−42	−58	−31
A2′	*41*	−1	−6	−16	−18	−23	*16*	−42	−14	−44
A3′	*53*	−11	18	16	−1	−18	*70*	10	−32	−32
A4′	*44*	−18	21	−14	−8	−14	*11*	14	−33	−52
A5′	*33*	−14	24	−6	2	−6	*12*	10	−10	−30
A6′	9	−24	4	−50	−27	−18	2	−32	−32	−42
A7′	16	15	15	−16	−3	29	1	7	8	−4
A8′	18	−10	−5	−27	−6	10	−14	−12	−32	3
A9′	4	−8	−2	16	−17	−14	19	2	−26	−14
A10′	*184*	−2	16	5	−50	−30	*51*	−26	−1	−68

[a] Data reported are the average % increases in proliferation, relative to control, as measured by ^{3}H-thymidine incorporation. Each **A′B′** combination is linked by a mixture of ten linking dicarboxylic acids. Ten of the most active mixtures (italic) were deconvoluted by resynthesis of the individual compounds.

Table 4. Mitogenic Activity of the Deconvoluted Mixtures (10 μM) of Library **13**[a]

	C1′	C2′	C3′	C4′	C5′	C6′	C7′	C8′	C9′	C10′
A2′B1′	5	15	12	2	4	12	10	2	*23*	−10
A3′B1′	−2	2	14	−24	−26	12	−75	−64	16	−2
A4′B1′	0	−24	2	−4	−21	−11	−18	−20	−18	−23
A5′B1′	12	*21*	16	*22*	1	0	−2	*22*	−7	0
A10′B1′	−8	−16	0	−15	−5	−54	−38	−41	0	−4
A2′B7′	*26*	−9	*26*	−25	7	−58	−48	−48	8	−6
A3′B7′	−8	−51	−19	−96	−22	−66	−97	−86	*24*	2
A4′B7′	10	−14	6	−46	−18	−72	−68	−58	−36	−30
A5′B7′	*32*	−1	45	−12	16	−51	−44	−6	*24*	−17
A10′B7′	−8	−34	19	−30	6	−58	−56	−34	7	0

[a] Data reported are the average % increases in proliferation, relative to control, as measured by ^{3}H-thymidine incorporation.

composition on functional activity. The mixture screening results (Table 3) indicate that many of these C_2-symmetrical compounds were able to support proliferation of the EPO-dependent UT-7 cells. The most active mixtures of library **13** contained either the 4-fluoro- or the 4-chlorophenethylamine (**B1′** or **B7′**) binding groups.

Deconvolution of the 10 most active mixtures containing the **B1′** and **B7′** subunits was conducted by resynthesis of the corresponding 100 individual compounds. These deconvoluted samples were screened in the cell proliferation assay (10 μM, 1% DMSO), and the results are presented in Table 4. Many of these agents were able to stimulate low levels of cell proliferation,[37] and the highest activity was observed for compounds containing the *N*-α-CBZ-lysine methyl ester (**A2′**) or the homologous *N*-α-CBZ-ornithine methyl ester (**A5′**). The optimal linking dicarboxylic acid (**C1′**−**C10′**) was dependent on the particular combination of the R^1-NH_2 and R^2-NH_2 substitutions, but, in general, the linear, more flexible linkers (i.e., **C1′**−**C4′**, **C9′**) led to greater cellular proliferation. The high levels of activity observed with the **A2′B1′**, **A2′B7′**, **A5′B1′**, and **A5′B7′** combinations are consistent with the most active structures of the iminodiacetic acid diamide screening results, illustrating the significance of the substituted lysine or ornithine binding groups. Many of the same R^1-NH_2 and R^2-NH_2 binding groups (especially the *N*-α-CBZ-lysine methyl ester)

(37) The negative values observed for many of the compounds can be attributed to the poor solubility of the dimeric isoindoline products or to possible cytotoxicity of these agents at 10 μM.

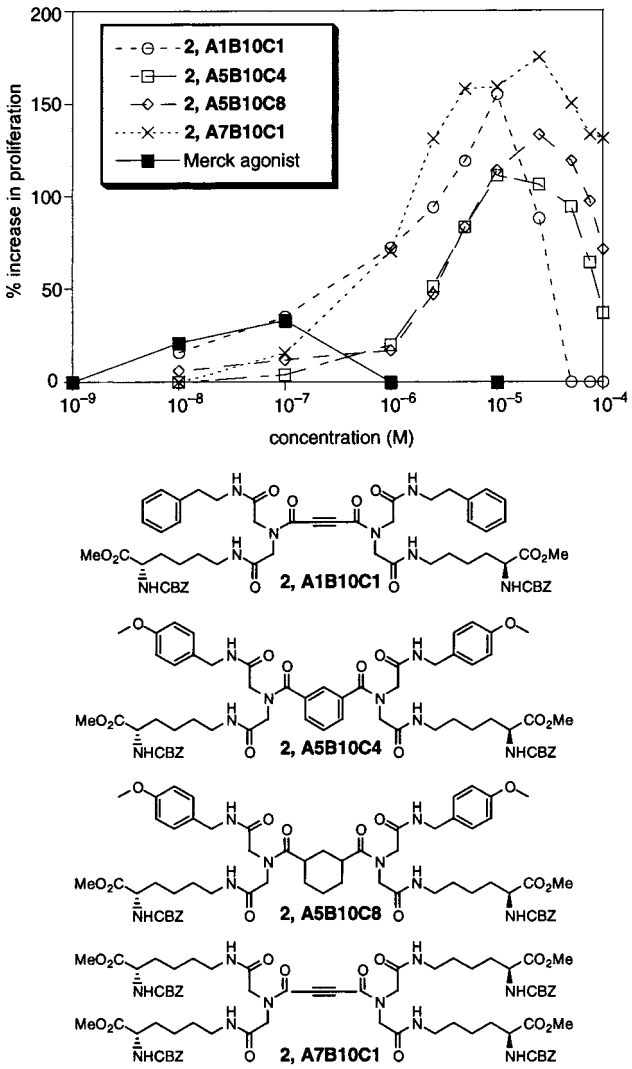


Figure 13. Concentration dependence of the stimulated increase in cell proliferation. UT-7 cells were incubated with varying concentrations (10^{-8}–10^{-4} M) of **2, A1B10C1**; **2, A5B10C4**; **2, A5B10C8**; **2, A7B10C1**; or the Merck agonist (10^{-9}–10^{-5} M) in 1% DMSO. The % increase in proliferation, relative to DMSO control, was determined by measuring the uptake of ^{3}H-thymidine.

were found to lead to similar activity when combined through linkers of different length and rigidity.

Dose-response curves were generated for the most active agents over a concentration range of 10^{-8}–10^{-4} M. Data for compounds **2, A1B10C1**; **2, A5B10C4**; **2, A5B10C8**; and **2, A7B10C1** are shown in Figure 13. Each of these compounds promotes a concentration-dependent increase in cell proliferation, beginning at submicromolar concentrations, that leads to a maximal 100–180% increase in stimulated growth at concentrations between 5 and 25 μM (Table 5). At higher concentrations, the observed proliferation begins to decrease presumably due to the saturation of EPOr with ligand, resulting in predominantly inactive 1:1 receptor–agent complexes, which has been observed with EPO and is expected of EPOr agonists.[38] To assess whether this effect was related to EPOr activation, the compounds listed in Table 5 were examined against a cell line that lacks the EPOr, FDC-P1.[39] Across a full concentration range (0.01–100 μM), no proliferation enhancement was observed (±15% of control). The unique concentration dependence of the cellular proliferation of the EPO-dependent UT-7 cell line and the lack of effect on the FDC-P1 cell line lacking an EPO receptor, combined with the competitive binding activity in the protein binding assay, suggest that the compounds are able to productively interact with EPOr and function as weak partial agonists. However, we cannot rule out the potential that the agents act at alternative, downstream intracellular sites not yet recognized. Nonetheless, this identification of small molecules that appear to act by promoting specific receptor dimerization and activation represents a remarkable achievement.[15] Although the discovery of antagonists of biological activity functioning through the disruption of protein–protein interactions has been achieved with increasing numbers and levels of success, the discovery of agonists functioning by promoting productive protein–protein interactions is extremely rare. The extent of stimulated cell growth is moderate, roughly 15–20% of the maximum response observed with EPO, but significant in comparison to the two previously reported EPOr agonists (Table 5). Notably, they are substantially more efficacious than the Merck agonist (15–20% versus 4% of the activity of EPO), and **2, A1B10C1** is equipotent with the Merck agonist at its maximal response dose of 100 nM and lower. Compounds **2, A1B10C1**; **2, A5B10C4**; **2, A5B10C8**; and **2, A7B10C1** (average MW = 1246) are considerably smaller than EMP1[8] (MW ca. 2100), which itself functions only as a dimer (Figures 1 and 14, MW ca. 4200), and the recently reported Merck nonpeptide EPOr agonist[12] (Figure 14, MW ca. 6400). They are synthesized in four steps from commercially available starting materials, allowing for easy derivatization and optimization. Key binding elements have been identified, potentially allowing for further reductions in the identified lead structures. The identification of these smaller, easily modified agents by a generalizable strategy represents a major step toward the development of a new generation of therapeutic agents.

The generation and screening of diverse libraries based on structurally unbiased or biased templates such as **1** or **6** using solution phase methods are straightforward and efficient approaches to the discovery of receptor binders which can subsequently be dimerized or oligomerized, allowing interactions

Table 5. Comparison of the Maximal Mitogenic Response of Selected Agents with EMP1, the Merck Agonist, and EPO

compound	maximal response (% increase in cell proliferation)	concentration (μM)
2, A1B10C1	155	10
2, A2B10C1	150	10[a]
2, A5B10C4	110	10
2, A5B10C8	133	25
2, A6B10C4	30	10[a]
2, A6B17C4	110	10[a]
2, A7B10C1	175	25
2, A7B10C4	95	5
2, A12B10C4	60	10[a]
2, A13B10C4	120	10[a]
Merck agonist	33	0.1
EMP1	670	0.5–10
EPO	812	0.01

[a] Highest concentration tested.

(38) Elliott, S.; Lorenzini, T.; Yanagihara, D.; Chang, D.; Elliott, G. *J. Biol. Chem.* **1996**, *271*, 24691. Schneider, H.; Chaovapong, W.; Matthews, D. J.; Karkaria, C.; Cass, R. T.; Zhan, H.; Boyle, M.; Lorenzini, T.; Elliott, S. G.; Giebel, L. B. *Blood* **1997**, *89*, 473.

(39) Dexter, T. M.; Garland, J.; Scott, D.; Scolnick, E.; Metcalf, D. *J. Exp. Med.* **1980**, *152*, 1036.

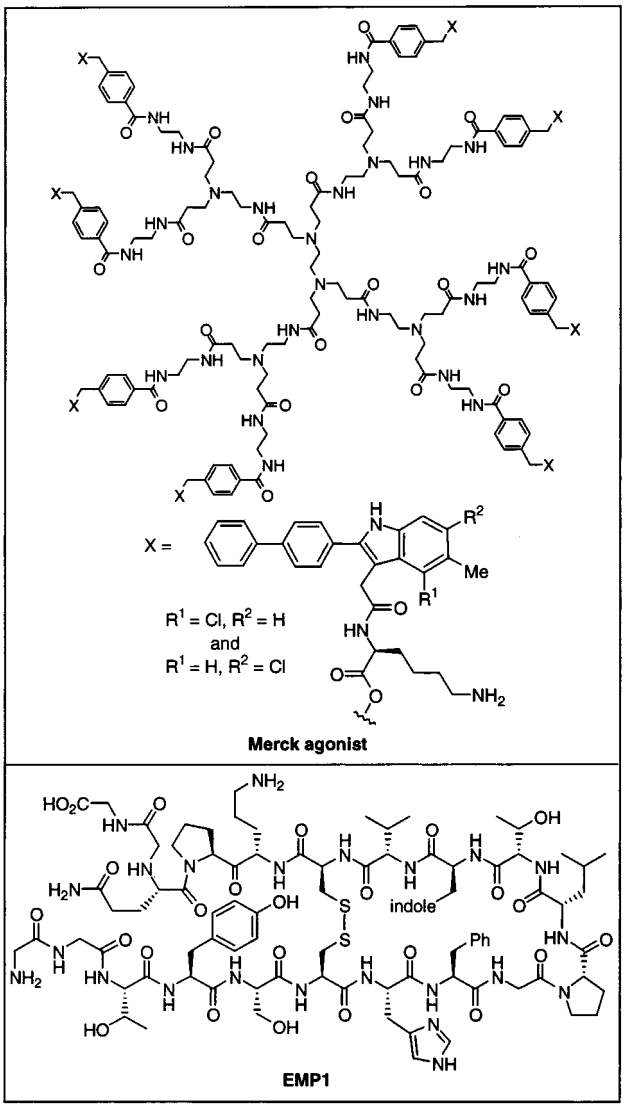


Figure 14. Structures of the Merck non-peptide agonist[12] and EMP1.[8]

with multiple protein binding sites leading to novel receptor agonists. This methodology can be readily extended to other receptor systems, including those activated by heterodimerization versus homodimerization, providing an even more general method for identifying novel small molecules which modulate protein−protein interactions. In the present example, the library subunits were chosen on the basis of the available structural information, expediting the lead generation process. The same strategy, however, has already been shown effective for the discovery of selective protein−protein antagonists in the absence of any specific structural information, utilizing randomized subunits known to have general binding interactions with protein surfaces.[40]

Conclusions

Through the screening of dimeric and higher order iminodiacetic acid diamide and isoindoline-5,6-dicarboxamide libraries, compounds were identified that function as competitors of EPO binding to its receptor. Many of the most active binders were further found to promote concentration-dependent proliferation of an EPO-dependent cell line, indicating their functional activity as partial agonists of EPO. This functional activity was observed with as few as two copies of the protein binding subunits, and some variability in the choice of the linker moiety was tolerated. They represent the third class of EPO partial agonists disclosed, but the first examples of relatively small compounds that function as EPO mimetics. Although their maximal functional activity is modest (15−20% that of EPO) and less than that reported for EMP1, they are substantially more efficacious than the Merck agonist. Their relative small size, the generalizable strategy for their discovery, and ease of synthesis makes them superb leads amenable to further optimization and characterization, and such studies are in progress.

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Supporting Information Available: Structures of the full set of subunits for libraries **3** and **4**, full details of the synthesis of library **13**, full characterization of **8**, **A1′**−**A10′**, matrix characterization of **9**, preparation and characterization of **13, A1′B1′C1′**−**10′** as individual compounds and the full mixture, and details of the cell proliferation assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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