

Use of combinatorial mutagenesis to select for multiply substituted human interleukin-3 variants with improved pharmacologic properties

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(Received 27 January 1999; revised 22 June 1999; accepted 11 August 1999)

A combinatorial mutagenesis strategy was used to create a collection of nearly 500 variants of human interleukin 3 (IL-3), each with four to nine amino acid substitutions clustered within four linear, nonoverlapping regions of the polypeptide. The variants were secreted into the periplasm of *Escherichia coli* and supernatants were assayed for IL-3 receptor-dependent cell proliferation activity. Sixteen percent of the variants, containing “region-restricted” substitutions, retained substantial proliferative activity through two rounds of screening. A subset of these was combined to yield variants with substitutions distributed through approximately half of the polypeptide. With one exception, “half-substituted” variants exhibited proliferative activity within 3.5-fold of native IL-3. A subset of the “half-substituted” variants was combined to yield “fully substituted” IL-3 variants having 27 or more substitutions. The combination of the substitutions resulted in a set of polypeptides, some of which exhibit increased proliferative activity relative to native IL-3. The elevated hematopoietic potency was confirmed in a methylcellulose colony-forming unit assay using freshly isolated human bone marrow cells. A subset of the multiply substituted proteins exhibited only a modest increase in inflammatory mediator (leukotriene C₄) release. The molecules also exhibited 40- to 100-fold greater affinity for the α subunit of the IL-3 receptor and demonstrated a 10-fold faster association rate with the α -receptor subunit. The multiply substituted IL-3 variants described in this study provide a unique collection of molecules from which candidates for clinical evaluation may be defined and selected. © 1999 International Society for Experimental Hematology. Published by Elsevier Science Inc.

Keywords: *Escherichia coli*—Hematopoietic cytokine—Leukotriene C₄—Protein engineering—Interleukin 3

Introduction

Interleukin 3 (IL-3) is a member of the hematopoietic cytokine family. It is a multilineage growth factor that stimulates the production of neutrophils, monocytes, eosinophils, erythroid cells, multipotent progenitors, and megakaryocytes [1,2]. In clinical settings requiring high-dose chemotherapy, IL-3 has been shown to stimulate the production of neutrophils and platelets. It has been studied clinically by itself and in combination with other growth factors [3]. IL-3 has been shown to potentiate and improve the effect of granulocyte colony-stimulating factor (G-CSF) for both neutrophil recovery and mobilization of hematopoietic progenitor cells for transplant [4,5]. At doses where IL-3 is effective in raising neutrophil and platelet counts, it also has inflammatory activity, possibly due to the release of histamine and sulfidoleukotrienes (leukotrienes C₄ [LTC₄], D₄ [LTD₄], and E₄ [LTE₄]) by eosinophils and basophils [6]. This feature of IL-3 has limited its clinical utility.

Creating protein variants with amino acid substitutions has been shown to be a powerful method to alter the biologic and biochemical properties of proteins, including several members of the hematopoietic cytokine superfamily: human growth hormone [7], interleukin 2 [8], and interferon α [9]. Furthermore, it has been found that multiple substitutions within these proteins may result in unique changes in biochemical properties or receptor binding kinetics [9–12]. Examples include consensus interferon, where the combination of 20 amino acid substitutions yielded a protein with unique set of biologic properties [9]; affinity-matured human growth hormone, where the combination of 15 amino acid substitutions resulted in a protein with a 400-fold increase in receptor binding [10]; and SC-65369, a truncated variant of IL-3 with 14 amino acid substitutions, which provided solubility properties suitable for a high-resolution nuclear magnetic resonance (NMR) structure to be determined [13].

In a recent study [14], 770 single site mutants of a truncated form of IL-3, IL-3_{15–125}, were constructed and evalu-

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ated for cell proliferation activity. It was found that a wide variety of substitutions could be made at most positions without loss of cell proliferative activity. These are defined as tolerant positions. In the present study, we created IL-3 variants with multiple substitutions at tolerant positions throughout the polypeptide. A subset of the variants exhibited significantly increased proliferative activity relative to native IL-3, and four of the variants have been shown to exhibit leukotriene release activity that is similar, i.e., within two- to threefold of native IL-3. These multiply substituted proteins comprise a library of variants for testing as pharmacologic agents for progenitor cell mobilization or the treatment of chemotherapy-induced neutropenia and thrombocytopenia. One of these, SC-55494 or daniplestim, has been described [15] and recently completed a Phase III clinical trial [16]. This report describes the method by which multiply substituted proteins with improved biologic properties were generated, using an approach that minimized the number of inactive proteins.

Materials and methods

DNA manipulation, plasmids, and expression in Escherichia coli

General DNA methods, bacterial hosts, and culture conditions have been described [14]. “Region-restricted” variants with nine or fewer substitutions were expressed as fusions with the LamB signal peptide under the control of the inducible *araBAD* promoter (pMON5988). The protein variants were secreted into the periplasm of *Escherichia coli* and extracted by osmotic shock [14]. The LamB signal peptide was removed as part of the secretion process, resulting in asparagine as the N-terminal amino acid. To permit further testing of selected variants, the genes were cloned into the vector pMON5847 from which proteins were produced in the *E. coli* cytoplasm [14,17]. After *in vitro* refold and purification, the protein concentration was determined by amino acid analysis. Protein variants produced in the cytoplasm were expressed with an additional alanine residue at their amino terminal end. Native IL-3_{1–133} is full length, recombinant human IL-3 produced in *E. coli* [14].

Combinatorial mutagenesis

To facilitate subsequent cloning and mutagenesis, the IL-3_{15–125} gene was engineered with unique *NcoI*, *EcoRV*, *NsiI*, *EcoRI*, and *HindIII* restriction sites. Four derivatives of pMON5988 were constructed in which the four regions of the IL-3_{15–125} gene (between adjacent pairs of restriction sites) were excised and replaced with a short oligonucleotide linker. The linker created a unique *NdeI* site and provided a screen for correct insertion of oligonucleotides coding for amino acid substitutions. The resulting deletion plasmids were designated pMON13356, pMON13357, pMON13358, and pMON13359, respectively. Variants with multiple substitutions in each of the four regions of the IL-3_{15–125} gene were created by cassette mutagenesis [18]. For each region, synthetic oligonucleotides were designed that restored the native coding sequence, except at positions that encoded the selected amino acid substitutions. Typically, the oligonucleotides were designed such that each complementary pair contained two or three amino acid substitutions. Separate pairs of oligonucleotides were designed to make all

of the possible combinations of the selected amino acid substitutions. Oligonucleotides were purified by polyacrylamide gel electrophoresis [19], 5'-phosphorylated by incubation with T4 kinase [20], and mixed at a concentration of 1 μ M in ligation buffer (25 mM Tris-HCl at pH 8.0, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 2 mM spermidine, 50 mM NaCl). After denaturing at 100°C, samples were permitted to cool slowly to 22°C. One picomole of each of the annealed oligonucleotide pairs was ligated with 0.2 pmol of plasmid DNA (pMON13356, pMON13357, pMON13358, or pMON13359), which had been digested with the appropriate restriction enzymes. Ligations were performed in 96-well microtiter plates at 22°C overnight, followed by digestion with *NdeI* to reduce parental vector background. The resulting plasmids were confirmed by restriction analysis. The genes encoding the IL-3 variants were analyzed by DNA sequencing. An additional amino acid substitution was identified in one of the “region-restricted” variants selected for further analysis. The region IV variant, R109E, K116V, N120H, A123E, also contained the conservative amino acid substitution T117S.

Proliferation assay for IL-3 variant proteins

Proliferative activity was determined using AML 193.1.3 cells [14]. In the initial screening assay, crude osmotic shock fractions containing the secreted “region-restricted” variant proteins were tested for proliferative activity at 1:10,000 and 1:50,000 dilutions. In the second screening of the “region-restricted” variants, osmotic shock fractions were assayed using a full 15-point dilution series. Activity of variants was defined as the concentration that gave 50% maximal proliferation (EC₅₀). The concentration of protein in osmotic shock fractions was determined by ELISA [14]. Activity of the secreted variants was expressed relative to that of unsubstituted IL-3_{15–125} produced under identical conditions.

To determine the cell proliferation activity of purified variants, AML 193.1.3 assays were performed in triplicate using a full 15-point dilution series. Potency of variants was defined as the concentration that gave EC₅₀. Relative potency was determined by dividing the EC₅₀ for native IL-3 by the EC₅₀ for the variant. From 1 to 12 preparations of each purified variant was assayed.

Human IL-3-mediated LTC₄

release from human mononuclear cells

Heparin-containing human blood was layered onto an equal volume of Ficoll-Paque (Pharmacia, Piscataway, NJ) medium (density 1.077 g/mL) and centrifuged at 300g for 30 minutes at 22°C. The band containing the mononuclear cells was adjusted to 50 mL with Dulbecco's phosphate-buffered saline (Gibco, Gaithersburg, MD) and centrifuged at 400g for 10 minutes at 22°C. The cell pellet was washed twice with HA buffer (20 mM HEPES, 125 mM NaCl, 5 mM KCl, 0.5 mM glucose, 0.025% bovine serum albumin) and centrifuged at 300g for 10 minutes. Cells were resuspended in HA buffer supplemented with 1 mM CaCl₂ and 1 mM MgCl₂, at a concentration of 1 \times 10⁶ cells/mL. The cells were transferred into a 96-well tissue culture plate, preincubated at 37°C for 10 minutes, and primed by the addition of various concentrations of cytokine (1.28 to 100,000 fM). After 15 minutes, cells were treated with 50 nM fMet-Leu-Phe (Calbiochem, La Jolla, CA) to activate leukotriene release and incubated for an additional 10 minutes. The incubation was terminated by centrifuging the plates at 350g at 4°C for 20 minutes. The supernatants were removed, and LTC₄ concentrations were determined by ELISA

(Cayman, Ann Arbor, MI). LTC₄ release activity was expressed relative to that of IL-3, which was used as a standard in each assay. The relative potency for each variant was calculated by dividing the EC₅₀ for native IL-3 by the EC₅₀ for the variant. For each variant, blood samples from at least three human donors were used in independent assays, and the mean was calculated, expressed relative to the activity of IL-3. *E. coli* osmotic shock fractions could not be used in the LTC₄ release assay due to high background levels. Hence, only purified proteins were tested in this assay.

Receptor binding studies

Analysis of binding of IL-3 and variants to IL-3R α /Fc (a fusion protein constructed by ligating the extracellular domain of the IL-3 α -receptor subunit [21] to a fragment of a modified mouse IgG2a Fc protein [22]) was performed on the BIAcore 2000 surface plasmon resonance instrument. Two flow cells were monitored simultaneously: a blank, which contained no rabbit anti-mouse Fc (RAMFc) reagent; and an active cell, which had RAMFc bound to the surface. The cells consisted of a gold surface to which a carboxymethylated dextran layer was bound. The dextran was activated for 7 minutes with a 1:1 mixture of N-ethyl-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), followed by washing with running buffer (for blank cell surface) or 0.05 mg/mL RAMFc solution (for active cell surface), respectively. The excess activated groups were deactivated with ethanolamine for 7 minutes.

During each run, a freshly immobilized or regenerated RAMFc surface was exposed to a 6-minute injection of the IL-3R α /Fc containing 5 mg/mL carboxymethylated dextran solution at a flow rate of 10 μ L/min. The surface was washed for 25 minutes with the running buffer, after which the flow rate was changed to 20 μ L/min and the IL-3 variant was injected for 3 minutes for the association phase. The sample was allowed to dissociate from the receptor for 2 minutes by running buffer alone across the bisensor chip. At this point, the chip was regenerated with a 3-minute injection of 1N formic acid, which stripped off the receptor while leaving the RAMFc intact. To determine the kinetic parameters, a series of concentrations for each variant was analyzed using a global fitting program supplied with the BIAcore system. This program fit the entire association and dissociation data for all concentrations simultaneously to yield k_a and k_d values. The K_d was obtained from dividing k_d by k_a .

High-affinity IL-3 receptor binding studies were performed on TF-1 cells as described previously [15].

Circular dichroism

Proteins were dissolved in 10 mM Tris buffer (pH 7.5). The concentration of samples was determined by measurement of A-280 on a Beckman DU-7400 spectrophotometer using theoretical extinction coefficients. To get an accurate protein concentration, the A-280 of samples was corrected for light scattering. The circular dichroism spectra of proteins were measured from 185 to 260 nm at 20°C using a Jasco 715 spectropolarimeter. The α -helical content of proteins was calculated based on the mean residue molecular ellipticity at 222 nm as given by Equation 1 [23]:

$$\% \text{ helix} = (2340 + \text{MRME}_{222}) / 30300 \quad (1)$$

where MRME₂₂₂ is the mean residue molecular ellipticity at 222 nm.

ELISA assays to determine antibody reactivity

The reactivity of IL-3 protein variants with an affinity-purified goat anti-IL-3 polyclonal antibody was evaluated using a sand-

wich ELISA as described previously [14]. The percent reactivity is the ratio of the concentration of variant protein determined in the ELISA to the protein concentration determined by amino acid composition.

CFU assay

IL-3 variants were assayed for CFU activity using CD34⁺ enriched human bone marrow as described previously [15]. Based on the number of colonies per 100,000 cells, two quantitative measures of performance in the CFU assay were calculated: relative response and relative concentration. The relative response is obtained by dividing the maximal response for the test sample by the maximal response of native IL-3. Relative response values >1 indicate a higher maximal response for the protein variant tested. The relative concentration is obtained by dividing the concentration for native IL-3 by the concentration for the test sample required to obtain the same response (computed at three response levels). Thus, the relative concentration is similar to a relative potency calculated using EC₅₀. Relative concentration values >1 indicate greater potency (lower concentration to obtain the same response). The signed rank test was used to assess whether the maximal response and the potency of the variants were significantly greater than native IL-3 (one-tailed at 0.05 level of significance).

Results

Mutagenesis strategy

We previously showed that a truncated form of IL-3 (IL-3_{15–125}) is highly accepting of single amino acid substitutions (at tolerant positions) without loss of cell proliferation activity [14]. We wanted to determine whether it was possible to construct extensively substituted IL-3 molecules as a route to create a library of unique IL-3 receptor agonists with an array of pharmacologic activities. To obtain a diverse yet manageable population of molecules, a limited number of substitutions at defined tolerant positions were chosen for combination. Substitutions at tolerant positions were chosen to increase the likelihood of generating proliferation active multisubstituted variant proteins. At each of 26 tolerant positions in truncated, IL-3_{15–125}, two amino acid substitutions were selected to replace the native amino acid (Fig. 1). In three instances (positions 59/60, 62/63, and 85/87), substitutions were made at one of the two positions indicated. In most cases, amino acids with a different chemical character from that of the native sequence were chosen for substitution, as illustrated by different colors in Figure 1. This was done to increase the diversity of the resulting multiply substituted proteins. For example, at position 29, a nonpolar Val (yellow) and a charged Arg residue (blue) were substituted for the polar Gln (green) in the native sequence.

The mutagenesis process was performed in two phases. In the first phase, cassette mutagenesis was used to create four to nine substitutions in each of four regions (I–IV) within the gene. The regions were defined by unique restriction sites and roughly corresponded to four helical segments of the protein. In the second phase of mutagenesis, multiple

Region I			Region II			Region III			Region IV		
Asn ¹⁸	Leu	Ile	Asn ⁵¹	Ser	Arg	Ala ⁷³	Gly	Leu	Arg ¹⁰⁹	Ser	Glu
Thr ²⁵	His	Gln	Arg ⁵⁵	Leu	Thr	Ser ⁷⁶	Ala	Val	Lys ¹¹⁶	Ser	Val
Gln ²⁹	Val	Arg	Glu ⁵⁹ Ala ⁶⁰	Leu ⁵⁹	Ser ⁶⁰	Lys ⁷⁹	Asp	Arg	Asn ¹²⁰	Gln	His
Leu ³²	Ala	Asn	Asn ⁶² Arg ⁶³	Val ⁶²	His ⁶³	Leu ⁸²	Val	Gln	Ala ¹²³	Ser	Glu
Phe ³⁷	Ser	Pro	Ser ⁶⁷	His	Asn	Leu ⁸⁵ /Leu ⁸⁷	Val ⁸⁵	Ser ⁸⁷			
Gly ⁴²	Ser	Ala	Gln ⁶⁹	Thr	Glu	Thr ⁹³	Arg	Ser			
Gln ⁴⁵	Val	Met				His ⁹⁸	Ile	Thr			
						Asp ¹⁰¹	Ala	Thr			
						Asn ¹⁰⁵	Ser	Gln			

Figure 1. The amino acids chosen for substitution at each position in each of the four regions (I–IV). For each region, the first column lists the amino acid present in native IL-3. The remaining columns illustrate the substituted amino acids at each position. For positions 59/60, 62/63, and 85/87, substitutions were made at one of the two positions. Color coding indicates the chemical class of each amino acid: nonpolar amino acids are yellow, polar amino acids are green, and charged amino acids are blue.

regions were combined to create IL-3 variants, with half of the polypeptide containing substitutions. The “half-substituted” proteins were combined to yield “fully substituted” variants with 26 or more amino acid substitutions. In addition, each of the “fully substituted” proteins were expressed with an amino terminal Ala, thus creating truncated variants with a total of 27 amino acid substitutions (see Materials and methods).

Screening of multiply substituted IL-3 variants for proliferative activity

In the first phase, variants containing substitutions in each of four different regions were expressed as fusions with the LamB signal peptide and secreted into the periplasm of *E. coli*. For the primary screen, two independent cultures from each cassette mutagenesis were grown in 1-mL cultures in 24-well tissue culture plates and induced for protein expression. Postinduction samples were fractionated by osmotic shock, and supernatants were screened for activity in the AML 193.1.3 cell proliferation assay at 1:10,000 and 1:50,000 dilutions. Variants having at least 30% of the [³H]thymidine incorporation relative to unsubstituted IL-3_{15–125} were selected for reinduction and tested in the secondary assay. Nearly 500 multiply substituted variants from the four regions were evaluated in the first screen, and approximately one third of the variants tested were found to be active. Clones expressing active variants were regrown and tested in secondary AML 193.1.3 assay (see Materials and methods). In the secondary screen, 79 of the variants containing “region-restricted” substitutions (or 16% of the total) demonstrated growth factor activity >30% of that seen for unsubstituted IL-3_{15–125}. Twelve of the 79 substituted genes were selected for further analysis. These particular “region-restricted” variants were selected for additional

combination based primarily on high proliferative activity and, to a lesser extent, on the diversity of amino acid substitutions. The substitutions within the 12 “region-restricted” proteins are shown in Figure 2A. Note that for each region there are three proteins, each with a unique set of site substitutions. Each set of substitutions is designated by a different color (green, blue, or magenta). The genes encoding these proteins were placed into appropriate vectors, and proteins were produced in an insoluble form in cytoplasmic inclusion bodies in *E. coli*. Using this vector, the proteins were expressed with an additional Ala at the N-terminus. To purify the proteins, inclusion bodies were solubilized in 6M guanidine HCl, refolded, and purified according to procedures described previously [14]. The AML 193.1.3 cell proliferation activity of the purified, “region-restricted” variants relative to native IL-3 is shown in Figure 2A. Examination of the proliferation activity indicated that this set of “region-restricted” variants possess similar activity to that of native IL-3.

Further combination of substitutions

In the second phase of the mutagenesis, variant genes were constructed by making combinations of the multisubstituted regions in the truncated IL-3 backbone. Region I + Region II (3 variants), Region II + Region III (2 variants) Region III + Region IV (3 variants), or Region I + Region IV (7 variants) combinations were constructed. These regions are adjacent either in the linear sequence of the protein or in the three-dimensional folded structure. A total of 15 “half-substituted” variants (with 11–15 amino acid substitutions in each) were expressed as inclusion bodies, and the proliferation activity of each protein was measured after solubilization, refold, and purification. Figure 2B shows a schematic diagram of several representative variants, with their corre-

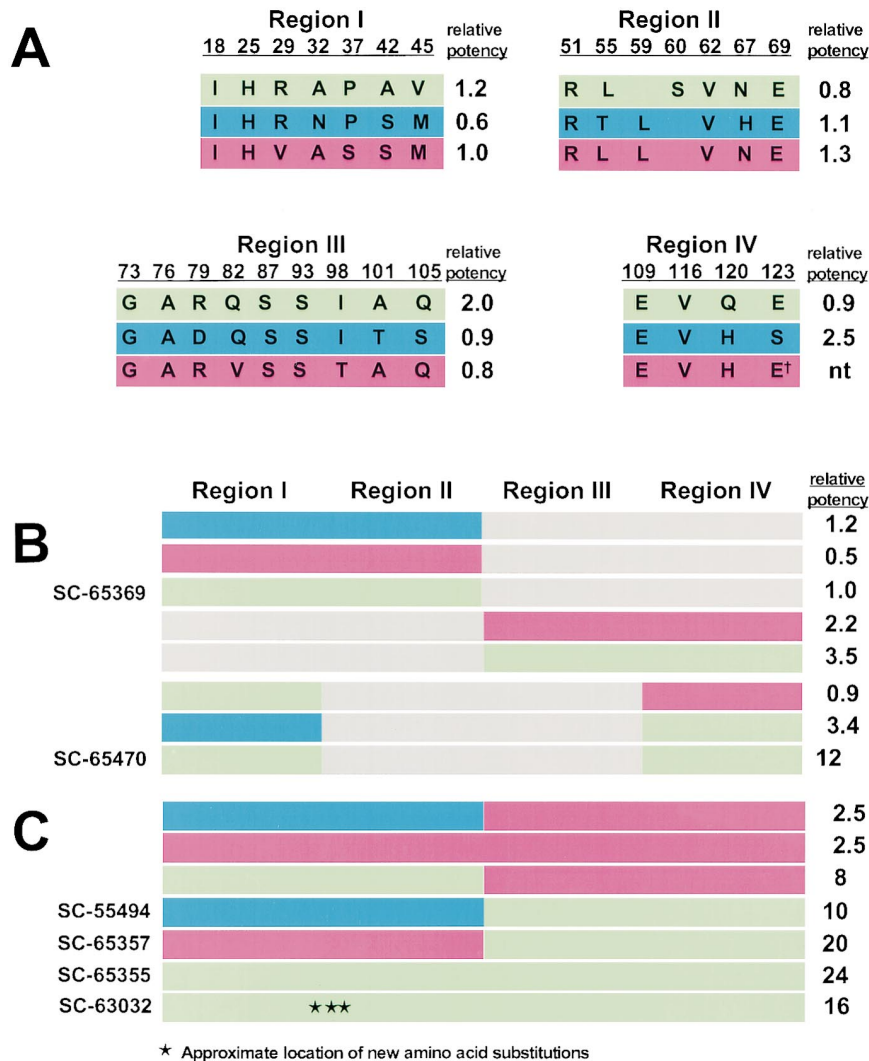


Figure 2. AML 193.1.3 cell proliferative activity relative to native IL-3. **(A)** Relative potency of the 12 “region-restricted” truncated variants compared to native IL-3. Single letter amino acid code is used to designate the substituted amino acids in each variant protein. Within each region, different colors are used to represent distinct sets of amino acid substitutions. The amino acids that are not listed correspond to those found in native IL-3. †An additional amino acid substitution was identified in this “region-restricted” variant at position 117 (see Materials and methods). **(B)** Relative potency of eight “half-substituted” variants compared to native IL-3. (Eight were selected for illustration of 15 proteins that were generated and tested). Color coding for each of the regions corresponds to the set of amino acid substitutions from **(A)**. Gray indicates that all amino acids in that region are those in unsubstituted IL-3_{15–125}. **(C)** Relative potency of seven “fully substituted variants” compared to native IL-3. The stars indicate the locations of the substitutions G42D, D46S, and E50D, which distinguish SC-63032 from SC-65355.

sponding cell proliferative activity relative to native IL-3. For each variant, the mean of at least three independent assays is reported. Fourteen of the 15 “half-substituted” variants that were generated retained proliferative activity within 3.5-fold of native IL-3. One of the “half-substituted” variants, SC-65470, had proliferation activity that was more than 3.5-fold greater than that of native IL-3. To attempt to generate additional variants with greater proliferative activity, more extensively substituted variant genes were constructed by combining all four of the regions, in different combinations, resulting in nine IL-3 variants with 27 amino acid substitutions (six of which are illustrated in Fig. 2C).

Interestingly, on combination of “half-substituted” proteins with other “half-substituted” proteins, several “fully substituted” proteins with 27 amino acids changes (e.g., SC-55494, SC-65357, and SC-65355) exhibited significantly elevated proliferation activity. Thus, it was observed that combining “half-substituted” variants yielded several “fully substituted” protein variants with substantially improved activity.

To evaluate the effect of increasing the number of substitutions beyond 27, additional amino acid changes were incorporated into one of the multiply substituted proteins, SC-65355. Substitution of a Gln at position 112 (a site that was

previously identified as tolerant [14]) caused ~60-fold decrease in proliferation activity. In another example, substitutions that were known to increase proliferative activity as single site substitutions (G42D, D46S, and E50D) were added to SC-65355, resulting in SC-63032 (Fig. 2C). The cell proliferative activity of SC-63032 decreased slightly relative to SC-65355, yet was still 16-fold greater than native IL-3. Thus, increasing the number of substitutions beyond 27 creates greater diversity in IL-3, yet in these examples, the proliferative activity was not increased by the additional substitutions. Due to increased cell proliferation activity compared to native IL-3, variants SC-65355, SC-65357, and SC-63032 (in addition to SC-55494) were chosen for further study.

Inflammatory mediator release activity of multiply substituted, truncated IL-3 variants

Purified preparations of multiply substituted variants, SC-65355, SC-55494, and SC-65357, were assayed for LTC₄ release activity using human mononuclear cells. As illustrated in Table 1, these variants exhibited leukotriene release activities that were not more than 3.3-fold greater than native IL-3. In contrast, the AML 193.1.3 cell proliferation activity for these variants was increased from 10- to 24-fold compared to native IL-3. The ability of SC-63032 to prime LTC₄ release was tested in a modified assay and was found to exhibit leukotriene release comparable to that obtained for SC-55494 (data not shown).

Circular dichroism studies

Circular dichroism studies were done to determine whether the overall, three-dimensional structure of the variants was consistent with that of native IL-3. The four multiply substituted variants tested (SC-55494, SC-65355, SC-65357, and SC-63032) were indistinguishable from native IL-3 or truncated IL-3_{15–125}, having virtually identical spectra with the

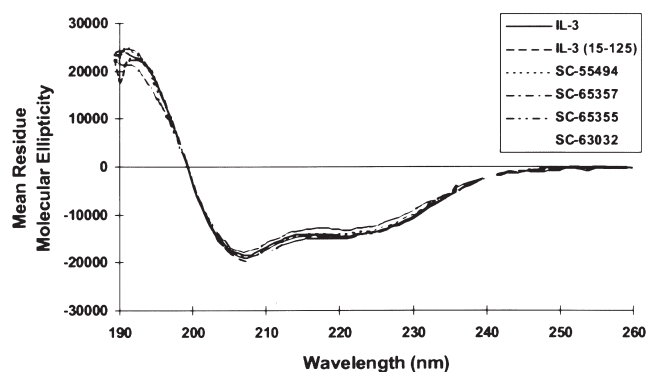


Figure 3. Circular dichroism spectra (190–260 nm) for multiply substituted variants compared to native IL-3 and unsubstituted IL-3_{15–125}.

double minimum at 208 and 222 nm characteristic of helical proteins (Fig. 3). The α -helical content of these proteins was calculated, and no significant differences were found. These similarities show that the large number of amino acid substitutions in these proteins do not substantially alter the secondary structure of the folded protein.

Receptor binding properties of multiply substituted proteins

The binding of the multiply substituted variants to the high-affinity IL-3 α / β receptor (IL-3R) complex on TF-1 cells was measured using competition binding studies (Table 2). A substantial (5- to 10-fold) increase in high-affinity binding was observed for the “fully substituted” proteins with improved proliferative activity (SC-55494, SC-65355, SC-65357, and SC-63032) compared to native IL-3.

To explore the basis for this improved binding to the high-affinity receptor, the kinetics of binding to a soluble form of the low-affinity α subunit of the IL-3 receptor complex (IL-3R α) was determined by surface plasmon resonance spectroscopy (Table 2). These results demonstrate that unsubstituted IL-3_{15–125} is only modestly improved over native IL-3 with respect to binding to the α subunit of the receptor. In contrast, the multiply substituted proteins with elevated proliferation activity exhibit an increased association rate, k_a , (6- to 9-fold) and decreased dissociation rate, k_d , (>5-fold) for the low-affinity (IL-3R α) receptor. The additional substitutions in SC-63032 (near the predicted α subunit contact site) lead to a further decrease in the dissociation rate compared to SC-65355. The low-affinity receptor binding (K_D) of SC-63032 is 100-fold tighter than native IL-3.

Antibody binding to multiply substituted variants

A measure of the difference between the multiply substituted IL-3 variants and truncated IL-3 variants with only one or two substitutions is the relative binding to these variants to polyclonal antibodies raised to native IL-3. The polyclonal antibody binding of variant proteins was compared in a sandwich ELISA format. The variant proteins

Table 1. Relative potency of multiply substituted variants compared to native interleukin 3

Compound	Relative potency*	
	LTC ₄ release	AML cell proliferation
IL-3	1	1
SC-65355	2.8 \pm 1.0 (n = 7)	24 (n = 10)
SC-55494	2.2 \pm 0.8 [†]	10 [†]
SC-65357	3.3 \pm 2.7 (n = 6)	20 (n = 12)
SC-63032	— [‡]	16 (n = 8)

n = number of replicate experiments.

*Activity relative to native interleukin 3 (IL-3). Leukotriene C4 (LTC₄) release from mononuclear cells \pm SD. Acute myelogenous leukemia (AML) proliferation activity of variants.

[†]Data from Thomas et al. [15].

[‡]The ability of SC-63032 to prime LTC₄ release was tested using a modified assay protocol and exhibited leukotriene release activity comparable to SC-55494.

Table 2. Comparison of IL-3R binding for multiply substituted variant proteins

Compound	High-affinity (α/β)	Low-affinity (α) soluble receptor		
	K_i (nM)	k_a (1/sM $\times 10^5$)	k_d (1/s $\times 10^{-3}$)	K_D (nM)
IL-3	3.0 \pm 0.3	1.1 \pm 0.3	96 \pm 8	910 \pm 250
IL-3 _{15–125}	4.35 \pm 2.33	3.3 \pm 0.4	170 \pm 21	510 \pm 72
SC-55494	0.38 \pm 0.05	9.3 \pm 2.3	20 \pm 1	23 \pm 6
SC-65355	0.65 \pm 0.25	9.4 \pm 1.2	17 \pm 2	19 \pm 1
SC-65357	0.64 \pm 0.38*	10.0 \pm 0.5	18 \pm 1.3	18.1 \pm 0.6
SC-63032	0.24 \pm 0.03	6.6 \pm 0.1	5.8 \pm 0.1	8.8 \pm 0.1

Values are the average \pm SD of ≥ 3 experiments.

*n = 2.

were captured by an immobilized anti-IL-3 antibody and detected with a peroxidase-labeled polyclonal antibody (Table 3). In this format, the polyclonal antibodies efficiently recognized the truncated IL-3 variants with one or two amino acid substitutions. In contrast, anti-IL-3 polyclonal antibodies did not recognize the multisubstituted variants. Conversely, when the ELISA plates were coated with polyclonal antibodies raised against one of the multisubstituted variants, SC-55494, no IL-3 binding was detected (data not shown). These studies suggest that whereas the amino acid contacts required for potent IL-3 receptor recognition are preserved in the multiply substituted proteins, the epitopes recognized by a neutralizing polyclonal antibody are not retained.

Colony-forming unit activity

To evaluate the hematopoietic activity of multiply substituted IL-3 variants, SC-65355 and SC-63032 were tested in a methylcellulose colony-forming unit (CFU) assay using CD34⁺ cells enriched from human bone marrow (Fig. 4). Both of the variants demonstrate increased activity in the CFU assay compared to native IL-3. Incubation with these proteins resulted in a greater number of colonies at all of the concentrations tested. For SC-65355 (n = 8), the average maximal response was 1.4-fold greater than for IL-3 (a significantly higher response level, $p = 0.03$), indicating a small but consistent increase in the number of hematopoietic progenitor cells are stimulated by SC-65355 compared to native IL-3. The increased potency of SC-65355 was

demonstrated by the decreased concentration of SC-65355 (ranging from 12.8- to 24.4-fold) that was needed to achieve the same response as native IL-3 at three levels (17%, 50%, and 83%) of the maximum response ($p < 0.02$). For SC-63032 (n = 7), the average maximal response was 1.8-fold greater than IL-3 ($p = 0.02$). Again for this protein, the average relative concentrations at three response levels indicated significantly greater potency (ranging from 27.7- to 68.1-fold, $p < 0.02$). Similar CFU activity results for SC-55494 have been described [15], thus demonstrating that this family of high-affinity IL-3 receptor agonists stimulates a higher frequency of hematopoietic progenitor cells than does native IL-3 at the same concentration. In addition, the maximal CFU response for SC-55494 is greater than that of native IL-3, indicating a greater number of colony-forming cells are stimulated to proliferate.

Discussion

In the present study, we used information regarding tolerant positions, and acceptable amino acid substitutions at these positions, to generate novel IL-3 receptor agonists. Variants with multiple changes in primary sequence were made. The results indicate that IL-3 receptor agonists with multiple substitutions possess unique biochemical and pharmacologic properties. The novel features of the bioactivity of these multiply substituted proteins could be due to the overall cumulative effect of combining multiple substitutions into a single molecule [9,11,12]. Alterations in one part of the protein may be independent of those in another, or they may complement, or fit with, changes in another portion, resulting in more potent proteins. In our work, both enhanced cell proliferation activity and CFU activity in multiply substituted proteins were observed. Substitutions in single regions of the protein resulted in proliferation activity that was only modestly better or the same as native IL-3. When these amino acid changes in single regions were combined in “half-substituted” proteins, proliferation activity was not increased significantly, except in one case. However, when the “half-substituted” proteins were combined to produce “fully substituted” versions, the proliferation activity was

Table 3. Relative binding of IL-3 variants in an IL-3 ELISA*

No. of substitutions	No. of molecules tested	Percent reactivity
1–2	40	22–365
13–14	6	0.2–8.2 [†]
27	9	<1.5 [†]

* Relative reactivity was measured in a ELISA using a goat polyclonal antibody raised against native IL-3.

[†]Significantly different ($p < 0.0001$) from the 1–2 substitution group using the Kruskal-Wallis test.

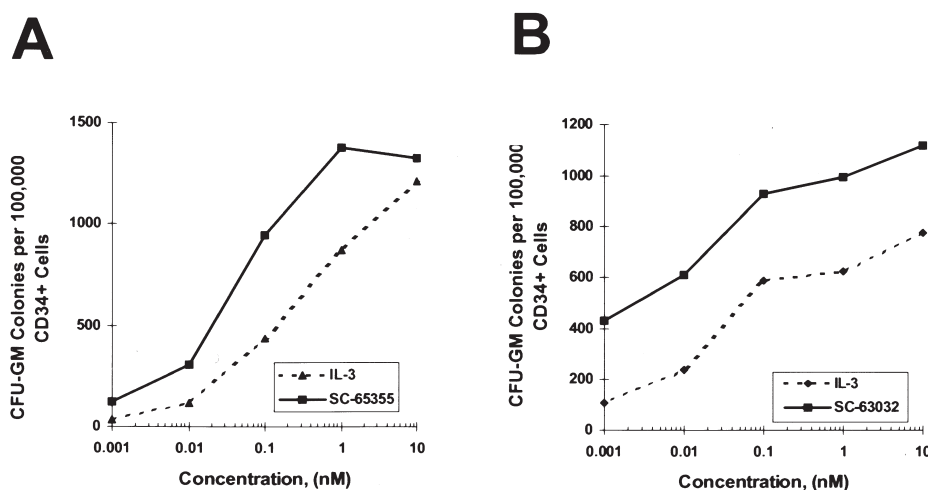


Figure 4. Methycellulose hematopoietic colony-forming unit (CFU) assay with human CD34⁺ bone marrow cells. (A) Number of CFUs per 100,000 CD34⁺ cells is the average of eight assays comparing SC-65355 and native IL-3. (B) Number of CFUs per 100,000 CD34⁺ cells is the average of seven assays comparing SC-63032 and native IL-3.

increased dramatically in several proteins and up to 24-fold in the case of SC-65355. The increased activity appears to be greater than what would be expected from simple additivity. This may be the consequence of substitutions distant in the linear sequence complementing one another in a unique way by their proximity in the folded, tertiary structure (Fig. 5.)

It was noted previously [24] that most tolerant positions in IL-3 are on the surface of the protein. NMR-based eluci-

dation of the IL-3 structure [13] indicates that the tolerant surface residues chosen for substitution in this study are dispersed and cover a large area. This is illustrated in Figure 5, where amino acids shown in magenta correspond to Region I substitutions; cyan, Region II; dark blue, Region III; and green, Region IV. The solvent accessible area of all the substituted residues in the proteins with 27 substitutions is approximately 40% of the total solvent accessible surface. This degree of change in solvent-exposed regions likely ac-

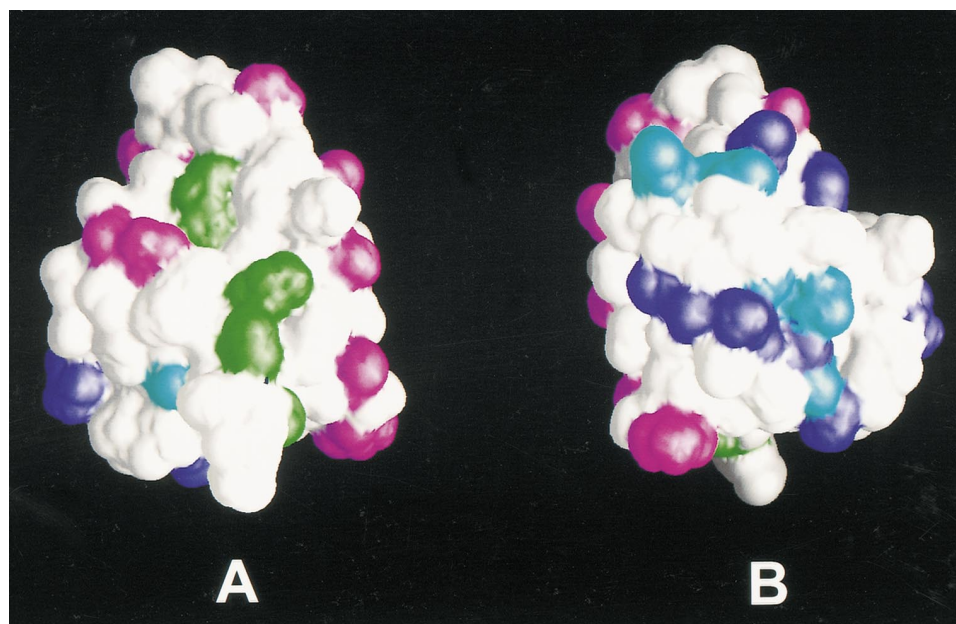


Figure 5. Solvent accessible surface model for truncated IL-3. Substituted residues in each of the four regions are illustrated in different colors: Region I, magenta; Region II, cyan; Region III, dark blue; and Region IV, green. (A) View of the structure with helices A and D in front. (B) The molecule in (A) is rotated 180° so that helices B and C are facing forward. The solvent accessible surface was calculated using GRASP [34] on a structural model of IL-3₁₅₋₁₂₅ [13,24].

counts for the lack of IL-3 antibody cross-reactivity to the multiply substituted proteins and provides further evidence that the exterior of the multiply substituted proteins is significantly different from that of native IL-3. It also is clear from Figure 5 that residues from different regions are in close proximity on the surface of the protein. The proximity of the substitutions in Regions I (magenta) and IV (green) in the three-dimensional folded structure of the protein may explain the complementary effects of these substitutions, as seen in increased proliferation activity of SC-65470, the only “half-variant” identified with >3.5-fold increased proliferation activity. Region I corresponds to the A and A' helices (amino acids 16–26 and 42–49, respectively) and Region IV to the D helix (amino acids 104–122), which together contribute to the binding to the α subunit of the IL-3 receptor.

The increase in receptor-mediated proliferation activity in clonal cell assays and in the hematopoietic CFU assay that is exhibited by several of the multiply substituted proteins may be due to their ~ 10 -fold increased affinity for the intact IL-3 α/β receptor (Table 2). Specifically, this may be the result of increased affinity (K_D) for the α subunit of the IL-3R i.e., 40- to 100-fold (Table 2). The increased affinity for the multiply substituted variants is due in large part to a ~ 10 -fold faster association rate compared to native IL-3. Two of the additional amino acid substitutions incorporated into SC-63032 (G42D and D46S) have been shown to individually increase binding of IL-3₁₅₋₁₂₅ to the low-affinity receptor IL-3R α as single substitutions (unpublished observation). These sites map in the A' helix at or near the predicted binding site for the α subunit of the IL-3 receptor complex [24]. Therefore, it is not surprising that the added substitutions in SC-63032 result in a threefold decrease of the dissociation rate from the α subunit as compared to SC-65355 (Table 2). The increase in binding affinity may enable beneficial pharmacologic outcomes for SC-55494, SC-65355, SC-65357, and/or SC-63032, such as a lowering in the efficacious dose and diminished side effects.

Interestingly, enhancement of binding of a ligand to its receptor does not always translate into increased receptor-mediated biologic activity. For instance, human growth hormone mutants with 400-fold increased receptor affinity have been generated; however, these variants do not demonstrate significantly increased bioactivity [25]. Several postreceptor binding events, such as intracellular signaling, also must occur for cell proliferation. Because proliferation was the essential criterion for selection of the IL-3 variants in these studies, the overall process of receptor binding, and postreceptor effects and subsequent biology, is expected to be encompassed in the variants that have been generated herein. An unexpected finding from this work is the improvement in the CFU activity, where several of the multiply substituted molecules not only were more potent than IL-3, but also stimulated an increased maximal response. One hypothesis for this improved activity is that the increased re-

ceptor binding affinity and/or low-affinity receptor association rate of the multisubstituted proteins promotes the proliferation of a type of hematopoietic precursor cell that does not proliferate in response to native IL-3.

The *in vivo* efficacy of one of these multiply substituted proteins, SC-55494 (daniplestim), was demonstrated initially in a nonhuman primate model of radiation induced myelosuppression alone [26] and in combination with G-CSF [27] and in a rhesus model of peripheral blood stem cell mobilization in combination with G-CSF [28]. Recently, encouraging results with the administration of daniplestim plus G-CSF in a Phase II clinical trial of peripheral blood stem cell mobilization were reported [29].

A potentially important property of a subset of the multiply substituted proteins is the observation that their leukotriene release activity remained similar, i.e., within two- to threefold, to that of native IL-3, whereas the proliferation activity increased substantially. The biologic basis of the separation in proliferation activity and leukotriene release is not understood. Perhaps different receptor subtypes (e.g., IL-3R subtypes) or accessory proteins are utilized for the two different biologic effects, and these multiply substituted proteins bind and signal preferentially through each receptor. The therapeutic implication for clinical utilization of a multiply substituted protein with an increased therapeutic ratio may be dramatic. Dose limiting toxicity is evident at higher doses of IL-3 due to flulike and inflammatory symptoms possibly associated with histamine and leukotriene release [3,6]. These inflammatory side effects may limit utilization of native IL-3 in the clinic.

The availability of this family of related protein variants provides the opportunity to optimize a number of additional properties that are likely to be useful for development of a successful protein therapeutic. These include production properties such as higher expression level; easier purification and formulation; enhanced stability; and longer *in vivo* half-life. Although most of these properties could not be assessed for the large number of variants examined in the initial screen, they can be investigated for the limited set of variants generated here. Daniplestim and other variants were found to have acceptable characteristics for recombinant protein production and therapeutic applications, in addition to their improved biologic profile. These improved physical properties also facilitated better understanding of structure/activity relationships for IL-3. In the course of these studies, a variant with appropriate solubility and stability properties for NMR studies (SC-65369) was identified. The solution structure [13,30] of this variant was determined. This structural data, together with information on residues critical for biologic activity, led to an improved understanding of the structure activity relationships for IL-3 [24].

A wide variety of mutagenesis approaches are available to modify the properties of proteins. In some cases, a great deal of structural and functional information already is available. In this situation, only a limited number of substi-

tutions may need to be generated to obtain the desired activity [31]. At the other end of the spectrum are mutagenesis studies in which little, if any, structural and/or functional information is available, and these situations frequently require screening a very large number (e.g., 10^4 to 10^{10}) of candidate proteins to identify those with suitable properties. Examples of these include shuffling and/or random mutagenesis strategies [32,33]. Prior to beginning our work on IL-3, little structural information was available. In initial IL-3 structure/activity studies [14], single-substituted IL-3 receptor agonists were identified as the result of a systematic approach of replacing residues with diverse amino acid substitutions and thus defining the acceptability (i.e., tolerance) of the substitutions with respect to proliferation activity. The tolerance map was the starting point for the work described within. A variety of substitutions then were combined to generate multiply substituted polypeptides. The choice to use a receptor-mediated cell-based proliferation assay to assess the biologic properties of substituted proteins limited the number of variant proteins that could be evaluated for practical reasons. The prior data on tolerance to amino acids substitution facilitated the choice of substitutions for combination in the multiply substituted variants. This strategy generated a reasonable fraction of active proteins that minimized the number of inactive proteins that were generated (16% of the “region-restricted” variants were active and all of the “half-substituted” and “fully substituted” variant proteins retained cell proliferation activity), thereby limiting the effort required to identify multiply substituted proteins with improved properties. Six of the initial nine “fully substituted” variant proteins were >6-fold more active in the AML 193.1.3 assay than native IL-3. Three of these variants and SC-63032 were studied more extensively. The elevated hematopoietic potency was confirmed in a methylcellulose CFU assay using freshly isolated human bone marrow cells. A subset of the multiply substituted proteins did not exhibit a correlative increase in inflammatory mediator (LTC_4) release. The method presented in this article provides a mechanism to generate proteins with diverse properties while only testing a limited number of variants.

Acknowledgments

We would like to acknowledge the technical expertise of the following: M. White and T. Spears for DNA sequencing; B. Thiele, M. Walker, L. Vu, and B. Joy for protein purification; A. Abegg, H. Tsai, A. Piltch, and R. Keith for bioactivity assays; J. Zobel, M. Jennings, C. Smith, G. Lange, and K. Duffin for protein analysis; J. Sebaugh for statistical analysis; and H. Day for administrative assistance. We thank J. Galluppi, G. Krivi, G. Bild, D. Tiemeier, Y. Feng, and L. Bell for helpful discussions.

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