Rapid Communication

Human Interleukin-6 Receptor Super-Antagonists With High Potency and Wide Spectrum on Multiple Myeloma Cells

By Elisabetta Sporeno, Rocco Savino, Laura Ciapponi, Giacomo Paonessa, Andrea Cabibbo, Armin Lahm, Kari Pulikki, Ren-Xiao Sun, Carlo Toniatti, Bernard Klein, and G. Ciliberto

Interleukin-6 (IL-6) is the major growth factor for myeloma cells and is believed to participate in the pathogenesis of chronic autoimmune diseases and postmenopausal osteoporosis. IL-6 has been recently shown to possess three topologically distinct receptor binding sites: site 1 for binding to the subunit specific chain IL-6Ra and sites 2 and 3 for the interaction with two subunits of the signaling chain gp130.

We have generated a set of IL-6 variants that behave as potent cytokine receptor super-antagonists by substitutions that abolish interaction with gp130 at either site 2 alone (site 2 antagonist) or at both sites 2 and 3 (site 2 + 3 antagonist). In addition, substitutions have been introduced in site 1 that lead to variable increases in binding for IL-6Ra up to 70-fold. IL-6 super-antagonists inhibit wild-type cytokine activity with efficacy proportional to the increase in receptor binding on a variety of human cell lines of different origin, and the most potent molecules display full antagonism at low molar excess to wild-type IL-6. When tested on a representative set of IL-6-dependent human myeloma cell lines, although site 2 super-antagonists were in general quite effective, only the site 2 + 3 antagonist Sant7 showed antagonism on the full spectrum of cells tested. In conclusion, IL-6 super-antagonists are a useful tool for the study of myeloma in vitro and might constitute, in particular Sant7, effective IL-6 blocking agents in vivo.

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substitutions at either of these two sites do not substantially affect binding to IL-6Rα but decrease interaction of the IL-6/IL-6Rα subcomplex with one of the two gp130 chains. The major consequence is impaired gp130 dimerization that results in the loss of biologic activity.29 Because IL-6 variants with these properties are still able to bind IL-6Rα efficiently, they can act as receptor antagonists on a variety of IL-6–responsive human cell lines.32 IL-6 variants carrying amino acid substitutions that improve the affinity of site 1 for IL-6Rα have also been generated. When these super-binder substitutions were combined with those of the antagonist, the resulting variant, named IL-6 super-antagonist, showed greater antagonistic potency.32

In this report we describe the generation of additional IL-6 super-antagonists with increasing affinity for IL-6Rα and deficient binding to gp130 at site 2 or sites 2 + 3. These molecules were tested on a variety of IL-6–responsive cell lines, several of which were IL-6–growth-dependent myeloma cells. This confirmed that our IL-6 variant meets the requirements of being both a potent antagonist at low doses and an IL-6 inhibitor for a wide spectrum of human myeloma cell lines.

MATERIALS AND METHODS

Plasmid Construction and Expression of Proteins

All superantagonists were obtained by polymerase chain reaction (PCR).24 In the case of Sant 5, Sant 7, and Sant 8, the regions containing the corresponding substitutions in the AB-loop were amplified using as template plasmids carrying the cDNAs encoding for hIL-6 variants D-6, A-8, and D-6/K-7,35 respectively. The primers used were designed in order to contain a 5' Bsr I site (spanning residues 38-40) and a 3' Xho I site (spanning residues 91-92).25,36 Amplified fragments were digested with Bsr I and Xho I and ligated to the pT7.7 plasmid27 carrying the Sant1 mutant32 digested with the same two enzymes. To construct Sant9, a 5'-Xho I/3'-Not I fragment spanning residues 91-184 was amplified by PCR using the pT7.7/DFRD plasmid as a template27 and introduced in the pT7.7/Sant5 plasmid previously digested with the same two enzymes. The identity of all mutants was verified by DNA sequencing.38 The sequence of the oligonucleotides used is available on request. The generation of mutants D-6, A-8, and K-7/D-6 has been reported in detail elsewhere.32 Wild-type hIL-6 and its variants were produced in Escherichia coli and purified as described.39

In Vitro Receptor Binding Assays

The soluble human IL-6Rα (sIL-6Rα) binding activity of hIL-6 superantagonists was assayed in enzyme-linked immunosorbent assay (ELISA)-based binding competition experiments.25,35,46 Commmunoprecipitations of sIL-6Rα and sgp130 in the presence of wild-type IL-6 and superantagonists were performed as described.29

Bioassays

Hep3B. Transcriptional activation of the CRP gene promoter in the human Hep3B hepatoma cell line was determined and quantified as described.25,31

A375. Cell growth was monitored by crystal violet staining as described.42

Human myeloma cell lines (HMCL). XG-1, XG-2, XG-4, and XG-6 HMCL were obtained by culturing freshly explanted malignant plasma cells from patients with terminal disease with a combination of IL-6 and granulocyte-macrophage (GM)-CSF.43 These HMCL have a plasma cell phenotype and the same Ig gene rearrangements as the patients' tumor cells. They were routinely cultured in RPMI 1640 supplemented with 5% fetal calf serum (FCS) and 1 ng/mL of recombinant IL-6.

HMCL proliferation assays. XG-1 Bioassays on XG-1 cells were performed as described.32 XG-2, XG-4 and XG-6. Cells were washed once with culture medium, incubated for 5 hours at 37°C in culture medium alone, and washed again twice. 10,000 cells in 100 μL of culture medium were then cultured in 96-well flat-bottomed microplates for 5 days with or without 100 pg/mL of recombinant IL-6 and with graded concentrations of various IL-6 receptor antagonists. Tritiated thymidine (0.5 μCi; 25 Ci/mmol/L; CEA, Saclay, France) was added for the last 8 hours of culture, and tritiated thymidine incorporation was determined as reported elsewhere.45

U266. Cells were grown at 5% CO₂ in RPMI 1640 medium supplemented with 15% FCS and their proliferation was monitored as thymidine incorporation. Briefly, U266 cells were washed twice and then plated in 96-well microtiter plates at a density of 10⁴ cells/well in RPMI 1640 and 1% FCS. Serial dilutions of either the various antagonists or the neutralizing anti–hIL-6 MoAb CLB-IL6-8 (CLB, Amsterdam, The Netherlands) were added to each well and cells were grown in a final volume of 200 μL of culture medium for 56 hours. Eight hours before ending the culture, 1 μCi of [methyl-³H]Thymidine (specific activity 83 Ci/mmol; Amersham, Slough, UK) was added to each well. Cells were then collected on glass filters and incorporated radioactivity was measured in a TopCount Microplate Scintillation Counter (Packard).

RESULTS

Generation of a Set of IL-6 Super-Antagonists

We have recently described the generation of an IL-6 receptor antagonist called DFRD, which carries the simultaneous substitution of four amino acids, two in the putative A helix Y31D/G35F and two in the putative C helix S118R/V121D.32 Using specific in vitro binding assays it was possible to show that when complexed with sIL-6Rα, DFRD maintains the ability to bind a single gp130 chain but does not trigger efficient gp130 dimer formation.29 Y31, G35, S118, and V121 therefore define one site of interaction between IL-6 and gp130 which we called site 2 to distinguish it from site 1 which binds IL-6Rα (Fig 1).32

Three aminoacid substitutions in the putative helix D were also obtained. Q175L, S176R, and Q183A (also called IRA),40,41 which together increase binding affinity for IL-6Rα approximately 4.5-fold.40 Combining the superbinder IRA substitutions with the antagonistic DFRD gave rise to another variant, called IL-6 Sant (for super-antagonist), which was able to antagonize IL-6 bioactivity in a variety of human cell lines with a potency 8- to 10-fold higher than that of DFRD.32 Complete growth inhibition of the strictly IL-6–dependent human XG-1 myeloma cell line was reached with 10 μg/mL of DFRD and 1 μg/mL of IL-6–Sant, respectively.42 This result showed that the potency of IL-6 receptor antagonists could be enhanced by increasing their affinity for IL-6Rα.

Molecular modeling of IL-6 has suggested that site 1 of interaction with IL-6Rα also comprises residues in the putative A-B loop. Indeed, we recently obtained additional su-
Fig 1. Distribution of super-binder and antagonistic substitutions on the surface of human IL-6. Shown is a schematic RIBBONS representation of the human IL-6 model highlighting residues that were mutated and that affect either the interaction with the hIL-6Ra (site 1) or the signal transducer gp130 (site 2 and 3). Whereas the antagonistic substitutions of the DFRD phenotype (Y31, G35, S118, V121) cluster in site 2 (red spheres) on helix A and C, mutations of pure super-binder character (blue spheres) are located in site 1: on Helix D, as present in IL-6 IRA (Q175, S176, Q183), or within the extended region of the AB-loop (K66, A68, E69, K70, Q75, S76) connecting helix A and B. A third group of residues conferring instead a combined super-binder/antagonistic property to the molecule (L57, E59, N60, light green spheres) resides within the small helical segment in the N-terminal part of the AB-loop. Besides being super-binder, the latter mutations most likely induce local structural alterations that, because of their close proximity to site 3, affect binding to g~130. Also indicated are wt residues W157, D160, T162 (dark green spheres), not mutated in this work, which have been shown to constitute part of Site 3.

Superbinder mutations by substituting residues 57 to 76 in the cytokine’s A-B loop. Three groups of superbinder substitutions in the A-B loop were, therefore, combined with those present in IL-6 Sant (now renamed Sant1) to generate novel IL-6 super-antagonists. The list of the new variants is in Table 1 together with the type and number of substitutions introduced. Figure 1 shows the topographical localization of the substituted residues on the putative three-dimensional model of human IL-6. The Sant9 variant was also generated to assess if super-antagonism can be obtained by A-B loop mutations in the absence of the D-helix IRA superbinder substitutions. Notably, the first group of superbinder substitutions in the A-B loop, namely L57D/E59F/N60W, map in close proximity of the region spanning residues 43-55, recently discovered to be an important structural determinant for signaling in IL-6 and shown to participate in the formation of site 3 of binding to gp130.

In Vitro Binding Properties of IL-6 Super-Antagonists

IL-6 super-antagonists were expressed in E coli and their receptor binding was measured in an in vitro IL-6/IL-6Ra binding assay. The results (Fig 2 and Table 1) indicate that the various IL-6 variants increase their ability to bind IL-6Ra: the most potent, Sant7 and Sant8 are approximately 15-fold stronger than Sant1. In addition, the evidence that Sant 9 is a superbinder in the absence of the three IRA substitutions, indicates that A-B loop substitutions can in-
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Table 1. Amino Acid Sequence and Binding Properties of IL-6 Receptor Super-Antagonists

<table>
<thead>
<tr>
<th>Mutant Protein</th>
<th>Y</th>
<th>G</th>
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The first row shows the amino acid present in wt IL-6 at the position indicated. For each mutant, all the amino acid substitutions in the respective positions are indicated; where no change is indicated, the wt residue is present. The receptor binding activity was calculated from displacement curves like the ones shown in Fig 2. The gp130 binding and dimerization activities were determined from immunoprecipitation experiments like the ones shown in Fig 3.

* Relative to wt.
† By in vitro immunoprecipitation experiments.

IL-6 superantagonists were also tested for their ability to interact with gp130 in vitro. We have recently established immunoprecipitation assays to determine gp130 binding by the IL-6/IL-6Ra subcomplex. In a first assay, Sepharose beads (Pharmacia, Uppsala, Sweden) coated with anti-myc MoAb were incubated with an myc-tagged preparation of sIL-6Ra and used to coimmunoprecipitate labeled gp130 in the presence of IL-6 variants. In this assay wild-type IL-6 was shown to bind gp130 at either site 2 or 3. Site 2 mutant DFRD maintains the ability to efficiently immunoprecipitate gp130 because of residual binding at site 3, whereas site 3 mutants T126D W157R/D160R or 2A maintain this ability because of binding at site 2. However, combined site 2 + 3 mutants lose this property. In a second assay, unlabeled sgp130-myc tagged is immobilized on Protein A-Sepharose beads coated with anti-myc MoAb. After washing, immunoprecipitations are performed in the presence of unlabeled sIL-6Ra, 35S-labeled flag-tagged sgp130, and IL-6 variants. Since flag-tagged 35S-sgp130 is not recognized by the anti-myc MoAb, its immunoprecipitation monitors the gp130 homodimerization. The results are in Fig 3. As expected, like the original antagonist DFRD none of the novel superantagonists is able to dimerize gp130 efficiently (Fig 3A). Interestingly, Sant7 has totally lost the ability to bind gp130 (Fig 3B), a feature characteristic of combined site 2 + 3 variants. From these results it is possible to conclude that L57/E59/N60 contribute to the efficient binding of gp130 at site 3. L57/E59/N60 reside within the small helical segment in the N-terminal part of the AB-loop (Fig 1). Therefore, we speculate that substitution at one or more of these residues results in a local structural change that, because of its close proximity to site 3 (Fig 1), affects binding of gp130. This conclusion is further strengthened by the observation that IL-6 mutants carrying the L57D/E59F/N60W substitutions, but not the DFRD mutations in site 2, can still bind gp130 but do not dimerize it.

IL-6 Super-Antagonists Show Increased Potency on a Variety of Human Cell Lines

IL-6 super-antagonists were tested in three different human cell lines: hepatoma Hep3B, where IL-6 induces transcription of a transfected C-reactive protein promoter; melanoma A375, where IL-6 induces growth arrest; and myeloma XG-1 cells, which are dependent on IL-6 for growth. We assayed first biologic activity in the absence of added wild-type IL-6. As expected, all variants were inactive even in high doses (not shown) as previously shown for DFRD and Sant1. However, when tested for their ability to inhibit IL-6, they all behaved as dose-dependent antago-
IL-6 Super-Antagonists Have a Wide Spectrum of Antagonism on Human Myeloma Cell Lines

To assess the spectrum of activity of IL-6 super-antagonists, they were tested on various human myeloma cell lines whose growth is dependent on IL-6. As with XG-1, XG-2, XG-4, and XG-6 have been recently derived from the peripheral blood of patients with plasma cell leukemia. Although XG-2 is only responsive to IL-6, XG-4 and XG-6, because of the efficient expression of the LIFR chain, can be growth stimulated also by the cytokines OncoM, LIF, and the combination of CNTF+ soluble CNTFRα, which all induce the formation of gp130-LIFR heterodimers that activate signal.

The entire set of IL-6 super-antagonists was tested on both cell lines in the absence and in the presence of IL-6 and the results are shown in Fig 5. On XG-4 and XG-6 all variants were biologically inactive and behaved as dose-dependent IL-6 antagonists. On the contrary, on XG-2 cells only Sant 7 was totally inactive, whereas the other molecules showed a significant, albeit strongly reduced, stimulatory growth effect. In line with this, only Sant 7 was capable of fully inhibiting growth. Worthy of note is that Sant7 differs from all the other super-antagonists because it is the only variant totally unable to interact in vitro with gp130 (Fig 2 and Table 1).

U266 myeloma cells do not depend on the addition of exogenous IL-6 for growth, but they do produce endogenous IL-6, which has been shown to stimulate cell proliferation via an autocrine loop that can be inactivated by antisense oligonucleotides to IL-6 mRNA or, less efficiently, by neutralizing antibodies to IL-6. A subset of super-antagonists were tested at increasing concentrations in the absence of exogenous IL-6 and their effect was compared to that of the neutralizing MoAb8. The results (Fig 6) show that all variants tested inhibit cell growth as measured by [3H]-thymidine incorporation. Their relative potency is comparable to that observed in other cell lines, with the most potent being Sant5 and Sant8 (not shown), followed by Sant 7 and then by the weak superbinder Sant 1. The extent of inhibition by IL-6 super-antagonists does not exceed 40%, a value identical to that obtained with the anti-IL-6 MoAb8.

DISCUSSION

The IL-6 receptor super-antagonists described in this report represent a class of molecules that fulfill many of the requirements for an effective neutralization of the cytokine pathogenic potential in MM. The main properties of the IL-
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Table 2. Antagonistic Potency of IL-6 Mutants on Different IL-6-Responsive Cell Lines

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Hep3B*</th>
<th>A375†</th>
<th>XG-1‡</th>
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<tr>
<td>DFRD</td>
<td>33.8 ± 8.6</td>
<td>24 ± 4</td>
<td>2,033 ± 578</td>
</tr>
<tr>
<td>Sant 1</td>
<td>4.6 ± 1.2</td>
<td>4 ± 1</td>
<td>230 ± 60</td>
</tr>
<tr>
<td>Sant 5</td>
<td>0.45 ± 0.12</td>
<td>0.25 ± 0.04</td>
<td>19.1 ± 4.4</td>
</tr>
<tr>
<td>Sant 7</td>
<td>1.2 ± 0.3</td>
<td>0.8 ± 0.07</td>
<td>61 ± 29</td>
</tr>
<tr>
<td>Sant 8</td>
<td>0.32 ± 0.05</td>
<td>0.12 ± 0.01</td>
<td>15 ± 1.3</td>
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<tr>
<td>Sant 9</td>
<td>2.5 ± 0.6</td>
<td>0.6 ± 0.09</td>
<td>225 ± 75</td>
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</table>

The molar excess of the various mutants necessary to give 50% inhibition in the various bioassays was calculated from inhibition curves like the ones shown in Fig 4.

* Hep3B human hepatoma cells were stimulated with 4 ng/mL of wt IL-6.
† A375 human melanoma cells were stimulated with 2 ng/mL of wt IL-6.
‡ XG-1 human myeloma cells were stimulated with 0.1 ng/mL of wt IL-6.

6 super-antagonists are (1) the capacity to antagonize IL-6 at low dosage: this is an absolute prerequisite if efficient neutralization in vivo is to be achieved in MM patients where the cytokine production is of several micrograms/day; and (2) the ability to antagonize IL-6 on a large spectrum of cytokine-responsive cells. This aspect is particularly relevant in MM where the variety of genetic changes and oncogene mutations that characterize myeloma may give rise to different cells, whose sensitivity to IL-6 diverges in both quantity and quality while still maintaining their IL-6 response. An extreme example is represented by U266, which maintain their growth and survival via the production of endogenous IL-6 and the activation of a positive autocrine loop. It has to be taken into account that in these latter cells, macromolecules are expected to be only partial antagonists because they are unable to interfere with the activation of growth stimulatory pathways triggered by the fraction of IL-6 that interacts with its receptors inside the endoplasmic reticulum. Indeed, it has been previously shown that antisense oligonucleotides to IL-6 mRNA are more efficient growth inhibitors than antibodies to the cytokine. Therefore, as anticipated

Fig 4. Inhibition of IL-6 activity on hepatoma, melanoma, and myeloma cells by IL-6 receptor superantagonists. (A) Human hepatoma Hep3B cells were transfected with a reporter plasmid containing secreted alkaline phosphatase (SEAP) gene under the control of the IL-6 responsive human C-reactive protein (CRP) promoter and stimulated with 4 ng/mL of IL-6 in the presence of increasing concentrations of the various antagonists. Sixty hours after induction, the medium was collected and the SEAP activity quantified as described. (B) Human melanoma A375 cells were plated in 96-well plates (2,000 cells/well) and cultured in the presence of 2 ng/mL of IL-6 and increasing amounts of the various antagonists. After 5 days, cell growth was monitored by crystal violet staining as described. (C) XG-1 cells were grown with 100 pg/mL of IL-6 and increasing concentration of antagonists. After 7 days, cell number was estimated by colorimetric determination of the hexosaminidase levels as described.
we find that the maximum degree of inhibition is identical for IL-6 super-antagonists and antibodies to IL-6.

In accordance with the expectations, the entire class of site 2 IL-6 super-antagonists is capable of fully antagonizing IL-6 in all tested cell lines, with the exception of XG-2. However, in this cell line full antagonism is obtained with the site 2 + 3 variant Sant7. Therefore, site 2 + 3 antagonism appears as the feature required to avoid leakage effects and a possible weak agonistic behavior on a minority, although certainly not negligible, population of MM cells. In conclusion, Sant7 thus represents a possible candidate for blocking IL-6 in vivo because it combines both potency and wide spectrum efficacy.

At the present time, we cannot provide a clear explanation for the different behavior of the super-antagonists on the various IL-6-responsive cell lines. The observed discrepancies are basically of two types: the fact that site 2 antagonists may seldom be ineffective (as discussed above for XG-2 cells) and the evidence that in the cell lines inhibitable by the entire class of molecules, their relative potency is not always identical; for example, Sant7 is more potent on XG-4 cells, equally potent on XG-6, but significantly less on XG-1, Hep3B, and A375 cells. This is likely to somehow mirror the biologic heterogeneity of the different cells. For myeloma cells, in particular, there is abundant evidence that they can vary in the number of IL-6 receptors and receptor recycling, in the production of soluble and biologically active IL-6Ra, in the coexpression of other receptor chains that may cooperate with gp130 to induce cell growth, and sometimes may accumulate genetic changes in both IL-6Ra and gp130, whose consequences for receptor assembly and signalling have not yet been fully explored. Therefore,
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myeloma cell death. Although we have not directly explored this issue, this is certainly open to investigation in suitable in vitro and in vivo models.

The recent study by Lu et al\(^2\) has led to two important conclusions. First, MoAbs to IL-6 stabilize the cytokine in a form biologically able to activate the membrane-bound receptors and are not able to reach therapeutic dosages in high IL-6 producers. Secondly, the daily production of IL-6 in MM patients was estimated, and resulted to be often up to 100 \(\mu\)g/d. IL-6 super-antagonists have a pharmacologic mechanism opposite to anti-IL-6 monoclonals and are therefore expected to leave the clearance of the wt cytokine unchanged. Furthermore, although it is not possible to make a precise calculation of the amount of super-antagonist required for full IL-6 inhibition in vivo, because this largely depends on still unknown pharmacokinetic properties and distribution, the potency displayed in vitro allows the prediction that effective dosages could be achieved also in patients with elevated production of the cytokine.

Finally, although in the present study we carefully analyze the effect of IL-6 super-antagonists on myeloma cells and discuss in detail their potential use for the therapy of MM, we should bear in mind that IL-6 has been shown to play a pathogenic role in several other disease such as lupus erythematosus, rheumatoid arthritis, and postmenopausal osteoporosis.\(^4\) In the light of this evidence and in view of the wide spectrum of activity of IL-6 super-antagonists, future efforts will have to be directed to assess their effect on a variety of other pathologies using suitable in vitro and in vivo models.

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IL-6 MUTANTS INHIBIT THE GROWTH OF MM CELLS

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Human interleukin-6 receptor super-antagonists with high potency and wide spectrum on multiple myeloma cells

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