Suppression of Chronic Myelogenous Leukemia Colony Growth by Interleukin-1 (IL-1) Receptor Antagonist and Soluble IL-1 Receptors: A Novel Application for Inhibitors of IL-1 Activity

By Zeev Estrov, Razelle Kurzrock, Meir Wetzler, Hagop Kantarjian, Mary Blake, David Harris, Jordan U. Guterman, and Moshe Talpaz

In this study, we investigated the role of interleukin-1β (IL-1β) in the malignant evolution of chronic myelogenous leukemia (CML) and the functional activity of IL-1 inhibitors. Bone marrow (BM) and peripheral blood (PB) low-density cells from 38 CML patients were studied in the colony-forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte colony culture assay. Samples from patients with early stage, interferon-α (IFN)-sensitive disease formed hematopoietic colonies in the presence of fetal calf serum (FCS), erythropoietin (Epo), and one of the following: granulocyte-macrophage colony-stimulating factor (10 ng/mL), IL-3 (15 ng/mL), both, or phytohemagglutinin-conditioned medium. The addition of IL-1β augmented IFN-sensitive CML colony growth in a dose-dependent manner at concentrations of 10 to 100 ng/mL. In sharp contrast, addition of the above growth factors did not augment the colony growth-promoting effect of FCS and Epo in samples from IFN-resistant patients; further, adherent cell fractionation or T-lymphocyte depletion attenuated the “autonomous” colony growth. Lysates of 2.5 × 10^6 low-density cells from each of six IFN-resistant and six IFN-sensitive CML patients and three normal volunteers were tested for intrinsic IL-1β content in an enzyme-linked immunosorbent assay and yielded a mean of 610 pg, 54.6 pg, and 49.4 pg of IL-1β, respectively. Interestingly, both soluble IL-1 receptors (sIL-1R) and IL-1 receptor antagonist (IL-1RA) at concentrations of 5 to 100 ng/mL (sIL-1R) and 10 to 500 ng/mL (IL-1RA) inhibited CML colony growth in a dose-dependent fashion, with maximal inhibition of 64% and 85%, respectively. A similar effect was noted with the use of anti-IL-1β neutralizing antibodies. These data implicate IL-1β in CML disease progression and suggest that the inhibitory effects of molecules such as sIL-1R and IL-1RA could conceivably be the basis of a novel therapeutic strategy against this disorder.

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with informed consent and were approved by the Human Experimentation Committee of our Institution.

CML patients were defined as IFN-sensitive if they achieved complete or partial hematologic remission. Patients who failed to demonstrate a decrease in their white blood cell (WBC) count to a level consistent with partial hematologic response and those who demonstrated an increase in count during the course of therapy were defined as having IFN-resistant CML.

Preparation of cells. Heparinized PB or BM cells were layered over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged at 400g for 20 minutes at 4°C to remove neutrophils and red blood cells (RBCs).

Low-density mononuclear cells were incubated in plastic tissue culture dishes or flasks (Falcon Plastics, Becton Dickinson, Oxnard, CA) with 10% fetal calf serum (FCS; Flow Laboratories, McLean, VA) in α-medium. The procedure was repeated until no more cells adhered to the tissue culture dishes. Nonadherent cells harvested in this way contained less than 3% monocytes, as confirmed by the following techniques: microscopic differential count of up to 200 cells prepared with Wright's stain, nonspecific (α-naphthyl butyrate) esterase staining, and immunocytochemical analysis using CD14 (My-4) monoclonal antibodies (MoAbs; Coulter Immunology, Hialeah, FL) to identify monocyte-promonocyte cells, as previously described. T cells were depleted from the nonadherent fraction by a panning technique. Briefly, nonadherent cells were incubated with saturating quantities (100 ng/10^6 cells) of CD3 MoAbs (Becton Dickinson) for 1 hour at 4°C. Labeled cells were washed three times to remove excess antibody before panning. Anti-Ig plates were prepared by incubating 100 × 15 mm plastic
culture plates (Falcon, Becton Dickinson and Co, Lincoln Park, NJ) with 5 mL affinity-purified rabbit antimouse Ig (Srotec Ltd, Oxford, UK) 100 μg/mL in phosphate-buffered saline (PBS) overnight at 4°C and washed with cold PBS before use. Antibody-labeled cells were resuspended in 5 mL PBS, 5% heat-inactivated FCS (10^6 cells/mL), and incubated over the coated plates for 1 hour at 4°C, after which the antibody-negative cells were recovered by gentle pipetting.

 Colony-forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) assay. The CFU-GEMM assay we used is a modification of the assay described by Fauser and Messmer. In brief, 2 × 10^5 nucleated low density BM or PB cells were cultured in 0.8% methylcellulose with Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY), 30% FCS (Flow), 5% leukocyte-conditioned medium prepared with phytohemagglutinin (PHA-LCM), recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) 10 ng/mL or IL-3 15 ng/mL or both (Immunex Corp, Seattle, WA) and human erythropoietin (Epo) 1.0 U/mL (British Columbia Cancer Research Institute, Vancouver, Canada). Recombinant IL-1β 10 to 100 U/mL (molecular weight [MW] 17,500; Boehringer Mannheim Biochemicals, Indianapolis, IN) was used in some experiments, and 100 ng/mL of anti-IL-1β neutralizing antibodies (Genzyme Corporation, Boston, MA) were used to negate its effect. In another set of experiments, sIL-1Rs (Immunex Corp) were added to cultures at concentrations of 0.1 to 100 ng/mL, and in similar cultures we tested the effect of IL-1RA (IL-1RA protein; The Upjohn Company, Kalamazoo, MI) at concentrations of 10 to 500 ng/mL. One milliliter of the culture mixture was placed in 35-mm Petri dishes (LUX, Nunc Inc, Naperville, IL) and incubated at 37°C with 5% CO₂, in air in a humidified atmosphere in duplicate. All cultures were evaluated after 14 days for the number of burst-forming unit-erythroid (BFU-E) colonies (defined as an aggregate of more than 500 hemoglobinized cells), or three or more erythroid subcolonies, CFU-granulocyte-macrophage (CFU-GM) colonies of 40 or more granulocyte or monocyte-macrophage cells or both, and mixed colonies containing all elements. Individual colonies were plucked from the cultures with a micropipette and analyzed for cellular composition.

 IL-1β enzyme-linked immunosorbent assay (ELISA). The ELISA assay was performed using an IL-1β ELISA Kit (Cistron Biotechnology, Pine Brook, NJ). To avoid exposure to tissue culture conditions that may affect IL-1 production, fresh low-density BM or PB cells (5 × 10^6) were frozen in 0.25 mL PBS at −20°C. All samples were thawed and frozen in liquid nitrogen three times and then centrifuged at 400g for 10 minutes to remove cell debris. Cell lysates and standard dilutions of IL-1β (20, 50, 100, 200, 500, and 1,000 pg/mL) were added to the test wells in duplicate and incubated for 2 hours at 37°C in 5% CO₂. The test wells were then washed three times with PBS and incubated with rabbit IL-1β antiserum for 2 hours, washed as previously described, and incubated for 30 minutes with goat antirabbit IgG conjugated to horse radish peroxidase enzyme. A substrate (0-phenylenediamine dissolved in 3% hydrogen peroxide solution) was added to the test wells after vigorous washing and then 0.4% sulfuric acid was added. The color intensity was read within 15 minutes at a wave length of 490 nm using a microplate autoreader (Model no. EL-330; Biotek Instruments, Virmoski, VT). The average net optical density (OD) of the standard IL-1β concentration was plotted. The amount of IL-1β in each sample was determined by interpolation from the standard curve.

 Statistical analysis. The statistical significance of differences between colony numbers was determined by Student's t-test, and the nonparametric test that corresponds to the parametric t-test (Mann-Whitney) was used to determine the statistical significance of differences between IL-1β concentrations.

 RESULTS

 Growth requirement of hematopoietic colonies from IFN-resistant and from IFN-sensitive CML patients. When studied in the CFU-GEMM colony culture assay, BM or PB low-density cells from most IFN-sensitive CML patients gave rise to BFU-E and CFU-GM colonies only in the presence of FCS, Epo, and one of the following: PHA-LCM, GM-CSF, IL-3, or GM-CSF plus IL-3. In contrast, in IFN-resistant patients, addition of colony-stimulating factors did not augment colony growth-promoting effect of FCS and Epo (Figs 1 and 2). This divergent growth pattern was prominent and the difference between the number of colonies...
colonies grown in the presence and absence of GF in the two groups of patients was statistically significant with the following GFs: PHA-LCM (P < .013 for BFU-E progenitors and P < .001 for CFU-GM), GM-CSF (P < .001 for BFU-E and P < .001 for CFU-GM), IL-3 (P < .015 for BFU-E and P < .001 for CFU-GM), and GM-CSF plus IL-3 (P < .007 for BFU-E and P < .003 for CFU-GM). A similar difference in colony growth pattern between the two groups of patients was observed also with CFU-GEMM colonies (data not shown). In the presence of exogenous GF, low-density cells from IFN-resistant and IFN-sensitive patients yielded similar numbers of colonies.

To evaluate the growth advantage of BM and PB cells from IFN-resistant CML patients, we studied the effect of IL-1β on hematopoietic colony formation from low-density cells from two IFN-resistant patients in the presence and absence of an exogenous growth factor (GM-CSF) after adherent cell fractionation and T-lymphocyte depletion (Fig 3). Hematopoietic colony proliferation from these two IFN-resistant patients became GF dependent when adherent cells, T lymphocytes, or both were depleted, indicating that the “autonomous” colony growth observed in BM or PB cultures from IFN-resistant patients was mediated through accessory cells.

The effect of the augmented production of IL-1β by IFN-resistant CML cells on CML stem cell proliferation. To further delineate the growth advantage of cells from IFN-resistant CML patients, we studied the effect of IL-1β on BFU-E and CFU-GM colony growth. When added to cultures in the absence of an exogenous growth factor (Table 2), IL-1β did not affect colony growth in cultures from IFN-resistant patients, whereas it stimulated colony proliferation of IFN-sensitive patients in a dose-responsive fashion (Fig 4). This stimulatory effect was statistically significant at an IL-1β concentration of 100 U/mL (P < .017 for BFU-E and P < .001 for CFU-GM colonies).

To test the hypothesis that IL-1β is overproduced in cells from IFN-resistant patients, thus providing a growth advantage either by enhancing the stimulatory effect of GFs that are already present in the culture system or by stimulating GF production, we first tested the effect of anti-IL-1β neutralizing antibodies on IFN-resistant CML “autonomous” colony proliferation. Anti–IL-1β neutralizing antibodies reduced colony numbers by 49% (BFU-E) and 55% (CFU-GM), and this effect was partially reversed by IL-1β (Fig 5).

We then measured the intracellular content of IL-1β by assaying lysates of cells from IFN-sensitive and IFN-resistant CML patients. It was shown that in contrast to IL-1α, IL-1β does not remain cell-associated and is se-

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Response to IFN</th>
<th>Mean No. of Colonies</th>
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<tbody>
<tr>
<td></td>
<td>BFU-E</td>
<td>CFU-GM</td>
</tr>
<tr>
<td>2</td>
<td>S</td>
<td>25.5</td>
</tr>
<tr>
<td>5</td>
<td>S</td>
<td>37</td>
</tr>
<tr>
<td>6</td>
<td>S</td>
<td>42.5</td>
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<tr>
<td>13</td>
<td>S</td>
<td>11</td>
</tr>
<tr>
<td>1</td>
<td>R</td>
<td>109</td>
</tr>
<tr>
<td>8</td>
<td>R</td>
<td>183</td>
</tr>
<tr>
<td>9</td>
<td>R</td>
<td>138.5</td>
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<tr>
<td>11</td>
<td>R</td>
<td>91</td>
</tr>
<tr>
<td>14</td>
<td>R</td>
<td>54.4</td>
</tr>
</tbody>
</table>

These experiments were performed on low-density cells in the presence of FCS and Epo and in the absence of exogenous GF. IL-1β was added to the culture at a final concentration of 100 U/mL. Mean numbers of colonies from duplicate cultures are presented.

Abbreviations: S, sensitive; R, resistant.
The data are presented as the percentage ± SD of mean colony numbers in control dishes. The control cultures were plated with the addition of IL-3 (patient 9) or GM-CSF (patient 25). The mean number of BFU-E colonies in the control dishes from patient 9 was 87, and the mean number of CFU-GM colonies was 121. In the control dishes from patient 25, the mean number of BFU-E colonies was 91 and the mean number of CFU-GM colonies was 134. 

**Extracts of IL-1r in Lysates From IFN-Sensitive and IFN-Resistant CML**

To evaluate the possible clinical usefulness of molecules that compete with IL-1β on the IL-1 receptor in CML, we examined the effect of sIL-1R and IL-1RA on CML hematopoietic colony-forming cells. In cultures from IFN-resistant CML patients, sIL-1R reduced BFU-E colony proliferation by 38% (P < .003) and CFU-GM colonies by 44% (P < .0025) at a concentration of 100 ng/mL. In the absence of exogenous GF, sIL-1R reduced BFU-E colonies by 61% (P < .001) and CFU-GM colonies by 64% (P < .0017) at the same concentration (Fig 6). Similarly, IL-1RA inhibited colony growth of cells obtained from two other IFN-resistant patients. At a concentration of 500 ng/mL, IL-1RA inhibited BFU-E colony proliferation by 40% (P < .002) and CFU-GM by 42% (P < .002) in the presence of colony-stimulating activity. In the absence of added GF, BFU-E growth was inhibited by 62% (P < .001) and CFU-GM by 65% (P < .001) (Fig 7), suggesting that the inhibitory effect exerted by these molecules can be partially reversed by an exogenous GF (GM-CSF or PHA-LCM). We then examined the effect of sIL-1R and IL-1RA on colony-forming cells from seven IFN-resistant CML patients, six IFN-sensitive CML patients, and six normal donors, with and without GF, in the presence and absence of IL-1β (Table 4). Both sIL-1R and IL-1RA inhibited CML BFU-E and CFU-GM colony growth. The inhibitory effect exerted by these molecules on normal hematopoietic colony proliferation was minimal. sIL-1R was significantly more inhibitory on IFN-resistant CML cells than on normal samples (P < .001 for BFU-E and P < .003 for CFU-GM in the absence of GF, and P < .005 for BFU-E and CFU-GM in the presence of GF). Similarly, IL-1RA was significantly more effective on IFN-resistant CML than on normal progenitors (P < .0015 for BFU-E and P < .001 for CFU-GM in the absence of GF, and P < .007 for BFU-E and P < .005 for CFU in the presence of GF). The

**Fig 5. The effect of anti-IL-1β neutralizing antibodies on "autonomous" colony growth from two IFN-resistant CML patients.**

The data are presented as the percentage ± SD of mean colony numbers in control dishes. The control cultures were plated with the addition of IL-3 (patient 9) or GM-CSF (patient 25). The mean number of BFU-E colonies in the control dishes from patient 9 was 87, and the mean number of CFU-GM colonies was 121. In the control dishes from patient 25, the mean number of BFU-E colonies was 91 and the mean number of CFU-GM colonies was 134. 

**Fig 6. The effect of sIL-1R on CML hematopoietic colony-forming cells in the presence and absence of exogenous GF.** The data are presented as the percentage ± SD of mean numbers of colonies in the control cultures. In patient 28 the mean number of BFU-E colonies in the presence of PHA-LCM was 158 and the mean number of CFU-GM colonies was 131. In the absence of PHA-LCM, the mean number of BFU-E colonies was 149 and the mean number of CFU-GM colonies was 125. In patient 29 the mean number of BFU-E colonies in the presence of GM-CSF was 104 and the mean number of CFU-GM colonies was 115. In the absence of GM-CSF, the mean number of BFU-E colonies was 98 and the mean number of CFU-GM colonies was 108.
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Fig 7. The effect of IL-1RA on CML hematopoietic colony-forming cells in the presence and absence of exogenous GF. The data are presented as the percentage ± SD of the mean number of colonies cultured in the presence of PHA-LCM (patient 35) and GM-CSF (patient 37). In patient 35 the mean number of BFU-E colonies grown in the presence of PHA-LCM was 123. In the absence of GM-CSF the mean number of BFU-E colonies was 119. In patient 37 the mean number of BFU-E colonies in the presence of PHA-LCM was 209. In the absence of GM-CSF, the mean number of BFU-E colonies was 179 and the mean number of CFU-GM colonies was 211.

colony growth inhibition of sIL-1R and IL-1RA was again less effective in the presence of GF (either GM-CSF or PHA-LCM) and the inhibitory effect was partially reversed by IL-1β.

DISCUSSION

In recent years, various studies have shown that CML cells have a growth advantage in culture. When CML blood cells were cocultured on a pre-established normal marrow-adherent layer, Ph' positive progenitors proliferated continuously. In contrast, their normal counterparts had to be activated by the addition of horse serum or factors such as IL-1 or platelet-derived GF (PDGF). Evidence for a CML-derived growth-stimulatory effect was presented in another study, in which the number of 35-day hematopoietic colonies from CML patients who later developed blastic crisis occurred, indicating that the biologic events abetting disease progression are already present in the advanced chronic stage.

We found that colony-forming cells from IFN-resistant patients proliferated in the presence of FCS and Epo alone. PHA-LCM, GM-CSF, and IL-3 at effective concentrations did not significantly increase hematopoietic colony numbers in samples from IFN-resistant patients but did increase colony numbers in samples from IFN-sensitive CML patients and from hematologically normal donors. Previous studies showed that almost all BFU-E and CFU-GM colonies from CML patients originate from the leukemic clone. Therefore, it is therefore unlikely that this divergent growth pattern results from dissimilar growth requirements of CML and normal progenitor colony-forming cells.

Because IFN-resistant CML patients are believed to represent a more advanced stage of disease progression than IFN-sensitive patients, it appears that an autocrine or paracrine mechanism might be operative. In agreement with previous studies, we found that neither CFU-GM nor BFU-E CML colonies proliferate autonomously. Depletion of adherent cells or T lymphocytes abrogated the "autonomous" colony proliferation of IFN-resistant CML, suggesting that CML progenitor colony-forming cells from these patients are GF dependent and proliferate in response to colony-stimulating factors released by accessory cells. However, myeloid colony-stimulating factors (GM-CSF, G-CSF, M-CSF, and IL-3) are not overexpressed in BM and PB mononuclear cells from CML patients. Therefore, a cytokine that induces production of GFs and/or enhances the effect of GFs already present in culture could mediate the growth advantage exhibited by IFN-resistant CML patients' cells. Because IL-1 possesses these properties, we tested the effect of IL-1β on colony-forming cells from IFN-sensitive patients in a dose-dependent manner, it had no effect on IFN-resistant CML colony growth. We assumed that IFN-sensitive cells had less effective in the presence of GM-CSF or PHA-LCM, sIL-1R was added at the initiation of culture at a concentration of 100 ng/mL, IL-1RA was added at a concentration of 100 ng/mL, and IL-1β was added at a final concentration of 100 U/mL.

Table 4. Percent of Growth Inhibition Induced by sIL-1R and IL-1RA on BFU-E and CFU-GM Colonies From IFN-Sensitive and IFN-Resistant Patients in the Presence and Absence of GF With and Without IL-1β

<table>
<thead>
<tr>
<th>Source of Sample</th>
<th>Soluble IL-R</th>
<th>IL-1RA</th>
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<tr>
<td></td>
<td>+GF</td>
<td>−GF</td>
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<tr>
<td>BFU-E</td>
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<tr>
<td>BFU-E</td>
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<td>+GM-CSF</td>
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<td>BFU-E</td>
<td>+GM-CSF</td>
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<tr>
<td>BFU-E</td>
<td>+IL-1β</td>
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<tr>
<td>BFU-E</td>
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<td>BFU-E</td>
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Data on patients 26 through 38 and six normal samples (four BM and two PB) are depicted. GFs used in these experiments were either GM-CSF or PHA-LCM. sIL-1R was added at the initiation of culture at a concentration of 100 ng/mL, IL-1RA was added at a concentration of 100 ng/mL, and IL-1β was added at a final concentration of 100 U/mL.
resistant CML colony-forming cells were maximally stimulated by endogenous IL-1β, and addition of exogenous IL-1β could not further increase colony growth as it did in IFN-sensitive CML. We have therefore tested the effect of anti-IL-1β neutralizing antibodies on BFU-E and CFU-GM colony-forming cells from two IFN-resistant patients and found a 50% inhibition of IFN-resistant CML colony growth. The addition of IL-1β partially reversed the inhibitory effect, thus proving its specificity. We then measured the amount of IL-1β in cell lysates and found that low-density cells from IFN-resistant patients produce significantly more IL-1β than cells from IFN-sensitive patients or cells from hematologically normal individuals. These data are meaningful even if both IL-1β and precursor IL-1α (pro-IL-1β) were measured in this assay, because pro-IL-1β has been reported to be partially active.66 The exceptionally low concentration of IL-1β in cell extracts from one IFN-resistant patient (patient 22) indicates that other growth-promoting mechanisms, such as lack of production of IL-1RA or release of different stimulatory cytokines, may enhance proliferation of the Ph-positive clone in certain individuals. Similar observations by Rambaldi et al.,66 who described nearly the same mechanism in acute myeloblastic leukemia, and by Otsuka et al., who found IL-1β mRNA in some CML monocyte samples,67 are in agreement with our results. Our data suggest that overproduction of IL-1β by cells from IFN-resistant CML patients provides the leukemic cells with a growth advantage and is probably a mechanism of disease progression in CML.

To further evaluate whether IL-1 inhibitory molecules affect CML progenitor cell proliferation, we tested the effect of sIL-1R and IL-1RA. sIL-1R is a truncated receptor that exists in solution as a monomer and binds 1 mol IL-1/μmol receptor.68 It has been found to be biologically active and it neutralized IL-1 in an animal model.69 IL-1RA was recently purified, sequenced, and cloned.70 This natural inhibitory molecule blocks the binding of IL-1α or IL-1β to IL-1 receptors and consequently inhibits different cellular activities that are mediated through binding of these cytokines to their receptor.71 We found that both sIL-1R and IL-1RA inhibited BFU-E and CFU-GM CML colony proliferation in a dose-dependent fashion and that their effect plateaued at 100 ng/mL and 500 ng/mL, respectively. Colony growth inhibition was more pronounced in cultures from IFN-resistant patients as compared with cultures from hematologically normal individuals (Table 4). The mild inhibitory effect exerted by sIL-1R and IL-1RA on CML progenitors from IFN-sensitive patients suggests that some of the IFN-resistant cells may be producing their own IL-1RA.

Recent data indicating that IFN inhibits IL-1 synthesis in mononuclear cells stimulated by IL-157 suggest that the inhibitory mechanisms of IFN in CML include suppression of IL-1. It is therefore possible that overproduction of IL-1 by CML cells may result in resistance to IFN, and inhibition of IL-1 may increase the efficacy of IFN in CML.

In conclusion, our observations indicate that CML progenitor proliferation may be reduced by blocking the interaction between IL-1β and its receptor. Based on these results, we intend to initiate a clinical trial investigating the use of IL-1RA in the treatment of CML.

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Suppression of chronic myelogenous leukemia colony growth by interleukin-1 (IL-1) receptor antagonist and soluble IL-1 receptors: a novel application for inhibitors of IL-1 activity

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