Agonists to the A3 adenosine receptor induce G-CSF production via NF-κB activation: A new class of myeloprotective agents

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Objective. The aim of this study was to evaluate the effect of CF101, a synthetic agonist to the A3 adenosine receptor (A3AR), on the production of granulocyte colony-stimulating factor (G-CSF). The ability of CF101 to act as a myeloprotective agent in chemotherapy-treated mice was tested.

Methods. CF101 was administered orally to naïve mice and its effect was studied on blood cell counts (coulter counter), serum G-CSF level (ELISA), bone marrow colony-forming cells (soft agar culture), and splenocytes' ability to produce ex vivo G-CSF. Protein extract was prepared from splenocytes and Western blot analysis was carried out to evaluate expression level of key proteins. In an additional set of experiments, CF101 was administered to mice 48 hours after cyclophosphamide treatment and blood cell counts as well as serum G-CSF levels were monitored.

Results. Oral administration of CF101 to naïve mice led to the elevation of serum G-CSF levels, an increase in absolute neutrophil counts (ANC), and bone marrow colony-forming cells. Splenocytes derived from these mice produced higher G-CSF level than controls. The molecular mechanisms underlying the events prior to G-CSF production included the upregulation of NF-κB and the upstream kinases phosphoinositide 3-kinase (PI3K), protein kinase B/Akt (PKB/Akt), and IKK. Accelerated recovery of white blood cells and neutrophil counts were observed in cyclophosphamide-treated mice following CF101 administration.

Conclusion. CF101 induced upregulation of the PI3K/NF-κB pathway leading to G-CSF production, resulting in myeloprotective effect in cyclophosphamide-treated mice. © 2002 International Society for Experimental Hematology. Published by Elsevier Science Inc.

Myelotoxicity is a severe and dose-limiting complication of chemotherapy. Drug-induced myelosuppression is a major toxic factor that limits the administration of larger, potentially more effective doses of chemotherapy. Granulocyte colony-stimulating factor (G-CSF), a hematopoietic growth factor, has become a standard supportive therapy for cancer patients with neutropenia. It stimulates the proliferation and differentiation of hematopoietic progenitors, leading to a re-

duction in the incidence of febrile neutropenia and acceleration of neutrophil recovery after chemotherapy or bone marrow transplantation [1]. G-CSF is rapidly cleared from the blood by an active neutrophil removal process which includes its binding to neutrophil cell-surface receptors and its subsequent removal from the peripheral blood via the metabolization process of neutrophils [2,3]. G-CSF synthesis and release is induced through the activation of the transcription factor NF-κB [4]. The latter is sequestered in the cytoplasm by the inhibitor proteins IκBα and IκBβ. NF-κB is released from the NF-κB/-IκB complex when IκB is phosphorylated by IκB kinase (IKK). Consequently, NF-κB translocates to the nucleus, where it acts as a functional transcription factor of G-CSF [5]. Another signaling path-

way that is involved with NF-κB regulation is the PI3K and its downstream effector PKB/Akt. PKB/Akt has specifically been shown to contribute to NF-κB regulation through association with and activation of IKK [6].

Several side effects have been associated with the use of G-CSF, including cutaneous adverse reactions and bone pain [7,8]. The use of G-CSF during hospitalization accounts for almost 8% of the total pharmacy budget [9,10]. Thus, new agents counteracting the myelosuppressive effects of chemotherapy are required.

Recently we demonstrated that adenosine induces G-CSF production and that this activity is mediated through the A3AR [11]. In vivo, adenosine and the synthetic A3AR agonists, IB-MECA and Cl-IB-MECA, acted as myeloprotective agents and prevented the myelotoxic effects of chemotherapy [12,13]. Adenosine, a ubiquitous purine nucleoside, is known to regulate a number of cellular activities such as cell growth and differentiation [14–16]. It is released into the extracellular environment from metabolically active or stressed cells and binds to selective G-protein–associated A1, A2$_A$, A2$_B$, and A3 membrane receptors [17,18]. The

A1 and A3 adenosine receptors are coupled with Gi proteins inhibiting adenylyl cyclase activity. The A3AR utilizes the α and βγ subunits to transmit extracellular signals to the cells' interior. The βγ heterodimer can regulate a diverse array of effector molecules including PI3K [19]. The A2$_A$ and A2$_B$ adenosine receptors are couples to Gs proteins and induce opposite effects via the activation of adenylyl cyclase [18].

In the present study we further investigated the ability of a synthetic A3AR agonist to induce G-CSF production and affect other hematological parameters. Moreover, the ability of an A3AR agonist to induce a myeloprotective effect has also been shown. The molecular mechanisms underlying the downstream signaling events following activation of A3AR in G-CSF–producing splenocytes was also described. Based on these findings, a pathway linking A3AR activation to NF-κB upregulation, leading to G-CSF production, is presented.

Materials and methods

Reagents
The A3 adenosine receptor agonist CF101, a GMP grade of the compound known generically as 1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-β-D-ribofuranuronamide) (IB-MECA), was synthesized for Can-Fite BioPharma by Albany Molecular Research Inc., Albany, NY, USA. For CF101, a stock solution of 10 mM was prepared in dimethylsulfoxide (DMSO) and further dilutions in culture medium or phosphate-buffered saline (PBS) were performed to reach the desired concentration.

RPMI, fetal bovine serum (FBS), and antibiotics for cell cultures were purchased from Beit Haemek, Haifa, Israel. Rabbit polyclonal antibodies for the following proteins were obtained as follows: PI3K p110α, phosphorylated P 75 β/Akt 1/2 at Ser473 (Cell Signaling Technology, Beverly, MA, USA); IKKα/β and goat polyclonal Actin (Santa Cruz Biotechnology Inc., San Diego, CA, USA); Anti-NF-κB p65 (RelA) (CEMICON International, Inc., Temecula, CA, USA; anti-murine G-CSF antibodies (PeproTech Inc., Rocky Hill, NJ, USA). Cyclophosphamide (CYP) was purchased from Taro Pharmaceutical Industries Ltd., Haifa Bay, Israel. Pyrrolidine dithiocarbamate (PDTC) and pertussis toxin (PT) were acquired from Sigma (St. Louis, MO, USA).

Mice
Male ICR mice (Harlan Laboratories, Jerusalem, Israel) aged 2 months, weighing an average of 25 g, were used. Standardized pelleted diet and tap water were supplied.

Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Rabin Medical Center, Petah Tikva, Israel.

Effect of CF101 on hematological parameters in naïve mice
To verify the optimal dose of CF101 required for the stimulation of the myeloid system, the drug was orally administered to ICR mice on two consecutive days. To test the specificity of CF101 response we utilized PT, which inhibits G-protein coupling to the receptor, and PDTC, an NF-κB inhibitor. The inhibitors were intraperitoneally injected 30 minutes prior to CF101 administration. Blood samples were withdrawn 24 hours after the second CF101 admin-
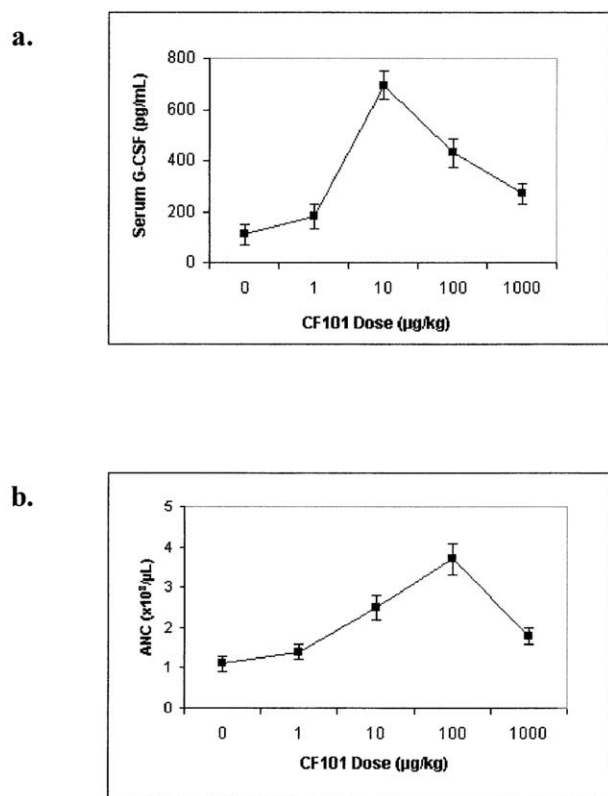

Figure 1. Effect of CF101 on serum G-CSF level and ANC in naïve mice—dose response curve. Mice were orally administered for two consecutive days with different doses of CF101. Twenty-four hours later, blood samples were withdrawn, neutrophils were counted, and G-CSF level was tested by ELISA. Data presented are the mean (± SD) of four independent experiments. Marked stimulation in the level of G-CSF (**a**) and neutrophils (**b**) is noted. The response is characterized by a bell-shaped curve.

istration. Spleens were removed and subjected to Lymphoprep (Nycomed AS, Oslo, Norway) for mononuclear cell separation. To collect bone marrow cell samples, femora were excised and cell suspensions were prepared.
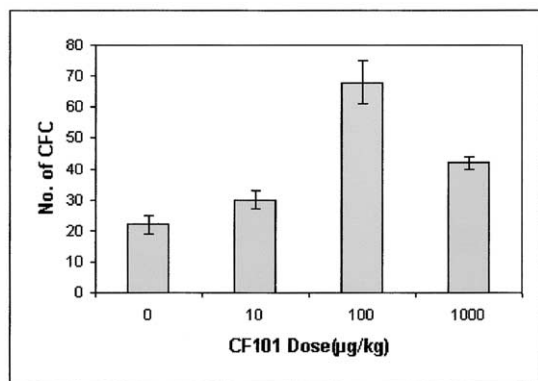
Each group contained 5 mice and experiments were repeated at least 4 times. The following parameters were evaluated:

Blood cell counts. White blood cell counts (WBC) were carried out in a Coulter counter and differential cell counts were performed on smear preparations stained with May-Grunwald-Giemsa solution.

G-CSF analysis in serum and in supernatants of cultured splenocytes. Serum samples for G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), and interleukin-1 (IL-1) level analysis were obtained and assayed by commercial murine ELISA kits from R&D Systems (Minneapolis, MN, USA).

To test G-CSF production by splenocytes from CF101-treated or control mice, mononuclear cells (5×10^6) were cultured in RPMI medium, containing 10% FBS. After 48 hours, culture supernatants were collected for G-CSF analysis.

Cloning of bone marrow cells in soft agar. To examine the number of colony-forming cells (CFC) in the bone marrow of CF101-treated or control mice, the soft agar technique of Pluznik and Sachs [20] was used. As a colony-stimulating factor we used Con. A splenocyte conditioned medium [21], which was added at a final concentration of 10% in the hard agar (0.5%) layer. Bone marrow cells (10^6) were seeded in a soft agar (0.37%) layer on top of the hard agar. Both agar layers were supplemented with 20% heat-inactivated fetal bovine serum. The cultures were incubated at 37°C in a CO_2-humidified incubator for 7 days. Colonies of more than 50 cells were counted.

Analysis of protein expression level in splenocyte extracts. To test PI3K, PKB/Akt, IKK, NF-κB, G-CSF, and actin, splenocytes were rinsed with ice-cold PBS and lysed in ice-cold lysis buffer (TNN buffer, 50 mM tris buffer, pH = 7.5, 150 mM NaCl, NP 40). Cell debris were removed by centrifugation at 7500*g* for 10 minutes. Supernatant was utilized for Western blot analysis. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the protein sample (50 μg) were separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), using 12% polyacrylamide gels. The resolved proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked with 1% bovine serum albumin and incubated with the relevant antibody (dilution 1:1000) for 24 hours at 4°C. Blots were then washed and incubated with a secondary antibody for 1 hour at room temperature. Bands were recorded using a BCIP/NBT color development kit (Promega, Madison, WI, USA). Data presented are representative of at least three different experiments.

Effect of CF101 on hematological parameters in neutropenic mice
To examine the myeloprotective effect of CF101, mice were injected intraperitoneally with 250 mg/kg body weight of CYP. CF101 (100 μg/kg body weight) was administered orally 48 hours and 72 hours following chemotherapy. Each group contained 5 mice and experiments were repeated 7 times.
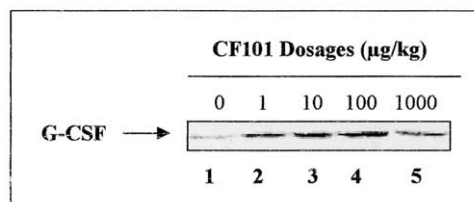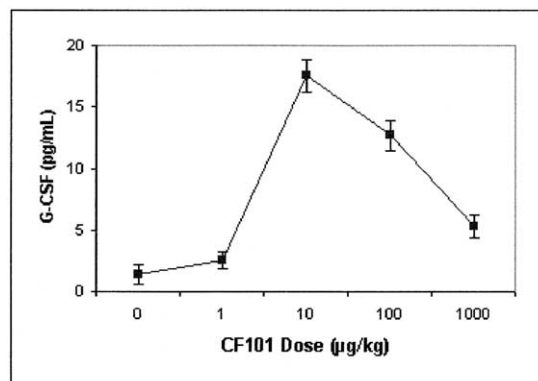

Figure 2. (a) CFC number and (b) G-CSF protein expression level in bone marrow cells derived from CF101- and vehicle-treated mice. Different doses of the drug were given orally for two consecutive days and 24 hours later bone marrow cells were separated from femurs. For CFC evaluation, cells were seeded in agar cultures as described in Materials and Methods section. Western blot analysis was carried out to measure G-CSF protein expression level. In the CF101-treated group, an elevation in bone marrow–derived CFC number was observed, correlated to increased G-CSF protein expression level. A bell-shaped response, peaking at 100 μg/kg, was observed.

Figure 3. G-CSF level secreted by splenocytes derived from CF101-treated mice. Mice were orally administered for two consecutive days with different doses of CF101. Twenty-four hours later, spleens were excised and splenocytes were separated by Lymphoprep and cultured for 48 hours without additional stimulation. The G-CSF level was evaluated in culture supernatants by ELISA. Stimulation of G-CSF production by splenocytes was observed peaking at a dose of 10 μg/kg.

Blood samples were taken 24, 48, 72, 96, 120, and 144 hours following CYP administration. WBC and absolute neutrophil counts (ANC) were obtained. Serum G-CSF level and CFC in the bone marrow were analyzed as described above. Colonies in the bone marrow cultures were counted after 7 days.

Statistical analysis
The results were statistically evaluated using the Student's *t*-test. Comparison between the mean value of different experiments was carried out. The criterion for statistical significance was $p < 0.05$.

Results

Effect of CF101 on the
hematological parameters of normal mice
Initially we determined, in naïve mice, the dose at which CF101 induced the maximal stimulatory effect on the myeloid system. In the CF101-treated group, serum G-CSF levels, bone marrow colonies, and ANC markedly increased compared with controls. The results produced a bell-shaped response, with G-CSF peaking at 10 μg/kg (Fig. 1a), while bone marrow CFCs and neutrophils peaked at 100 μg/kg (Figs. 1b and 2a). As the increase in bone marrow CFCs may result from G-CSF induction, we tested G-CSF expression in bone marrow cells derived from control and CF101-treated mice. As shown in Figure 2b, a noticeable increase in the G-CSF expression level was observed, with a pattern of response similar to that seen with the serum G-CSF, neutrophils, and bone marrow CFCs.

We could not detect any change in serum level of GM-CSF, SCF, and IL-1 in the CF101-treated mice.

To determine whether CF101 stimulated G-CSF production by splenocytes, the latter were cultured ex vivo for 48

hours and culture supernatants were collected and tested for G-CSF by ELISA. Figure 3 depicts a marked increase in G-CSF production in the cultures of splenocytes derived from CF101-treated mice. As with the bone marrow and serum, a bell-shaped curve was observed.

Given that NF-κB mediates the transcriptional activation of the gene that encodes G-CSF, we investigated the possible role of this transcription factor in CF101-induced G-CSF production. We first examined the level of key proteins downstream to the activation of A3AR, known to induce NF-κB upregulation. Immunoblot analysis of splenocyte protein extracts from CF101-treated mice revealed a dose-dependent upregulation of PI3K, phosphorylated PKB/Akt, IKK, NF-κB, and G-CSF (Fig. 4). The level of all proteins (excluding IKK) peaked at a dose of 100 μg/kg CF101 (lane 4) and decreased at 1000 μg/kg CF101 (lane 5), similar to that of G-CSF level. The level of the reference housekeeping protein, actin, remained constant in all samples (Fig. 4, bottom). The NF-κB inhibitor PDTC counteracted the effect of CF101 and prevented the upregulation of NF-κB, G-CSF, and neutrophil counts (Fig. 5a and b). To further examine whether splenocyte response to CF101 is A3AR mediated, we tested the effect of the Gi protein inactivator PT on NF-κB protein level. PT reversed the capability of CF101 to upregulate NF-κB (Fig. 5c).

In a different set of experiments, we tested the time-dependent kinetics of serum G-CSF and ANC following oral administration of 100 μg/kg CF101.

An eightfold increase in serum G-CSF level was seen 24 hours after the second CF101 administration (Fig. 6a). This was followed by a dramatic drop in G-CSF level, 24 hours later. The response of the neutrophils showed a different pattern, peaking 48 hours after CF101 administration, decreasing gradually, and returning to normal values after 120 hours (Fig. 6b).

In contrast to the marked effect on the granulocytic lineage, no effect was observed on red blood cells or platelet counts. At no time during the above experiments did we observe any evidence of toxicity or any other side effects.

Effect of CF101 on the
hematological parameters of neutropenic mice
The results in the naïve mice showing myelostimulatory effect of CF101 prompted us to test its efficacy as a myeloprotective agent in chemotherapy-treated mice. CYP alone induced a nadir lasting 48 to 120 hours after treatment. In the group treated with CYP + CF101, WBC and ANC were higher than the nadir values of the CYP-treated group. Full recovery was noted after 144 hours in the group treated with CYP + CF101 (Fig. 7a and b). Serum G-CSF level dramatically increased in the CYP- and the CYP + CF101–treated groups. However, after 144 hours, a decline in G-CSF level was noted in the group treated with the combined therapy (Fig. 7c). Analysis of bone marrow CFCs revealed a decrease in the number of CFCs in the CYP-treated group and
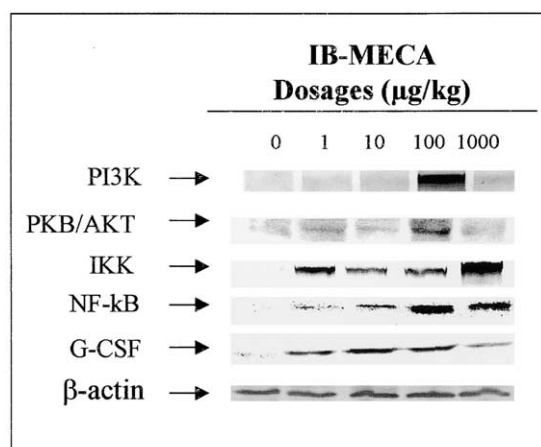

Figure 4. Western blot analysis of proteins extracted from splenocytes derived from CF101-treated mice. Mice were orally administered for two consecutive days with different doses of CF101. Twenty-four hours later, spleens were excised and proteins were extracted. Signaling proteins upstream to G-CSF including PI3K, PKB/Akt, IKK, and NF-κB were analyzed and were compared to the level of the housekeeping protein β-actin.
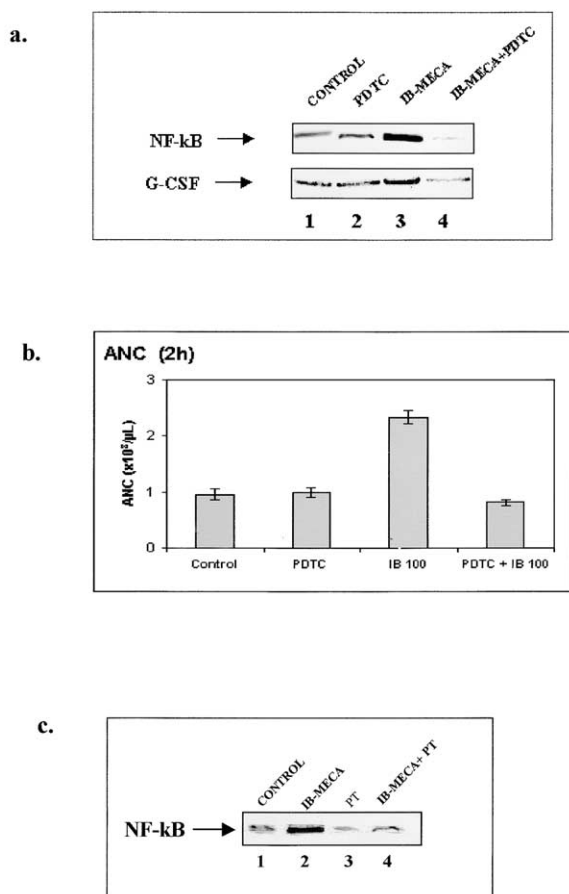
a.

b.

c.

Figure 5. Western blot analysis of proteins extracted from splenocytes derived from mice treated with the NF-κB inhibitor PDTC or with PT. Mice were orally administered for two consecutive days with 100 μg/kg CF101. Thirty minutes prior to each treatment, PDTC (200 mg/kg) or PT (125 μg/kg) were injected intraperitoneally. Twenty-four hours after the second CF101 administration, spleens were excised and proteins were extracted. In the PDTC-treated mice, neutrophil counts were analyzed and compared to those of the control group. PDTC and PT reversed the effect of CF101 and downregulated NF-κB expression level **(a & c)**. Furthermore, PDTC counteracted G-CSF expression level and neutrophil counts when administered prior to CF101 **(a & b)**.

a marked increase in the group treated with CYP + CF101 (vs control $p < 0.01$; vs CYP $p < 0.001$) (Fig. 7d).

Discussion

The present study shows that CF101 induces G-CSF production, which in turn stimulates myeloid progenitor cell expansion in the bone marrow, increases the WBC and ANC in the peripheral blood, and acts as a myeloprotective agent in chemotherapy-treated mice.

CF101 is a stable adenosine derivative as result of a substitution at the 2-position and at the N6 and 5′ positions of adenosine (Fig. 8). This structure protects the molecule against rapid metabolization by adenosine deaminase and further enhances its affinity to A3AR, while having low affinity to the $A2_A$ or
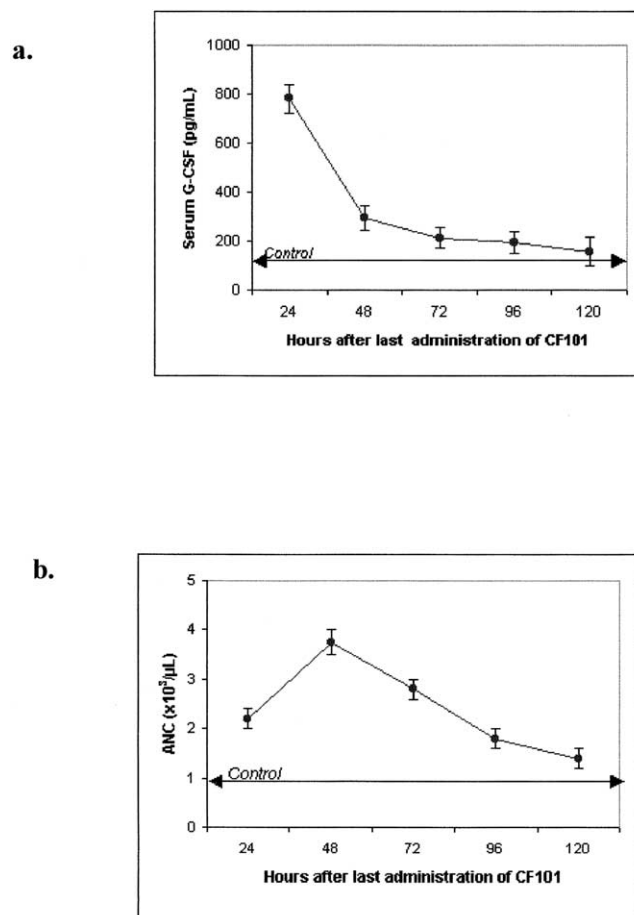
a.

b.

Figure 6. Serum G-CSF level and ANC in naïve mice treated with 100 μg/kg CF101—time response curve. Mice were orally administered for two consecutive days with 100 μg/kg CF101. Twenty-four hours later, ANC and G-CSF levels were analyzed.

$A2_B$ adenosine receptors [22,23]. We found that the half-life time of CF101 in mice was 1.3 hours (unpublished data), sufficient to induce a systemic effect after oral administration.

Evidence in this study supports the notion that CF101 induces G-CSF production. G-CSF was found to be highly expressed in bone marrow cells and splenocytes derived from CF101-treated mice. Moreover, serum G-CSF level and ANC markedly increased. However, serum level of other related cytokines remained unchanged in the CF101-treated mice, indicating specific stimulation of G-CSF production.

A salient finding of this study was that each of the hematological parameters examined reacted in a bell-shaped curve after CF101 treatment, namely, an increase up to a dose of 100 μg/kg followed by a decrease when the dose was further raised. This may be explained by the affinity profile of CF101 to the different adenosine receptors. Although possessing a high affinity to the A3 receptor, when the concentration of the agonist is increased, it may also activate the $A2_A$ and $A2_B$ receptor, resulting in a contrasting effect. This assumption is supported by our earlier studies [12] showing that IB-
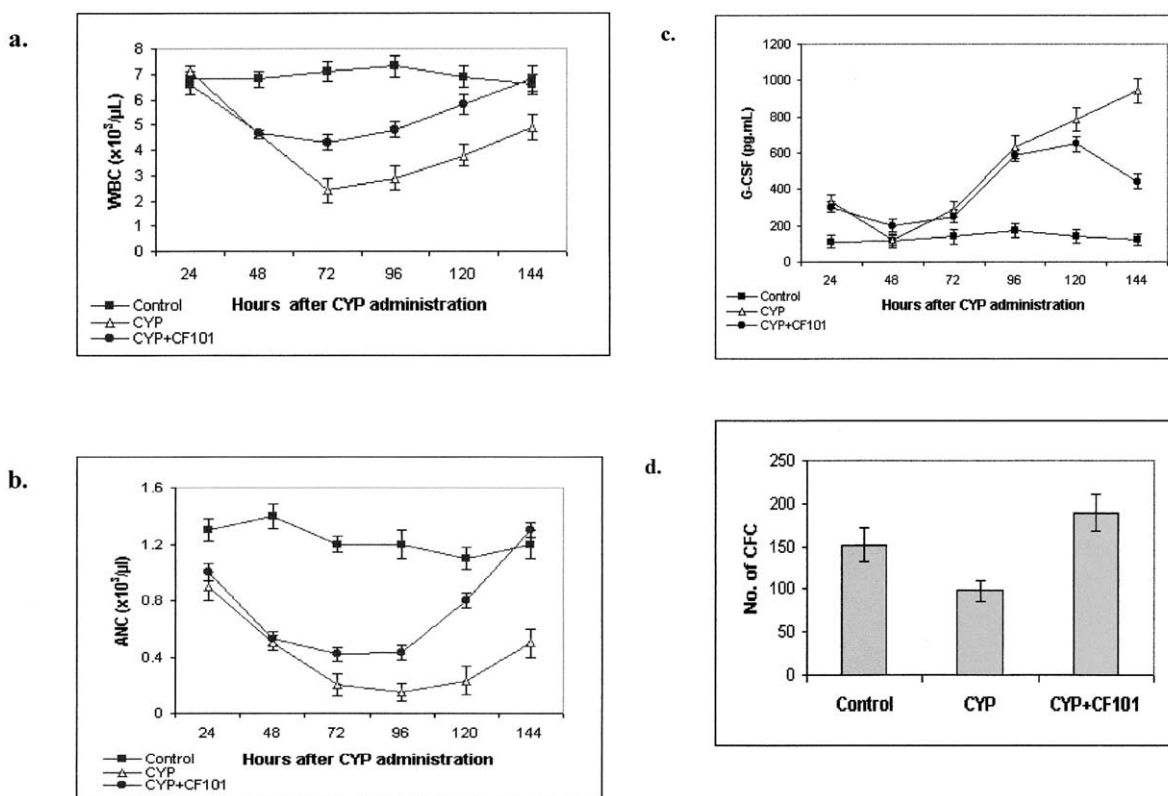
Figure 7. Effect of CF101 on the hematological parameters in mice rendered neutropenic by CYP. Mice were pretreated with CYP (200 mg/kg body weight) and following 48 hours and 72 hours, CF101 (100 μg/kg) was orally administered. At different time points, WBC **(a)**, ANC **(b)**, serum G-CSF level **(c)**, and bone marrow CFCs **(d)** were evaluated. Each group contained 5 mice and 7 experiments were carried out. Results of one representative experiment are shown.

MECA's stimulatory effect on bone marrow cell proliferation was reversed at high concentrations of this agonist.

Interestingly, an inverse correlation between G-CSF and neutrophil numbers was observed in the time response studies, namely, when neutrophils reached their maximal counts, the serum G-CSF level dramatically decreased. This paradox is thought to be associated with receptor-mediated clearance of G-CSF by neutrophils, which are removed from the blood to the liver during metabolization, carrying G-CSF [2,3].

The elevation in ANC is probably a result of the increase in the G-CSF level, which induces proliferation, differentiation, and maturation of myeloid progenitors and their subsequent migration to the peripheral blood. This notion is supported by the increase in bone marrow CFC, derived from CF101-treated mice, indicating an expansion of the myeloid progenitor subpopulation.

Splenocytes derived from CF101-treated mice showed an upregulation of G-CSF expression. In light of recent reports showing that the NF-κB family plays an important role in G-CSF transcription [4], we evaluated a possible mechanism that links the activation of A3AR to NF-κB. Our data demonstrated an upregulation of PI3K and its downstream target PKB/Akt. The latter was shown [5,6,24] to phosphorylate and activate IKK, which is responsible for

the phosphorylation of IκB. This, in turn, frees NF-κB to translocate to the nucleus, where it regulates gene transcription [5]. Indeed, our findings demonstrate an increased expression level of IKK and NF-κB, indicating that G-CSF
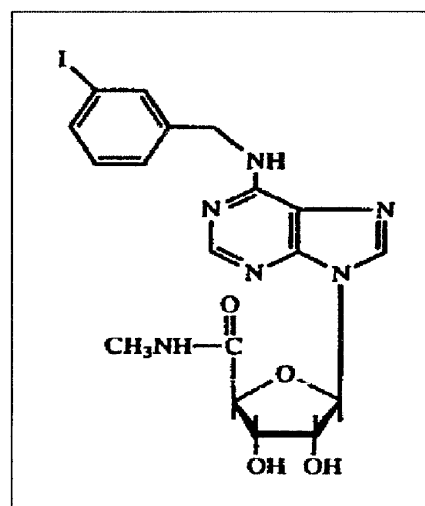

Figure 8. Chemical structure of CF101. 1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-β-D-ribofura-nuronamide (IB-MECA).

upregulation may result from the activation of the PI3K/ NF-κB pathway. This conclusion is supported by the observations of others [25,26] who demonstrated that G-CSF production is mediated via the release of NF-κB and its subsequent translocation to the nucleus. The A3AR-NF-κB pathway was further confirmed in this study by treating the mice with PT, a Gi-protein inactivator, which acts by preventing the coupling of the receptor to the Gi-protein. Splenocytes derived from mice that were treated with CF101 and PT were unable to upregulate NF-κB level. Moreover, the NF-κB inhibitor PDTC, known to suppress the release of IκB from the latent cytoplasmic form of NF-κB, counteracted the effect of CF101 and prevented increase in NF-κB level. There are several lines of evidence in the literature showing that activation of a Gi-protein-coupled receptor induces up-

regulation of PI3K and PKB/Akt. However, only Gao et al. [27] indicated that A3AR activation induces an increase in PKB/Akt level in RBL basophilic leukemia cells. RBL and splenocytes belong to the hematopoietic lineage. It is interesting to note that they both responded to CF101 by elevation of PKB/Akt expression. The data presented in this study support a model in which CF101 can promote G-CSF production through a transcriptional mechanism that includes upregulation of NF-κB (Fig. 9).

The ability of CF101 to induce G-CSF production, as was evidenced in the spleen, bone marrow, and peripheral blood, led us to assume that CF101 may counteract chemotherapy-induced myelotoxicity. Indeed, administration of CF101 to mice rendered neutropenic by cyclophosphamide treatment resulted in accelerated recovery of peripheral
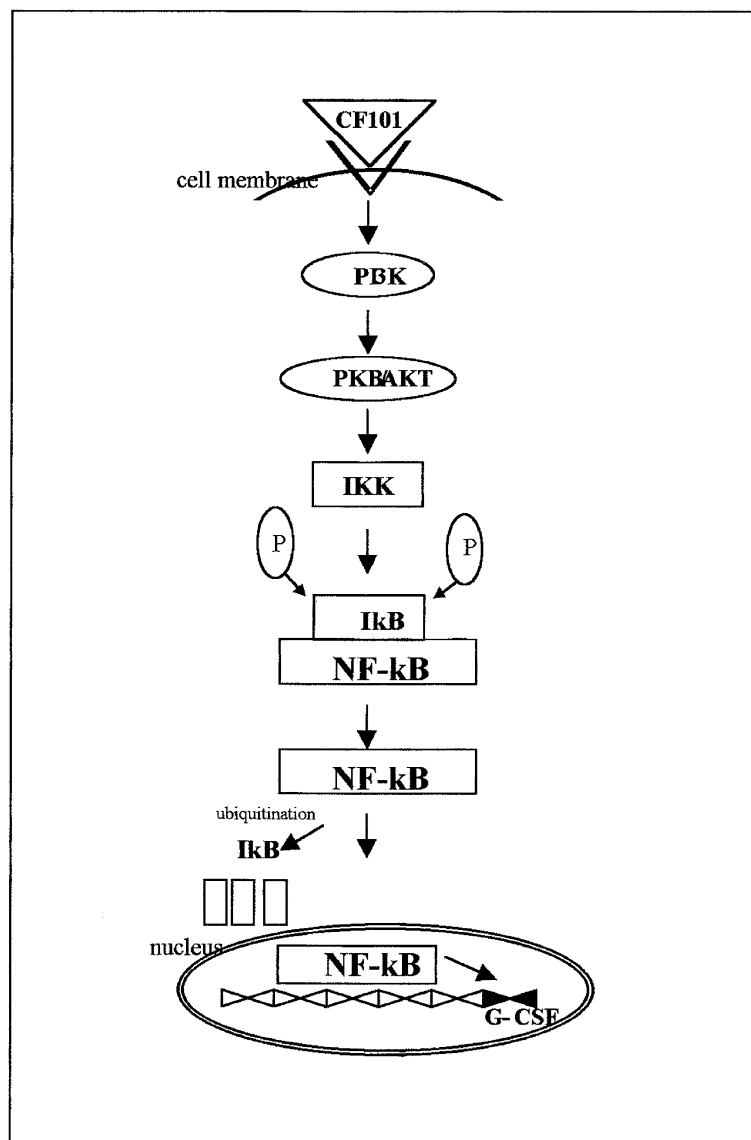

Figure 9. Proposed signaling pathway for G-CSF production in splenocytes. Binding of CF101 to A3AR induces upregulation of PI3K and PKB/Akt, which subsequently activates IKK. The latter phosphorylates IκB, and thus frees NF-κB, which translocates to the nucleus and induces G-CSF transcription.

WBC and ANC. These effects were inversely correlated with G-CSF levels. A drop in G-CSF level was observed on day 6, when ANC went up. These findings support the above-mentioned notion that G-CSF is absorbed by receptors present on neutrophils and then removed from the peripheral blood [2,3]. An additional possibility to account for the increased neutrophil counts in the CYP + CF101–treated group is the ability of CF101 to prolong neutrophil survival. It has been earlier shown [28] that the induction of the PI3K-NF-κB signaling pathway correlates with reduced apoptosis and prolongation of survival time of neutrophils.

Our previous studies showed that activation of A3AR on tumor cells inhibits cell growth in vitro and suppresses tumor development in experimental murine models of melanoma and colon carcinoma [12,13,29,30]. Others demonstrated that agonists to A3AR induce cytoprotective effects (i.e., cerebroprotective activity following chronic administration of IB-MECA to gerbils with cerebral ischemia [31], cardioprotective activity during prolonged simulated ischemia by rescuing injured myocytes and protection against doxorubicin-induced cardiotoxicity [32–34]) and anti-inflammatory effects [35].

In view of these findings, CF101 can be described as a representative of a unique family of compounds exerting differential effects on tumor and normal cells. In addition, as a small, orally bioavailable molecule, CF101 may be suggested as a candidate for development as a myeloprotective agent.

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