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Review paper

A3 adenosine receptor as a target for cancer therapy

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Targeting the A3 adenosine receptor (A3AR) by adenosine or a synthetic agonist to this receptor (IB-MECA and Cl-IB-MECA) results in a differential effect on tumor and on normal cells. Both the adenosine and the agonists inhibit the growth of various tumor cell types such as melanoma, colon or prostate carcinoma and lymphoma. This effect is specific and is exerted on tumor cells only. Moreover, exposure of peripheral blood mononuclear cells to adenosine or the agonists leads to the induction of granulocyte colony stimulating factor (G-CSF) production. When given orally to mice, the agonists suppress the growth of melanoma, colon and prostate carcinoma in these animals, while inducing a myeloprotective effect via the induction of G-CSF production. The de-regulation of the Wnt signaling pathway was found to be involved in the anticancer effect. Receptor activation induces inhibition of adenylyl cyclase with a subsequent decrease in the level of protein kinase A and protein kinase B/Akt leading to activation of glycogen synthase kinase-3*β*, a key element in the Wnt pathway. The oral bioavailability of the synthetic A3AR agonists, and their induced systemic anticancer and myeloprotective effect, renders them potentially useful in three different modes of treatment: as a stand-alone anticancer treatment, in combination with chemotherapy to enhance its therapeutic index and myelprotection. It is evident that use of the A3AR agonist for increasing the therapeutic index of chemotherapy may also invariably give rise to myeloprotection and vice versa. The A3AR agonists are thus a promising new class of agents for caner therapy. [© 2002 Lippincott Williams & Wilkins.]

Key words: A3 adenosine receptor, adenosine, cancer therapy, myeloprotection.

Why A3 adenosine receptor (A3AR)? A historical perspective

The realization that A3AR may be a target for cancer therapy is the result of extensive research that has its routes in a clinical observation. It is known that tumor metastases are extremely rare in muscle tissue, not withstanding the fact that it constitutes about 65–70% of lean body mass. This observation led to research that has its purpose to decipher the physiological basis for this intruiging phenomenon. It was found that muscle cells secrete small molecules (MF) which inhibit growth of tumor cells.[1,2] This growth inhibitory effect was observed on a broad range of different tumor cell lines *in vitro*, such as melanoma, carcinoma, leukomia and lymphoma. Remarkably, these small molecules had an opposite effect on normal cells including bone marrow cells, fibroblasts and muscle cells, inducing these cells to proliferate. When administered to melanoma or colon carcinoma-bearing mice, these muscle-derived small molecules inhibited tumor growth (Figure 1 and 2) and when administered concomitantly with chemotherapy, they exerted a myeloprotective effect (Figure 3). Interestingly, these effects were exerted upon oral administration. In summary, these small, muscle-secreted molecules had a dual activity with a differential effect on tumor cells, on the one hand, and normal cells such as bone marrow cells, on the other hand.

Within the framework of the efforts to characterize these small molecules, it was shown that muscle cells released adenosine which exhibited the dual effect of inhibiting tumor cell growth and inducing proliferation of bone marrow cells, however only *in vitro*.[3] We have further shown that the *in vitro* dual effect of adenosine was mediated via the A3AR.[4] However, oral administration of adenosine to mice was not able to evoke this dual effect observed with the muscle-released small molecules. At that stage it was realized that muscle-conditioned medium contains active components, other than adenosine, which are responsible for the *in vivo* antitumor effect. We thus conducted additional experiments to explore the possibility that A3AR agonists are secreted by the

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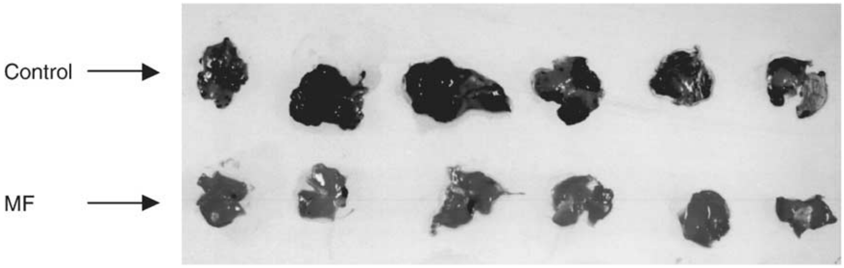

Figure 1. MF inhibits the development of lung melanoma metastatic foci in mice. C57BL/6J mice were i.v. inoculated with B16-F10 melanoma cells. The mice were treated daily orally with MF. On day 15, the mice were sacrificed, lungs were removed and tumor foci were counted (control group 68 ± 5.2; MF treated 34 ± 2.6).

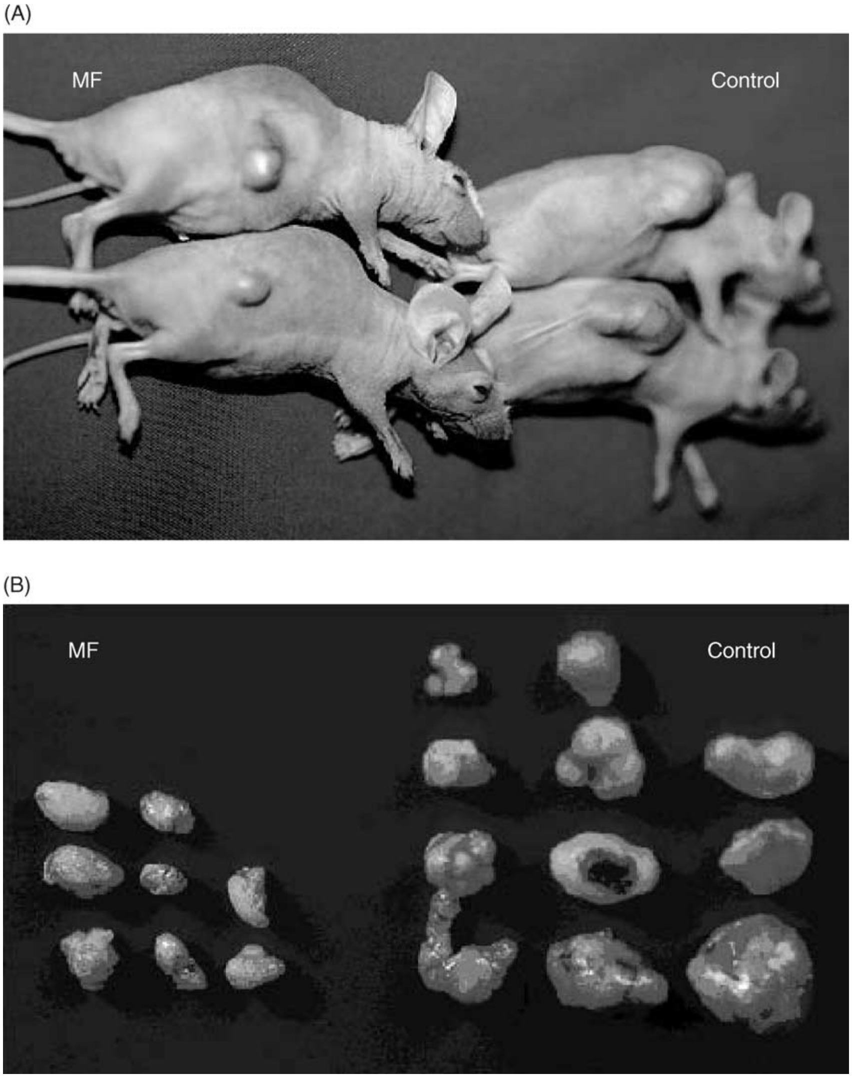

Figure 2. MF Inhibits the development of HCT-116 human colon carcinoma. BALB/c origin nude mice were s.c. inoculated with HCT-116 human colon carcinoma cells. Mice were treated daily orally with MF. On day 35 mice were sacrificed, tumors were removed and weighed. (a) A comparison between representative mice in the control (left) and MF treated (right) group. (b) A comparison between weight of tumor lesions that were excised from the control (left, 90.4 ± 21.2 mg) or treated (right, 23.1 ±3.3 mg) group.

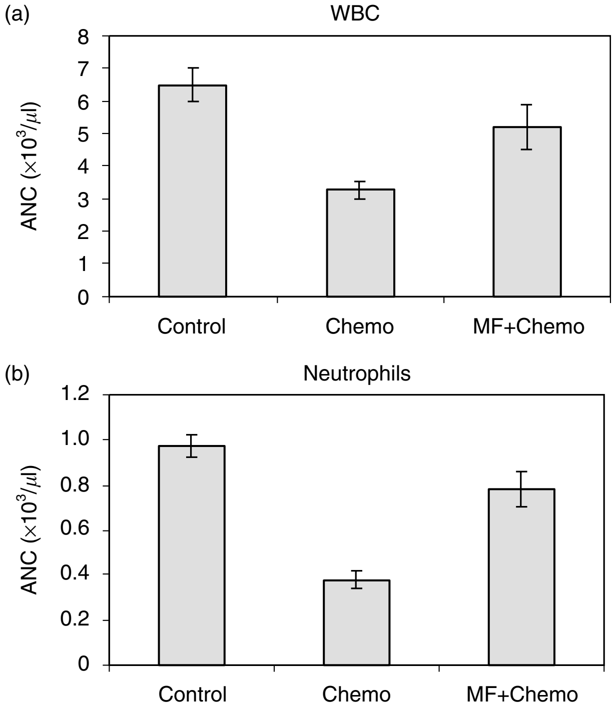

Figure 3. Effect of MF on the hematological parameters in mice rendered neutropenic by CYP. The effect of MF on the myeloid system in mice treated with CYP was evaluated. CYP alone decreased the number of WBC and neutrophils. MF, administered 2 days after chemotherapy, increased the number of total WBC count (a) and restored the percentage of neutrophils (b).

muscle cells, attributing to the *in vivo* effect. Indeed, when we blocked the A3 receptor on tumor or normal cells and subsequently exposed those cells to muscle conditioned medium (pretreated by adenosine deaminase to remove adenosine), it did not inhibit tumor growth, nor stimulated normal cell proliferation. These data led to the conclusion that muscle conditioned medium, in addition to adenosine, contains agonists to the A3AR which are responsible for its antitumor as well as the stimulatory effect toward normal cells.[4]

This provided the rationale for using the A3AR as a target for anticancer and myeloprotective treatments. Synthetic agonists to this receptor, IB-MECA (1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purine-9-yl]-*N*-methyl-β-D-ribofuranuronamide) and Cl-IB-MECA (2-chloro-N^6-(3-iodobenzyl)-adenosine-5¢-*N*-methyluronamide), chemically synthesized by Jacobson *et al.*,[5] were found to exert the same dual effect and were thus selected for further studie.[6,7] It was demonstrated that these agonists have qualitatively the same activity as the natural agonist secreted by the muscle cells, as will be shown bellow.

The A3AR

The A3AR is one out of four cell membrane receptors which bind adenosine and are classified as A1, A2A, A2B and A3 (8,9). The A3AR has a topology of typical Gi-protein cell surface receptor with seven α-helical membrane spanning domains. Its C-terminus tail contains high serine and threonine residues which are rapidly phosphorylated by GRKs **[Define?]**, thus leading to rapid desensitization. It was recently found that receptor internalization takes place after 8 min while receptor recycling takes place after 34.6 min.[10,11] Upon activation of A3AR, adenylyl cyclase activity and cAMP formation are inhibited, leading to decreased level of the effector protein kinase A. Activation of phospholipase C and D and mobilization of Ca^{2+} from intracellular and extracellular sources were reported following receptor activation.[9] The level of A3ARs in different tissues was found to be low, with the exception of testis, eosinophils and basophils, which showed massive expression. Thus, most of the studies which describe A3AR characteristics were carried out with transfected rat or human cells.[10,12] Recently, the groups of Borea[13,14] and Suh *et al.*[15] showed that tumor cells such as human A375 melanoma, human Jurkat T cell lymphoma and murine pineal tumour cells highly express A3ARs on the cell surface. On the other hand, Zhao *et al.* reported that during normal embryo development no expression of A3AR was found, except for the aorta and heart. When the A3AR gene was overexpressed in smooth, cardiac and skeletal muscle lineages during early embryogenesis in knockout or wild-type mice, it was lethal to the emryos.[16] Taken together, it seems that low receptor expression is a characteristic of most normal tissues, while tumor cells show high expression, which suggest this receptor as a target for the induction of tumor growth arrest.

The anticancer effect of A3AR agonists and its mechanism

The synthetic agonists, IB-MECA and Cl-IB-MECA, possess high affinity to the A3AR. The two molecules are adenosine derivatives as result of a substitution at the 2-position and at the N^6 and 5¢ positions of adenosine (Figure 4). This structure protects against rapid metabolization by adenosine deaminase and further enhances the affinity to A3AR, while having low affinity to the A1, A2A or A2B adenosine receptors.[5]

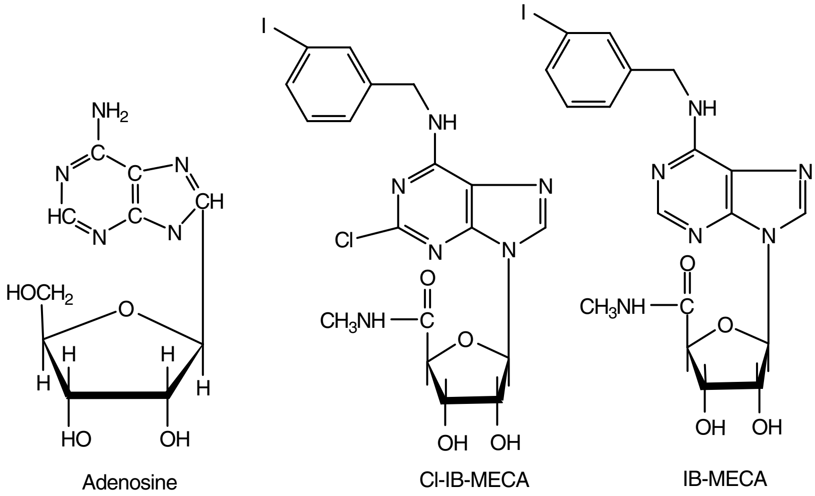

Figure 4. Chemical structure of the two synthetic A3AR agonists, IB-MECA and Cl-IB-MECA, compared to the natural ligand adenosine.

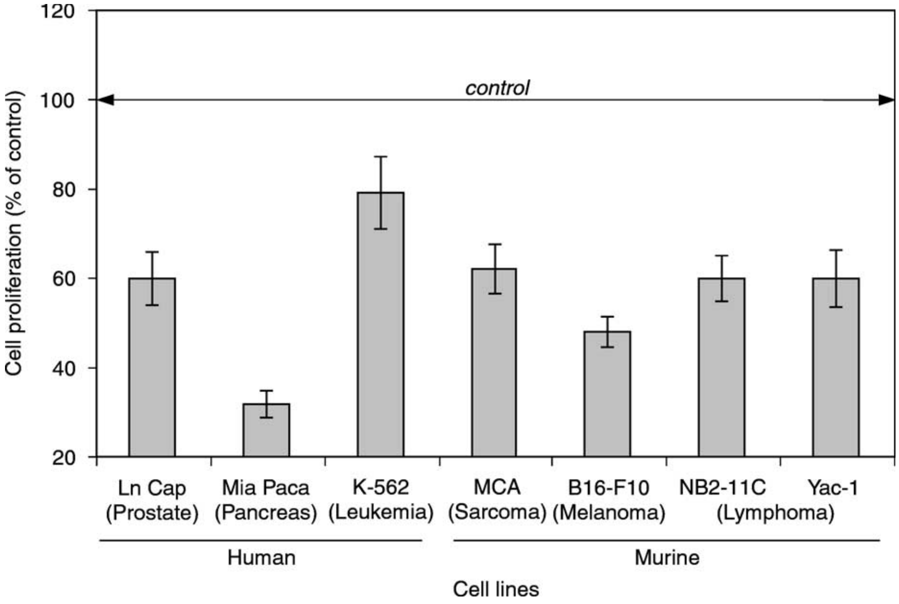

Figure 5. IB-MECA inhibits the proliferation of various tumor cell lines. Tumor cells were exposed to IB-MECA (10 μM) for 48 h. Proliferation was measured by [^{3}H]thymidine incorporation assay.

The two agonists were found to have a potent anticancer effect when used *in vitro* at low concentrations (0.01–10 μM). Exposure of various tumor cell to the A3AR agonists inhibited prolifertion of these cells (Figure 5). The mechanism was found to involve inhibition of telomerase activity and a cell cycle arrest in the G_0/G_1 phase of the cell cycle leading to a cytostatic effect.[6,17] Additionally, it was found that agonists to the A3AR cause tumor growth inhibition by de-regulating the Wnt signaling pathway.[18] The Wnt signaling pathway, active during embryogenesis and tumorigenesis, leads to cell cycle progression and cell proliferation. Glycogen synthase kinase (GSK)-3β plays a key role in this pathway by phosphorylating β-catenin, leading to its ubiquitination. Upon activation of the Wnt pathway GSK-3β is deactivated and β-catenin is not phosphorylated, and thus accumulates in the cytoplasm. β-catenin, which is then translocated to the nucleus, associates with Lef/Tcf inducing the transcription of cell cycle

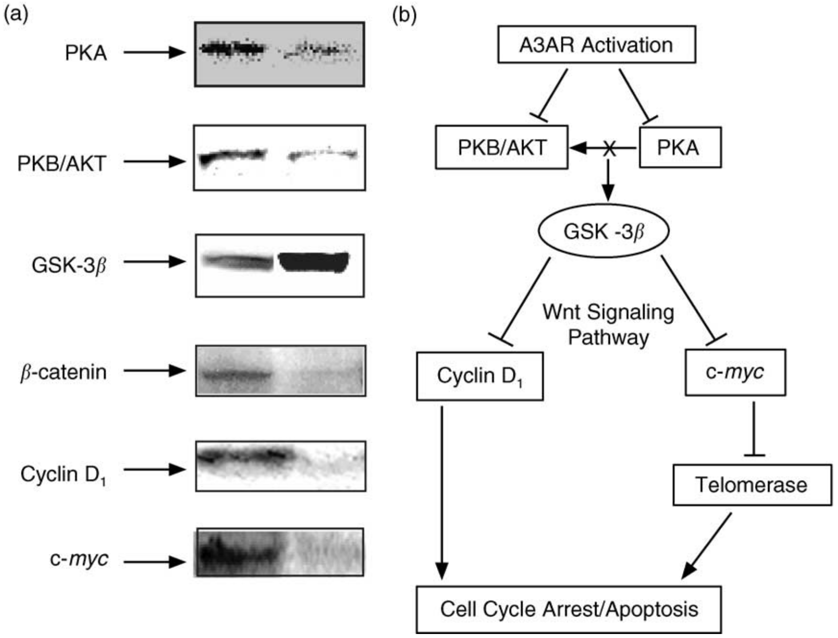

Figure 6. A3AR agonists induce de-regulation of the Wnt signaling pathway. (a) B16-F10 melanoma cells were treated with 10 nM IB-MECA. Protein extracts were prepared and subjected to Western blot analysis. (b) Suggested signaling pathway which takes place upon A3AR activation by IB-MECA.

progression genes such as c-*myc* and cyclin D_1. Upon exposure of the tumor cells to the A3AR agonist IB-MECA, the expression of PKA and B (PKA and PKB/Akt, respectively), key elements downstream to cAMP, known to phosphorylate and inactivate GSK-3β, was inhibited. Consequently, it yielded an increase in the GSK-3β level, followed by destabilization of β-catenin and subsequent suppression of cyclin D_1 and c-*myc* (Figure 6). The specificity of this response was demonstrated when an antagonist to A3AR, 5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate (MRS-1523), reversed the increase in the GSK-3β level, counteracting IB-MECA's effect on melanoma cell growth.

In three different experimental tumor models in mice, including syngeneic (B16-F10 melanoma in C57Bl/6J mice) and xenograft models (HCT-116 human colon carcinoma and PC-3 human prostate carcinoma in nude mice), IB-MECA or Cl-IB-MECA inhibted tumor growth when administered orally at low dosages (5–100 μg/kg). The tumor-inhibitory effect was of the same magnitude as that seen with a standard chemotherapy protocol. When given in combination with chemotherapy, a synergistic effect was seen yielding an overall larger effect than either these agonists or chemotherapy alone (Figure 7).

The myeloprotective effect of A3AR agonists

Synthetic A3AR agonists exhibited a meylostimulatory effect both *in vitro* and *in vivo*. This was manifested in the induction of G-CSF production by mononuclear cells derived from bone marrow or spleen. *In vivo*, IB-MECA or Cl-IB-MECA acted as myeloprotective agents and counteracted the myelotoxicity induced by chemotherapy.[4,17] Administration of the compounds to mice that were pretreated with cyclophosphamide (CYP) resulted in an accelerated recovery of white blood cell (WBC) and neutrophil counts (Figure 8). As shown for the anticancer effect, IB-MECA and Cl-IB-MECA exerted the myeloprotective effect at a therapeutic window of 5–100 μg/kg body weight.

The potential for future cancer therapy

Agonists to the A3AR possess unique characteristics which make them attractive future anticancer and myeloprotective agents. The molecules are orally bioavaiable, non-toxic and have therapeutic effect at low dosages. A3AR agonists can be used in the treatment of cancer in a number of treatment modalities: monotherapy to combat cancerous

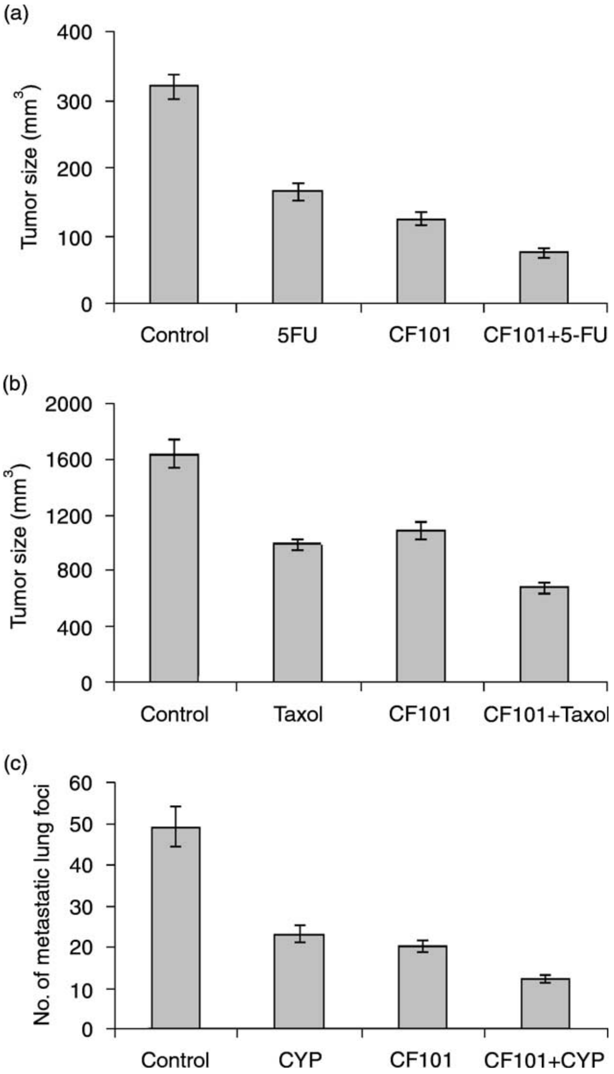

Figure 7. A3AR agonists synergise with chemotherapy to inhibit tumor development in melanoma, colon carcinoma and prostae cancer in mice.

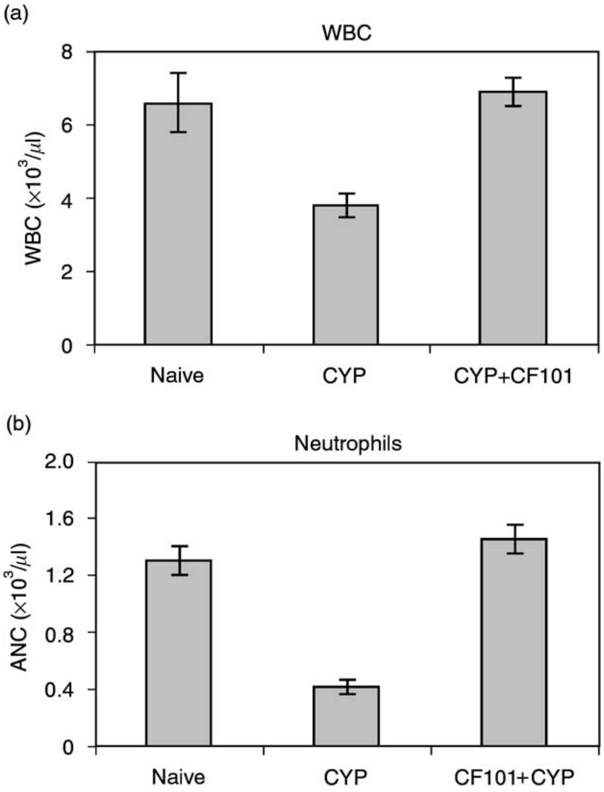

Figure 8. Effect of IB-MECA on WBC and neutrophil numbers in mice rendered neutropenic by CYP. CYP alone decreased the number of WBC and neutrophils. IB-MECA, administered 2 days after CYP, increased the number of total WBC counts (a) and restored the percentage of neutrophils (b).

conditions, combination with chemotherapy to increase the therapeutic index of the cytotoxic agent and at the same time to act as a myeloprotective agent, and maintanace treatment following aggressive therapies such as radio- or chemotherapy. In other studies,[19–21] A3AR agonists (at similar dosages) were shown to induce additional beneficial effects. These included neuroprotective activity following chronic administration of IB-MECA to gerbils with cerebral ischemia,[19] cardioprotective activity during prolonged simulated ischemia by rescuing injured myocytes[20] and anti-inflammatory effects.[21] These additional effects, while being worthy therapeutic targets by their own rights, may also benefit cancer patients and thus increase the atractiveness of using these agents in cancer therapy.

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