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The A3 Adenosine Receptor as a New Target for Cancer Therapy and Chemoprotection

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Adenosine, a purine nucleoside, acts as a regulatory molecule, by binding to specific G-protein-coupled A_1, A_{2A}, A_{2B}, and A_3 cell surface receptors. We have recently demonstrated that adenosine induces a differential effect on tumor and normal cells. While inhibiting *in vitro* tumor cell growth, it stimulates bone marrow cell proliferation. This dual activity was mediated through the A3 adenosine receptor. This study showed that a synthetic agonist to the A3 adenosine receptor, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyl-uronamide (Cl-IB-MECA), at nanomolar concentrations, inhibited tumor cell growth through a cytostatic pathway, i.e., induced an increase number of cells in the G0/G1 phase of the cell cycle and decreased the telomeric signal. Interestingly, Cl-IB-MECA stimulates murine bone marrow cell proliferation through the induction of granulocyte-colony-stimulating factor. Oral administration of Cl-IB-MECA to melanoma-bearing mice suppressed the development of melanoma lung metastases (60.8 ± 6.5% inhibition). In combination with cyclophosphamide, a synergistic antitumor effect was achieved (78.5 ± 9.1% inhibition). Furthermore, Cl-IB-MECA prevented the cyclophosphamide-induced myelotoxic effects by increasing the number of white blood cells and the percentage of neutrophils, demonstrating its efficacy as a chemoprotective agent. We conclude that A3 adenosine receptor agonist, Cl-IB-MECA, exhibits systemic anticancer and chemoprotective effects. © 2001 Academic Press

Key Words: **A3 adenosine receptor; melanoma; bone marrow; synthetic A3 agonists; neutrophils; G-CSF.**

INTRODUCTION

Conventional cancer treatments have many modalities, all directed at killing tumor cells or preventing their proliferation. The most common approach is chemotherapy, which is not selective toward tumor cells, but damaging to normal cells too. Bone marrow myeloid cells are the first to be adversely affected by chemotherapy, leading to a decline in the number of peripheral blood granulocytes and in some cases even to neutropenic fever. Thus, treatment differentiating between tumor and normal cells is required.

Recently, we showed, *in vitro,* that adenosine inhibits the proliferation of various tumor cell lines, while maintaining the growth of normal cells, such as bone marrow cells [1, 2]. Adenosine is a ubiquitous nucleoside present in all body cells. It is released from metabolically active or stressed cells and subsequently acts as a regulatory molecule. It binds to cells through specific A_1, A_{2A}, A_{2B}, and A_3 G-protein-associated cell surface receptors, thus acting as a signal transduction molecule by regulating the levels of adenylyl cyclase and phospholipase C [3, 4]. However, the utilization of adenosine as a therapeutic agent is restricted, as it rapidly metabolizes to inosine and AMP and thus, its ability to exert a systemic effect is limited.

Pharmacological studies by our group, using antagonists to the adenosine receptors, revealed that the A3 adenosine receptor (A3AR) plays a key role in the adenosine-induced inhibition of tumor cell proliferation, simultaneously stimulating bone marrow cell growth [2, 5, 6]. Agonists to the A3AR act similarly to adenosine, while having the advantage of being stable, nondegradable, and bioavailable molecules.

In this study, we used Cl-IB-MECA, an A3 adenosine receptor agonist, and examined its differential effect on tumor and normal cells. *In vitro* studies showed that Cl-IB-MECA inhibits the proliferation of a melanoma cell line while stimulating bone marrow cell growth. When administered orally to mice, it inhibited the development of melanoma lung metastases and acted as a chemoprotective agent. This dual activity suggests Cl-IB-MECA's application as an anti-cancer treatment by itself or as an adjuvant to chemotherapy with a myeloprotective effect.

MATERIALS AND METHODS

Tumor and normal cells. B-16-F10 melanoma cells were used. Cells were maintained in RPMI medium containing 10% fetal bovine serum (FBS) and were transferred to a freshly prepared medium twice weekly. Normal bone marrow cells were obtained from the

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C57BL/6J mice femur and were disaggregated by passing through a 25-G needle.

Drugs. The A3 adenosine receptor agonist 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyl-uronamide (Cl-IB-MECA) was used. Cl-IB-MECA was dissolved in dimethylsulfoxide and then dilutions were obtained in RPMI medium. Cl-IB-MECA was kindly donated by the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program. The A3 adenosine receptor antagonist (5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate) (MRS-1523) was used to prove the specific binding of Cl-IB-MECA to the A3AR.

All agents were introduced to the culture medium at the time of plating.

Anti-murine G-CSF antibodies (rabbit antiserum purified by Protein A chromatography, Cytolab LTD, Weizmann Science Park, Israel) were used. Cyclophosphamide was purchased from Taro Pharmaceutical Industries Ltd. (Haifa Bay, Israel).

Cell proliferation assays. [^{3}H]Thymidine incorporation assay was used to evaluate cell growth. B-16-F10 melanoma cells (1.5×10^4/ml) or bone marrow cells (1.5×10^6/ml) were incubated with Cl-IB-MECA at a concentration of 100 and 10 nM, in 96-well microtiter plates for 48 h. To test whether Cl-IB-MECA exerted its effect on tumor or normal cells through binding to the A3AR, MRS-1523 (10 nM), an antagonist to the A3AR, was added to the cell cultures in the presence of Cl-IB-MECA. Cultures of B-16 melanoma or bone marrow cells that were incubated in the presence of MRS-1523 only served as controls. For the last 18 h of incubation, each well was pulsed with 1 μCi [^{3}H]thymidine. Cells were harvested and the [^{3}H]thymidine uptake was determined in an LKB liquid scintillation counter (LKB, Piscataway, NJ). These experiments were repeated at least 10 times. Since counts per minute (cpm) values may differ from one experiment to another, in order to compare between the various studies, cpm of Cl-IB-MECA-treated samples were calculated as the percentage of cpm of untreated (control) samples. According to this analysis, control values are 100%.

Cell cycle analysis. Flow cytometric analysis of the cell cycle of B-16 melanoma cells was carried out by propidium iodide staining according to Krishan [7]. Cells (1.5×10^4/ml), were cultured in RPMI medium supplemented with 10% FBS in the presence of Cl-IB-MECA (10 nM) for 24 h at 37°C in a CO_2 incubator. Cells cultured in RPMI supplemented with 10% FBS served as controls. At the end of the incubation period, the cells were washed three times with PBS and resuspended in staining buffer containing 0.1% bovine serum albumin (BSA), 50 μg/ml propidium iodide (PI), 0.1% Triton X-100, and 1 mg/ml RNAase (boiled for 10 min). Samples were examined after 30 min of staining on a FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA). Induction of apoptosis can be detected by reduced fluorescence of the nuclear dye PI with the appearance of an hypodiploid peak at a lower fluorescence values. The cell cycle studies were repeated at least 10 times.

Telomeric signal analysis. B16-F10 melanoma cells were incubated in the absence or presence of 10 nM Cl-IB-MECA for 48 h. Thirty minutes prior to harvest, cells were treated with Colcemid (final concentration, 0.04 μg/ml, GIBCO BRL). At the end of the incubation period, the culture content was centrifuged and the cell pellet was treated with hypotonic solution (KCl, 0.06 M) for 20 min, and then fixed in a mixture of acetic acid and methanol (1:3, v/v). Slides were prepared following the standard air drying procedure [8].

The cytological preparations from the control as well as treated cultures were used for fluorescence *in situ* hybridization (FISH) experiments to evaluate the amount of telomeric signals in cells.

For FISH analysis, the cytological preparations were hybridized with a biotin-labeled telomeric DNA probe according to the manufacturer's protocol (Oncor, Inc., Gaithersburg, MD) with slight modifications [9]. All slides were coded and analyzed using a fluorescence microscope (Nikon, Mellville, NY) equipped with FITC and PI filters (Chroa Technology Corp., Brattleboro, VT). A minimum of 200 interphase nuclei was analyzed.

The percentage telomeric area in the interphase nuclei in FISH preparations was quantified by a software package (Metaview Imaging system version 3.6a, Universal Imaging Co., Westchester, PA). From each sample, at least 50 interphase nuclei were quantified and the mean value of percentage telomeric area compared to the total nuclear area was calculated. The values of the treated groups were compared with those of control after decoding of the slides.

Effect of antibodies against murine granulocyte-colony-stimulating factor (G-CSF) on the response of bone marrow cells to Cl-IB-MECA. To test whether Cl-IB-MECA induced bone marrow cell proliferation via its capability to stimulate G-CSF production, anti-G-CSF antibodies (rabbit antiserum purified by Protein A chromatography (Cytolab Ltd., Weizmann Science Park, Israel); 100 μg/ml, titer 1:16,000) were added to a culture of bone marrow cells in the presence of Cl-IB-MECA (10 nM). Cell proliferation was measured as described above. As control served cells that were incubated with anti-G-CSF only.

In vivo studies. Male C57BL/6J 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).

To examine Cl-IB-MECA's capability of inhibiting tumor growth *in vivo,* we used the artificial lung metastasis model in which 2.5×10^5 B16-F10 melanoma cells were inoculated to mice intravenously (i.v.). In these experiments the activity of Cl-IB-MECA was compared with the recognized cyclophosphamide (CYP) regimen of 50 mg/kg body weight. In addition, therapy of Cl-IB-MECA combined with CYP, was tested. In all experiments where CYP was used, it was administered 1 day after tumor inoculation via intraperitoneal injection.

Mice were divided into four groups (each contained 20 mice) and were treated daily via per os administration according to the following protocol (designated as regimen A):

1. control group—vehicle only;
2. 6 μg/kg body weight Cl-IB-MECA;
3. chemotherapy—50 mg/kg CYP;
4. 50 mg/kg CYP + 6 μg/kg body weight Cl-IB-MECA

Mice were sacrificed after 15 days, lungs removed and black metastatic foci were counted using a dissecting microscope.

To test the myeloprotective effect of Cl-IB-MECA, blood samples were withdrawn from the above-mentioned groups 96, 120, and 144 h following the treatment with CYP. Blood cell counts were carried out in a Coulter counter and differential cell counts were performed on smear preparations stained with May–Grunvald–Giemsa solution.

Statistical analysis. The *in vitro* and *in vivo* results were statistically evaluated using the Student's *t* test. For statistical analysis, comparison between the mean value of different experiments was carried out. The criterion for statistical significance was $P < 0.05$.

RESULTS

Cl-IB-MECA Inhibits Tumor Cell Growth and Stimulates Bone Marrow Cell Proliferation in Vitro through Specific Binding to A3AR

Cl-IB-MECA exerted a dose-dependent statistically significant inhibitory effect on the growth of B16-F10 melanoma cells, as measured by [^{3}H]thymidine incorporation. Proliferation of untreated melanoma cells is presented as 100 ± 4.3%. In the presence of Cl-IB-MECA, melanoma cell proliferation was inhibited and reached 60 ± 7.5 and 72 ± 8.5% of control values ($P < 0.001$ for both concentrations) at 100 and 10 nM Cl-IB-MECA, respectively (Fig. 1).

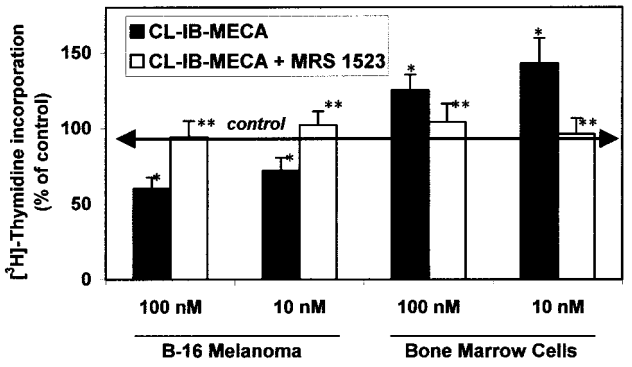

FIG. 1. Cl-IB-MECA induced a dose dependent inhibitory effect on the proliferation of B16-F10 melanoma cells, while stimulating bone marrow cell growth. The A3 adenosine receptor antagonist MRS-1523 (10 nM) neutralized the inhibitory/stimulatory effect of Cl-IB-MECA. Cell proliferation was measured by [^{3}H]thymidine incorporation assay. *$P < 0.002$ relating to a comparison between Cl-IB-MECA with respect to the control. **$P < 0.002$ relating to a comparison between Cl-IB-MECA + MRS with respect to Cl-IB-MECA alone.

An opposite effect was obtained when murine bone marrow cells were incubated in the presence of Cl-IB-MECA. Proliferation of bone marrow cells was stimulated and reached 125 ± 10.4 and 143 ± 16.5% of control values ($P < 0.001$ for both concentrations) at 100 and 10 nM Cl-IB-MECA, respectively (Fig. 1).

In the presence of Cl-IB-MECA and the A3 antagonist MRS-1523, proliferation of melanoma cells was not inhibited and was similar to that of control untreated cells ($P < 0.001$ for 10 and 100 nM concentrations). MRS-1523 neutralized also the stimulatory effect of Cl-IB-MECA on the murine bone marrow cells (100 nM, $P < 0.002$, and 10 nM; $p < 0.001$).

These results demonstrate that the inhibitory or stimulatory activity of Cl-IB-MECA were both mediated through A3AR.

Cl-IB-MECA Affects Cell Cycle and Telomeric Signal

Cell cycle analysis of B16-F10 melanoma cells treated with 100 nM Cl-IB-MECA, revealed an hypodiploid peak at the lower fluorescence values demonstrating 10.1% of apoptotic cells. Cell cycle analysis of B16-F10 melanoma cells treated with Cl-IB-MECA (10 nM) for 24 h, revealed a statistically significant ($P < 0.002$) increased percentage of cells in the G0/G1 phase with a decreased percentage in the S and G2 phases of the cell cycle (Fig. 2). We did not observe an hypodiploid peak at the lower fluorescence values, i.e., no apoptosis at this Cl-IB-MECA concentration was noted.

Although the maximal inhibitory effect was demonstrated at a concentration of 100 nM, we preferred to continue the experiments with the lower dose of Cl-IB-MECA (10 nM) since at this concentration, a more pronounced stimulatory effect toward bone marrow cells was observed.

Telomeric signal analysis of B16-F10 melanoma cells incubated with 10 nM Cl-IB-MECA revealed a significant ($P < 0.01$) reduction in the percentage telomeric area in comparison to control (Figs. 3a and 3b).

The above results proved that Cl-IB-MECA (at a 10 nM concentration) inhibits tumor cell growth through a cytostatic rather than an apoptotic pathway.

Cl-IB-MECA Exerts Its Stimulatory Activity on Bone Marrow Cells via G-CSF Production

The stimulatory effect of Cl-IB-MECA on murine bone marrow cell proliferation was examined in the presence and absence of anti-G-CSF antibodies. Anti-G-CSF antibodies (0.05 μg/ml), partially canceled the stimulatory effect of Cl-IB-MECA. At a higher antibody concentration (0.5 μg/ml), the stimulatory effect on bone marrow cell proliferation was completely neutralized (Fig. 4). This supported the assumption that the stimulatory effect of Cl-IB-MECA is mediated by its capability to induce G-CSF production.

Cl-IB-MECA Inhibits Tumor Cell Growth in Vivo and Protects against Myelotoxicity Following Chemotherapy

In this set of experiments, we evaluated Cl-IB-MECA's ability to inhibit the growth of lung metastases in mice inoculated with B16-F10 melanoma cells. The effect of Cl-IB-MECA alone or in combination with cyclophosphamide was examined.

In the mice inoculated with B16-F10 melanoma cells and treated with Cl-IB-MECA alone, a statistically significant inhibition in the development of lung metastatic foci (60.8 ± 6.5%, $P < 0.001$) was observed. Moreover, a combined treatment of Cl-IB-MECA and CYP resulted in an additive inhibitory effect on the number of lung metastatic foci (CYP alone: 47 ± 5.6%, $P < 0.001$; CYP + Cl-IB-MECA: 78.5 ± 9.1%, $P < 0.0001$) (Fig. 5).

Mice treated with cyclophosphamide alone exhibited a decline in the number of peripheral blood leukocytes and neutrophils. Administration of Cl-IB-MECA following chemotherapy, restored the number of white blood cells (Fig. 6a) and the percentage of neutrophils (Fig. 6b) to normal values, after 120 and 144 h, respectively.

DISCUSSION

This study showed that Cl-IB-MECA induced a differential effect on tumor and normal cells. It inhibited the growth of B16-F10 melanoma cells, while promoting the proliferation of bone marrow cells. Although the maximal inhibitory/stimulatory effect was around 40%, it has a highly statistical significance since the

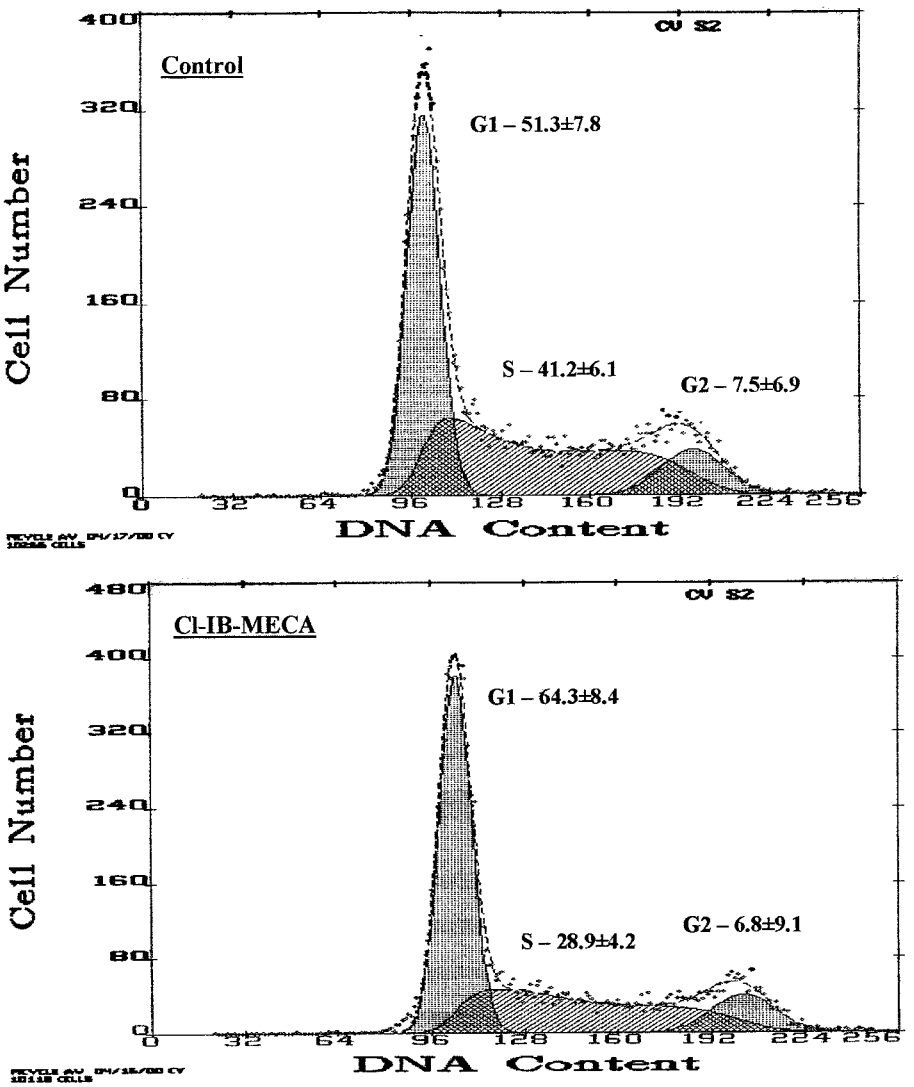

FIG. 2. Cl-IB-MECA induces a cytostatic effect on B16-F10 melanoma cell growth. Flow cytometric analysis of the cell cycle status of B16-F10 melanoma cells following incubation with 0.01 μM Cl-IB-MECA resulted in an increase in the number of cells in the G0/G1 phase and a decrease in the S phase. Cells were stained with propidium idodide.

experiments were repeated at least 10 times. The MRS-1523 antagonist [22–24] reversed these inhibitory and stimulatory effects, demonstrating that Cl-IB-MECA's dual activity was mediated through A3AR. Cl-IB-MECA was first synthesized by the group of Jacobson *et al.* [22], which reported that the agonist possesses a high affinity to the rat A3 adenosine receptor with a K_i value of 0.33 (while having K_i values of 820 and 1590 nM for the A1 and A2A adenosine receptors, respectively). In the present study we used 10 and 100 nM Cl-IB-MECA which induced the inhibitory/stimulatory effects at concentrations much lower than the K_i values for the A1 and A2 receptors. Thus we may conclude that the effect of Cl-IB-MECA was specifically mediated through the A3 adenosine receptor. Although Cl-IB-MECA was synthesized as a rat A3 adenosine receptor agonist, we presume that it has similar affinity to the murine A3 receptor due to an identity of 91% between the two receptors. This was concluded following a comparison between receptor sequence of both species, utilizing the Blast Pub Med engine.

Cl-IB-MECA, at a concentration of 10 nM, induced a cytostatic effect on melanoma cell growth. It elicited inhibition of [^{3}H]thymidine incorporation, increased number of cells in the G0/G1 phase of the cell cycle, demonstrating that Cl-IB-MECA acts as a cytostatic agent.

Supporting these findings are those of Brambilla *et al.*, who demonstrated that activation of A3 receptors led to a cytostatic effect [13]. CHO cells transfected with human A3 receptor cDNA and incubated in the presence of IB-MECA or Cl-IB-MECA, induced a cytostatic effect on cell growth, by arresting the cells at the G2/M phase of the cell cycle.

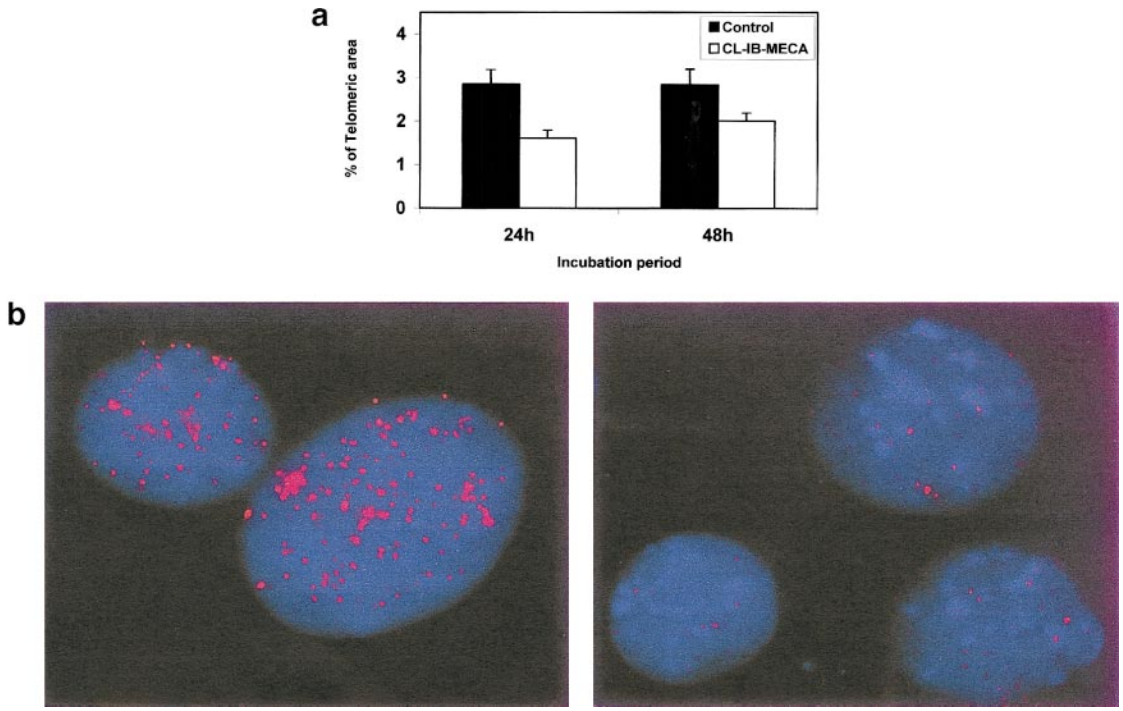

FIG. 3. (a) Cl-IB-MECA reduces the telomeric signal in B-16 melanoma cells. Quantitation of the telomeric signal in cytological preparations treated with Cl-IB-MECA showed a significant ($P < 0.01$) reduction in the percentage telomeric area in comparison to control. (b) FISH preparations with telomeric DNA in B-16 melanoma cells treated with 0.01 μM Cl-IB-MECA. Note more signals in the left panel (control) compared to the right panel (treated).

In the present study Cl-IB-MECA, at a concentration of 100 nM, induced apoptosis in the B-16 melanoma cells. Adenosine or its agonists, at micromolar concentrations, have been reported to inhibit cell growth through an apoptotic mechanism including HL-60 promyelocytic leukemia, U-937 histiocytic lymphoma cells [14], cardiomyocytes [15], and rat astroglial cells [16]. Thus, it can be concluded that the effect of adenosine or its agonists on cell proliferation depends upon its extracellular concentration; i.e., while at low nanomolar concentrations cell cycle arrest is induced, at the micromolar range apoptosis takes place.

Furthermore, a reduced telomeric signal was observed in the melanoma cells following incubation with A3AR agonists. Telomeres are specialized structures consisting of repeat arrays of TTAGGGn located at the ends of chromosomes and are essential for their stability. In the majority of normal somatic cells, telomeres shorten with each cell division, serving as a checkpoint for cell cycle progression and regulating cell senescense and apoptosis [17].

Accumulating evidence indicates that telomerase ac-

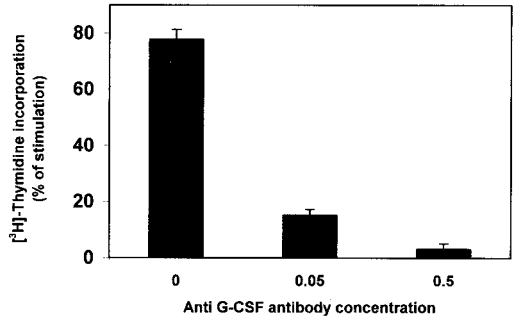

FIG. 4. Antibodies to G-CSF neutralized the stimulatory effect of Cl-IB-MECA on bone marrow cell proliferation (measured by [^{3}H]thymidine incorporation assay) in a dose-dependent manner.

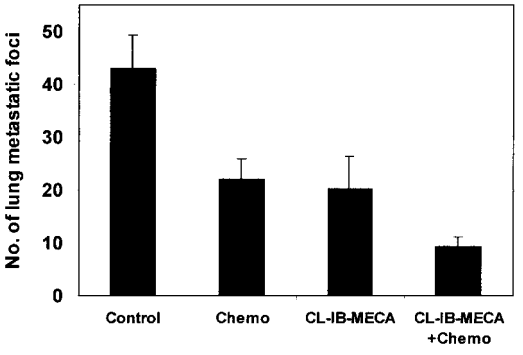

FIG. 5. The effect of Cl-IB-MECA alone or in combination with cyclophosphamide on the development of lung metastatic melanoma in mice was examined. A statistically significant decrease in the number of melanoma lung foci ($P < 0.001$) was observed by Cl-IB-MECA alone (Fig. 4). Moreover, Cl-IB-MECA synergized with cyclophosphamide and induced a marked inhibition ($P < 0.0001$) in the number of melanoma lung foci.

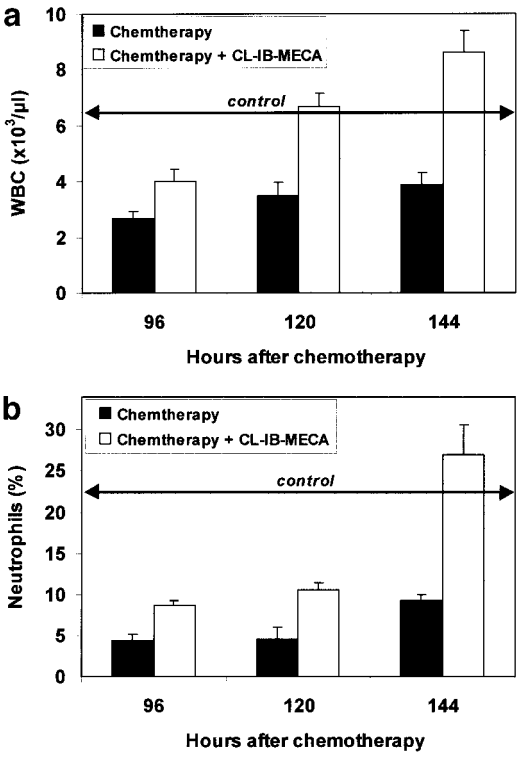

FIG. 6. The effect of Cl-IB-MECA on the myeloid system in mice inoculated with B16-melanoma cells and treated with cyclophosphamide, was evaluated. Cyclophosphamide alone decreased the number of leukocytes and neutrophils. Cl-IB-MECA, administered after chemotherapy, restored the number of total white blood cell count (a) and the percentage of neutrophils (b). Each group contained 20 mice and three experiments were carried out.

tivity is reactivated in cancer and immortal cells [18, 19]. Increased telomerase activity was found during progression of melanocytic naevi to malignant melanoma, in cutaneous malignant melanoma lesions and in uveal melanoma [20, 21]. We suggest that activation of the senescence pathway by Cl-IB-MECA may act as an additional mode of inhibiting melanoma cell growth.

We confirmed the *in vitro* anti-cancer effect by *in vivo* studies. Oral administration of Cl-IB-MECA to mice resulted in the suppression of melanoma lung metastases. Moreover, an additive inhibitory effect was observed when Cl-IB-MECA was administered in combination with cyclophosphamide. In previous studies we administered IB-MECA, an A3 adenosine receptor agonist similar in its structure to Cl-IB-MECA, to melanoma-bearing mice in three differentt modes, i.e., intraperitoneal, subcutaneous, and per os. Similar inhibitory effect on melanoma growth *in vivo* was observed following the three treatment modes (unpublished data). As the oral route has the most convenient clinical application, we have chosen to further treat the mice via the oral route. Indeed in a recent study [22] we showed that IB-MECA suppressed the development of lung metastatic foci when administered daily orally to mice inoculated with B-16-F10 melanoma cells. The systemic activity exerted by Cl-IB-MECA following its per os administration demonstrated the high bioavailabilty of the compound. Cl-IB-MECA is a stable molecule due to a substitution at the 2-position and at the N^6 and 5′ positions of adenosine. This structure protects the molecule against rapid metabolization *in vivo* by adenosine deaminase and further enhances its affinity to A3AR. Van Schaick *et al.* [23] reported that the serum half-life time of Cl-IB-MECA, when administered to rats, was 30 min. This is sufficient time for the molecule to be transported from the digestive tract to the circulation, thereby exerting a systemic effect. To the best of our knowledge this is the first time that *in vivo* efficacy with Cl-IB-MECA or any A3 adenosine agonist is exhibited through per os administration. Cl-IB-MECA was earlier shown by Von Lubitz *et al.* to possess *in vivo* efficacy. Chronic treatment of mice, in a dose range similar to the one we used in the present study, resulted in a neuroprotective effect [24, 25].

Interestingly, we observed that Cl-IB-MECA induced a positive stimulatory effect on normal murine bone marrow cells. This activity was mediated *in vitro* through a mechanism entailing the induction of G-CSF production. Moreover, Cl-IB-MECA *in vivo* counteracted chemotherapy-induced myelotoxicity by increasing the number of white blood cells and neutrophils. G-CSF is clinically used to reduce the length of neutropenia following chemotherapy and bone marrow transplantation. It stimulates the proliferation and differentiation of hematopoietic progenitors and also controls the functional activities of neutrophils and macrophages [26, 27]. Thus, we may suggest that Cl-IB-MECA, through its capability to induce G-CSF production, exerted the *in vivo* chemoprotective effect.

Our results led us to conclude that Cl-IB-MECA has a contrasting effect on normal (stimulation) and tumor (inhibition) cells. Agonists to A3AR have previously been shown to mediate opposing effects on various cell types and this has even been defined as a “paradoxical” phenomenon [28]. A3AR activation, by agonist concentrations in the nanomolar range has been found to mediate functions such as cerebroprotective activity following chronic administration of IB-MECA to gerbils with cerebral ischemia [29]; cardioprotective activity during prolonged simulated ischemia by rescuing injured myocytes [30]; and an anti-inflammatory effect [31]. However, micromolar concentrations of the agonists were shown to induce cytotoxic effects via an apoptotic pathway, as detailed above.

Thus, it may be suggested that activation of A3AR mediates a variety of activities, including cell growth regulation and induction of cytokine production. The anti-cancer and chemoprotective activities, combined with the cardio- and neuro-protective effects of A3AR agonists suggest the use of these small, highly bioavailable molecules as agents to combat cancer.

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