

# Homeostatic Action of Adenosine A<sub>3</sub> and A<sub>1</sub> Receptor Agonists on Proliferation of Hematopoietic Precursor Cells

MICHAL HOFER,<sup>\*,1</sup> MILAN POSPIŠIL,<sup>\*</sup> VLADIMÍR ZNOJIL,<sup>†</sup> JIŘINA HOLÁ,<sup>\*</sup> DENISA ŠTREITOVÁ,<sup>\*</sup> AND ANTONÍN VACEK<sup>\*</sup>

*\*Laboratory of Experimental Hematology, Institute of Biophysics, v.v.i., Academy of Sciences of the Czech Republic, Brno, Czech Republic; and †Institute of Pathological Physiology, Medical Faculty, Masaryk University, Brno, Czech Republic*

Two adenosine receptor agonists, *N*<sup>6</sup>-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA) and *N*<sup>6</sup>-cyclopentyladenosine (CPA), which selectively activate adenosine A<sub>3</sub> and A<sub>1</sub> receptors, respectively, were tested for their ability to influence proliferation of granulocytic and erythroid cells in femoral bone marrow of mice using morphological criteria. Agonists were given intraperitoneally to mice in repeated isomolar doses of 200 nmol/kg. Three variants of experiments were performed to investigate the action of the agonists under normal resting state of mice and in phases of cell depletion and subsequent regeneration after treatment with the cytotoxic drug 5-fluorouracil. In the case of granulopoiesis, IB-MECA 1) increased by a moderate but significant level proliferation of cells under normal resting state; 2) strongly increased proliferation of cells in the cell depletion phase; but 3) did not influence cell proliferation in the regeneration phase. CPA did not influence cell proliferation under normal resting state and in the cell depletion phase, but strongly suppressed the overshooting cell proliferation in the regeneration phase. The stimulatory effect of IB-MECA on cell proliferation of erythroid cells was observed only when this agonist was administered during the cell depletion phase. CPA did not modulate erythroid proliferation in any of the functional states investigated, probably due to the lower demand for cell production as compared with granulopoiesis. The results indicate opposite effects of the two adenosine receptor agonists on proliferation of hematopoietic cells and suggest the plasticity

and homeostatic role of the adenosine receptor expression. *Exp Biol Med* 233:897–900, 2008

**Key words:** adenosine receptors; hematopoiesis; cell proliferation

## Introduction

Extracellular adenosine has been found to regulate a variety of physiological functions, including cell proliferation through ligation to four known adenosine receptors denoted A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub>. In most tissues, more adenosine receptor subtypes are coexpressed in the same cell type. These G-protein-coupled receptors differ in their abilities to stimulate or inhibit adenylate cyclase and to influence functioning of the second messenger systems, such as calcium and potassium channels, phospholipases, and mitogen-activating kinases, modulating different cell functions (1–4). We have shown previously that activation of the adenosine receptor signaling via elevation of extracellular adenosine under *in vivo* conditions stimulates hematopoiesis in normal and myelosuppressed mice (5–8). Later it was shown that similar effects can be induced by the adenosine analog *N*<sup>6</sup>-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA), considered as the selective agonist of adenosine A<sub>3</sub> receptors (9–12). These findings have implied a role for adenosine A<sub>3</sub> receptors in the positive control of hematopoiesis. Because hematopoietic cell renewal systems are under both stimulatory and inhibitory control, it has been logical to consider the existence of the opposite adenosine receptor activity that plays role in negative control. Our experiments investigating the action of various adenosine receptor agonists on the cycling of hematopoietic progenitor cells *in vivo* have revealed that A<sub>1</sub> receptor activated by its selective agonist *N*<sup>6</sup>-cyclopentyladenosine (CPA) is the most promising candidate for a negative feedback mediator at the level of adenosine receptor signaling (13, 14).

This work was supported by the Academy of Sciences of the Czech Republic (grant AV0Z50040507) and by the Grant Agency of the Czech Republic (grants 305/06/0015 and 305/08/0148).

<sup>1</sup> To whom correspondence should be addressed at Laboratory of Experimental Hematology, Institute of Biophysics, v.v.i., Academy of Sciences of the Czech Republic, Královopolská 135, CZ-61265 Brno, Czech Republic. E-mail: hofer@ibp.cz

Received February 6, 2008.  
Accepted February 29, 2008.

DOI: 10.3181/0802-RM-43  
1535-3702/08/2337-0897\$15.00  
Copyright © 2008 by the Society for Experimental Biology and Medicine

The studies presented in this paper stem from the above data and describe the actions of isomolar doses of IB-MECA and CPA on granulocytic and erythroid cell proliferation in the bone marrow of mice subjected to the treatment with these two agonists under different functional states of the target cell systems. The results indicate the opposing and homeostatic mode of action of adenosine A<sub>3</sub> and A<sub>1</sub> receptors, and suggest the plasticity of adenosine receptor expression reflecting the requirements of the cell system to maintain the steady state.

## Materials and Methods

**Mice.** Male (CBAx57Bl/10)F<sub>1</sub> mice 3 months old and weighing an average of 30 g were used. Standardized pelleted diet and HCl-treated tap water were given *ad libitum*. The mice were kept under controlled illumination conditions (12:12-hr light:dark cycle), and the temperature was maintained at 22° ± 1°C. The use and treatment of the animals followed the European Community Guidelines as accepted principles for the use of experimental animals. The experiments were performed with the approval of the Institute's Animal Care and Use Committee.

**Drugs.** N<sup>6</sup>-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA), the agonist of adenosine A<sub>3</sub> receptors, and N<sup>6</sup>-cyclopentyladenosine (CPA), the agonist of adenosine A<sub>1</sub> receptors, were dissolved initially in dimethylsulfoxide and diluted with sterile saline and injected intraperitoneally (ip) in single isomolar doses of 200 nmol/kg in a volume of 0.2 ml. The final concentration of dimethylsulfoxide was 2%. 5-Fluorouracil (5-FU) was diluted in saline and injected ip in a single dose of 100 mg/kg in a volume of 0.2 ml. The corresponding drug vehicles were used for control injections. All drugs were obtained from Sigma (St. Louis, MO).

**Hematological Methods.** Mice were sacrificed by cervical dislocation. The femurs were dissected, and marrow cells were flushed from the bone. Numbers of nucleated cells of the bone marrow were determined using a Coulter Counter (model ZF; Coulter Electronics, UK). Differential counts were performed on marrow smears stained with the May-Grünwald-Giemsa method. For granulocytic lineages, myeloblasts through myelocytes were designated as proliferative cells, and metamyelocytes through segmented stages designated as nonproliferative cells. For erythroid lineages, proerythroblasts through basophilic erythroblasts were classified as proliferative cells, and polychromatic and orthochromatic erythroblasts as nonproliferative cells. Total counts of granulocytic or erythroid cells per femur represent the sum of proliferative and nonproliferative cells.

**Statistics.** The data are given as means ± SEM. Experiments were repeated two to four times, and the data were pooled. The Mann-Whitney rank-sum test was used for comparison of the effects, and Holm's method was applied to correct for multiple comparisons. The significance level was set at  $P < 0.05$ .

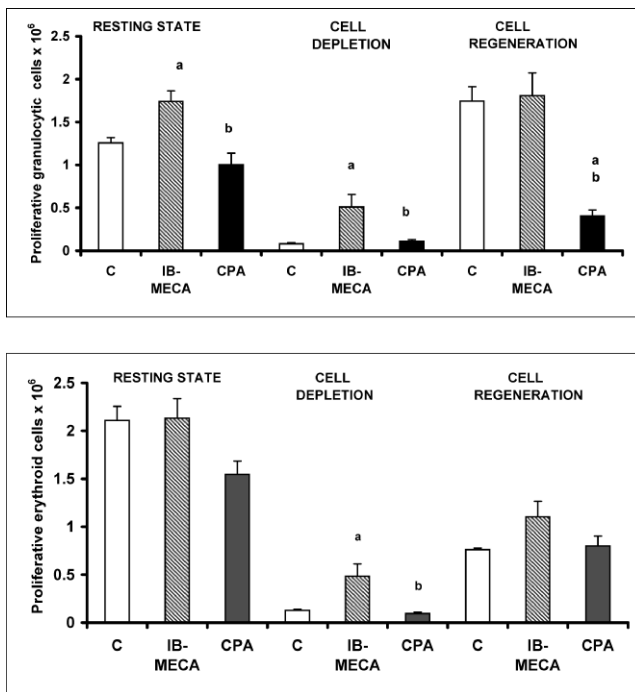
## Results

Experiments included investigations of the *in vivo* effects of the two adenosine receptor agonists IB-MECA and CPA, which activate selectively adenosine A<sub>3</sub> and A<sub>1</sub> receptors, respectively, on murine bone marrow hematopoiesis under normal resting state and under conditions of bone-marrow depletion and regeneration after administration of the cytotoxic drug 5-FU 3 or 7 days before sampling of femoral bone marrow. In each variant of the experiments, the mice were treated either with the vehicle (control) or with the adenosine receptor agonists given in isomolar doses of 200 nmol/kg twice daily for 2 days before the sampling of bone marrow, which followed 24 hrs after the last injection of the vehicle or drug. Morphological criteria were used to determine the proliferative cells of the granulocytic and erythroid cell lineages. The choice of drug doses was based on our former experiments showing that they induced opposite effects on the cycling of murine hematopoietic progenitor cells (13).

The agonists' effects on the proliferative granulocytic cells are presented in the upper part of Figure 1. In control mice treated with vehicle in the cell-depletion phase, cell counts were strongly decreased to 6.4% of the resting norm, but overshot the norm in the regeneration phase by about 39%. Differences between these three control values were significant at  $P < 0.01$ . Treatment with IB-MECA increased the counts of proliferative granulocytic cells moderately but significantly by a factor of 1.38 under normal resting state, strongly increased counts of these cells by a factor of 6.30 in the cell-depletion phase, but did not influence cell counts in the cell-regeneration phase. Treatment with CPA did not significantly influence counts of proliferative granulocytic cells under conditions of normal resting state or in the cell-depletion phase, but strongly decreased counts of these cells to 23% of the overshooting control in the cell-regeneration phase.

The agonists' effects on the proliferative erythroid cells are shown in the lower part of Figure 1. As in case of granulocytic cells, proliferative erythroid cells in control mice are strongly decreased to 6% of the resting norm in the cell-depletion phase. Compared with the granulocytic cells, counts of proliferative erythroid cells of control mice in the cell-regeneration phase did not overshoot or even attain the norm. Nevertheless, differences between these three control values are significant at  $P < 0.01$ . Treatment with IB-MECA significantly increased counts of proliferative erythroid cells by a factor of 3.79 in the cell-depletion phase but did not significantly influence the counts of these cells in the normal resting state or in the cell-regeneration phase. Treatment with CPA did not significantly influence the counts of proliferative erythroid cells in any of the functional states investigated, including the regeneration phase.

Effects of the treatments on the total counts of granulocytic and erythroid cells, that is, the sum of the proliferative and non-proliferative cells in the femoral bone marrow are summarized in Table 1. No significant effects of



**Fig. 1.** Counts of proliferative granulocytic and proliferative erythroid cells (mean  $\pm$  SEM) in femoral bone marrow of mice. Animals were treated with vehicle (C) or IB-MECA and CPA given ip at isomolar doses of 200 nmol/kg twice daily (at 0800 and 1400 hrs) for 2 days before bone marrow sampling, which occurred 24 hrs after the last injection of the vehicle or drugs. Effects of such treatments were investigated in normal resting mice and in mice whose bone marrow cells were either depleted (day 3 after 5-FU administration) or regenerating (Day 7 after 5-FU administration). Each control group contained 25 to 30 mice, with 10 to 15 mice in each group treated with IB-MECA or CPA. Statistical significance:  $P < 0.01$  vs. Control (a) or IB-MECA-treated mice (b).

IB-MECA on the total counts of cells in either lineage was observed in spite of its above-described stimulatory effects on the counts of proliferative cells. This indicates that the enhanced fluxes of cells through the multiplicative pool are associated with enhanced effluxes of cells from the maturation pool. The only significant effect on total cell

counts has been found in the granulocytic lineage in bone marrow of mice treated with CPA in the regeneration phase after 5-FU administration. Here, the decrease of total cell counts is the logical consequence of the inhibition of cell proliferation, which leads to the exhaustion of the maturation and storage pools of the bone-marrow granulocytic cells.

## Discussion

When considering effects of IB-MECA and CPA on granulocytic bone marrow cells, it is evident that the two agonists influence in an opposite manner the proliferation of the cells, depending on the functional state of the cell systems. The agonist of the  $A_3$  receptors IB-MECA enhances strongly granulocytic cell proliferation in the cell-depletion phase and moderately increases proliferation of these cells under normal resting state. The agonist of the  $A_1$  receptors, CPA, is under these conditions ineffective but strongly inhibits granulocytic cell proliferation that overshoots the norm in the cell-regeneration phase. Evidence of opposite effects of different adenosine receptor subtypes is not surprising. Cronstein *et al.* (15) have shown that human neutrophils possess at least two adenosine receptor subtypes ( $A_1$  and  $A_2$ ) and that occupancy of  $A_1$  receptors induces pro-inflammatory effects, i.e., promotes neutrophil adherence to endothelium, whereas occupancy of  $A_2$  receptors induces anti-inflammatory effects like inhibition of neutrophil adherence and generation of toxic oxygen metabolites. Lee and Emala (16) have found that adenosine  $A_1$  receptor activation reduces, while  $A_3$  receptor activation worsens renal ischemia-reperfusion injury.

A further important result of our experiments is the variable effectiveness of the adenosine receptor agonists used under different functional states of the cell populations investigated. Because the effects of the agonists are dependent on the expression of the relevant receptors (17–20), our data suggest the plasticity of this function in the target tissue. Thus, the results support the assumption of the homeostatic role of the adenosine receptor expression

**Table 1.** Total Counts of Granulocytic and Erythroid Cells ( $\times 10^6$ ) in Femoral Bone Marrow under Various Experimental Conditions<sup>a</sup>

| Bone marrow sampling | Treatment          |                    |                                  |
|----------------------|--------------------|--------------------|----------------------------------|
|                      | Vehicle - control  | IB-MECA            | CPA                              |
| Resting state        |                    |                    |                                  |
| Granulocytic cells   | 9.835 $\pm$ 0.336  | 10.007 $\pm$ 0.526 | 9.157 $\pm$ 0.551                |
| Erythroid cells      | 10.807 $\pm$ 0.473 | 10.004 $\pm$ 0.527 | 9.474 $\pm$ 0.707                |
| Cell depletion       |                    |                    |                                  |
| Granulocytic cells   | 2.977 $\pm$ 0.147  | 2.482 $\pm$ 0.253  | 3.168 $\pm$ 0.366                |
| Erythroid cells      | 2.546 $\pm$ 0.131  | 2.963 $\pm$ 0.341  | 2.587 $\pm$ 0.282                |
| Cell regeneration    |                    |                    |                                  |
| Granulocytic cells   | 4.244 $\pm$ 0.456  | 4.570 $\pm$ 0.531  | 1.193 $\pm$ 0.267 <sup>a,b</sup> |
| Erythroid cells      | 3.633 $\pm$ 0.296  | 3.609 $\pm$ 0.302  | 3.781 $\pm$ 0.397                |

<sup>a</sup>Data (mean  $\pm$  SEM) were obtained in the same experiments as used for investigation of proliferative cells and shown in Figure 1. Each control group contained 25 to 30 mice, with 10 to 15 mice in each group treated with IB-MECA or CPA. Statistical significance:  $P < 0.01$  vs. Control (a) or IB-MECA-treated mice (b).

reflected by the action of the agonists. From this point of view, adenosine A<sub>3</sub> receptors can be classified as activators of the positive control, whereas adenosine A<sub>1</sub> receptors can be classified as activators of the negative control of the proliferation of hematopoietic cells.

Taking into account these results, it is possible to discuss some peculiarities and differences in the response of the granulocytic and erythroid cells to the action of adenosine receptor agonists. The cell proliferation-stimulating effects of IB-MECA in the cell-depletion phase are lineage-independent and thus indicate high expression of adenosine A<sub>3</sub> receptors under conditions of increased demand in both cell systems for replenishing the strongly depleted bone marrow cells. Evidence that IB-MECA stimulates cell proliferation under normal resting state only in the granulocytic system and not in the erythroid system can be related to the emergency role of the short-lived (several hours) mature granulocytic cells in the defense mechanisms and thus to a higher demand for production of these cells as compared with the long-lived erythrocytes (more than 30 days in mice) (21). Therefore, in the granulocytic lineage, higher expression of adenosine A<sub>3</sub> receptors linked with the mechanism of positive control should be expected. Similar differences in the physiology of the both lineages can determine the effects of CPA investigated in the cell-regeneration phase. As shown in the experiments presented here, this agonist of adenosine A<sub>1</sub> receptors inhibits proliferation of granulocytic cells, overshooting the resting norm. Thus, higher expression of adenosine A<sub>1</sub> receptors is evidently linked with mechanisms of negative control activated to protect the tissue against damage, which can arise under hyperproliferation of granulocytic cells. Ineffectiveness of CPA to modulate proliferation of erythroid cells under normal resting state and in the cell-regeneration phase can be explained by the lower requirements for proliferation in this cell lineage, which under regeneration does not attain the threshold for activation of the feedback loop represented by the expression of adenosine A<sub>1</sub> receptors.

In conclusion, the findings presented here indicate the potency of adenosine A<sub>3</sub> and A<sub>1</sub> receptors to modulate in an opposite manner proliferation of hematopoietic cells, the plasticity of adenosine receptor expression, and its role in maintaining homeostasis in the hematopoietic cell renewal system. These findings can contribute to a better understanding of possible curative strategies utilizing adenosine receptor agonists in the modulation of hematopoietic functions.

1. Abbracchio MP, Burnstock G. Purinergic signalling: Pathophysiological roles. *Jap J Pharmacol* 78:113–145, 1998.
2. Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Ådén U, Svenningsson P. Adenosine receptor signaling in vitro and in vivo. *Drug Develop Res* 52:274–282, 2001.
3. Jacobson KA, Gao Z-G. Adenosine receptors as therapeutic targets. *Nature Rev Drug Discov* 5:247–264, 2006.

4. Schulte G, Fredholm BB. Signalling from adenosine receptors to mitogen-activated protein kinases. *Cell Signall* 15:813–827, 2003.
5. Hofer M, Pospíšil M, Netíková J, Znojil V, Vácha J. Granulocyte colony-stimulating factor and drugs elevating extracellular adenosine act additively to enhance the hemopoietic spleen colony formation in irradiated mice. *Physiol Res* 48:37–42, 1999.
6. Hofer M, Pospíšil M, Znojil V, Vacek A, Weiterová L, Holá J, Vácha J. Drugs elevating extracellular adenosine promote regeneration of haematopoietic progenitor cells in severely myelosuppressed mice: their comparison and joint effects with the granulocyte colony-stimulating factor. *Eur J Haematol* 68:4–11, 2002.
7. Pospíšil M, Hofer M, Znojil V, Vácha J, Netíková J, Holá J. Synergistic effect of granulocyte colony-stimulating factor and drugs elevating extracellular adenosine on neutrophil production in mice. *Blood* 86:3692–3697, 1995.
8. Pospíšil M, Hofer M, Vacek A, Netíková J, Holá J, Znojil V, Weiterová L. Drugs elevating extracellular adenosine enhance cell cycling of hematopoietic progenitor cells as inferred from the cytotoxic effects of 5-fluorouracil. *Exp Hematol* 29:557–562, 2001.
9. Bar-Yehuda S, Madi L, Barak D, Mittelman M, Ardon E, Ochaion A, Cohn S, Fishman P. Agonists to the A<sub>3</sub> adenosine receptor induce G-CSF production via NF-κB activation: A new class of myeloprotective agents. *Exp Hematol* 30:1390–1398, 2002.
10. Fishman P, Bar-Yehuda S, Farbstein T, Barer F, Ohana G. Adenosine acts as a chemoprotective agent by stimulating G-CSF production: A role for A<sub>1</sub> and A<sub>3</sub> adenosine receptors. *J Cell Physiol* 183:393–398, 2000.
11. Hofer M, Pospíšil M, Vacek A, Holá J, Znojil V, Weiterová L, Štreitová D. Effects of adenosine A<sub>3</sub> receptor agonist on bone marrow granulocytic system in 5-fluorouracil-treated mice. *Eur J Pharmacol* 538:163–167, 2006.
12. Hofer M, Pospíšil M, Znojil V, Holá J, Vacek A, Štreitová D. Adenosine A<sub>3</sub> receptor agonist acts as a homeostatic regulator of bone marrow hematopoiesis. *Biomed Pharmacother* 61:356–359, 2007.
13. Pospíšil M, Hofer M, Vacek A, Znojil V, Pipalová I. Effects of stable adenosine receptor agonists on bone marrow hematopoietic cells as inferred from the cytotoxic action of 5-fluorouracil. *Physiol Res* 53:549–556, 2004.
14. Pospíšil M, Hofer M, Vacek A, Holá J, Pipalová I, Znojil V. N<sup>6</sup>-cyclopentyladenosine inhibits proliferation of murine hematopoietic progenitor cells in vivo. *Eur J Pharmacol* 507:1–6, 2005.
15. Cronstein BN, Levin RI, Phillips M, Hirschhorn R, Abramson SB, Weissmann G. Neutrophil adherence to endothelium is enhanced via adenosine A<sub>1</sub> receptors and inhibited via adenosine A<sub>2</sub> receptors. *J Immunol* 148:2201–2206, 1992.
16. Lee HT, Emala CW. Protective effects of renal ischemic preconditioning and adenosine pretreatment: role of A<sub>1</sub> and A<sub>3</sub> receptors. *Am J Physiol Ren Physiol* 278:F380–F387, 2000.
17. Black RG, Guo Y, Ge Z-D, Murphree SS, Prabhu SD, Jones WK, Bolli R, Auchampach JA. Gene dosage-dependent effects of cardiac-specific overexpression of the A<sub>3</sub> adenosine receptor. *Circ Res* 91:165–172, 2002.
18. Dhalla AK, Shryock JC, Shreeniwas R, Belardinelli L. Pharmacology and therapeutic applications of A<sub>1</sub> adenosine receptor ligands. *Curr Top Med Chem* 3: 369–385, 2003.
19. Dougherty C, Barucha J, Schofield PR, Jacobson KA, Liang BT. Cardiac myocytes rendered ischemia resistant by expressing the human adenosine A<sub>1</sub> or A<sub>3</sub> receptor. *FASEB J* 12:1785–1792, 1998.
20. Madi L, Ochaion A, Rath-Wolfson L, Bar-Yehuda S, Erlanger A, Ohana G, Harish A, Merimski O, Barer F, Fishman P. The A<sub>3</sub> adenosine receptor is highly expressed in tumor versus normal cells: potential target for tumor growth inhibition. *Clin Cancer Res* 10:4472–4479, 2004.
21. Nečas E, Šefc L, Brecher G, Bookstein N. Hematopoietic reserve provided by spleen colony-forming units (CFU-S). *Exp Hematol* 23:1242–1246, 1995.