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# $N^6$-Cyclopentyladenosine inhibits proliferation of murine haematopoietic progenitor cells in vivo

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**Abstract**

Effects of $N^6$-cyclopentyladenosine (CPA), the selective adenosine $A_1$ receptor agonist, on bone marrow haematopoietic progenitor cells for granulocytes and macrophages (CFC-GM) were investigated by utilizing the model of haematopoietic damage induced by 5-fluorouracil. Experiments were performed in vivo on $B10CBAF_1$ mice. A single i.p. injection of CPA at the optimum dose of 200 nmol/kg administered 22 h before a single injection of 5-fluorouracil (100 mg/kg, i.p.) protected CFC-GM against the cytotoxic damage as determined 4 days later. Isomolar doses of the selective agonists for adenosine $A_{2A}$ receptors, i.e. 2-*p*-(2-carboxyethyl)-phenethylamino-5′ -*N*-ethylcarboxamidoadenosine, and for adenosine $A_3$ receptors, i.e. $N^6$-(3-iodobenzyl)adenosine-5′ -*N*-methyluronamide, did not induce such effects. Because 5-fluorouracil is a cell cycle-specific drug damaging mainly cells in the S-phase, protective effects of CPA can be explained by its inhibitory action on the cell cycling. This interpretation was confirmed by experiments demonstrating that repeated administration of CPA in the hyperproliferation phase of the recovering haematopoiesis after 5-fluorouracil treatment inhibited transiently restoration of CFC-GM counts.


*Keywords:* Adenosine receptor; $N^6$-Cyclopentyladenosine; Haematopoietic progenitor cell; 5-Fluorouracil; (Mouse)

## 1. Introduction

Adenosine exhibits specific extracellular signalling activity in the regulation of a variety of cell functions including growth and proliferation (see reviews by Abbracchio, 1996; Schulte and Fredholm, 2003). Studies performed in our laboratories during the last decade have shown that extracellular adenosine receptors can participate also in the control of haematopoiesis. It has been demonstrated that elevation of extracellular adenosine under in vivo conditions induced by the combined administration of dipyridamole, a drug inhibiting the cellular uptake of adenosine, and AMP, the adenosine prodrug, enhances haematopoiesis in normal and myelosuppressed mice and synergizes with the effects of granulocyte colony-stimulating factor (Hofer et al., 1997, 1999, 2001, 2002; Pospíšil et al., 1995, 1998; Weiterová et al., 2000). It has been noted that these haematopoiesis-stimulating effects are pleiotropic and result from enhanced cycling of haematopoietic progenitor cells (Pospíšil et al., 2001). The possibility to utilize adenosine receptor signalling in enhancement of haematopoiesis has been confirmed by experiments of Fishman et al. (2000) demonstrating the curative effects of adenosine alone on haematopoiesis in cyclophosphamide-treated mice and, in addition, by data showing the haematopoiesis-stimulating effects of an adenosine $A_3$ receptor agonist under similar conditions (Bar-Yehuda et al., 2002).

The above findings demonstrated the role of adenosine signalling in the positive control of haematopoiesis. The results presented here represent novel findings indicating that action of adenosine receptors can participate also in the mechanisms of negative control by inhibiting the cycling of haematopoietic progenitor cells. Such an action could be

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inferred from the experiments showing that $N^6$-cyclopentyladenosine, a potent agonist for adenosine $A_1$ receptors (Klotz, 2000), protects progenitor cells committed to granulocyte-macrophage development when administered prior to 5-fluorouracil, a drug which is preferentially toxic to rapidly proliferating cells, and furthermore from findings demonstrating the suppressive effects of $N^6$-cyclopentyladenosine on the recovery of the progenitor cells when given after 5-fluorouracil treatment.

## 2. Materials and methods

### 2.1. Animals

B10CBA$F_1$ male mice aged 3 months and weighing in average 30 g were obtained from the breeding facility of the Medical Faculty, Masaryk University, Brno, Czech Republic. The mice were kept under controlled conditions; standardized pelleted diet and HCl-treated tap water were available ad libitum. The use and treatment of 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 ethics committee.

### 2.2. Drugs and their administration

The following adenosine receptor agonists were used: $N^6$-cyclopentyladenosine (CPA), the adenosine $A_1$-selective receptor agonist; 2-*p*-(2-carboxyethyl)-phenethylamino-5′ -*N*-ethylcarboxamidoadenosine (CGS 21680), the adenosine $A_{2A}$-selective agonist; $N^6$-(3-iodobenzyl)adenosine-5′ -*N*-methyluronamide (IB-MECA), the adenosine $A_3$-selective agonist. CPA and CGS 21680 were dissolved in water, IB-MECA initially in dimethyl sulphoxide. All drug solutions were further diluted with sterile saline and injected i.p. in a volume of 0.2 ml. The final concentration of dimethyl sulphoxide was 2%. The corresponding drug vehicles were used for control injections. The variable drug doses used and schemes of their administration are given under Results. 5-Fluorouracil was diluted in saline and injected i.p. at a single dose of 100 mg/kg in a volume of 0.2 ml. All the drugs used were obtained from Sigma (St. Louis, MO, USA).

### 2.3. Haematological methods

The mice were sacrificed by cervical dislocation. The femurs were removed and the marrow cells were harvested by standard procedures. Numbers of nucleated cells of the femoral marrow were determined using a Coulter Counter (Model ZF, Coulter Electronics, UK). Standard procedures were used for the in vitro assay of the femoral clonogenic cells (Heyworth and Spooncer, 1993). Briefly, granulocyte-macrophage colony-forming cells (CFC-GM) were assayed using a semisolid plasma clot technique. Femoral marrow cell suspensions were plated in triplicate and incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$. CFC-GM were scored after 7-day incubation as colonies containing 50 or more cells. The numbers of CFC-GM per femur were calculated.

### 2.4. Statistics

The data are given as means±S.E.M. Experiments were repeated two to four times and the data were pooled. The sets of experiments investigating the time or dose dependence of the effects were subjected to Kruskal–Wallis analysis of variance. Two-group data were compared using the Mann–Whitney rank sum test. The significance level was set at $P<0.05$.

## 3. Results

Experimental protocol for investigating protective action of CPA on 5-fluorouracil effects consisted of observations of the counts of nucleated cells and of CFC-GM in the femoral marrow of mice on day 4 after the single i.p. injection of 5-fluorouracil. This cytotoxic drug alone at a dose of 100 mg/kg decreased the femoral marrow cellularity to 31% and CFC-GM to 14% of values found in non-treated mice (Table 1). CPA was administered in single i.p. injections at a dose of 200 nmol/kg at different intervals before 5-fluorouracil administration, viz. 10, 16, 22, and 28 h. In another set of experiments, CPA was administered in single i.p. injections at doses of 100, 200, 300, and 400 nmol/kg 22 h prior to 5-fluorouracil administration. The effects were evaluated on the percentage base. As shown in Figs. 1 and 2, effects of CPA were dose- and time-dependent and exhibited bell-shaped dependences. The significant influence of the dose and time factors on the observed effects in both the indices, i.e. marrow cellularity and CFC-GM counts, was confirmed by the use of Kruskal–Wallis analysis of variance ($P<0.01$). The significant protective effect of CPA on 5-fluorouracil toxicity was obtained when administering this adenosine receptor agonist at a dose of 200 nmol/kg 22 h before 5-fluorouracil administration. Because of the possibility that the protective effects of CPA

Table 1
Haematopoietic indices in non-treated and 5-fluorouracil alone-treated mice

| | Non-treated mice | Day 4 after 5-fluorouracil |
|---|---|---|
| Nucleated cells per femur ($\times 10^{-3}$) | 17,840±1580 | 5453±215 |
| CFC-GM per femur | 17,304±1465 | 2361±175 |

The data are given as arithmetic means±S.E.M. from 20 non-treated mice and from all mice receiving 5-fluorouracil alone ($n$=86) and used in investigations of the protective effects of CPA on the 5-fluorouracil cytotoxicity. 5-Fluorouracil was injected i.p. at a dose of 100 mg/kg.

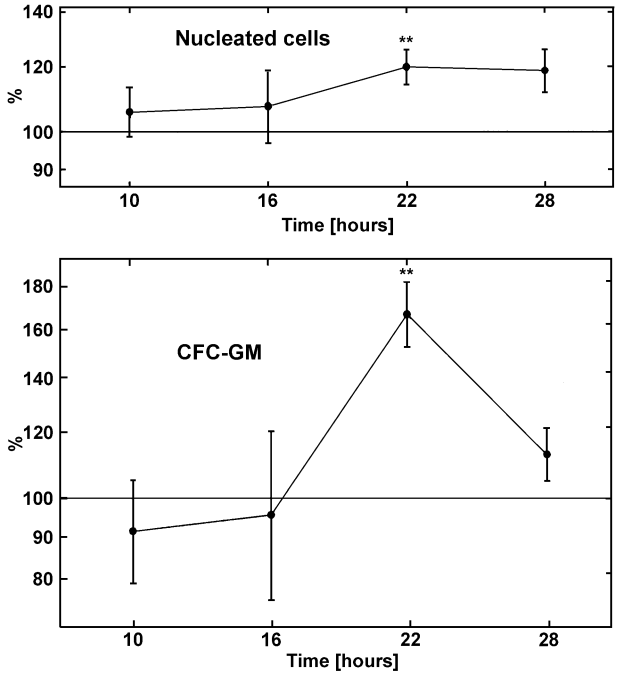


Fig. 1. Dependence of the counts of femoral marrow nucleated cells and of CFC-GM on the time interval between the i.p. administration of 200 nmol/kg of CPA and 5-fluorouracil (100 mg/kg, i.p.), as determined on day 4 after the 5-fluorouracil treatment. The data were expressed as percent changes related to values obtained in simultaneously examined 5-fluorouracil-treated mice injected with vehicle instead of CPA (100%). The points represent geometric means±S.E.M. for groups investigated at the intervals of 10 ($n$=14), 16 ($n$=10), 22 ($n$=27), and 28 ($n$=10) h. Similar numbers of mice treated with the vehicle instead of CPA were used. Statistical significance: **$P$<0.01 vs. vehicle-treated mice (Mann–Whitney test).

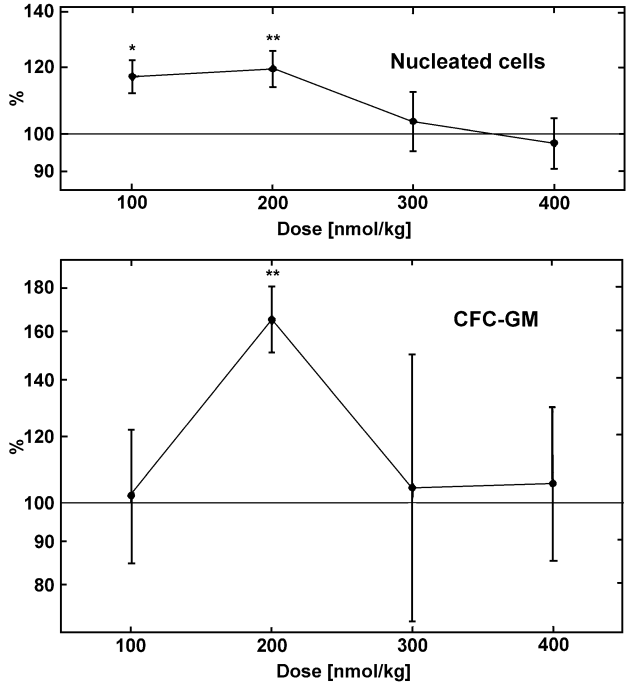


Fig. 2. Effects of different doses of CPA administered i.p. 22 h before 5-fluorouracil (100 mg/kg, i.p.) on the counts of femoral marrow nucleated cells and of CFC-GM determined on day 4 after 5-fluorouracil treatment. The data were expressed as percent changes related to values obtained in simultaneously examined 5-fluorouracil-treated mice injected with vehicle instead of CPA (100%). The points represent geometric means±S.E.M. for groups treated with CPA at doses of 100 ($n$=14), 200 ($n$=27), 300 ($n$=10), and 400 ($n$=7) nmol/kg. Similar numbers of mice treated with the vehicle instead of CPA were used. Statistical significance: **$P$<0.01, *$P$<0.05 vs. vehicle-treated mice (Mann–Whitney test).

observed after 5-fluorouracil action could be a consequence of the numerical expansion of progenitor cells which outlasts up to the period after 5-fluorouracil treatment, separate experiments were performed investigating the counts of nucleated bone marrow cells and of CFC-GM in vehicle- and CPA-treated mice at the time of the administration of 5-fluorouracil in the above experiments. No quantitative effects in both haematopoietic indices were observed in CPA-treated mice when compared to mice receiving only vehicle (data not given). Thus, the sensitivity of the cell population to 5-fluorouracil is determined by the functional state of the cell population, i.e. probably at a particular phase of the cell cycle. The protective effects were more expressive in the progenitor cell counts compared to those of nucleated cells. For this reason, the effects observed in the values of nucleated cells can be interpreted as a consequence of the feeding of the maturing pool of bone marrow cells from the surviving committed progenitor cells.

The effects of CPA given at a dose of 200 nmol/kg 22 h before 5-fluorouracil administration were compared with those of CGS 21680 and IB-MECA administered at isomolar doses in the same time interval before 5-fluorouracil treatment. While CGS 21680 was ineffective, IB-MECA induced an opposite effect, i.e. a sensitization towards the 5-fluorouracil action (Table 2).

When investigating the effects of CPA given after 5-fluorouracil treatment under conditions of the recovering haematopoiesis, multiple i.p. administration of CPA at doses of 200 nmol/kg was used. Two experimental variants were investigated. Because the highest proliferation pressures

Table 2
Comparison of CPA, CGS 21680, and IB-MECA effects on nucleated cells and CFC-GM per femur

| | Nucleated cells (%) | CFC-GM (%) |
|---|---|---|
| CPA (27) | 120±6[a] | 165±15[a] |
| CGS 21680 (14) | 99±6 | 116±18 |
| IB-MECA (20) | 75±3[a] | 67±7[a] |

All adenosine receptor agonists were administered i.p. at single isomolar doses of 200 nmol/kg 22 h before single i.p. injection of 5-fluorouracil at a dose of 100 mg/kg. Haematological indices of the femoral bone marrow were determined on day 4 after 5-fluorouracil treatment. The effects were expressed as percent of values obtained in mice treated with 5-fluorouracil and injected with vehicle instead of adenosine receptor agonists (100%) and are reported as geometric means±S.E.M. Numbers of drug-treated mice are given in parentheses; similar numbers of mice treated with vehicle were used.

[a] Statistical significance: $P$<0.01 vs. vehicle-treated mice (Mann–Whitney test).

should be expected to occur soon after induction of the damage by 5-fluorouracil, CPA was administered twice daily on days 1–4 in the experiment 1. In experiment 2, CPA was given once daily from day 4 to day 7 after 5-fluorouracil treatment. In both experiments the effects were evaluated 24 h after the last injection of CPA. As shown in Table 3, CPA administration significantly decreased CFC-GM counts as compared to values found in mice treated with 5-fluorouracil alone. After CPA treatment, CFC-GM counts decreased to 26% in experiment 1, to 56% in experiment 2. The counts of nucleated cells were not significantly influenced.

Furthermore, an experiment was performed to answer the question of the reversibility of the observed inhibitory effects of CPA on CFC-GM counts when administering this drug after 5-fluorouracil treatment. The variant of the experiment 1 described in Table 3 was used and the haematopoietic indices were assayed on day 8 after 5-fluorouracil administration, i.e. 3 days after the last injection of vehicle or CPA. Mean values (±S.E.M., $n$=5) of femoral nucleated cells in vehicle- and CPA-treated mice were $14{,}763 \pm 1209 \times 10^3$ and $16{,}054 \pm 1041 \times 10^3$, respectively, those of CFC-GM counts 18,001±1342 and 14,937±814, respectively. These data suggest a rapid regeneration of bone marrow haematopoiesis with values approaching the norm (for normal values see Table 1). There are no statistically significant differences between the values found in vehicle- and CPA-treated mice. Concerning the rate of regeneration, mean values of femoral nucleated cells in vehicle- and CPA-treated mice increased from day 5 to day 8 after 5-fluorouracil administration by factors of 2.5 and 2.4, respectively, those of CFC-GM counts by factors of 8.6 and 27.6, respectively (for values obtained on day 5 after 5-fluorouracil administration see Table 3). The results show that CPA-induced decrease of CFC-GM counts in 5-fluorouracil-pretreated mice is a transient response and is compensated by an enhanced cell proliferation in the rebound phase.

Table 3
Effects of CPA treatment after 5-fluorouracil administration on nucleated cells and CFC-GM per femur

| | | Vehicle | CPA |
|---|---|---|---|
| Experiment 1 | Nucleated cells per femur ($\times 10^{-3}$) | 5823±824 | 6549±722 |
| | CFC-GM per femur | 2097±781 | 541±227[a] |
| Experiment 2 | Nucleated cells per femur ($\times 10^{-3}$) | 21,122±1647 | 23,038±2188 |
| | CFC-GM per femur | 12,544±1049 | 7036±746[b] |

All mice were injected i.p. with a single dose of 5-fluorouracil at a dose of 100 mg/kg. Experiment 1: mice were treated i.p. with CPA at doses of 200 nmol/kg twice daily on days 1–4 after 5-fluorouracil injection. Experiment 2: mice were treated i.p. with CPA at doses of 200 nmol/kg once daily on days 4–7 after 5-fluorouracil injection. Effects were assessed 24 h after the last injection of vehicle or CPA. The data are given as arithmetic means±S.E.M. from 8 to 10 mice per group.

[a] Statistical significance: $P$<0.05 vs. vehicle-treated mice (Mann–Whitney test).

[b] Statistical significance: $P$<0.01 vs. vehicle-treated mice (Mann–Whitney test).

## 4. Discussion

The model of 5-fluorouracil toxicity, as used in our study, has been widely employed in solving problems referring to proliferative activity of haematopoietic cells (Down et al., 1997; Hodgson et al., 1982; Molineux et al., 1994; Neta et al., 1996). This model is based on the knowledge that 5-fluorouracil, a nucleoside analogue, is incorporated into DNA during S-phase of the cell cycle and induces death, and that quantification of this toxicity allows to detect the cycling state of the cell population. Thus, an increase of cell damage after administration of 5-fluorouracil indicates rapid cell cycling and conversely, a decrease of cell damage, i.e. cell protection, suggests slower cell cycling. The reason for investigating the cytotoxic effects on day 4 after 5-fluorouracil administration is the probable prolonged action of this drug. As shown by Chadwick and Rogers (1972), active metabolites of 5-fluorouracil persist in tissues of mice for 3 days and can enter into cells even before the S-phase. Thus, it is the rate of proliferation and not necessarily cell cycle specificity which determines 5-fluorouracil susceptibility (Harrison and Lerner, 1991). Furthermore, there exists evidence that recovery processes reflected in the size of the CFC-GM pool after 5-fluorouracil treatment at the dose used begin to become manifested after an interval of 5 days (Hodgson et al., 1982). Consequently, haematopoietic indices determined on day 4 after 5-fluorouracil administration represent the measure of the degree of damage. From these points of view, the observed protective effects of CPA can be explained by the ability of this adenosine receptor agonist to inhibit the proliferation of haematopoietic progenitor cells. Bell-shaped dose–response dependence of this effect can be due to the loss of the selectivity of the agonist at higher doses. The occurrence of the protective effects of CPA after a lag period of 22 h is most probably conditioned by the kinetics of the processes in the messenger pathways between the initial signalling events at G-protein-coupled receptors and cell cycle modulating processes including DNA synthesis. We did not find any data describing the time dependence of the action of negative regulators on cell cycling. Only some papers dealing with the positive action on cell cycle can be mentioned. Rozengurt (1982) reported that the nonselective adenosine receptor agonist 5′ -*N*-ethylcarboxamidoadenosine induced in quiescent cultures of Swiss 353 cells a rapid initial increase of cAMP levels and the following enhancement of DNA synthesis manifested most expressively after the lag period of about 20 h. Nečas et al. (1976) observed transiently enhanced cycling of haematopoietic stem cells in mice about 20 h after administration of isoproterenol, which stimulates adenylyl cyclase through β1-adrenergic receptors. Similar time-dependent and transient cell cycle

enhancing effects in the compartments of haematopoietic progenitor cells of mice were induced by the cytokine interleukin-1 (Neta et al., 1996). It cannot be excluded that the effects of adenosine receptor agonists in vivo are mediated via modulation of the balance in the regulatory cytokine network. The time limitation of the protective effects of CPA, which has been shown in our experiments, suggests the reversibility of the proposed cell cycle modulating action and thus the rapid activation of mechanisms providing the control of the cell population size in the haematopoietic cell renewal system.

Indicative of the selective action of CPA at the level of adenosine $A_1$ receptors were experiments comparing its effects with those induced by the isomolar doses of specific agonists for adenosine $A_{2A}$ and $A_3$ receptors. The sensitizing effects of IB-MECA, the agonist of adenosine $A_3$ receptors, can be due to the enhancement of proliferation in the investigated cell system. Such a conclusion is consistent with observations of Bar-Yehuda et al. (2002) demonstrating the stimulatory effects of IB-MECA on haematopoietic reconstitution in myelosuppressed mice. Thus, in terms of the cell cycling, signals induced by the activation of adenosine $A_1$ and $A_3$ receptor subtypes seem to be engaged in the control of haematopoiesis as counteracting regulatory mechanisms. Such a possibility remains to be further analysed.

Experiments investigating the effects of CPA administered after 5-fluorouracil treatment were performed to test the efficiency of its cell cycle inhibiting activity under the situation of recovering haematopoiesis. In particular, the progenitors of granulocytes are subjected to strong proliferation pressures due to the demand for production of mature cells. To overcome the effects of the proliferation pressures, multiple administration of CPA was used. Assuming that CPA inhibits cell cycling, its action should lead to a decrease of CFC-GM counts. Such an effect was observed. This effect is reversible and can be considered as cytostatic rather than cytotoxic. Effects of CPA given after 5-fluorouracil administration demonstrated a further interesting phenomenon, i.e. the absence of its inhibitory action on the recovery of maturing cell compartments reflected in femoral marrow cellularity. It is likely that CPA acts differently at various levels of the age structure of the haematopoietic cell system.

Many studies performed in vitro indicated that signalling from adenosine receptors to mitogen-activated protein kinases modulated cell proliferation. Some of these investigations have demonstrated inhibitory effects of the adenosine $A_1$ receptors on proliferation of various non-tumour and tumour cells (see review by Schulte and Fredholm, 2003). The only observation related to the haematopoiesis was that of Fishman et al. (2000) who assessed a moderate stimulatory action of CPA on cycling of bone marrow cells in vitro when using an assay of [$^3$H]thymidine incorporation. However, such an experiment can give information only for the cycling in the proliferative pool of the differentiated precursor cells because of the very low incidence of progenitor cells (less than 1%) in the bone marrow cell population (Novak and Necas, 1994).

To our knowledge, the findings presented here provide the first evidence for the inhibitory action of CPA on the cycling of haematopoietic progenitor cells. It has to be noted that the described in vivo effects of CPA are comparable to those induced by the transforming growth factor-β, a potent inhibitor of the cell cycle for many cell types including haematopoietic ones (Fortunel et al., 2000). Thus, CPA can be attached to the list of negative regulators of haematopoiesis.

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