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ORIGINAL ARTICLE

Effect of the Cdk-inhibitor roscovitine on mouse hematopoietic progenitors in vivo and in vitro

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Abstract Myelosuppression is one the most frequent side effects of chemotherapy. New agents that more selectively target cancer cells have been developed in attempt to improve the effects and to decrease the side effects of cancer treatment. Roscovitine is a purine analogue and cyclin-dependent kinase inhibitor. Several studies have shown its cytotoxic effect in cancer cell lines in vitro and in xenograft models in vivo. In this study, we investigated the effect of roscovitine on hematopoietic progenitors in vitro and in vivo in mice. The clonogenic capacity of hematopoietic progenitors was studied using burst-forming unit-erythroid (BFU-E), colony-forming unit granulocyte, macrophage (CFU-GM) and colony-forming unit granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM). In vitro, bone marrow cells were exposed to roscovitine (25–250 μM) in Iscove's modified Dulbecco's media for 4 h or to roscovitine (1–100 μM) in MethoCult media for 12 days. No effect on colony formation was observed after exposure to roscovitine for 4 h; however, concentration- and cell type-dependent effects were observed after 12 days. Roscovitine in concentration of 100 μM inhibited the growth of all types of colonies, while lower concentrations have shown differential effect on hematopoietic progenitors. The most sensitive were CFU-GEMM, followed by BFU-E and then CFU-GM. In vivo, mice were treated with single dose of roscovitine (50, 100 or 250 mg/kg) and the effect on bone marrow was studied on day 1, 3, 6, 9 or 12 after the treatment. In the second part of experiment, the mice were treated with roscovitine 350 mg/kg/day divided into two daily doses for 4 days. The bone marrow was examined on day 1 and 5 after the last dose of roscovitine. On day 1, BFU-E decreased to less than 50% of the controls ($P = 0.019$). No decrease in BFU-E formation was observed on day 5. No significant effect was observed on CFU-GM and CFU-GEMM growth after the treatment with multiple doses of roscovitine. Single doses of roscovitine or dimethylsulfoxide did not affect the colony formation. We also studied the distribution of roscovitine to the bone marrow after a dose of 50 mg/kg was administered intraperitoneally. Only 1.5% of the drug was detected in the

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bone marrow. Thus, the roscovitine effect on hematopoietic progenitors in bone marrow in vivo is only transient. One reason may be that only a small fraction of roscovitine reaches the bone marrow. Another explanation may be the short half-life observed for roscovitine that might not allow enough cell exposure to the drug. However, the toxicity of roscovitine to hematopoietic progenitors in vitro is within the same exposure range as cytotoxicity to cancer cells. Thus, precaution should be taken in clinical trials, especially when combinations with myelosuppressive cytostatics are used.

Keywords Myelosuppression · Hematopoietic progenitors · Mouse · Cdk-inhibitor · Clonogenic assay

Introduction

Chemotherapy remains one of the cornerstones of cancer therapy; however, adverse effects are limiting factors for many treatment schedules. Myelosuppression is one of the most frequent and serious adverse effects of conventional cytostatics and frequently limits successful treatment.

The search for novel agents that target specifically pathological processes of human cancer cells has lead to the discovery of small molecules that modulate cell cycle and apoptosis. These are tightly coupled processes and are balanced in multicellular organisms. Tumor development is characterized by alteration in the control machinery of the cell proliferation, differentiation and death.

Cyclin-dependent kinases (Cdks) are key enzymes involved in cell-cycle regulation. They have also been associated with transcription, neural and muscular functions, and apoptosis [13, 21]. Cdks and cofactors, including cyclins and endogenous Cdk inhibitors (Cdki), compose the complexes that regulate the phosphorylation of substrates required for cell-cycle progression [27]. The activity of Cdks can be modulated by small molecules that bind to their ATP-binding pockets or by altering the composition of the Cdk/Cdki complexes by different mechanisms [26]. Inhibition of Cdks may result in cell-cycle arrest, induction of differentiation, apoptosis or inhibition of transcription depending on which specific Cdk is modulated, the growth state of cell, the presence or absence of specific cell-cycle components and the tissue type [33].

Roscovitine is a purine analogue that selectively inhibits the activity of Cdks including Cdc2, Cdk2 and Cdk5 and the extracellular kinases such as Erk1 and Erk2 [17]. It acts by competing with the ATP-binding site of the kinases [4].The cytotoxic effect of roscovitine has been studied in several cancer cell lines in vitro and in animal models of cancer xenografts [16, 23]. The potential capacity of this drug for treatment of different types of cancer, including central nervous system tumors, has been suggested [19, 31, 32]. Severe and prolonged immunosuppression after the treatments with purine analogues has been observed [1, 2, 11, 25]. No severe adverse effects of roscovitine have been reported yet. It has been demonstrated that treatment with roscovitine had no significant effect on peripheral blood cells obtained from a mouse model of glomerulonephritis, though a tendency toward a decrease in leukocyte counts was noticed [6]. However, the effect of roscovitine on hematopoietic progenitors has not been reported so far.

In this study, we investigated the clonogenic capacity of bone marrow hematopoietic progenitors after the treatment with roscovitine in vitro and in vivo in Balb/C mice.

Materials and methods

Chemicals

The chemicals used in this study were purchased as follows: dimethylsulfoxide (DMSO) from Sigma, Stockholm, Sweden; roscovitine from LC Laboratories, Woburn, USA; RPMI 1640 medium, phosphate-buffered saline (PBS), Iscove's modified Dulbecco's medium (IMDM), fetal bovine serum (FBS) from Invitrogen AB, Stockholm, Sweden; Methocult GF M3434, MethoCult M3534 and MethoCult M3334 from StemCell Technologies Inc., Vancouver, Canada; heparin 5,000 IU/ml from LeoPharma A/S, Ballerup, Denmark. All other chemicals and solvents were of analytical grade and purchased from Merck, Germany.

Animals

All animal experiments were approved by the regional ethics committee for animal research in accordance with the Animal Protection Act, the Animal Protection Regulation and the Regulation of the Swedish National Board for Laboratory Animals.

Female Balb/C mice 8–12 weeks old, with a weight of 18–25 g, were purchased from B&K Universal, Sweden. The mice were allowed to adapt to their surroundings for 1 week before starting the treatment. The animals were kept in fully acclimatized room at constant temperature and humidity on a 12-h light/dark cycle and were fed standard pellet and water ad libitum.

Treatment and sampling

Pharmacokinetics and distribution of roscovitine to bone marrow

Roscovitine was dissolved in DMSO in a concentration of 50 mg/ml. Roscovitine/DMSO was further diluted with sodium chloride 9 mg/ml (1:9 v/v) and immediately administered intraperitoneally (i.p.) in a dose of 50 mg/kg. Plasma and bone marrow were sampled at 10, 20 and 30 min and 1, 2, 3, 4, 6 and 8 h. Two animals were used for each time point. Blood was collected by heart puncture into tubes containing 20 µl heparin (5,000 IU/ml) and centrifuged. Plasma was transferred to new tubes and proteins were precipitated with acetonitrile and centrifuged. Clear supernatant was transferred to new tubes and stored at −20°C until assay. Femurs were removed and cleaned; bone marrow was flushed out with 0.15 ml of sodium chloride 9 mg/ml and single-cell suspension was prepared by gentle flushing through needle and syringe. Nucleated cells were counted using Türk solution and bone marrows were stored at −20°C until assay.

In vivo treatment with roscovitine

In vivo experiments were divided into two parts. In the first part, the mice were treated with a single dose of roscovitine of 50, 100 or 250 mg/kg and the bone marrow was examined using clonogenic assay on days 1, 3, 6, 9 and 12 after the treatment. Three mice were used for each time and dose point. In the second part, the mice were treated with roscovitine with a dose of 350 mg/kg/day divided into two daily doses for four consecutive days. Bone marrow was harvested for clonogenic assay on day 1 and 5 after the last dose of roscovitine. Mice treated with DMSO were used for assessment of the effect of the solvent and the untreated animals served as controls. Five mice were used for each treatment and time point; eight mice were used as untreated controls. Roscovitine was diluted in DMSO and before i.p. administration mixed with NaCl 9 mg/ml (1:9 v/v). The final concentration of DMSO was 10%.

Treatment of bone marrow cells in vitro

In the first part, single-cell suspensions of bone marrow cells from untreated mice were prepared in IMDM supplemented with 20% FBS (IMDM/FBS). For viability assessments using AlamarBlue, 2×10^5 cells/well were plated in black 96-well microplates. For clonogenic assay, 2×10^5 cells/ml were transferred in round bottom sterile tubes. Roscovitine was dissolved in DMSO and several concentrations were prepared in order to reach the same concentration of DMSO in the final incubation media. The cells were incubated with roscovitine in two different schedules: A—Cells from three mice were incubated with roscovitine in concentrations of 25, 50 or 100 µM or DMSO for 1, 3, 6, 12 and 24 h. B—Cells from four mice were incubated with roscovitine in concentrations of 25, 50, 100 or 250 µM or DMSO for 4 h. The concentration of DMSO in all samples was 0.3%. Cells incubated with medium/FBS were used as controls. Then, the cells were washed once in IMDM/FBS and plated in methylcellulose-based medium. In schedule A, colony-forming unit granulocyte, macrophage (CFU-GM) and burst-forming unit-erythroid (BFU-E) were examined separately in MethoCult M3534 and MethoCult M3334, respectively. In schedule B, CFU-GM, BFU-E and colony-forming unit granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) were examined in MethoCult M3434.

In the second part, five mice were used for the experiment. Roscovitine with a final concentration of 1, 10, 25 or 100 µM or DMSO was added to MethoCult GF M3434 and bone marrow cells were added and plated as below. The concentration of DMSO in all the samples was 0.3%. Cells grown in MethoCult without the addition of roscovitine or DMSO served as the controls.

Roscovitine analysis

Roscovitine concentration in plasma and bone marrow was analyzed using high-performance liquid chromatography methods as published previously [29].

AlamarBlue viability assay

Crude bone marrow cells were incubated with roscovitine or DMSO as above. After 2 h of incubation with a drug or solvent, AlamarBlue was added with a final concentration of 10% for the last 2 h of incubation. Fluorescence was read using FLUOstar Optima (BMG Labtech GmbH, Offenburg, Germany).

Clonogenic assay

Nucleated marrow cells were plated in 1.1 ml MethoCult medium in 35 mm Petri dishes in duplicates. Dishes were incubated at 37°C with 5% CO_2 and 95% humidity. Colonies were scored using an inverted microscope. CFU-GM were defined as consisting of 50 or more cells, and BFU-E as consisting of 30 or

more cells. Twenty thousand nucleated cells in MethoCult M3534 and 2×10^5 nucleated cells in MethoCult M3334 were plated and scored at day 7 for CFU-GM and BFU-E, respectively. Twenty thousand nucleated cells were plated in MethoCult M3434 and scored for CFU-GM, BFU-E and CFU-GEMM on day 12.

Pharmacokinetic analysis

The concentration–time curves were adjusted to data sets via non-linear iterative least square regression analysis. The pharmacokinetic parameters were calculated using WINNONLIN ver 5.01 (Pharsight Corporation, Mountain View, CA, USA). Bone marrow data were derived after adjustment of concentrations to 10^9 cells (approx. 1 g of tissue). Area under the concentration–time curve (AUC) was estimated using both trapezoidal rule and Winnonlin program.

Statistical analysis

Values are expressed as the mean ± SD. Statistic analysis was performed using non-parametric Kruskal–Wallis and Mann–Whitney test, whenever appropriate. *P*-value of less than 0.05 was considered as the level of significance.

Results

Effect of roscovitine on bone marrow cells in vitro

The viability of bone marrow cells incubated with roscovitine for 4 h decreased in a concentration-dependent manner. Roscovitine 250 μM decreased the viability of bone marrow cells to 70% compared to the controls ($P = 0.015$). (Fig. 1)

Colony formation decreased in a concentration- and time-dependent manner, when bone marrow cells were exposed to roscovitine until 24 h (Fig. 2a, b). Both CFU-GM and BFU-E were affected; however, erythroid colonies were more sensitive and their growth was blocked by incubation with roscovitine of 50 and 100 μM for 12 h. A decrease in colony formation was observed in the controls at 12 and 24 h of incubation. DMSO exposure did not affect the colony formation compared to the controls. However, the formation of CFU-GM, BFU-E and CFU-GEMM was not significantly affected by incubation with roscovitine for 4 h, despite the fact that the concentration of roscovitine was increased up to 250 μM (data not shown).

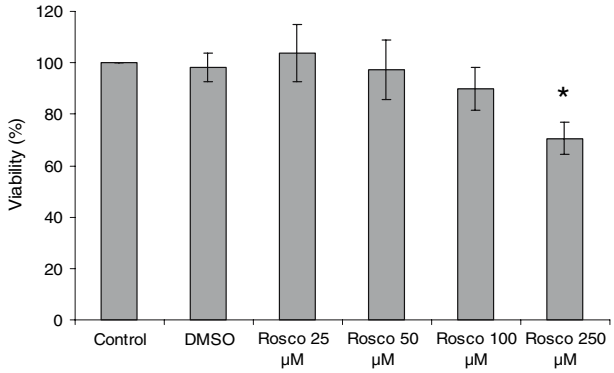

Fig. 1 Viability of bone marrow cell after exposure to roscovitine. Bone marrow cells were incubated with roscovitine (*Rosco*) for 4 h. Viability was assessed using AlamarBlue assay and expressed as % of the control. Results are presented as mean ± SD of three animals. *Indicates significant level of $P < 0.05$

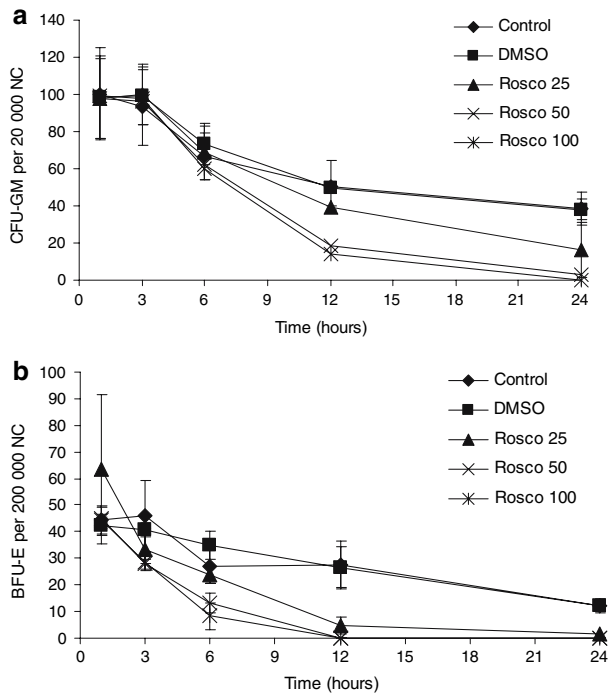

Fig. 2 Effect of roscovitine on hematopoietic progenitors in vitro. Bone marrow cells were incubated with roscovitine (*Rosco*) in concentrations of 25, 50 or 100 μM or *DMSO* for 1, 3, 6, 12 and 24 h. Cells incubated with medium supplemented with FBS were used as *controls*. Then the cells were washed from drug and plated in methylcellulose-based medium. *CFU-GM* (**a**) and *BFU-E* (**b**) were counted at day 7 using inverted microscope. Results are expressed as mean ± SD from three animals

In the next step of the experiment, roscovitine was added to the MethoCult medium and the bone marrow cells were exposed to roscovitine during the period of colony growth. The suppression of colony formation was dependent on cell type and concentration. CFU-

Table 1 Plasma and bone marrow pharmacokinetic parameters following i.p. administration of roscovitine 50 mg/kg

	AUC_{trapez} (nmol/l·hr)	AUC_{inf} (nmol/l·h)	C_{max} (nmol/l)	Cl (l/h)	V_d (l)	$t_{½}$ (h)
Plasma	282,518	275,781	202,014	0.05	0.015	0.82
BM	4,096	4,588	4,946	0.62	0.54	0.61

BM bone marrow, *AUC* area under the concentration–time curve, AUC_{trapez} AUC estimated using trapezoidal rule, AUC_{inf} AUC derived using Winnonlin analysis, C_{max} estimated maximum concentrations, *Cl* clearance, V_d apparent volume of distribution, $t_{½}$ half-life

GM growth was blocked at 100 μM roscovitine, but was not affected by lower tested concentrations of roscovitine (Fig. 3a). BFU-E formation was decreased by roscovitine at 25 μM and completely inhibited at 100 μM (Fig. 3b). CFU-GEMM growth was completely stopped by roscovitine at concentrations of 25 and 100 μM and decreased by 10 μM roscovitine, but this decrease was not significant (Fig. 3c).

Pharmacokinetics of roscovitine

Pharmacokinetic parameters are presented in Table 1. Fig. 4 presents plasma AUC after 50 mg/kg roscovitine is administered i.p. Only about 1.5% of roscovitine reached the bone marrow.

Effect of roscovitine on hematopoietic progenitors in vivo

Cellularity of bone marrow expressed as number of nucleated cells per femur was not affected by roscovitine, regardless of whether it was administered as a single dose or multiple doses (data not shown).

Single dose of roscovitine of 50, 100 or 250 mg/kg did not affect the clonogenic capacity of the bone marrow at any time point examined (data not shown).

Treatment with multiple doses of roscovitine of 350 mg/kg/day divided into two daily doses for 4 days transiently affected the BFU-E growth. At day 1 after the last dose of roscovitine, BFU-E decreased to less than 50% of the controls ($P = 0.019$). No decrease in BFU-E formation was observed at day 5 (Fig. 5b). No significant effect on CFU-GM and CFU-GEMM growth was observed after the treatment with multiple doses of roscovitine (Fig. 5a, c). DMSO did not affect the colony formation.

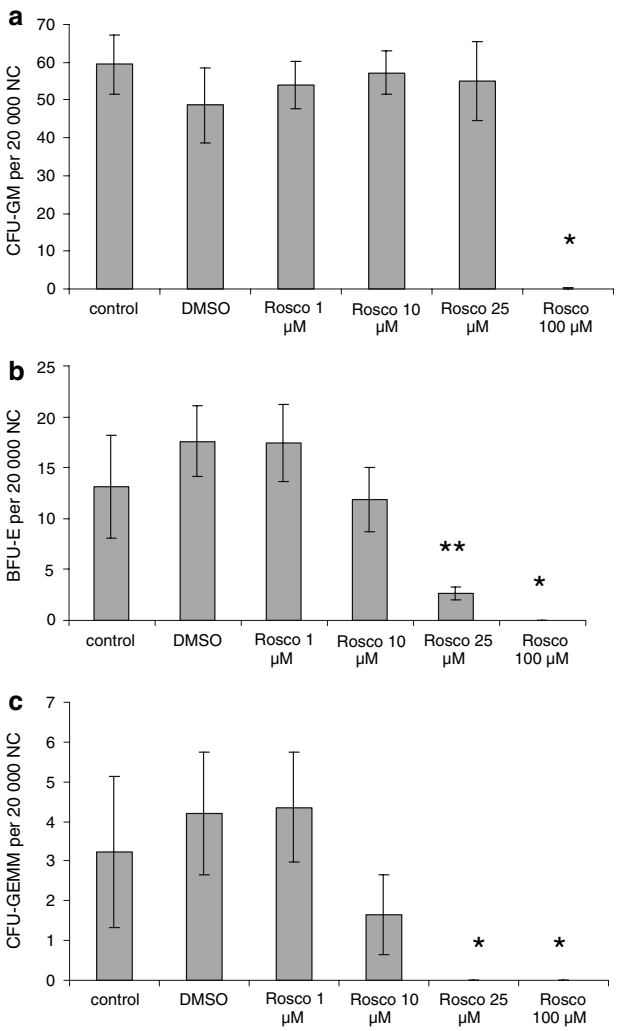

Fig. 3 Effect of roscovitine on colony formation in vitro. Roscovitine (*Rosco*) at final concentration of 1, 10, 25 or 100 μM or *DMSO* was added to methylcellulose-based media. Twenty thousand nucleated bone marrow cells were added. *CFU-GM* (**a**), *BFU-E* (**b**) and *CFU-GEMM* (**c**) were counted at day 12 using inverted microscope. Results are expressed as mean ± SD from five mice. *Indicates significant level of $P < 0.05$, **Indicates significant level of $P < 0.01$

Discussion

Myelosuppression is one of the most frequent side effects of cytostatics, and thus a limiting factor in cancer therapy. Supportive treatment has been developed during the last decades aiming to decrease pancytopenia-related complications. Substantial advances in molecular and cellular biology have lead to the identification of several molecules that play a critical role in cell cycle, signal transduction and apoptosis. These molecules have been also recognized as potential therapeutic targets, and new agents of different classes with diverse mechanisms of action have been synthesized.

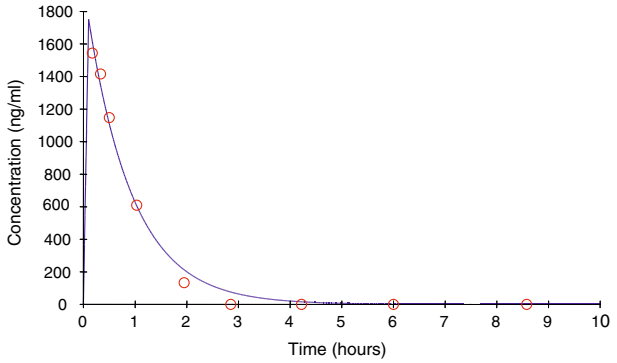

Fig. 4 Area under the concentration–time curve in plasma after roscovitine 50 mg/kg administered i.p. Each time point represents one animal within the same series

Roscovitine is a purine analogue and selective Cdki [4, 18]. The drug has been studied in a variety of cellular and animal models and several reports support the efficacy of this compound as an anti-tumoral agent used alone or in combination with other therapies [16, 17–20, 31, 32]. An oral formulation of the drug (CYC202) is currently being evaluated in clinical trials of cancer and other diseases such as glomerulonephritis [17].

In the present study, we investigated the effect of roscovitine on bone marrow cells in vitro and in vivo in mice.

Roscovitine was shown to decrease the viability of crude bone marrow cells in a concentration-dependent manner. After 4 h of incubation with roscovitine, the viability of cells incubated with 250 μM decreased to 70%. A majority of the bone marrow cells consist of myeloid cells at different stages of maturation. A recently published paper has shown that roscovitine has induced apoptosis in mature neutrophils in vitro [24]. Roscovitine-induced apoptosis was time dependent and most pronounced at 20 h of incubation. However, the concentration used in that study was 12 times lower than that in our study, which may explain the shorter time to effect in our study. We did not address the type of cell death induced by roscovitine in our experiments. Incubation with roscovitine up to 250 μM for 4 h did not affect the clonogenic ability of the hematopoietic progenitors. Four hours of incubation may be too short for roscovitine to exert its effect; however, longer incubation decreased the clonogenic capacity of the controls (Fig. 2), which is probably due to lack of relevant growth factors in the media supplemented with FBS. This is in agreement with decreased viability of primary hematopoietic cells cultured in vitro without appropriate growth factors [24].Thus, when the cells were incubated with roscovitine for up to 24 h, the additive effect of roscovitine and deprivation of growth

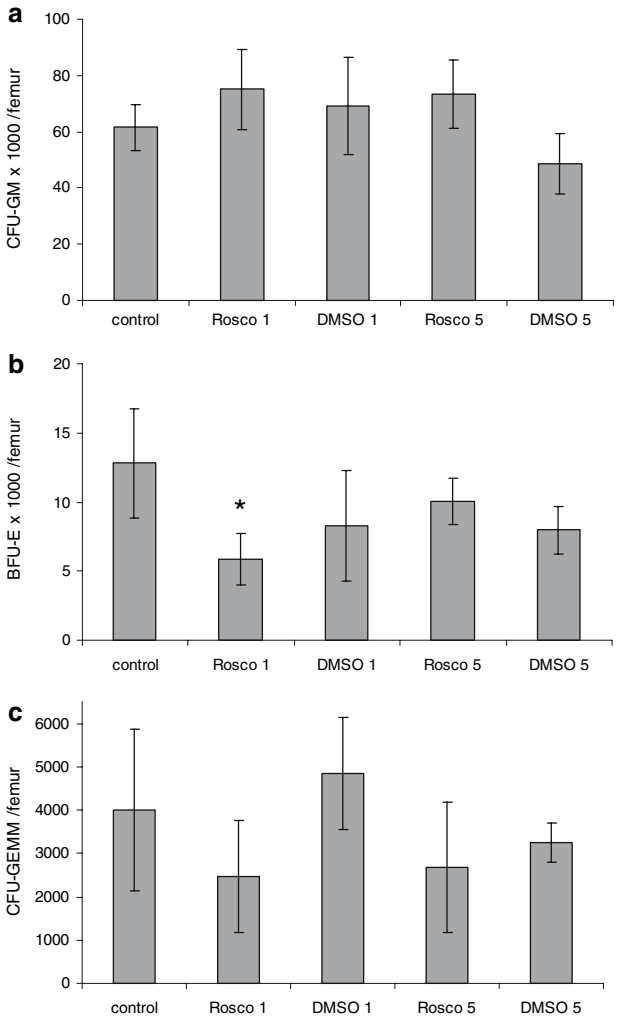

Fig. 5 Effect of roscovitine on hematopoietic progenitors in vivo. Mice were treated with roscovitine (*Rosco*) in dose 350 mg/kg/day divided into two daily doses for 4 days. Mice treated with *DMSO* served as the controls for assessment of the effect of the solvent. Untreated animals were used as *controls*. Bone marrow was examined 1 and 5 days (numerals *1* or *5*) after the last dose of roscovitine. *CFU-GM* (**a**), *BFU-E* (**b**) and *CFU-GEMM* (**c**) were counted on day 12 using inverted microscope. Each group consisted of five mice, control group of eight mice. Results are expressed mean ± SD. *Indicates significant level of $P < 0.05$

factors cannot be excluded. Therefore, we examined the colony formation after addition of roscovitine to semisolid MethoCult media containing recombinant growth factors. Decrease in colony growth was observed and was dependent on cell type and concentration. BFU-E colonies were more sensitive than CFU-GM, but the most sensitive were CFU-GEMM. The higher sensitivity of erythroid progenitors has been observed for other cytotoxic drugs and xenobiotics compared to granulocyte–macrophage progenitors [3, 15]. Corazza et al. postulate that these agents might exert an effect on cytokines (e.g., erythropoietin, trans-

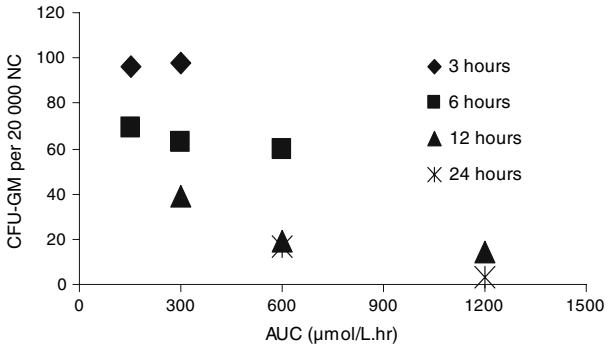

Fig. 6 The influence of time on the effect of roscovitine on hematopoietic progenitors in vitro. Cells were incubated with roscovitine (Rosco) for 3, 6, 12 and 24 h. Area under the concentration–time curve (*AUC*) has been estimated by trapezoidal rule. The same *AUC*s resulted from different time and concentration combinations

forming growth factor-beta, granulocyte macrophage colony-stimulating factor and/or macrophage inflammatory peptide-alfa) on stroma cells that interact with hematopoietic progenitors or any step in the production of hemoglobin [3].

Roscovitine has a long half-life in media (96 h at 37°C, unpublished observation). Thus, the estimated in vitro AUCs in our experiments for start concentration of 10 µM is 1,620 µM·h, for start concentration of 25 µM is 4,050 µM·h and for 100 µM is 16,200 µM·h, respectively. Several in vitro studies on inhibitory activity of roscovitine in cancer cell lines has been published [12, 14, 16, 22, 28]. When reported IC50 and incubation times are recalculated to AUC, the exposure to roscovitine that inhibits hematopoietic progenitors is within the same range as exposure that inhibits tumor cells. The cytotoxic effect of roscovitine has also been found in human peripheral blood mononuclear cells in vitro [7].

The time of exposure to roscovitine is an important factor and affects roscovitine-induced inhibition of hematopoietic progenitors. Lower roscovitine concentration with longer incubation time results in higher inhibition of colony formation compared to higher concentration of roscovitine with shorter incubation time, despite the fact that both concentration and time combinations give the same AUC, as shown in Fig. 6 for CFU-GM. A similar relationship was observed for BFU-E. This differs from others cytostatics, in which the linear relationship between the AUC and cytotoxicity independent of incubation times has been shown [9, 10].

The anti-tumor effect of roscovitine has been demonstrated in xenograft models [23, 28]. However, no significant effect on peripheral blood cell has been reported in vivo. In a study of Gherardi et al., only a slight decrease in leukocyte count was observed in a mouse model of glomerulonephritis [6].

In our study, we examined the effect of roscovitine on the clonogenic capacity of bone marrow in vivo in mice. Single dose of roscovitine up to 250 mg/kg did not affect the colony formation of bone marrow cells. This was unexpected since the total dose of 250 mg/kg administered i.p. has been reported to reduce tumor size by 85% in xenograft model of Ewing's sarcoma [28].

We decided to examine the pharmacokinetics of roscovitine since the solvent and administration way may influence bioavailability and distribution of the drugs. Our results are in agreement with the published data in mouse, rat and human [5, 22, 23, 30]. Roscovitine has also been shown to be distributed well to different organs [23, 30]; however, the distribution to bone marrow has not been reported yet. In this study, the fraction of roscovitine that reached the bone marrow was only about 1.5%.

The dose of roscovitine used in the multiple dose schedule was estimated using the pharmacokinetic data. The target plasma AUC was based on the published data by Raynaud aiming to reach exposure equivalent to roscovitine 500 mg/kg administered orally [23]. Treatment twice daily for four consecutive days has lead to only the transient decrease in BFU-E at day 1, but no decrease was observed 5 days after the last dose of roscovitine. CFU-GM and CFU-GEMM were not affected by the treatment.

Similar results were described with olomoucine, which is a Cdki and analogue of roscovitine [8]. The authors reported the effect of olomoucine in a spontaneous dog melanoma model. No side effects of the therapy were observed by standard clinical and laboratory analyses, including undetectable myelosuppression, hepatotoxicity, nephrotoxicity and neurotoxicity.

In our study, mice treated with roscovitine twice daily displayed changes in the fur, which acquired a rough look after the second or third injection. One mouse died several hours after the third dose of roscovitine. However, no pathologic changes were found at the autopsy. The appearance of the fur returned to normal by the end of the treatment. We can only speculate about the cause of the observed changes, but such effects have not been observed in the DMSO controls.

Thus, roscovitine's effect on the hematopoietic progenitors in the bone marrow in vivo is only transient. This could be explained by the fact that only a small fraction of roscovitine reaches the bone marrow. Another explanation may be that the short half-life observed for roscovitine might not allow enough cell exposure to the drug. However, the toxicity of roscovitine to hematopoietic progenitors in vitro is within the same exposure range as cytotoxicity to cancer cells.

Therefore, precaution should be taken in clinical trials, especially when combinations with standard and myelosuppressive cytostatics are used.

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