



## The Influence of Amifostine Used Alone or in Combination with 2-Chlorodeoxyadenosine on Normal and Chronic Myelogenous Leukemia Granulocyte-Macrophage Progenitor Cells *in Vitro*

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**Abstract.** We evaluated the influence of amifostine used alone or in combination with 2-chlorodeoxyadenosine (2-CdA) on the colony growth of normal and chronic myeloid leukemia (CML) granulocyte-macrophage progenitor cells (CFU-GM) in semisolid culture *in vitro*. Amifostine at a concentration of 1 mg/ml was either added directly to the culture medium of normal and CML CFU-GM, or mononuclear cells (MNCs) were first preincubated with amifostine at the same concentration, washed in Iscove's modified Dulbecco minimum essential medium (IDMEM) and then added to the culture medium. Amifostine used alone inhibited the growth of CML CFU-GM colonies to a higher degree than those of normal CFU-GM, but the differences were not statistically significant. Amifostine preincubated with MNCs and used together with the highest concentration of 2-CdA significantly inhibited the colony growth of CML CFU-GM as compared to 2-CdA alone ( $p < 0.05$ ). In contrast, the colony growth inhibition of normal CFU-GM was not significantly lower compared to 2-CdA used alone. Our studies suggest that 2-CdA used together with amifostine is more toxic to leukemic CFU-GM than to their normal counterparts.

**Key words:** amifostine; 2-CdA; interaction; CFU-GM; chronic myelogenous leukemia; culture *in vitro*.

### Introduction

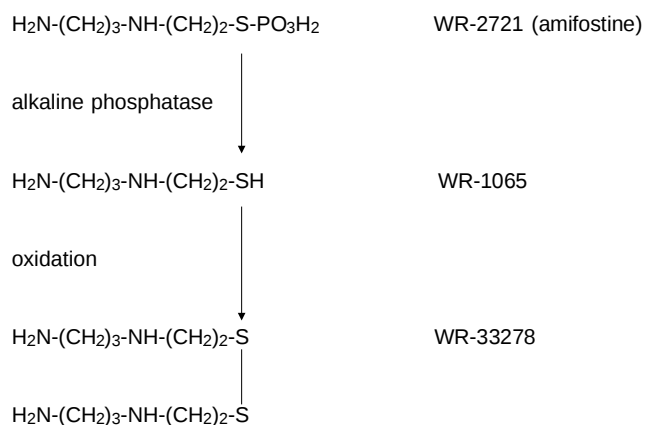
All antineoplastic drugs used in the treatment of hematological disorders have several adverse effects in which the cytotoxic action on normal tissue plays a great role. Myelosuppression is one of the dose-limi-

ting toxicities which is usually manifested as neutropenia and thrombocytopenia. The protection of normal hematopoietic progenitor cells from the cytotoxic effect of chemotherapeutic agents as well as ionizing radiation has been the topic of many recent studies. It seems that amifostine a new cytoprotective agent, offers

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Abbreviations used: 2-CdA – 2-chlorodeoxyadenosine, CML – chronic myeloid leukemia, AML – acute myeloid leukemia, CLL – chronic lymphocytic leukemia, NHL – non-Hodgkin's lymphoma, CFU-GM – colony forming unit-granulocyte-macrophage, BFU-E – burst forming unit-erythroid, CFU-GEMM – colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte, G-CSF – granulocyte-colony stimulating factor, GM-CSF – granulocyte-macrophage-colony stimulating factor, IL – interleukin 3, MNCs – mononuclear cells, IDMEM – Iscove's modified Dulbecco minimum essential medium, HBSS – Hank's balanced salt solution, PF – protection factor.

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**Fig. 1.** Metabolism of amifostine

a possibility to protect normal bone marrow cells from the toxicity of alkylating drugs and free radicals generated by ionising radiation<sup>6, 7, 12</sup>.

Amifostine (WR-2721, Ethiol) is an inorganic thiophosphate compound, chemically known as aminothiophosphate. It is a prodrug, dephosphorylated in the tissue by alkaline phosphatase to pharmacologically active metabolite, the free thiol (WR-1065) (Fig. 1). The conversion is much more effective in normal tissue than in tumor<sup>22</sup>. Although amifostine was developed in the 1950s as a radioprotective agent in a classified nuclear warfare project, it is currently known that it can prevent DNA-damage caused also by several cytotoxic drugs. In both animal and clinical studies, amifostine has decreased radiation- or chemotherapy-induced renal and neurological toxicity associated with cisplatin, cyclophosphamide and vinblastine therapy of advanced and metastatic solid tumors<sup>5, 32</sup>. Moreover, it has been widely reported to prevent myelosuppression by efficiently lessening neutropenia, reducing the frequency of thrombocytopenia episodes and shortening the time to platelet recovery<sup>12</sup>. There are also data which suggest that amifostine stimulates formation of multipotent as well as erythroid bone marrow progenitors, and it has been proposed as a therapeutic agent in the treatment of low-risk myelodysplastic syndromes (MDS) where most complications are related to cytopenia<sup>18, 30</sup>. Until now, however, there have been few studies concerning the cytoprotective effect of amifostine against antineoplastic drugs used in the treatment of animal or human leukemias<sup>1, 15, 20</sup>.

Clinical observations indicate that a new halogenated analog of purine, 2-chlorodeoxyadenosine (2-CdA), plays an important role in the treatment of many lymphoproliferative disorders, including chronic lymphocytic leukemia (CLL), hairy cell leukemia and B- and T-non-Hodgkin lymphomas (NHL)<sup>10, 23, 24, 25</sup>. Recent

data have demonstrated that this drug is also active in myeloid malignancies, both in acute myeloid leukemia (AML) and chronic myelogenous leukemia (CML)<sup>29, 31</sup>. In addition, 2-CdA has been used in combination with cytokines such as IFN- $\alpha$ , IFN- $\gamma$  or TNF- $\alpha$ <sup>13, 27</sup>. There are data suggesting that 2-CdA can be used together with chlorambucil in patients with CLL as well as with mitoxantrone or dexamethasone in the treatment of refractory and recurrent low grade NHL<sup>3, 26</sup>. Although the toxicity of 2-CdA is relatively low, the main adverse effect of its use is associated with myelosuppressive action and the danger of development of opportunistic infections<sup>2</sup>. On the other hand, amifostine demonstrated protection against chlorambucil or mitoxantrone, drugs which were used in combination with 2-CdA<sup>33</sup>.

Therefore, it would be interesting to investigate the interaction of amifostine and 2-CdA and discover whether this interaction can have any clinical implication. The aim of our study was to evaluate the effect of amifostine used either alone or in combination with 2-CdA on normal and CML granulocyte-macrophage colony forming cells (CFU-GM) in semisolid cultures *in vitro*.

## Materials and Methods

**Patients studied.** Specimens of bone marrow were collected from 10 patients with CML in the chronic phase. The mean age of patients was 42.8 years (range 28–71). All patients were newly diagnosed and the diagnoses were based on standard clinical, morphological, cytochemical and cytogenetic criteria. None of the patients had received cytotoxic drugs before the collection of bone marrow for the study.

Normal bone marrow specimens were obtained from 10 hematologically normal patients. The mean age of these patients was 46.5 years (range 19–69). All specimens were collected with the consent of the donor.

**Amifostine and 2-CdA.** Amifostine (Ethiol) was kindly provided by Schering-Plough (USA). 2-CdA was synthesized according to the method described by KAZIMIERCZUK et al.<sup>11</sup> and was provided by the Foundation of Development of Diagnostics and Therapy (Warsaw).

**Assay for normal and CML CFU-GM.** The assay for CFU-GM was based on the method described by ISCOVE et al.<sup>9</sup> in our modification<sup>27</sup>. Briefly, bone marrow were collected into preservative-free heparine (Sigma, England). The mononuclear cells (MNCs) were obtained from marrow by layering over lymphoprep (Histopaque 1077, Sigma, England) and washed twice in Hank's balanced salt solution (HBSS).

The MNCs were suspended in 0.2 ml of fetal calf serum (FCS; Gibco Ltd., Scotland) which was a 20% of total volume and then 20% of Iscove's modified Dulbecco minimum essential medium (IDMEM; Gibco Ltd., Scotland), 55% methyl cellulose (Fluorochem, Switzerland) and 5% growth factors: 20 ng/ml G-CSF (Neupogen, Roche, Switzerland), 25 ng/ml GM-CSF (Leucomax, Novartis, Switzerland) and 40 ng/ml IL-3 (Sigma, England) were added.

In the first part of our studies, MNCs were suspended in FCS at the concentration of  $5 \times 10^5$  cells/ml and were plated with 1 mg/ml of amifostine used either alone or together with 2-CdA at the following concentrations: 10, 20 and 40 nM. MNCs as well as the agents were added directly to the culture.

In the second part of the experiment, MNCs were first suspended in IDMEM supplemented with 10% autologous plasma, diluted to  $1 \times 10^5$  cells/ml and preincubated with 1 mg/ml of amifostine at the temperature of 37°C for 15 min. Afterwards, preincubated cells were resuspended in FCS at the concentration of  $5 \times 10^5$  cells/ml and were plated either without or with 2-CdA at the concentrations of 10, 20 and 40 nM under the same conditions as in the first part of the experiment.

At the same time, MNCs were plated only with 2-CdA at the concentrations of 10, 20 and 40 nM as well as without any agents as control cultures.

All cultures were incubated for 14 days at 37°C in an atmosphere of 5% CO<sub>2</sub> in air and examined with an inverted microscope. Then aggregates containing 40 or more cells were scored as colonies. The colonies were separated from cultures and, after centrifuging, the cells were stained according to the May-Grunwald-Giemsa method for the confirmation of their morphology.

**Statistical analysis.** Statistical differences between experimental and control cultures were evaluated by Student's *t*-test at the level of significance  $p < 0.05$ .

IC<sub>80</sub> value was defined as the concentration of agents that achieved 80% inhibition of colony formation. Protection factor (PF) was defined by the ratio of IC<sub>80</sub> for a given antineoplastic drug with and without amifostine addition. PF > 1.0 indicates cytoprotective effect<sup>33</sup>.

## Results and Discussion

For the last few years numerous preclinical and clinical studies have shown that amifostine protects normal tissue but not tumors against the toxic effects of alkylating agents and cisplatin<sup>5, 6, 22</sup>. Further laboratory studies have demonstrated that amifostine can also protect normal primitive hematopoietic progenitors

against the toxicity of other classes of anticancer agents with diverse mechanisms of action, such as anthracyclines and mitoxantrone<sup>17</sup>. Some authors have observed that amifostine selectively protects human granulocyte-macrophage progenitor cells (CFU-GM) from the cytotoxic effect of the cyclophosphamide derivative 4-hydroperoxycyclophosphamide or mafosfamide without compromising antitumor effect and it may be used as one of several methods available for bone marrow purging before autografting<sup>4</sup>.

In our present *in vitro* studies, we at first evaluated the influence of amifostine used alone on the colony growth of normal and CML CFU-GM. We observed that amifostine directly added to the culture significantly decreased the number of both types of colonies as compared to the control ( $p < 0.05$ ) (Table 1). In contrast, preincubation of mononuclear cells with amifostine significantly inhibited only the growth of colonies formed by CML CFU-GM as compared to the control. In the case of normal CFU-GM the differences were not statistically significant ( $p > 0.05$ ) (Table 2). The growth of CML CFU-GM colonies was inhibited to a higher degree than normal CFU-GM, although the differences were also not statistically significant ( $p > 0.05$ ) (Table 2). These findings confirm our previous observations that amifostine given alone does not change the survival time of mice with leukemias L1210 and P388<sup>19</sup>. In contrast, List et al.<sup>18</sup> demonstrated in their *in vitro* studies that, in the absence of antineoplastic exposure, amifostine or its active metabolite enhanced the number of CFU-GEMM and BFU-E colonies; however, the differences were not statistically significant. In addition, a greater protection was afforded for erythroid bursts<sup>17</sup>. On the other hand, however, in 2 of 18 patients with MDS treated with amifostine, blast counts increased with irreversible transformation to acute leukemia<sup>16</sup>.

Amifostine (WR-2721) does not or only minimally exerts a protective activity by itself and, as a prodrug, it must be *in vitro* dephosphorylated to the active metabolite WR-1065 by adding to the culture medium either alkaline phosphatase or 10–20% of non-heat inactivated autologous serum or plasma<sup>7</sup>. On the other hand, the WR-1065 generated *in vitro* is the substrate for diamine oxidase present in serum or plasma. This enzyme can catabolize WR-1065 to acrolein and hydrogen peroxide; each of these alone or in combination can be toxic to cells in culture<sup>21</sup>. It is possible that the colony growth inhibition observed in our *in vitro* studies was connected with this effect.

We have also demonstrated that amifostine used in combination with 2-CdA, either directly added to the culture or after preincubation with MNCs, inhibited the

**Table 1.** The effect of amifostine directly added to the culture on normal and CML CFU-GM

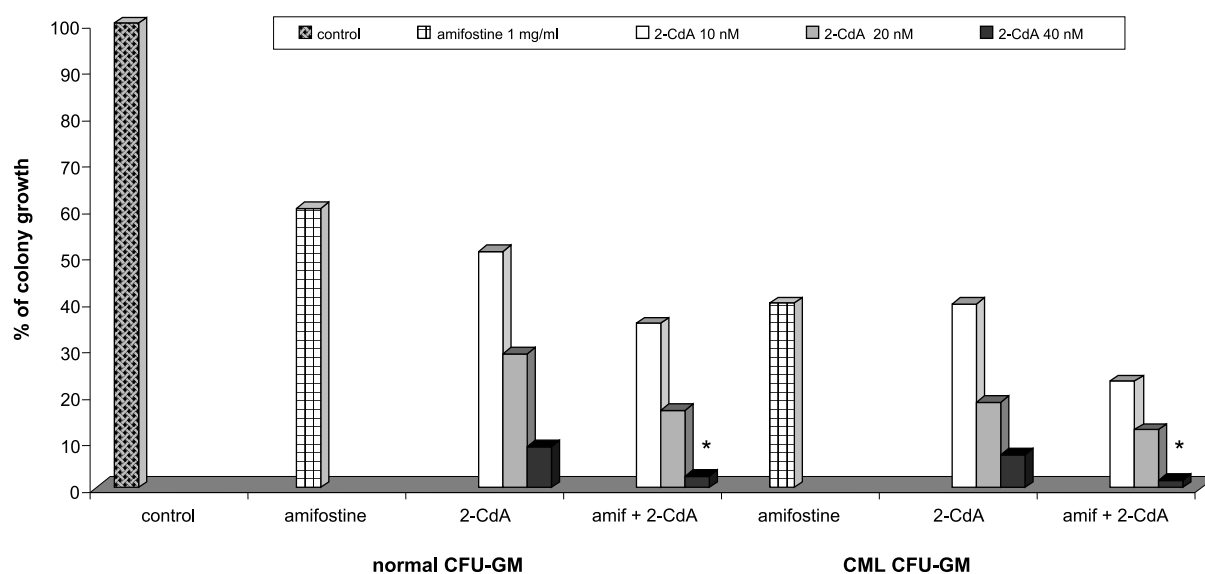
| Type of cells            | Control                | Agent               |                     |                     |                    |                     |                    |                  | Statistical analysis for 1–8 (p<0.05)                   |
|--------------------------|------------------------|---------------------|---------------------|---------------------|--------------------|---------------------|--------------------|------------------|---------------------------------------------------------|
|                          |                        | amifostine 1mg/ml   | 2-CdA               |                     | amifostine + 2-CdA |                     |                    |                  |                                                         |
|                          |                        |                     | 10 nM               | 20 nM               | 40 nM              | 2+3                 | 2+4                | 2+5              |                                                         |
|                          | 1                      | 2                   | 3                   | 4                   | 5                  | 6                   | 7                  | 8                |                                                         |
| Normal CFU-GM (A)        | 47.2±5.4<br>(38–62)    | 28.4±6.5<br>(20–47) | 24.0±6.6<br>(18–34) | 13.6±4.9<br>(10–24) | 3.3±1.8<br>(2–6)   | 16.7±3.3<br>(11–24) | 7.9±1.1<br>(6–10)  | 0.7±0.4<br>(0–2) | 1:2, 1:6,<br>1:7, 1:8,<br>2:5, 2:7,<br>2:8, 5:8         |
| CML CFU-GM (B)           | 111.4±15.7<br>(89–164) | 44.2±6.5<br>(30–68) | 44.0±7.4<br>(35–69) | 20.5±5.0<br>(14–31) | 8.7±1.4<br>(2–10)  | 25.6±6.4<br>(12–32) | 13.9±4.7<br>(9–15) | 1.2±0.4<br>(0–3) | 1:2, 1:6,<br>1:7, 1:8,<br>2:4, 2:5,<br>2:7, 2:8,<br>5:8 |
| Statistical analysis A:B | p<0.01                 | p>0.05              | p>0.05              | p>0.05              | p<0.05             | p>0.05              | p>0.05             | p>0.05           |                                                         |

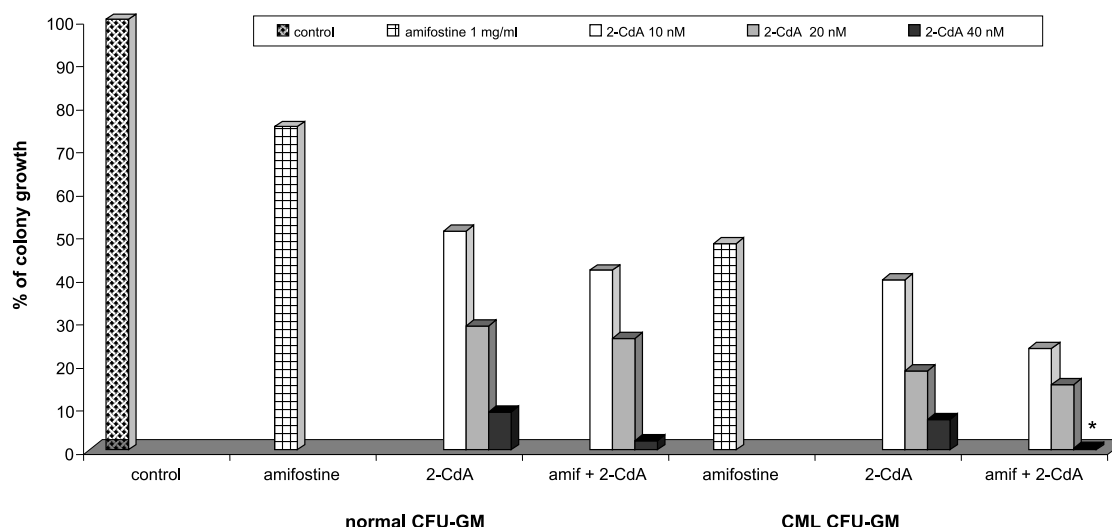
Values are expressed as a mean colony number ± SEM, per  $5 \times 10^5$  MNCs, ranges in parentheses.

**Table 2.** The effect of amifostine preincubated with MNCs, used alone or prior to 2-CdA on normal and CML CFU-GM

| Type of cells            | Control                | Agent               |                     |                     |                    |                     |                     |                  | Statistical analysis for 1–8 (p<0.05)                       |
|--------------------------|------------------------|---------------------|---------------------|---------------------|--------------------|---------------------|---------------------|------------------|-------------------------------------------------------------|
|                          |                        | amifostine 1mg/ml   | 2-CdA               |                     | amifostine + 2-CdA |                     |                     |                  |                                                             |
|                          |                        |                     | 10 nM               | 20 nM               | 40 nM              | 2+3                 | 2+4                 | 2+5              |                                                             |
|                          | 1                      | 2                   | 3                   | 4                   | 5                  | 6                   | 7                   | 8                |                                                             |
| Normal CFU-GM (A)        | 47.2±5.4<br>(38–62)    | 35.4±6.6<br>(28–54) | 24.0±6.6<br>(18–34) | 13.6±4.9<br>(10–24) | 3.3±1.8<br>(2–6)   | 19.7±4.8<br>(14–35) | 12.3±2.2<br>(9–20)  | 1.0±0.8<br>(0–2) | 1:6, 1;7,<br>1:8, 2:4,<br>2:5, 2:8                          |
| CML CFU-GM (B)           | 111.4±15.7<br>(89–164) | 53.5±6.3<br>(43–68) | 44.0±7.4<br>(35–69) | 20.5±5.0<br>(14–31) | 8.7±1.4<br>(2–10)  | 26.3±5.0<br>(16–36) | 16.9±2.3<br>(12–23) | 0.5±0.7<br>(0–3) | 1:2, 1:6,<br>1:7, 1:8,<br>2:4, 2:5,<br>2:6,2:7,<br>2:8, 5:8 |
| Statistical analysis A:B | p<0.01                 | p>0.05              | p>0.05              | p>0.05              | p<0.05             | p>0.05              | p>0.05              | p>0.05           |                                                             |

Values are expressed as a mean colony number ± SEM, per  $5 \times 10^5$  MNCs, ranges in parentheses.

**Fig. 2.** The influence of amifostine added directly to the culture on normal and CML CFU-GM. The results are expressed as a percentage of colony number as compared to the control. \*Values statistically significant vs. 2-CdA used alone



**Fig. 3.** The influence of amifostine used prior to 2-CdA on normal and CML CFU-GM. The results are expressed as a percentage of colony number as compared to the control. \*Values statistically significant vs. 2-CdA used alone

**Table 3.** The comparison of IC<sub>80</sub> for 2-CdA with and without amifostine and protection factor for normal and CML CFU-GM

| Type of cells | Without preincubation           |              |      | With preincubation              |              |      |
|---------------|---------------------------------|--------------|------|---------------------------------|--------------|------|
|               | IC <sub>80</sub> (nM) for 2-CdA |              | PF   | IC <sub>50</sub> (nM) for 2-CdA |              | PF   |
|               | – amifostine                    | + amifostine |      | – amifostine                    | + amifostine |      |
| Normal CFU-GM | 28.07                           | 18.23        | 0.69 | 28.07                           | 25.02        | 0.89 |
| CML CFU-GM    | 19.24                           | 12.79        | 0.66 | 19.24                           | 14.28        | 0.74 |

PF – protection factor.

growth of both types of colonies in a manner dependent on 2-CdA dosage (Figs. 2, 3). Amifostine directly added to the culture and used with 2-CdA at the concentration of 40 nM inhibited the growth of normal and CML CFU-GM colonies as compared to 2-CdA used alone ( $p < 0.05$ ) (Fig. 2). However, preincubation of MNCs with amifostine prior to the adding of the highest concentration of 2-CdA significantly inhibited only the growth of CML CFU-GM colonies. In this case, the colony growth inhibition of normal CFU-GMs was not significantly lower as compared to 2-CdA used alone ( $p > 0.05$ ) (Fig. 3). These findings are in agreement with our previous studies in which we demonstrated that 2-CdA used alone or in combination with IFN- $\alpha$  or IFN- $\gamma$  inhibited to a higher degree the growth of CML CFU-GMs than their normal counterparts<sup>27</sup>. Similar observations have been made by other investigators. MARTENS and HAGENBEEK<sup>20</sup> have demonstrated in preclinical studies that amifostine allows the use of escalated doses of chemo- and radiotherapy without reducing the anti-leukemic efficacy<sup>20</sup>. The data presented by AN et al.<sup>1</sup> indicate that amifostine not only protects normal hema-

topoietic progenitors from the cytotoxic effect of nitrogen mustard, but also sensitizes BCR/ABL+ leukemic progenitors to this agent. CAPIZZI<sup>5</sup> has demonstrated that pretreatment with amifostine protects normal marrow progenitor cells (CFU-GM, CFU-GEMM and BFU-E) from the cytotoxic effect of carboplatin, paclitaxel, doxorubicin, daunorubicin and mitoxantrone therapy. This author has also shown that amifostine reduces radiation- or chemotherapy-induced toxicity in such tissues as bone marrow, heart, intestine, kidney, liver, skin and lung<sup>5, 6</sup>.

It is important, however, that the mechanisms of action of all the cytostatics described above are different from the mechanism of 2-CdA used in our studies. The mechanism of 2-CdA action has not been yet fully explained, but it may be connected with apoptosis<sup>28</sup>. The precise mechanism by which amifostine can promote colony formation and cell survival is also not clear, but it may relate in part to the capacity of low molecular weight thiols to protect cells from peroxidation of membrane lipids, analogous to the antioxidant properties described for bcl-2<sup>8</sup>. CAPIZZI<sup>5</sup> has suggested

that WR-1065 reduces apoptosis caused by several agents and the mechanisms of this effect include binding of WR-1065 to DNA and nuclear proteins, thereby altering the structure of the internucleosomal region of chromatin and rendering it less vulnerable to degradation. Alternatively, cytoprotection of WR-1065 can be connected with its polyamine-like structure. It is known that polyamines are important regulators of cellular proliferation and differentiation, the metabolism of which is tightly regulated by hematopoietic cytokines<sup>14</sup>.

We have also shown that protection factor for 2-CdA used together with preincubated amifostine was higher in the case of normal CFU-GM than CML CFU-GM (0.89, 0.74, respectively), but the differences were not statistically significant ( $p > 0.05$ ) (Table 3). In both cases, PF was below 1.0 and we could not confirm the cytoprotective effect of amifostine.

In conclusion, we showed that amifostine added either directly to the culture or after preincubation with MNCs did not protect the normal CFU-GM progenitors. The agent significantly inhibited the colony growth of CML CFU-GM as compared to the control. Although CML CFU-GM colonies were inhibited to a higher degree than normal CFU-GM ones, the differences were not statistically significant. Furthermore, amifostine preincubated with mononuclear cells and used prior to 2-CdA at the concentration of 40 nM significantly inhibited the colony growth of CML CFU-GM as compared to 2-CdA used alone. In contrast, the colony growth of normal CFU-GM was not significantly lower. We suggest that 2-CdA combined with amifostine is more toxic to leukemic CFU-GM than their normal counterparts. Finally, we suggest that, despite promising results of cytoprotective action of amifostine in the treatment of solid tumors, its application in hematological disorders is still not fully explained. The protective effect of amifostine against 2-CdA in human myeloid leukemias is doubtful, though it may have an application in the treatment of other disorders, such as myelodysplastic syndromes or for bone marrow purging before autografting in CML patients.

**Acknowledgment.** The authors would like to thank Prof. Paweł Grieb (Foundation for Development of Diagnostics and Therapy, Warsaw, Poland), who kindly supplied 2-chlorodeoxyadenosine. We are also grateful to Schering-Plough Company, which kindly provided amifostine (Ethyol).

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Received in October 1999

Accepted in March 2000