

INDUCTION OF CASPASE 3 AND MODULATION OF SOME APOPTOTIC GENES IN HUMAN ACUTE PROMYELOCYTIC LEUKEMIA HL-60 CELLS BY CARBOPLATIN WITH AMIFOSTINE

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Induction of caspase 3 and modulation of some apoptotic genes in human acute promyelocytic leukemia HL-60 cells by carboplatin with amifostine. M. MIROWSKI, M. RÓŻALSKI, U. KRAJEWSKA, E. BALCERCZAK, W. MŁYNARSKI, R. WIERZBICKI. *Pol. J. Pharmacol.*, 2003, 55, 227–234.

The influence of carboplatin alone and carboplatin in combination with cytoprotective agent amifostine on the growth, caspase 3 activity and some apoptotic genes expression was investigated *in vitro* in human acute promyelocytic leukemia HL-60 cells. Proliferation of HL-60 cells exposed to carboplatin dropped down with increasing dose of the drug. This effect was slightly higher when carboplatin was used in combination with amifostine. The cytotoxic index (IC₅₀) was estimated as 6.6 and 4.4 × 10⁻⁴ M (after 24 h) and 3.3 and 2.5 × 10⁻⁵ M (after 48 h) for carboplatin and carboplatin with amifostine, respectively. This effect was accompanied by induction of caspase 3 activity. HL-60 cells treated with carboplatin alone showed about 120-fold increase in caspase 3 activity. Combination of carboplatin with amifostine induced the enzyme activity up to 280 times. Furthermore, the expression of *bcl-2*, *c-myc* and *bax* genes involved in apoptosis as well as *p65*, which function in this process is unknown, were determined. Semi-quantitative RT-PCR showed a decrease in *bcl-2* and an increase in *bax*, *c-myc* and *p65* expression in HL-60 cells treated with carboplatin in combination with amifostine as compared to the cells treated only with carboplatin.

We conclude that amifostine may potentiate carboplatin therapeutic efficiency towards human acute promyelocytic leukemia cells.

Key words: *HL-60 cell line, carboplatin, amifostine*

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Abbreviations: AFC – *amidofluorocoumarin*, CM – *culture medium*, GAPDH – *glyceraldehyde-3-phosphate dehydrogenase*, HL-60 – *human acute promyelocytic leukemia cells*, ICE – *interleukin-1-beta-converting cysteine protease*, MPCR – *multiplex polymerase chain reaction*, PCR – *polymerase chain reaction*, RT-PCR – *reverse transcriptase polymerase chain reaction*

INTRODUCTION

Amifostine (s-2[3-aminopropylamino]-ethyl phosphothioic acid, WR2721, Ethylol) is a pro-drug converted by alkaline phosphatase to active dephosphorylated form (WR1065) which penetrates the cell membrane by both active and passive diffusion mechanism [5, 37]. It was proven that it efficiently reduced the extent of DNA damage caused by free radicals [11, 22]. Amifostine is accumulated more rapidly in various normal tissues than in tumors [37] and could be used clinically to limit the side effect of chemotherapy on normal tissues [7], while not affecting the therapeutic efficacy on tumors [32, 33]. This phenomenon is probably connected with the fact that normal tissues are more vascularized than neoplastic tissues. Furthermore, in normal tissues greater ability of dephosphorylation of amifostine to the free thiol may be caused by a more neutral intracellular environment than in some tumors [5, 6]. The drug is also clinically used to reduce the toxic effect of radiation, nitrogen mustard, cisplatin, carboplatin, cyclophosphamide, carmustine, mephalan and 5-fluorouracil on hematopoietic progenitor cells [35] and cumulative renal toxicity associated with platinum drugs administration in patients suffering from lung cancer [29]. Amifostine is able to significantly decrease the hematological and non-hematological toxicity of cyclophosphamide, carboplatin or cisplatin [3, 9, 14].

Recently, more attention has been paid to the effect of amifostine in the treatment of hematologic malignancies such as myelodysplastic syndrome (MDS) and acute myeloblastic leukemia (AML) for potentiating the effects of cytotoxic agents [26]. Better therapeutic efficacy was shown for the combination of platinum drugs and amifostine [15, 16, 33]. Elevation of antitumor action by amifostine was shown for carboplatin and 5-fluorouracil in colon 26 tumors in BALB/c mice [34] as well as in human ovarian cancer xenografts OVCAR-3, A2780

and FMa growing subcutaneously in the nude mice [15]. Carboplatin [cis-diammine(cyclobutane-1,1-dicarboxylato)platinum(II)], as a second-generation platinum drug, has less nephrotoxicity and ototoxicity but more myelotoxicity than cisplatin, the first-generation platinum drug [30].

Amifostine has been reported to delay cell-cycle progression in cultured cells [10, 12] and affect the expression of *c-myc* gene [18]. North et al. [25] have proven that WR1065 affects the activity of P53 protein. They showed that exposure of cultured cells to this compound induced accumulation and activation of P53 protein.

The aim of this study was to evaluate the influence of carboplatin with amifostine on the growth of HL-60 promyelocytic leukemia cells *in vitro*, on the induction of apoptosis and the expression of some genes connected with this process.

MATERIALS and METHODS

Cell line

Human acute myeloblastic leukemia cells (HL-60 line) were used. The cells were grown in culture medium (CM) containing RPMI 1640 (Biomed, Poland), supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% fetal calf serum (Sigma) inactivated at 56°C for 30 min. Cell culture was started with about 5×10^5 HL-60 cells/ml and carried out in disposable 24-well plastic plates (Nunc) in a CO₂ incubator at 37°C over three days. After this time, wells containing HL-60 cells were supplemented with fresh CM. Three days later, the cells were pipetted into sterile centrifuge tubes (Nunc) and collected by centrifugation (50 × g, 6 min). The cells were resuspended in fresh CM and seeded onto sterile plastic plates.

Antiproliferative effect of carboplatin and amifostine

At the early stage of the work, the effect of amifostine alone on the growth of HL-60 cells was determined. The cells were seeded onto 6-well plate at the density of about 0.7×10^6 cells in 2 ml of CM per well. On the next day, amifostine solution (lyophilized powder dissolved in CM) was added to final concentration from 10^{-6} to 10^{-2} M (0.5 ml) and the cells were incubated for 24 and 48 h. After these time intervals, the cells were stained with try-

pan blue and counted under a microscope with the use of Bürker's hemocytometer.

To evaluate the cytotoxic effect of carboplatin in combination with amifostine, HL-60 cells (0.7×10^6 in 2 ml CM per well) were preincubated with amifostine (10^{-2} M) for 30 min. Then the cells were centrifuged ($100 \times g$, 6 min), cell pellet was resuspended in fresh CM and seeded onto 6-well-plate. Next, the cells were treated with increasing doses of carboplatin (from 10^{-7} to 10^{-3} M, diluted with CM). After 4 h incubation period, cell suspension was centrifuged. The cells were resuspended in 3 ml of fresh CM and further grew on 6-well plate in a CO₂ incubator at 37°C. The number of cells was determined 24 and 48 h after exposure to the drug.

The control contained the untreated cells to which instead of the drugs equal volume of CM was added.

In the case of exposure to carboplatin alone, the cells were preincubated in CM without amifostine and treated as above.

Determination of caspase 3 activity in cellular lysates

HL-60 cells after treatment with carboplatin and/or amifostine were used for determination of caspase 3 activity by fluorimetric immunosorbent enzyme assay (FIENA, Roche). Cellular lysates were prepared from HL-60 cells (2×10^6) 24 h after their treatment with amifostine (10^{-2} M) and carboplatin at IC₅₀ dose. Untreated HL-60 cells were used as a control. The cells were pipetted to sterile tubes (10 ml) and centrifuged ($100 \times g$, 6 min, 4°C). All sediments were resuspended in 7 ml of cold 0.01 M phosphate buffer containing 0.9% NaCl (PBS, without Ca²⁺ and Mg²⁺). After centrifugation, to the tubes containing cell sediments, 200 µl of cold 10 mM dithiothreitol (DTT) was added, the mixture was briefly vortexed and incubated for 1 min in ice bath. Next, the samples were carefully vortexed and pipetted to 2 ml Eppendorf tubes. After centrifugation ($9000 \times g$, 1 min), the supernatant (cellular lysate) was used directly for enzyme activity determination.

Caspase 3 activity assay, based on the capture of caspase 3 from HL-60 cellular lysates by a monoclonal antibody, was done according to manual provided by the manufacturer (Cat. No. 2 012 952 Roche). Caspase 3 activity was proportional to the fluorochrome (amidofluorocoumarin, AFC) formed

from the substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (Ac-DEVD-AFC). The generated free fluorescence products were determined fluorometrically at $\lambda_{max} = 505$ nm. Caspase 3 activity was expressed as nmoles of AFC released/ 10^6 cells.

RNA extraction

Total RNA was isolated from HL-60 cells (about 10^5) treated with carboplatin or carboplatin with amifostine (IC₅₀, 24 h of growth) by Total RNA Prep Plus Minicolumn Kit (A&A Biotechnology, Poland) based on RNA isolation method developed by Chomczyński and Sacchi [8]. The isolated RNA had an A_{260/280} ratio of 1.6–1.8.

cDNA synthesis

cDNA was synthesized by RevertAid™ cDNA Synthesis Kit (Fermentas, Lithuania). Reaction mixture (total volume of 20 µl) contained 1 µl of total RNA (3 µg), 1 µl of oligo(dT) 18 primer (0.5 µg) and 8 µl of deionized, nuclease-free water. The mixture was spun down and incubated at 70°C for 5 min, then chilled on ice and the following components were added: 4 µl of 5× reaction buffer, 1 µl of ribonuclease inhibitor (20 U), 4 µl of 10 mM dNTPs mix. The mixture was incubated at 37°C for 5 min and 1 µl of RevertAid M-MuLV reverse transcriptase (200 U) was added. The mixture was incubated at 42°C for 60 min. The reaction was stopped by heating at 70°C for 10 min.

bcl-2, *c-myc*, *ICE* and *p53* genes expression

The expression of human *bcl-2*, *c-myc*, *ICE*, *p53* and housekeeping *GAPDH* genes were done by multiplex PCR (MPCR) with the use of human apoptosis set 1 detection kit (hAPO1-MPCR, Biosource International, USA) according to the manufacturer's manual. The kit provides the optional primer/buffer system, which will enhance specific, multiple amplification. In MPCR, "hot start" DNA polymerase (Qiagen, Germany) was used.

p65 and *bax* genes expression

For *p65* expression, the primers were designed on the basis of sequences of short N-terminal fragments of total P65 molecule (5'-GGTCCACGGC-GGACCGGT-3') and its CNBr-peptide migrated in electrophoresis as a band with 51 kDa (5'-GACCCCGAGAACGTGGTGC-3') [2, 23].

For *box* expression, the primers (foreword: 5'-CA-GCTCTGAGCAGATCATGAAGACA-3' and reverse: 5'-GCCCCATCTTCTTCCAGATGGTGAG-3' were designed on the basis of the article by Mori et al. [24].

As the PCR control, β -actin cDNA was amplified using the primers 5'-TGTATGCCTCTGGTC-GTACCAC-3' and 5'ACAGAGTACTTGCGCT-CAGGAG-3' [31].

PCR

PCR mixture contained 1.5 mM MgCl₂, dNTPs mix, 0.5 U Taq Polymerase, reaction buffer and 0.5 μ M of each primer. DNA was amplified in 35 cycles using the parameters: denaturation (94°C; 30 s), annealing (57°C; 30 s); extension (72°C; 30 s). Following PCR, the amplicons were analyzed by gel electrophoresis with ethidium bromide staining and scanning in a densitometer for semi-quantitative analysis (LKB Image Master). Expression of the investigated genes was determined by normalizing their expression against the expression of reference (housekeeping) *GAPDH* (921 bp) or β -actin (562 bp) genes. The areas of stained DNA bands were determined and the ratio of products of investigated genes to housekeeping gene products was calculated.

Statistical analysis

Data are presented as mean values \pm SD. Statistical significances were calculated by Student's *t*-test or Mann-Whitney test.

RESULTS

HL-60 cells in culture were treated with amifostine at concentration from 10⁻⁶ to 10⁻² M and further incubated for 24 or 48 h. As can be seen in Table 1, the growth of HL-60 cells stayed at the same level. Only in the case of 24-h incubation with the lowest amifostine concentration, statistically non-significant stimulation of cell proliferation was noticed. The highest amifostine concentration (10⁻² M) was selected for further experiments with carboplatin influence on the growth of HL-60 cells.

HL-60 cells pretreated with amifostine were then incubated with carboplatin. After washing out of the drug, the cells were further grown for 24 h. The growth of HL-60 cells dropped down with increasing dose of carboplatin (10⁻⁷ – 10⁻³ M). This

Table 1. The effect of amifostine on HL-60 cells in culture. Mean values \pm SD from 5 experiments are presented as a percentage of the cell number in comparison to untreated cells to which instead of the drug equal volume of CM was added (control)

Growing time of amifostine-treated cells (h)	Amifostine concentration (M)				
	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴	10 ⁻³	10 ⁻²
24	120 \pm 6	101 \pm 7	103 \pm 3	110 \pm 4	102 \pm 6
48	102 \pm 2	91 \pm 8	99 \pm 4	95 \pm 3	92 \pm 4

Table 2. The effect of carboplatin and carboplatin with amifostine (10⁻² M) on the growth of HL-60 cells. Mean values \pm SD from 5 experiments are presented as a percentage of the cell number in comparison to untreated cells (control). Growing time: 24 h. * Differences statistically significant (Student's *t*-test)

Drug	Carboplatin concentration (M)				
	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴	10 ⁻³
Carboplatin	95 \pm 10	98 \pm 11	85 \pm 6	74 \pm 7	40 \pm 4
Carboplatin with amifostine	94 \pm 11	93 \pm 9	78 \pm 8	66 \pm 4*	21 \pm 3*

Table 3. The cytotoxic effect (IC₅₀) of carboplatin and carboplatin with amifostine on the growth of HL-60 cells. Mean values \pm SD from 5 experiments are presented

Drug	Growing time (h)			
	24		48	
	IC ₅₀ (10 ⁻⁴ M)	p	IC ₅₀ (10 ⁻⁵ M)	p
Carboplatin	6.6 \pm 1.4		3.3 \pm 0.7	
Carboplatin with amifostine	4.4 \pm 0.3	> 0.05	2.5 \pm 1.0	> 0.1

p – statistical significance of differences (Mann-Whitney test)

effect was deeper when HL-60 cells were pretreated with amifostine. In the case of carboplatin at concentration 10⁻³ M this effect was the highest (about 50% of inhibition, Tab. 2).

The cytotoxic index IC₅₀ (concentration of the drug that induces 50% inhibition of cell growth) was calculated for cultured HL-60 cells. After 24 h of the growth, IC₅₀ for carboplatin was estimated as 6.6 \times 10⁻⁴ M, whereas for carboplatin with amifostine as 4.4 \times 10⁻⁴ M. Cytotoxic effect was clearly higher when the cells were grown for 48 h (Tab. 3).

Table 4. The caspase 3 activity in lysates of HL-60 cells treated with amifostine, carboplatin and carboplatin with amifostine. The values are expressed as nmols of amidofluorocoumarin (AFC) released/10⁶ cells and in relative activity. Mean values ± SD from 3 experiments are presented. Growing time: 24 h

Caspase 3 activity	Untreated cells (control)	Amifostine	Carboplatin	Carboplatin with amifostine
AFC (nmols)	11 ± 0.8	13 ± 0.9	1320 ± 52	3065 ± 94
Relative activity	1 ± 0.1	1.2 ± 0.15	120 ± 2.4	278 ± 12

Table 5. The ratio of amplicons of the investigated genes to reference (*GAPDH* or β -*actin*) genes. Mean values ± SD from 5 experiments are presented. Growing time: 24 h. * Differences statistically significant (Student's *t*-test)

Investigated/reference genes	Carboplatin	Carboplatin with amifostine
<i>bcl-2/GAPDH</i>	0.75 ± 0.01	0.29 ± 0.01*
<i>c-myc/GAPDH</i>	0.41 ± 0.02	0.67 ± 0.03*
<i>bax/β-actin</i>	0.50 ± 0.01	0.64 ± 0.01*
<i>p65/β-actin</i>	0.58 ± 0.02	0.86 ± 0.03*

The analysis of caspase 3 activity in HL-60 cells treated with carboplatin at a dose equal to IC₅₀ showed that these cells had 120-times higher activity in comparison to untreated cells. When HL-60 cells were preincubated with amifostine before carboplatin treatment, caspase 3 activity was even 280-times higher. The cells incubated with amifostine alone showed no differences in the enzyme activity compared to untreated cells (Tab. 4).

In further studies, the activity of some genes responsible for regulation of apoptosis was analyzed by multiplex RT-PCR. The expression of *bcl-2*, *c-myc*, *ICE* and *p53* was determined. In experiments with carboplatin and carboplatin with amifostine, no *ICE* and *p53* genes expression was observed (Fig. 1). The ratio between *bcl-2/GAPDH* amplicons dropped down from 0.75 (carboplatin) to 0.29 (combination of carboplatin with amifostine). On the contrary, *c-myc/GAPDH* ratio showed growing tendency from 0.41 to 0.67, respectively (Tab. 5).

Another multiplex PCR was carried out to get information about the levels of *bax* (638 bp) and *p65* (130 bp) genes expression (Fig. 2). The ratio

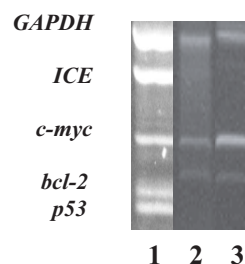


Fig. 1. An example of multiplex PCR (MPCR) for *p53* (204 bp), *bcl-2* (233 bp), *c-myc* (371 bp), *ICE* (658bp) and reference *GAPDH* (921 bp) genes in HL-60 cells treated with carboplatin (3) and carboplatin with amifostine (2). Positive control included in the kit (1)

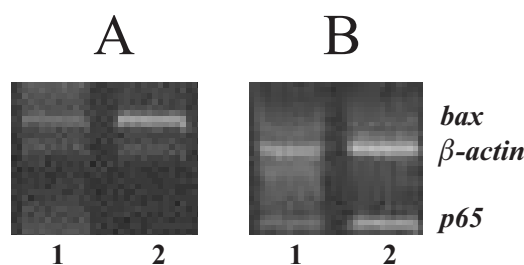


Fig. 2. An example of multiplex PCR (MPCR) for *bax* (638 bp) (A), *p65* (130 bp) (B) and reference β -*actin* (562 bp) genes in HL-60 cells treated with carboplatin (1) and carboplatin with amifostine (2)

between *bax/β-actin* amplicons had increasing tendency from 0.5 (carboplatin) to 0.64 (combination of carboplatine with amifostine). Similarly, *p65/β-actin* ratio went up from 0.58 to 0.86, respectively (Tab. 5).

DISCUSSION

There are some data showing that treatment with amifostine does not affect the therapeutic efficiency of some anti-cancer drugs on tumors. Apart from the cytoprotective role of amifostine in solid tumors, it also can be used in the treatment of hematologic malignancies, such as myelodysplastic syndrome (MDS) and acute myeloblastic leukemia (AML) for potentiating the effects of cytotoxic agents [26]. Korycka and Robak [17] 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 granulocyte-macrophage progenitor cells (CML CFU-GM) *in vitro*. Amifostine used alone inhibited the growth of CML CFU-GM colonies to

a higher degree than those of normal CFU-GM. Furthermore, amifostine used together with 2-chlorodeoxyadenosine showed stronger inhibition of the colony growth of CML CFU-GM as compared to 2-CdA alone.

We have evaluated the influence of amifostine or carboplatin alone and carboplatin in combination with amifostine on survival rate of HL-60 cells *in vitro*. Taking into account that WR1065 generated from amifostine *in vitro* may be a substrate for diamine oxidase, which is present in fetal calf serum, we decided to use amifostine at a concentration of 10^{-2} M. However, after preincubation with amifostine, the cells were washed with fresh CM and then carboplatin was added. After next incubation, this compound was also washed out by CM and the cells were further grown for 24 or 48 h. Estimation of IC_{50} showed that amifostine significantly potentiated carboplatin cytotoxic effect.

In our study, amifostine alone practically had no influence on cell growth. Similar observation has been published by Marańda et al., [19] who noticed that amifostine given alone did not change the survival time of mice with leukemias L1210 and P388. On the contrary, carboplatin showed dose-dependent decrease of cell growth. This effect was deeper if before carboplatin treatment HL-60 cells were pretreated with amifostine. It is our opinion that preincubation of HL-60 cells with amifostine enhances cytotoxic effect of carboplatin. In the clinical practice, amifostine is usually given intravenously before treatment with cytostatic drugs. The mechanism by which amifostine can potentiate antiproliferative effect of carboplatin is not clear, but it may relate, in part, to ability of amifostine to delay cell-cycle progression, which was reported by others for cultured cells [10, 12]. Cell-cycle delay by amifostine and DNA-damage generated by carboplatin may be a strong and synergistic signal to programmed cell death. This hypothesis was confirmed in our study by increase in caspase 3 activity, *bax* and *c-myc* genes expression and decrease of *bcl-2* gene expression. Additionally, diamine oxidase present in the serum can catabolize amifostine to acrolein and hydrogen peroxide, which can be toxic to cells in culture [21], but it should not be the case in our study because the cells after short exposure to amifostine were put into fresh CM.

In further studies, we aimed to get more information about the possible stimulation of apoptosis

in HL-60 cells by the compounds described above. Apoptosis is a major form of cell death, characterized initially by a series of stereotypic morphological changes. The most common is the DNA fragmentation, which is a relatively late event during programmed cell death. An earlier, almost unique event, is the exposure of phosphatidylserine on the outer leaflet of the plasma membrane bilayer. The initiation of all intracellular events seems to be connected with the activation of specific proteases called caspases (cysteiny-aspartic-acid-proteases). Special role in this process seems to be played by caspase 3 because its active form is responsible for the cleavage and breakdown of several cellular components related to DNA repair and regulation. We have noticed that the cell treatment with carboplatin stimulates the caspase activity about 120 times. However, when HL-60 cells were treated with combination of carboplatin and amifostine, activity of this enzyme was stimulated about 280 times. On the contrary Capizzi [7] suggested that the active metabolite of amifostine, WR-1065, may reduce the apoptosis caused by several agents. This involves binding of WR-1065 to nuclear proteins, thereby altering the structure of the internucleosomal regions of chromatin and rendering it less vulnerable to degradation.

Apoptosis requires activation of some specific genes. The *bcl-2*, *ICE*, *p53*, *c-myc* and *bax*, among others, belong to these genes. In the present study, expression of these genes in HL-60 cells treated with carboplatin and with carboplatin in combination with amifostine was determined semi-quantitatively. Expression of *bcl-2* gene drastically dropped down from 0.75 (carboplatin treatment) to 0.29 (carboplatin in combination with amifostine). Protein product of *bcl-2* has been shown to block apoptosis in experimental systems [28]. The mechanism of the blocking is unknown, although biochemical studies have implicated this protein in the regulation of cell redox potential [13]. Genetic evidence indicates that *bcl-2* suppresses apoptosis [4]. Expression of *bax*, another gene from this family, in our study showed growing tendency. It is known that *bax* plays a role in the regulation of apoptosis induced by chemotherapy and other stimuli [36] and its expression is usually connected with induction of programmed cell death [27]. Similar growing tendency was also noticed for *p65*, the biological role of which is still unknown, and for *c-myc*. The *c-myc* oncogene is known as a transcriptional

regulator and its higher expression correlates with susceptibility to apoptosis [1]. Amifostine has been reported to repress the expression of *c-myc* gene [18].

North et al. [25] showed that exposure of cultured cells to WR1065 induced accumulation and activation of P53 protein. We were not able to get amplification of *p53* gene in HL-60 cells. Usually, loss of *p53* function correlates with the decreased sensitivity to chemotherapy in a variety of human tumors. The observed induction of apoptosis in our experiment is, probably, connected with a *p53*-independent mechanism. Negative results were also obtained for *ICE*, which encodes the interleukin-1- β -converting cysteine protease and induces apoptosis in various cell types when it is overexpressed [20]. Taking into account that caspase 3 activity was significantly stimulated in our experiments, the lack of *ICE* expression suggests that protease encoding by *ICE* is not participating in activation of the investigated caspase.

On the basis of obtained results, we can conclude that cytoprotective agent, amifostine, stimulates cytotoxic effect of carboplatin on HL-60 cells and intensifies pro-apoptotic action of the drug. This observation leads to suggestion that amifostine may potentiate therapeutic effect of this cytostatic on acute promyelocytic leukemia cells.

Acknowledgments. The work was supported by grants no. 502-13-844 and 502-13-843 from the Medical University of Łódź, Poland. Mrs. E. B. was supported by L'Oréal and UNESCO fellowship "For women in science".

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Received: December 23, 2002; in revised form: March 17, 2003.