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Report

Effects of WR-2721 and cyclophosphamide on the cell cycle phase specificity of apoptosis in mouse bone marrow

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Elucidation of the mechanisms of action of the thiol and alkylating agents on normal cells requires the knowledge of their cell cycle phase specificity in terms of their ability to induce apoptosis. The effects of *S*-2-/3-aminopropylamino/ethyl phosphorothioic acid (WR-2721, Amifostine) and cyclophosphamide (CP) on apoptosis and cell cycle progression were assessed in the mouse bone marrow. Adult male Swiss mice were treated with WR-2721, at a dose of 400 mg/kg body weight, and/or CP, at a dose of 200 mg/kg body weight. Application of the laser scanning cytometry APO-BRDU assay, a two-color staining method for labeling of DNA breaks and cellular DNA, allowed an identification of apoptotic and non-apoptotic cells, and their position with respect to their cell cycle phase. Temporary alterations in the number of apoptotic cells and also all bone marrow cells, including apoptotic and non-apoptotic ones, were determined throughout the 240-h period after treatment of mice with WR-2721 and/or CP. These drugs, given alone, affected apoptotic cell death and caused deregulation of the cell cycle in the bone marrow. WR-2721, applied 30 min prior to CP administration, resulted in a suppressing effect on apoptosis and the cell cycle perturbation triggered in normal bone marrow cells by the alkylating drug. The patterns of changes in the frequency of apoptotic cells and the number of apoptotic and non-apoptotic bone marrow cells, observed in all phases of the cell cycle, were dependent on the agent(s) given and the time interval after WR-2721 and/or CP administration. [© 2002 Lippincott Williams & Wilkins.]

Key words: Apoptosis, bone marrow cells, cell cycle, cyclophosphamide, laser scanning cytometry, WR-2721.

Introduction

Apoptosis is an active process of cell death in which the cell itself designs and executes the program of its own demise and subsequent body disposal.[1] Apoptosis is basically characterized by cellular shrinkage, marked condensation and margination of chromatin, nuclear and cellular fragmentation with well preserved cell organelles, and formation of apoptotic bodies. Apoptotic cells and apoptotic bodies are ingested and digested by neighboring cells or macrophages without eliciting any inflammatory response.[2,3] The control of cell death is as important as the regulation of cell proliferation. A delicate balance between apoptosis and cell proliferation is responsible for the maintenance of cellular homeostasis.[4,5] Apoptotic cell death plays a crucial role in the normal function of the hematopoietic system.[6,7]

Apoptosis is accepted as the mode of cell death occurring as the result of chemotherapy.[8] Chemotherapeutic drugs act on neoplastic cells, but also damage normal cells and especially cell populations that have rapid cell turnover, e.g. hematopoietic cells.[9] Therefore, protection of normal cells from the cytotoxic effects of chemotherapy is a most challenging issue. *S*-2-/3-aminopropylamino/ethyl phosphorothioic acid (WR-2721, Amifostine) has been developed to protect normal cells against the toxicities of chemotherapeutic agents.[10,11]

Little is known about induction of apoptosis in normal hematopoietic cells by the aminothiol WR-2721 and cyclophosphamide (CP, 2-[bis-/2-chloroethyl/-amino]-tetrahydro-2H-1,2,3-oxaza-phosphorine-2-oxide), one of the alkylating drugs widely used in chemotherapy.[12,13] Understanding the mechanisms of action of these agents requires knowledge of their cell cycle phase specificity, in terms of their ability to induce apoptosis.

Recently, a new laser scanning cytometry (LSC) assay has been introduced for identifying the cell cycle phase of the cells which undergo apoptosis in

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response to different agents. This method relies on bivariate analysis of cellular DNA content and the *in situ* detection of DNA strand breaks.[14,15] Apoptotic DNA degradation results in the presence of extensive DNA cleavage.[16–18] The 3′-hydroxyl ends in DNA breaks can be analyzed by attaching to them fluorescein isothiocyanate (FITC)-tagged bromolated deoxyuridine triphosphate nucleotides (Br-dUTP), in a reaction catalyzed by exogenous terminal deoxynucleotidyl transferase (TdT). The simultaneous measurement of DNA content, using propidium iodide (PI), makes it possible to identify apoptotic and also non-apoptotic cells and position them with respect to their cell cycle phase.[19–21]

The aim of the present study was to assess the cell cycle phase specificity of apoptosis and any changes in the cell cycle induced in the mouse bone marrow after application of the aminothiol WR-2721 and/or the alkylating drug CP, using LSC analysis of DNA strand breaks and cellular DNA content.

Materials and methods

Animals

The adult male Swiss mice (Animal Breeding Unit, Cracow, Poland) employed in the experiments weighted from 29 to 32 g. All mice were kept under constant environmental conditions with a 12/12 h light/dark cycle. They were fed standard granulated chow and given drinking water *ad libitum*.

Chemicals

WR-2721 (Amifostine) was obtained from the Institute of General Chemistry, Warsaw Agricultural University. CP (Endoxan) was from Asta Pharma (Hamburg, Germany).

Doses and sampling intervals

WR-2721 was given at a single, sublethal dose of 400 mg/kg body weight, diluted in 200 μl aqua pro injectione (Polfa, Starogard Gdanski, Poland). CP was applied at a single, sublethal dose of 200 mg/kg body weight, dissolved in 300 μl physiological saline (Polfa). WR-2721 was administered alone and 30 min prior to CP application. The solutions were freshly prepared, directly before the mice were injected. The i.p. route of administration was used in all experiments. The control group consisted of untreated mice. Animals were killed by cervical dislocations at 3, 6, 12, 24, 48, 96 and 240 h after administration of WR-2721 and/or CP, and bone marrow samples were withdrawn. Each experimental group, at each time point after the drug(s) application, contained four or five mice.

Preparation procedure and fixation of bone marrow cells

Bone marrow cells from both femurs were flushed out with PBS (BioMed, Lublin, Poland) and dispersed by gentle pipetting. Finally, the cells were suspended in 500 μl of PBS. After adding 5 ml of 1% (w/v) formaldehyde (Chemicals, Lublin, Poland) in PBS, the cell suspension was placed on ice for 15 min. Then, cells were centrifuged for 5 min at 80 *g* and the supernatant was discarded. The cells were washed in 5 ml of PBS and pelleted by centrifugation. The washing and centrifugation steps were repeated once more. The bone marrow cells were then resuspended in 500 μl of PBS and, after adding 5 ml of ice-cold 70 % (v/v) ethanol, stored at -20°C until use.

Bone marrow cell counting

Bone marrow cells were counted using a Reichert (Buffalo, NY) camera. The number of cells was calculated in both femurs.

APO-BRDU assay

An APO-BRDU kit (Phoenix Flow Systems, San Diego, CA), a two-color staining method for labeling of DNA breaks and total cellular DNA to detect apoptotic cells, was used. The kit consisted of washing, reaction and rinsing buffers for processing individual steps in the assay, TdT, Br-dUTP, FITC-tagged anti-BrdU antibody for labeling of DNA breaks, and PI/RNase A solution for counter staining of the total DNA.

APO-BRDU protocol

The bone marrow cell suspension, containing approximately 1×10^6 cells, was centrifuged for 5 min at 145 *g* and the 70% ethanol was removed by

aspiration. The cell pellet was resuspended with 1 ml of wash buffer, centrifuged as before and the supernatant was removed by aspiration. The wash buffer treatment was repeated, and the cell pellet resuspended in 50 μl DNA labeling solution containing 10 μl TdT reaction buffer, 0.75 μl TdT, 8 μl Br-dUTP and 32.25 μl distilled H_2O. The cells were incubated in the DNA labeling solution for 60 min at 37°C in a temperature-controlled bath. At the end of the incubation time, 1 ml of rinse buffer was added to each tube, the cells were centrifuged and the supernatant removed by aspiration. The cell rinsing with 1 ml of the rinse buffer was repeated and then the cell pellet was resuspended in 100 μl of the antibody solution containing 5 μl of FITC-labeled anti-BrdU antibody suspended in 95 μl of the rinse buffer. The cells were incubated with the fluoresceinated antibody solution in the dark for 30 min at room temperature. Then, 500 μl of the PI/RNase A solution was added to the tube containing 100 μl of the antibody solution and the cells were incubated in the dark for 30 min at room temperature. After incubation, the cells were spread on a microscopic slide by centrifugation at 110 *g* for 6 min (Shandon, Pittsburgh, PA), then covered by a coverslip and subjected to cell fluorescence measurement.[14,22]

LSC analysis of bone marrow cells

The fluorescence of individual cells was measured by LSC (CompuCyte, Cambridge, MA). For the cell cycle analyses and DNA strand break detection, fluorescence was exited with 488 nm laser line, and green fluorescence of FITC-conjugated anti-BrdU antibody was measured at 530 nm and red fluorescence of PI at above 600 nm.[14,21] At least 5000 cells were analyzed per slide.

Statistical evaluation

The frequency of apoptotic cells (Figure 1A) and the number of all bone marrow cells, including apoptotic and non-apoptotic ones (Figure 1B), were determined in each phase of the cell cycle.[19–21] The statistical significance for the data was calculated by an analysis of variance and Duncan's new multiple-range test.

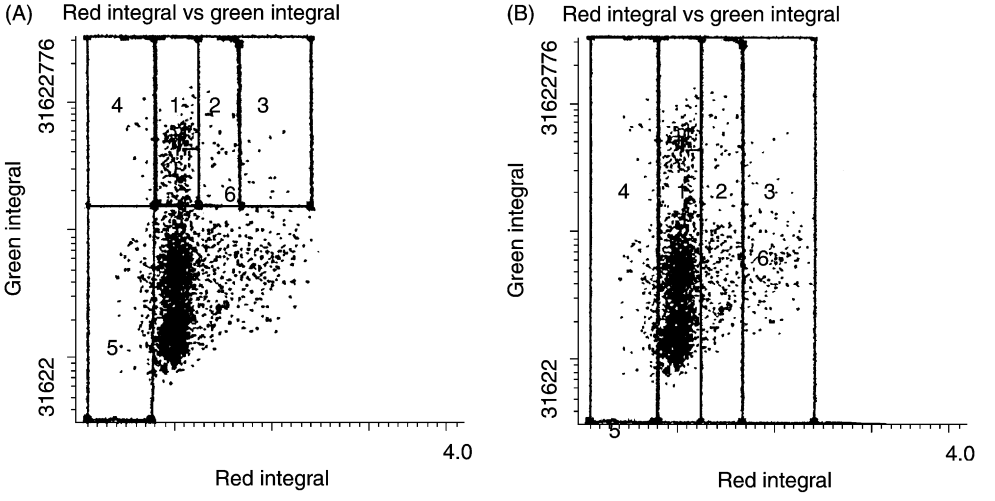

Figure 1. Dot-plot of FITC green fluorescence (green integral) versus PI red fluorescence (red integral) of bone marrow cells. LSC cell cycle distribution of both apoptotic cells and all bone marrow cells including apoptotic and non-apoptotic ones, found at 3 h after treatment of the male mouse with CP, at a dose of 200 mg/kg body weight. (A) Detection of apoptotic cells using the LSC APO-BRDU assay based on the bivariate analysis of apoptosis-associated DNA strand breaks and cellular DNA content. Five subpopulations of apoptotic cells were made on the basis of DNA strand break FITC-labeling and cellular DNA staining with PI: (1) G_1/G_0 apoptotic cells, (2) S phase apoptotic cells, (3) G_2/M apoptotic cells, (4–5) early and late apoptotic sub-G_1 cells, respectively, and (6) all apoptotic bone marrow cells. (B) Determination of the cell cycle position of apoptotic and non-apoptotic cells using the LSC APO-BRDU assay. Four subpopulations of both apoptotic and non-apoptotic cells were made on the basis of apoptosis-associated DNA strand break FITC-labeling and the total content of cellular DNA staining with PI: (1) G_1/G_0 cells, (2) S phase cells, (3) G_2/M cells, (4–5) sub-G_1 cells and (6) all bone marrow cells, including apoptotic and non-apoptotic ones.

Results

Cell cycle position of apoptotic and non-apoptotic bone marrow cells

The effects of the aminothiol WR-2721 and the chemotherapeutic drug CP on apoptotic cell death and cell cycle progression, determined by using LSC assay based on simultaneous analysis of cellular DNA content and the *in situ* detection of DNA strand breaks, were observed in the mouse bone marrow at 3, 6, 12, 24, 48, 96 and 240 h after the agent(s) administration (Figure 2). The groups of mice show statistically significant differences ($p < 0.05$) both in terms of the frequency of apoptotic cells and the number of all bone marrow cells, including apoptotic and non-apoptotic ones, analyzed with respect to their cell cycle phase position. All data are presented as mean ± SD.

G_1/G_0 apoptotic cells

In relation to the controls, the number of G_1/G_0 apoptotic bone marrow cells was calculated to be significantly increased at 3, 12, 24 and 240 h after CP administration, at 3, 6, 12 and 24 h in mice which WR-2721 and CP were given, and at 3 h in those treated with WR-2721 alone. As compared with mice which received WR-2721 and CP, the percentage of apoptotic cells observed in the G_1/G_0 phase of the cell cycle was significantly lower at 6 h, and significantly higher at 3, 12 and 240 h after treatment of mice with CP alone (Figure 2A).

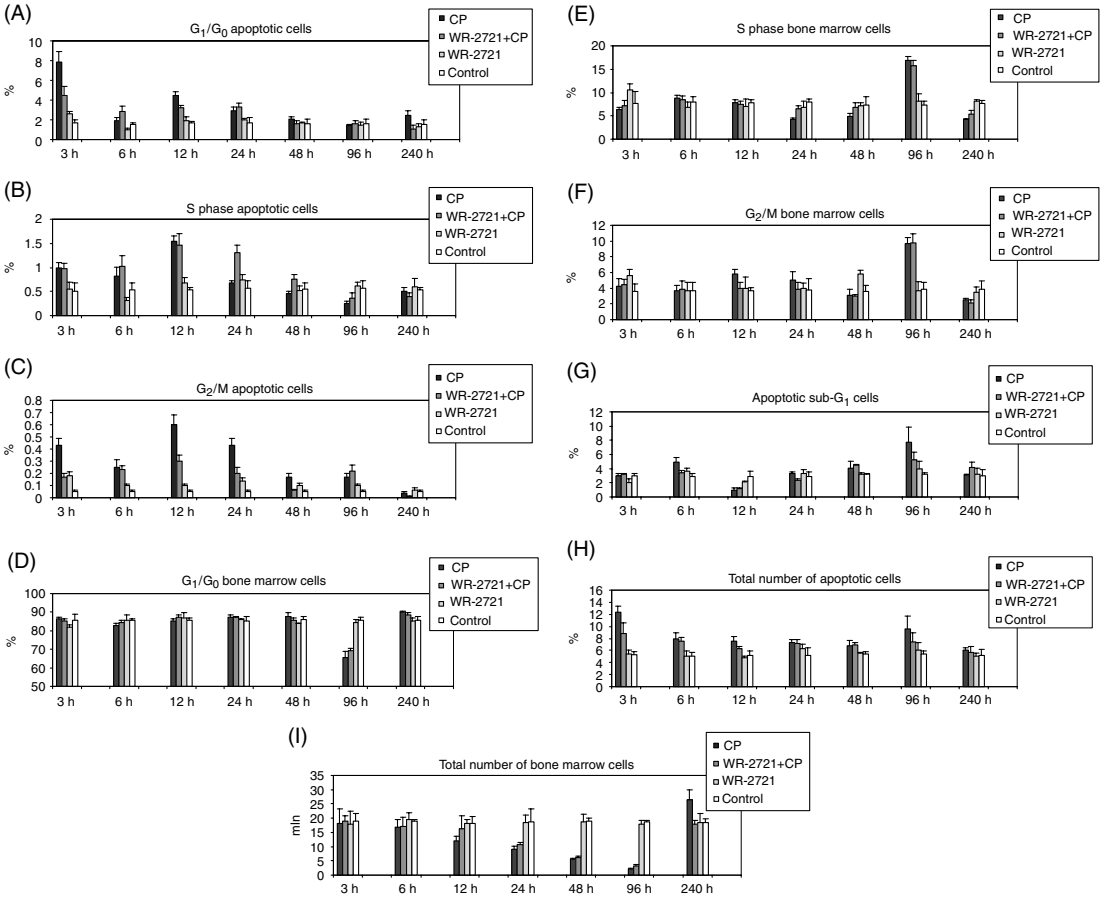

Figure 2. Effects of WR-2721 and CP on apoptosis and cell cycle progression determined in the mouse bone marrow by LSC simultaneous analysis of cellular DNA content, and the *in situ* detection of DNA strand breaks allowing to identify apoptotic and non-apoptotic cells and to reveal their cell cycle position. Temporary changes in the frequency of apoptotic cells (A–C and G), and also all bone marrow cells, including apoptotic and non-apoptotic ones (D–F) found in the particular phases of the cell cycle, as well as the total frequency of apoptotic cells (H) and the total number of bone marrow cells (I) determined throughout the 240-h period after treatment of mice with WR-2721 and/or CP. All data are presented as mean ± SD.

S phase apoptotic cells

In comparison with the controls, the number of S phase apoptotic bone marrow cells was found to be significantly greater at 3, 6 and 12 h after CP administration, and at 3, 6, 12 and 24 h after treatment of mice with WR-2721 prior to CP injection, but significantly smaller at 96 h in those which received CP alone. The percentage of apoptotic cells detected in the S phase of the cell cycle was significantly lower at 24 and 48 h in mice treated with CP only than in those given both WR-2721 and CP (Figure 2B).

G_2/M apoptotic cells

As compared with the controls, the number of G_2/M apoptotic bone marrow cells was assessed to be significantly increased at 3, 6, 12, 24, 48 and 96 h after treatment of mice with either CP or WR-2721 alone, and also at 3, 6, 12, 24 and 96 h after administration of both WR-2721 and CP, but significantly decreased at 240 h in mice given WR-2721 and CP. The percentage of apoptotic cells observed in the G_2/M phase of the cell cycle was significantly higher at 3, 12, 24, 48 and 240 h in mice which received CP only than in those injected with both WR-2721 and CP (Figure 2C).

G_1/G_0 bone marrow cells

As compared with the controls, the number of G_1/G_0 bone marrow cells, including apoptotic and non-apoptotic ones, was observed to be significantly smaller at 3 h in mice treated with WR-2721 alone, and at 96 h in mice injected with CP alone and in those which received both WR-2721 and CP, whilst the percentage of G_1/G_0 cells was significantly higher at 240 h after CP administration (Figure 2D).

S phase bone marrow cells

In relation to the controls, the number of S phase apoptotic and non-apoptotic bone marrow cells was significantly increased at 3 h after WR-2721 administration, and at 96 h in mice which received CP alone and in those treated with WR-2721 and CP, whilst the number of S phase cells was found to be significantly reduced at 24, 48 and 240 h after CP application, and also at 240 h in mice given both WR-2721 and CP. The percentage of cells in the S phase of the cell cycle was significantly lower at 24 and 48 h in mice injected with CP alone than in those which applied WR-2721 and CP (Figure 2E).

G_2/M bone marrow cells

In comparison with the controls, the number of G_2/M apoptotic and non-apoptotic bone marrow cells was significantly increased at 3 and 48 h after WR-2721 application, and at 12 and 96 h after CP administration, whilst in mice given WR-2721 and CP, the percentage of G_2/M cells was significantly increased at 96 h and significantly reduced at 240 h after the injections. The number of cells in the G_2/M phase of the cell cycle was observed to be significantly greater at 12 h in mice treated with CP alone than in those treated with WR-2721and CP (Figure 2F).

Sub-G_1 bone marrow cell population

In relation to the controls, an increased number of sub-G_1 bone marrow cells was found at 6 and 96 h after CP administration, and at 48 and 96 h after treatment with both WR-2721 and CP, and a decreased number of sub-G_1 cells was encountered at 12 h in mice injected with CP only and in those which received both WR-2721 and CP. As compared with mice given WR-2721 and CP, the percentage of cells in sub-G_1 population appeared to be significantly higher at 6 and 96 h in those treated with CP alone (Figure 2G).

Total number of apoptotic cells

In comparison with the untreated control mice, the total number of apoptotic bone marrow cells was found to be significantly increased at 3, 6, 12, 24 and 96 h after CP administration, and at 3, 6 and 96 h after treatment of mice with WR-2721 and CP. The percentage of apoptotic cells was significantly higher at 3 and 96 h in mice that received CP only than in those treated with both WR-2721 and CP (Figure 2H).

Total number of bone marrow cells

As compared with the controls, the total number of bone marrow cells in both femurs appeared to be significantly reduced at 24, 48 and 96 h after WR-2721 and CP treatment, and at 12, 24, 48 and 96 h after CP administration, but significantly increased at 240 h in mice given CP only. In relation to mice treated with both WR-2721 and CP, the total number of bone

marrow cells was significantly decreased at 12 h and significantly increased at 240 h in those which received CP alone (Figure 2I).

Discussion

The results of the present investigations have shown the cell cycle phase specificity of the aminothiol WR-2721 and the alkylating drug CP, in terms of induction of apoptosis, and changes in cell cycle progression caused in the mouse bone marrow by the drug(s). Using LSC analysis of cellular DNA content and the *in situ* detection of DNA strand breaks, temporary alterations in the frequency of apoptotic cells and the number of all bone marrow cells, including apoptotic and non-apoptotic ones, were found in the particular phases of the cell cycle, throughout the 240-h period after WR-2721 and/or CP administration.

CP, a nitrogen mustard analog, is able to form alkylated products with two nucleophilic sites. 4-Hydroxy-CP, acrolein and phosphoramide mustard are the major metabolites of CP that can be considered for its cytotoxic activity. The reactive alkylating agents can bind covalently to a variety of molecules. CP metabolites can react with available groups of amino acids, proteins and peptides, such as NH_2, COOH, SH, and with the primary phosphate, hydroxyl and amino groups of the bases of nucleic acids; nevertheless, the most important site of binding is to DNA. The biological effects of CP are generally considered to originate from damage to cellular DNA.[22]

CP application, at a dose of 200 mg/kg body weight, triggered apoptosis in the mouse bone marrow. On the basis of the presence of DNA strand breaks in apoptotic cells, it was demonstrated that CP induced apoptosis in all phases of the cell cycle. LSC analysis of apoptosis revealed that during the 240-h period, CP had major preference for triggering of apoptotic cell death in G_2/M cells, but was also effective in induction of apoptosis in G_1/G_0 and S phase bone marrow cells. Thus, the pattern of alterations in the number of apoptotic cells appeared to be different in the particular phases of the cell cycle and in the sub-G_1 population. The apoptotic sub-G_1 cell population represented cells, which undergoing apoptosis lost DNA, by shedding apoptotic bodies, which contained the nuclear fragment(s). Temporary alterations in the total number of apoptotic cells, in which detected FITC labeling of DNA strand breaks, reflect fluctuations in the number of apoptotic ones, detected in the particular phases of the cell cycle, and in the sub-G_1 cell population. The similar effect of CP on apoptosis induction, determined by using a microscopic analysis of immunocytochemically stained apoptotic bone marrow cells, was reported by Mazur and Czyzewska.[13]

Following treatment of mice with CP, progression of bone marrow cells through the cell cycle was markedly perturbed. Throughout the 240-h period after CP administration, the frequency of bone marrow cells, including apoptotic and non-apoptotic ones, which DNA stained with PI, fluctuated in all phases of the cell cycle, and especially in the G_2/M and S phases. It is assumed that apoptosis initiated by DNA damage can cause cell cycle deregulation and cell cycle perturbation can lead to apoptotic cell death. The interactions between cell proliferation and cell death appear to be linked to cell cycle checkpoints, which monitor DNA damage.[1,4,5]

WR-2721, an analog of cysteamine, is a phosphorylated aminothiol pro-drug that is dephosphorylated *in vivo* by membrane-bound alkaline phosphatase to its active metabolite WR-1065 (2-/3-aminopropylamino/ethanothiol). It is known, that the free thiol WR-1065 is consequently oxidized to the symmetrical disulphide WR-33278, or mixed disulfides with endogenous thiols or thiol-containing proteins, or can also be metabolized to cysteamine and others sulfide compounds containing SH groups that may have additional protective properties. The hydrogen atom donation by the sulfhydryl groups, and inactivation of the charged carbonium ions of activated alkylating drugs through a nucleophilic attack, are believed to be responsible for the protective action from alkylation.[23–25] However, the metabolic pathways of WR-2721 leading to protection against triggering of apoptosis and cell cycle disturbance by CP, have not yet been clarified.

WR-2721 administration, at a dose of 400 mg/kg body weight, 30 min prior to treatment of mice with CP, resulted in a suppressing action of the aminothiol on induction of apoptosis in bone marrow cells by the alkylating drug. The differences in temporary changes of the number of apoptotic cells observed in the particular phases of the cell cycle, and in sub-G_1 cell population, during the 240-h period, surely resulted from different cell turnover and hematopoietic recovery of the bone marrow cell population occurring in mice which received CP only and in those pre-treated with WR-2721 prior to CP administration. The effects of WR-2721 and CP on 'delayed apoptosis' induced in the erythropoietic system were demonstrated by Mazur and Bławat.[12] The results have shown that WR-2721 can play an important role in the rescue of hematopoietic cells

from CP-induced apoptosis. It is accepted that the active metabolites of WR-2721 are able to modify apoptosis induction, by restoring cellular redox status, depleted by the highly electrophilic metabolites of CP.[26]

The modulatory action of WR-2721 on cell cycle disturbance caused in the mouse bone marrow by CP was shown. The correlation between cell proliferation, apoptotic cell death and cellular redox status is strong, but the mechanisms by which such regulation occur are not well understood.[3,26]

WR-2721 administration without subsequent CP application affected apoptosis induction and cell cycle progression in the mouse bone marrow. Thus, modification of cellular metabolism by the sulfhydryl metabolites of WR-2721, applied alone, can also lead to triggering apoptotic cell death and cell cycle deregulation.

The influence of CP on apoptosis and cell cycle progression ultimately affected the size of the bone marrow cell population. WR-2721 effectively prevented changes in the number of bone marrow cells caused by the alkylating drug. The pattern of changes in the total number of bone marrow cells coincides with the patterns of alterations in the number of leukocytes, granulocytes, lymphocytes and reticulocytes observed in the peripheral blood of mice which received CP alone, and those treated with WR-2721 and CP, respectively.[27]

In conclusion, WR-2721, and especially CP, when given alone, affected apoptotic cell death and cell cycle progression in the mouse bone marrow. WR-2721, applied 30 min prior to CP administration resulted in a suppressing effect on apoptosis and cell cycle disturbance, induced in bone marrow cells, by the alkylating agent. These studies *in vivo* have shown, for the first time, the cell cycle phase specificity of WR-2721 and CP, in terms of triggering of apoptosis in normal bone marrow cells. Taking into consideration the clinical aspects of the present findings, the possibility of diminishing CP-induced apoptosis and cell cycle perturbation in normal hematopoietic cells, using Amifostine, could offer more effective chemotherapy.

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