

In vitro* Effects of New Generation Oxazaphosphorines on Human Promyelocytic Leukemia Cells

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Mafosfamide cyclohexylamine salt (D-17272), 4-hydro-peroxy-cyclophosphamide (D-18864) and glufosfamide (D-19575, β -D-glucose-isophosphoramidate mustard) are new generation oxazaphosphorine agents. The present investigation was undertaken to determine the activity of these three oxazaphosphorines in human promyelocytic leukemia HL-60 cells. The research was conducted using the spectrophotometric MTT assay and the electronic Beckman Coulter and microscopy methods. Functional and morphological changes were observed after exposure of HL-60 cells to the oxazaphosphorine agents. The various patterns of temporary alterations in cell viability, size and count, and also in the frequency of leukemic cells undergoing mitotic catastrophe, apoptosis and necrosis, were shown. Different leukemic cell responses to the action of the three oxazaphosphorines were evaluated. These are the first data comparing the *in vitro* activity of D-17272, D-18864 and D-19575 against human promyelocytic leukemia cells.

Key words: oxazaphosphorines, cell viability, volume and count, mitotic catastrophe, apoptosis, necrosis.

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Oxazaphosphorines represent an important class of anticancer alkylating agents. Cyclophosphamide, ifosfamide and trofosfamide are commonly used in the treatment of a wide variety of solid tumors and hematological malignancies. New generation oxazaphosphorine agents have been developed in an attempt to improve the efficacy of chemotherapy. Mafosfamide cyclohexylamine salt, 4-hydro-peroxy-cyclophosphamide and glufosfamide (Fig. 1) are new oxazaphosphorines. An assessment of the activity of these alkylating agents against pathological cells is of key importance in chemotherapy (STYCZYŃSKI *et al.* 2002a; ZHANG *et al.* 2005a; LIANG *et al.* 2007; JACOB *et al.* 2008; GIRAUD *et al.* 2010; MAZUR *et al.* 2011, 2012a).

Cell viability, mitotic catastrophe, cell death, and cell size and count are accepted important parameters for determining and characterizing the

anticancer activity of various chemotherapeutic agents (ROSS 1976, 1981a, 1981b; KASPERS *et al.* 1995; MAKIN & HICKMAN 2000; BECKER *et al.* 2002; BORST & ROTTENBERG 2004; MANSILLA *et al.* 2006; RICCI & ZONG 2006; DE BRUIN & MEDEMA 2008; SUN & PENG 2009). Nevertheless, available information on the antileukemic potential of the new oxazaphosphorines is scarce. The *in vitro* activity of mafosfamide cyclohexylamine salt, glufosfamide, and 4-hydro-peroxy-cyclophosphamide has only been studied in childhood acute leukemia cells, human lymphoblastoid cells, human histiocytic lymphoma U937 cells, human acute lymphoblastic leukemia MOLT-4 cells, and human acute myeloblastic leukemia ML-1 cells (STYCZYŃSKI *et al.* 2006b; GOLDSTEIN *et al.* 2008; MAZUR *et al.* 2009, 2010, 2012b; OPYDO-CHANEK *et al.* 2010, 2011).

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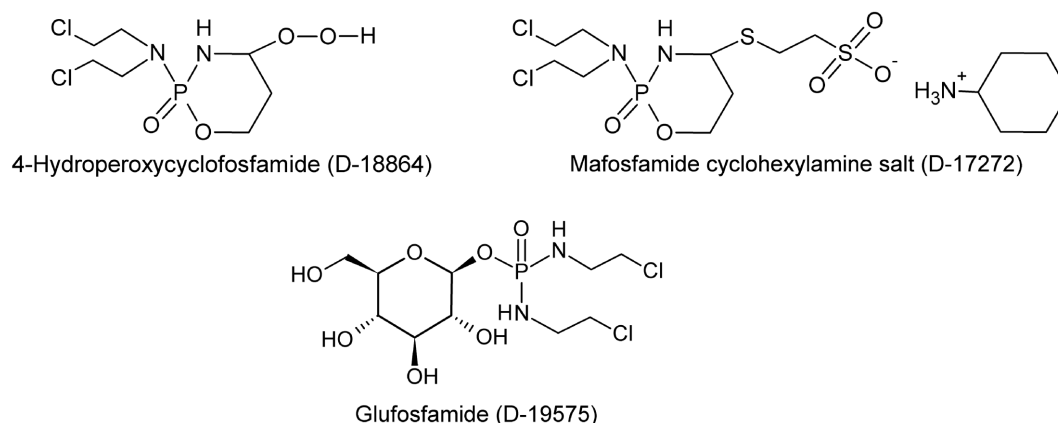


Fig. 1. The chemical structures of the three oxazaphosphorine agents D-18864, D-17272, and D-19575.

The leukemic cell response to the action of the new oxazaphosphorines has not yet been completely explained and is still under investigation.

The aim of the present investigation was to evaluate and compare the *in vitro* activities of mafosfamide cyclohexylamine salt, 4-hydro-peroxy-cyclophosphamide and glufosfamide, against human promyelocytic leukemia HL-60 cells. After oxazaphosphorine application, temporary changes in leukemic cell viability, size and count and also in the frequency of HL-60 cells undergoing mitotic catastrophe, i.e. the process preceding cell death (MANSILLA *et al.* 2006), and two major types of programmed death, apoptosis and necrosis (KAUFMANN & EARNSHAW 2000; MAKIN & HICKMAN 2000; BORST & ROTTENBERG 2004; HENDRIQUEZ *et al.* 2008; SUN & PENG 2009), were analyzed.

Material and Methods

Cells

Human promyelocytic leukemia HL-60 cells (American Type Culture Collection, Rockville, MD, USA) were maintained in RPMI 1640 medium (Gibco BRL Life Technologies), supplemented with 10% fetal calf serum (GIBCO BRL Life Technologies), 2 mM L-glutamine (Sigma Aldrich), and antibiotic antimycotic solution (AAS, Sigma Aldrich). AAS contained 20 units of penicillin, 20 µg streptomycin and 0.05 µg amphotericin B. Every third day, HL-60 cells were passaged. The cells grew at 37°C in an atmosphere of 5% CO₂ in air (HERAcell incubator, KendroLab).

Chemicals

Mafosfamide cyclohexylamine salt (D-17272), 4-hydro-peroxy-cyclophosphamide (D-18864) and

glufosfamide (D-19575, β-D-glucose-isophosphoramidate mustard) were obtained from NIOMECH (Bielefeld, Germany). D-17272, D-18864 and D-19575 were dissolved in *aqua pro injectione* (Polpharma). All solutions were freshly prepared directly before treatment of the leukemic HL-60 cells.

Oxazaphosphorine doses and cell treatment

After a dilution of the cell suspension to a density of 15×10^4 cells/ml medium, HL-60 cells were subjected to a 60 min oxazaphosphorine agent exposure. D-18864 was given at a dose of 10 µg/ml medium, D-19575 at a dose of 100 µg/ml medium, and D-17272 was applied in two doses: 10 µg/ml and 100 µg/ml medium. The control material consisted of untreated HL-60 cells. After a 60 min treatment with the oxazaphosphorine agent, leukemic cells were centrifuged at 1000 rpm (MPW-360R centrifuge, Med. Instruments) for 10 min, and the supernatant was discarded. The cells were then washed in 2 ml of PBS (BioMed) and pelleted by centrifugation for 7 min. The wash and centrifugation were repeated once more and the cells were resuspended in the complete RPMI 1640 medium.

Analyses of HL-60 cells after oxazaphosphorine application

Temporary changes occurring in human promyelocytic leukemia HL-60 cells were determined at 24, 48, and 72 h after cell exposure to the oxazaphosphorine agent. At these three time intervals, the cell viability, volume and count, and also the frequency of cells undergoing mitotic catastrophe, apoptosis and necrosis, were analyzed.

In vitro spectrophotometric MTT assay

In viable, metabolically active cells, the tetrazolium ring of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is cleaved, yielding formazan crystals. Changes in the metabolic activity of cell populations result in a concomitant change in the amount of formazan formed.

MTT (Sigma-Aldrich) was dissolved in RPMI 1640 medium, at a concentration of 5 mg/ml, and filtered through a 0.2 μm filter. 100 μl of the yellow MTT solution was added to each well of a 24-well plate, containing 1 ml of the cell suspension, and the cells were incubated at 37°C with 5% CO₂. The blank solution was prepared according to the above procedure using complete medium without cells. After the three-hour incubation period, the resulting formazan crystals were dissolved with 1 ml of acidified isopropanol (0.05 N HCl in absolute isopropanol). Absorbance of the obtained solution was measured at a wavelength of 570 nm using a Pharmacia Ultrospec III spectrophotometer (Pharmacia). The extent of MTT conversion in cells was also expressed as a percentage value of the control.

Measurement of HL-60 cell size and count

The Beckman Coulter method of cell sizing and counting is based on the detection of an electrical pulse which results from the passage of each cell through an aperture. The amplitude of the produced electrical pulse depends on the cell volume. The number of pulses indicates the cell count.

Samples of HL-60 cell suspension were taken from flasks and immediately diluted in ISOTON II (Beckman Coulter filtered electrolyte solution based on 0.9 % saline). 500 μl of the cell suspension was added to 4.5 ml of ISOTON II. After the dilution of the leukemic cell suspension, individual HL-60 cells were measured using a Z2 Coulter counter (Beckman Coulter, USA). The volume and count distribution of HL-60 cells was obtained using a counter equipped with a 100 μm diameter orifice. The flow rate was 500 μl / 12.5 sec. The range for cell measurement was determined as 268-7346 fL. The cell volume and count was analyzed at 832-7346 fL. The instrument was calibrated using 10 μm diameter latex beads (Beckman Coulter CC size standard). The mean cell volume and the cell count were determined using Z2 Accu-Comp software (Beckman Coulter, USA).

Microscopy analysis of HL-60 cells

HL-60 cell suspension, eventually diluted in HBBS (GIBCO BRL Life Technologies), containing approximately 2×10^5 cells, was added into

a cytospin chamber and centrifuged at 1000 rpm (MPW-350R centrifuge, Med. Instruments) for 6 min, at 4°C. After air drying, the prepared cytopspins were fixed in a mixture of ethanol and acetic acid solution (9:1), at room temperature for 30 min. Immediately before staining, the slides with the fixed cells were rinsed with PBS (BioMed). The cytopspins were stained with a dye solution containing 1 $\mu\text{g/ml}$ of 4'-6'-diamino-2-phenylindole (DAPI) and sulforhodamine 101 (Sigma Aldrich), for 3-5 min. The slides were then rinsed in PBS and the cytopspins were mounted under a coverslip in a drop of PBS.

Three cytopspins from each experimental group, stained with DAPI and sulforhodamine 101, were examined under 400x magnification, using a Jena-val epifluorescent microscope (Carl Zeiss, Germany). Based on the morphology of leukemic cells, the frequency of HL-60 cells undergoing mitotic catastrophe, apoptosis, and necrosis was calculated. The incidence of cells with mitotic catastrophe, apoptotic and necrotic cells among 9000 HL-60 cells (3000 cells per slide) was determined.

The cells undergoing mitotic catastrophe express multiple mitotic abnormalities, such as mis-segregation and condensation of chromosomes, and chromosome alignment defects. Mitotic catastrophe is also characterized by the formation of multinucleated giant cells with uncondensed abnormal nuclei, and also micronuclei (MANSILLA *et al.* 2006; RICCI & ZONG 2006). The features of apoptosis include cell shrinkage, chromatin condensation, nuclear fragmentation, and cell disassembly into apoptotic bodies. Characteristic features of necrosis are cell swelling, complete morphological cell disintegration, membrane rupture and the release of cell content (RAFFRAY & COHEN 1997; PROSKURYAKOV *et al.* 2002; RICCI & ZONG 2006; DE BRUIN & MEDEMA 2008). The morphological criteria described above discriminated between leukemic cells which underwent mitotic catastrophe, apoptosis, and necrosis.

Statistical evaluation

Statistical significance of differences in the amount of formazan formed, the cell volume and count, and in the frequency of cells undergoing mitotic catastrophe, apoptosis, and necrosis, were evaluated by an analysis of variance and Duncan's new multiple range test. A difference with $P < 0.05$ was considered statistically significant. The results were confirmed by three independent experiments carried out in triplicate.

Results

Effects of oxazaphosphorines on HL-60 cells

We determined the influence of new generation oxazaphosphorines D-17272, D-18864 and D-19575 on human promyelocytic leukemia HL-60 cells. The effects of these three oxazaphosphorine agents on cell viability (Table 1, Fig. 2), cell size (Table 2, Fig. 3) and count (Table 3), the induction of mitotic catastrophe (Table 4, Fig. 4), the triggering of apoptosis (Table 5, Fig. 4) and necrosis (Table 6, Fig. 4) were compared.

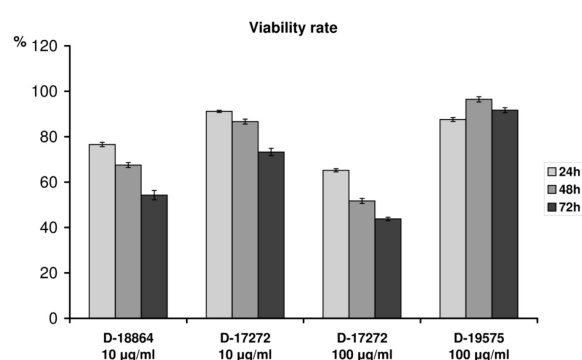


Fig. 2. Effects of oxazaphosphorines on the viability rate of HL-60 cells. The extent of MTT conversion to formazan in HL-60 cells, expressed as a percentage value of the control.

Cell viability

The leukemic cell viability was correlated with the optical density of the formazan solution. In comparison with the controls, the optical density of the formazan solution decreased 24h, 48h, and 72h after treatment of HL-60 cells with D-18864, D-17272, and at 24h and 72h after leukemic cell exposure to the action of D-19575. Among HL-60 cells treated with the oxazaphosphorines, the smallest values of the optical density of the formed formazan were found following D-17272 application at a dose of 100 µg/ml. The values of the optical density of the formazan solution appeared greater when D-17272 was given at a dose of 10 µg/ml medium, as compared with values obtained after D-18864 application at the same dose. Moreover, greater optical density of formazan was observed after treatment of HL-60 cells with D-19575 than when D-17272 was applied at the same dose of 100 µg/ml medium (Table 1). The viability rate, expressed as a percentage value of the control, appeared to be distinctly decreased in HL-60 cells treated with D-18864, and especially in leukemic cells exposed to the action of D-17272 at a dose of 100 µg/ml medium (Fig. 2).

Cell size

In relation to the controls, the mean volume of HL-60 cells increased in all the remaining experi-

Table 1

The optical density of formazan solution determined in HL-60 cells after their exposure to the oxazaphosphorine agents

Group characteristic			Time intervals after cell exposure to oxazaphosphorines		
			24h	48h	72h
No.	Agent given	Agent dose	Optical density of formazan solution ($\times 10^{-2}$)		
			Mean \pm SD	Mean \pm SD	Mean \pm SD
I	D-18864	10 µg/ml	2, 3, 4, 5, 48h, 72h 16.25 \pm 0.97	2, 3, 4, 5, 24h, 72h 26.71 \pm 1.09	2, 3, 4, 5, 24h, 48h 31.91 \pm 2.03
II	D-17272	10 µg/ml	1, 3, 4, 5, 48h, 72h 19.35 \pm 0.48	1, 3, 4, 5, 24h, 72h 34.27 \pm 1.08	1, 3, 4, 5, 24h, 48h 43.08 \pm 1.64
III	D-17272	100 µg/ml	1, 2, 4, 5, 48h, 72h 13.84 \pm 0.69	1, 2, 4, 5, 24h, 72h 20.45 \pm 1.11	1, 2, 4, 5, 24h, 48h 25.75 \pm 0.74
IV	D-19575	100 µg/ml	1, 2, 3, 5, 48h, 72h 18.59 \pm 0.88	1, 2, 3, 24h, 72h 38.16 \pm 1.20	1, 2, 3, 5, 24h, 48h 53.92 \pm 1.09
V	Control		1, 2, 3, 4, 48h, 72h 21.23 \pm 1.31	1, 2, 3, 24h, 72h 39.57 \pm 1.15	1, 2, 3, 4, 24h, 48h 58.82 \pm 1.32

The data are presented as mean values \pm standard deviation.

Statistically significant differences at $P < 0.05$

Differences between experimental groups: different from Group I – 1; Group II – 2; Group III – 3; Group IV – 4; Group V – 5.

Differences within each experimental group: different from 24h – 24h; 48h – 48h; 72h – 72h.

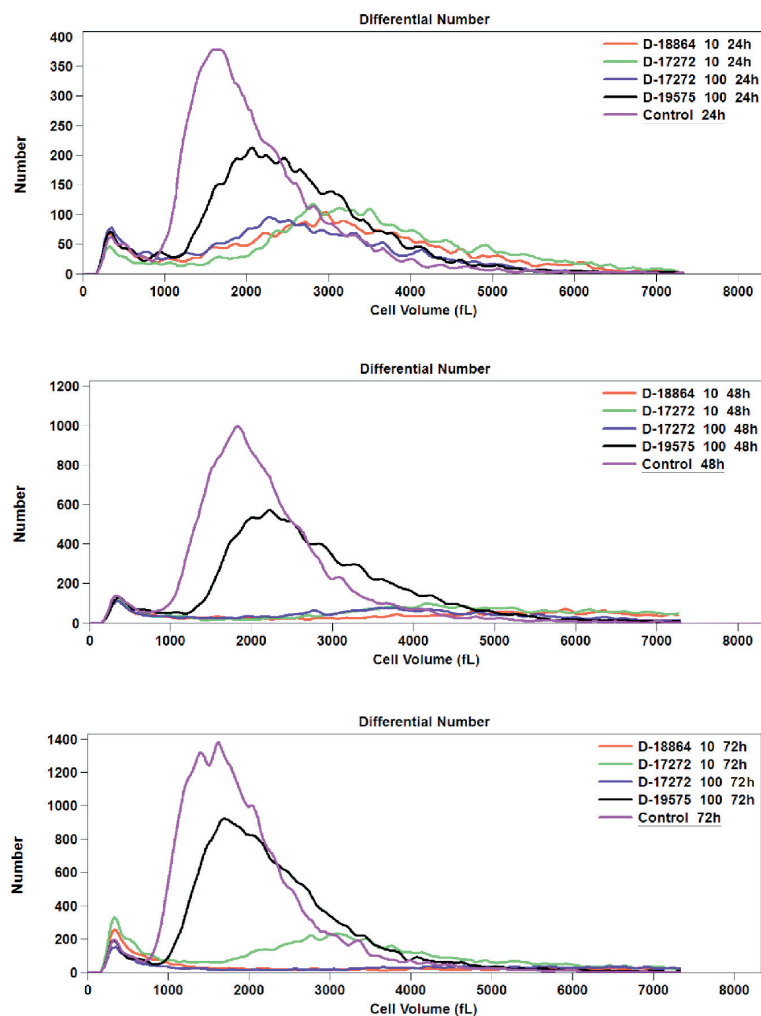


Fig. 3. The mean volume of HL-60 cells after their exposure to the action of three oxazaphosphorine agents. The peaks on the left represent cellular debris, presumably apoptotic bodies and necrotic cell fragments, which were excluded from the analysis of HL-60 cell volume. 10 – the oxazaphosphorine agent applied at a dose of 10 $\mu\text{g/ml}$ medium; 100 – the oxazaphosphorine agent applied at a dose of 100 $\mu\text{g/ml}$ medium.

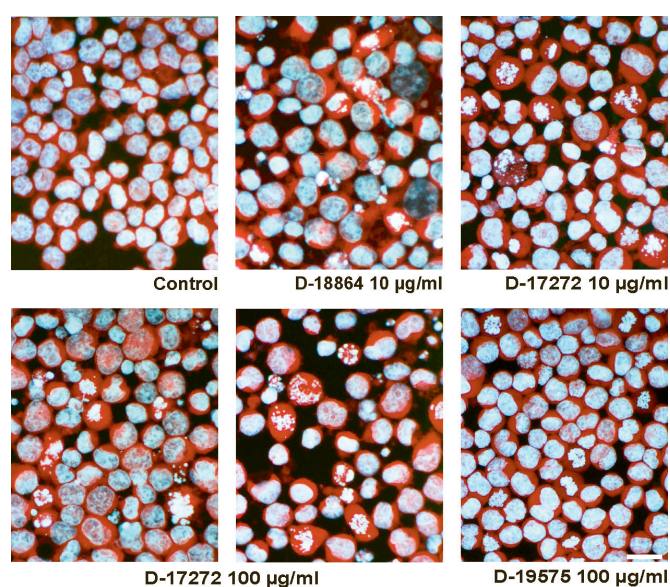


Fig. 4. Effects of oxazaphosphorines on human promyelocytic leukemia HL-60 cells. Morphology of HL-60 cells observed 72h after their exposure to the action of the three oxazaphosphorine agents D-18864, D-17272 and D-19575. The leukemic HL-60 cells, stained with the DNA specific fluorochrome DAPI and the protein fluorochrome sulforhodamine 101, are visible under a Zeiss fluorescence microscope. Hallmarks of mitotic catastrophe, apoptosis, and necrosis were given in the methodology section. Scale bar – 20 μm .

mental groups and at all three time intervals, i.e. 24h, 48h, and 72h after exposure to oxazaphosphorines. Among the oxazaphosphorine tested, the smallest mean volume of HL-60 cells was observed when D-19575 was applied at a dose of 100 $\mu\text{g/ml}$ medium. Larger values of mean cell volume

were found 48h after D-18864 and D-17272 application, at a dose of 10 $\mu\text{g/ml}$ medium, and 72h after leukemic cell exposure to D-17272 at a dose of 100 $\mu\text{g/ml}$ medium, compared to values obtained at the remaining time intervals (Table 2).

Table 2

The mean volume of HL-60 cells after their exposure to oxazaphosphorine agents

Group characteristic			Time intervals after cell exposure to oxazaphosphorines		
			24h	48h	72h
No.	Agent given	Agent dose	Mean Cell Volume (fL)		
			Mean \pm SD	Mean \pm SD	Mean \pm SD
I	D-18864	10 $\mu\text{g/ml}$	2, 3, 4, 5, 48h, 72h 3 246 \pm 73	2, 3, 4, 5, 24h, 72h 4 594 \pm 80	2, 3, 4, 5, 24h, 48h 3 842 \pm 36
II	D-17272	10 $\mu\text{g/ml}$	1, 3, 4, 5, 48h 3 534 \pm 49	1, 3, 4, 5, 24h, 72h 4 473 \pm 63	1, 3, 4, 5, 48h 3 557 \pm 88
III	D-17272	100 $\mu\text{g/ml}$	1, 2, 4, 5, 48h, 72h 2 882 \pm 28	1, 2, 4, 5, 24h, 72h 3 923 \pm 90	1, 2, 4, 5, 24h, 48h 4 381 \pm 87
IV	D-19575	100 $\mu\text{g/ml}$	1, 2, 3, 5, 48h, 72h 2 629 \pm 28	1, 2, 3, 5, 24h, 72h 2 789 \pm 27	1, 2, 3, 5, 24h, 48h 2 333 \pm 28
V	Control		1, 2, 3, 4, 48h, 72h 2 106 \pm 26	1, 2, 3, 4, 24h, 72h 2 227 \pm 15	1, 2, 3, 4, 24h, 48h 1 933 \pm 22

For explanation of signs see Table 1.

Table 3

The count of HL-60 cells after their exposure to oxazaphosphorine agents

Group characteristic			Time intervals after cell exposure to oxazaphosphorines		
			24h	48h	72h
No.	Agent given	Agent dose	Cell count ($\times 10^3$)		
			Mean \pm SD	Mean \pm SD	Mean \pm SD
I	D-18864	10 $\mu\text{g/ml}$	2, 4, 5, 48h, 72h 167.99 \pm 9.31	2, 4, 5, 24h, 72h 150.42 \pm 6.26	2, 4, 5, 24h, 48h 96.50 \pm 4.13
II	D-17272	10 $\mu\text{g/ml}$	1, 3, 4, 5, 72h 194.23 \pm 5.20	1, 3, 4, 5, 72h 208.37 \pm 10.91	1, 3, 4, 5, 24h, 48h 432.26 \pm 8.20
III	D-17272	100 $\mu\text{g/ml}$	2, 4, 5, 72h 158.33 \pm 6.85	2, 4, 5, 72h 151.00 \pm 7.97	2, 4, 5, 24h, 48h 102.68 \pm 7.09
IV	D-19575	100 $\mu\text{g/ml}$	1, 2, 3, 5, 48h, 72h 278.73 \pm 10.92	1, 2, 3, 5, 24h, 72h 637.00 \pm 20.97	1, 2, 3, 5, 24h, 48h 1 036.55 \pm 16.58
V	Control		1, 2, 3, 4, 48h, 72h 345.95 \pm 22.07	1, 2, 3, 4, 24h, 72h 846.75 \pm 17.72	1, 2, 3, 4, 24h, 48h 1 315.65 \pm 35.01

For explanation of signs see Table 1.

Table 4

The frequency of HL-60 cells undergoing mitotic catastrophe after oxazaphosphorine application

Group characteristic			Time intervals after cell exposure to oxazaphosphorines		
			24h	48h	72h
No.	Agent given	Agent dose	Frequency of cells undergoing mitotic catastrophe (%)		
			Mean \pm SD	Mean \pm SD	Mean \pm SD
I	D-18864	10 μ g/ml	2, 3, 5, 48h, 72h 5.74 \pm 0.57	3, 4, 5, 24h, 72h 8.87 \pm 1.70	2, 3, 4, 5, 24h, 48h 12.50 \pm 1.49
II	D-17272	10 μ g/ml	1, 3, 4, 5, 48h, 72h 4.46 \pm 0.51	3, 4, 5, 24h, 72h 9.67 \pm 1.05	1, 3, 4, 5, 24h, 48h 15.12 \pm 1.19
III	D-17272	100 μ g/ml	1, 2, 4, 5, 48h, 72h 3.33 \pm 0.21	1, 2, 5, 24h, 72h 6.42 \pm 1.05	1, 2, 4, 5, 24h, 48h 10.60 \pm 1.04
IV	D-19575	100 μ g/ml	2, 3, 5, 72h 6.21 \pm 0.68	1, 2, 5, 72h 5.47 \pm 0.71	1, 2, 3, 5, 24h, 48h 1036.55 \pm 16.58
V	Control		1, 2, 3, 4 0.92 \pm 0.18	1, 2, 3, 4 1.04 \pm 0.17	1, 2, 3, 4 1.09 \pm 0.27

For explanation of signs see Table 1.

Cell count

In comparison with the controls, the cell count of HL-60 cells decreased in all the remaining experimental groups and at all time intervals (24, 48h, and 72h) after oxazaphosphorine application. Among HL-60 cells, the highest cell number was encountered when D-19575 was given at a dose of 100 μ g/ml medium. After the treatment of HL-60 cells with D-17272 at two doses of 10 μ g/ml and 100 μ g/ml medium, the cell count appeared to be reduced in a dose-dependent manner. D-18864 given at a dose of 10 μ g/ml caused a greater decrease of the leukemic cell number than D-17272 applied at the same dose (Table 3).

Mitotic catastrophe

In comparison with the controls, the yield of mitotic catastrophe increased in all the remaining experimental groups and at all three time intervals (24h, 48h, and 72h) after HL-60 cell exposure to the tested oxazaphosphorines. The frequency of the leukemic cells undergoing mitotic catastrophe increased over time after application of D-18864 and D-17272, and decreased over time when D-19575 was given. The highest yield of mitotic catastrophe was observed 72h after the treatment of HL-60 cells with D-17272 at a dose of 10 μ g/ml medium (Table 4).

Apoptosis

In relation to the controls, the frequency of apoptotic HL-60 cells increased in all the remaining experimental groups and at all three time intervals (24h, 48h, and 72h) after leukemic cell exposure to the oxazaphosphorine agents. Among the oxazaphosphorines tested, the lowest frequency of HL-60 cells undergoing apoptosis was found when D-19575 was applied. The effects of D-17272 on apoptosis-induction in HL-60 cells was dose-dependent. The frequency of apoptotic cells was lower after D-17272 application at a dose of 10 μ g/ml medium than when D-18864 was given at the same dose (Table 5).

Necrosis

In comparison with the controls, a higher frequency of HL-60 cells undergoing necrosis was found in all the remaining experimental groups and at all three time intervals (24h, 48h, and 72h) after the application of oxazaphosphorine. Among the tested oxazaphosphorines, the lowest frequency of necrotic cells was found after exposure of HL-60 cells to the action of D-19575. After treatment of the leukemic cells with D-17272 at two doses of 10 μ g/ml and 100 μ g/ml medium, the yield of necrosis increased in a dose-dependent

Table 5

The frequency of HL-60 cells undergoing apoptosis after oxazaphosphorine application

Group characteristic			Time intervals after cell exposure to oxazaphosphorines		
			24h	48h	72h
No.	Agent given	Agent dose	Frequency of cells undergoing apoptosis (%)		
			Mean \pm SD	Mean \pm SD	Mean \pm SD
I	D-18864	10 μ g/ml	2, 4, 5, 48h, 72h 3.74 \pm 0.95	2, 3, 4, 5, 24h, 72h 4.59 \pm 1.01	2, 4, 5, 24h, 48h 6.04 \pm 1.44
II	D-17272	10 μ g/ml	1, 3, 4, 5 2.56 \pm 0.36	1, 3, 4, 5 2.70 \pm 0.54	1, 3, 4, 5 3.10 \pm 0.78
III	D-17272	100 μ g/ml	2, 4, 5, 72h 3.23 \pm 0.67	1, 2, 4, 5, 72h 3.66 \pm 0.75	2, 4, 5, 24h, 48h 5.53 \pm 0.80
IV	D-19575	100 μ g/ml	1, 2, 3, 5 1.20 \pm 0.29	1, 2, 3, 5 1.32 \pm 0.18	2, 3, 5 1.46 \pm 0.25
V	Control		1, 2, 3, 4 0.54 \pm 0.13	1, 2, 3, 4 0.60 \pm 0.18	2, 3, 4 0.64 \pm 0.19

For explanation of signs see Table 1.

Table 6

The frequency of HL-60 cells undergoing necrosis after oxazaphosphorine application

Group characteristic			Time intervals after cell exposure to oxazaphosphorines		
			24h	48h	72h
No.	Agent given	Agent dose	Frequency of cells undergoing necrosis (%)		
			Mean \pm SD	Mean \pm SD	Mean \pm SD
I	D-18864	10 μ g/ml	2, 4, 5, 48h, 72h 5.31 \pm 1.18	2, 3, 4, 5, 24h 6.74 \pm 1.25	2, 3, 4, 5, 24h 7.57 \pm 1.04
II	D-17272	10 μ g/ml	1, 4, 5, 72h 4.08 \pm 0.32	1, 3, 5 4.33 \pm 1.26	1, 3, 5, 24h 5.18 \pm 1.29
III	D-17272	100 μ g/ml	4, 5, 72h 4.63 \pm 1.03	1, 5, 72h 4.62 \pm 0.95	1, 2, 4, 5, 24h, 48h 6.21 \pm 1.21
IV	D-19575	100 μ g/ml	1, 2, 3, 5, 48h, 72h 2.31 \pm 0.36	1, 5, 24h 3.97 \pm 0.85	1, 3, 5, 24h 4.28 \pm 0.95
V	Control		1, 2, 3, 4 0.87 \pm 0.21	1, 2, 3, 4 0.91 \pm 0.24	1, 2, 3, 4 1.01 \pm 0.20

For explanation of signs see Table 1.

manner. Necrotic death was induced in HL-60 cells to a greater degree when D-18864 was given at a dose of 10 μ g/ml than when D-17272 was applied at the same dose (Table 6).

Discussion

In the present study, the activity of the new generation oxazaphosphorines, D-17272, D-18864 and D-19575, against human promyelocytic leu-

kemia HL-60 cells was evaluated. Various patterns of temporary changes in cell viability, size and count, and in the frequency of cells undergoing mitotic catastrophe and programmed cell death, were found at 24h, 48h and 72h after exposure of HL-60 cells to the action of these three oxazaphosphorine agents. The results obtained in the present investigation and previous studies point to the varied action of D-18864, D-17272, and D-19575 on pathological hematopoietic cells. The damage caused by these alkylating agents in HL-60 cells was manifested as decreased cell viability, different cell volume distribution, reduced cell count, induction of mitotic catastrophe, and triggering apoptotic and necrotic cell death. The decreased viability rate and a reduced count of MOLT-4 cells and ML-1 cells treated with oxazaphosphorines were also observed by OPYDO-CHANEK *et al.* (2011) and MAZUR *et al.* (2012b). The effects of D-18864, D-17272 and D-19575 on mitotic catastrophe-induction in U937 cells was demonstrated by MAZUR *et al.* (2009). The influence of the cyclophosphamide analogue mafosfamide on triggering apoptotic death in human lymphoblastoid cells was reported by GOLDSTEIN *et al.* (2008). The size distribution of MOLT-4 cells, U-937 cells and ML-1 cells exposed to oxazaphosphorines was shown by OPYDO-CHANEK *et al.* (2010, 2011) and MAZUR *et al.* (2012b). The human leukemic cell response to the action of D-17272, D-18864 and D-19575 found in the previous and present investigations appeared to be dependent on the agent tested and its dose, the time intervals after oxazaphosphorine application and the cell line used (MAZUR *et al.* 2009, 2010, 2012b; OPYDO-CHANEK *et al.* 2010, 2011).

The alterations observed in the leukemic cells following their exposure to D-18864, D-17272 and D-19575 surely resulted from abnormal processes occurring at the molecular, biochemical, and cellular levels. The exact mechanisms of action of the three oxazaphosphorine agents are almost entirely unknown (BODY & YULE 2000; ZHANG *et al.* 2005b; LIANG *et al.* 2007). What is known is that D-18864, 4-hydro-peroxy-cyclophosphamide generates its active principle 4-hydroxy-cyclophosphamide (4-OH-CP). D-17272, a mafosfamide cyclohexylamine salt, generates 4-OH-CP. Phosphoramidate mustard and acrolein are the major reactive alkylating agents of the two prodrugs D-18864 and D-17272. β -D-glucose-isophosphoramidate mustard, D-19575, contains the directly reactive alkylating moiety, isophosphoramidate mustard. These reactive alkylating agents are responsible for the cytotoxicity of oxazaphosphorines. The anticancer activity of D-18864, D-17272 and D-19575 is believed to also be dependent on the pharmacokinetic and pharmacody-

namic properties of these alkylating agents due to their different chemical structure (BODY & YULE 2000; ENGEL *et al.* 2000; SEKER *et al.* 2000; ZHANG *et al.*, 2005a, 2005b; LIANG *et al.* 2007; MAZUR *et al.* 2011, 2012a).

In summary, the results of the present study are the first data comparing the potential of the three oxazaphosphorines, D-17272, D-18864, and D-19575, to induce mitotic catastrophe and programmed death, and affect the viability, size and count of human promyelocytic leukemia HL-60 cells. An elucidation of the mechanisms responsible for the various activities of the new generation oxazaphosphorine agents against the different types of pathological hematopoietic cells can provide the rationale for their optional use in chemotherapy.

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