

Comparative Effects of New Generation Oxazaphosphorines on the Size and Viability of Human Acute Myeloblastic Leukemia Cells*

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Mafosfamide cyclohexylamine salt (D-17272), 4-hydro-peroxy-cyclophosphamide (D-18864), and β -D-glucose-isophosphoramidate mustard (D-19575, glufosfamide) are three new generation oxazaphosphorine agents. The aim of the present study was to compare the cell response to the action of these three oxazaphosphorines. The experiments were performed *in vitro* on human acute myeloblastic leukemia ML-1 cells. After exposure of ML-1 cells to the oxazaphosphorines, the size, viability and count of these cells were determined. The research was conducted using the spectrophotometric MTT assay and the electronic Beckman Coulter method. The temporary changes in the ML-1 cell size, viability and count, were dependent on the oxazaphosphorine agent tested, its dose, and the time intervals after its application. Among the three oxazaphosphorine agents, D-18864 proved to be the most cytotoxic, and D-19575 was characterized by the lowest cytotoxicity. The results suggest the possibility of using the electronic sizing and counting method and the MTT assay as a rapid *in vitro* test for assessing leukemic cell sensitivity to the action of new potential chemotherapeutic agents.

Key words: Human leukemic ML-1 cells, oxazaphosphorines, cytotoxicity, cell size, viability, and count.

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Oxazaphosphorines are a class of alkylating agents, widely used in chemotherapy. However, to improve the therapeutic index of this class of chemotherapeutic agents, new generation oxazaphosphorines have been developed. Mafosfamide cyclohexylamine salt (D-17272), 4-hydro-peroxy-cyclophosphamide (D-18864), and β -D-glucose-isophosphoramidate mustard (D-19575, glufosfamide) are new oxazaphosphorine derivatives. The cell response to the action of these alkylating compounds is still under experimental and clinical investigation (BODY and YULE 2000; ENGEL *et al.* 2000; SEKER *et al.* 2000; BLANEY *et al.* 2005; ZHANG *et al.* 2005a, 2005b;

LIANG *et al.* 2007; GIRAUD *et al.* 2010; MAZUR *et al.* 2011).

The influence of the new generation oxazaphosphorines on pathological hematopoietic cells is not completely known yet. Available information on the cytotoxic activity of D-17272, D-18864, and D-19575 in human leukemic cells is still scarce (STYCZYŃSKI *et al.* 2000a, 2000b; GOLDSTEIN *et al.* 2008; MAZUR *et al.* 2009, 2010; OPYDO-CHANEK *et al.* 2010a, 2010b).

Therefore, the objective of the present study was to evaluate *in vitro* cytotoxicity of three new oxazaphosphorine agents on human acute myelo-

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blastic leukemia ML-1 cells. Temporary changes in the size, viability and count of ML-1 cells subjected to the exposure of D-17272, D-18864, and D-19575, were analyzed using the electronic Beckman Coulter method and the MTT assay.

Material and Methods

Cells

Human acute myeloblastic leukemia ML-1 cells (European Collection of Cell Culture, ECACC, Sigma Aldrich, Catalogue No. 88113007) were maintained in RPMI 1640 (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. Leukemic ML-1 cells were passaged every third day. The cells grew exponentially at 37°C in an atmosphere of 5% CO₂ in air (HERAcell incubator, KendroLab). The cultures were periodically tested for *Mycoplasma* infection.

Chemicals

Mafosfamide cyclohexylamine salt (D-17272, CAS No. 84210-80-0), 4-hydro-peroxy-cyclophosphamide (D-18864, CAS No. 39800-16-3) and glufosfamide (D-19575, β -D-glucose-isophosphoramidate mustard, CAS No. 132682-98-5) 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 ML-1 cells.

Agent doses and cell treatment

After a dilution of the cell suspension to a density of 15×10^4 cells/ml medium, ML-1 cells were exposed for 30 min to the oxazaphosphorine agent. D-18864 was applied at a dose of 10 $\mu\text{g}/\text{ml}$ medium, D-19575 at a dose of 100 $\mu\text{g}/\text{ml}$ medium, and D-17272 was given at doses of 10 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$ medium. The control material consisted of untreated ML-1 cells. After 30 min treatment with the oxazaphosphorine agent, leukemic ML-1 cells were centrifuged for 10 min at 1000 rpm and the supernatant was discarded. Then the cells were 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 resuspended in the complete RPMI 1640 medium.

ML-1 cell analysis

Temporary alterations occurring in human leukemic ML-1 cells were assessed at 24h, 48h, and 72h after the cell exposure to the oxazaphosphorine agent. At these three time intervals, the size, viability and count of ML-1 cells, were determined.

Beckman Coulter method of cell sizing and counting

The electronic Beckman Coulter method was applied to determine ML-1 cell size and count. The Beckman Coulter method of cell sizing and counting is based on the detection and measurement of changes in electrical resistance produced by a cell suspended in a conductive liquid traversing through a small aperture. The passage of each individual cell momentarily modulates the impedance of the electrical path between two electrodes located on each side of the aperture. An electrical pulse, suitable for sizing and counting, results from the passage of each cell through the aperture. While the amplitude of the produced electrical pulse depends on the cell volume, the number of pulses indicates the cell count.

Samples of ML-1 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 ML-1 cell suspension, individual leukemic cells were measured using the Z2 Coulter counter (Beckman Coulter, USA). The count and volume distributions of ML-1 cells were obtained using the counter equipped with a 100 μm diameter orifice. The flow rate was 500 $\mu\text{l} / 12.5$ sec. The range for cell measurement was determined as 162-10 370 fL. The cell volume and count was analyzed at 851-10 370 fL. The instrument was calibrated using 10 μm diameter latex beads (Beckman Coulter CC size standard). The mean cell volume, modal peak and the cell count, were determined using Z2 AccuComp software (Beckman Coulter, USA).

MTT viability assay

The *in vitro* MTT test was designed for spectrophotometrically determining the viability of cell populations. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is the key component used in the MTT assay. In viable, metabolically active cells, the tetrazolium ring 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. The cells were then incubated at 37°C with 5% CO₂. A 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), and 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.

Statistical evaluation

Statistical significance of differences in the mean cell volume and the mean amount of formazan formed as well as in the mean cell count, were evaluated by an analysis of variance and the Duncan's new multiple range test.

Results

Effects of oxazaphosphorines on ML-1 cells

The influence of three new generation oxazaphosphorines on human acute myeloblastic

leukemia ML-1 cells, was determined. The effects of D-18864, D-17272, and D-19575, on the leukemic cell size (Table 1 and Table 2, Fig. 1), and their viability (Table 3, Fig. 2) and count (Table 4), observed at 24h, 48h, and 72h after the oxazaphosphorine agent application, were compared.

Mean volume of ML-1 cells (Table 1, Fig. 1)

In comparison with the controls, the mean volume of ML-1 cells was increased in all the experimental groups and at all three time intervals 24h, 48h, and 72h after the leukemic cell exposure to the action of the oxazaphosphorine agents. Among ML-1 cells treated with the oxazaphosphorine agents, the greatest mean volume was obtained when D-17272 was given at a higher dose of 100 $\mu\text{g}/\text{ml}$ medium, and the smallest mean volume of the leukemic cells was observed after D-19575 application, at the same dose. The increased mean volume of ML-1 cells exposed to D-17272 at two doses, 10 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$ medium, appeared to be dose-dependent. The mean volume was found to be smaller in ML-1 cells treated with D-17272, at a dose of 10 $\mu\text{g}/\text{ml}$ medium, than in those exposed to D-18864 at the same dose.

Modal peak of the volume distribution curve (Table 2, Fig. 1)

In relation to the controls, greater values in the modal peaks of the volume distribution curves were found in ML-1 cells exposed to the action of the oxazaphosphorines, except for the value obtained

Table 1

The mean volume of ML-1 cells after their exposure to the oxazaphosphorine agents

Group characteristic			Mean cell volume (fL)		
			24h	48h	72h
No.	agent given	agent dose	mean \pm SD	mean \pm SD	mean \pm SD
I	D-18864	10 $\mu\text{g}/\text{ml}$	2,3,4,5,48h,72h 3729 \pm 75	2,3,4,5,24h,72h 5114 \pm 74	2,3,4,5,24h,48h 3926 \pm 89
II	D-17272	10 $\mu\text{g}/\text{ml}$	1,3,4,5,48h,72h 3385 \pm 51	1,3,4,5,24h,72h 2961 \pm 85	1,3,4,5,24h,48h 2440 \pm 49
III	D-17272	100 $\mu\text{g}/\text{ml}$	1,2,4,5,48h,72h 3902 \pm 62	1,2,4,5,24h,72h 6033 \pm 87	1,2,4,5,24h,48h 5238 \pm 67
IV	D-19575	100 $\mu\text{g}/\text{ml}$	1,2,3,5,48h,72h 2629 \pm 53	1,2,3,5,24h,72h 2819 \pm 72	1,2,3,5,24h,48h 2322 \pm 90
V	control		1,2,3,4,72h 2290 \pm 37	1,2,3,4,72h 2309 \pm 96	1,2,3,4,24h,48h 2199 \pm 91

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

Statistically significant differences at $P < 0.05$

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

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

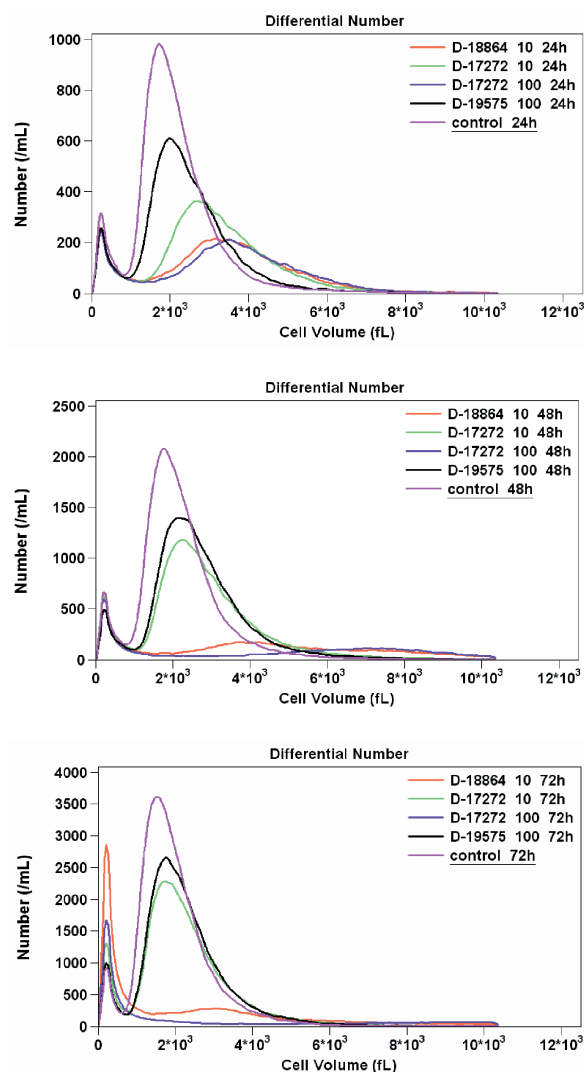


Fig. 1. The volume of ML-1 cells exposed to the oxazaphosphorines. The volume distribution curves of ML-1 cells analyzed with the Beckman Coulter counter. The peaks on the left represent cellular debris, presumably apoptotic bodies and necrotic cell fragments, which were excluded from the analysis of the ML-1 cell volume.

at 72h after D-17272 application at a higher dose of 100 $\mu\text{g}/\text{ml}$ medium. The patterns of temporary changes in the modal peak of the volume distribution curve are correlated with those of the mean volume of ML-1 cells.

ML-1 cell viability (Table 3, Fig. 2)

The cell viability is correlated with the optical density of formazan solution. Compared with the controls, the optical density of formazan solution decreased 24h, 48h, and 72h after the treatment of ML-1 cells with D-18864, D-17272, and at 24h and 72h after their exposure to the action of D-19575. Among ML-1 cells treated with the oxazaphosphorine agents, the values of the optical density of formazan solution were found to be greater following D-19575 application at a dose of 100 $\mu\text{g}/\text{ml}$, and when D-17272 was given at a dose of 10 $\mu\text{g}/\text{ml}$ medium, and smaller values were obtained when D-18864 was applied at a dose of 10 $\mu\text{g}/\text{ml}$ and D-17272 at a dose of 100 $\mu\text{g}/\text{ml}$ medium.

The viability rate indicating the extent of MTT conversion to formazan, and expressed as the percentage values of the controls, appeared to be distinctly reduced in ML-1 cells exposed to D-18864 at a dose of 10 $\mu\text{g}/\text{ml}$ and especially in those treated with D-17272 at a dose of 100 $\mu\text{g}/\text{ml}$ medium.

ML-1 cell count (Table 4)

In comparison with the controls, the cell count of ML-1 cells decreased in all experimental groups and in all three time intervals at 24h, 48h, and 72h after the leukemic cell exposure to the action of the oxazaphosphorine agents. Among ML-1 cells treated with the oxazaphosphorine agents, the smallest cell number was found when D-17272 was given at a higher dose of 100 $\mu\text{g}/\text{ml}$ medium, and the greatest number of the leukemic cells was encountered after D-19575 application, at the same dose. A reduced

Table 2

The modal peaks of the volume distribution curves of ML-1 cells exposed to the action of D-17272, D-18864, and D-19575

Group characteristic			Modal peak (fL)		
No.	agent given	agent dose	24h	48h	72h
I	D-18864	10 $\mu\text{g}/\text{ml}$	3181	3628	3222
II	D-17272	10 $\mu\text{g}/\text{ml}$	2735	2290	1925
III	D-17272	100 $\mu\text{g}/\text{ml}$	3506	7437	871
IV	D-19575	100 $\mu\text{g}/\text{ml}$	1965	2168	1804
V	control		1682	1804	1601

Table 3

The optical density of formazan solution determined after ML-1 cell exposure to the oxazaphosphorine agents

Group characteristic			Optical density of formazan solution ($\times 10^{-1}$)		
			24h	48h	72h
No.	agent given	agent dose	mean \pm SD	mean \pm SD	mean \pm SD
I	D-18864	10 $\mu\text{g/ml}$	2,4,5,48h,72h 2.13 \pm 0.08	2,3,4,5,24h 3.16 \pm 0.07	2,3,4,5,24h 3.09 \pm 0.09
II	D-17272	10 $\mu\text{g/ml}$	1,3,5,48h,72h 2.26 \pm 0.13	1,3,5,24h,72h 4.24 \pm 0.14	1,3,4,5,24h,48h 4.92 \pm 0.25
III	D-17272	100 $\mu\text{g/ml}$	2,4,5,48h,72h 2.13 \pm 0.05	1,2,4,5,24h 2.92 \pm 0.06	1,2,4,5,24h 2.86 \pm 0.10
IV	D-19575	100 $\mu\text{g/ml}$	1,3,5,48h,72h 2.45 \pm 0.14	1,3,24h,72h 4.27 \pm 0.15	1,2,3,5,24h,48h 5.13 \pm 0.12
V	control		1,2,3,4,48h,72h 2.57 \pm 0.07	1,2,3,24h,72h 4.33 \pm 0.17	1,2,3,4,24h,48h 5.49 \pm 0.09

For explanation of signs, see Table 1.

Table 4

The count of ML-1 cells after their exposure to the oxazaphosphorine agents

Group characteristic			Cell count ($\times 10^3$)		
			24h	48h	72h
No.	agent given	agent dose	mean \pm SD	mean \pm SD	mean \pm SD
I	D-18864	10 $\mu\text{g/ml}$	2,4,5,48h,72h 169.99 \pm 5.06	2,3,4,5,24h,72h 221.18 \pm 5.75	2,3,4,5,24h,48h 300.76 \pm 8.18
II	D-17272	10 $\mu\text{g/ml}$	1,3,4,5,48h,72h 225.88 \pm 6.40	1,3,4,5,24h,72h 632.50 \pm 12.97	1,3,4,5,24h,48h 1049.91 \pm 14.08
III	D-17272	100 $\mu\text{g/ml}$	2,4,5,72h 159.03 \pm 3.83	1,2,4,5,72h 155.59 \pm 5.86	1,2,4,5,24h,48h 134.62 \pm 4.57
IV	D-19575	100 $\mu\text{g/ml}$	1,2,3,5,48h,72h 281.39 \pm 10.50	1,2,3,5,24h,72h 704.49 \pm 38.92	1,2,3,5,24h,48h 1150.73 \pm 79.70
V	control		1,2,3,4,48h,72h 374.63 \pm 14.07	1,2,3,4,24h,72h 818.78 \pm 63.04	1,2,3,4,24h,48h 1373.67 \pm 15.68

For explanation of signs, see Table 1.

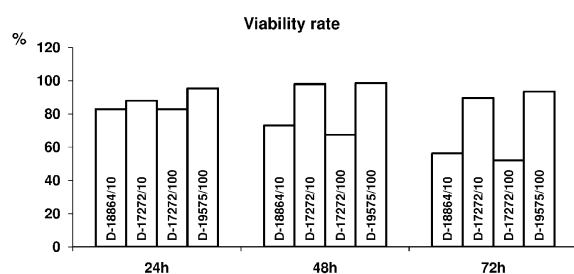


Fig. 2. Effects of the oxazaphosphorines on the viability rate of ML-1 cells. The extent of MTT conversion to formazan in ML-1 cells expressed as a percentage value of the control. D-18864/10 - D-18864 applied at a dose of 10 $\mu\text{g/ml}$ medium; D-17272/10 - D-17272 applied at a dose of 10 $\mu\text{g/ml}$ medium; D-17272/100 - D-17272 applied at a dose of 100 $\mu\text{g/ml}$ medium; D-19575/100 - D-19575 applied at a dose of 100 $\mu\text{g/ml}$ medium.

count of ML-1 cells exposed to D-17272 at two doses, 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ medium, appeared to be dose-dependent. The cell number was observed to be greater in ML-1 cells treated with D-17272, at a dose of 10 $\mu\text{g/ml}$ medium, than in those exposed to D-18864 at the same dose.

Discussion

The results of the present study have shown the human acute myeloblastic leukemia ML-1 cell response to the action of three new generation oxazaphosphorines D-17272, D-18864, and D-19575. Temporary alterations in the size, viability and count of ML-1 cells, observed 24h, 48h and 72h

after their exposure to the oxazaphosphorines agents, were dependent on the agent tested, its dose, and the time intervals after its application. Among the oxazaphosphorine agents, D-18864 appeared to be the most cytotoxic, and D-19575 was characterized by the lowest cytotoxicity. The variable leukemic cell responses to the action of the oxazaphosphorines, were found.

The cytotoxic effects of the oxazaphosphorines, D-17272, D-18864, and D-19575, surely resulted from their various action on human leukemic ML-1 cells. However, the precise mechanisms responsible for cytotoxicity of these three oxazaphosphorine agents on pathological hematopoietic cells have not yet been completely elucidated. It is accepted that phosphoramidate mustard and acrolein are the major reactive alkylating agents of D-17272 and D-18864. D-19575 contains the direct alkylating moiety, isophosphoramidate mustard. The reactive alkylating compounds are involved in the toxicologic reactions because they can bind to a variety of cellular molecules disturbing the normal processes occurring in the cell. Moreover, the cytotoxicity of these oxazaphosphorines is dependent on their pharmacokinetic and pharmacodynamic properties (BODY and YULE 2000; ZHANG *et al.*, 2005a, 2005b; LIANG *et al.* 2007; GIRAUD *et al.* 2010; MAZUR *et al.* 2011).

The changes in the metabolic activity and cell viability, the volume and count of leukemic ML-1 cells following D-17272, D-18864, and D-19575 application can result from various alterations occurring in these cells at the molecular, biochemical and morphological levels. In the previous publication it has been shown that damage caused by the oxazaphosphorine agents in the hematopoietic cells was manifested as DNA breakage, cell cycle disruption, cell proliferation inhibition, mitotic catastrophe and programmed cell death – induction (MAZUR 2002; MAZUR *et al.* 2009, 2010; OPYDO-CHANEK *et al.* 2010a, 2010b).

To summarize, the results of the present investigations indicated that three analyzed parameters, cell volume, viability, and cell count are important in determining and comparing cytotoxic effects observed in human leukemic cells after their exposure to the new generation oxazaphosphorines. In conclusion, the electronic sizing and counting method and MTT assay can be used to study the anticancer activity of newly synthesized and tested, potential chemotherapeutic agents.

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