In vitro cytotoxicity testing of new generation oxazaphosphorines against human histiocytic lymphoma cells

Małgorzata Opydo-Chanek, Lidia Mazur* & Marta Stojak
Department of Experimental Hematology, Jagiellonian University, Gronostajowa 9, 30-387 Cracow, Poland

Received 26 March 2013

Oxazaphosphorines belong to a group of alkylating agents. Mafosfamide cyclohexylamine salt (D-17272), 4-hydro-peroxy-cyclophosphamide (D-18864) and glufosfamide (D-19575, β-D-glucose-isophosphoramide mustard) are new generation oxazaphosphorines. The objective of the present study was to compare the cytotoxic action of these oxazaphosphorine compounds against human histiocytic lymphoma U937 cells. The chemical structures of the oxazaphosphorines were responsible for the different responses of U937 cells. The cytotoxic effects of D-17272, D-18864, and D-19575 on U937 cells depended on the agent tested, its dose, and the time intervals after the oxazaphosphorine application. Among the oxazaphosphorine agents, D-18864 appeared to be the most cytotoxic, and D-19575 was characterized by the lowest cytotoxicity. The in vitro cytotoxic activities of the oxazaphosphorines were strongly associated with their cell death inducing potential.

Keywords: Apoptosis and necrosis, Cell count, Cell viability, Cytotoxicity, Mitochondrial membrane potential, Oxazaphosphorines, U937 cells

The nitrogen mustard derivatives were introduced into the clinic in the second half of the 20th century. Cyclophosphamide, the most successful nitrogen mustard analog, and its isomer ifosfamide, belong to a class of anticancer drugs, known as oxazaphosphorines. These alkylating agents are widely used for treating a variety of human malignancies. Nevertheless, the design and development of new oxazaphosphorines as potential chemotherapeutic agents are of key importance for enhancing therapeutic efficacy. New oxazaphosphorine analogs such as mafosfamide cyclohexylamine salt (D-17272), 4-hydro-peroxy-cyclophosphamide (D-18864) and glufosfamide (D-19575, β-D-glucose-isophosphoramide mustard) have been synthesized and tested. However, information concerning the anticancer potential of new oxazaphosphorine compounds is still scarce.

Chemotherapeutic agents exert their cytotoxic effects on pathological cells by disturbing the cellular processes, and by triggering cell death. In recent years, the cell death inducing potential of anticancer agents has become a topic of interest. Apoptosis and necrosis are two distinct types of programmed cell death occurring during chemotherapy. A wide range of anticancer drugs can affect cell viability and induce programmed death of pathological cells by triggering mitochondrial pathways.

The mode of action of newly synthesized oxazaphosphorines on pathological hematopoietic cells is not yet completely known. Thus, the objective of the present study is to assess and compare the in vitro cytotoxic activity of the three oxazaphosphorines, D-17272, D-18864, and D-19575 against human histiocytic lymphoma U937 cells. The cell viability and mitochondrial membrane potential (ΔΨm), the yield of apoptosis and necrosis, and the cell count, have been analyzed.

Materials and Methods

Cells—Human histiocytic lymphoma U937 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. U937 cells were passaged every third day. The cells grew...
exponentially at 37 °C in an atmosphere of 5% CO₂ in air (HERAcell incubator, KendroLab).

**Oxazaphosphorine agents**—Mafosfamide cyclohexylamine salt (D-17272), 4-hydro-peroxy-cyclophosphamide (D-18864) and glufosfamide (β-D-glucose-isophosphoramid mustard, D-19575) were obtained from NIOMECH (Bielefeld, Germany). D-17272, D-18864 and D-19575 were dissolved in aqua pro injectione (Polpharma, Poland). All solutions were freshly prepared directly before treatment of U937 cells.

**Agent doses and cell treatment**—After a dilution of the cell suspension to a density of 15×10⁷ cells/mL, U937 cells were exposed for 30 min to the oxazaphosphorine agent. D-18864 was given at a dose of 5 µg/mL medium, D-19575 at a dose of 50 µg/mL medium, and D-17272 was applied at two doses of 5 µg/mL and 50 µg/mL medium. The control material consisted of untreated U937 cells. After 30 min treatment with the oxazaphosphorine agent, U937 cells were centrifuged (MPW-360R centrifuge, Med. Instruments) for 10 min at 1000 rpm and the supernatant was discarded. The cells were washed in 2 mL of PBS (BioMed) and pelletted by centrifugation. The wash and centrifugation were repeated once more and the cells resuspended in the complete RPMI 1640 medium.

**Analyses of U937 cells after the oxazaphosphorine application**—Temporary changes occurring in the amount of formazan formed, mitochondrial membrane potential, the yield of apoptosis and necrosis, and the cell count, were determined 24h, 48h and 72h after the exposure of U937 cells to the action of oxazaphosphorine agents.

**In vitro spectrophotometric MTT assay**—4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma Aldrich) was dissolved in the complete RPMI 1640 medium, at a concentration of 5 mg/mL, and filtered through a 0.2 µm filter. MTT solution (200 µL) was added to each well of a 12-well plate containing 2 mL of the cell suspension, and the cells were incubated at 37 °C with 5% CO₂. The blank solution was prepared according to the procedure described above, using the complete RPMI 1640 medium without U937 cells. After 2h incubation period, the resulting formazan crystals were dissolved with 2 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).

**Flow cytometry analysis of mitochondrial membrane potential**—JC-1 (5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazol-carboxyanine iodide) was stored as a stock solution in dimethyl sulfoxide (DMSO, Sigma Aldrich) at a concentration of 1 mg/mL. A final concentration of JC-1 staining solution was 10 µg/mL. The U937 cell suspension, containing 5×10⁷ cells/mL, was centrifuged for 7 min at 1000 rpm. The cells were washed twice in 2 mL of HBSS (GIBCO BRL Life Technologies), and resuspended in 990 µL of the complete RPMI 1640 medium. Then, 10 µL of JC-1 staining solution was added, and the cells were incubated at 37 °C in the dark. After the 15 min incubation period, the cells were centrifuged for 7 min at 1000 rpm, and washed twice in 2 mL of HBSS. Finally, the supernatant was discarded and the cells were resuspended in 500 µL of HBSS. The fluorescence of JC-1 was analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Red emission of JC-1 aggregates was detected in fluorescence channel 2 (FL2) and green emission of JC-1 monomers in fluorescence channel 1 (FL1). The fluorescence emission shift of JC-1 from red to green was analyzed using CellQuest Pro software (Becton Dickinson).

**Flow cytometry Annexin V-FITC / PI assay**—Dual staining of U937 cells with fluoresceinated annexin V (annexin V-FITC) and propidium iodide (PI) was performed according to the manufacturer’s instruction (Calbiochem). Briefly, the cell suspension, containing 5×10⁷ cells, was centrifuged for 5 min at 1000 rpm, at room temperature. The supernatant was discarded and the cells were resuspended in 500 µL of cold PBS. After the centrifugation, the cells were resuspended in 500 µL of cold binding buffer. Then 1.25 µL of annexin V-FITC staining solution was added, and the cells were incubated in the dark for 15 min, at room temperature. Following the incubation, the cells were centrifuged for 5 min at 1000 rpm and the supernatant was discarded. Cell pellet was resuspended in 500 µL of cold binding buffer, and 10 µL of PI solution, at a concentration of 30 µg/mL, was added. Cell samples were placed on ice, away from light, and FITC and PI fluorescence was immediately measured using FACSCalibur flow cytometer (Becton Dickinson). The frequency of live cells, early apoptotic cells, and also the frequency of late apoptotic and necrotic cells, were determined using CellQuest Pro software (Becton Dickinson).
**Light microscopy observation**—U937 cell suspension, eventually diluted in PBS, containing approximately 2×10^5 cells, was added into a cytopsin chamber and cytocentrifuged for 6 min at 1000 rpm, at 4 °C. After air drying, the prepared cytopsins were fixed in methanol for 15 min, at room temperature. The Hemacolor set was used to stain U937 cells, according to the manufacturer’s instruction (Merck Chemicals). Morphological observations were carried out under a light microscope at a magnification of 1000x.

**Electronic cell counting**—The cell count was determined using a Z2 Coulter counter (Beckman Coulter, USA). Samples of U937 cell suspension were taken from flasks and immediately diluted in ISOTON II (Beckman Coulter filtered electrolyte solution based on 0.9 % saline). Cell suspension (500 µL) was added to 4.5 mL of ISOTON II. After the dilution of the cell suspension, the cell count was measured. The cell counter was 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 count was analyzed at 775–7346 fL using Z2 AccuComp software (Beckman Coulter, USA).

**Statistical evaluation**—The results were confirmed by three independent experiments carried out in duplicate or triplicate. All the data obtained, the amount of formazan formed which was expressed as a percentage value of the control, the yield of the cells expressing high and low mitochondrial membrane potential, the frequency of live cells and those undergoing early apoptosis, late apoptosis and necrosis, and also the cell count, are presented as the mean ± SD. The statistical significance for the data was evaluated by an analysis of variance and Duncan’s new multiple range test. \( P < 0.05 \) were considered statistically significant.

**Results**

**Effects of oxazaphosphorines on U937 cells**—The effects of D-17272, D-18864, and D-19575 on human histiocytic lymphoma U937 cells were evaluated. The influence of the three oxazaphosphorine agents on the cell viability (Fig. 1) and mitochondrial membrane potential (Fig. 2), the frequency of cells undergoing apoptosis and necrosis (Figs 3 and 4), and the cell count (Fig. 5) was found.

**Cell viability (Fig. 1)**—The influence of the oxazaphosphorines on the viability rate of U937 cells was shown. In comparison with the controls, the cell viability rate decreased 24, 48, and 72 h after D-18864 and D-17272 application, and increased only 24 h after the treatment of U937 cells with D-19575. The values of the cell viability rate appeared to be greater when D-17272 was given at a dose of 5 µg/mL medium, as compared with the values obtained after D-18864 application, at the same dose. The cell viability decreased in a dose-dependent manner in U937 cells, at 48 and 72 h after their treatment with D-17272, at two doses of 5 and 50 µg/mL medium. Moreover, a greater cell viability rate was found after the treatment of U937 cells with D-19575 than when D-17272 was applied at the same dose of 50 µg/mL medium.

**Mitochondrial membrane potential (Fig. 2)**—Among the three oxazaphosphorines, only the two agents, D-18864 and D-17272, distinctly reduced mitochondrial membrane potential of U937 cells. In relation to the controls, the values of \( \Delta \psi_m \) decreased 24, 48, and 72 h after the exposure of U937 cells to the action of D-18864 and D-17272. The mitochondrial membrane potential of U937 cells was affected to a greater extent after the application of D-18864 at a dose of 5 µg/mL medium than when D-17272 was given at the same dose. The values of \( \Delta \psi_m \) decreased in U937 cells, in a dose-dependent manner, after their exposure to the action of D-17272.

**Apoptosis and necrosis—induction (Fig. 3)**—It was observed that the three oxazaphosphorines triggered apoptosis and necrosis in U937 cells. After the application of D-18864, D-17272, and D-19575, the
Fig. 2—The effects of the oxazaphosphorine agents on the mitochondrial membrane potential of U937 cells. [(A) Representative dot plot of JC-1 green (FL-1) and red (FL-2) fluorescence. Gate R1 indicates cells with high Δψm and gate R2 – cells with low Δψm. (B) Frequency of U937 cells with high and low Δψm. The mean values of Δψm were determined by three independent experiments performed in duplicate. Values not significantly different at P<0.05 according to Duncan’s test: *, **, between groups treated with the oxazaphosphorine agents, + between the time points, # compared to the control].

Fig. 3—The effects of the oxazaphosphorine agents on apoptosis and necrosis – induction in U937 cells, were determined using the flow cytometry fluoresceinated annexin V / propidium iodide (annexin V-FITC / PI) assay. (A) Representative dot plot of U937 cells stained with a combination of fluoresceinated annexin V and propidium iodide. (B-D) The frequency of live cells (annexin V-FITC negative / PI negative, B), early apoptotic cells (annexin V-FITC positive / PI negative, C) and late apoptotic and necrotic cells (annexin V-FITC positive / PI positive, D) [Values are mean ± SD for duplicate measurements from three independent experiments. Values not significantly different at P<0.05 according to Duncan’s test: *, +, between the groups of U937 cells treated with the oxazaphosphorine agents, # between the time points, * compared to the control].
Fig. 4—Morphology of the control U937 cells (A) and those exposed to the action of D-17272, at a dose of 50 µg/mL medium. U937 cells were observed under a light microscope (original magnification: 1000x). Hemacolor was used to stain the cells. (B) One apoptotic U937 cell expressing cellular shrinkage, chromatin condensation and nuclear fragmentation (long arrow). (C) Three necrotic U937 cells with characteristic features including cell swelling and morphological cell disintegration (arrowhead).

Fig. 5—The count of U937 cells exposed to the action of the three oxazaphosphorine agents, D-18864, D-17272, and D-19575, was determined using the electronic Beckman Coulter method. The number of U937 cells was calculated per 1 mL medium. [Values are mean ± SD for triplicate measurements from three independent experiments. Values not significantly different at P<0.05 according to Duncan’s test: * between the groups of U937 cells treated with the oxazaphosphorine agents, # compared to the control].

frequency of both live cells (Fig. 3B) and early apoptotic cells (Fig. 3C), and the frequency of late apoptotic and necrotic cells (Fig. 3D), were assessed.

Live cells (Fig. 3B)—Compared to the controls, the frequency of live U937 cells decreased after the application of D-18864 and D-17272. Among the oxazaphosphorines tested, the lowest frequency of live cells was found when D-17272 was given at a dose of 50 µg/mL medium. D-17272 caused a smaller decrease of live cells than when D-18864 was given at the same dose. After the treatment of U937 cells with D-17272 at two doses of 5 and 50 µg/mL medium, the yield of early apoptosis increased in a dose-dependent manner. D-19575 did not distinctly increase the frequency of early apoptotic U937 cells.

Early apoptotic cells (Fig. 3C)—In comparison with the controls, the frequency of early apoptotic U937 cells increased 24, 48, and 72 h after the application of D-18864 and D-17272. The frequency of early apoptotic cells was lower after D-17272 application at a dose of 5 µg/mL medium than when D-18864 was given at the same dose. After the treatment of U937 cells with D-17272 at two doses of 5 and 50 µg/mL medium, the yield of early apoptosis increased in a dose-dependent manner. D-19575 did not distinctly increase the frequency of early apoptotic U937 cells.

Late apoptotic and necrotic cells (Fig. 3D)—Compared to the controls, the frequency of late apoptotic and necrotic cells increased 24 and 72 h after U937 cell exposure to the action of all three oxazaphosphorines, and also 48 h after the application of D-17272 at a dose of 50 µg/mL medium, and when D-18864 was given at a dose of 5 µg/mL medium. Among the oxazaphosphorines tested, the highest frequency of late apoptotic and necrotic cells was observed in U937 exposed to D-17272 at a dose of 50 µg/mL.

Oxazaphosphorine influence on cell morphology (Fig. 4)—Based on the morphological features of U937 cells exposed to the action of D-18864, D-17272 and D-19575, it can be stated that the cells underwent apoptosis and necrosis. The microscopic analysis confirmed that apoptotic and necrotic cells were mainly observed after the application of D-17272 at a dose of 50 µg/mL medium, and D-18864 at a dose of 5 µg/mL medium.

Cell count (Fig. 5)—The influence of D-18864, D-17272 and D-19575 on the count of U937 cells was observed. In relation to the controls, the cell count decreased 24, 48, and 72 h after the application of three oxazaphosphorine agents. Among U937 cells treated with the oxazaphosphorines, the lowest cell count was found when D-17272 was given at a dose of 50 µg/mL.
medium, and the highest cell count was encountered after D-19575 application at the same dose. A reduced count of U937 cells when exposed to D-17272, at two doses of 5 and 50 µg/mL medium, appeared to be dose-dependent. The cell count was found to be greater in U937 cells treated with D-17272, at a dose of 5 µg/mL medium, than in those exposed to D-18864 at the same dose.

Discussion

In vitro cytotoxic activity of the oxazaphosphorines— The results of the present study demonstrated the cytotoxic effects of the new generation oxazaphosphorines on the human histiocytic lymphoma U937 cells. After the application of D-18864, D-17272, and D-19575, different patterns of temporary changes in the cell viability, mitochondrial membrane potential, the frequency of cells undergoing apoptosis and necrosis, and the cell count, were found. The in vitro cytotoxic activity of D-17272, D-18864, and D-19575 against the U937 cells was dependent on the agent tested, its dose, and the time intervals after the oxazaphosphorine application. Among the oxazaphosphorines, D-18864 appeared to be more active than D-17272, and D-19575 was responsible for the weakest cytotoxic activity against U937 cells. In the previous investigations, different cytotoxicity of these alkylating agents was observed in human acute lymphoblastic leukemia MOLT-4 cells, human acute myeloblastic leukemia ML-1 cells, and human promyelocytic HL-60 cells.

Influence of oxazaphosphorines on cell viability— Cell viability is an important parameter characterizing cytotoxic activity of potential anticancer agents. Using the MTT assay, temporary changes in the viability rate of U937 cells exposed to the oxazaphosphorines have been shown. In the previous studies, temporary alterations in the viability were observed in MOLT-4, ML-1, and HL-60 cells treated with D-17272, D-18864, and D-19575, at the greater doses of 10 and 100 µg/mL medium. The new generation oxazaphosphorines, especially D-18864 and D-17272, caused a decrease in the viability of human pathological hematopoietic cells.

Influence of oxazaphosphorines on mitochondrial membrane potential—Mitochondrial membrane potential is an important parameter of mitochondrial function. A decrease of $\Delta\psi_m$ has been accepted to occur during apoptotic and necrotic cell death. With the use of JC-1, temporary alterations in $\Delta\psi_m$ were found in U937 cells exposed to the action of three oxazaphosphorines. The mitochondrial membrane potential of U937 cells was distinctly affected by D-18864 and D-17272. The obtained results indicate that these new oxazaphosphorine agents trigger the mitochondrial pathways of programmed cell death.

Apoptosis and necrosis - induction by oxazaphosphorines—The cell surface exposure of phosphatidylserine, and the plasma membrane impairment allow us to determine cells undergoing apoptotic and necrotic cell death. The yield of apoptosis and necrosis occurring in human histiocytic lymphoma U937 cells exposed to the action of the three oxazaphosphorines was assessed using the annexin V-FITC/PI assay. Using fluoresceinated annexin V as a specific marker for the cell surface exposure of phosphatidylserine, and propidium iodide as a marker for the plasma membrane impairment, temporary alterations in the frequency of early apoptotic cells and in the frequency of late apoptotic and necrotic cells were found. The influence of the cyclophosphamide analogue mafosfamide on induction of apoptotic death in human lymphoblastoid cells was also reported by Goldstein et al.

The oxazaphosphorine agents can exert their cytotoxic activities by triggering apoptotic and necrotic cell death.

Influence of oxazaphosphorines on cell count—Temporary changes in the cell count allow us to assess cell sensitivity to the action of different agents. In the present study, the electronic Beckman Coulter method was used to determine that the oxazaphosphorines distinctly affected the count of U937 cells. The reduced count of human acute lymphoblastic and myeloblastic leukemia cells exposed to the action of the oxazaphosphorines, were also observed. Temporary changes in the cell count can result from cell cycle disruption, inhibition of cell proliferation, and bolstered cell death processes.

Mechanisms of action of new oxazaphosphorines—It is accepted that the cytotoxic effects of the oxazaphosphorines on human pathological cells depend mainly on the active alkylating agents. Phosphoramid mustard and acrolein are the major reactive compounds of D-18864 and D-17272. The oxazaphosphorine agent D-19575 contains the direct alkylating moiety, isophosphoramide mustard. The active alkylating agents react with available groups of amino acids, proteins, and peptides
such as -SH, -COOH, -NH₂, and with the primary phosphate, hydroxyl, and amino-groups of nucleic acids. The main action target of the alkylating agents is considered to be DNA. Moreover, mitochondria play a central role in response to DNA damage. It is accepted that DNA breakage can elicit death signaling pathways that can lead to disruption of the mitochondrial membrane potential, and ultimately to mitochondrial dysfunction and cell death. It has been reported that DNA damage was caused by glufosfamide in U937 cells, and by mafosfamide in human lymphoblastoid cells. Despite the above data on the action of alkylating agents, the precise mechanisms responsible for the cytotoxic effects caused by D-18864, D-17272, and D-19575 in human pathological cells are not yet completely known.

Conclusion

The findings of the present study are the first available data which compare temporary changes in human pathological cells are surely dependent on the chemical structure of these oxazaphosphorine compounds. A better understanding of the action of the new oxazaphosphorines against human pathological cells is important for the optional use of these agents in chemotherapy.

Acknowledgement

The Study was supported by Research Projects K/ZDS/001720 and K/ZDS/001959. The authors thank Urszula Klaput for technical assistance.

References


