ACTA BIOLOGICA CRACOVIENSIA Series Zoologia 54: 33–38, 2012

In Vitro Response of Human Pathological Hematopoietic Cells to Fludarabine Phosphate

Małgorzata Opydo-Chanek, Marta Stojak and Lidia Mazur[1]

Department of Experimental Hematology, Jagiellonian University, Gronostajowa 9, 30-387 Cracow, Poland

Accepted November 5, 2012

The present study was undertaken to determine a possible influence of fludarabine (fludarabine phosphate, F-ara-AMP) on the cell viability and count. The experiments were performed *in vitro* on human acute lymphoblastic MOLT-4 cells, human acute myeloblastic ML-1 cells, and human histiocytic lymphoma U-937 cells. The research was conducted using the spectrophotometric and Beckman Coulter methods. The cell viability was analyzed using MTT assay. The cell count was detected using an electronic Z2 Coulter counter. Temporary changes in the cell viability and count were assessed at 24h and 48h after F-ara-AMP application. The *in vitro* activity of fludarabine phosphate against MOLT-4, ML-1, and U-937 cells was compared. F-ara-AMP applied at the four concentrations - 250 nM, 500 nM, 750 nM, and 1 μM - distinctly decreased the viability and count of the pathological hematopoietic cells. The effects of F-ara-AMP on MOLT-4, ML-1, and U-937 cells were dependent on the tested agent and its dose, the time intervals after the agent application, and the cell line used. ML-1 and U-937 cells appeared to be more resistant than MOLT-4 cells to the action of fludarabine phosphate. The *in vitro* response of the three human pathological hematopoietic cell lines to the F-ara-AMP action, was shown.

Key words: human pathological hematopoietic cells, fludarabine phosphate, *in vitro* cell response

INTRODUCTION

Fludarabine is an adenine nucleotide analog. Fludarabine, marketed as fludarabine phosphate (F-ara-AMP) under the trade name Fludara, is a chemotherapy agent (Hood and Finley, 1991; Plunkett and Saunders, 1991; Keating et al., 1994; Montillo et al., 2006, Ewald et al. 2008). Fludarabine phosphate has been extensively used to successfully treat various hematological malignancies (Ross et al., 1993; Keating et al., 1994; Montillo et al., 2006). Nevertheless, some aspects of the action of fludarabine are as yet unclear. Available information on biological properties of fludarabine and its activity against different pathological hematopoietic cells, is still scarce.

Cell viability and count are the important parameters characterizing anticancer activity of chemotherapeutic drugs (Kaspers et al., 1995; Borowicz et al., 2012). The MTT assay and Beckman Coulter

[1] lidia.mazur@uj.edu.pl

PL ISSN 0001-530X

method are accepted to be the sensitive, simple and rapid methods which make it possible to determine and compare *in vitro* viability and mortality in cultured cell lines, and cell sensitivity to various agents.

The purpose of the present study was to evaluate the *in vitro* cell response of the three human pathological hematopoietic cell lines to the action of fludarabine phosphate. Temporary changes in the viability and count of human acute lymphoblastic cells, human acute myeloblastic cells, and human histiocytic lymphoma cells subjected to the action of F-ara-AMP were compared using the MTT assay and Beckman Coulter method.

MATERIALS AND METHODS

Cells

Human acute lymphoblastic MOLT-4 cells, human acute myeloblastic ML-1 cells (ECACC, European Collection of Cell Cultures, UK), and 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. The cells were passaged every third day. These cells grew expotentially at 37°C in the atmosphere of 5% CO_2 in air (HERAcell incubator, KendroLab).

Agent doses and treatment

After a dilution of the cell suspension to a density of $15x10^4$ cells/ml, MOLT-4, ML-1, and U937 cells were exposed to the action of fludarabine phosphate (F-ara-AMP, Fludara, Schering), which was applied at the four concentrations - 250 nM, 500nM, 750 nM, and 1 µM. The control material consisted of untreated cells.

Analysis of cell response to F-ara-AMP

Temporary alterations occurring in the cells were analyzed at 24h and 48h after the fludarabine phosphate application. At these two time intervals, the viability and count of U-937, ML-1, and MOLT-4 cells, were assessed. The cell viability was analyzed using MTT assay, and the cell count was detected using an electronic Beckman Coulter method (Mazur et al., 2012).

Spectrophotometric MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, 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 24-well plate, containing 1 ml of the cell suspension, and the cells were incubated at 37°C with 5% CO_2. Blank solution was prepared according to the above procedure using complete medium without cells. After the three-hour incubation period, 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 to formazan in the cells reflects their viability. The value of the formazan formed was also expressed as a percentage value of the control.

Electronic cell counting

Samples of the 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 cell suspension, individual cells were measured using a Z2 Coulter counter (Beckman Coulter, USA). The cell count was detected using the counter equipped with a 100 µm diameter orifice. The instrument was calibrated using 10 µm diameter latex beads (Beckman Coulter CC size standard). The flow rate was 500 µl / 12.5 sec. The cell count was determined using Z2 AccuComp software (Beckman Coulter, USA) and calculated per 1 ml of medium.

Statistical evaluation

Statistical significance of differences in the amount of formazan formed, and in the cell count, were

evaluated by an analysis of variance and the 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

The influence of fludarabine phosphate on MOLT-4, ML-1, and U-937 cells was evaluated. The various patterns of temporary changes in the optical density of formazan solution which reflects the cell viability (Table 1, 2, and 3, Fig.1) and in the human pathological hematopoietic cell count (Table 4, 5, and 6), were observed at 24h and 48h after F-ara-AMP application.

TABLE 1. The optical density of formazan solution determined in MOLT-4 cells after their exposure to the action of fludarabine phosphate

Experimental group		Time intervals after cell exposure to F-ara-AMP	
		24h	48h
No.	Drug dose	Optical density of formazan solution ($x10^{-2}$)	
		Mean ± SD	Mean ± SD
I	Control	2,3,4,5,48h 5.52 ± 0.17	2,3,4,5,24h 13.50 ± 0.15
II	250 nM	1,4,5,48h 4.51 ± 0.71	1,3,4,5,24h 12.45 ± 0.28
III	500 nM	1,5 4.27 ± 0.50	1,2,4,5 3.97 ± 0.58
IV	750 nM	1,2,5,48h 4.02 ± 0.40	1,2,3,5,24h 1.97 ± 0.42
V	1 μM	1,2,3,4,48h 3.35 ± 0.18	1,2,3,4,24h 1.23 ± 0.08

The values are presented as the mean ± standard deviation. A difference with $P < 0.05$ was considered statistically significant using analysis of variance and Duncan's new multiple range test. Differences between experimental groups were indicated as different from Group I - 1; Group II - 2; Group III - 3; Group IV - 4; Group V - 5; Differences within each experimental group between the two time points were given as different from 24h - 24h; 48h - 48h.

TABLE 2. The optical density of formazan solution determined in ML-1 cells after their exposure to the action of fludarabine phosphate

Experimental group		Time intervals after cell exposure to F-ara-AMP	
		24h	48h
No.	Drug dose	Optical density of formazan solution ($x10^{-2}$)	
		Mean ± SD	Mean ± SD
I	Control	2,3,4,5,48h 17.73 ± 0.27	2,3,4,5,24h 38.03 ± 0.87
II	250 nM	1,3,4,5,48h 16.90 ± 0.35	1,3,4,5,24h 36.43 ± 0.84
III	500 nM	1,2,4,5,48h 14.47 ± 0.68	1,2,4,5,24h 30.33 ± 1.20
IV	750 nM	1,2,3,48h 12.99 ± 0.36	1,2,3,5,24h 24.70 ± 0.63
V	1 μM	1,2,3,48h 12.82 ± 0.27	1,2,3,4,24h 21.78 ± 0.83

For explanation see Table 1.

TABLE 3. The optical density of formazan solution determined in U-937 cells after their exposure to the action of fludarabine phosphate

Experimental group		Time intervals after cell exposure to F-ara-AMP	
		24h	48h
No.	Drug dose	Optical density of formazan solution ($x10^{-2}$)	
		Mean ± SD	Mean ± SD
I	Control	2,3,4,5,48h 19.01 ± 0.30	3,4,5,24h 46.12 ± 1.05
II	250 nM	1,3,4,5,48h 18.24 ± 0.61	3,4,5,24h 45.94 ± 0.85
III	500 nM	1,2,4,5,48h 16.12 ± 0.40	1,2,4,5,24h 36.70 ± 0.36
IV	750 nM	1,2,3,5,48h 14.34 ± 0.36	1,2,3,5,24h 29.74 ± 0.29
V	1 μM	1,2,3,4,48h 13.27 ± 0.29	1,2,3,4,24h 25.60 ± 0.60

For explanation see Table 1.

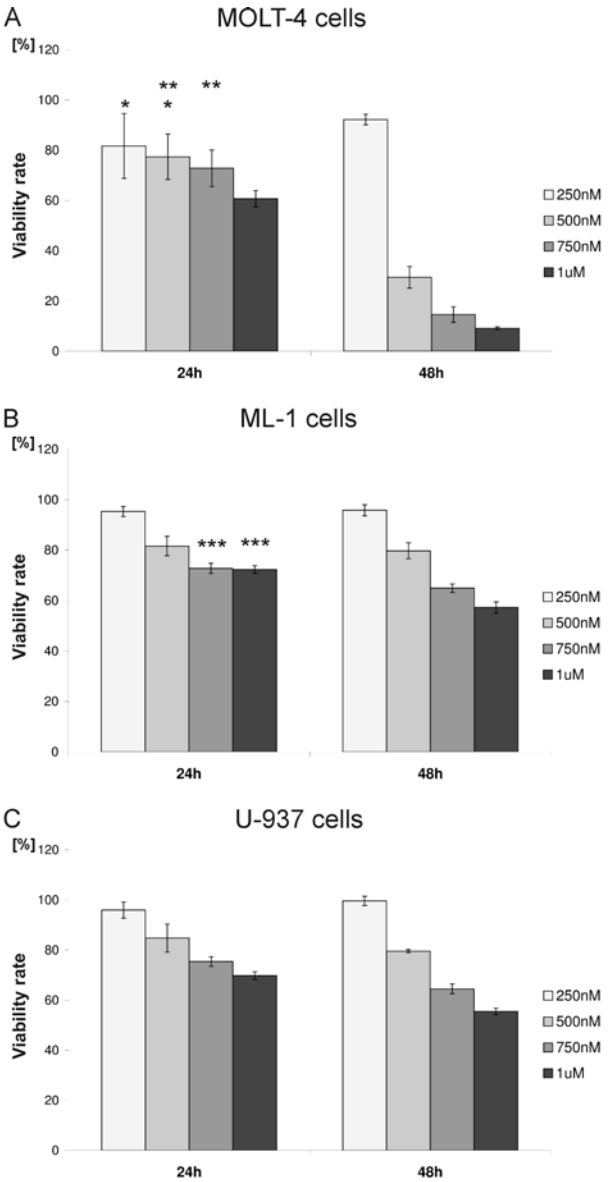

Fig. 1. Effects of fludarabine phosphate on the viability rate of MOLT-4 cells **(A),** ML-1 cells **(B)** and U-937 cells **(C).**
The values are presented as the mean ± standard deviation. A difference with $P < 0.05$ was considered statistically significant using analysis of variance and Duncan's new multiple range test. Values not significantly different at $P<0.05$: *, **, *** - between the particular groups of cells treated with fludarabine phosphate at different doses.

Cell viability

The optical density of formazan solution determined in MOLT-4, ML-1, and U-937 cells exposed to the action of F-ara-AMP is given in Table 1, 2, and 3, respectively. The viability rate of the human pathological hematopoietic cells is shown in Fig.1.

In comparison with the controls, the optical density of formazan solution decreased in the remaining experimental groups of MOLT-4 cells (Table 1), ML-1 cells (Table 2), and U-937 cells (Table 3) treated with fludarabine phosphate, but a significant decrease of the amount of formazan formed in U-937 cells was not observed at 48h after the application of this adenine nucleotide analog (Table 3). Following the exposure of the pathological hematopoietic cells to the action of F-ara-AMP, the optical density of formazan solution (Table 1, 2, 3) and the viability rate (Fig. 1) appeared to be reduced in a dose- and time dependent manner.

Cell count

The counts of MOLT-4, ML-1 and U-937 cells treated with F-ara-AMP are given in Table 4, 5 and 6, respectively.

In relation to the controls, the cell count decreased in all the remaining experimental groups of the human pathological hematopoietic cells, at two time intervals 24h and 48h after the application of fludarabine phosphate. The count of MOLT-4 cells (Table 4), ML-1 cells (Table 5), and U-937 cells (Table 6) treated with F-ara-AMP, decreased in a dose- and time dependent manner.

DISCUSSION

The effects of fludarabine phosphate on human acute lymphoblastic MOLT-4 cells, human acute myeloblastic ML-1 cells, and human histiocytic lymphoma U937 cells, were compared. It has been demonstrated that F-ara-AMP applied at the four concentrations - 250 nM, 500 nM, 750 nM and 1μM, distinctly affected the viability and count of MOLT-4, ML-1, and U-937 cells. The influence of fludarabine phosphate on the human pathological hematopoietic cells was dependent on the tested agent and its dose, the time intervals after the agent application, and the cell line used. MOLT-4 cells appeared to be more sensitive than ML-1 and U-937 cells to the action of F-ara-AMP. The different effects of fludarabine phosphate on the three human pathological hematopoietic cell lines are

TABLE 4. The count of MOLT-4 cells after their exposure to the action of fludarabine phosphate

Experimental group		Time intervals after cell exposure to F-ara-AMP	
		24h	48h
No.	Drug dose	Cell count (x10^3)	
		Mean ± SD	Mean ± SD
I	Control	2,3,4,5,48h 300.82 ± 6.16	2,3,4,5,24h 567.91 ± 5.71
II	250 nM	1,3,4,5,48h 250.90 ± 5.58	1,3,4,5,24h 388.30 ± 27.11
III	500 nM	1,2,4,5,48h 233.30 ± 2.90	1,2,4,5,24h 279.13 ± 4.67
IV	750 nM	1,2,3,5,48h 215.16 ± 2.74	1,2,3,5,24h 257.52 ± 2.89
V	1 μM	1,2,3,4,48h 204.76 ± 4.12	1,2,3,4,24h 242.55 ± 4.31

For explanation see Table 1.

TABLE 5. The count of ML-1 cells after their exposure to the action of fludarabine phosphate

Experimental group		Time intervals after cell exposure to F-ara-AMP	
		24h	48h
No.	Drug dose	Cell count (x10^3)	
		Mean ± SD	Mean ± SD
I	Control	2,3,4,5,48h 284.61 ± 12.69	2,3,4,5,24h 688.58 ± 18.28
II	250 nM	1,3,4,5,48h 251.67 ± 7.29	1,3,4,5,24h 551.94 ± 19.82
III	500 nM	1,2,5,48h 222.32 ± 6.86	1,2,4,5,24h 424.20 ± 6.21
IV	750 nM	1,2,48h 211.22 ± 9.77	1,2,3,5,24h 357.14 ± 24.00
V	1 μM	1,2,3,48h 208.10 ± 2.62	1,2,3,4,24h 293.51 ± 19.79

For explanation see Table 1.

TABLE 6. The count of U-937 cells after their exposure to the action of fludarabine phosphate

Experimental group		Time intervals after cell exposure to F-ara-AMP	
		24h	48h
No.	Drug dose	Cell count (x10^3)	
		Mean ± SD	Mean ± SD
I	Control	2,3,4,5,48h 281.35 ± 13.30	2,3,4,5,24h 684.73 ± 21.79
II	250 nM	1,4,5,48h 227.65 ± 5.73	1,3,4,5,24h 508.33 ± 46.18
III	500 nM	1,4,5,48h 220.13 ± 4.09	1,2,4,5,24h 384.35 ± 18.11
IV	750 nM	1,2,3,5,48h 200.20 ± 8.55	1,2,3,5,24h 325.66 ± 13.28
V	1 μM	1,2,3,4,48h 174.22 ± 6.79	1,2,3,4,24h 267.12 ± 15.04

For explanation see Table 1.

surely dependent on its metabolism, pharmacokinetic properties, and the cell status (Plunkett et al., 1990, 1993; Gandhi and Plunkett, 2002).

Fludarabine phosphate is a water-soluble prodrug that is rapidly converted to the free nucleoside 9-β-D-arabinosyl-2-fluoroadenine (F-ara-A). F-ara-A is transported into the cell and it is then rephosphorylated via deoxycytidine kinase to the 5'-triphosphate derivative, F-ara-ATP. This principal active form of fludarabine has multiple mechanisms of action, which are mostly directed toward DNA. F-ara-ATP competitively inhibits DNA synthesis via inhibition of DNA polymerase, ribonucleotide reductase, DNA primase, and DNA ligase. F-ara-ATP prevents elongation of DNA strands through direct incorporation into DNA as a false nucleotide. Partial inhibition of RNA polymerase II and resultant reduction in protein synthesis may also occur. It is assumed that effects of F-ara-ATP on DNA, RNA and protein synthesis all contribute to inhibition of DNA synthesis and cell growth. The cytotoxic effects of fludarabine occur primarily in the S-phase of cell division but this adenine nucleotide analog is also active against non-proliferating cells. Nevertheless, the mechanisms

of action of F-ara-ATP have not been completely characterized (Gandhi and Plunkett, 2002; Plunkett et al., 1990, 1993, 2003; Ewald et al., 2008).

To summarize, differences in the potential of fludarabine phosphate to cause temporary alterations in the viability and count of MOLT-4, ML-1, and U-937 cells have been found. The *in vitro* response of the three lines of pathological hematopoietic cells to the action of F-ara-AMP is shown. In conclusion, the elucidation of biological properties of fludarabine and its activity against different human pathological hematopoietic cells can considerably improve its clinical application.

ACKNOWLEDGEMENTS

The authors wish to thank Urszula Kłaput for her excellent technical assistance.

This work was supported by Research Project K/ZDS/001720.

REFERENCES

Borowicz, P., L. Mazur, M. Opydo-Chanek, K. Wojcieszek, M. Stojak, K. Blicharski, and G. Zwierzyńska. 2012. Combined action of new generation anticancer agents. XXI International Symposium of Polish Network of Molecular and Cellular Biology UNESCO/PAS "Molecular and Physiological Aspects of Regulatory Processes of the Organism", Cracow, 220-221.

Ewald, B., D. Sampath, and W. Plunkett. 2008. Nucleoside analogs: molecular mechanisms signaling cell death. *Oncogene* 27: 6522-6537.

Gandhi, V., and W. Plunkett. 2002. Cellular and clinical pharmacology of fludarabine. *Clin. Pharmacokinet.* 41: 93-103.

Hood, M.A., and R.S. Finley. 1991. Fludarabine: a review. *Ann. Pharmacother.* 25: 518-524.

Kaspers, G.J.L., A.J.P. Veerman, R. Pieters, I. Van Zantwijk, K. Hahlen, and E.R. Van Wering. 1995. Drug combination testing in acute lymphoblastic leukemia using the MTT-assay. *Leukemia Res.* 19: 175-181.

Keating, M.J., S. O'brian, L.E. Robertson, H. Kantarjian, M. Dimopulos, P. McLaughlin, F. Cabanillas, V. Gregoire, L.Y. Yang, V. Gandhi, E. Estey, and W. Plunkett. 1994. The expanding role of fludarabine in hematologic malignancies. *Leukemia Lymphoma* 14: 11-16.

Mazur, L., M. Opydo-Chanek, K. Wojcieszek, M. Stojak and U. Niemeyer. 2012. Comparative effects of new generation oxazaphosphorines on the size and viability of human acute myeloblastic leukemia cells. *Folia biol. (Kraków)* 60: 35-40.

Montillo M., F. Ricci, and A. Tedeschi. 2006. Role of fludarabine in hematological malignancies. *Expert Rev. Anticancer Ther.* 6: 1141-1161.

Plunkett, W., V. Gandhi, P. Huang, L.E. Robertson, L.Y. Yang, V. Gregoire, E. Estey, and M.J. Keating. 1993. Fludarabine: pharmacokinetics, mechanisms of action, and rationales for combination therapies. *Semin. Oncol.* 20: 2-12.

Plunkett, W., P. Huang, and V. Gandhi. 1990. Metabolism and action of fludarabine phosphate. *Semin. Oncol.* 17: 3-17.

Plunkett, W., and P.P. Saunders. 1991. Metabolism and action of purine nucleoside analogs. *Pharmacol. Ther.* 49: 2339-2368.

Ross, S.R., D. McTavish, and D. Faulds. 1993. Fludarabine: a review of its pharmacological properties and therapeutic potential in malignancy. *Drugs* 45: 737-759.