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## Comparative Cytotoxicity of Fludarabine in Human Peripheral Blood Mononuclear Cells and in the Promyelocytic Leukemia Cell Line (HL60)\*

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**Abstract:** Fludarabine is an inhibitor of DNA/RNA synthesis with antitumor activity on lymphoproliferative malignancies. We investigated the dose-dependent cytotoxic effects of the drug on human peripheral blood mononuclear cells, the HL60 cell line and the differentiated HL60 cells. Dimethylformamide was used for the neutrophilic differentiation of HL60 cells and the MTT assay was used to estimate drug cytotoxicity. Undifferentiated tumor cells had higher proliferation rates compared to the differentiated subline. There was 57% cell death in HL60 cells at a dose of 5 µg/ml of the drug. On the other hand, in differentiated HL60 cells and nonmalignant cells, cell death did not exceed 40% after exposure to 500 µg/ml. These results demonstrated that proliferation rate and differentiation status may have a contribution in fludarabine cytotoxicity.

**Key Words:** HL60, MTT assay, fludarabine, cytotoxicity, differentiation.

### Introduction

Fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine monophosphate, F-ara-AMP) is a purine nucleoside analogue effective in various lymphoproliferative disorders, in particular chronic lymphocytic leukemia (CLL). It is dephosphorylated to F-ara-A, which enters the cell by active transport and then accumulates as the 5'-triphosphate, F-ara-ATP (1-3). This active metabolite has been shown to inhibit DNA synthesis by inhibiting ribonucleotide reductase, DNA polymerase, DNA primase and DNA ligase. Thus, incorporation of fludarabine nucleotides into DNA in proliferating cells highly correlates with drug induced cell death (4-7). Termination of RNA chain elongation and inhibition of RNA synthesis is a unique feature of the drug that may be important for cytotoxicity on non-dividing cells (8,9). In addition, recent studies have suggested that fludarabine-induced cytotoxicity observed in quiescent cells, such as CLL, may be related to caspase activation (10,11). Cell specificity of the fludarabine has been attributed either to

selective transport into leukemic lymphocytes or to enhanced phosphorylation to the active form in these cells (12).

There is an inverse relationship between proliferation and maturation (13,14). That is, cells lose their proliferative capacity before or while acquiring the mature phenotype. Human leukemia cell line HL60 can be induced to differentiate to mature myeloid phenotype by a variety of agents (15,16). For example, cells exposed to dimethylformamide acquire the features of a mature neutrophil. The present study compared dose-dependent effects of fludarabine between normal myeloid cells, leukemic cell line (HL60) and differentiated HL60 cells.

### Materials and Methods

#### Cell Culture

Peripheral mononuclear cells were isolated from a healthy donor by Ficoll-Hypaque density gradient and cultured immediately at a concentration of  $4 \times 10^6$

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cells/ml in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified incubator containing 5% CO<sub>2</sub>. HL60 cells (human promyelocytic cell line) were maintained under the same conditions at a density of 4 x 10<sup>5</sup> cells/ml. For inducing differentiation, HL60 cells were cultured in RPMI 1640 (without phenol red) supplemented with 20% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. These cells were treated with 100 mM dimethylformamide for 5 days. Differentiated HL60 cells were then cultured under the same conditions as parental HL60 cells at a density of 8 x 10<sup>5</sup> cells/ml and cytotoxicity was evaluated on the same day. Proliferation rates of parental and differentiated HL60 cells were determined after the indicated period.

**MTT Assay**

Fludarabine monophosphate was obtained from Schering AG, Germany. The drug was diluted in a culture medium and dose-dependent cytotoxic effects were studied by culturing cells with 0.5–1000 µg/ml fludarabine monophosphate. MTT assay was used to evaluate cell viability after drug exposure. Briefly, 50 µl of cell suspension was seeded in 96-well plates and 50 µl of drug added to each well. After 48 h of incubation, 25 µl of MTT solution (1 mg/ml final concentration) was added to each well and the plates were incubated for a further 4 h. The produced formazan was solubilized by

adding 80 µl of lysing buffer of 23% SDS dissolved in a solution of 45% DMF (pH 4.7). After an overnight incubation at 37 °C, the optical densities (OD) at a wavelength of 540 nm were measured using the microplate reader (Spectramax Plus, Molecular Devices, UK). Cells incubated in culture medium alone served as a control for cell viability (untreated wells). All assays were performed in quadruplicate and mean ± SD values were used to estimate inhibition rate. The inhibition rate (% of cell death) was calculated as (1 - OD of treated cells/OD of untreated cells) x 100.

**Results**

**Cytotoxicity of Fludarabine on HL60 Cells**

The dose-response curve of fludarabine on HL60 cells is presented in Figure 1. The dose of 5 µg/ml of fludarabine produced 57% cell death in tumor cells. At increasing doses, cell viability decreased slightly and a plateau phase occurred on the drug-response curve. The percentage of cell death was 69% at the dose of 20 µg/ml and it did not exceed 71% at the highest dose.

**Cytotoxicity of Fludarabine on Differentiated HL60 Cells**

The cytotoxic effect of fludarabine on differentiated HL60 cells is presented in Figures 2 and 3. The dose of 5 µg/ml of fludarabine produced only 5% cell death in these cells. There was a moderate effect of the drug at the dose

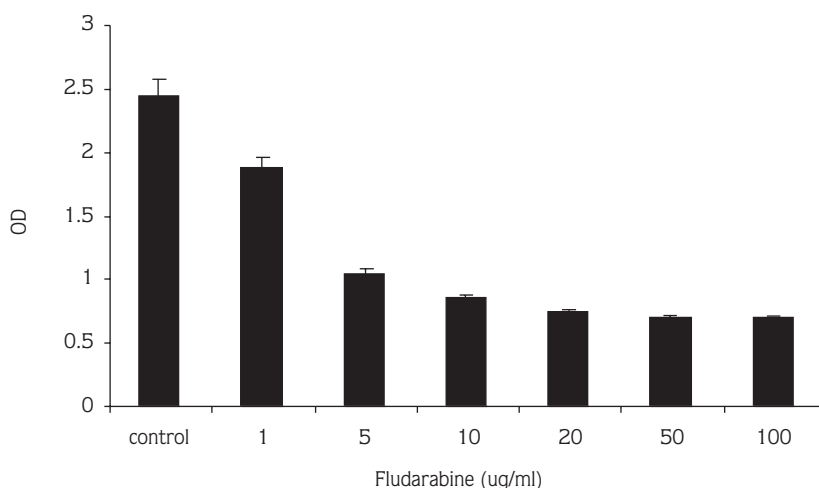


Figure 1. Cytotoxicity of fludarabine on HL60 cells. OD values represent mean ± SD of 4 wells.

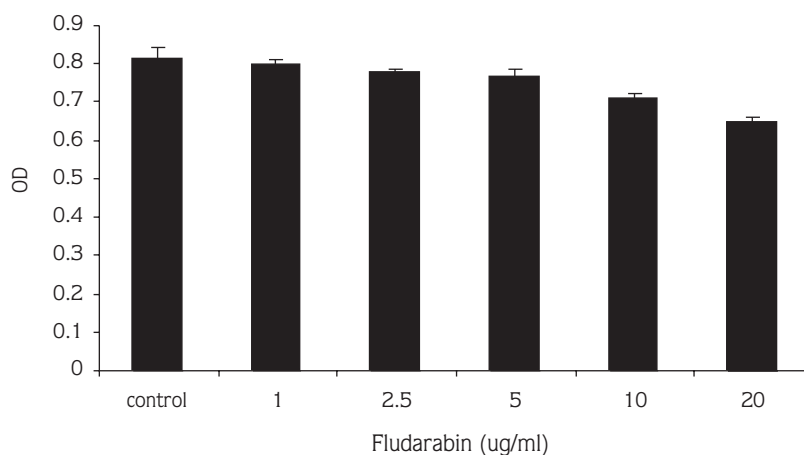


Figure 2. Cytotoxicity of fludarabine (1-20 µg/ml) on differentiated HL60 cells. OD values represent mean ± SD of 4 wells.

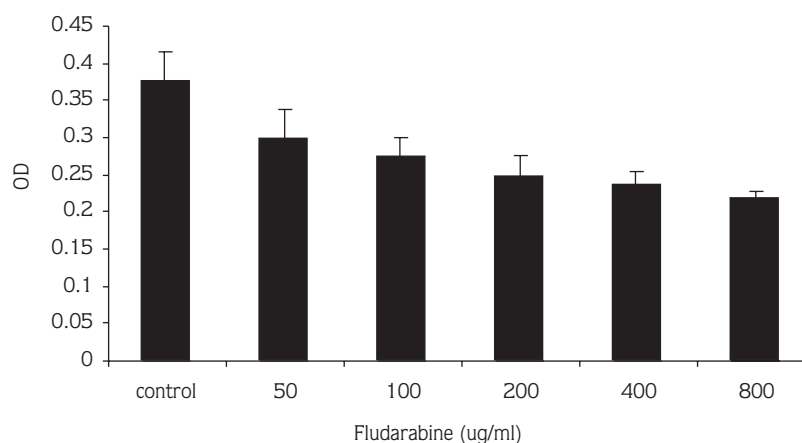


Figure 3. Cytotoxicity of fludarabine (50-800 µg/ml) on differentiated HL60 cells. OD values represent mean ± SD of 4 wells.

of 50 µg/ml, which was estimated as 20% cell death. The differentiated HL60 cells failed to reach 50% inhibition rate at increasing doses. The percentage of cell death was only 42% at the dose of 800 µg/ml.

#### Cytotoxicity of Fludarabine on Normal Mononuclear Cells

The cytotoxic effect of the drug on normal mononuclear cells is presented in Figures 4 and 5. Fludarabine did not produce an obvious cytotoxicity in a dose-dependent manner on normal cells. Both doses of 5 µg/ml and 50 µg/ml of the drug produced 22% cell death.

The percentages of cell death at the doses of 500 µg/ml and 1000 µg/ml were 33% and 37%, respectively.

#### Discussion

This study addresses the dose-dependent cytotoxicity of fludarabine in cells of different origin and/or maturation state. Fludarabine has a cell cycle specific cytotoxic effect targeted at DNA synthesis, which accounts for the killing of dividing cells. Here, we report cytotoxic effects of the drug on proliferating HL60 cells and the differentiated subline. It has been reported that induction to differentiation in immature myeloid cells

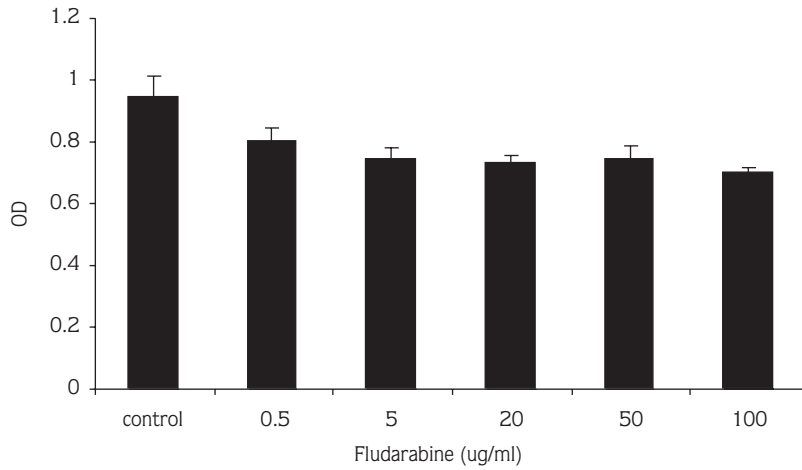


Figure 4. Cytotoxicity of fludarabine (0.5 -100 µg/ml) on normal mononuclear cells. OD values represent mean ± SD of 4 wells.

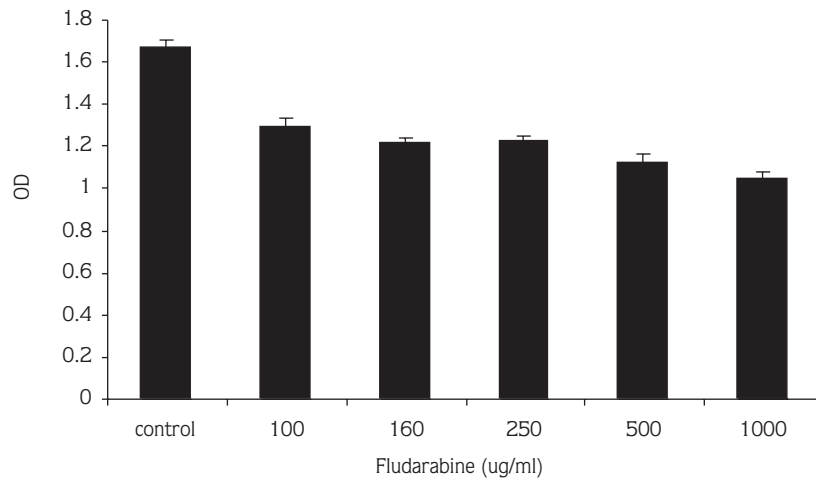


Figure 5. Cytotoxicity of fludarabine (100 -1000 µg/ml) on normal mononuclear cells. OD values represent mean ± SD of 4 wells.

increases coinciding with a decrease in proliferation rate (13). In our study, the proliferation rate of HL60 cells was 3-fold higher than that of differentiated HL60 cells. The decreased proliferation rate of differentiated HL60 cells was in accordance with this switch in the cycle. Dimethylformamide induces differentiation in less than 60% of HL60 cells at 96 h (15). It was also shown that, in culture conditions containing 10% fetal bovine serum differentiation rate into neutrophils did not exceed 40% in myeloid cells (13). In our experiments, the morphologic evaluation (granule content and cell size) on flow cytometry showed that the differentiation rate was 30%. It is noteworthy that even at low ratios of

differentiated cells, insensitivity to the drug was demonstrated.

We tested the fludarabine dose-response curve, which included the clinically effective plasma drug levels (17). Accordingly, at the dose of 5 µg/ml, more than 50% cell death was achieved in undifferentiated HL60 cells. Additionally, in freshly isolated tumor cells obtained from a CLL patient, the cell death was 43% at the same dose. The percentage of cell death reached 69% at the dose of 50 µg/ml (data not shown). A concordance between dose-response curves of undifferentiated HL60 cells and the patient’s leukocytes is evident. These results may be

critical with respect to the cell specificity and to the cytotoxicity of the drug at low doses in tumor cells. The efficiency in lymphoid cells confirms the importance of fludarabine as being the gold standard in chemotherapy.

González-Cid et al. have shown that fludarabine induces apoptosis in normal human lymphocytes in a dose-dependent manner and the apoptotic index has been found below 20% at 5 µg/ml of drug concentration (18). We showed that normal mononuclear cells were resistant to the cytotoxicity of fludarabine even at a dose of 1000 µg/ml.

Deoxycytidine kinase is the dominant kinase for phosphorylation and activation of fludarabine (3,19). It has been reported that plasma deoxycytidine levels increased after chemotherapy. Consecutively, it causes inhibition of deoxycytidine kinase activity resulting in decreased fludarabine cytotoxicity (20). In the same study, HL60 cells treated with fludarabine (10 µM; ~3 µg/ml) and deoxycytidine (0.1-32.4 µM) were shown to survive in a dose-dependent manner where exposure to

deoxycytidine alone did not affect cell proliferation or viability. Therefore, it seems likely that the initial poor activity of fludarabine against either differentiated HL60 cells or normal lymphocytes could not be attributed to failure of drug activation.

In conclusion, (i) the impact of tumor cell proliferation rate is critical for the extent of dose-dependent fludarabine cytotoxicity; (ii) differentiation to mature phenotype is associated with fludarabine insensitivity, and (iii) insensitivity to the drug in normal and non-dividing lymphoid cells should be taken into consideration when studying drug effects on leukocytes.

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