

## DNA-PK, ATM AND MDR PROTEINS INHIBITORS IN OVERCOMING FLUDARABINE RESISTANCE IN CLL CELLS

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**Aim:** To perform the comparative study of the effects of DNA-dependent protein kinase (DNA-PK) inhibitors vanillin and NU7026, ataxia telangiectasia mutated kinase (ATM)/ATM and Rad3 related (ATR) kinase inhibitor caffeine and multidrug resistance (MDR) protein modulator cyclosporine A (CsA) on fludarabine resistant and sensitive lymphocytes from chronic lymphocytic leukemia (CLL) patients. **Methods:** Cells sensitivity *in vitro* was determined with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). DNA-PKs and ATM expression in CLL cells was evaluated using Western blotting. Multidrug transporter protein expression and function was assessed by flow cytometry. Pro- or anti-apoptotic genes (*BAX*, *LICE BCL-2*, *BCL-XS FLICE*, *FAS*, *TRAIL*) expression on mRNA level was evaluated. **Results:** Caffeine, vanillin, NU7026 and CsA increased fludarabine cytotoxicity against fludarabine-resistant CLL cells samples in comparison with sensitive cell samples. However, fludarabine-sensitive CLL samples were sensitized with inhibitors to a greater extent compared with resistant CLL samples. ATM expression increased in fludarabine-resistant CLL samples, but no apparent correlation between DNA-PKs level and fludarabine sensitivity *in vitro* or sensitization effect of DNA-PK inhibitors were observed. Fludarabine-resistant CLL lymphocytes showed tendency for depressed MDR efflux and decreased level of mRNA of pro-apoptotic gene *BCL-XS*. **Conclusion:** Absence of any definite conformity between fludarabine-resistant cell susceptibility to combined action of fludarabine and inhibitors, and molecular pathways that might be involved in this process does not exclude drugs synergy in fludarabine-resistant cells that could be used for overcoming resistance to nucleoside analogs in CLL.

**Key Words:** fludarabine, resistance, vanillin, caffeine, NU7026, leukemia.

Fludarabine-based therapies are considered to be the most adequate and widely applied for treatment of patients with chronic lymphocytic leukemia (CLL) [1–4]. However, some patients develop drug resistance through variable pathways detectable even at diagnosis or more often as the disease progresses and acquires drug insusceptibility (relapse, refractory disease) [5–6]. In accordance with known molecular basis for fludarabine activity (inhibition of DNA repair by multiple underlying mechanisms) and chemotherapy resistance [7], the application of DNA repair inhibitors is among novel strategies to circumvent it, in addition to new drug combinations [8].

DNA-dependent protein kinase (DNA-PK) and ataxia telangiectasia mutated kinase (ATM)/ATM and Rad3 related (ATR) kinases are among key proteins required for nonhomologous end joining (NHEJ) and homologous recombination pathway. It was demonstrated that inhibitors of these molecules could be applied to overcome the drug and ionizing radiation resistance. Besides that it is known that cells, which are not sensitive to some drugs, can be sensitized by the modifier of multidrug resistance-associated protein of ABC family cyclosporin A (CsA), although

ABC proteins (e.g. P-gp) are not the main nucleoside analogue fludarabine transporters in CLL cells [9].

We hypothesized that inhibiting DNA-PK and ATM would sensitize CLL cells to drug-induced DNA damage depending on the initial sensitivity to fludarabine. Therefore, in the current work we studied the response of drug resistant and sensitive CLL cells to fludarabine in the presence of the inhibitors known for the above-mentioned activity with parallel assessment of some molecular pathways involved in this interaction.

### MATERIALS AND METHODS

**Patient samples.** Peripheral blood was obtained from CLL patients. All patients signed the informed consent. Diagnosis was established in compliance with the standard clinical and laboratory data according to the International Workshop Criteria [10].

Mononuclear cells were separated by density gradient centrifugation. Freshly isolated leukemic cells were cultured at a seeding density of  $2 \times 10^6$  cells/ml in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% FBS at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells viability (trypan blue exclusion) was > 95%.

**Drugs and reagents.** Fludarabine (Fludarabel®, Belmedpreparaty, Republic of Belarus) was tested in the dose of 5 µg/ml that is comparable to the therapeutic drug concentration in blood. Different types of DNA-PK inhibitors were applied. Small molecule NU7026 (2-(morpholino-4-yl)-benzo[c]chromen-4-one) and less-specific inhibitor vanillin (4-hydroxy-3-methoxybenzaldehyde) were tested for drug sensitization

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**Abbreviations used:** ATM – ataxia telangiectasia mutated kinase; ATR – ATM and Rad3 related kinase; CLL – chronic lymphocytic leukemia; CsA – cyclosporin A; DNA-PK – DNA-dependent protein kinase; MDR – multidrug resistance; MFI – median fluorescence intensity; MTT – 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NHEJ – nonhomologous end joining.

effect. NU7026 was a generous gift from Dr. Barbara Durkacz (New-Castle upon Tune University, England). Caffeine (Tathchimpharmpreparaty, Russia) was used as ATM/ATR kinases inhibitor. NU7026 was dissolved in anhydrous dimethylsulfoxide, vanillin and caffeine were dissolved in distilled water. NU7026 was used in all drug combination experiments at final concentrations of 10  $\mu$ M, vanillin — 200  $\mu$ M, caffeine — 7  $\mu$ M. Inhibitors-induced cell death did not exceed 20%. Final concentration of MDR protein modulator CsA (Novartis Pharmaceutical Corporation, NJ) was 0.5  $\mu$ M. All other reagents were from Sigma (USA), unless otherwise stated.

**Cytotoxic assay.** The cell sensitivity *in vitro* was determined with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). Briefly, human leukemia cells ( $2 \times 10^6$ /ml) were seeded into 96-well culture plate containing drug(s) and solvent controls. After 48 h of incubation 20  $\mu$ l of MTT solution (5 mg/ml in phosphate buffer saline) was added to each well, and the cells were incubated for 4 h. The formazan crystals were dissolved with 100  $\mu$ l of acidic isopropanol and the absorbance of each well was read at 540 nm in a plate reader. Results were expressed as a percentage of control. In the case of inhibitor sensitization effect testing, the results were standardized in respect to cell viability under inhibitor action alone.

**Western blotting.** DNA-PKs and ATM expression in CLL cells was evaluated using Western blotting. Whole cell extracts were prepared and loaded onto Tris-Acetate 3–8% denaturing polyacrylamide gradient gels using standard protocols. Samples were transferred onto nitrocellulose membrane and probed with antibodies against DNA-PKs, ATM and actin. Anti-mouse HRP-linked secondary antibodies were used. ECL reagent (Amersham, UK) was used for luminescence reaction activation. Results quantification was performed with a Fuji LAS-3000 luminescent image analyzer system.

**Flow cytometric assessment of multidrug transporter protein expression and function.** P-gp expression was analyzed using the protein-specific antibody FITC-conjugated anti-P-gp 170 (clone 17F9) (BD Pharmingen, USA). Protein expression was quantitated as percentage of protein positive cells. The median fluorescence intensity (MFI) was recorded as the median fluorescence channel shift (specific antibodies/internal control). MFI was categorized as follows: negative (MFI < 1.5) and positive (MFI > 1.5).

Rhodamine 123 was used as substrate for MDR protein function assay. CsA modulates the MDR protein function. Functional activity of MDR proteins was estimated as efflux in the presence and absence of a modulator: Efflux = [cells with low fluorescence intensity [with modulator] — cells with low fluorescence intensity [without modulator]] (%). Fluorescence intensity considered to be low when MFI < 1.5.

**Assessment of MDR protein gene expression.** Total RNA extraction was performed using Gen Elute Mammalian Total RNA Miniprep Kit according to the manufacturer instructions. The quantification and

$A_{260}/A_{280}$  ratio were obtained using spectrophotometer Gene Quant RNA/DNA Calculator (GE Healthcare). Total RNA was also visualized by agarose gel electrophoresis. cDNA was synthesized with Advantage RT-for-PCR Kit (BD, USA) according to the manufacturer instructions. As internal control RNA isolated from healthy people lymphocytes was used. The variations between samples in the quality of RNA extracted and subsequent cDNA synthesis were normalized by their relative quantities of *Gus*. IM-9 cell line was used as control sample for quantification of studied gene in CLL cells. Primers sequences: *MDR1* forward primer — AGG AAG ACA TGA CCA GGT ATG C, reverse primer: CCA ACA TCG TGC ACA TCA AAC, TaqMan: FAM CCT GGC AGC TGG AAG ACA AAT ACA CAA BHQ1.

#### **Assessment of pro- and anti-apoptotic genes.**

Apoptosis genes expression on mRNA level was assessed using kit from Maxim Biotech (USA) according to the instructions.

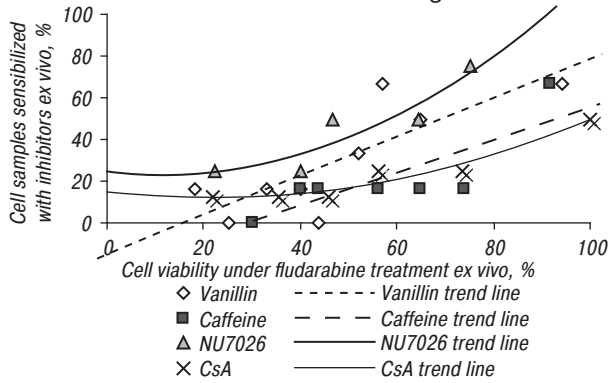
**Statistical data analysis.** Nonparametric statistics were used, including Spearman rank correlation and the Mann — Whitney test. Statistical data were tested for significance by 2-tailed analysis. To estimate the modulator sensitization frequency in resistant and sensitive cells we applied  $\chi^2$  statistics. The result of tests was considered statistically significant if the  $P \leq 0.05$ . All statistical analysis was performed using the software package Statistica 6.0.

## **RESULTS**

**Inhibitors synergy with fludarabine in drug resistant CLL cells.** Total number of cell samples isolated from CLL patients was 115. Variability of cell response to drugs alone or in combinations was observed. Sensitization frequency in cells co-treated with fludarabine and inhibitors is presented on Fig. 1. The overall frequency of sensitized samples increased with the decreasing drug sensitivity. According to the fludarabine cell sensitivity *in vitro* samples were classified as sensitive (less than 30% of viable cells,  $n = 30$ ) and resistant (more than 70% of survived CLL lymphocytes,  $n = 21$ ) cases. All other cases ( $n = 64$ ) had intermediate sensitivity (cell viability 30–70%). Caffeine sensitized 50% of fludarabine-resistant cell samples. DNA-PK inhibitors (NU7026 and vanillin) enhanced drug cytotoxicity in 27 and 33% resistant samples, respectively, and 43% of fludarabine-resistant cell samples were sensitized with CsA. At the same time, caffeine and CsA were absolutely inactive in fludarabine sensitive samples. DNA-PK inhibitors treatment combined with fludarabine decreased cell survival in 8 and 16% of studied CLL sensitive cases.

DNA-PK and ATM inhibitors sensitized fludarabine-resistant CLL lymphocytes in 1.2 folds ( $P < 0.05$ ). CsA showed the most pronounced sensitization effect among drug resistant cells (1.4 folds,  $P < 0.05$ ). However, quantitative sensibilization effect was more expressed in drug sensitive CLL cells: 1 out of 5 sensitive CLL samples sensitized with inhibitors showed extremely high (100-fold) increase of sensitivity, the

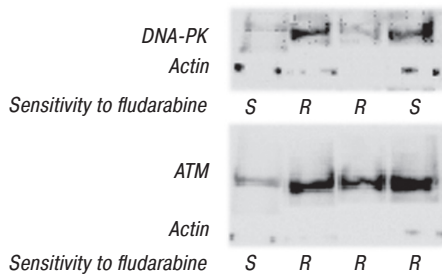
other fludarabine sensitive CLL samples were sensitized with inhibitors 3.5-fold on average.



**Fig. 1.** Overall sensitization frequency in cells co-treated with fludarabine and inhibitors. CLL cells were treated with fludarabine (14  $\mu$ M) alone or in combination with vanillin (200  $\mu$ M), caffeine (7  $\mu$ M), NU7026 (10  $\mu$ M) or CsA (0.5  $\mu$ M). Each point represents average data on 5 to 9 patient samples. Frequency was calculated as the ratio of sensitized CLL samples number to the total number of studied samples *in vitro*

**DNA-PKs level and ATM expression level in CLL cells.** DNA-PKs level was studied in *ex vivo* lymphocytes from 12 B-CLL patients. DNA-PKs concentration in *ex vivo* cell extracts varied 10-fold. However, there was not any apparent correlation between the DNA-PKs level and fludarabine sensitivity *in vitro* and sensitization effect of DNA-PK inhibitors.

ATM expression was assessed in 10 CLL samples of intact malignant lymphocytes. In contrast to DNA-PKs, ATM expression level was 2-fold higher in fludarabine resistant CLL samples compared with sensitive ones ( $P < 0.05$ ) (Fig. 2).



**Fig. 2.** ATM and DNA-PK expression in fludarabine sensitive (S) and resistant (R) CLL samples. DNA-PKs and ATM expression in CLL cells was evaluated by Western blotting. Membranes were probed with antibodies against DNA-PKs, ATM and actin. Anti-mouse HRP-linked secondary antibodies were used. ECL reagent was used for luminescence reaction activation. Results quantification was performed with a Fuji LAS-3000 luminescent image analyzer system

**Fludarabine sensitivity of CLL cells in respect to MDR protein expression and function.** As the percentage of P-gp positive cells was unreliable parameter because of P-gp trace amount, we used MFI and Kolmogorov-Smirnov D statistics. MFI median did not reveal any difference in drug sensitive and resistant cells. Rhodamine 123 efflux features the intensity of P-gp function. Intensification of P-gp function was shown in drug sensitive cells compared with resistant subset (Table 1). More pronounced suppression of MDR protein function by CsA was shown in fludarabine sensitive CLL subset.

**Table 1.** MDR protein/gene expression and function in CLL cells with different response to fludarabine *ex vivo*

MDR status parameters	CLL lymphocytes response to fludarabine <i>ex vivo</i>		p
	drug sensitive	drug resistant	
P-gp positive cells.%	0.72 (0.34–0.80)	0.25 (0.19–1.93)	0.56
P-gp MFI. a.u.	1.88 (1.67–3.31)	2.06 (1.52–2.47)	0.29
P-gp efflux: number of cells with weak fluorescent dye accumulation.%	51 (37–74)	28 (23–39)*	0.04
The extent of CsA suppression of MDR proteins activity, fold	2.76 (2.06–3.77)	1.63 (1.58–1.68)*	0.04

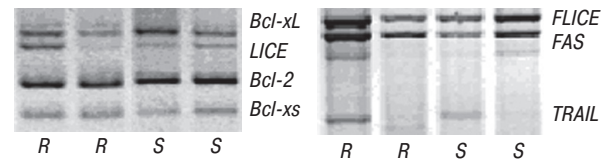
Note: \*  $p < 0.05$ .

**Expression of mRNA of molecules involved in apoptosis in CLL cells.** No difference in mRNA expression levels was found between fludarabine resistant and sensitive CLL lymphocytes for pro-apoptotic genes *TRAIL*, *FAS*, *FLICE* and for anti-apoptotic genes *BCL-2* and *BCL-XL*. However, mRNA level of pro-apoptotic *BCL-XS* was increased in sensitive samples (Table 2, Fig. 3).

**Table 2.** Apoptosis gene mRNA quantification in fludarabine-sensitive and fludarabine-resistant CLL subsets

Apoptosis gene	The relative intensity of mRNA expression in	
	fludarabine sensitive CLL lymphocytes	fludarabine resistant CLL lymphocytes
Mitochondrial apoptosis pathway		
<i>BCL-XL</i>	0.26 (0.25–0.33)	0.22 (0.13–0.30)
<i>BCL-2</i>	0.45 (0.44–0.52)	0.44 (0.41–0.61)
<i>BCL-XS</i>	0.15 (0.15–0.16)	0.07 (0.06–0.12)*
<i>LICE</i>	0.08 (0.06–0.34)	0.19 (0.19–0.20)
Receptor-induced apoptosis pathway		
<i>FLICE</i>	0.72 (0.42–0.99)	0.67 (0.62–0.79)
<i>FAS</i>	0.23 (0–0.45)	0.03 (0–0.1)
<i>TRAIL</i>	0.06 (0–0.10)	0.20 (0.11–0.38)

Note: \*  $p < 0.01$ .



**Fig. 3.** Pro- and anti-apoptotic mRNA gene expression in fludarabine sensitive (S) and resistant (R) CLL samples. Multiplex polymerase chain reaction was used for genes expression analysis. It was assessed with kit from Maxim Biotech. Expression of *GAPDH* gene was used as control

Furthermore, mRNA of pro-apoptotic genes was investigated in CLL samples in connection with different response to inhibitors: cells sensitized to fludarabine by all studied inhibitors and CLL lymphocytes unresponsive to the inhibitors. Not sensitized cells from fludarabine-resistant and sensitive samples had elevated expression levels of *BAX* and *LICE* (procaspase 3) mRNA and decreased mRNA level of pro-apoptotic agent *BCL-XS* and anti-apoptotic *BCL-XL*. No difference in mRNA expression of *FAS*, *FASL*, *TRAIL*, *FLICE* genes was shown between sensitized and not sensitized CLL subgroups.

## DISCUSSION

Fludarabine cytotoxicity is mediated by the DNA synthesis and repair inhibition. In the current work we hypothesized that repair of DNA damage induced by fludarabine may be mediated in part *via* DNA-PK, ATM and probably *via* MDR. Therefore DNA-PK, ATM and may be MDR inhibitors would sensitize cells to this agent.

Taking into account that ATR activity is downregulated in non-cycling CLL cells, we consider that caffeine as ATM inhibitor sensitized CLL cells to fludarabine most frequently (50% of resistant samples). DNA-PK inhibitors vanillin and NU7026 sensitized resistant lymphocytes in 27 and 33% of samples, respectively. CsA increased fludarabine cytotoxicity in 43% of resistant samples. Fludarabine activity in drug sensitive CLL lymphocytes was enhanced by caffeine and CsA not more than in 16 and 8%, respectively. But DNA-PK inhibitors were entirely inactive in CLL cells responded to fludarabine. However, small number of fludarabine sensitive samples (5 out of 30) that were able to respond to inhibitors were sensitized with them to a greater extent than initially fludarabine resistant CLL samples.

Previously, it was demonstrated that fludarabine activity was potentiated in the presence of DNA-PK inhibitor NU7441 by 2–14 folds, while in our study potentiating effect depended on the initial fludarabine sensitivity and varied greatly from 1.1 to more than 100 fold. Sensitization effect of NU7441 was shown in 73% of studied cases [11], though our study established response dependence on initial drug sensitivity rate.

We didn't demonstrate any correlation between DNA-PKs expression and fludarabine sensitivity, and inhibitor sensitization. However, increased ATM level was detected in fludarabine-resistant cells. In other studies it was shown that DNA-PKs levels correlated with DNA-PK activity though varied 50-fold between cases. NU7441 sensitized CLL cells to chlorambucil and fludarabine, and abrogated drug-induced autophosphorylation of DNA-PKs at Ser<sup>2056</sup>. In addition, high DNA-PK levels were even predictive for reduced treatment-free interval [12]. Moreover, human fibroblasts pretreated with DNA-PKs inhibitors vanillin and Wortmannin showed increased levels of chromosome breakages and became more sensitive to fludarabine-induced cell death. An active role of NHEJ pathway was also suggested from the analysis of Chinese hamster cell lines. XR-C1 (DNA-PKs-deficient) and XR-V15B (Ku80-deficient) cells showed hypersensitivity to fludarabine as was detected by the increased frequency of chromosome aberrations, decreased mitotic index and impaired survival rates [13]. At the same time, no significant induction of homologous recombination after fludarabine treatment was shown. Taken together these data and our results underlined the heterogeneity of studied molecular properties of leukemia cells in CLL patients and the necessity of development of differentiated treatment approach in future.

It is known that MDR proteins expression could be one of the reasons for drug resistance development. Some data evidence that fludarabine has capacity to overcome the negative effect of MDR overexpression. Therefore, response to fludarabine doesn't correlate with P-gp status. These data support our findings concerning decreased P-gp function and its impaired CsA suppression in fludarabine resistant CLL cells. Moreover, it is necessary to take into consideration equilibrative and concentrative nucleoside transporter (ENT, CNT) family

as far as fludarabine accumulation in CLL cells is mostly mediated by ENT and hCNT3 type transporters. Their biologic activity was clearly correlated with fludarabine cytotoxicity. It reveals a role of ENT-mediated uptake in drug responsiveness in patients with CLL [14, 15].

As fludarabine may induce apoptosis in a CD95/Fas receptor, FADD, IAPs and caspase-8-independent manner by activation of the mitochondrial cell death pathway [16, 17], our results concerning *FAS* and *TRAIL* mRNA content in CLL lymphocytes with different fludarabine sensitivity could be in line with these data.

The role of BCL-2, BAX, BAG-1, and MCL-1 proteins in CD5/CD20-positive B-CLL cells has already been elucidated. BCL-2 expression was decreased after fludarabine treatment. MCL-1 expression was increased in fludarabine-resistant cells and seemed to be a remarkable protein for the inhibition of the apoptotic process in CLL. After fludarabine treatment, BAG-1 expression was increased in fludarabine-resistant cells [18]. Though the apoptotic program induction depends on the subtle mitochondrial protein balance, *BCL-XS* was the only gene in our study that showed the difference in fludarabine resistant and sensitive cells. Cells non-sensitized with DNA-PK and ATM inhibitors had elevated expression of *BAX* and *LICE* (procaspase 3) mRNA and decreased mRNA level of pro-apoptotic *BCL-XS* and anti-apoptotic *BCL-XL* mRNA.

According to our data drug cell response *ex vivo* is an integral test for cellular molecular biological properties defining the treatment course outcome [19]. However, exact underlying mechanisms of drug resistance are still obscure, and have some peculiarities in individual patients. In this connection the idea of new approaches to overcoming drug resistance on the basis of evaluation of differentiated resistant and sensitive cell responses to variable agents should not be neglected.

Unfortunately, molecular profiling of leukemia cells does not always correlate with their drug sensitivity, and cannot give ultimate predictive answer for treatment choice. These data validate the known concept of targeting DNA repair mechanisms in therapy. Therefore, the novel observation that fludarabine-resistant cells may be susceptible to the action of DNA repair inhibitors more often than fludarabine-sensitive cells, could be of experimental and, in future, of certain clinical significance. At least, our *in vivo* studies of CLL patients' early response to fludarabine-based therapy demonstrated that vanillin *in vitro* was more often active in cells obtained from early non-responders compared with cells from early responders though cells of *in vivo* responders were more sensitive to fludarabine *in vitro* [20].

As a whole, in the current work variable association between studied genes expression in CLL cells and fludarabine sensitivity was demonstrated. Possibly additional investigations of inhibitors activity may clarify the situation. Anyway, this fact does not cancel inhibitors' synergy with fludarabine in drug resistant lymphoid cells that can be used to overcome

resistance to nucleoside analogs in treatment of lymphoid malignancies. Evidently, the usage of more individual approach to standard regimens is needed at the present moment. Therefore, direct evaluation of cellular fludarabine sensitivity and its modulation by variable stimulators and inhibitors of different signaling pathways may be more preferable than estimation of expression of genes and proteins known for their predictive significance.

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