

PHOTODYNAMIC RESPONSIVENESS OF HUMAN LEUKEMIA Jurkat/A4 CELLS WITH MULTIDRUG RESISTANT PHENOTYPE

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Background: Photodynamic therapy (PDT) is considered as a possible alternative approach to overcoming multidrug resistance (MDR). Analysis of cross-resistance to PDT in cells with different MDR pathways and resistance levels seems to be advantageous for elucidating the general mechanisms of cancer cell resistance to various treatment modalities. **Aim:** The aim of the study was to clarify whether the Jurkat/A4 leukemia cells with MDR phenotype are cross-resistant to PDT. **Methods:** Human T-cell acute lymphoblastic leukemia line Jurkat and Jurkat/A4 subline with MDR phenotype were used. 5-Aminolevulinic acid (ALA) and Photolon (a complex of chlorine-e6 and polyvinylpyrrolidone; PL) or gold nanocomposite of PL were applied as photosensitizers. The cells were pretreated with photosensitizers and exposed to laser radiation at corresponding wavelengths. The phototoxicity was assessed in trypan blue exclusion test. The hypodiploid cell fraction was analyzed by flow cytometry of propidium iodide-stained cells. Expression of genes related to PDT resistance was analyzed by microarray technique with Affymetrix U133A chips. **Results:** ALA-mediated PDT resulted in dose-dependent cell death in both lines, the relative photodynamic efficacy in Jurkat/A4 cells being inferior to that in the parental Jurkat cells. There was no correlation between phototoxicity and apoptosis induction both in Jurkat and Jurkat/A4 cells. PL-mediated general phototoxicity in Jurkat cells amounted up to 75% at the maximal photosensitizer dose with about 40% of apoptotic death fraction. PL-phototoxicity in Jurkat/A4 cells was considerably lower. In contrast to Jurkat cells, PL-gold composite did not increase the efficacy of photosensitization as compared to free PL in Jurkat/A4 cells. **Conclusions:** Multidrug-resistant Jurkat/A4 cells exhibit reduced sensitivity to phototoxic effect in comparison with parental Jurkat cells independently of nature of the photosensitizer being assayed.

Key Words: photodynamic therapy, leukemia cells, multidrug resistance, apoptosis.

Multidrug resistance (MDR) is one of the main problems limiting the efficacy of cancer chemotherapy. The predominant MDR mechanism is associated with the overexpression of certain transmembrane proteins such as P-glycoprotein (P-gp) and other members of ABC transporter family providing for the increased efflux of chemotherapeutics out of cancer cells. Several approaches seem to be useful in overcoming MDR phenotype. Among them are the design of chemotherapeutic agents with low affinity to ABC transporters, the target therapy, and the use of some physical methods of therapy.

Photodynamic therapy (PDT) is based on the application of a non-toxic photosensitizer that accumulates selectively in cancer cells. Once excited by laser-emitted light of appropriate wavelength, the photosensitizer transmits the energy to molecular oxygen with formation of singlet oxygen and other reactive oxygen species, which elicit a potent cytotoxic effect. Since the basic principle of PDT is different from that of the conventional chemotherapy, PDT has been considered an alternative approach to overcoming MDR phenotype [1, 2]. In fact, some studies showed that PDT is effective in the treatment of MDR cancer cells [1]. The cross-

resistance to chemotherapeutic drugs and several photosensitizers, in particular porphyrin derivatives has been also reported [3]. Nevertheless, in several studies cross-resistance to PDT in MDR leukemia cells has not been confirmed [4]. It should be also noted that in MDR cancer cells the advantages of PDT treatment may be associated with the mechanisms other than directly related to the P-gp overexpression.

Jurkat/A4 cells with MDR phenotype were obtained upon the treatment of human T-cell acute lymphoblastic leukemia Jurkat cells with the agonistic anti-CD95 mAb [5]. It has been demonstrated earlier that Jurkat/A4 cells are cross-resistant to apoptosis induced by a broad spectrum of clinically relevant chemotherapeutic drugs [5, 6] and to X-ray exposure [7]. Since MDR of Jurkat/A4 cells involves the mechanisms other than P-gp mediated efflux [8], analysis of PDT effects in these cells undertaken in our study seems to be of considerable interest.

Therefore, the aim of the study was to clarify whether the multidrug resistant Jurkat/A4 leukemia cells possess the cross-resistance to photodynamic treatment.

MATERIALS AND METHODS

Cells. Human T-cell acute lymphoblastic leukemia line Jurkat was obtained from the National Collection of Cell Lines of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (Kyiv, Ukraine). The Jurkat/A4 cell subline was generated earlier as previously described [5]. The cells were cultured

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Abbreviations used: ALA – 5-aminolevulinic acid; ALA-PDT – ALA-induced PDT; MDR – multidrug resistance; PDT – photodynamic therapy; P-gp – P-glycoprotein; PL – Photolon.

in RPMI-1640 medium (Sigma, USA) supplemented with 2 mM L-glutamine and 10% fetal calf serum (Sigma, USA) at 37 °C and 5% CO₂. Both cultures were passaged every 3–4 days upon reaching maximum cell density.

Photosensitizers. 5-Aminolevulinic acid (ALA) and two chlorine e₆ based formulations — Photolon (PL) (RUE Belmedpreparaty, Belarus) and its conjugate with gold nanospheres of 45 nm in diameter, were used as photosensitizers.

Photodynamic treatment and cytotoxicity. Cells in log-phase were treated with photosensitizers in Hanks' solution without phenol red. ALA was assayed at concentrations 0.1 mM, 0.5 mM and 1 mM. Cells were incubated with ALA for 4 h at 37 °C allowing for ALA conversion to protoporphyrin IX and then were exposed to the radiation of helium-neon laser LG-111 at the wavelength of 633 nm with energy density of 25 J/cm². Upon exposure to laser radiation, the cells were transferred to complete nutrient medium and cultured for 18 h at 37 °C. PL was assayed at 0.1 µg/mL, 0.25 µg/mL and 0.5 µg/mL by chlorine e₆. Cells were incubated with PL for 1.5 h, washed out thrice with non-colored Hanks' solution and exposed to semiconductor laser at λ = 658 nm with energy density of 1 J/cm². The exposed cells were transferred to the fresh medium and cultured for 18 h at 37 °C. PL-gold composite with gold nanoparticles concentration of 10 µg/mL was assayed under the same conditions as PL. Cell viability was assessed by trypan blue exclusion test.

Apoptosis estimation. Apoptosis in Jurkat and Jurkat/A4 cells was assessed by flow cytometry. The cells were resuspended in hypotonic lysis buffer containing 0.1% sodium citrate, 0.1% Triton X-100, 5 µg/ml propidium iodide. 250 µg/ml of RNase A was added to each sample, and the cells were stained for 15 min at 37 °C. Flow cytometry was performed on a BD™ FACSCalibur system (Becton Dickinson, USA). Forward and sideways light scattering provided the elimination of dead cells and debris. The fluorescence of propidium iodide-stained cells was measured. The data were analyzed using CellQuest software package (BD "Biosciences", USA). Sub-G₁ (<2*N* ploidy) cell population was considered as apoptotic. The net apoptosis percentage following subtraction of spontaneous apoptosis in non-treated cells was calculated.

Microarray analysis. The gene expression profiles were evaluated with Affymetrix U133A chips (Santa Clara, CA, USA) as previously described [9]. The protocol for processing the RNA, amplifying and labeling fragments, hybridizing material on the microarray, and scanning was similar to the standard Affymetrix protocol for GeneChip® expression analysis. Expression of the genes under study in Jurkat and Jurkat/A4 cells was compared based on our data set (MIAM-Express Database, accession number E-MEXP-530) processed with the aid of Microarray Suite software. Binary log ratios and fold changes were calculated by comparing signals in Jurkat/A4 and Jurkat cells. The expression data on *CD3G* gene encoding for T-cell specific membrane protein were given as internal con-

trol. The arbitrary 2-fold-change cutoff was set for our analysis to decide whether the gene was differentially expressed in Jurkat/A4 vs. Jurkat cells.

Statistical analysis. The data on cell phototoxicity and apoptosis were obtained in triplicate experiments. *t* test was used for statistical analysis. A value of *p* < 0.05 was accepted as statistically significant. The microarray data were analyzed with the aid of Microarray Suite software.

RESULTS

ALA as well as both chlorine e₆ compositions (PL and PL-gold composite) have been shown to be devoid of dark (without light) cytotoxicity (data not shown). The distinct concentration-dependent cell death was demonstrated in Jurkat cells pretreated for 4 h with ALA at the doses in the range of 0.1–1.0 mM followed by the exposure to radiation of helium-neon laser (Fig. 1). ALA-mediated PDT-induced cell death was also evident in Jurkat/A4 cells although the death fractions in Jurkat/A4 cells in ALA dose range used were significantly lower than in the parental Jurkat cell line (54% vs. 80% for the maximal dose).

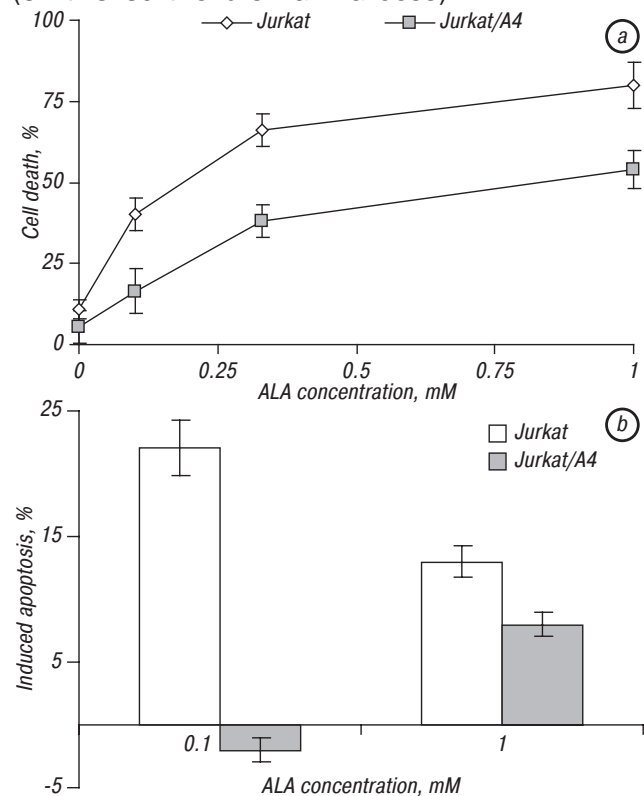


Fig. 1. Percentage of cell death (a) and induced apoptosis (b) in Jurkat and Jurkat/A4 cells pretreated with ALA and exposed to laser radiation at λ = 633 nm. Cell death was estimated by trypan blue exclusion. Apoptosis was determined by flow cytometry of propidium iodide-stained cells. Each point represents the means ± S.D. of triplicate samples

As shown in Fig. 2, both cell lines were also sensitive to PL-mediated PDT. However, the patterns of such sensitivity were different. In Jurkat cells, PL-mediated PDT-induced cell death was clearly dose-dependent while in Jurkat/A4 cells the death fraction was maximal at 0.25 µg/mL and did not increase further with increasing PL concentration. Again, as in ALA-mediated PDT tests,

the maximal death fraction in Jurkat cells was significantly higher than in Jurkat/A4 cells (77% vs. 23%).

The effects of PL as a photosensitizer were further compared to those of PL composite with gold nanospheres. PDT-induced cell death in Jurkat cells treated with PL-gold composite exceeded that PL, but in Jurkat/A4 cells, the effects of PL-gold composite were not superior to those of PL.

Therefore, Jurkat/A4 cells proved to be less susceptible to PDT-induced cell damage mediated both by ALA and PL applied in the form of two different preparations.

Then, the contribution of apoptosis into the overall Jurkat and Jurkat/A4 cell death in PDT tests was analyzed. As shown in Fig. 1, the general pattern of ALA-mediated PDT-induced apoptosis in Jurkat and Jurkat/A4 cells was similar with an absolute percentage of hypodiploid cells in both cell lines being only a small part of the total cell death fraction (13% vs. 80% in Jurkat cells and 8% vs. 54% in Jurkat/A4 cells at 1 mM ALA). As opposed to this, the pronounced apoptotic effect was evident in PL-mediated PDT tests in Jurkat and Jurkat/A4 cells with relatively high percentage of apoptotic fraction making up to at least half of the total death fraction at the similar photosensitizer concentrations (Fig. 2). The treatment with PL-gold composite tended to decrease apoptosis percentage as compared with that in PL-treated cells.

To gain insight into mechanisms of the different PDT responsiveness, gene-expression profiling studies of parental Jurkat cells and resistant Jurkat/A4 cells were performed. The data relevant to genes involved in PDT sensitivity/resistance are presented in the Table. Both Jurkat and Jurkat/A4 cell lines showed the typical feature of T-cells: high signal intensities for CD3g. Among 10 genes that had been reported previously

as been involved in sensitivity/resistance to PDT only *FECH*, *NFE2L2*, *GPX4*, *LDLR*, and *RUNX3* were expressed in studied cells at meaningful levels. However, the differences in their expression between Jurkat/A4 and parental cell line were not significant.

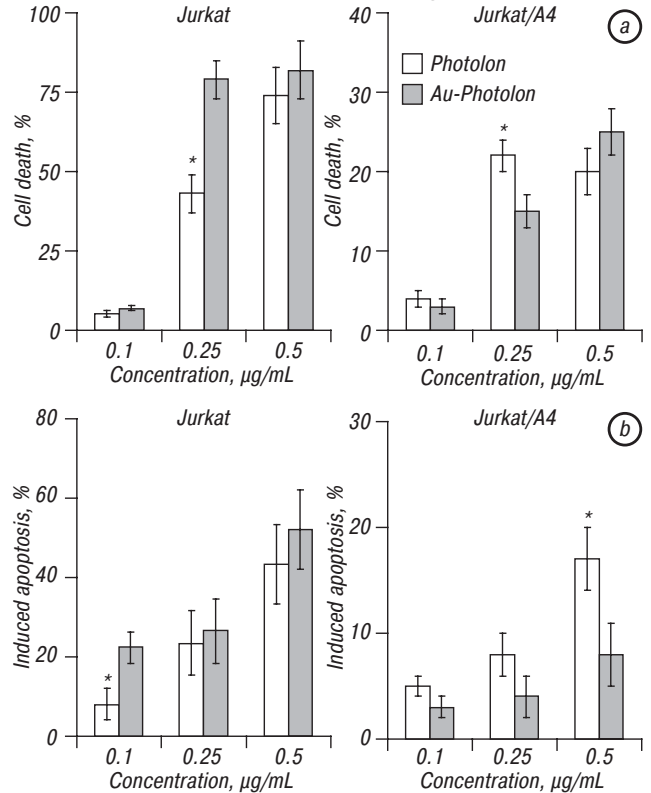


Fig. 2. Percentage of cell death (a) and induced apoptosis (b) in Jurkat and Jurkat/A4 cells pretreated with PL or PL-gold nanocomposite and exposed to laser radiation at $\lambda = 658$ nm. Cell death was estimated by trypan blue exclusion. Apoptosis was determined by flow cytometry of propidium iodide-stained cells. Each point represents the means \pm S.D. of triplicate samples. * $p < 0.05$ PL vs. PL-gold

Table. Expression of genes associated with sensitivity/resistance to PDT in Jurkat/A4 cells as compared to parental Jurkat cells*

Affix-ID	Gene symbol	Entrez gene name	Signal intensity from Jurkat/A4 cells	Signal evaluation	Fold change, Jurkat/A4 vs. Jurkat Binary log ratio	Folds	Change p value
203116_s_at	<i>FECH</i>	Ferrochelatase (protoporphyrin)	512.8	P	0.4	1.32	0.000023
			402.8	P	0.2	1.15	0.094279
209735_at	<i>ABCG2</i>	ATP-binding cassette, sub-family G (WHITE), member 2	72.5	A	0.2	1.15	0.429141
			28.1	A	-0.5	0.71	0.500000
201146_at	<i>NFE2L2</i>	Nuclear factor (erythroid-derived 2)-like 2	289.5	P	-0.1	0.93	0.757415
			295.8	P	-0.1	0.93	0.500000
201106_at	<i>GPX4</i>	Glutathione peroxidase 4 (phospholipid hydroper-	1281.8	P	0.1	1.07	0.366593
			918.9	P	-0.3	0.81	0.981872
208711_s_at	<i>CCND1</i>	Cyclin D1	18.3	A	-1.3	0.41	0.500000
			9.9	A	-1.2	0.44	0.145682
200953_s_at	<i>CCND2</i>	Cyclin D2	144.3	P	1.4	2.64	0.00013
			116.2	P	1.5	2.83	0.001077
201700_at	<i>CCND3</i>	Cyclin D3	801.0	P	-0.2	0.87	0.981872
			1907.5	P	0.3	1.23	0.232549
211607_x_at	<i>EGFR</i>	Human epidermal growth factor receptor precursor	7.6	A	-0.1	0.93	0.703911
			6.9	A	0.2	1.15	0.203871
214786_at	<i>MAP3K1</i>	Mitogen-activated protein kinase kinase 1	19.6	A	1.3	2.46	0.232549
			17.3	A	0.1	1.07	0.118009
205680_at	<i>MMP10</i>	Matrix metalloproteinase 10 (stromelysin 2)	1.9	A	-0.1	0.93	0.500000
			16.4	A	2.2	4.59	0.378880
202068_s_at	<i>LDLR</i>	Low density lipoprotein receptor (familial hyper-	132.8	P	0.5	1.41	0.105663
			434.2	P	0.2	1.15	0.500000
204197_s_at	<i>RUNX3</i>	Runt-related transcription factor 3	146.0	P	0.4	1.32	0.000101
			181.7	P	0.6	1.52	0.002032
206804_at	<i>CD3G</i>	CD3g molecule, gamma (CD3-TCR complex)	627.4	P	0.4	1.32	0.015426
			664.6	P	0.1	1.07	0.500000

*Two replicate values given for each gene were obtained independently with three-month interval. In Microarray Analysis Suite software Wilcoxon's test was used to generate detected calls; signal evaluation: when $p < 0.05$ – transcripts are present (P), when $p > 0.05$ – absent (A). A transcript was considered differentially expressed in Jurkat/A4 vs. Jurkat cells when increased or decreased more than 2.0-fold in both replicates with Log Ratio p -value threshold $p < 0.05$ for increased expression and $p > 0.95$ for decreased expression (one-sided Wilcoxon's rank test)

DISCUSSION

PDT is a modern treatment modality that may be advantageous in therapy of some forms of cancer [10]. Moreover, PDT has been considered an alternative approach to overcoming MDR phenotype [2]. Nevertheless, the mechanisms contributing to the PDT-induced death of cancer cells with MDR phenotype have not been elucidated yet. The cross-resistance of MDR cells to PDT has been also the question of controversy. It is not known to which extent the apoptosis induction contributes to PDT-induced cell death.

Two different photobiologically active substances were assayed in our PDT study. ALA is not a photosensitizer *per se* and converts to the true photosensitizer, protoporphyrin IX, in the cells by the heme biosynthesis pathway. The tumor selectivity of ALA-mediated PDT-induced cell death is believed to be associated in part with the increased accumulation of protoporphyrin IX in cancer cells due to decreased activity of ferrochelatase, a rate-limiting enzyme in heme biosynthesis pathway. PL represents the complex of chlorine e_6 photosensitizer with polyvinyl pyrrolidone at a 1:1 ratio. Thus, the substances used here as photosensitizers differed in their photophysical properties, biological mechanisms, accumulation and selectivity patterns [11]. Therefore, it seemed informative to compare PDT activity in terms of cell death and apoptosis induction in the leukemic cells studied.

Jurkat/A4, a stable subline of Jurkat cells with acquired MDR phenotype, is a useful model for studying the apoptotic resistance [5, 9, 12]. The previous studies demonstrated certain defects in realization of apoptosis in these MDR cells [6] while underlying mechanisms of such defects have not been elucidated yet. Since PDT effects of both ALA and chlorine e_6 do not seem to be associated with the mechanisms related to the P-gp overexpression [4, 13], it was of interest to analyze PDT-induced cell death and apoptosis in MDR cells possessing the cross resistance towards the broad spectrum of chemotherapeutic agents and physical factors that is not presumably determined by P-gp dependent mechanisms [8].

Since ALA-PDT demonstrated cross-resistance to chemotherapy in several cell types [4, 14], it was important to clarify whether ALA-PDT is effective in the treatment of Jurkat/A4 cells with MDR phenotype. In fact, our findings demonstrated the cross resistance of Jurkat/A4 cells to PDT-induced cell phototoxicity mediated by both ALA and PL. Nevertheless, the ratio of inferred photosensitizer concentrations resulting in the same PDT-induced cell phototoxicity in resistant Jurkat/A4 and parental Jurkat cells is many times less than the corresponding ratios in assays with various classes of chemotherapeutic agents [5]. Similarly, less than two-fold reduction of the responsiveness to ALA-mediated PDT was earlier found out in B-cell malignant Raji cells with the acquired six-fold resistance to doxorubicin [15].

We have also assayed photodynamic efficacy of PL nanocomposite with colloid gold taking into account the tropism of the colloid gold to cancer cells [16]. It was earlier shown that conjugation with gold nanoparticles

increases the photodynamic effects of PL and hemo-toporphyrin in leukemic cells [17, 18]. Therefore, it was interesting to find out whether such activation explained usually by a better transportation of drugs into the treated cells [19] would affect the photodynamic responses of MDR leukemic cells. As it is seen from Fig. 2, in our experiments with Jurkat/A4 cells, the activity of PL-gold composite was not superior to that of free PL.

The mechanisms of the cell death induced by two studied photosensitizers were analyzed. Whereas the contribution of apoptosis in ALA-mediated phototoxicity was small, PL-mediated death of Jurkat and Jurkat/A4 cells was characterized by relatively large apoptotic fraction suggesting greater role of apoptosis in PL-mediated as compared with ALA-mediated phototoxicity.

The involvement of several genes such as *FECH*, *ABCG2*, *NFE2L2*, *GPX4*, *CCND1*, *EGFR*, *MAP3K1*, *MMP10*, *LDLR*, and *RUNX3* into sensitivity/resistance to PDT has been recently proposed [20–22]. In our microarray study, only five genes from the list of those supposedly involved into PDT resistance, namely *FECH*, *NFE2L2*, *GPX4*, *LDLR*, and *RUNX3*, were expressed at meaningful levels, although the differences in their expression between Jurkat/A4 and parental cell line were not significant. Recently, cyclin D1 involvement [23] was found to be relevant to the development of squamous cell carcinoma resistance to PDT, but we have not detected *CCND1* expression in both Jurkat and Jurkat/A4 cell lines. We hypothesized that other D-type cyclins may substitute for cyclin D1 in leukemia cells under study. Indeed, both cell lines expressed high levels of *CCND2* and *CCND3*. Furthermore, the expression of *CCND2* but not *CCND3* increased significantly in the Jurkat/A4 vs. Jurkat cells. As cyclin D2 is commonly overexpressed in hematological malignancies [24, 25], it would be intriguing to investigate the possible role of *CCND2* in PDT resistance of leukemia cells.

To sum up, Jurkat/A4 cells with MDR phenotype turned out to be moderately cross-resistant to PDT-induced cell death and apoptosis irrespective of whether ALA or PL was used as a photosensitizer. However, the relative contribution of apoptotic cell death into the overall phototoxicity of these two substances differs considerably. The further analysis of PDT cytotoxicity in susceptible and resistant leukemic cells may be useful for elucidating the underlying general mechanisms of PDT effects and phenomenon of cross resistance.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest.

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