

EMOXIPINE MODULATES CONCENTRATION-DEPENDENT EFFECTS OF CYTARABINE AND CYCLOCYTIDINE ON ACTIVATION OF HUMAN T CELLS

ABSTRACT

Title. Emoxipine modulates concentration-dependent effects of cytarabine and cyclocytidine on activation of human T cells.

Introduction. Both cytarabine and cyclocytidine are used in the treatment of acute myeloid leukemia. Well known that cytarabine and other related cytosine-based nucleoside analogues are being toxic to tumor cells by increasing levels of cellular oxidative stress as it could be abrogated by antioxidants. However, very little is known both about both the effects of combinations of antimetabolites with antioxidants on the cytotoxic innate and adaptive immune cells and whether lymphocytes toxicity affects its anticancer efficiency.

Aim. To estimate effects of cytarabine and cyclocytidine with emoxipine on *in vitro* activated human T cells at concentrations reached during *in vivo* treatment with high doses, conventional doses and low doses.

Materials and methods. T cells derived from blood donors were activated *in vitro* in cell culture medium alone or supplemented with cytarabine 0.1-10.0 μM or cyclocytidine 0.1-10.0 μM . Cell characteristics were assessed by flow cytometry.

Results. Only cytarabine 1.0-10.0 μM had both antiproliferative and proapoptotic effects. Additionally, these cytarabine concentrations increased the γIFN -producing by $\text{CD3}^+\text{CD4}^+$ T cells and did not affect the release of this cytokine by $\text{CD3}^+\text{CD8}^+$ T cells. In contrast, the lowest concentration (0.1 μM) did not have or showed minor antiproliferative or cytotoxic effects, did not alter the release of γIFN . Cyclocytidine did not affect viability of normal peripheral blood mononuclear cells but decreased the proliferative capacity of activated normal T cells in dose-dependent manner. Additionally, cyclocytidine altered the percentage of γIFN -producing proliferative $\text{CD3}^+\text{CD8}^+$ cytotoxic T cells for any concentration tested (0.1, 1.0, 1 and 10.0 μM) meanwhile highly suppressed the number of the whole amount of $\text{CD3}^+\text{CD8}^+$ cells and did not affect the release of cytokines by $\text{CD3}^+\text{CD4}^+$ T cells.

The study of the expression of the CD107a marker showed a significant stimulating effect of 10 μM of cytarabine on the activation of subpopulations of T-lymphocytes (CD3^+) and cytotoxic T-lymphocytes ($\text{CD3}^+\text{CD8}^+$).

Keywords: cytarabine; cyclocytidine; oxidative stress; emoxipine; T cells

1. INTRODUCTION

One of the most important groups of drugs in oncology is cytosine-based nucleoside analogs. Cytarabine (ara-C) is an antitumor drug based on pyrimidine nucleoside and

arabinose, widely used in the treatment of leukemia. Ara-C is mainly used in acute promyelocytic leukemia (APL), non-Hodgkin's lymphoma (NHL) and chronic myelocytic leukemia [1]. Ara-C can be used alone or in combination with other antitumor agents such as daunorubicin, doxorubicin, thioguanine or vincristine. Ara-C acts on rapidly dividing cells and inhibits DNA synthesis in the S-phase of the cell cycle, as well as prevents the transition of cells from the G1 phase to the S-phase. The exact mechanism of action of cytarabine triphosphate has not been clarified, but it appears to inhibit DNA polymerase by competing with deoxycytidine triphosphate [8]. In addition to antitumor activity, CIT also has a toxic side effect.

Cyclocytidine is an anhydro form of ara-C which resists enzymatic deamination due to undergoing slow hydrolysis to ara-C what allows effective plasma levels of ara-C to be maintained for 6 hours following cyclocytidine administration [1]. Although in recent years progress has been made in improving cancer therapy, the treatment for each patient remains a complex problem, in this case for selecting optimal personalized treatment approach needs to be taken into account numerous parameters of the patient's condition and course of malignant process [2-7]. Last years the phenomena of involvement of the immune system in the implementation of the antitumor effect of cytostatic therapy have been experimentally identified, theoretically justified and proved on clinical material [8-10]. Chemotherapeutic drugs can enhance an immune response of the host against the tumor in addition to killing cancer cells by direct cytotoxicity. The immune system makes a crucial contribution to the effectiveness of treatment, involving mechanisms of innate and adaptive immunity and a wide range of cytokines that provide cytotoxic effects on tumor cells. Numerous preclinical and clinical studies have shown that the effectiveness of many chemotherapy drugs depends on the preservation of the functional well-being of the immune system [11-13].

An important aspect limiting the use of cytostatic drugs is that these drugs have undesirable side effects due to possible impact on the host cell's genetic apparatus. In this regard, it is attractive to search for substances or their combinations (with antioxidants, in particular), the use of which will lead to a decrease in intoxication [14]. The widespread use of antioxidant drugs in clinical practice is hindered by their insufficiently studied interaction with elements of the modern generally accepted scheme of complex chemotherapeutic treatment of cancer patients. And although a number of studies reflect the possibility of increasing the antitumor effectiveness of individual cytostatics when they are combined with antioxidants,

Numerous original research articles have focused on the topic of whether supplemental antioxidants administered during chemotherapy can protect normal tissue without adversely influencing tumor damage. Due to variation in study design, intervention protocol, type of cancer, timing of observation, inclusive criteria, statistical analysis, and chemotherapy scheme develops uncertainty to make definitive conclusion regarding the risk of decreased tumor control because of administering supplemental antioxidant during chemotherapy. Previous *in vitro* studies have shown that cytarabine and other related cytosine-based nucleoside analogues are being toxic to tumour cells by increasing levels of cellular oxidative stress as it could be abrogated by antioxidants [15]. On the contrary recent review definitely concludes that that antioxidant when given concurrently (a) do not interfere with chemotherapy, (b) enhance the cytotoxic effect of chemotherapy, (c) protects normal tissue and (d) increases patient survival and therapeutic response [16-18]. The results obtained cannot be transferred to all components of complex chemotherapeutic treatment and need further detailed studies concerning specific schemes of combined use.

However, very little is known both about both the effects of combinations of antimetabolites with antioxidants on the cytotoxic innate and adaptive immune cells and whether lymphocytes toxicity affects its anticancer efficiency.

In the present study we therefore investigated the *in vitro* effects of various cytarabine and cyclocytidine concentrations, and emoxipine on activated T cells.

2. MATERIAL AND METHODS

2.1 Cell donors and preparation of peripheral blood mononuclear cells

The studies have been conducted according to the rules of the local Ethics Committee (Committee on Bioethics of the Republic of Belarus) and buffy coats were derived from healthy blood donors after informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation (Histopaque-1077; «Sigma», Germany; specific density 1.077) from buffy coats from 15 healthy blood donors (median age 39 years; 7 male and 8 female) as reported in [19].

2.2 Drugs

Cytarabine (cytosine β -D-arabinofuranoside) and cycloctidine (cytosine 2,2'-anhydro- β -D-arabinofuranoside) were synthesized as described in our previous research [20].

Cytarabine, cycloctidine, and emoxipine were dissolved in ddH₂O to obtain a concentration of 10 mM before aliquoted. All drugs were stored at -80 °C. Drugs were thawed on the same day they were used in experiments and based on studies of *in vivo* levels the drugs were tested at the following concentrations that are relevant to low-toxicity treatment: cytarabine and cycloctidine 0.1 μ M [21-23] and at 10 μ M and 1 μ M corresponding to high-dose therapy [24, 25]. Emoxipine was added always at equimolar concentrations.

2.3 Cell culture

PBMC were suspended in pre-warmed medium RPMI-1640 (Bio-Whittaker, USA), with 10% FBS (Gibco, Germany), 2 mM L-glutamine (Bio-Whittaker, USA) [19], 1% antibiotic-antimycotic solution (Gibco, Germany) alone or supplemented with cytarabine 0.1-10.0 μ M or cycloctidine 0.1-10.0 μ M and cultured in 96-well culture plates at a final concentration of 2.0×10^5 cells/well during 48 hours (viability and proliferation analyses), or during 72 hours (intracellular γ IFN production analysis) or at a final concentration of 1.0×10^7 cells/mL during six days (mitogen-induced proliferation analysis).

T lymphocytes were activated with 2.5 μ g/mL of phytohemagglutinin (PHA, Sigma, Germany). Drugs were prepared from frozen stock solutions the same day as the experiments. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ before cells/supernatants were harvested [19].

2.4 Flow cytometric analysis of viability, proliferation and γ IFN production

Flow cytometry was performed by Cytotflex (Beckman Coulter, USA). For each sample at least 10 000 cells were counted [26]. All results were analyzed by CytExpert Software (VWR International, LLC, USA).

2.5 Viability and proliferation assay

PBMC dissolved in PBS were stained strictly according to the manufacturer's instructions in the Annexin A5 FITC/7-AAD Kit (BeckmanCoulter, CША); thereafter cells were washed and cultures prepared as described above. The cells were harvested after 48 hours and stained for 15 minutes with 10 μ L Annexin A5-FITC and 20 μ L 7-AAD. Cells were further incubated for 15 minutes in dark and thereafter washed in ice-cold 1% BSA/PBS before two-color flow cytometric analysis.

To assess the proliferative capacity after stimulation with PHA cells were stained with 7 μ M carboxyfluorescein succinimidyl ester (CFSE, Fluka, Germany) for 5 minutes in dark at room temperature. The staining reaction was stopped by 2-fold centrifugation in a cold medium

RPML-1640 with 25 mM HEPES, 2 mM L-glutamine, 1% streptomycin-penicillin-neomycin and 10% inactivated FBS [27].

The number of proliferating and non-dividing T-cell subpopulations was registered on the 6th day of culture by flow cytometry using PC7-conjugated anti-CD3, PC5-conjugated anti-CD8 (BeckmanCoulter, USA). The proliferation of T-lymphocytes and their subpopulations was estimated as the percentage of non-dividing (CFSE_{high}) and proliferating (CFSE_{low}) T-cells.

2.6 Intracellular γ IFN production assay

Intracellular γ IFN production assay was performed as reported in [28]. Briefly, spontaneous and PHA-induced intracellular production of γ -interferon (γ IFN) was evaluated after 72 hours of cells culture. To quantify the level of intracellular production of γ IFN, 10 ng/mL of phorbol 12-myristate 13-acetate (Sigma, Germany), 1 μ g/mL of calcium salt of ionomycin (Cayman Chemicals, USA), and 10 μ g/mL of brefeldin A (Cayman Chemicals, USA) were added 4 hours before the end of cultivation. Thereafter the following anti-human antibodies were added: PC7-conjugated anti-CD3, PC5-conjugated anti-CD8 (BeckmanCoulter, USA). Cells were fixed for 10 minutes with a 4% solution of *p*-formaldehyde in saline. Cells were further centrifuged for 5 minutes at 1500 rpm and thereafter PE-conjugated anti- γ IFN (Beckman Coulter, USA) was added.

2.7 Cytotoxicity assay

Cytotoxic capacity of PBMC was evaluated with target human tumor cell line K562. K562 target cells were labeled with CFSE at 7 μ M concentration to discriminate target cells from effector cells. Then, effector cells (PBMC) were incubated with CFSE labeled K562 target cells at effector-to-target (E : T) ratio 5 : 1 in 96-well plates. The cells were cultured in 150 μ L culture media with interleukin-2 (IL-2, Fluka, Germany) as cytotoxicity stimulator alone or supplemented with cytarabine 1.0 mM or cycloctidine 1.0 mM or its combination with emoxipine 1 mM. After coculture for 4 hours at 37 °C, 5% CO₂, the cell mixture was stained with 5 μ L of propidium iodide (PI, Invitrogen, Germany) for 15 min in the dark. The non-viable tumor cells were identified as CFSE+PI+K562 cells. PBMC cytotoxicity was calculated as the ratio of the percentage of cell death K562 in co-culture with IL-2-stimulated PBMC to non-stimulated cells [29].

2.8 CD107a Degranulation Assay

CD107a expression on effector cells (PBMC or NK cells) was measured to analyze lymphocytes degranulation. Lymphocytes were incubated with or without K562 cells as described above. Following a 4-hour culture, cell mixture was stained with monoclonal antibodies against CD8-FITC, CD107a-PE, CD3-APC, and CD56-PC7 (R&DSsystems, Beckman Coulter, USA). The NK and T cells were gated as CD56⁺ and CD3⁺ cells respectively, and cytotoxic T lymphocytes were further enumerated as CD3⁺CD8⁺ cells. To determine the CD107a expression of cells, CD107a positive rate of effector cells was analyzed [30].

2.9 Statistics

Statistical analysis performed using R Statistical Software (Foundation for Statistical Computing, Vienna, Austria). The median, lower and upper percentiles (25th and 75th percentiles) were used for descriptive statistics of the study groups. The statistically significant differences between the compared groups were determined using nonparametric U-Mann-Whitney criterion and Wilcoxon criterion. The differences were considered statistically significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

Cycloctidine do not affect viability of normal peripheral blood mononuclear cells, whereas cytarabine has a small, but statistically significant, antiproliferative and proapoptotic effect

PBMC derived from healthy blood donors ($n = 15$) were cultivated *in vitro* during 48 hours in medium alone or medium supplemented with cytarabine 0.1-10.0 μM or cycloctidine 0.1-10.0 μM . The viability (Figure 1; Annexin-AAD assay) of PBMC were then analyzed by flow cytometry. Cycloctidine did not cause any statistically significant alteration of peripheral blood mononuclear cells viability.

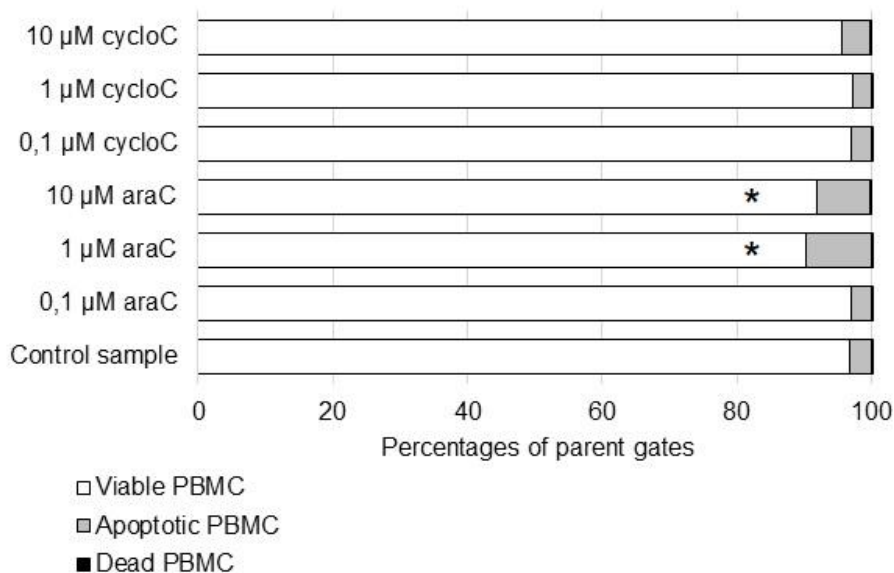


Fig. 1. Viability of peripheral blood mononuclear cells after exposure to cytarabine or cycloctidine

*Significant from normal control, * $P < 0.05$*

A small, but statistically significant, decrease in viability was detected after exposure to cytarabine (1.0-10.0 μM).

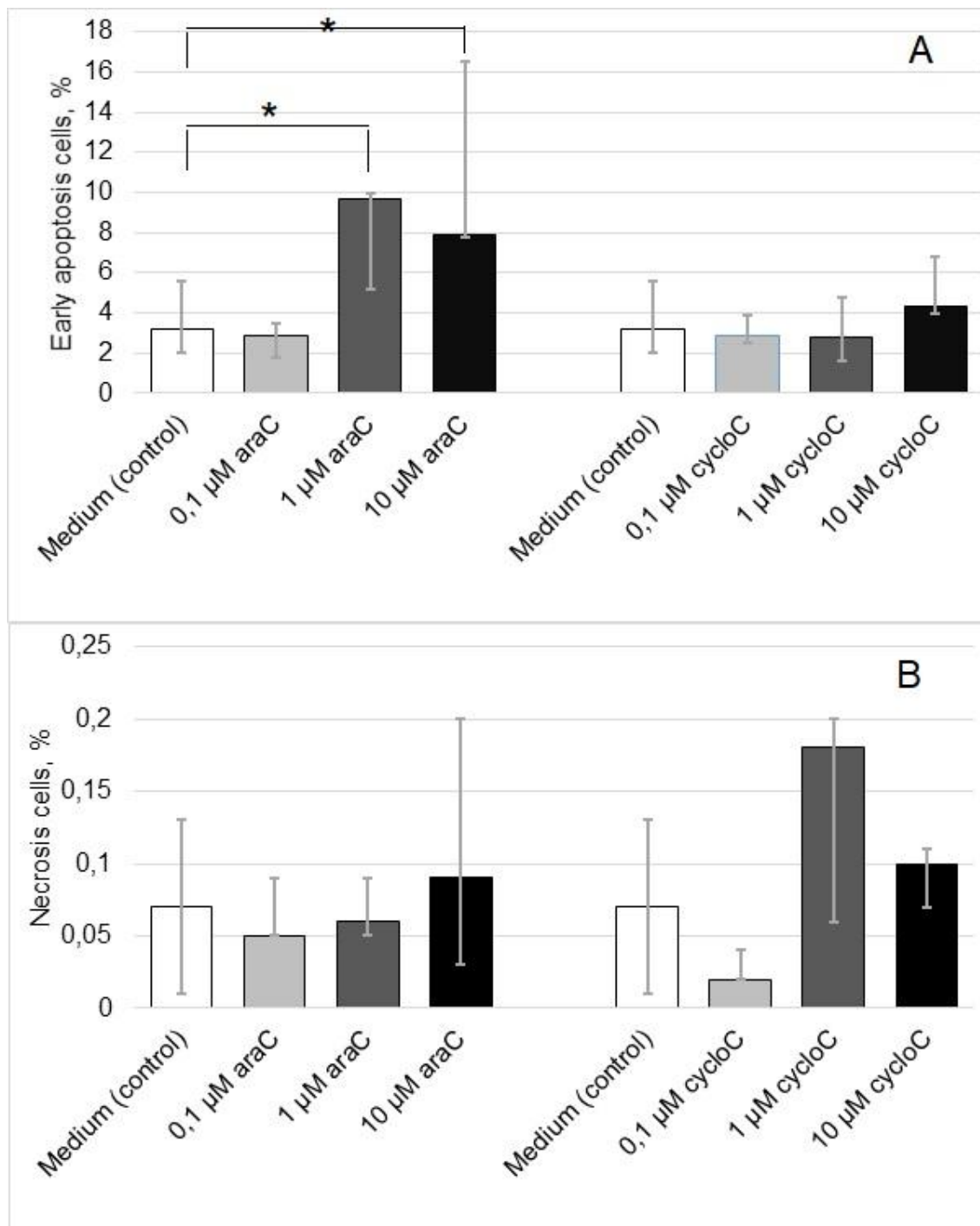


Fig. 2. Number of apoptotic (A) and necrotic (B) cells (%) after exposure to cytarabine or cycloctidine

*Significant from normal control, * $P < 0.05$*

An increased fraction of early apoptotic cells was then detected together with the decreased viability in the cytarabine-containing cultures (Figure 2A); an observation suggesting that the

decreased viability is caused by drug-induced apoptosis. Cycloctidine at 0.1-10.0 μM concentrations did not cause any statistically significant alteration on PBMC viability.

Both cytarabine and cycloctidine affect proliferative capacity of activated normal T cells in dose-dependent manner

PBMC derived from healthy blood donors ($n=15$) were activated *in vitro* culture with anti-CD3 plus anti-CD8 during six days of culture in medium alone or medium supplemented with cytarabine 0.1-10.0 μM or cycloctidine 0.1-10.0 μM .

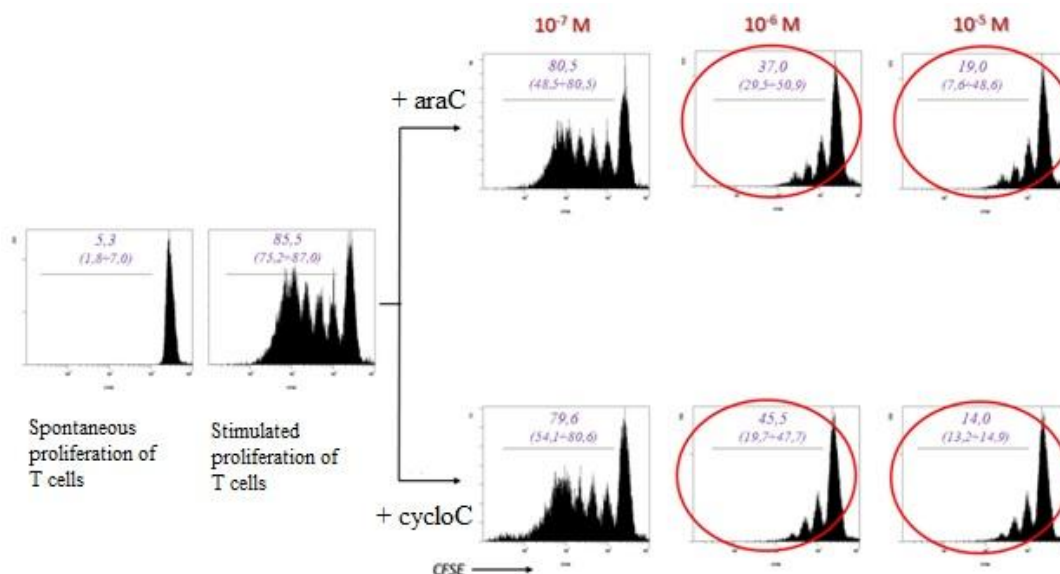


Fig. 3. Proliferation of activated T lymphocytes after exposure to cytarabine or cycloctidine

The proliferation (Figure 3; the CFSE proliferation assay) of $\text{CD3}^+\text{CD4}^+$ and $\text{CD3}^+\text{CD8}^+$ T cells were then analyzed by flow cytometry. Both cytarabine and cycloctidine at 1.0-10.0 μM concentrations showed a dose-dependent suppressive effect on stimulated T cell proliferation. A strong and statistically significant decrease in proliferation from 85.5 (75.2-87.0)% to 19.0 (7.6-48.6)% was detected after exposure to 10.0 μM cytarabine. Similarly, when cycloctidine at a higher concentration of 10.0 μM was present in medium the decrease in proliferation from 85.5 (75.2-87.0)% to 14.0 (13.2-14.9)% was detected. The suppressive effect on stimulated T cell proliferation was shown both for $\text{CD3}^+\text{CD4}^+$ T helper lymphocytes as well as $\text{CD3}^+\text{CD8}^+$ cytotoxic T cells (Figure 4).

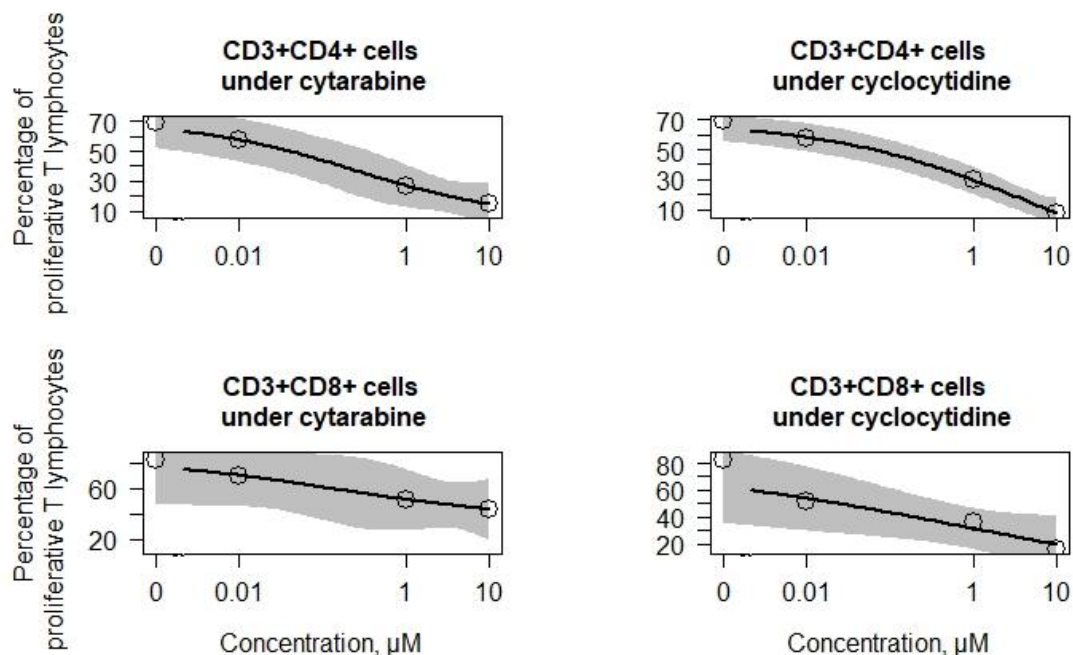


Fig. 4. Proliferation of activated subpopulations of T lymphocytes after exposure to cytarabine or cycloctidine

The suppression of stimulated proliferation of peripheral blood lymphocytes, be it observed, is developed against the background of a pronounced cytopathic effect of cells, manifested in 97.4-98.8% of cell death; an observation suggesting that the decreased viability is caused by activation-induced apoptosis.

The intracellular γIFN production by stimulated T cells is altered by both cytarabine and cycloctidine

Normal PBMCs derived from 15 healthy individuals were cultivated *in vitro* during 72 hours in medium alone or medium supplemented with drugs. Production of γIFN was not altered for non-stimulated cultures containing various concentrations of cytarabine and cycloctidine. Only after PHA-stimulated activation the number of γIFN -producing CD3^+ T cells showed an expected dose-dependent reduction caused by cytarabine (0.1, 1.0 and 10.0 μM) but not by cycloctidine in general.

The response of γIFN -producing $\text{CD3}^+\text{CD4}^+$ T helper lymphocytes and $\text{CD3}^+\text{CD8}^+$ cytotoxic T cells on the exposure to cytarabine or cycloctidine differed. The percentage of proliferative $\text{CD3}^+\text{CD4}^+$ T cells in total fraction of PBMCs reduced to 16.5% of control after exposure to 10.0 μM cytarabine whereas the number of γIFN -producing $\text{CD3}^+\text{CD4}^+$ T cells showed a high reduction to 61.2% of control after exposure to the lowest 0.1 μM concentration of cytarabine. Next, the intracellular γIFN production by stimulated $\text{CD3}^+\text{CD4}^+$ T cells after exposure to 1.0-10.0 μM cytarabine is altered not so dramatically: to 49.8 and 43.6% of control respectively (Figure 5). Thus the $\text{CD3}^+\text{CD4}^+\gamma\text{IFN}^+ : \text{CD3}^+\text{CD4}^+$ ratio was significantly increased by cytarabine from 0.7 to 2.6 in dose-dependent manner.

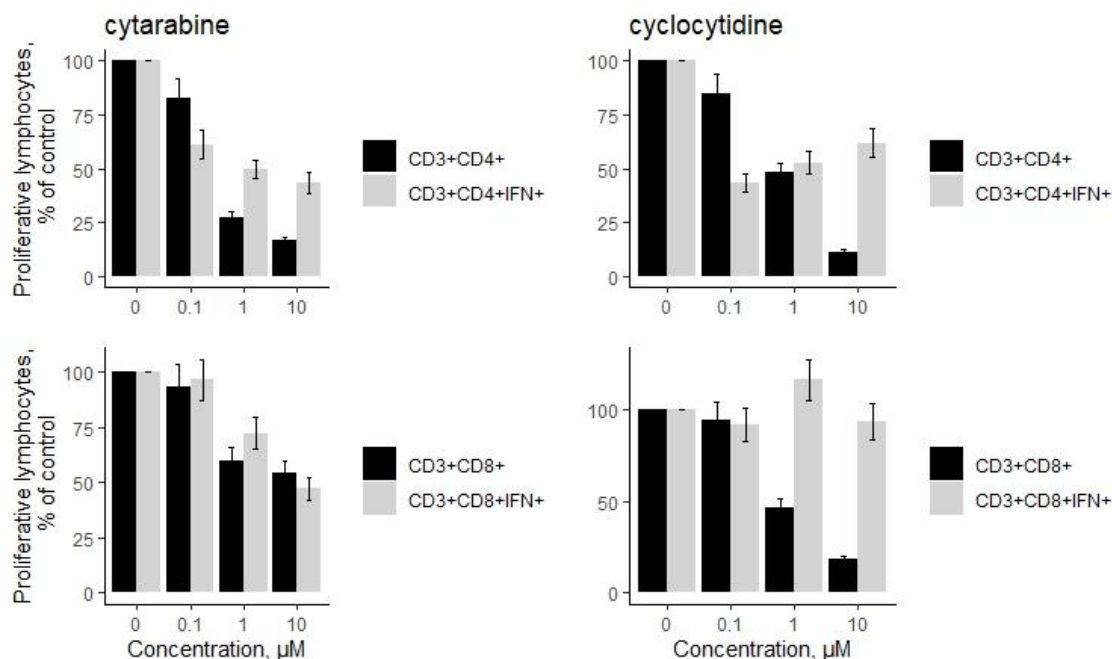


Fig. 5. Intracellular γIFN production of activated subpopulations of T lymphocytes after exposure to cytarabine or cycloctidine

Cycloctidine had a similar increasing effect on the $\text{CD3}^+\text{CD4}^+\gamma\text{IFN}^+ : \text{CD3}^+\text{CD4}^+$ ratio but only when testing the highest concentration (10.0 μM).

Contrariwise the percentage of γIFN -producing $\text{CD3}^+\text{CD8}^+$ cytotoxic T cells after the exposure to cytarabine is altered as much as the whole population of cytotoxic T cells and the $\text{CD3}^+\text{CD8}^+\gamma\text{IFN}^+ : \text{CD3}^+\text{CD8}^+$ ratio is not changed. In contrast, cycloctidine altered the percentage of γIFN -producing proliferative $\text{CD3}^+\text{CD8}^+$ cytotoxic T cells for any concentration tested (0.1, 1.0, 1 and 10.0 μM) meanwhile highly suppressed the number of the whole amount of $\text{CD3}^+\text{CD8}^+$ cells. Therefore, the $\text{CD3}^+\text{CD8}^+\gamma\text{IFN}^+ : \text{CD3}^+\text{CD8}^+$ ratio was significantly increased by cycloctidine from 0.97 to 5.1 in dose-dependent manner.

Emoxipine affect the cytotoxicity of non-activated human T cells under cultivation with cytarabine

For evaluating the effects of cytarabine and cycloctidine and their combination with emoxipine on spontaneous and IL-2-stimulated cytotoxicity of lymphoid cells in relation to the K562 tumor cell line, the percentage of dead K562 cells detected as CFSE+PI+K562 cells was used.

Cytarabine inhibits the unstimulated cytotoxicity of PBMCs by 2.1 times ($p < 0.05$), and its combination with emoxipine weakens this inhibitory effect by 57.6%. Cycloctidine, as well as its combination with emoxipine, does not affect cytotoxicity under experimental conditions. Evaluation of the effects of cytarabine and cycloctidine and their combinations with emoxipine on the IL-2 stimulated cytotoxicity of PBMCs did not reveal statistically significant differences, but there was also a trend in the inhibitory effect of the compounds (Table 1).

Table 1. Number of non-viable CFSE+PI+K562 (%) in co-cultures with unstimulated and IL-2-stimulated peripheral blood mononuclear cells after exposure to cytarabine or cycloctidine and emoxipine (data presented as median, 25%-75% quantile)

Compound	Spontaneous cytotoxicity	IL-2 stimulated cytotoxicity
Control	19,7 (15,6÷22,0)	41,1 (40,0÷43,2)
Cytarabine	9,2 * (7,0÷10,7)	38,8 (36,2÷42,4)
Cyclocytidine	18,8 (15,2÷21,7)	39,9 (38,7÷41,1)
Emoxipine	19,7 (16,8÷19,9)	38,3 (37,1÷39,8)
Cytarabine with emoxipine	14,5 (5,8÷32,9)	39,3 (34,3÷44,1)
Cyclocytidine with emoxipine	21,5 (15,0÷26,3)	38,4 (35,6÷42,6)

Significant from normal control, * $P < 0.05$

Co-cultured cells were incubated for 4 hours with 10^{-6} M cyclocytidine or cytarabine separately or together with 10^{-6} M emoxipine. Each value represents the median, 25th, and 75th percentile of dead K562 cells, calculated as a % of the total cell population.

Cytarabine significantly increased IL-2-stimulated CD107a expression for CD3⁺ T-lymphocytes (by 2.1 times) and cytotoxic CD3⁺CD8⁺ T-lymphocytes (by 47.5%), but not for natural killer cells (CD56⁺). The observed effect increased after emoxipine was added to the culture mixture.

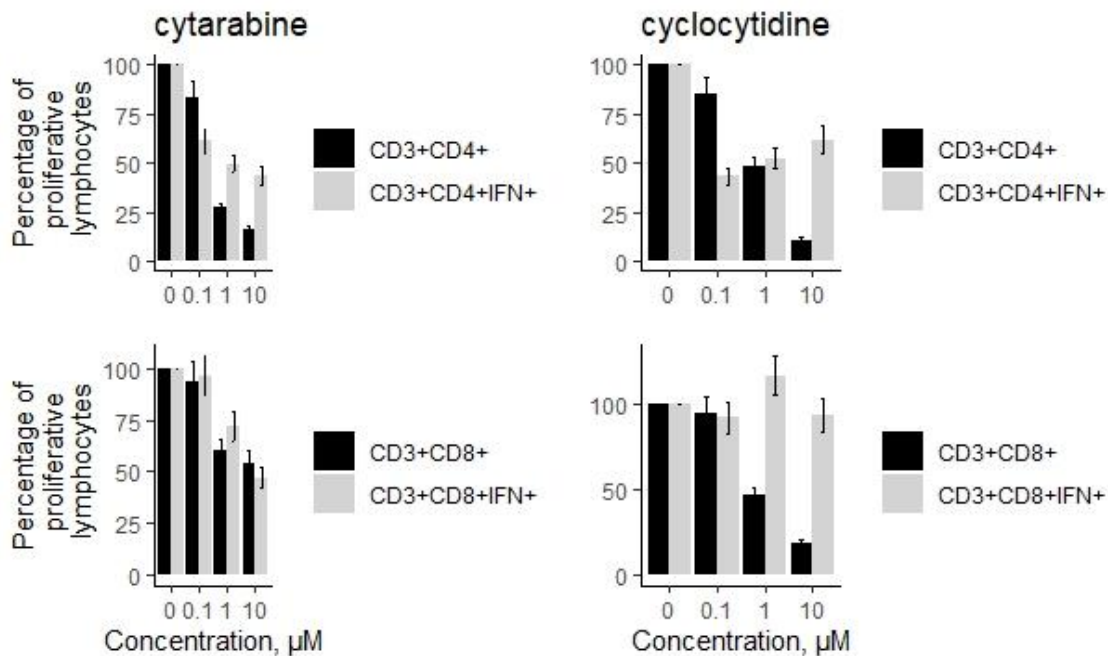


Fig. 6. Percentage (compared to control: PBMC + K562) of CD107a⁺ lymphoid cells in co-culture with K562 cell line when cultured after exposure to cytarabine or cyclocytidine with/without emoxipine

Significant from normal control, * $P < 0.05$

Thus, when co-culturing cells with 10^{-6} M cytarabine and 10^{-6} M emoxipine, the specific weight of CD3⁺CD107a⁺ cells increased by 3.5 times compared to the control (MPC+K562), and in the case of CD3⁺CD8⁺CD107a⁺ cells-by 2.0 times (Figure 6).

4. DISCUSSION

It is known that anticancer therapy is accompanied by significant side effects, which significantly affects the course, prognosis and effectiveness of treatment of diseases [31, 32]. Many of them are directly related to the processes of free radical oxidation, which significantly increase during chemotherapy of tumors. Thus, a violation of the balance between free radicals and the antioxidant system can increase intoxication and even lead to toxic damage to healthy organs and tissues, which turns out to be a limiting factor in deciding whether to continue therapy. It should be taken into account that the processes of lipid peroxidation have already been activated in oncological patients [33]. However, the widespread use of antioxidant drugs in clinical practice is hindered by their insufficiently studied interaction with elements of the modern generally accepted scheme of complex chemotherapeutic treatment of cancer patients [34-36]. And although a number of studies suggest the possibility of increasing the antitumor effectiveness of individual cytostatics when they are combined with antioxidants, the results obtained cannot be transferred to all components of complex chemotherapeutic treatment and need further detailed studies concerning specific schemes of combined use [37, 38]. In the present work, a strong antioxidant emoxipine was used to protect healthy non-malignized cells when antimetabolites were exposed to tumor culture. Peripheral blood lymphocytes were used as a model of healthy cells, which, along with this, were a source for subsequent modeling of the immune response to a tumor. At the same time, results were obtained indicating the possibility of using antioxidants both to protect healthy cells from death under conditions of oxidative stress caused by antitumor chemotherapy, and as a modulator of antitumor activity of cytotoxic T-lymphocytes.

5. CONCLUSION

In the present study, neither modified nucleosides nor their combination with emoxipine caused changes in the IL-2-stimulated cytotoxicity of lymphoid cells in relation to the K562 tumor cell line, which does not contradict the known data. However, by the expression of the CD107a marker, we were able to prove a pronounced effect of cytarabine on the activation of a subpopulation of T-lymphocytes (CD3⁺) and cytotoxic T-lymphocytes (CD3⁺CD8⁺), which was potentiated by emoxipine.

The obtained results indicate the possibility of direct protection of cytotoxic lymphocytes from death under conditions of oxidative stress caused by antitumor chemotherapy with the antioxidant emoxipine. Cycloctidine did not have a pronounced effect, while in combination with emoxipine, there was a tendency to increase the expression of CD107a on CD3⁺ T-lymphocytes (by 69.6%) and cytotoxic CD3⁺CD8⁺ T-lymphocytes (by 43.4%).

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. the products used for this research are commonly and predominantly use products in our area of research and country. there is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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