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Biological Evaluation of a Novel 2'-Fluoro Derivative 5-Azacytidine as a Potent DNA Methyltransferase Inhibitor

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Abstract

Background: DNA methyltransferases (DNMTs) are key epigenetic regulatory enzymes involved in the expression of many genes and are considered as an attractive target for cancer treatment, especially hematological malignancies. Therefore, promising DNMT inhibitors characterized by low toxicity, target activity and high selectivity are crucial for the development of new cancer therapy and research on the inhibitory mechanism. We had previously demonstrated that the novel 2'-fluoro-2'-deoxy-arabinofuranosyl 5-azacytosine nucleoside (2'F-araAC) showed high antiproliferative activity *in vitro* and increased hydrolytic stability compared to the known agents like azacitidine and decitabine. **Objective:** The objective of the present study was to investigate the effect of novel 2'F-araAC as potent anti-leukemia agent and DNMTs inhibitor on nuclear extract of the HCT-116 human colorectal cell line and P388 and L1210 mouse leukemia cell lines. **Methods**: The DNMTs activity was evaluated using the fluorometric DNMT Activity Quantification Kit (Abcam) and were reported as the percentage of control. Nuclear proteins were extracted from HCT-116 cell line using the Nuclear Extraction Kit (Abcam). To explore the mechanism of anti-leukemic activity of 2'F-araAC, cell cycle and apoptosis analyses were performed on P388 and L1210 cell lines. **Results:** It has been shown that the DNMTs activity was significantly reduced at 1 and 10 μM of 2'F-araAC compared to controls. Moreover, 2'F-araAC can induce G2/M cell cycle arrest and apoptosis in P388 and L1210 mouse leukemia cell lines as shown by flow cytometry method. Apoptosis was 54.53% and 43.35% for 2'F-araAC vs. 2.88% and 5.25% for the control P388 and L1210 cell lines, respectively. **Conclusions:** Thus, our study presents a new and promising compound to further develop new epigenetic regulators to be used as antitumor agents.

Keywords

Drug Discovery, Decitabine, 2'-Fluorodeoxy-Arabino Analog,
DNA Methyltransferase, Enzyme Inhibition, Cell Cycle, Apoptosis

1. Introduction

Myelodysplastic syndromes are a heterogeneous group of blood cell disorders arising at the hematopoietic stem cell level, which are manifested by cytopenias due to ineffective hematopoiesis, myeloid cell dysplasias and the risk of transformation to acute myeloid leukemia (AML) [1]-[3]. The use of agents affecting pathogenetic processes (DNA hypermethylation) represents one of the most promising strategies to treat myelodysplastic syndrome (MDS) [4]-[7]. The significance of DNA methylation is emphasized by the growing number of human diseases. Abnormal DNA methylation associated with increased DNMT expression or activity has been found in many different diseases, especially cancer. DNMT inhibition can contribute to demethylation and silent gene expression.

The addition of methyl groups is implemented by the DNMT enzyme family. Three DNMTs (DNMT1, DNMT3A, and DNMT3B) are requisite to establish and maintain DNA methylation patterns [8] [9]. Two additional enzymes (DNMT2 and DNMT3L) may also have more specialized, but related functions. DNMT1 is likely responsible for maintaining established DNA methylation patterns, whereas DNMT3A and DNMT3B are likely to mediate the establishment of new or *de novo* DNA methylation patterns. DNMT3L has been found to be a catalytically inactive regulatory factor of DNA methyltransferases necessary for DNMT3A and DNMT3B functioning. In cancer cells, not only DNMT1 can be responsible for maintaining abnormal gene hypermethylation but also DNMT3B can share this function. In mammalian cells, methylation is commonly triggered in the DNA CpG sections at the C-5 position of the cytosine residue. The methylated CpG sites specifically arranged form the DNA methylation profile [8] [9]. The methylation profile is established *de novo* by DNMT3A and DNMT3B and supported in each round of replication by Dnmt1 [10]-[12]. Many tumors show CpG site hypermethylation in the promoter regions of tumor suppressor genes and genes responsible for DNA repair. This results in the inactivation of these genes, as well as in common DNA hypomethylation causing genome instability [13]-[15].

Patients with MDSs have been found to show increased methylation of the p15INK4b gene encoding a protein inhibitor of cyclin-dependent kinases 4 and 6, which regulates the passage of G1 phase of the cell cycle [16]-[18]. Methylation of this gene is noted in more than 50% of cases, mostly in patients with elevated bone marrow blast cell levels (RAEB). In addition to the p15 gene, the genes encoding the CDH1, HIC1 and ER proteins are in the methylated state [19]. DNA methyl transferase inactivation can result in silent gene rebalancing and cell differentiation [20] [21].

Azacitidine (AZA, 5-azacytidine, Vidaza®) and decitabine (DAC, 2'-deoxy-5-azacytidine, Dacogen®), two hypomethylating agents, are used in the treatment of high-risk myelodysplastic syndromes (MDS) and acute myeloid leukemia patients (AML) [22]-[24]. These agents as cytidine analogues (**Figure 1**) are incorporated in place of cytosine during replication: about 80% - 90% of AZA is incorporated into the RNA and to a lesser degree into the DNA; and DAC - into the DNA.

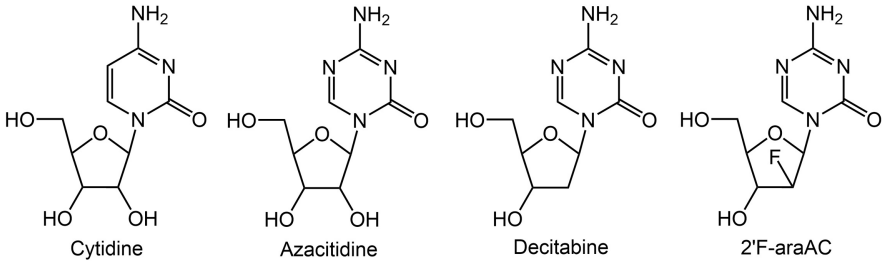

Figure 1. Chemical structures of cytidine and its biologically active 5-azacytosine derivatives.

Due to the substitution of a carbon atom with a nitrogen atom, the methyl group attachment becomes irreversible. DNMT remains bound to DNA molecules, which contributes to the depletion of its intracellular deposits. Decitabine can decrease DNMT1 and DNMT3A expression, reversing abnormal transcription activation, while azacitidine only targets DNMT1 [23]. DNA replication subsequent cycles result in a weaker methylation. Silent gene function recovery induces a cellular differentiation repair [25].

AZA and DAC, being highly effective, are still fiercely toxic and mutagenic in their effects. The search for direct DNMT inhibitors that along with their antitumor activity would be moderate in effect on normal cells is a pressing challenge of developing new highly effective anticancer agents [26] [27].

The introduction of a fluorine atom, which has a prominent effect on the physical, chemical and biological characteristics of these compounds, seems to be a promising area of the molecule modification [28]-[31]. We have previously demonstrated that 2'(3')-fluorine-containing nucleoside of 5-azacytosine is characterized by good solubility, high chemical stability, improved pharmacological properties, and is less toxic compared to AZA [32].

The *in vitro* anti-leukemia activity assessment of the novel 2'F-araAC (4-amino-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-1,3,5-triazine-2(1H)-one, **Figure 1**) showed that the new fluoro analog as well as AZA as a comparator, selectively and dose-dependently suppressed viability of acute myelogenous leukemia KG-1 cells, acute monocytic leukemia TPH-1 and acute promyelocytic leukemia HL-60 cells. At the same time, EC_{50} (median effective concentration) values were 0.35 μM (KG-1), 0.51 μM (TPH-1), 0.58 μM (HL-60) for the novel fluoro analog and 3.66 μM (KG-1), 4.25 μM (TPH-1), 5.37 μM (HL-60) for AZA suggesting a 9 to 10-fold increase in the specific cytotoxic effect of 2'F-araAC compared to control [32].

The antileukemic activity of 2'F-araAC was examined in comparison with DAC in the P388D1 mice leukemia model on females in the dose range of 0.5 - 10 mg/kg. There was a significant increase in life expectancy compared to controls from 77% to 176%, which was evidence of a dose-dependent effect. A comparative analysis of the antitumor activity of 2'F-araAC and decitabine in equimolar effective doses (2 mg/kg) indicates significant effectivity for 2'F-araAC: mean survival time of animals is 30% higher and lifespan extension is 53% higher [33]-[35].

The assessment of physical and chemical characteristics of the synthesized compounds showed [32] that the novel AZA analogue with a fluorine atom at C(2') with arabino configuration had a better hydrolytic stability compared to the known agent AZA which might help to avoid a partial loss of its biological activity during the expected half-life.

Apparently, 2'F-araAC, like 2'-deoxy-5-azacytidine, is phosphorylated inside the cell at first to 5'-monophosphate and then to 5'-triphosphate. The active form of 2'F-araAC is incorporated into DNA, thereby providing the drug with the direct cytotoxic potential and inhibiting DNA methylation. Since aberrant DNA methylation at CpG islands within promoter regions of individual tumor suppressor genes is considered as one of the main mechanisms regulating the phenotype of tumor myeloblasts in MDS/AML, it is postulated that DNA hypomethylation under the influence of decitabine is able to induce re-expression of silenced tumor suppressor genes. It can be assumed that 2'F-araAC acts in a similar way.

Based on previous studies, the inhibitory activity of 2'F-araAC against total DNMTs and its effect on the cell cycle and apoptosis has been studied in this work.

2. Materials and Methods

2.1. Reagents

AZA and DAC were supplied by the Research and Production Center "HimFarmSintez", Institute of Bioorganic Chemistry of the National Academy of Sciences of Belarus, Republic of Belarus; 4-amino-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-1,3,5-triazine-2(1H)-one (2'F-araAC) was synthesized according to the method described in [32]. HCT-116 human colorectal carcinoma cell line, L-1210 mouse lymphocytic leukemia cell line and P-388 murine lymphoid neoplasm cell line were obtained from the Russian Collection of Cell Cultures (Institute of Cytology, Russian Academy of Sciences).

2.2. DNA Methyltransferase Activity Inhibition Assessment

HCT-116 cancer cells were grown to 70% - 80% confluence in McCoy's 5A (Sigma M4892) medium, 10% fetal bovine serum (Capricorn FBS-12A) and 1% Antibiotic Antimycotic solution (Sigma A5955), and then removed with 0.25% trypsin/EDTA solution (Capricorn TRY-2B), washed from trypsin with potassium phosphate buffer centrifuging for 5 minutes at 1000 rpm [36].

The nuclear protein was isolated using the Nuclear Extraction Kit (Abcam, ab113474). Cell pellet (31×10^6) was resuspended in a pre-extraction buffer

(containing DTT and 1:1000 protease inhibitors), incubated for 10 minutes on ice and vortexed vigorously for 10 seconds and centrifuged for 1 minute at 12,000 rpm. The supernatant was removed and an extraction buffer was added to the precipitate. It was incubated on ice for 15 minutes and vortexed for 5 seconds every 3 minutes. Then it was sonicated for 10 seconds. It was then centrifuged for 10 minutes at 14,000 rpm. A supernatant containing the nuclear protein was collected.

The amount of nuclear protein was quantified using the Bradford Assay Kit (Abcam, ab102535) according to manufacturer's instructions. Absorbance was measured at 595 nm using a Tecan Infinite M200 plate reader. The DNMTs Activity Quantification Kit (Fluorometric) (Abcam, ab113468) was used to analyze DNA methyltransferases activity. The analysis was performed according to manufacturer's instructions.

The stock solution of the studied compounds of 2'F-araAC, DAC and AZA was diluted to final concentrations of 1 μM and 10 μM with buffer for analysis. The concentration of the initial solvent in the reaction medium was no more than 1%. The reaction medium contained 7 μg of nuclear protein, 1 μM and 10 μM of the studied compounds, and the Adomet reaction buffer containing S-adenosylmethionine coenzyme. The control reaction medium contained 7 μg of nuclear protein and the Adomet reaction buffer, as well as 25 ng and 50 ng of DNMT enzyme and the Adomet reaction buffer. The reaction was carried out at 37˚C, 120 min in filmed plate strips. Fluorescence was measured on the Tecan Infinite M200 plate reader at Ex/Em = 530/590 nm. The degree of DNA methyltransferases activity inhibition was calculated by the equation:

Inhibition (%) = [1 − ((RFU, inhibitor-containing sample − RFU, blank)/(RFU, non-inhibitor-containing sample - RFU, blank))] × 100%.

where RFUs are relative fluorescence units.

2.3. *In Vitro* Cell Cycle Effects

L1210 and P388 cells were placed in a 6-well plate with a density of 3×10^5 per well. The tested compounds were added at concentrations equal to IC_{50} (prespecified) for 48 hours. Then the cells were washed from the culture medium by centrifugation at 1000 rpm for 5 minutes, rewashed with PBS, and fixed with ice ethanol under permanent shaking and incubated at −20˚C for 24 hours. The samples were afterwards washed from alcohol by centrifugation, rewashed with PBS, and a solution containing 100 μg/ml of RNase A and 50 μg/ml of propidium iodide (PI) was added; incubated for 40 minutes in the dark and measured on the Cytomics FC 500 Beckman Coulter flow cytometer (FL4 channel for PI). The results were analyzed using Kaluza 2.0 software (Beckman Coulter) [37].

2.4. Apoptosis Assay

L1210 and P388 cells were placed in a 6-well plate in quantities of 3×10^5 per well. Test compounds were added at concentrations equal to IC_{50} (prespecified in the

MTT assay for L1210 – 2'F-araAC, DAC and AZA at a concentration of 0.03 μM, 0.1 μM and 2.5 μM, respectively; for P388 2'F-araAC, AZA and DAC at a concentration of 0.3 μM, 0.3 μM and 5.0 μM, respectively) for 48 hours. The cells are then washed from the culture medium by centrifugation at 1000 rpm for 5 minutes, re-washed with a phosphate buffer, and the Annexin V-AlexaFluor488 and PI dyes were added from the Dead Cell Apoptosis Kit with Annexin V for Flow Cytometry (Invitrogen, V13245) were added as described in the manufacturer's instructions; they were incubated for 15 minutes in the dark and measured on the Cytomics FC 500 Beckman Coulter flow cytometer (FL1 for Annexin V-AlexaFluor488 and FL4 for PI) [38].

3. Results

DNMT inhibitors are being developed as potential anticancer drugs. Two nucleoside DNMT inhibitors, such as AZA and DAC, have been FDA approved for clinical use to treat myelodysplastic syndromes and acute myeloid leukemia. Despite being highly effective, their use is restricted by poor bioavailability, chemical instability and serious side effects.

The search for new analogues similar in effectiveness but with better stability and less severe toxicity would be a solution to the problem of azacitidine resistance. The introduction of fluorine into the molecule of natural compounds changes their physical, chemical and pharmacological characteristics, which are closely linked with their biological functions. It facilitates the changing of the pharmaceutical profile of these compounds in a wide range, which explains their broad use.

It is worth noting that that the fluorine atom and proton have close Van der Waals radii (1.35 and 1.20 Å, respectively) and, therefore, there are no additional steric obstacles in the molecule. Substitution of the 2'-hydroxyl group for a fluorine atom gives an analog similar to 2'-deoxy-5-azacytidine. In addition, the fluorine atom is the most electronegative substituent and, given that there are other electronegative substituents in the pentofuranose ring, it should have a powerful effect on the furanose ring conformation. All these factors will determine the physical, chemical and biological properties of fluorodeoxy analogs. In 2013, we synthesized 2'(3')-fluorinated analogues of 5-azacytidine [32] and examined their biological properties. Considering the 2'F-araAC long-term benefits, we examined the inhibitory activity of the analogue in relation to DNMT.

3.1. DNA Methyltransferase Inhibition Assay

The level of DNMTs inhibition was determined in the nuclear extract of the HCT-116 human colorectal cell line because the aberrant gain of DNA methylation at CpG islands is frequently observed in colorectal tumors [39]. The nuclear extract was exposed to varying concentrations of 2'F-araAC, DAC and AZA for 120 minutes. Protein extracts were analyzed according to the DNMT Activity Quantification Kit (Fluorometric) (Abcam, ab113468) protocol. Known

DNA methyltransferase inhibitors DAC and AZA were used as comparators.

Figure 2 shows calculated data on the inhibitory activity of AZA, DAC and 2'F-araAC at concentrations of 1 μM and 10 μM with respect to DNMT contained in the HCT-116 cell nuclear extract. The experimental results showed that the DNMT activity was remarkably inhibited by the tested compounds. At 10 μM concentration of 2'F-araAC the level of DNMT activity vs. control was the lowest. At the same time, AZA and DAC inhibited DNMT activity by 75% and 86%, respectively, of the value of the inhibition by 2'-fluorodeoxy-arabino analog at a concentration of 10 μM. It should be added that the ability of the studied compounds to diminish the DNA methylation activity can be achieved at low doses. Azacitidine at a concentration of 1 μM showed a 2-fold weaker DNA methylation activity compared to 2'F-araAC, while for DAC it was 1.7-fold weaker compared to fluoro analog.

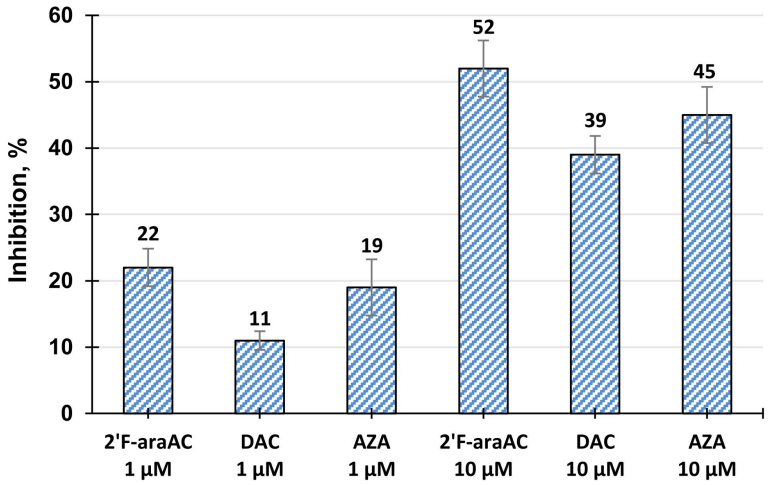

Figure 2. Inhibition (%) of DNA methyltransferase activity (mean ± SD; n = 2).

We assume that the compound may have metabolism similar to decitabine. In particular, once incorporated into DNA, decitabine is recognized as a substrate by DNMTs, namely DNMT1, but due to the presence of an N5 rather than C5 atom, irreversible formation of a covalent bond between DNA and DNMT occurs. Under low concentrations, this activity depletes DNMTs and results in global DNA hypomethylation while under high concentrations, it additionally results in double-strand DNA breaks and cell death [40] [41].

Experimental results indirectly allowed us to suggest the binding between the carbon-6 atom of the cytosine ring and the catalytic site of DNMT resulting in enzyme inactivation.

3.2. Effect of 2'F-araAC, AZA and DAC on the Cell Cycle of P388 and L1210 Cells

The inhibition of the cell cycle resulting in the delay of the cancer cells proliferation is an underlying mechanism of action of antitumor agents.

As previously shown, 2'F-araAC demonstrated antiproliferative activity against several types of human hemoblastosis cell lines (KG-1, TPH-1, HL-60, K562) [32]. It is interesting that 2'F-araAC was strongly cytotoxic against acute myelogenous leukemia cells at a concentration of 0.1 μM, whereas the threshold limit for AZA was 1.0 μM. The saturation effect was achieved with increased concentration of the tested nucleoside to 1.0 μM. The Aza saturating concentration was much greater (10 μM) compared to 2'F-araAC [32].

The effect of the novel 2'-fluoro analog on the cell cycle was examined in comparison AZA and DAC. Cell cycle experiments were performed using P388 cells treated with 2'F-araAC, DAC and AZA at concentrations of 0.3 μM, 0.3 μM and 5.0 μM, respectively. L1210 murine lymphoma cells were treated with 2'F-araAC, DAC and AZA at concentrations of 0.03 μM, 0.1 μM and 2.5 μM based on their IC_{50}. The cells were exposed to the compounds for 48 h. Treated cells were harvested, fixed in ethanol, stained with propidium iodide and analyzed for cell cycle distribution by flow cytometry.

As seen from the results (Table 1 and Table 2), the novel 2'-fluoro analog significantly disrupted the proliferation of P388 and L1210 cells.

Table 1. Effects of 2'F-araAC, AZA and DAC on P388 cell cycle.

Compounds	Concentration, μM	G1, %	S, %	G2/M, %
P388 (control)	DMSO	22.99 ± 2.69	61.45 ± 3.45	15.56 ± 2.10
AZA	5.0	28.99 ± 0.51	58.59 ± 0.71	12.43 ± 0.21
DAC	0.3	34.80 ± 0.08	52.28 ± 1.68	12.93 ± 1.59
2'F-araAC	0.3	5.60 ± 3.07	46.76 ± 1.18	47.64 ± 2.33

Table 2. Effects of 2'F-araAC, AZA and DAC on L1210 cell cycle.

Compounds	Concentration, μM	G1, %	S, %	G2/M, %
L1210 (control)	DMSO	30.28 ± 4.32	60.16 ± 2.78	9.57 ± 1.80
AZA	2.5	29.48 ± 0.13	62.82 ± 0.11	7.72 ± 0.23
DAC	0.1	35.12 ± 1.92	49.10 ± 1.18	15.79 ± 0.75
2'F-araAC	0.03	23.39 ± 3.15	57.90 ± 1.36	18.72 ± 2.21

2'F-araAC significantly changed the cell cycle distribution of cells P388, causing a 4-fold decrease in the fraction of G1 cells compared to control. Whereas, AZA and DAC slightly increased the fraction of G1 cells in concentrations of 5.0 μM and 0.3 μM and concomitantly reduced for all other cell cycle phases, respectively.

Unlike AZA and DAC, 2'F-araAC increased the percentages of G2/M phase almost three times with a concomitant decrease in the S-phase population (Table 1, Figure 3(a)). This experiment suggested that new fluoro analog induces G2/M-phase cell cycle arrest at concentration of 0.3 μM.

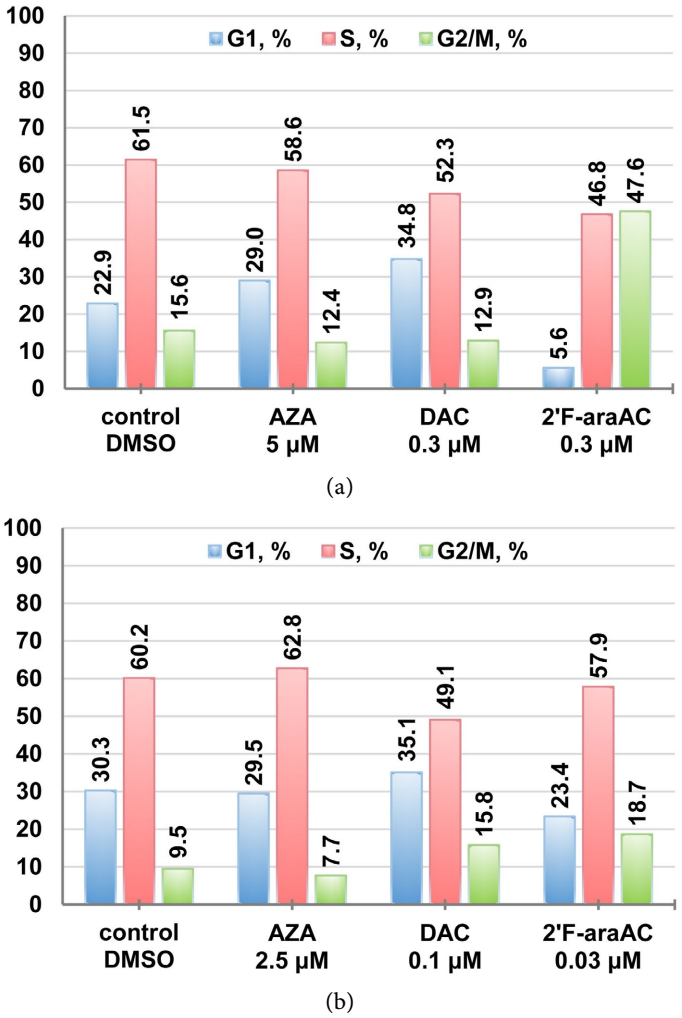

Figure 3. Effect of AZA, DAC and 2'F-araAC on the cell cycle of of P388 mouse lymphoid neoplasm cell line (a) and L1210 (b).

The L1210 mouse lymphocytic leukemia cell cycle analysis is shown in Figure 3(b). The results are summarized in Table 2. For L1210 cells, lower concentrations of the studied compounds were used to diminish cytotoxic effects of the drugs. Treatment for 48 hours with 2'F-araAC decreased fraction of G1 cells and caused the increase in the percentages of cells in the G2/M phase from 9.57% (DMSO) to 18.72% (0.03 μM). AZA at a concentration of 2.5 μM had no significant effect on the cell cycle. Similar to 2'F-araAC, however, at a lower concentration, DAC (0.1 μM) increased the fractions of G2/M cells to 15.79%. Moreover, DAC prolonged the G1 phase with a concomitant decrease in the S phase (Table 1).

Our results have shown that P388 and L1210 cells treated with 2'F-araAC at a dose of 0.3 and 0.03 μM, respectively, contributed to a G2/M phase arrest suggesting that the inhibition of cell cycle progression could be one of the mechanisms through which fluoro analog might influence cancer cell proliferation. Inhibition of cell proliferation of rapidly growing cancer cells is a compelling argument for the development of an anticancer drug on the basis of 2'F-araAC.

3.3. 2'F-araAC, AZA and DAC Increase Apoptosis Markers in P388 and L1210 Cells

Apoptosis is crucial in the elimination of mutated neoplastic and hyperproliferating neoplastic cells from the system and is therefore considered a protective mechanism impeding cancer progression [42].

The fact that 2'F-araAC induces P388 and L1210 cell cycle disruption prompted us to examine the effects produced by this compound on cell apoptosis level. In the case of P388 the tested concentrations were 0.3 μM for 2'F-araAC, 5 μM for AZA and 0.3 μM for DAC. L1210 cells were treated with 0.03 μM of 2'F-araAC, 2.5 μM of AZA and 0.1 μM of DAC. The concentrations were chosen on the bases of there IC_{50}. Both cell lines were treated for 48 hours. Apoptotic cells were determined by flow cytometry using Annexin V-FITC/PI double labeling. On the histograms at **Figure 4** and **Figure 5**, we can see that all investigating compounds in the effective concentrations (IC_{50}) cause early (B4 quadrant) and late apoptosis (B2 quadrant), whereas the amount of necrotic cells is small.

Apoptotic cells percentages were 29.41% for AZA, 42.16% for DAC and 54.53% for 2'F-araAC vs. 2.88% for the control P388 cell culture. Less than 1% of cell population treated with all compounds showed necrotic signs (**Figure 4**).

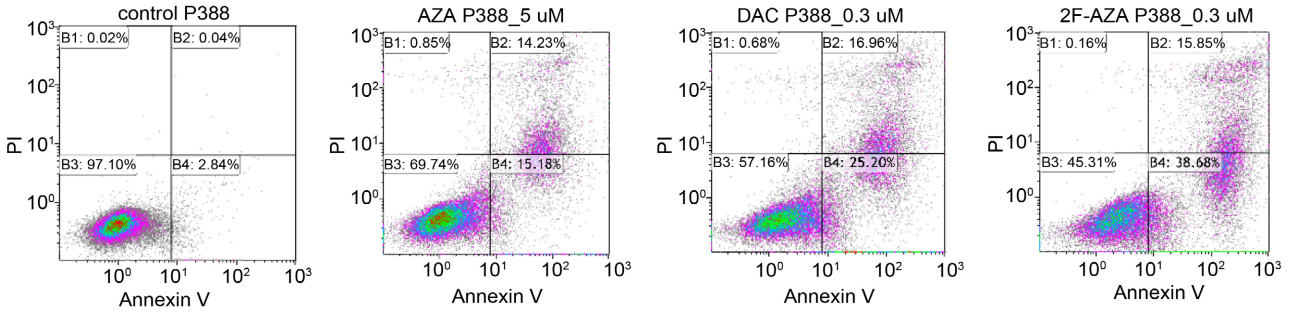

Figure 4. Effect of AZA, DAC and 2'F-araAC on apoptosis in murine lymphocytic leukemia lines P388 (B1 quadrant-necrosis; B2 quadrant-late apoptosis; B3 quadrant-living cells; B4 quadrant-early apoptosis).

Apoptotic cells percentages were 30.21% for AZA, 43.00% for DAC and 43.35% for 2'F-araAC vs. 5.25% for the control L1210 cell culture. Less than 2% of cell population treated with all compounds showed necrotic signs (**Figure 5**).

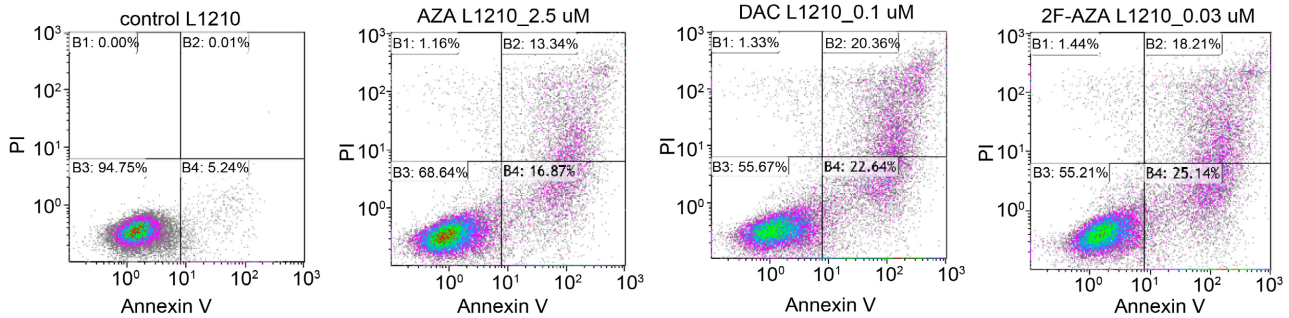

Figure 5. Effect of AZA, DAC and 2'F-araAC on apoptosis in murine lymphocytic leukemia lines L1210 (B1 quadrant-necrosis; B2 quadrant-late apoptosis; B3 quadrant-living cells; B4 quadrant-early apoptosis).

The P388 and L1210 cells showed different viability under the influence of 2'F-araAC, which could be species-specific. A greater cell death of P388 cell line caused by 2'F-araAC vs. AZA and DAC was due to a greater percentage of cells undergoing early apoptosis (Figure 6). Early apoptotic cells percentages were 38.7% for 2'F-araAC vs. 15.2% and 25.2% for AZA and DAC, respectively. For L1210 cells fluoro analog and DAC were similar in their effects on apoptosis level. Of note, the small quantity of necrotic cells appeared after the 2'F-araAC treatment of both cell lines is not more than 1.44% suggesting no significant cytotoxic effect of 2'F-AZA on these sublines.

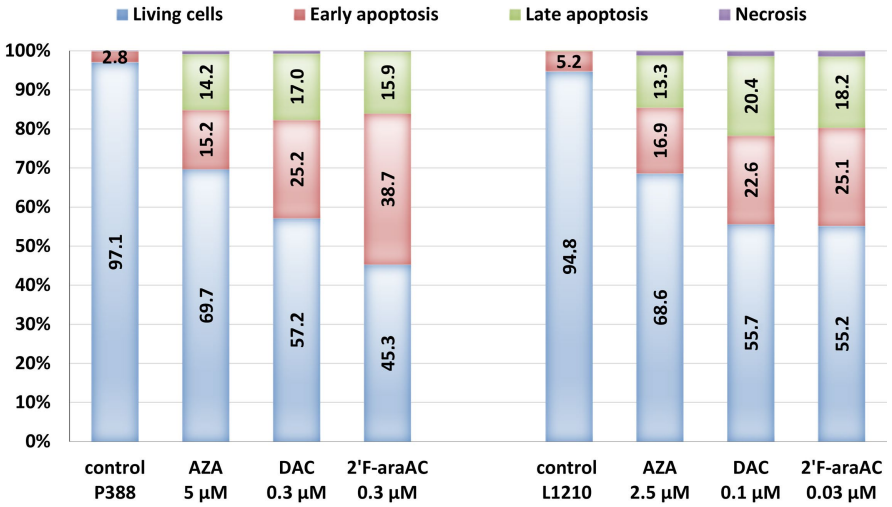

Figure 6. Percent of apoptosis and necrosis induced by compounds in P388 and L1210 cell line.

The obtained data suggest common cell death mechanisms of action of 2'F-araAC, AZA and DAC and clearly different effect on the cell cycle. It can be assumed that the novel azacitidine 2'-fluorodeoxy-arabino analog inhibits the proliferation of P388 and L1210 cells through G2/M cell cycle arrest and apoptosis induction.

4. Conclusions

2'-Fuoro-2'-deoxy-arabinofuranosyl 5-azacytosine nucleoside has been shown to exhibit high inhibitory activity against HCT-116 human colorectal cancer cell line DNA methyltransferases causing depletion of DNA methyltransferases and DNA hypomethylation and showing superior results compared to known analogues azacitidine and decitabine. In addition, 2'F-araAC induces G2/M cell cycle arrest and apoptosis in P388 and L1210 murine leukemia lymphocytic cells.

To summarize, it can be assumed that the biological activity of the novel 2'-fluoro analog 5-azacytidine demonstrates a powerful mechanism for cell cycle arrest and apoptosis induction through inhibition of DNMT activity.

The results have demonstrated that azacitidine 2'-fluorodeoxy-arabino analog might be a promising molecule for the development of a novel anti-cancer agent.

Further *in vivo* studies are therefore needed to assess its potential antiproliferative and antitumor activity.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Corey, S.J., Minden, M.D., Barber, D.L., Kantarjian, H., Wang, J.C.Y. and Schimmer, A.D. (2007) Myelodysplastic Syndromes: The Complexity of Stem-Cell Diseases. *Nature Reviews Cancer*, **7**, 118-129. https://doi.org/10.1038/nrc2047

[2] Steensma, D.P. and Bennett, J.M. (2006) The Myelodysplastic Syndromes: Diagnosis and Treatment. *Mayo Clinic Proceedings*, **81**, 104-130. https://doi.org/10.4065/81.1.104

[3] Ehrlich, M. (2002) DNA Methylation in Cancer: Too Much, but Also Too Little. *Oncogene*, **21**, 5400-5413. https://doi.org/10.1038/sj.onc.1205651

[4] Feinberg, A.P. (2018) The Key Role of Epigenetics in Human Disease Prevention and Mitigation. *New England Journal of Medicine*, **378**, 1323-1334. https://doi.org/10.1056/nejmra1402513

[5] Kiziltepe, T., Hideshima, T., Catley, L., Raje, N., Yasui, H., Shiraishi, N., *et al*. (2007) 5-Azacytidine, a DNA Methyltransferase Inhibitor, Induces ATR-Mediated DNA Double-Strand Break Responses, Apoptosis, and Synergistic Cytotoxicity with Doxorubicin and Bortezomib against Multiple Myeloma Cells. *Molecular Cancer Therapeutics*, **6**, 1718-1727. https://doi.org/10.1158/1535-7163.mct-07-0010

[6] Karahoca, M. and Momparler, R.L. (2013) Pharmacokinetic and Pharmacodynamic Analysis of 5-Aza-2'-Deoxycytidine (Decitabine) in the Design of Its Dose-Schedule for Cancer Therapy. *Clinical Epigenetics*, **5**, Article No. 3. https://doi.org/10.1186/1868-7083-5-3

[7] Seelan, R.S., Mukhopadhyay, P., Pisano, M.M. and Greene, R.M. (2018) Effects of 5-Aza-2'-Deoxycytidine (Decitabine) on Gene Expression. *Drug Metabolism Reviews*, **50**, 193-207. https://doi.org/10.1080/03602532.2018.1437446

[8] Okano, M., Bell, D.W., Haber, D.A. and Li, E. (1999) DNA Methyltransferases Dnmt3a and Dnmt3b Are Essential for De Novo Methylation and Mammalian Development. *Cell*, **99**, 247-257. https://doi.org/10.1016/s0092-8674(00)81656-6

[9] Yagi, M., Kabata, M., Tanaka, A., Ukai, T., Ohta, S., Nakabayashi, K., *et al*. (2020) Identification of Distinct Loci for De Novo DNA Methylation by DNMT3A and DNMT3B during Mammalian Development. *Nature Communications*, **11**, Article No. 3199. https://doi.org/10.1038/s41467-020-16989-w

[10] Gershman, A., Sauria, M.E.G., Guitart, X., Vollger, M.R., Hook, P.W., Hoyt, S.J., *et al*. (2022) Epigenetic Patterns in a Complete Human Genome. *Science*, **376**, eabj5089. https://doi.org/10.1126/science.abj5089

[11] Kato, Y., Kaneda, M., Hata, K., Kumaki, K., Hisano, M., Kohara, Y., *et al*. (2007) Role

of the Dnmt3 Family in De Novo Methylation of Imprinted and Repetitive Sequences during Male Germ Cell Development in the Mouse. *Human Molecular Genetics*, **16**, 2272-2280. https://doi.org/10.1093/hmg/ddm179

[12] Sun, J., Yang, J., Miao, X., Loh, H.H., Pei, D. and Zheng, H. (2021) Proteins in DNA Methylation and Their Role in Neural Stem Cell Proliferation and Differentiation. *Cell Regeneration*, **10**, Article No. 7. https://doi.org/10.1186/s13619-020-00070-4

[13] Jurkowska, R.Z., Jurkowski, T.P. and Jeltsch, A. (2010) Structure and Function of Mammalian DNA Methyltransferases. *ChemBioChem*, **12**, 206-222. https://doi.org/10.1002/cbic.201000195

[14] Lyko, F. (2017) The DNA Methyltransferase Family: A Versatile Toolkit for Epigenetic Regulation. *Nature Reviews Genetics*, **19**, 81-92. https://doi.org/10.1038/nrg.2017.80

[15] Herman, J.G. and Baylin, S.B. (2003) Gene Silencing in Cancer in Association with Promoter Hypermethylation. *New England Journal of Medicine*, **349**, 2042-2054. https://doi.org/10.1056/nejmra023075

[16] Ye, F. and Li, N. (2019) Role of P15(INK4B) Methylation in Patients with Myelodysplastic Syndromes: A Systematic Meta-Analysis. *Clinical Lymphoma Myeloma and Leukemia*, **19**, e259-e265.

[17] Chen, H. and Wu, S. (2002) Hypermethylation of the p15(INK4B) Gene in Acute Leukemia and Myelodysplastic Syndromes. *Chinese Medical Journal*, **115**, 987-990.

[18] Chim, C.S., Tam, C.Y.Y., Liang, R. and Kwong, Y.L. (2001) Methylation of P15 and P16 Genes in Adult Acute Leukemia. *Cancer*, **91**, 2222-2229. https://doi.org/10.1002/1097-0142(20010615)91:12<2222::aid-cncr1252>3.3.co;2-i

[19] Aggerholm, A., Holm, M.S., Guldberg, P., Olesen, L.H. and Hokland, P. (2005) Promoter Hypermethylation of *p15*^{INK4B}, *HIC1*, *CDH1*, and *ER* Is Frequent in Myelodysplastic Syndrome and Predicts Poor Prognosis in Early-Stage Patients. *European Journal of Haematology*, **76**, 23-32. https://doi.org/10.1111/j.1600-0609.2005.00559.x

[20] Brenner, C. and Fuks, F. (2006) DNA Methyltransferases: Facts, Clues, Mysteries. In: *Current Topics in Microbiology and Immunology*, Springer, 45-66. https://doi.org/10.1007/3-540-31390-7_3

[21] Kar, S., Deb, M., Sengupta, D., Shilpi, A., Parbin, S., Torrisani, J., *et al*. (2012) An Insight into the Various Regulatory Mechanisms Modulating Human DNA Methyltransferase 1 Stability and Function. *Epigenetics*, **7**, 994-1007. https://doi.org/10.4161/epi.21568

[22] Derissen, E.J.B., Beijnen, J.H. and Schellens, J.H.M. (2013) Concise Drug Review: Azacitidine and Decitabine. *The Oncologist*, **18**, 619-624. https://doi.org/10.1634/theoncologist.2012-0465

[23] Hu, C., Liu, X., Zeng, Y., Liu, J. and Wu, F. (2021) DNA Methyltransferase Inhibitors Combination Therapy for the Treatment of Solid Tumor: Mechanism and Clinical Application. *Clinical Epigenetics*, **13**, Article No. 166. https://doi.org/10.1186/s13148-021-01154-x

[24] Krečmerová, M. and Otmar, M. (2012) 5-Azacytosine Compounds in Medicinal Chemistry: Current Stage and Future Perspectives. *Future Medicinal Chemistry*, **4**, 991-1005. https://doi.org/10.4155/fmc.12.36

[25] Ghavifekr Fakhr, M., Farshdousti Hagh, M., Shanehbandi, D. and Baradaran, B. (2013) DNA Methylation Pattern as Important Epigenetic Criterion in Cancer. *Genetics Research International*, **2013**, 1-9. https://doi.org/10.1155/2013/317569

[26] Cheng, J.C., Matsen, C.B., Gonzales, F.A., Ye, W., Greer, S., Marquez, V.E., *et al*.

(2003) Inhibition of DNA Methylation and Reactivation of Silenced Genes by Zebularine. *Journal of the National Cancer Institute*, **95**, 399-409. https://doi.org/10.1093/jnci/95.5.399

[27] Zhou, L., Cheng, X., Connolly, B.A., Dickman, M.J., Hurd, P.J. and Hornby, D.P. (2002) Zebularine: A Novel DNA Methylation Inhibitor That Forms a Covalent Complex with DNA Methyltransferases. *Journal of Molecular Biology*, **321**, 591-599. https://doi.org/10.1016/s0022-2836(02)00676-9

[28] Gillis, E.P., Eastman, K.J., Hill, M.D., Donnelly, D.J. and Meanwell, N.A. (2015) Applications of Fluorine in Medicinal Chemistry. *Journal of Medicinal Chemistry*, **58**, 8315-8359. https://doi.org/10.1021/acs.jmedchem.5b00258

[29] Van Den Boogaart, J.E., Kalinichenko, E.N., Podkopaeva, T.L., Mikhailopulo, I.A. and Altona, C. (1994) Conformational Analysis of 3'-Fluorinated A(2' - 5') A(2'-5') A Fragments. *European Journal of Biochemistry*, **221**, 759-768. https://doi.org/10.1111/j.1432-1033.1994.tb18789.x

[30] Bozhok, T.S., Sivets, G.G., Baranovsky, A.V. and Kalinichenko, E.N. (2016) Synthesis of Novel 6-Substituted Thymine Ribonucleosides and Their 3'-Fluorinated Analogues. *Tetrahedron*, **72**, 6518-6527. https://doi.org/10.1016/j.tet.2016.08.065

[31] Gu, D., Dong, K., Jiang, A., Jiang, S., Fu, Z., Bao, Y., *et al*. (2022) PBRM1 Deficiency Sensitizes Renal Cancer Cells to DNMT Inhibitor 5-Fluoro-2'-Deoxycytidine. *Frontiers in Oncology*, **12**, Article 870229. https://doi.org/10.3389/fonc.2022.870229

[32] Bozhok, T.S., Kalinichenko, E.N., Kuz'mitskii, B.B. and Golubeva, M.B. (2016) Synthesis, Hydrolytic Stability, and Antileukemic Activity of Azacytidine Nucleoside Analogs. *Pharmaceutical Chemistry Journal*, **49**, 804-809. https://doi.org/10.1007/s11094-016-1375-4

[33] Zilberman, R., Bozhok, T., Panibrat, A., Savin, A., Vinnikova, O. and Kalinichenko, E. (2021) *In vivo* Study of Antileukemic Activity of New Fluorine Substituted Decitabine Analogue on Male P388D1 Tumor-Bearing Mice. In: *Book of Abstracts VII International Conference* "*Chemistry, Structure and Function of Biomolecules*", Institute of Bioorganic Chemistry NAS of Belarus, 64.

[34] Zilberman, R.D., Bozhok, T.S., Panibrat, O.V., Savin, A.O., Vinnikova, O.V. and Kalinichenko, E.N. (2021) Antitumor Activity of the 2'-Fluoro-Substituted Derivative of Decitabine in In-Vivo Experiments on the Model of Transplantable Hemoblastosis P388D1. In: *Proceedings of the International Scientific and Practical Conference* "*Modern Technologies in Medical Education*", Belarusian State Medical University, 2090-2093.

[35] Bozhok, T.S., Golubeva, M.B., Zilberman, R.D. and Kalinichenko, E.N. (2015) New Fluorine-Containing Analogue of Azacitidine: Cytotoxic Activity and Acute Toxicity. https://doi.org/10.13140/RG.2.1.1031.7843

[36] Freshney, R.I. (2010) Culture of Animal Cells. Wiley. https://doi.org/10.1002/9780470649367

[37] Riccardi, C. and Nicoletti, I. (2006) Analysis of Apoptosis by Propidium Iodide Staining and Flow Cytometry. *Nature Protocols*, **1**, 1458-1461. https://doi.org/10.1038/nprot.2006.238

[38] Rieger, A.M., Nelson, K.L., Konowalchuk, J.D. and Barreda, D.R. (2011) Modified Annexin V/propidium Iodide Apoptosis Assay for Accurate Assessment of Cell Death. *Journal of Visualized Experiments*, **50**, Article ID: 2597. https://doi.org/10.3791/2597

[39] Sanaei, M. and Kavoosi, F. (2022) Effect of 5'-Fluoro-2'-Deoxycytidine, 5-Azacytidine, and 5-Aza-2'–Deoxycytidine on DNA Methyltransferase 1, CIP/KIP Family,

and INK4A/ARF in Colon Cancer HCT-116 Cell Line. *International Journal of Cancer Management*, **14**, e110419. https://doi.org/10.5812/ijcm.110419

[40] Qin, T., Jelinek, J., Si, J., Shu, J. and Issa, J.J. (2009) Mechanisms of Resistance to 5-Aza-2'-Deoxycytidine in Human Cancer Cell Lines. *Blood*, **113**, 659-667. https://doi.org/10.1182/blood-2008-02-140038

[41] Yang, X., Lay, F., Han, H. and Jones, P.A. (2010) Targeting DNA Methylation for Epigenetic Therapy. *Trends in Pharmacological Sciences*, **31**, 536-546. https://doi.org/10.1016/j.tips.2010.08.001

[42] Elmore, S. (2007) Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology*, **35**, 495-516. https://doi.org/10.1080/01926230701320337