In vitro and *in vivo* antileukemic effect of novel dimers consisting of 5-fluorodeoxyuridine and arabinofuranosylcytosine

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Various amphiphilic heterodinucleoside phosphates containing 1-β-D-arabinofuranosylcytosine (ara-C) and 5-fluorodeoxyuridine (5-FdUrd) have recently been synthesized in order to increase the efficacy of ara-C and 5-FdUrd. Employing growth inhibition and growth recovery assays, we evaluated the in vitro effects of four of these dimers (No. 2, 2A, 3, 10) in L1210 and P388D1 murine leukemia cells. Although ara-C and 5-FdUrd appeared equimolar in all dimers, their contribution to the cytotoxicity of these agents was different. Thus, the liberation of ara-C and 5-FdUrd from their dimeric origin and their subsequent metabolic activation had a different course. In another set of experiments, we examined the in vivo effects of these agents in mice. The dimer with the highest cytotoxicity in vitro exerted the lowest acute toxicity and yielded the lowest therapeutic effect in vivo. The obtained data indicate that dimers with slower liberation of ara-C and 5-FdUrd were less cytotoxic, but prolonged liberation of both antimetabolites protected them from inactivation and extended the time period of therapeutic action. Some of the dimers exceeded the synergistic effects yielded by simultaneous application of both ara-C and 5-FdUrd. The significantly higher therapeutic potential of these new antitumor agents indicates that further studies are warranted.

Key words: Arabinofuranosylcytosine; 5-Fluorodeoxyuridine; Heterodinucleoside phosphates; L1210 murine leukemia cells; P388D1 murine leukemia cells.

Antimetabolites are generally structural analogues of naturally occurring intracellular metabolic intermediates essential for the normal function of a cell (pyrimidines or purines). Such similarities allow these drugs or their metabolites to serve as substrates for key intracellular enzymes. The substrate substitution ultimately results in the inhibition of key enzymes necessary for synthesis of folic acid, pyrimidines or purines for DNA or RNA formation in neoplastic cells. Antimetabolites like ara-C and 5-FdUrd are being used in the treatment of hematological malignancies [1, 2] or colorectal carcinomas [3]. Both compounds inhibit DNA and RNA synthesis as well as reparative DNA synthesis. Intracellular activation of these antimetabolites is required for their cytotoxic and therapeutic effect. The phosphorylation of 5-FdUrd by thymidine kinase induces the formation of an active metabolite (5-FdUrd-5'-monophosphate) that forms a stable complex with thymidylate synthetase. Its enzymatic activity is inhibited by this step, resulting in depletion of deoxythymidine triphosphate which serves as a precursor for DNA synthesis. 5-FdUrd monophosphate is an intermediate in the synthesis of 5-FdUrd triphosphate which is capable of being incorporated into DNA structures. Consequently, this leads to the formation of DNA-chain breaks [4]. Ara-C is being activated to its cytotoxic and therapeutically active form, ara-C-5'-triphosphate (ara-CTP) which inhibits DNA synthesis and induces apoptosis [5,6].

The extensive inactivation of 5-FdUrd and ara-C in patients represents a significant limit for the therapeutic outcome of these agents. The effect of 5-FdUrd suffers from its degra-

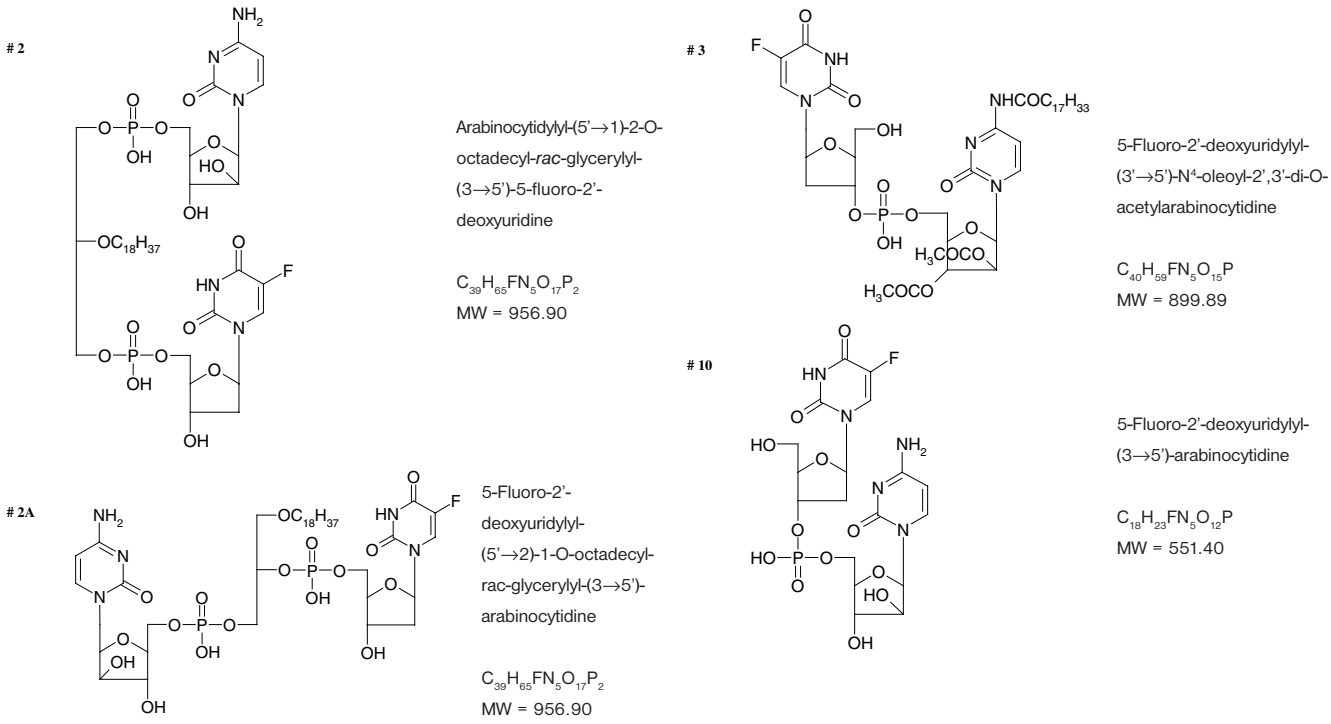

Figure 1 Structural formulas of dimers No. 2, 2A, 3, and 10.

dation to dihydro-5-fluorouracil [9], while Ara-C is deaminated to its inactive metabolite arabinofuranosyluracil (ara-U), which is caused by cytidine deaminase [7,8]. This problem has already been addressed through synthesis of many ara-C analogs that are more stable towards deamination [10,11].

In order to enhance the cytotoxic effects of 5-FdUrd and ara-C, a new strategy of masking nucleoside phosphates by the synthesis of amphiphilic heterodinucleoside phosphates has been developed by Schott and coworkers. These dimers (No. 2, 2A, 3, 10) act as duplex drugs as they contain two active moieties with different mechanisms of action which are activated after uptake in the target cell. This combination of powerful antitumor agents should increase the efficacy of 5-FdUrd and ara-C and might also be able to overcome drug resistance which still remains the major problem in cancer chemotherapy [12-14]. Due to their amphiphilic structure, an enhanced cellular uptake and a different drug distribution can be expected since monophosphorylated nucleosides (5-FdUMP and ara-CMP) could be formed directly in the tumor cell after enzymatic cleavage of the dimer. Thus, the molecule would not have to pass the first phosphorylation step, which is known to be rate limiting. Consequently, low activities of nucleoside-5´-monophosphate kinases might be circumvented by the dimers, resulting in increased antitumor effects.

The interest in dimeric compounds containing ara-C and 5-FdUrd is further motivated by the fact that both agents demonstrated synergistic action in both experimental models and clinical tests [15-17]. Additionally, it was shown in a number of experiments that some of these dimers are capable of overcoming resistance in various human cancer cell lines [18-20].

The main goal of this work was to compare the effects of the monomers ara-C and 5-FdUrd with the cytotoxic activity of the dimers at equimolar concentrations. In growth inhibition and growth recovery assays, the in vitro activity of the dimers in L1210 and P388D1 leukemia cell lines were compared with the effect of combined 5-FdUrd and ara-C treatment. Employing in vivo experiments, acute toxicity and therapeutic potential of these compounds were determined in L1210- and P388D1-leukemia bearing mice. Again, these parameters were compared to the data obtained with a combination of 5-FdUrd and ara-C.

Materials and methods

Drugs and chemicals

The investigated dimers No. 2 [arabinocytidylyl-(5'→ 1)-2-O-octadecyl-rac-glycerylyl-(3 → 5')-5-fluoro-2'-deoxyuridine], No. 2A [5-fluoro-2'deoxyuridylyl-(5'→ 2)-1-O-octadecyl-rac-glycerylyl-(3→5')-arabinocytidine], No. 3[5-fluoro-2'deoxyuridylyl-(3'→ 5')-N^4-oleoyl-2',3'-di-O-acetylarabinocytidine], and No. 10 [5-fluoro-2'deoxyuridylyl-(3→5')-arabinocytidine] were synthesized and provided by Prof. H. Schott, University of Tuebingen, Germany (structural formulas are depicted in Fig.

1). Ara-C (1-β-D-arabinofuranosylcytosine) and 5-FdUrd (5-fluorodeoxyuridine) were purchased from Sigma-Aldrich Handels GmbH, Vienna, Austria. All other chemicals and reagents were commercially available and of highest purity.

Cell culture

The L1210 and P388D1 murine leukemia cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were maintained at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO, Grand Island Biological Co., Grand Island, NY, USA), 100 U/ml Penicillin G, 100 µg/ml streptomycin, and 2 mM L-glutamine (Sebac, Germany) in a humidified atmosphere containing 5% CO_2. Cells in the logarithmic phase of growth were used for all studies described.

Growth recovery assay

Exponentially growing L1210 and P388D1 cells ($0.5x10^6$ per ml) were seeded in tissue culture flasks and incubated with 200 nM ara-C and/or 5-FdUrd for 24h at 37°C. Subsequently, drugs were washed out and cells were resuspended in fresh medium and cultivated for another 3 days. Viable cell number was determined daily.

Growth inhibition assay

Exponentially growing L1210 and P388D1 cells ($0.2x10^6$ per ml) were incubated with increasing concentrations of dimers under cell culture conditions. Since all dimers contain ara-C and 5-FdUrd in a 1:1 ratio (Fig. 1), cytotoxicity was compared to an equimolar mixture of ara-C and 5-FdUrd and among the dimers themselves. Cell counts and IC_{50} values were determined after 24, 48 and 72h. Viability of cells was determined by staining with Trypan blue. Results were calculated as number of viable cells.

Animals

Inbred DBA/2J mice of both sexes (weighing 18-22g) were obtained from the breeding facility of the Cancer Research Institute of SAS (Bratislava, Slovak Republic). The animals were reared under standard conditions; six mice were housed in a cage. Food and water were provided *ad libitum*. The workstation is certificated for performing scientific research on animals. The Ethics Committee of the Cancer Research Institute approved the *in vivo* experiments. These experiments were performed in full adherence with the European Community Guidelines principles for the care and use of laboratory animals.

Acute toxicity of drugs

Acute toxicity of the compounds was monitored through registration of body weight of the experimental animals on daily basis during the whole time of experiment [21]. Various doses of drugs were applied in selected application regimes during the experiment in order to determine the acute toxicity of dimers No. 2, 2A, 3, and 10 as lethal and sub-lethal effects. A dose regime with sub-lethal effects on the experimental mice was determined for every drug for designing a therapeutic protocol.

In vivo antileukemic activity

L1210- and P388D1-leukemia bearing mice were used as antileukemic models of therapy [21-23]. The experimental animals were intraperitoneally implanted with L1210 ($1x10^5$ cells per mouse) or P388D1 ($1x10^6$ cells per mouse) murine leukemia. Antileukemic treatment started 24h later. The indicated doses of drugs were administered intraperitoneally (0.5 ml per mouse). Treatment schedules and doses were chosen according to previous experiments and corresponded to non-lethal doses. Animals were weighed daily and observed for the development of ascites and leukemia related death. Mean survival time (MST) and percentage of increase of life span (% ILS) were calculated and compared with MST of untreated control groups. Statistical significance of the difference was calculated and the effect of all therapeutic regimens was evaluated using a standard *t*-test for unpaired observations.

Statistical calculations

IC_{50} values of drugs were calculated using the CalcuSyn software for Windows. Statistical significance of the obtained results was determined by unpaired *t*-test.

Table 1 IC_{50} values of ara-C, 5-FdUrd, and dimers determined in L1210 [A] and P388D1 [B] cell lines after 24, 48, and 72 hours of incubation

L1210 [A]	IC_{50}-values (nmol/l)		
Compounds	24 h	48 h	72 h
AraC	99.9	49.8	52.8
5-FdUrd	> 200	2.5	3.7
AraC+5-FdUrd	28.1	1.8	3.9
Dimer 2	99.0	63.6	24.8
Dimer 2A	77.3	22.2	17.3
Dimer 3	44.9	36.0	19.4
Dimer 10	38.0	5.4	5.8

P388D1 [B]	IC_{50}-values (nmol/l)		
Compounds	24 h	48 h	72 h
AraC	116.6	67.0	41.9
5-FdUrd	>200	9.8	12.6
AraC+5-FdUrd	138.3	9.8	10.9
Dimer 2	>200	62.4	51.0
Dimer 2A	>200	25.7	9.2
Dimer 3	>200	22.0	15.6
Dimer 10	>200	19.3	18.5

Results

Growth recovery assay

Exponentially growing L1210 and P388D1 cells were pretreated with ara-C and/or 5-FdUrd for 24 hours (using an equimolar concentration of 200 nM). Subsequently, cells were transferred to fresh medium and their viability was recorded after 24, 48, and 72 hours. During this period, cells exposed to ara-C renewed their proliferation in fresh medium easily and were similar to control cell cultures. In contrast, cells ex-

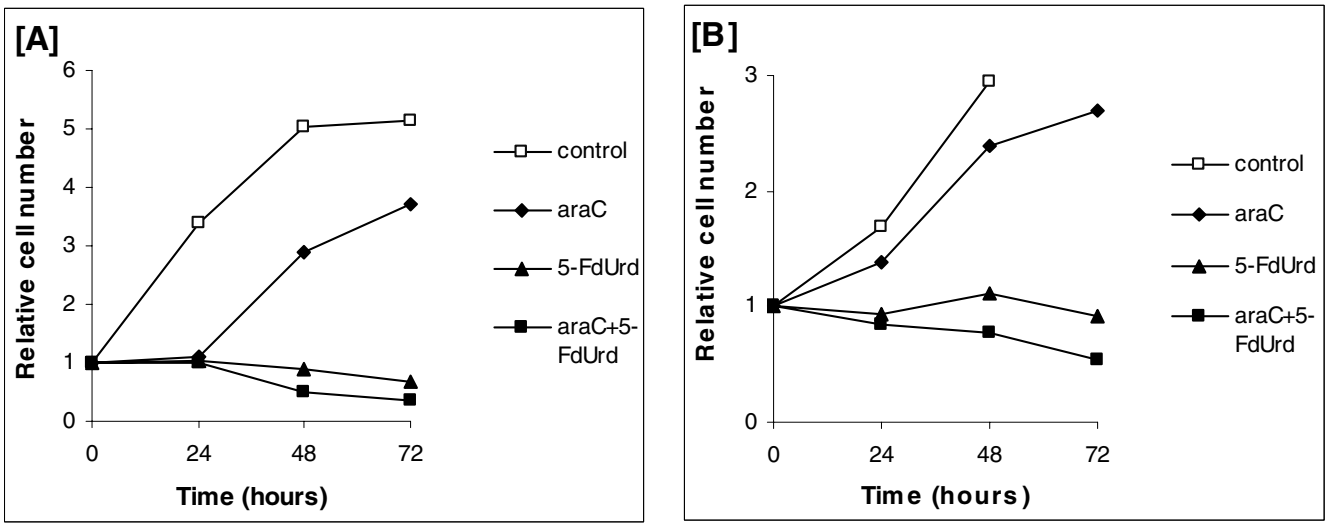

Figure 2 Effect of 24 hrs of pretreatment with ara-C (200 nM) and/or 5-FdUrd (200 nM) on the subsequent growth of L1210 [A] and P388D1 [B] cells in fresh medium after 24, 48, and 72h as evaluated by a growth recovery assay.

posed to 5-FdUrd did not renew their proliferation. Simultaneous application of both ara-C and 5-FdUrd led to only marginally increased cytotoxicity when compared to samples pretreated with 5-FdUrd. In all, 24 hours of exposure to ara-C or 5-FdUrd had a significantly different effect on tumor cells, a fact that was reflected in different cytotoxic profiles of these compounds (Fig. 2).

Growth inhibition assay

After 24 hours of incubation, the cytotoxic effects of 5-FdUrd were lower than those of ara-C. However, after 48 and 72 hours, cytotoxicity of 5-FdUrd became significantly higher than those of ara-C in both cell lines. Ara-C yielded only cytostatic effects, whereas 5-FdUrd exerted significant cytotoxicity. Results are summarized in Table 1.

Differences in cytotoxicity (IC_{50} values) between dimers and simultaneous application of ara-C and 5-FdUrd correspond with the ability of dimers to liberate one or both antimetabolites bonded in the dimer in relation to the applied dose and depending on the time period after application. The cytotoxic activity of the dimers increased gradually from No. 2 in the following order: No. 2< No. 2A< No. 3< No. 10. Both components of dimer No. 10 (ara-C and 5-FdUrd) liberated and became active. Only partial liberation/activation of antimetabolites contributed to the cytotoxic activity of dimers No. 2A and 3. The cytotoxic activity of dimer No. 2 was mostly due to liberation/activation of ara-C. Cytotoxicity of the dimers was similar in L1210 and P388D1 cells, but L1210 cells were more sensitive and their response to drug exposition was more prompt which correlates with their higher proliferation activity.

Non-lethal exposure of mice to dimers

A sub-lethal dose regime was established for every dimer. Weight decrease of animals should not exceed 30% of their original weight at the beginning of the experiment (results are depicted in Figure 3) as this represents a limit for therapeutic use in mice. Cytotoxicity of the dimers significantly decreased in the following order: No. 2 > No. 2A > No. 3 >

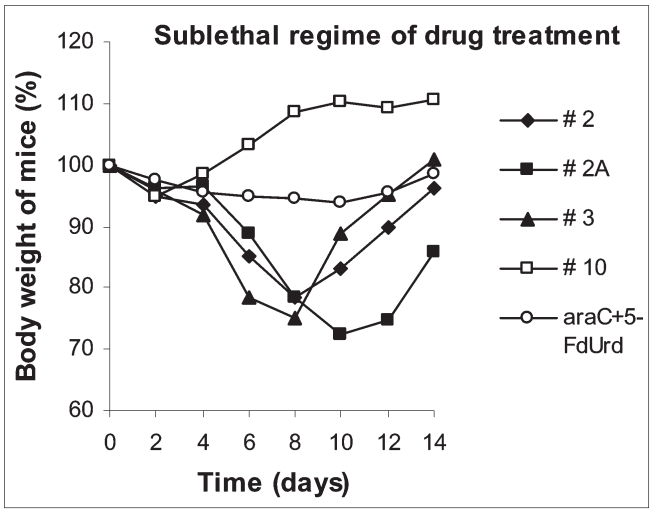

Figure 3 Non-lethal effect of dimers No. 2, 2A, 3, and 10 evaluated at different doses/regimes in mice. The toxic/reversible effects are presented in the enclosed graph as a progressive decrease/increase of body weight. Treatment resulting in more than 30% loss of original body weight was lethal for the experimental mice.

Compound	Schedule (days)	Dose/day (μmol/kg)	Cumulative dose (μmol/kg)
ara-C+5-FdUrd	1-10	20	200
dimer # 2	1;3;5;7	20	80
dimer # 2A	1;3;5;7;9	80	400
dimer # 3	1-5	80	400
dimer # 10	1-10	20	200

Table 2 Antileukemic effect of ara-C + 5-FdUrd, and dimers No. 2, 2A, 3, and 10 on the survival of L1210- [A] and P388D1-leukemia bearing mice [B].

[A]

Compound	N	Schedule (days)	Dose/day (μmol/kg)	Cumulative (μmol/kg)	MST (days ± SD)	ILS (%)	Significance (p-value)
Saline	6	–	–	–	8.7 ± 0.7	–	–
AraC+5-FdUrd	6	1; 4	80+80	160+160	10.7 ± 0.5	23.99	0.0002
Dimer 2	6	1; 4	80	160	14.2 ± 0.4	63.44	<0.0001
Dimer 2A	6	1; 4	80	160	12.4 ± 0.9	43.25	<0.0001
Dimer 3	6	1; 4	80	160	11.1 ± 1.1	27.80	0.0011
Dimer 10	6	1; 4	80	160	10.5 ± 1.3	21.11	0.0108

[B]

Compound	N	Schedule (days)	Dose/day (μmol/kg)	Cumulative (μmol/kg)	MST (days ± SD)	ILS (%)	Significance (p-value)
Saline	6	–	–	–	20.6±1.4	–	–
AraC+5-FdUrd	6	1; 3;5;7;9	40+40	200+200	35.5±5.4	72.3	0.0002
Dimer 2	6	1; 3;5;7;9	40	200	*toxic death	–	–
Dimer 2A	6	1; 3;5;7;9	40	200	21.7±1.0	5.6	0.0905
Dimer 3	6	1; 3;5;7;9	40	200	21.3±1.2	3.4	0.2337
Dimer 10	6	1; 3;5;7;9	40	200	20.8±0.8	0.9	0.9469
Dimer 10	6	1; 3;5;7;9	80	400	22.5±2.1	9.2	0.0196
Dimer 10	4	1-10	40	400	33.7±5.7	63.6	0.0013
Dimer 10	3	1-10	**40	400	42.3±14	105.3	0.0084

* toxic death – mice died during the drug treatment
** dimer was adminstered as a daily dose divided into two sub-doses (20 μmol/kg each) at morning and afternoon
MST – mean survival time
ILS – increase of life-span

No. 10. Applied doses and intervals of application were the two main factors contributing to the observed toxicity. Several doses and therapeutic schemes were tested for dimers and antimetabolites in control mice and optimal therapeutic regimens were developed.

Antileukemic effect of drugs in leukemia-bearing mice

L1210-bearing mice are marked by cells with a high rate of proliferation and by a relatively short course of the disease (untreated control animals usually die within 7-10 days after cell implantation). P388D1-bearing mice are typical of slowly proliferating cells resulting in a much longer course of disease (untreated control animals usually die within 18-22 days after cell implantation).

In L1210-leukemia, the dimers demonstrated a very good therapeutic potential during their application to experimental animals even when they were administered only at days 1 and 4 after implantation with a cumulative dose of 160 μmol/kg (Table 2A). Dimer No. 2 was the most effective drug. However, its high acute toxicity did not allow us to achieve similar results in any experiment employing P388D1 leukemia cells as a therapeutic model. In L1210-leukemia bearing mice, the therapeutic potential of the dimers increased in the following order: No. 10 < No. 3 < No. 2A < No. 2 which is completely controversial to the order of the determined *in vitro* cytotoxicity of the dimers (Table 1).

The P388D1-leukemia model was found to be not equally suitable for the used therapeutic regime (application of dimers at days 1, 3, 5, 7, and 9; cumulative dose of 200 μmol/kg; Table 2B). Regarding dimer No. 2, this cumulative dose was lethal for experimental animals. Concerning dimers No. 2A and 3, it was sub-lethal and on the border of any therapeutic usefulness. Dimer No. 10 was significantly less toxic (Fig. 3) – therefore, we were able to increase its cumulative dose to 400 μmol/kg and to apply this quantity at days 1-10 (40 μmol/kg as a single dose or 20 μmol/kg twice per day, Table 2). Generally, an increase of doses or numbers of doses improved the therapeutic outcome of dimer No. 10 in this model.

Discussion

Antineoplastic chemotherapy is not uniformly successful as various types of cancer in humans are either intrinsically resistant to treatment, acquire resistance during therapy, or as the chemotherapy cannot be successfully be delivered in cytotoxic concentrations to the tumor site. A wide variety of biochemical and physiologic phenomena has been observed that can modulate the efficacy of antineoplastic drugs. These include reduced drug uptake by the tumor cell, enhanced drug efflux, enhanced intracellular metabolism or detoxification of the chemotherapeutic agent that limits tumor cell toxicity. In the present study, we evaluated the effects of several new amphiphilic heterodinucleoside phosphates containing ara-C and 5-FdUrd on L1210 and P388D1 murine leukemia cells in comparison to the monomeric anticancer drugs ara-C and 5-FdUrd. We found that some of these dimers exert stronger antitumor effects when compared to the clinically used monomers.

Four dimers (No. 2, 2A, 3, and 10) were investigated for their *in vitro* cytotoxicity and *in vivo* therapeutic activity, and the obtained results were compared with an equimolar combination of ara-C and 5-FdUrd. The *in vitro* experiments were also used for determining the contribution of ara-C or 5-FdUrd components of dimers towards cytotoxicity of the particular dimer. It was expected that cytotoxicity could only be exerted after liberation of at least one of the antimetabolites, followed by subsequent phosphorylation to the active triphosphate (ara-CTP and/or 5-FdUTP). Since the cytotoxicity profiles of ara-C and 5-FdUrd are different, it can be assumed which of the monomers is activated and which one is not contributing towards antitumor activity.

The obtained results revealed that dimer No. 10 (Fig. 2, Table 1) exerts a cytotoxic activity comparable to a combination of ara-C and 5-FdUrd. The cytotoxic profile of dimer No. 2 reflects a significant contribution of ara-C and hardly any cytotoxic effect of 5-FdUrd. Regarding dimers No. 2A and 3, both components (ara-C and 5-FdUrd) contributed to their cytotoxicity, although their overall activity was low.

The therapeutic potential of dimers No. 2, 2A, 3, and 10 was investigated in L1210- and P388D1-bearing mice. Again, equimolar mixtures of ara-C and 5-FdUrd served as control values. The best therapeutic outcome in L1210-leukemia bearing mice was obtained with dimer No. 2 (Table 2A, 2B, Fig. 3). These findings are quite remarkable since this agent showed the lowest *in vitro* activity.

The increased antileukemic activity of these novel compounds underpins the protection of ara-C and 5-FdUrd against metabolic inactivation while they are incorporated in the dimers. On the other hand, the cytotoxic potential of the monomeric drugs can only be demonstrated after their liberation and activation by phosphorylation to active triphosphate molecules since ara-CTP and/ or 5-FdUTP are responsible for the activity of the dimers. The *in vivo* experiments confirmed the therapeutic activity of monomers and dimers in L1210 leukemia.

However, these drugs were not active in P388D1-leukemia bearing mice at the used dosage. Regarding dimer No. 10, changed conditions of therapy (increased cumulative dose and shortened intervals between individual drug applications) resulted in improved therapeutic outcome (Table 2B). The observed toxicity did not allow the administration of a similar therapeutic regime with dimers No. 2, 2A, and 3.

According to the results in Table 2A, dimers 2 and 2A may liberate monophosphates of ara-C and 5-FdUrd. As these dimers are stereo isomers, the difference in ILS indicates a different rate of hydrolysis and, consequently, different kinetics in terms of formation of active triphosphates (ara-CTP and 5-FdUTP). This assumption is also supported by the data shown in Tables 1A and 1B. The hydrolysis of dimer No. 3 and 10 possibly results in the formation of 5-FdUrd-3'-monophosphate that requires additional removal of a single phosphate group for its activation. As the only difference between dimer No. 3 and 10 is acetylation of the ara-C amino group and two free hydroxyl groups in the sugar moiety, it can be concluded that acetylation provides additional protection to the ara-C molecule, resulting in an increased ILS. The effect of ara-C acetylation is reversed under *in vitro* conditions (Tables 1A and 1B) probably because of the necessity of hydrolysis (deacetylation) of ara-C liberated from dimer 3, delaying the *in vitro* formation of ara-CTP.

The findings presented here demonstrate that these new agents may be beneficial for leukemia therapy since their administration could prevent resistance [18-20, 24] and therapeutic effects are likely to become synergistic in combined chemotherapy [17]. In addition, the activity of monomers could be prolonged because of their protection against metabolic degradation while being a part of the dimers. We believe that these results will serve as stimuli for further investigation of these promising new compounds.

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