Cytotoxicity and Antileukaemic Activity of New Duplexes Linking 3-C-Ethynylcytidine and 5-Fluorodeoxyuridine

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Abstract. *The cytotoxic and antineoplastic potential of two new duplex drugs, ECyd-5-FdU and ECyd- lipid- 5-FdU, were compared with the activity of the parent single-nucleoside analogues, 3-C-ethynylcytidine (ECyd) and 5-fluorodeoxyuridine (5-FdU), either applied as monotherapy or simultaneously in equimolar concentrations simulating their ratio in a duplex drug. Murine leukaemia L1210 cells were used for comparative in vitro tests of the duplex and the single drugs. The tested substances were evaluated for their cytotoxicity, combinatory potential and revitalisation properties. Additionally, an in vivo model of leukaemia L1210-bearing mice of the DBA/2J strain was used for testing of acute toxicity and antileukaemic activity using various chemotherapeutic regimes. Based on the results of this study, the suitability of ECyd and 5-FdU for forming a duplex drug was discussed from the perspective of their expected synergistic anticancer activities. We found an improvement of chemotherapy outcomes of the new duplex drugs tested by comparing their in vitro cytotoxicity and an increase of the time of survival of experimental leukaemia-bearing mice in a statistically significant manner.*

Antimetabolites represent a significant group of antineoplastic agents that possess a characteristic specificity towards target structures in tumour cells (1-3), compared to other groups of cytostatics, such as alkylating agents. A precise understanding of the mechanisms of action of individual anticancer agents is essential for their use in chemotherapy in various chemotherapeutic regimes (4-6). It is accepted that the antineoplastic potential of antimetabolites

in chemotherapy is limited by the short time of their therapeutic action in an organism due to their pharmacokinetic parameters, the necessity of their phosphorylation to an active anabolite and also their metabolic inactivation (7). Synthesis of new prodrug-like derivatives, analogues and conjugates of antimetabolites represents an option for new substances with improved pharmacokinetics and pharmacodynamics (8-11). This means that new prodrugs would either act in an organism for a longer time and, therefore, would show higher bioactivity compared to a parent compound, or that the activation will take place only in tumour cells as a result of improved selectivity, or that the metabolic degradation of the prodrug, specifically in its activated form, will be decreased. All of these features, together with suitable forms of drug administration as part of an optimal combined-chemotherapy protocol, should lead to an optimised therapeutic application of such new antimetabolite prodrugs.

The concept of combined chemotherapy is essential, being advantageous over singe chemotherapy regimens due to possible synergistic effects of the applied chemotherapeutic agents. However, when therapeutic combinations are not properly selected, the combined antitumour action may only be additive or may even be antagonistic. A goal of preclinical studies of potential antineoplastic agents is to gather enough data from experimental models that would be of use in creating therapeutic regimes with the best possible therapeutic outcome due to a suitable combination of anticancer drugs and an optimal regime of administration. Both *in vitro* and *in vivo* techniques and models make a comparison between cytotoxic/cytostatic effects in tissue culture models and therapeutic outcome in experimental animals possible (5, 6, 12). When a substance with significant cytotoxicity *in vitro* does not demonstrate the expected therapeutic potential *in vivo*, and when this is not because of metabolic degradation or lack of bio-activation, then the problem may be that an unsuitable therapeutic regime was used. All of these concerns have led to the search

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for ways to increase the therapeutic potential of antineoplastic agents, including antimetabolites. Synthesis of new duplex drugs is one option in that search (13-17).

This rationale was applied during the present study of new synthetic duplex drugs linking the antimetabolite 5-fluorodeoxyuridine 5-FdU and ECyd directly *via* phosphodiester bonding 3-c-ethynylcytadine (5-FdU-ETC) or indirectly over a glycerine lipid backbone (ECyd-lipid-5-FdU) (Figure 1). The duplex drugs were synthesised based on the idea that combining these two active antimetabolites in one molecule should ensure higher therapeutic outcome because of their synergism when they attack molecular targets of malignant cells at the same time. Consequently, the cytotoxic/cytostatic potential of these substances in L1210 cell culture was related to the results of antileukaemic therapy of experimental mice bearing leukaemia L1210 cells. The data were analysed from the viewpoint of suitability/non-suitability of the parent antimetabolites ECyd and 5-FdU for conjugation in a duplex drug and also considering the usefulness of using a lipid backbone as a part of ECyd-lipid-5-FdU. These two duplex drugs differ in the presence of a lipid backbone in ECyd-lipid-5-FdU that increases the lipophilicity of the hydrophilic nucleoside analogues. Additionally, based on cytotoxicity data obtained *in vitro*, the study determined a therapeutic regime for optimal manifestation of the therapeutic potential of the duplex drug in experimental leukaemia-bearing mice.

Materials and Methods

Drugs. The two investigated duplex drugs 5-FdU-ECyd [5-fluoro-2'-deoxyuridylyl-(3→5')-3-*C*-ethynylcytidine] and Ecyd-lipid-5-FdU [3-*C*-ethynylcytidylyl-(5'→1)-2-*O*-octadecyl-rac-glycerylyl-(3→5')-5-fluoro-2'-deoxyuridine], as well as the nucleoside analogue ECyd (3-*C*-ethynylcytidine) were synthesized by a Professor H. Schott at the Department of Organic Chemistry, University of Tuebingen, Germany. 5-FdU and all other chemicals and reagents used were obtained commercially and were of the highest purity.

Cell culture. Murine leukaemia cell line L1210 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum (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 an atmosphere of 5% CO_2 in humidified air at 37˚C. Exponentially growing cells were used in all experiments. The presented data always represent a mean of three or more determinations. Standard deviations were within 5%.

Growth inhibition assay. The cytotoxic/cytostatic potential of the tested substances (5-FdU, ECyd, 5-FdU-ECyd and ECyd-lipid-5-FdU) was determined *in vitro* using the model of L1210 cells in the phase of their exponential growth (1×10^5 cells/ml). The presented data always represent a mean of three or more determinations.

Standard deviations were within 5%. The cells in a growth medium were exposed to 5 nM or 10 nM of the tested substance for 48 h. Cell viability was than determined using Trypan blue and was expressed as a percentage relative to the viability of the control cells. Because both tested duplex drugs had 5-FdU and ECyd as parts of their molecules (Figure 1) in a 1:1 ratio, their cytotoxicity was compared to the cytotoxicity of 5-FdU plus ECyd mixture in equimolar concentrations. The cytotoxic potential that was determined for both duplex drugs was related to the cytotoxicity of the 5-FdU plus ECyd mixture and was expressed as a multiple of their decreased cytostatic potential. Additionally, a combination index (CI) was calculated for 5-FdU and ECyd in a single application and in a combination according to the method of Chou and Talalay (18). A CI value smaller than 1 represents synergism of activities of the compounds in a mixture, while a CI value larger than 1 indicates antagonism of the action of substances that are being combined for the cytostatic test.

Growth recovery assay. The growth recovery assay (5, 6, 19) consisted of two phases. In the first phase, inhibition of cellular proliferation in exponentially growing cells of leukaemia L1210 (cell density 5×10^5 cells/ml) was induced by cultivating the cells with ECyd (200 nM/l), 5-FdU (100 nM/l), 5-FdU-ECyd (100 nM/l) and ECyd-lipid-5-FdU (100 nM/l). In the second phase of the assay, after an interval of cultivation with an antimetabolite (24, 48 and 72 h), the cells were washed and cultivated in a drug-free medium (1×10^5 cells/ml) for 10-12 days. During the drug-free cultivation, cell viability was determined daily (days 1-12) using Trypan blue and the time at which proliferation of the drug-affected or drug-damaged cells was renewed was determined. The presented data always represent a mean of three or more determinations. Standard deviations were within 5%.

Animals. Inbred DBA/2J mice of both genders, weighing 18-22 g, were obtained from the breeding facility of the Cancer Research Institute of SAS (Bratislava, Slovak Republic). There were six animals for every experimental condition tested. The animals were reared under standard conditions. The host laboratory was 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 guideline principles for the care and use of laboratory animals.

Acute toxicity of drugs. Acute toxicity of the tested substances in experimental animals significantly limits the use of any substance in therapy. The dose that did not cause a weight loss of more than 30% of the original weight was determined for all substances used in the experiments. This led to the determination of the maximal tolerated dose (MaxTD) for all tested compounds. The limit of 30% weight loss was selected as such weight loss is induced by a sub-lethal dose of a substance. A further weight loss leads to toxic side- effects and is usually lethal for experimental animals.

An experimental model with leukaemia L1210- bearing DBA mice was employed. Drugs were applied intraperitoneally in a single administration of ECyd (10 μM/kg), 5-FdU-Ecyd (10 μM/kg per day), ECyd-lipid-5-FdU (10 μM/kg) and 5-FdU (100 μM/kg). Additionally, the toxicity of both duplex drugs was tested at repeated administration of 5 μM/kg per day on days 1, 3 and 5.

5´-FdU, 5-fluorodeoxyuridine
ECyd, 3'-ethynylcytidine
P, phosphodiester linakge
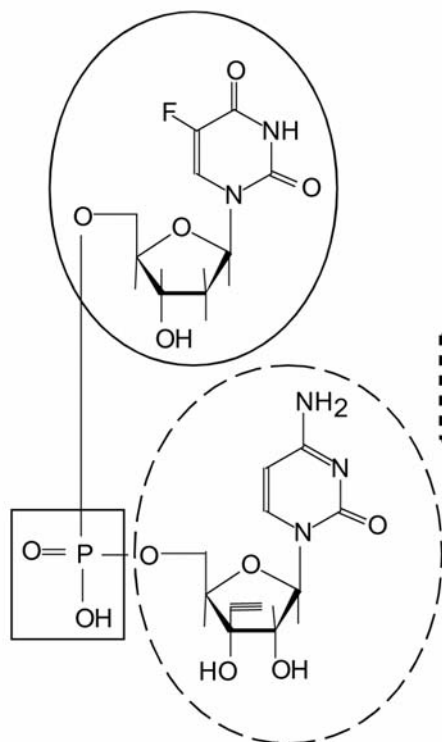L, hydrophobic glycerol lipid backbone

5-FdU-ECyd
5-fluoro-2'-deoxyuridylyl-(3→5')-3-C-ethynylcytidine
MW: 575
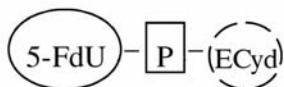
ECyd-lipid-5-FdU :
3`-C-ethynylcytidylyl-(5'→1)-2-O-octadecyl-rac-glycerylyl-(3→5')-5-fluoro-2'-deoxyuridine
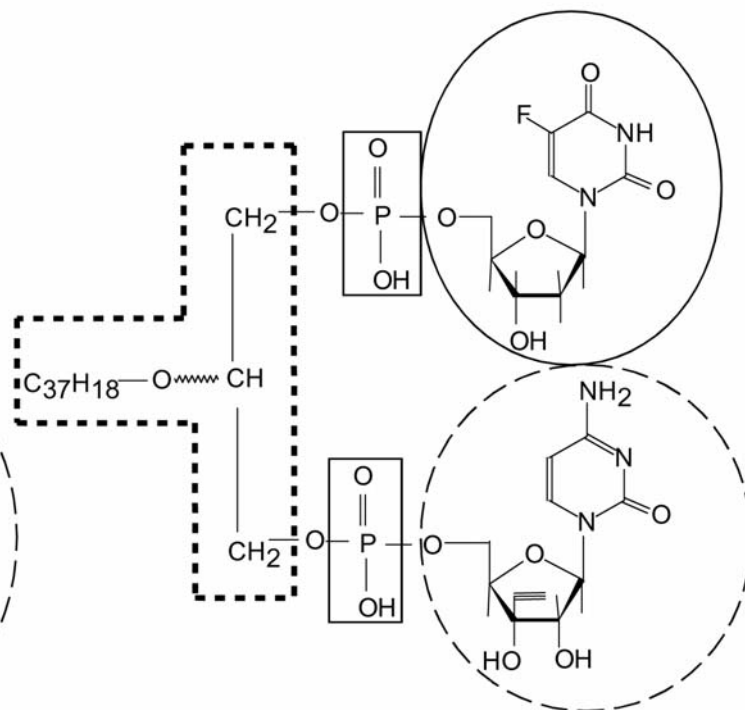MW: 981
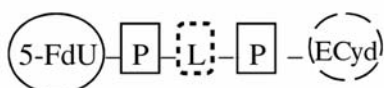
Figure 1. *Structural formulas of duplex drugs and nucleoside analogs used in this study.*

Antileukaemic activity in vivo. L1210 leukaemia-bearing mice were used as an antileukaemic therapeutic model. The experimental animals received intraperitoneally implanted L1210 murine leukaemia cells (1×10^5 cells per mouse). Antileukaemic treatment started 24 h 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 MaxTD. Animals were weighed daily and observed for development of ascites. Leukaemia-related deaths were recorded. The therapeutic effect of the substances was evaluated by the increase in the mean survival time (MST) of the treated mice with implanted leukaemia L1210 cells in comparison to the implanted but untreated control mice, which determined the percentage increase of life span (ILS).

Index of cytotoxic/therapeutic activity of a drug. The cytotoxic potential of the drugs was determined as an IC_{50} value in *in vitro* experiments and their therapeutic efficacy was determined as the dose of the particular drug that prolonged the time of survival of experimental leukaemia-bearing mice in a statistically significant manner (TED). The index of cytotoxic and therapeutic activity (ICTA) of each drug was calculated as the ratio IC_{50}/TED. ICTA characterized each drug in relation to its cytotoxic potential in the treatment of leukaemia. ICTA for cisplatin was arbitrary selected as a reference and was assigned a value of 100%. ICTA values for other substances or drugs were expressed as percentages in relation to this reference. The IC_{50} values of drugs were calculated using the CalcuSyn software for Windows (Biosoft, Cambridge, UK).

Results

Growth inhibition assay. Lower cytotoxic effect was observed with ECyd than with the equimolar concentration of 5-FdU, for two concentrations, 5 nM/l and 10 nM/l. The difference was significant as cytotoxic inhibition through ECyd compared to 5-FdU was 2.5 times lower at the concentration of 5 nM and 3.6 times lower at the concentration of 10 nM (Table I). Moreover, decreased cytotoxicity was also observed with both duplex drugs compared to the cytotoxicity of equimolar mixtures of ECyd and 5-FdU (5 nM or 10 nM of duplex drug compared to 5 + 5 nM and 10 + 10 nM of the parent antimetabolites, respectively). The cytotoxicity of 5-FdU-ECyd was decreased 1.4- and 1.5-fold, respectively, for the two concentrations given above. The decrease of cytotoxicity was even more significant with ECyd-lipid-5-FdU, for which respective decreases of 8.1- and 8.3-fold were recorded (Table I).

Evaluation of the cytotoxic potential of the combination of ECyd and 5-FdU applied simultaneously in comparison to the calculated cytotoxicity sum of both antimetabolites applied separately (18) resulted in a strong synergism of cytotoxicity of both drugs. CI values were 0.525 and 0.711, respectively (Table I).

Growth recovery assay. All substances were examined in growth recovery assay experiments. The results of the first phase of the assay are shown in Figure 2A. A cytotoxic effect of 5-FdU and of both duplex drugs was obtained at the same concentration of 100 nM. In contrast, a comparable cytotoxic effect of ECyd was observed only at the concentration of 200 nM. This finding confirmed the higher cytotoxic potential in L1210 cells of 5-FdU compared to ECyd as presented in Table I. Growth recovery or renewal of proliferation during the 12-day cultivation in a drug-free cultivation medium is followed up in the previously drug-exposed cells during the second phase of the assay. The data obtained with L1210 cells exposed to the tested substances for 24, 48 or 72 h are presented in Figure 2B-D, respectively. Additional characteristics of the tested antimetabolites were therefore demonstrated, as cells exposed to 5-FdU renewed their proliferation within 4-6 days and cells exposed to ECyd within 7-9 days of cultivation in a drug-free medium. In the case of the duplex drugs, neither growth recovery nor proliferation renewal was detected even 12 days after the transfer of cells into drug-free conditions. These results demonstrated that cells exposed to 5-FdU renew their proliferation faster than cells exposed to ECyd. This observation serves as a base for the expectation that therapeutic effects *in vivo* may be more significant with the therapy using ECyd than 5-FdU despite the higher cytotoxicity of 5-FdU compared to ECyd (see Table I).

Table I. *Antiproliferative potential of 5-FdU, ECyd, 5-FdU-ECyd, ECyd-lipid-5-FdU monitored 48 hours after L1210 cells exposure to an individual substance or to their combination. Data are the means of at least three determinations. Standard deviations were within ±5%.*

Treatment	Concentrations (nM)	Cell viability		*CI value
		% Of control	Relative value	
Control	/	100.0	/	
5-FdU	5	24.5	1.0× (1)	
ECyd	5	60.2	2.5× (1)	
5-FdU + ECyd	5+5	11.5	1.0× (2)	0.525 (S)
5-FdU-ECyd	5	16.7	1.4× (2)	
ECyd-lipid-5-FdU	5	90.4	8.1× (2)	
5-FdU	10	14.5	1.0× (1)	
ECyd	10	51.6	3.6× (1)	
5-FdU + ECyd	10+10	7.8	1.0× (2)	0.711 (S)
5-FdU-ECyd	10	11.8	1.5× (2)	
ECyd-lipid-5-FdU	10	65.0	8.3× (2)	

*CI, Combinatory index calculated according the method described in the text; (S), synergism (CI<1.0): (1) Anti-proliferative effect of Ecyd was 2.5-fold, respectively 3.6-fold lower in an comparison to 5-FdU. (2) Anti-proliferative effect of 5-FdU-ECyd was 1.4-fold, respectively 1.5-fold lower in comparison to 5-FdU+ECyd combination; anti-proliferative effect of ECyd-lipid-5-FdU was 8.1-fold, respectively 8.3-fold lower in comparison to 5-FdU+ECyd combination.
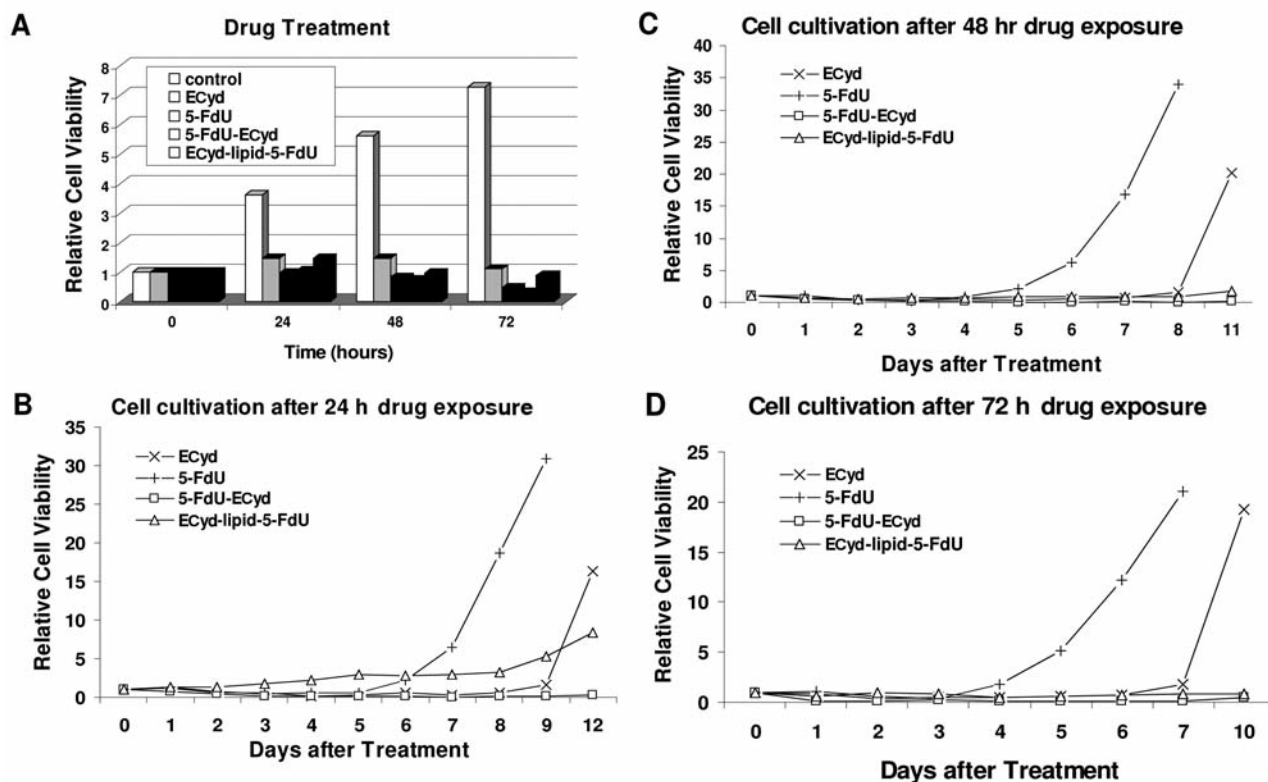
Figure 2. *Growth recovery assay. Murine leukaemia cells L1210 were in the first phase of the experiment exposed with antimetabolites ECyd (200 nM/L), 5-FdU (100 nM), 5-FdU-Ecyd (100 nM), Ecyd-lipid-5-FdU (100 nM) for 24, 48 or 72 h to stop cell proliferation (A). In the second phase of the experiment, the non-proliferating cells were transferred to a fresh drug-free medium for investigation of their ability to renew proliferation during the following 10-12 days' cultivation (B, C, D). Data are presented as the mean from three independent experiments. Standard deviations were within ±5%.*

The results with the duplex drugs were even more encouraging. This may have been caused by a synergistic action of ECyd and 5-FdU and justified the synthesis of these duplex drugs. The results of ECyd, 5-FdU and both duplex drugs led to the expectation that adequately improved therapeutic profiles of the duplex drugs *in vivo* (a prolongation of a remission and difference of a relapse phase of leukaemia) as therapeutic outcomes are likely to correspond well with the results obtained in the growth recovery assay.

Acute toxicity of drugs. Acute toxicity of 5-FdU, ECyd, 5-FdU-ECyd and ECyd-lipid-5-FdU was monitored as weight change of experimental animals during days 1-7 and days 1-13 of the experiment as shown in Figure 3. The toxicity of the substances decreased in the following order: ECyd>5-FdU-ECyd>ECyd-lipid-5-FdU>>5-FdU. 5-FdU demonstrated the lowest toxicity despite the fact that was administered in a 10-fold higher concentration than the other substances (Figure 3A). An increase of administrated doses of the duplex drugs from one to three led to an

increase in toxicity of the ECyd-lipid-5-FdU. ECyd-lipid-5-FdU became more toxic at repeated administrations than did 5-FdU-ECyd (Figure 3B).

Antileukaemic activity in vivo. The study of antileukaemic activity of all substances was based on knowledge of their MaxTD. Consequently, no toxicity-related death was observed (Table II). The therapeutic effect of the substances tested at the same experimental condition (dose and administration) increased in the following order: ECyd-lipid-5-FdU=5-FdU<5-FdU-ECyd<ECyd (Table II). The observed ILS values were statistically significant ($p<0.5$) for all tested compounds when compared to survival length of the untreated control group. The 10-fold increase of 5-FdU dose (100 μmol/kg/day) did not result in any improved therapeutic outcome. However, the therapeutic outcome improved with duplex drugs when a different administration regime was used (5 μmol/kg on days one, three and five). Both duplex drugs were significantly less effective in therapy when administered at a single dose only (10 μmol/kg/day) in comparison to ECyd. However, the therapeutic outcome with
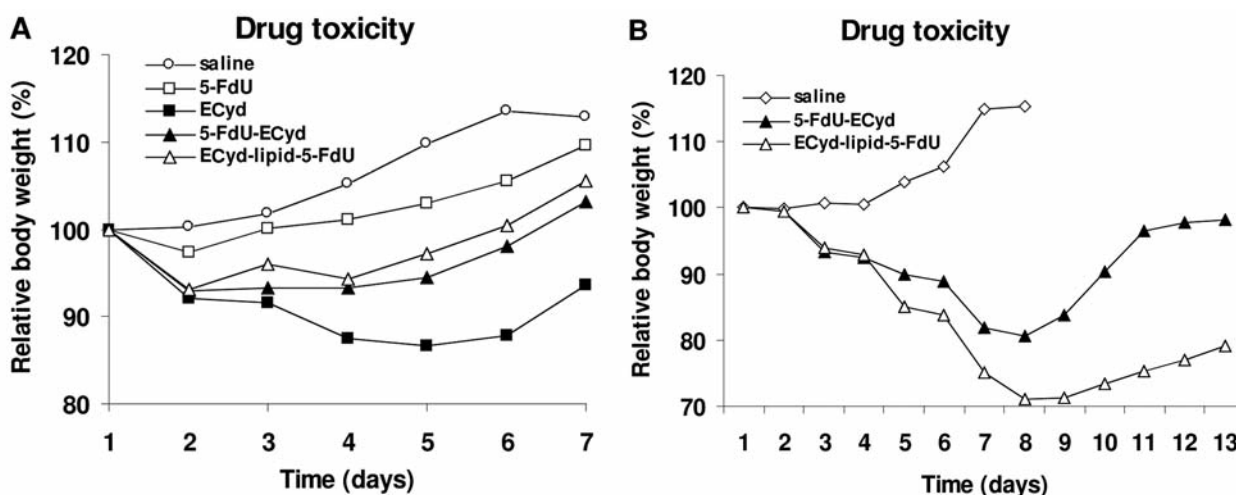
Figure 3. *Acute toxicity of the tested substances was monitored as a decrease of the weight of experimental animals after a daily administration of a single dose of 5-FdU (100 μmol/kg per day), ECyd (10 μmol/kg per day), 5-FdU-ECyd (10 μmol/kg per day), ECyd-lipid-5-FdU (10 μmol/kg per day) during a 7-day period (days 1-7 of the experiment, A). Similarly, toxicity of duplex drug 5-FdU-ECyd and ECyd-lipid-5-FdU was monitored as a weight loss during a period of 13 days (days 1-13 of the experiment, B). The applied doses of both duplex drug were 5 μmol/kg at day 1, 3 and 5 (a cumulative dose of 15 μmol/kg was administered). Six or more animals were treated in every group. Data are presented as the average of determinations. Standard deviations were significantly less than 5%.*

Table II. *Antileukemic effect of tested drugs on the survival of leukaemia L1210-bearing mice.*

Drug	Administration (days)	Dose/day (μmol/kg)	Cumulative dose (μmol/kg)	Leukemia-related death*	
				MST (days±SD)	ILS (%)
Control	/	/	/	8.25±0.61	0.0
ECyd	1	10	10	13.21±1.35	60.12
5-FdU	1	10	10	9.85±2.06	19.39
5-FdU	1	100	100	9.75±0.76	18.18
5-FdU-ECyd	1	10	10	11.05±1.38	33.94
ECyd-lipid-5-FdU	1	10	10	9.80±0.77	18.79
Control	/	/	/	9.00±0.61	0.0
ECyd	1; 3; 5	5	15	14.0±0.00	64.71
5-FdU	1; 3; 5	5	15	10.0±1.41	17.66
5-FdU-ECyd	1; 3; 5	5	15	14.5±0.77	61.11
ECyd-lipid-5-FdU	1; 3; 5	5	15	14.2±1.33	58.33

There were 6 mice in every experimental group. ILS, Increase of life-span (%); MST, mean survival time (± SD); *no 'toxic death' occurred.

duplex drug treatment became comparable with that of the ECyd treatment when the duplex drugs were administered in three doses (5 μmol/kg on days one, three and five).

ICTA. Table III gives the ICTA values for the substances tested, relative to the ICTA of cisplatin. The order of the loss of therapeutic potential in the applied experimental conditions was as follows: 5-FdU>araC>5-FdU-ECyd>ECyd >ECyd-lipid-5-FdU>>cisplatin. These results clearly illustrated that antimetabolites lose their cytotoxicity in the course of their presence in a biological system during

chemotherapy. This was even more obvious in the comparison with cisplatin that retains its chemotherapeutic potential in a much more significant extent. The results showed that only 0.6-4.0% of the cytotoxic potential of antimetabolites are actually applied during the course of chemotherapy. However, this percentage increased in the case of the tested duplex drugs. The applied cytotoxic potential was 0.9 % for 5-FdU-ECyd and 8.6% for ECyd-lipid-5-FdU. The value of the actual applied-in-chemotherapy cytotoxic potential was notably improved in the case of ECyd-lipid-5-FdU. An inclusion of a lipid moiety

Table III. *Index of cytotoxic and therapeutic activity (ICTA).*

Drug	IC$_{50}$ (μM)	TED (μmol/kg)	Index IC$_{50}$/TED	Standard cis-Pt (%)
cis-Pt*	0.2334	10	2.3×10^{-2}	100
araC*	0.0327	200	1.6×10^{-4}	0.7
5-FdU	0.0020	15	1.3×10^{-4}	0.6
Ecyd	0.0091	10	9.1×10^{-4}	4.0
5-FdU-Ecyd	0.0022	10	2.2×10^{-4}	0.9
Ecyd-lipid-5-FdU	0.0201	10	2.0×10^{-3}	8.6

IC$_{50}$, Cytotoxicity expressed as the drug concentration that inhibits cell proliferation by 50%; TED, therapeutic dose prolonged survival of leukaemia L1210-bearing mice in a statistically significant manner ($p \leq 0.5$). cis-Pt standard, IC$_{50}$/TED determined for cis-Pt (2.3×10^{-2}) used as a standard value (100%). The values calculated for other substances are compared to the standard (as % of the standard). *cis-Pt and araC are standards used for *in vitro* and *in vivo* experiments.

into the duplex drug structure is justified as it results in approximately 10-fold higher application of cytotoxic potential of the substance in chemotherapy compared to 5-FdU-Ecyd that does not have a lipid backbone in the molecule (Figure 1).

Discussion

The present study evaluated new duplex drugs, based on two types of experimental results. First, *in vitro* techniques were used with regard to cytotoxic potential CI and growth recovery assay. Secondly, *in vivo* investigations using experimental animals were performed regarding acute toxicity, antileukaemic effects, and suitable therapeutic regime. The therapeutic properties of the duplex drugs were compared with those of the parent antimetabolites 5-FdU and ECyd, applied simultaneously. The aim of these comparisons was to evaluate which of the characteristics of the duplex drugs are additive or synergistic in their nature and which are antagonistic.

The data shown in Table III indicated that the selection of parent antimetabolites for a synthesis of duplex drugs was rational. This is because of the generally accepted fact that antimetabolites often demonstrate high cytotoxicity in various tissue cultures but their anticancer activity *in vivo* is usually much lower than expected if judged simply according to their *in vitro* cytotoxicity. For better quantification of this low efficiency of antimetabolites in chemotherapy, the comparison with cisplatin was used. The data from the experiments performed demonstrate that the ICTA value for the antimetabolites is approximately 1/100 of the ICTA value for cisplatin (Table III). Consequently the antimetabolites represent substances that demonstrate excellent *in vivo* cytotoxicity in antileukaemic action. The

combination of the two antimetabolites, ECyd and 5-FdU, demonstrated high cytotoxicity, resulting in their synergistic action (CI<1, Table I). However, a similar cytotoxic effect was not observed with the duplex drugs. Decrease in cytotoxicity was especially pronounced with ECyd-lipid-5-FdU (Table I). However, this may not be important for the use of these duplex drugs in chemotherapy of leukaemia as the growth recovery assay demonstrated that cells exposed to the duplex drugs (for 48 or 72 h) do not renew cellular proliferation (Figure 2). This was different from the behaviour of cells exposed to ECyd or 5-FdU.

Acute toxicity (Figure 3), chemotherapeutic potential and an effective therapeutic regime (Table II) were evaluated in *in vivo* experiments using experimental mice. Again, the duplex drugs were compared to ECyd and 5-FdU in all aspects tested. Acute toxicity determined in mice was significantly higher for ECyd than for 5-FdU. This is a disadvantage for the combination of both parent nucleosides as in duplex drugs both substances are present in equimolar concentration. Consequently, the dose of the mixture of both parent nucleoside analogues was limited due to MaxTD value determined for ECyd. Consequently, at this dose, 5-FdU was administered in a relatively low dose regarding its toxicity and its therapeutic activity (Figure 3A, Table II). The second discrepancy in administering ECyd and 5-FdU in a combination was the fact that these antimetabolites are usually administered in different therapeutic regimes. While ECyd demonstrated its chemotherapeutic effect even after a single dose administration (Table II), 5-FdU requires administration over a period of several days (6). Consequently, the usefulness of combined therapy may be enhanced if toxicity and the required application time-length of the combined substances are similar. Otherwise, if there is a large difference between these parameters in the drugs used in combined chemotherapy regime, the therapeutic outcome of such combination may be diminished.

The present study evaluated the benefit of introducing a lipid backbone into the structure of a duplex drug. The presence of a lipid backbone in the structure of ECyd-lipid-5-FdU improved the antileukaemic activity compared to 5-FdU-ECyd, which did not have a lipid residue in its molecular structure (Figure 1). These findings are similar to those published previously on dimers consisting of 5-FdU and arabinosylcytosine, where the introduction of glycerol-based hydrophobic moiety also benefited experimental leukaemia-bearing mice (6). Both duplex drugs demonstrated low cytotoxicity *in vitro*, as shown by their low ICTA values relative to the ICTA value for cisplatin (Table III). ECyd-lipid-5-FdU had a markedly higher ICTA value than did 5-FdU-ECyd, suggesting higher involvement of the antileukaemic potential of ECyd-lipid-5-FdU compared to 5-FdU-ECyd *in vivo*.

The reason for the better antileukaemic performance of ECyd-lipid-5-FdU may be explained by comparing their

molecular structure (Figure 1). The introduction of glycerol backbone derivatized with a long chain carbon residue in the structure ECyd-lipid-5-FdU results in a significant increase of lipophilicity compared to 5-FdU-ECyd. It is possible that this change improves the cellular uptake of ECyd-lipid-5-FdU compared to 5-FdU-ECyd. Moreover, in the structure of 5-FdU-ECyd, both parent nucleosides are connected through phosphodiester linkage. During hydrolysis of 5-FdU-ECyd, one of the nucleosides is necessarily left without a phosphate residue at the 5' position of the sugar moiety. This position is a place where activation of the nucleoside occurs by forming a monophosphate and, since monophosphate formation is a rate-limiting process, 5-FdU-ECyd is disadvantaged compared to ECyd-lipid-5-FdU. This is because ECyd-lipid-5-FdU hydrolysis may liberate both parent nucleosides in the form of a nucleoside monophosphate and, consequently, the formation of an active triphosphate is increased.

In conclusion, the results and the techniques used in the present study indicate a method which may be useful in selecting and developing substance combinations with anticancer activity that would promote their synergistic action not only in their cytotoxicity but also mainly in their action in experimental animals and, possibly, even in patients. The developed approach may be used to assess the suitability of specific chemotherapy combinations of particular drugs that would enable the prediction of possible actions of a designed combination in relation to its synergism or antagonism during chemotherapy.

Acknowledgements

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