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Acute Leukemias

The interaction of gemcitabine and cytarabine on murine leukemias L1210 or P388 and on human normal and leukemic cell growth in vitro

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ABSTRACT

Background and Objectives. **Gemcitabine (dFdC) is a new nucleoside antimetabolite of deoxycytidine that resembles cytarabine (Ara-C) in both its structure and metabolism. Little is known about dFdC efficacy in hematologic malignancies, either as a single drug or in combination with other drugs. In this study we have tried to determine whether the cytotoxic effect of Ara-C can be increased by using it in combined therapy with dFdC.**

Design and Methods. **In the *in vivo* part of our study, mice bearing L1210 or P388 leukemia were treated with dFdC and Ara-C. The drugs were administered alone and in combination according to the following schedules: Ara-C and dFdC at the same time, dFdC before Ara-C, and Ara-C before dFdC. The efficacy of the therapy against leukemia (defined as the increase in lifespan, ILS) was assessed as the percentage of the median survival time (MST) of the treated group (T) in relationship to that of the control group (C): $ILS=[(MST_C / MST_T) -1]x100$. In the *in vitro* part of our study, normal granulocyte-macrophage colony-forming unit (CFU-GM) cells as well as CFU-GM cells obtained from patients with chronic myeloid leukemia (CML) were incubated either with dFdC or Ara-C alone or with adequate concentrations of a combination of these drugs.**

Results. **The *in vivo* experiment revealed that in both leukemias tested, combined therapy with dFdC given before Ara-C and dFdC given at the same time with Ara-C were more effective than monotherapy with either dFdC or Ara-C. The other treatment schedule (Ara-C before dFdC) did not significantly prolong the survival time of the treated mice bearing L1210 or P388 leukemia as compared with the treatment with dFdC alone. The *in vitro* experiments showed that dFdC used together with Ara-C acted additively on normal as well as CML CFU-GM cells. Furthermore, the drugs used jointly inhibited the growth of colonies formed by CML CFU-GM cells to a significantly higher degree than normal CFU-GM and the differences were statistically significant in the case of the combination of highest concentrations.**

Interpretation and Conclusions. **Gemcitabine increased the activity of Ara-C. As these agents incorporate into DNA blocking chain elongation, and moreover, dFdC influences the cytotoxicity of Ara-C, our results could be explained by the drugs acting at these levels. dFdC used jointly with Ara-C may have an important clinical implication in the treatment of CML and other hematologic malignancies in future.**

Key words: gemcitabine, cytarabine, leukemias, *in vitro* cultures, interactions

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Cytarabine (Ara-C) is one of the most effective drugs for the treatment of acute myeloid and lymphoblastic leukemias (AML, ALL) as well as for chronic myelogenous leukemia (CML). This drug is used either alone or in combination with other cytotoxic agents. The cytotoxicity of Ara-C is closely correlated to its uptake and intracellular conversion to its active metabolite, the 5'-triphosphate (Ara-CTP). The intracellular accumulation of Ara-CTP is a multistep process in which phosphorylation of Ara-C by deoxycytidine kinase (dCK) is rate-limited.[1] Several previous investigations have revealed that fludarabine (Fara-A) and cladribine (2-CdA), due to inhibition of ribonucleotide reductase, increase the activity of dCK, leading to a higher rate of Ara-CTP accumulation.[2,3] Hence, increasing Ara-CTP production by dCK has been a major goal in chemotherapy.

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a fascinating new analog of deoxycytidine that contains two fluorine atoms in the 2' position of the deoxyribose sugar. It resembles Ara-C in both its structure and metabolism.[4,5] dFdC exerts its cytotoxic effect after phosphorylation by dCK to the active metabolite triphosphate dFdC (dFdCTP), but in comparison to Ara-CTP the relative concentration of dFdCTP is higher and is retained for longer periods of time because of the mechanism of *masked chain termination* of DNA exhibited by dFdC.[6,7] Moreover, dFdC may inhibit DNA synthesis by acting at different sites – either by inhibition of DNA polymerase or by incorporation into DNA and blockage of chain elongation or inhibition of the enzymes ribonucleotide reductase and deoxycytidine deaminase, and also by incorporation of dFdC into RNA.[8,9]

Little is known about the efficacy of dFdC in hematologic malignancies, either given alone or in combination with other drugs. Our previous studies proved that dFdC in combination with 2-CdA has an additive action on murine leukemias L1210 and P388.[10] In the present study, we tried to determine whether the cytotoxic effect of Ara-C can be potentiated by using it in combined therapy with dFdC.

Design and Methods

The in vivo study

Animals. For these experiments 144 male CD2F1 strain mice were used. The animals weighed 24-27g, were aged 8-12 weeks, and were purchased from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wroclaw, Poland). They were given standard laboratory food and water *ad libitum*. The animals were divided into 6 groups of 6 for each leukemia line.

Leukemias. Leukemia cell lines L1210 and P388 were kindly provided by the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wroclaw) and were maintained by serial passages in the ascitic fluid of CD2F1 mice. Leukemic cells from the fluid were resuspended in 0.9% sodium chloride so that 10^6 L1210 or P388 cells were injected intraperitoneally (i.p.) into CD2F1 recipients.

Therapeutics. dFdC (Gemcitabine) was kindly supplied by Eli Lilly & Co., Indianapolis, IN, USA as a dry powder. Solutions for the experiments were prepared in isotonic saline and stored at 5°C. Cytarabine (Cytosar, Upjohn) was diluted in 0.9% solution sodium chloride just before injection. The drugs were delivered in a volume of 0.01 mL/g mouse weight. The control group of mice received equivalent volumes of 0.9% sodium chloride.

Antileukemic assay. Animals received 10^6 L1210 or P388 leukemic cells i.p. on day 0. All treatments were i.p. and were initiated on the next day. Gemcitabine was administered at a dose of 20 mg/kg on days 1, 4, 7 and 10. Ara-C was given at a dose of 1,000 mg/kg on days 1, 4, and 7. Drugs were given alone and in combinations according to the regimens given below:

1) dFdC and Ara-C at the same time (dFdC on days 1, 4, 7, 10 and Ara-C on days 1, 4, 7);
2) dFdC before Ara-C: sequential therapy (dFdC on days 1, 4, 7, 10 and Ara-C on days 11, 14, 17);
3) Ara-C before dFdC: sequential therapy (Ara-C on days 1, 4, 7 and dFdC on days 8, 11, 14, 17).

Drugs doses were chosen according to ref. #11-14. The survival of the animals was recorded for daily for a minimum of 60 days. The median survival time (MST) was assessed according to Geran's method:[15] MST= (x+y)/2, where x denotes the earliest day when the number of dead animals is ≥ N/2; y denotes the earliest day when the number of dead animals is ≥ (N/2)+1; and N denotes the number of animals in the group. The efficacy of the therapy against leukemia (defined as the increase in lifespan, ILS) was assessed as the percentage of the median survival time of the treated group (T) in relationship to that of the control group (C): ILS= $[(MST_C / MST_T) -1] \times 100$. It was assumed that a 25% prolongation of the survival time of the treated group compared with that of the control group indicates antineoplastic activity of the drug. As each experiment was repeated twice (6 mice per group), data are reported for 12 animals per dose-group.

The in vitro study

Patients studied. Specimens of peripheral blood or bone marrow were collected from 10 patients with CML in the chronic phase. The mean age of patients was 44.8 years (range 29-73). All patients were newly diagnosed and their diagnoses were based on standard clinical, morphologic, cytochemical and cytogenetic criteria. None of the patients had received cytotoxic drugs before the collection of bone marrow for the study. Normal bone marrow specimens were obtained from 10 newly diagnosed patients with Hodgkin's disease. The mean age of these patients was 45.5 years (range 19-67). All specimens were collected with the consent of the patients.

Assay for normal and CML CFU-GM. The assay for colony-forming unit granulocyte-macrophage (CFU-GM) was based on the method described by Iscove *et al*.[16] with our modification.[17] Peripheral blood or bone marrow were collected into preservative-free heparin. Mononuclear cells (MNCs) were obtained from blood or marrow by layering over lymphoprep (Histopaque 1077) and washed twice in Hanks balanced salt solution (HBSS). The MNCs, at a concentration of 5×10^5cells/mL, were suspended in 20% fetal calf serum (FCS, Gibco, Ltd., Scotland) to which 20% Iscove's modified Dulbecco minimum essential medium (IDMEM, Gibco, Ltd., Scotland), 55% of methyl cellulose and 5% of growth factors, 20ng/mL G-CSF (Neupogen), 25 ng/mL GM-CSF (Leucomax) and 40ng/mL IL-3 were added.

In the first part of the *in vitro* study the MNCs were plated with dFdC at the following concentrations: 0.25nM, 0.5 nM and 1 nM. At the same time the MNCs were also plated with Ara-C at the concentrations of 10^{-8}, 10^{-7} and 10^{-6} M. The concentrations of these agents were chosen according to results from other studies.[17-20,21]

In the second part of the experiment dFdC and Ara-C were added to the culture media using the following combinations of concentrations:

1) 0.25 nM of dFdC and 10^{-8} M of Ara-C;
2) 0.5 nM of dFdC and 10^{-7} M of Ara-C;
3) 1.0 nM of dFdC and 10^{-6} M of Ara-C.

All cultures were incubated on 35 mm Ø Petri dishes (Nunc, Denmark) for 14 days at 37°C, in an atmosphere of 5% of CO_2 in air and afterwards they were examined with an inverted microscope. Aggregates containing 40 or more cells were scored as colonies. In order to confirm the morphology of colonies, after having been separated from cultures and centrifuged, the cells were stained according to the May-Grünwald-Giemsa method.

Statistical analysis. In the *in vivo* study, statistical analysis was performed using the Mann-Whitney-test. In the *in vitro* study, statistical differences between the experimental and control cultures were evaluated by Student's t-test, using a level of statistical confidence $p<0.05$.

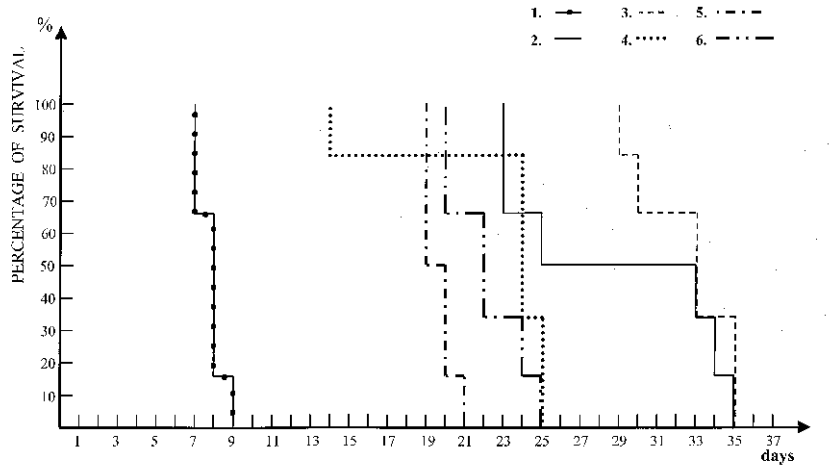

Figure 1. Percentage of survival of mice bearing L1210 leukemia as a function of time. 1. Untreated; 2. treated with Ara-C and dFdC at the same time; 3. treated with dFdC before Ara-C; 4. treated with Ara-C before dFdC ; 5. treated with Ara-C; 6. treated with dFdC.

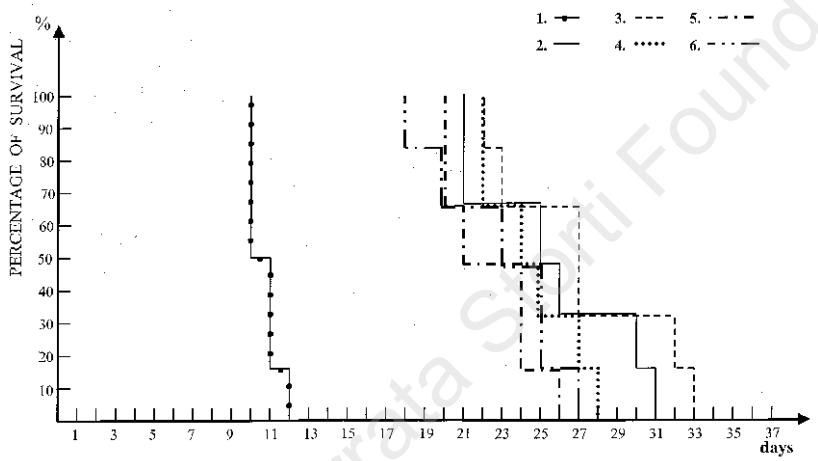

Figure 2. Percentage of survival of mice bearing P388 leukemia as a function of time. 1. Untreated; 2. treated with Ara-C and dFdC at the same time; 3. treated with dFdC before Ara-C; 4. treated with Ara-C before dFdC; 5. treated with Ara-C; 6. treated with dFdC.

Interactions. IC_{50} values were defined as the concentration of agents that achieved 50% inhibition of colony formation. The interaction index as a ratio between the observed and expected values (O/E) was used to estimate subadditive, additive or synergistic interaction. An observed/expected ratio > 1.2 was defined as subadditive, 0.8-1.2 as additive and < 0.8 as a synergistic effect.[22]

Results

The in vivo part of the study

The survival time of the treated mice bearing L1210 or P388 leukemia was significantly longer than that of the untreated animals (p=0.00395) (Figures 1 and 2).

In both leukemias tested, the most effective treatment was the schedule in which dFdC was given before Ara-C. The prolongation of the survival time of the treated mice (ILS) compared to that of the untreated one was 312.5% in the case of L1210 and 157% in the case of P388 leukemia (Table 1). Moreover, the prolongation of the survival time of the animals bearing L1210 or P388 leukemia treated with this regimen compared with the mice receiving Ara-C or dFdC separately was statistically significant (p=0.00358, p=0.00376 for L1210 leukemia and p=0.039, p=0.042 for P388 leukemia; respectively). In addition, both in L1210 and P388 leukemia, the survival time of mice receiving Ara-C with dFdC simultaneously was also significantly prolonged as compared to that of the animals receiving Ara-C or dFdC separately (p=0.01, p=0.037 for L1210 leukemia, and p=0.042, p=0.042 for P388 leukemia; respectively). However, in the case of L1210 and P388 leukemia, the survival time of mice treated with Ara-C + dFdC was similar (but not significantly different, p=0.149, p=0.107; respectively) to the survival time of mice treated with dFdC before Ara-C (Figures 3 and 4).

In the case of L1210 leukemia, the regimen in which Ara-C was given before dFdC did not statistically prolong the survival time of the treated mice as

Table 1. ILS values for the treated mice bearing L1210 or P388 leukemia.

	Ara-C	*dFdC*	*Ara-C + dFdC*	*dFdC before Ara-C*	*Ara-C before dFdC*	*0.9% NaCl*
ILS (%) L1210	143.7	175	262.5	312.5	200	-
ILS (%) P388	114.3	128.6	142.9	157.1	133.3	-

ILS: prolongation of survival time of treated mice compared with the control group.

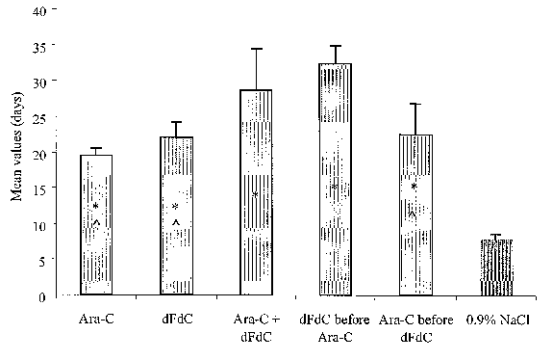

Figure 3. Antileukemic effect of Ara-C and dFdC on murine L1210 leukemia.
Statistical significance as compared with the control group (values are shown in the text).
^Statistical significance as compared with the group treated with dFdC before Ara-C (values are shown in the text).

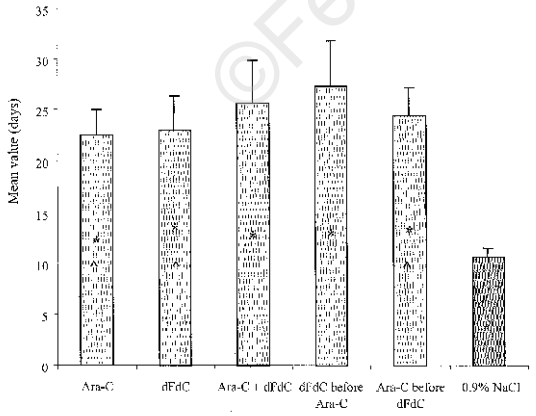

Figure 4. Antileukemic effect of Ara-C and dFdC on murine P388 leukemia.
****statistical significance as compared with the control group (values are shown in the text).***
^statistical significance as compared with the group treated with dFdC before Ara-C (values are shown in the text)

compared with that of the animals receiving Ara-C or dFdC separately (p=0.23, p=0.631; respectively) (Figure 4). Similarly, this schedule was not more effective than monotherapy with either Ara-C or dFdC (p=0.23, p=0.423; respectively) in the case of mice bearing P388 leukemia. (Figure 3)

The in vitro part of the study

All three concentrations of dFdC used separately inhibited the growth of normal as well as CML CFU-GM colonies as compared to the controls, in a dose dependent manner (Figure 5). Statistically significant colony growth inhibition of normal CFU-GM cells was obtained when dFdC was used at the concentrations of 0.5 nM or 1 nM (p=0.002, p=0.0015; respectively). In contrast, the colony growth of CML CFU-GM was significantly inhibited only after the use of the highest concentration of this agent (p=0.0014) (Table 2). The IC_{50} value for dFdC was lower for CML CFU-GM than for normal cells, however, the differences were not significant (Table 3).

Statistically significant colony growth inhibition of normal and CML CFU-GM was also observed when Ara-C was added separately to the culture medium at the concentration of 10^{-7} M or 10^{-6} M (p<0.05) (Table 2).

dFdC used together with Ara-C inhibited the growth of normal as well as CML CFU-GM colonies as compared to the controls, in a dose-dependent manner (Figure 5). Statistically significant colony growth inhibition of normal and CML CFU-GM was observed after the use of all three combinations of concentrations (p<0.05) (Table 2). Furthermore, when these agents were used together at the highest concentrations, statistically significant differences were also shown between the colony growth inhibition of normal and CML CFU-GM colonies (p=0.015) (Figure 5). dFdC used together with Ara-C had additive action on both normal CFU-GM and CML CFU-GM . However, the O/E ratio was lower for normal than CML CFU-GM colony forming cells (0.88 and 0.94; respectively) (Table 3).

Discussion

The aim of this study was to investigate the influence of Ara-C given in combination with dFdC on murine leukemias L1210 and P388 as well as on normal and leukemic cell growth *in vitro*. Our previous experiments had shown that in murine leukemias L1210 and P388 the combined therapy, with dFdC given before 2-chlorodeoxyadenosine (2-CdA), was more effective than monotherapy with either dFdC or 2-CdA. That study had proved the additive action of dFdC and 2-CdA on both leukemias tested.[10] Our further in vitro study also revealed that dFdC used together with 2-CdA acted in an additive way on both normal and CML CFU-GM progenitors. Moreover, combined therapy with higher concentrations of both drugs inhibited the growth of CML CFU-GM colonies to a greater degree as compared to inhibition of colony growth of normal CFU-GM cells (the paper submitted for publication).

The cytotoxic activity of Ara-C is dependent on intracellular conversion to its active metabolite, 5'-triphosphate Ara-CTP. Intracellular accumulation of

Table 2. The effect of gemcitabine (dFdC) used alone or in combination with cytosine arabinoside (Ara-C) on normal and CML CFU-GM in culture in vitro (values are expressed as a mean number ±SEM, per 5x10^5 MNCs, ranges in parentheses).

Agent		*dFdC*			*Ara-C*			*dFdC + Ara-C*		
	Control	*0.25 nM*	*0.5 nM*	*1 nM*	*10^{-8}M*	*10^{-7}M*	*10^{-6}M*	*2+5*	*3+6*	*4+7*
Type of cells	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Normal CFU-GM	52.0±3.0 (49-58)	46,5±4.2 (46-53) p=0.256	33.5±6.5 (30-43) p=0.002	21.5±2.5 (18-25) p=0.0015	48.5±4.6 (41-55) p=0.266	40.3±7.4 (28-43) p=0.005	24.5±2.4 (20-28) p=0.0005	36.8±4.0 (43-39) p=0.0013	27.8±2.8 (20-32) p=0.0003	14.3±1.2 (9-19) p=0.0002
CML CFU-GM	86.5±10.0 (74-104)	70.8±9.8 (60-89) p=0.07	61.0±8.3 (56-70) p=0.058	34.0±3.5 (30-36) p=0.0014	72.3±10.2 (58-92) p=0.204	65.0±6.5 (57-7) p=0.021	42.8±6.3 (31-56) p=0028	57.3±4.3 (49-62) p=0.0014	49.8±3.8 (38-48) p=0.0045	9.2±0.9 (9-11) p=0.0012

p value – comparison with control

Table 3. The comparison of IC_{50} and type of interaction for normal and CML CFU-GM colony formation.

Type of cells	*IC_{50}*		*IC_{50}*		*O/E*
	dFdC (nM)	*Ara-C (M)*	*dFdC+Ara-C*		*dFdC+Ara-C*
Normal CFU-GM	0.92	0.96x10^{-6}	0.63	0.23x10^{-6}	0.88 (A)
CML CFU-GM	0.82	0.98x10^{-6}	0.68	0.28x10^{-6}	0.94 (A)

A: additive effect; IC_{50}: the concentration of agents that achieved 50% inhibition of colony formation; O/E: observed/expected survival index (interaction index).

Ara-CTP is a multistep process, the first step of which, phosphorylation of Ara-C to its monophosphate by deoxycytidine kinase (dCK), is rate –limiting.[1,23] Moreover, Ara-C uptake is proportional to extracellular drug concentration only up to 10-20 mM Ara-C and further dose escalation does not improve its efficacy.[24] Therefore, augmentation of dCK activity plays a major role in the potentiation of Ara-CTP production.

Several recent reports have shown that the use of fludarabine or 2-CdA, agents which inhibit ribonucleotide reductase, can increase the activity of dCK and Ara-CTP accumulation in combined therapy with Ara-C.[2,3] Furthermore, it has been proven, that Ara-CTP accumulation by leukemic blasts is enhanced by

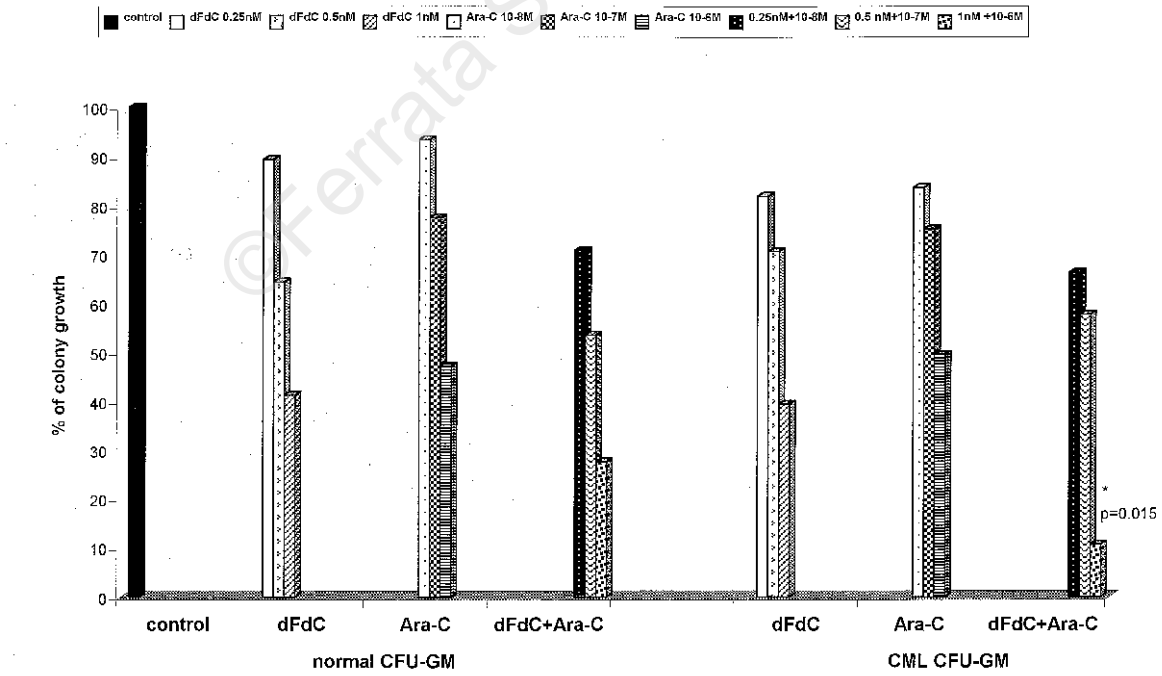

Figure 5. Percentage of colony growth of normal and CML CFU-GM after the use of dFdC and Ara-C alone or in combination in culture *in vitro*.
Statistical significance as compared to normal CFU-GM.

prior infusion of fludarabine or 2-CdA.[2,3,25]

dFdC shows a close resemblance to Ara-C but has major quantitative and qualitative differences from the latter compound with regard to biological and clinical effects.[4,5,26] dFdC is active both *in vitro* against many solid tumors and leukemic cell lines[13,27] and *in vivo* as monotherapy for non-small cell lung cancer, pancreatic cancer, peripheral T-cell lymphomas, Hodgkin's disease and non-Hodgkin's lymphomas.[28-31] After entering the cell, dFdC is activated by phosphorylation to gemcitabine monophosphate (dFdCMP), catalyzed by dCK, and subsequently to gemcitabine triphosphate (dFdCTP).[5,6] The accumulation of dFdC phosphorylated forms is higher and the intracellular T1/2 of dFdCTP is much longer than that of Ara-C.[26,32] Moreover, dFdCDP can decrease the pool of dCTP in different cell lines by the inhibition of ribonucleotide reductase.This gives dFdC a major advantage over Ara-C because dCK inhibition by dCTP is reduced, allowing a higher rate of phosphorylation.[32] In addition, dFdCTP can be incorporated into the DNA, followed by one more deoxynucleotide, after which DNA polymerization stops.[9] This contrasts with Ara-C triphosphate, for which termination of DNA strand elongation is observed at the incorporation site. Furthermore, exonuclease activity is unable to excise dFdCMP, in contrast to Ara-C monophosphate.[8,9,26] dFdC is also more potent than Ara-C in the induction of apoptosis in leukemic cells and in the activation of expression of c-jun and c-fos genes that could be involved in the signal transduction pathway of programmed cell death.[33] Both Ara-C and dFdC are incorporated into DNA blocking chain elongation, but in contrast to Ara-C which is incorporated preferentially into repairing DNA, dFdC is incorporated into replicating DNA, so these agents used together could co-operate at different DNA sites.[34,35] This could expain the results of our study.

It seems that the scheduling of drugs is relevant for their interactions. This is based on the drugs' mechanisms of action. The cytotoxic activity of Ara-C is dependent on its intracellular conversion to the active metabolite – Ara-CTP. But the fact that dFdC can decrease the pool of dCTP by inhibition of ribonucleotide reductase gives dFdC a major advantage over Ara-C because dCK inhibition by dCTP is reduced, allowing a higher rate of phosphorylation of Ara-C and facilitating the incorporation of Ara-CTP into the DNA chain.[7,35] Moreover, the therapeutic efficacy of Ara-C is limited by the rapid elimination of intracellular Ara-CTP from target cells. The prolonged retention of dFdCTP may help to overcome this disadvantage.[26]

In conclusion, our present experiments revealed that, in both leukemias tested, combined therapy with dFdC given before Ara-C and dFdC given at the same time with Ara-C, was more effective than monotherapy with either dFdC or Ara-C. The other treatment schedule (Ara-C before dFdC) did not significantly prolong the survival time of the treated mice bearing L1210 or P388 leukemia as compared to treatment with dFdC alone. Moreover, we observed that dFdC as well as Ara-C used alone inhibited colony growth of normal and CML CFU-GM cells in a dose-dependent manner as compared to the controls. Used in combination, these drugs showed an additive effect on normal and CML CFU-GM progenitor cells. Furthermore, the combined therapy inhibited the colony growth of CML CFU-GM cells to a significantly higher degree than that of normal CFU-GM cells and the differences were statistically significant at the highest concentrations.

As these agents incorporate into DNA blocking chain elongation and moreover dFdC influences the cytotoxicity of Ara-C, our results could be explained by the fact that drugs interfere at these levels.

Potential implications for clinical practice

We suggest that in the treatment of patients with CML as well as other hematologic malignancies the combined therapy with dFdC and Ara-C could be more effective than monotherapy. It may have an important clinical implication. However , further preclinical and clinical studies are necessary to confirm this suggestion.

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Contributions and Acknowledgments

ELM designed the study, did the in vivo experiments and some of the in vitro ones and wrote the paper. AK participated in the in vitro experiments and in preparing the manuscript. TR was responsible for the conception and design of the study, interpretation of the data and critical revision of manuscript. The authors are listed in an order that reflects their individual contribution to the study.

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Disclosures

Conflict of interest: none

Redundant publications: This work was presented in part at the Fourth Congress of the European Haematology Association, June 9-12, 1999, Barcelona. Spain and published in abstract form in Haematologica 84, EHA-4 Abstract Book, June 1999, p. 55, abstract PO-0235.

Manuscript processing

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