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Comparison of antineoplastic activity of 2′,2′-difluorodeoxycytidine and cytosine arabinoside against human myeloid and lymphoid leukemic cells

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2′,2′-difluorodeoxycytidine (known as dFdC, Gemcitabine and LY188011) is a new analog of deoxycytidine which has demonstrated excellent antineoplastic activity against many kinds of solid tumors and leukemic cell lines. We were interested in the comparison of the antineoplastic activity of this new antimetabolite with cytosine arabinoside (ARA-C) against HL-60 myeloid, RPMI-8392 B-lymphoid and Molt-3 T-lymphoid leukemic cell lines. Our *in vitro* experiments showed that dFdC was a more potent cytostatic drug than ARA-C against all the leukemic lines with IC_{50} ranging from 3 to 10 nM for dFdC and from 26 to 52 nM for ARA-C for a 48 h exposure. The cytotoxicity of both drugs was evaluated by clonogenic assay and dFdC was found to be 100 times more potent than ARA-C against all the leukemic cell lines for both a 2 h and a 24 h exposure. The recovery of DNA synthesis after drug removal was much slower for dFdC than for ARA-C. However, in contrast to cytostatic and cytotoxicity results ARA-C was a more potent inhibitor of DNA synthesis than dFdC for all the leukemic cell lines for short exposure. Uptake and elimination of the drugs showed that dFdC accumulated to a higher degree in the leukemic cells than ARA-C and that elimination of this difluoro analog was slower than that of ARA-C. These results indicate that dFdC has more potent *in vitro* antileukemic activity than ARA-C.

Key words: Cellular pharmacology, cytosine arabinoside, 2′,2′-difluorodeoxycytidine, leukemia.

Introduction

Cytosine arabinoside (ARA-C), a deoxycytidine (CdR) analog, is well established for the treatment of acute myeloid leukemia.[1,2] 2′,2′-difluorodeoxycytidine (dFdC) is a new and very interesting antimetabolite of CdR in which the two hydrogen atoms in the 2′ position of the deoxyribose sugar have been replaced by two fluorine atoms.[3,4] In order to be active inhibitors of DNA synthesis, both ARA-C and dFdC follow the metabolic path of CdR, being first phosphorylated to their monophosphate form by CdR kinase and then converted into their triphosphate form by other kinases.[5–8] Cells deficient in CdR kinase are resistant to the action of both ARA-C and dFdC.[6] The exact mechanisms of action of dFdC are not fully understood but appear to be related to the inhibition of DNA synthesis and inhibition of ribonucleotide reductase.[9]

Heinemann *et al.*[6] have reported that dFdC is a more potent cytotoxic agent against Chinese hamster ovary (CHO) cells than ARA-C. Other workers have shown that dFdC is active against many solid tumors. Several reports have shown that dFdC is a potent inhibitor of growth against different leukemic cell lines.[5,9,10] Since dFdC has shown better cytotoxic activity than ARA-C in K562 leukemic cells we wanted to compare the antineoplastic effect of dFdC and ARA-C against human leukemic cells of different phenotypes: myeloid (HL-60), B-lymphoid (RPMI-8392) and T-lymphoid (Molt-3). We also have investigated the cellular pharmacology of these antimetabolites in the leukemic cells in an attempt to elucidate the mechanisms by which dFdC produces its antineoplastic activity.

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Materials and methods

Materials

ARA-C was obtained from the Upjohn Company of Canada. [5-^{3}H]ARA-C was obtained from Moravek Biomedicals Inc (Brea, CA). dFdC was obtained by Lilly Research Laboratories (Indianapolis, IN) and [^{3}H]dFdC was synthesized by Amersham Canada Ltd (Oakville, Ontario) and purified by HPLC using a Spherisorb ODS C18, 5 μm, 0.4 cm × 25 cm (Chromatography Science, Montreal) with a 5 mM ammonium formate pH 4.3 buffer containing 5% methanol. The purified product was then assayed with cytidine deaminase and the appearance of a peak corresponding to [^{3}H]dFdU was identified, thus assuring that product was really [^{3}H]dFdC. [^{3}H-methyl]thymidine (20 Ci/mmol) was obtained from Dupont Canada Inc (Mississauga, Ontario).

Cell culture

Human HL-60 myeloid leukemic cells were obtained from Dr R. Gallo, National Cancer Institute (Bethesda, MD). Human RPMI-8392 B-lymphoid and human Molt-3 T-lymphoid leukemic cell lines were obtained from the Institute for Medical Research (Camden, NJ) and American Type Tissue Culture Collection (Rockville, MD) respectively. The cell lines were maintained in suspension culture in minimal essential medium (MEM) containing non-essential amino acids (GIBCO, Grand Island, NY) and 10% heat-inactivated fetal calf serum (Flow Laboratories, Mississauga, Ontario). The doubling time for the cell lines was between 20 and 24 h.

Inhibition of cell growth and colony assay

For the growth inhibition studies 5 ml of leukemic cells suspended in MEM medium containing 5% dialyzed fetal calf serum (10^5/ml) were placed in a tissue culture flask with the indicated concentration of ARA-C or dFdC. At 48 h the cells were counted with a model Z Coulter Counter. The IC_{50} were determined by approximating the concentration of drug that inhibits by 50% the cellular growth of the leukemic cell lines.

The proliferative viability of the leukemic cells after exposure to ARA-C or dFdC was determined by cloning in soft agar. At the termination of drug exposure the cells were centrifuged and suspended in drug-free medium. An aliquot of 150–200 cells was placed in 2 ml of 0.3% soft agar medium containing 16% serum. After incubation for 14–16 days at 37°C in 5% CO_2 incubator, the number of colonies (>500 cells) was counted. The cloning efficiency of the control cells was in the range 40–60%.

DNA synthesis measurement

The rate of DNA synthesis was measured by the incorporation of [^{3}H-methyl]thymidine into DNA. 10^5 cells were suspended in MEM containing 5% dialyzed serum and incubated with different concentrations of the corresponding drugs for 4 h. At termination of drug exposure 1 μCi of [^{3}H-methyl]thymidine (20 Ci/mmol) was added to the medium and 1 h later the cells were put on GF/C glass fiber filter, washed with 5% TCA, 0.9% NaCl and methanol. The filters containing the DNA fraction were then dried and assayed for radioactivity in scintillation fluid (Omnifluor, Dupont).

DNA synthesis recovery measurement

In order to measure the rate of DNA synthesis recovery the cells were first incubated with 1 μM of the corresponding drugs for 2 h and then washed free of drug and suspended in MEM medium containing 5% dialyzed serum. At each time point 1 μCi [^{3}H-methyl]thymidine (20 Ci/mmol) was added to 2×10^5 cells. The cells were then incubated for 1 h with the isotope and assayed for DNA synthesis as described above.

Uptake and retention of ARA-C and dFdC in the leukemic cells

For these experiments leukemic cell lines were suspended in MEM containing 5% dialyzed serum to a density of 1.5×10^5 cells/ml. The cells were then incubated with 1 μM of either [^{3}H]ARA-C or [^{3}H]dFdC for 1, 2 and 4 h. For the measurement of the total uptake of drugs at each time point the cells were put on a GF/C glass fiber filter and washed twice with 0.9% NaCl, dried and assayed for

radioactivity. For the nucleotide retention assay the cells were centrifuged after 4 h of incubation with the radioactive drugs and resuspended in fresh medium. At time points 1, 2 and 4 after washing the cells were placed on a GF/C glass fiber filter and washed twice with 0.9% NaCl, dried and assayed for radioactivity.

Results

The first aspect of this study was to compare the growth inhibitory actions of ARA-C and dFdC against leukemic cell lines of different phenotypes. Table 1 gives the concentration that produced 50% growth inhibition (IC_{50}) for a 48 h exposure to these two analogs against human HL-60 (myeloid), Molt-3 (T-lymphoid) and RPMI-8392 (B-lymphoid) leukemic cell lines. The growth of the leukemic cells was inhibited more by dFdC than ARA-C at same concentration of both drugs. dFdC showed a better growth inhibition effect than ARA-C on HL-60 and RPMI-8392 as compared with Molt-3. The IC_{50} values of ARA-C on HL-60 and RPMI-8392 cells were 47 nM and 52 nM respectively, as compared with 5.5 and 3.0 nM for dFdC. The IC_{50} value of dFdC for the Molt-3 leukemic cell line was about 2.6-fold lower than that of ARA-C. These results show clear evidence that dFdC is a more potent cytostatic agent than is ARA-C against the different leukemic cell lines.

We performed clonogenic assays to determine the cytotoxicity produced by different concentrations of ARA-C and dFdC for different exposure times on the three leukemic cell lines. Tables 2 and 3 show the results obtained after 2 h and 24 h exposure times, respectively. Table 2 shows that dFdC was about 100-fold more cytotoxic than ARA-C on all the leukemic cells for a 2 h exposure. A concentration of 1.0 μM of ARA-C produced 30%, 25% and 37% cytotoxicity against HL-60, RPMI-8392 and Molt-3 cells respectively, whereas dFdC at a concentration 100 times less produced about the same level of cell kill. Similar results were obtained for an incubation time of 24 h with different concentrations of both drugs; it took a 100 times more ARA-C to achieve a level of cytotoxicity similar to that of dFdC against the different types of leukemic cells (Table 3). dFdC at a concentration of 0.1 μM produced >99% cell kill whereas to achieve the same effect with ARA-C it was necessary to increase the concentration up to 10 μM (results not shown).

The inhibition of DNA synthesis in the different types of leukemic cells produced by different concentrations of dFdC and ARA-C for a 4 h exposure is shown in Table 4. At a concentration of 0.01 μM ARA-C produced 40–55% DNA

Table 1. Comparison of growth inhibition by ARA-C and dFdC after a 48 h treatment on myeloid HL-60, lymphoid RPMI-8392 and Molt-3 leukemic cell lines

Drug	IC_{50} (nM)[a]		
	HL-60	RPMI-8392	Molt-3
ARA-C	47 ± 10	52 ± 11	26 ± 11
dFdC	5.5 ± 0.9	3.0 ± 0.3	10 ± 3

[a] IC_{50} are means of three experiments in duplicate ± SE.

Table 2. Clonogenic survival of HL-60, RPMI-8392 and Molt-3 leukemic cell lines to the cytotoxic action of ARA-C or dFdC for a 2 h exposure

Treatment (2 h)	Concentration (μM)	Cytotoxicity (%)[a]		
		HL-60	RPMI-8392	Molt-3
ARA-C	0.1	22 ± 8	18 ± 6	12 ± 4
	1.0	30 ± 9	25 ± 11	37 ± 12
	10.0	47 ± 11	40 ± 6	58 ± 14
dFdC	0.01	29 ± 9	15 ± 10	36 ± 16
	0.10	57 ± 14	48 ± 11	51 ± 15
	1.00	68 ± 9	75 ± 18	67 ± 12

Values are expressed as percentage of survival relative to control.
[a] Mean ± SE ($n = 3$).

Table 3. Clonogenic survival of HL-60, RPMI-8392 and Molt-3 leukemic cell lines to the cytotoxic action of ARA-C or dFdC for a 24 h exposure

Treatment (24 h)	Concentration (μM)	Cytotoxicity (%)[a]		
		HL-60	RPMI-8392	Molt-3
ARA-C	0.01	21 ± 7	16 ± 2	18 ± 11
	0.1	36 ± 18	18 ± 6	23 ± 6
	1.0	49 ± 10	74 ± 4	81 ± 9
dFdC	0.001	58 ± 12	20 ± 5	22 ± 6
	0.010	76 ± 16	92 ± 6	73 ± 2
	0.100	>99	>99	>99

Values are expressed as percentage of survival relative to control.
[a] Mean ± SE ($n = 3$).

synthesis inhibition whereas dFdC produced only 17–34% inhibition. Both drugs showed about the same level of DNA synthesis inhibition at a concentration of 0.1 μM with values ranging from 74% to 84%.

We measured the recovery of DNA synthesis following the incubation of the leukemic cells with 1 μM of dFdC or ARA-C for 2 h (Figure 1). At time 0 the inhibitions of DNA synthesis were comparable for all three leukemic cell lines. Cells treated with ARA-C showed a significant recovery during the 6 h interval following the wash-out which was up to 30% for HL-60 and Molt-3 cells and up to 80% for RPMI-8392 cells. With dFdC the recovery was very low or non-existent for the three leukemic cell lines during the 6 h interval.

We investigated the total uptake and rate of elimination of dFdC and ARA-C in the leukemic cell lines. The results showed that there was a greater accumulation of the phosphorylated forms of dFdC into all leukemic cells as compared with ARA-C during 4 h of incubation with 1 μM of the drugs (Figure 2). Accumulation of dFdC into the cells increased during the 4 h incubation. This result was not seen with ARA-C which reached almost the maximum value during the first hour of incubation. The elimination of ARA-C and dFdC was different in the three leukemic cell lines used. As shown in Figure 2 ARA-C was rapidly eliminated from the cells within the first hour following wash-out of the drug from the medium. This rapid decrease was not found for dFdC which showed a slow rate of elimination for 4 h in each of the leukemic cell lines.

Table 4. Inhibition of DNA synthesis produced by ARA-C and dFdC on HL-60, RPMI-8392 and Molt-3 leukemic cell lines following a 4 h exposure

Drug	Concentration (μM)	Inhibition of DNA synthesis (%)[a]		
		HL-60	RPMI 8392	Molt-3
ARA-C	0.01	55 ± 9	40 ± 11	54 ± 5
	0.10	83 ± 2	78 ± 5	84 ± 4
dFdC	0.01	34 ± 7	20 ± 10	17 ± 7
	0.10	78 ± 4	74 ± 4	75 ± 4
	1.00	90 ± 2	88 ± 2	89 ± 2

Cells were incubated for 4 h with drug followed by 1 h incubation with [^{3}H-methyl]thymidine.

[a] Mean ± SE (n = 3).

Discussion

Since the currently used chemotherapy for acute leukemias still fails to cure all patients it is important to search for new effective drugs. dFdC was found to have cytostatic activity in leukemic cells comparable with that of ARA-C.[8,10,11] In addition dFdC was found to be active against many types of solid tumors.[11] dFdC acts as a terminator of DNA chain elongation and is also incorporated at internucleotide linkage.[8,12] Both of these mechanisms probably account for its lethal effect on cells. dFdCDP inhibits the catalytic action of ribonucleotide reductase[8,9] and thus blocks the *de novo* deoxynucleotide biosynthesis resulting in the lowering of the intracellular concentration of deoxycytidine triphosphate (dCTP). This reduction in dCTP will produce less feedback inhibition on CdR kinase and an increased phosphorylation of dFdC. Since dFdC was observed to be more active than ARA-C in CHO tumor cells we wanted to investigate in depth its antineoplastic action against human leukemic cells of different phenotypes.

According to our results dFdC did not show marked selectivity against different types of leukemic cells with respect to inhibition of growth, but was more active than ARA-C for each cell line. For a 48 h exposure (Table 1) we obtained IC_{50} values for dFdC between 3 and 10 nM (the B-cell line being the most sensitive) and for ARA-C between 26 and 52 nM (the B-cell and myeloid cell lines being the least sensitive). Plunkett *et al.*[8] reported an IC_{50} for K562 leukemic cell line of 5 nM for dFdC and of 7 nM for ARA-C for a 72 h exposure time and of 270 nM and 90 nM respectively for a 2 h exposure. Other reports showed that both of these antimetabolites produce about the same extent of inhibition of growth on CCRF-CEM T-cell leukemia.[11] The differences between these reports and our results may be related to differences in methodology or cell type since our results showed that dFdC was a better inhibitor of growth than ARA-C for a 48 h exposure on all the leukemic cell lines (Table 1). The loss of clonogenicity for all the leukemic cell lines was dependent on drug concentration and exposure time for both ARA-C and dFdC. dFdC was approximately 100 times more cytotoxic than ARA-C (Tables 2 and 3). Exposure for 24 h of the leukemic cells to 0.1 μM dFdC produced >99% cell kill whereas the same concentration of ARA-C produced only 18–36% cytotoxic effect on the leukemic cells (Table 3). At identical concentrations of both drugs, ARA-C showed greater inhibition

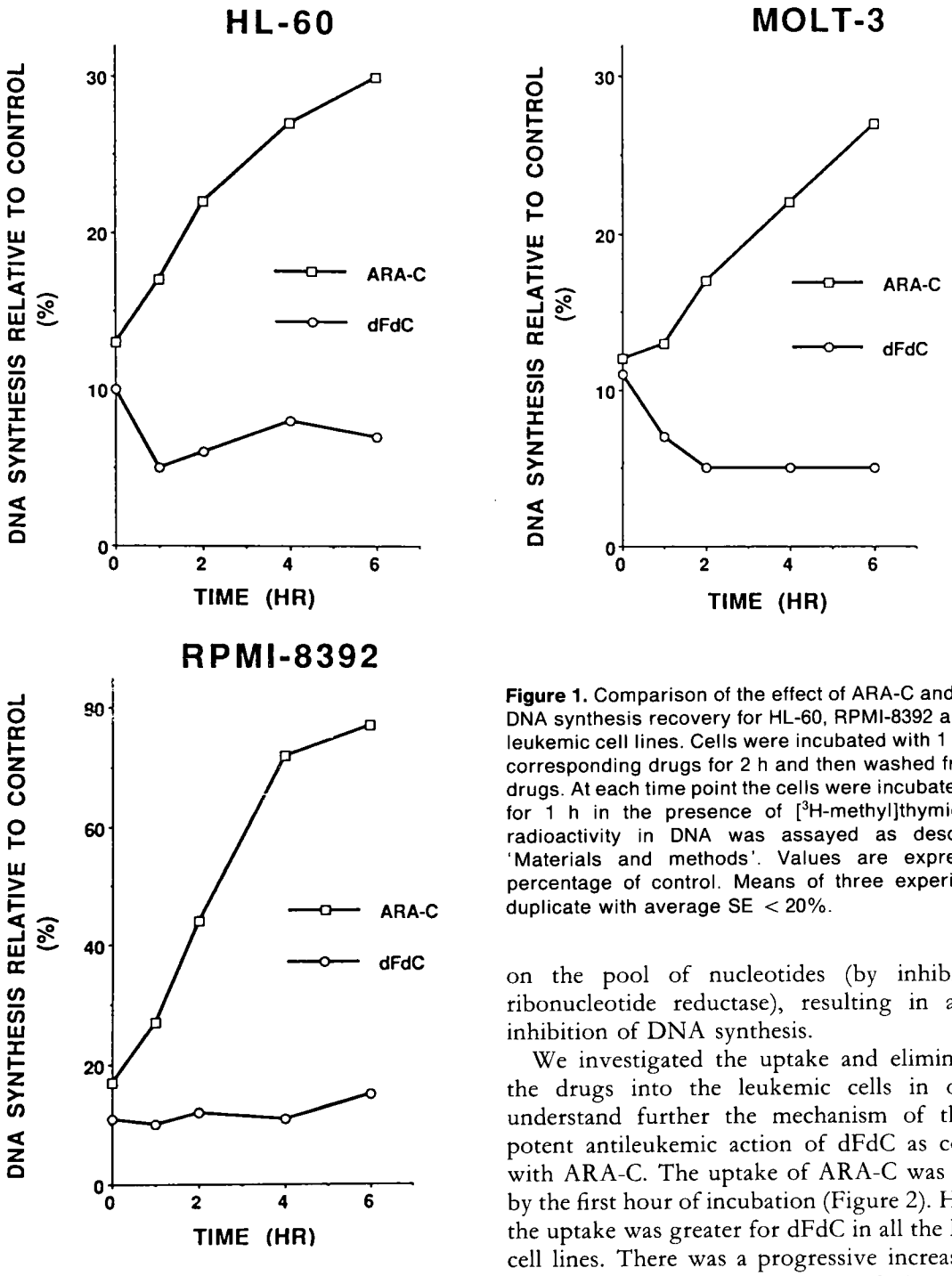

Figure 1. Comparison of the effect of ARA-C and dFdC on DNA synthesis recovery for HL-60, RPMI-8392 and Molt-3 leukemic cell lines. Cells were incubated with 1 μM of the corresponding drugs for 2 h and then washed free of the drugs. At each time point the cells were incubated at 37°C for 1 h in the presence of [^{3}H-methyl]thymidine and radioactivity in DNA was assayed as described in 'Materials and methods'. Values are expressed as percentage of control. Means of three experiments in duplicate with average SE < 20%.

of DNA synthesis than dFdC at 0.01 μM, but not at 0.1 μM (Table 4). We also observed that the duration of this inhibition was longer for dFdC (Figure 1). Heinemann *et al.*[6] have also observed this longer inhibition of DNA synthesis for dFdC in CHO cells. Thus it appears that the cytotoxic effect of dFdC is related not only to its incorporation into the DNA, but also to its action on the pool of nucleotides (by inhibition of ribonucleotide reductase), resulting in a longer inhibition of DNA synthesis.

We investigated the uptake and elimination of the drugs into the leukemic cells in order to understand further the mechanism of the more potent antileukemic action of dFdC as compared with ARA-C. The uptake of ARA-C was maximal by the first hour of incubation (Figure 2). However, the uptake was greater for dFdC in all the leukemic cell lines. There was a progressive increase in the amount of radioactivity retained in the cells following the next few hours of incubation with the drug which was significantly higher for [^{3}H]dFdC than [^{3}H]ARA-C.

All the leukemic cells used in our experiments showed a longer retention of dFdC than ARA-C, the latter analog being eliminated very rapidly from the cells (Figure 2). The T-cell (Molt-3) and myeloid (HL-60) leukemic cell lines showed a more rapid

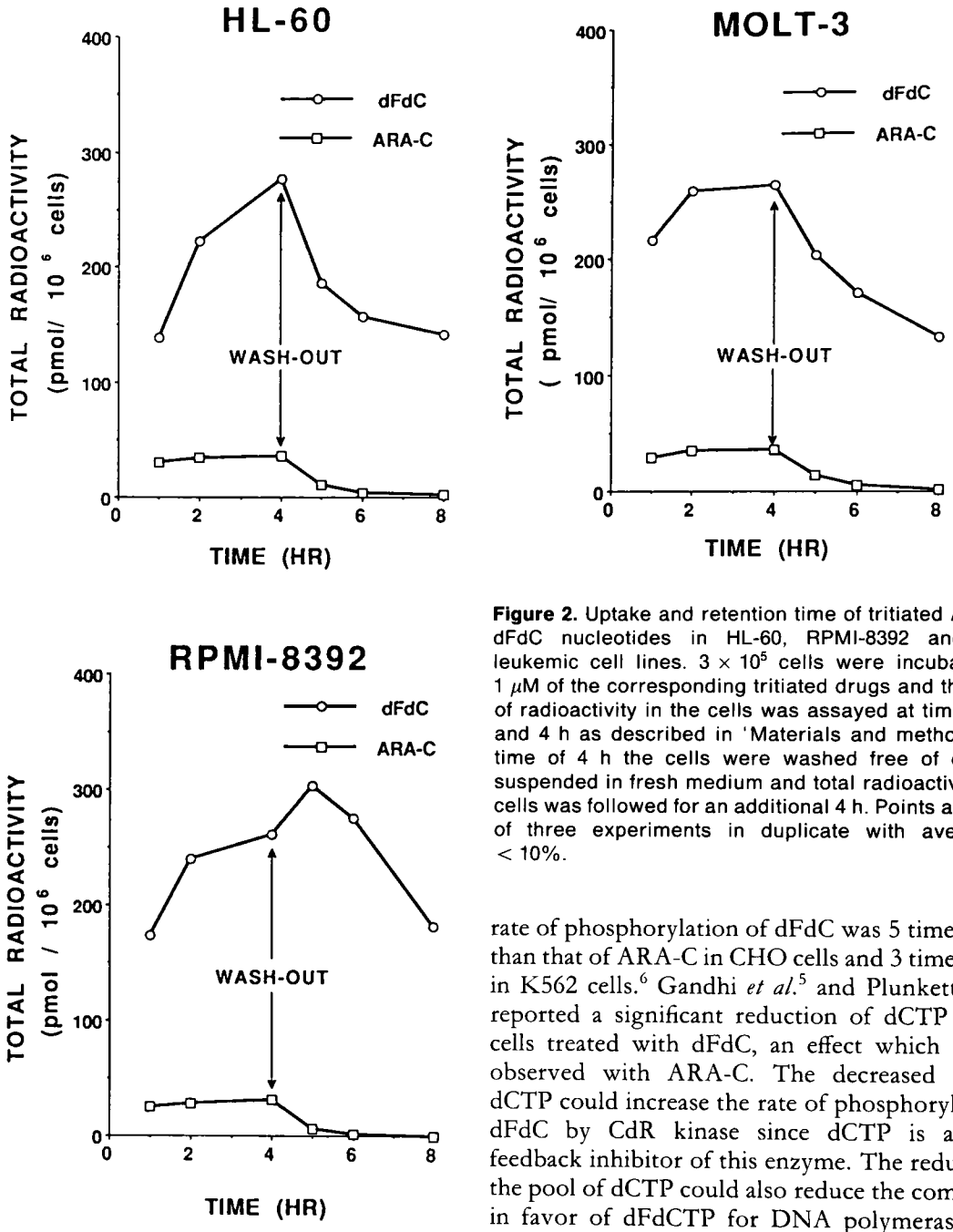

Figure 2. Uptake and retention time of tritiated ARA-C or dFdC nucleotides in HL-60, RPMI-8392 and Molt-3 leukemic cell lines. 3×10^5 cells were incubated with 1 μM of the corresponding tritiated drugs and the uptake of radioactivity in the cells was assayed at times of 1, 2 and 4 h as described in 'Materials and methods'. At a time of 4 h the cells were washed free of drug and suspended in fresh medium and total radioactivity in the cells was followed for an additional 4 h. Points are means of three experiments in duplicate with average SE < 10%.

elimination of dFdC than the B-cell (RPMI-8392) line and this correlated with a longer elimination of this analog in this particular cell type. In CHO cells the uptake and retention of dFdC is also much greater than that of ARA-C.[6]

The greater uptake and longer half-life for dFdC as compared with ARA-C could be related to many factors. Some workers showed that dFdC was a better substrate than ARA-C on CdR kinase. The rate of phosphorylation of dFdC was 5 times higher than that of ARA-C in CHO cells and 3 times higher in K562 cells.[6] Gandhi *et al.*[5] and Plunkett *et al.*[8,9] reported a significant reduction of dCTP pool in cells treated with dFdC, an effect which was not observed with ARA-C. The decreased pool of dCTP could increase the rate of phosphorylation of dFdC by CdR kinase since dCTP is a potent feedback inhibitor of this enzyme. The reduction in the pool of dCTP could also reduce the competition in favor of dFdCTP for DNA polymerase alpha, which would give dFdC an advantage over ARA-C.

Nucleoside transport may also play a role with respect to drug action. At low concentrations (1 μM) of drug the facilitated diffusion could be a rate-limiting factor owing to the limited number of carrier sites, but this may not be true for high extracellular concentration (>10 μM), where passive diffusion of the drugs predominates.[14,15] Greater phosphorylation of dFdC may be due to a better transport than that of ARA-C at low concentrations, permitting a greater access to CdR

kinase. Heinemann *et al.*[6] have shown that the initial rate of membrane transport was 65% higher for dFdC than for ARA-C in CHO cell line deficient in CdR kinase.

Several investigators have shown that the elimination of the dFdCTP varies slightly depending on the cell line used, but was always longer than that of ARA-CTP.[5,8,10,13] The longer half-life of dFdC in cells may be related to the different phosphated forms of this analog which could produce an inhibition of some of the degradative enzymes.

Our *in vitro* data show that dFdC is a more cytotoxic analog than ARA-C for leukemic cells of different phenotypes. Since the retention time correlates with the duration of clinical remission[16,17] dFdC may be a very good candidate for clinical trials since it shows longer retention time than ARA-C. Optimal dose scheduling in patients may prove that dFdC is a more effective drug than ARA-C in acute leukemia.

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