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EFFECT OF FLUDARABINE AND ARABINOSYLCYTOSINE ON MULTIDRUG RESISTANT CELLS

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ABSTRACT

Background and Objective. Anthracyclines are first-line drugs in the treatment of acute leukemia, but the sensitivity of leukemic cells to anthracyclines can be downmodulated by multidrug resistance (MDR) transport proteins like Pgp. Pgp overexpression is negatively related to treatment response. Alternative drugs may be required to overcome the MDR problem.

Methods. Arabinosylcytosine (ara-C) and 9-β-D-arabinofuranosyl-2-fluoro-adenine monophosphate (fludarabine, F-ara) were tested alone and in combination in four pairs of leukemia and tumor non-MDR and MDR cell lines. Toxicity was assayed by growth inhibition with the microcultured MTT assay.

Results. MDR cells were more sensitive than or as sensitive as non-MDR cells to ara-C and to F-ara alone. The resistance index to ara-C was decreased upon pre-exposure of the MDR cells to low-dose F-ara (10 ng/mL), showing that the combination of ara-C and F-ara was more active on MDR cells than on non-MDR parental ones.

Interpretation and Conclusions. Neither sensitivity to ara-C nor sensitivity to F-ara was influenced by Pgp overexpression These data provide a rationale for more extensive and more intensive testing of combinations of ara-C and F-ara in Pgp-mediated MDR acute leukemia. In relapsed/resistant and in secondary acute leukemias, increasing the dose of ara-C and combining ara-C with F-ara might be more rewarding than administering anthracyclines or other Pgp-processable compounds.

Key words: multidrug resistance, acute leukemia, chemotherapy, anthracycline, fludarabine, arabinosylcytosine

Several transmembrane proteins, including the 170 Kd glycoprotein (P170 or Pgp) coded by the mdr-1 gene that belongs to the ATP-binding cassette of the transporter gene superfamily, the multidrug resistance-related protein (MRP) and the lung resistance-related protein (LRP), may influence the intracellular concentration and distribution of several antitumor cytotoxic drugs such as Vinca alkaloids, epipodophyllotoxin, taxol, anthracenedione and anthracycline derivatives.[1-5] The anthracyclines are first-line drugs for the treatment of acute non-lymphocytic leukemia (ANLL) and leukemic cell sensitivity to anthracyclines is likely to be a major determinant of treatment response.[6] The other first line drug for ANLL is arabinosylcytosine (ara-C), which is not processed by any of the multidrug resistance transport proteins that have been identified so far. The pathways followed by ara-C are different. A large portion of the drug is deaminated and neutralized outside the cell.[7] A small amount of ara-C enters the cell through a cytidine kinase pathway and is partly deaminated and partly phosphorylated to form a pseudonucleoside (ara-C triphosphate or ara-CTP) that interferes with DNA polymerases and with DNA formation itself.[7,8] Resistance to ara-C is mainly associated with a high level of ara-C deamination or with a low level of ara-CTP formation.[7-9]

9-β-D-arabinofuranosyl-2-fluoro-adenine monophosphate (fludarabine, F-ara) was originally developed during a systematic search for compounds that interfere with adenosine deaminase (ADA), with the aim of increasing deoxynucleotide concentration to toxic levels in neoplastic lymphocytes.[10,11] However, F-ara is also phosphorylated and the resulting nucleoside (F-ara-ATP) inhibits DNA synthesis and is incorporated into DNA, leading to DNA strand breaks and enhancing apoptosis.[12-14] Moreover, F-ara potentiates ara-C metabolism in leukemic cells due to an indirect effect of F-ara-ATP on deoxycytidine kinase, which is the rate limiting enzyme for the conversion of ara-C to its active form ara-CTP.[15-18] F-ara was shown to be active in chronic lymphocytic leukemia and in malignant

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lymphoma,[12] but its effect on ara-C metabolism has also been exploited in the treatment of acute non lymphocytic leukemia with promising results.[17,19-22] For that reason we investigated the cytotoxic effect of F-ara alone and in combination with ara-C in non-MDR and MDR tumor cell lines.

Materials and Methods

Drugs

Arabinosylcytosine was obtained from Upjohn S.A. (Puurs, Belgium) and F-ara came from Schering A.G. (Berlin, Germany). Both drugs were dissolved in distilled water at 1 mg/mL and aliquots were stored at –20°C.

Cell lines

We used four pairs of human tumor cell lines, each pair consisting of the parental sensitive line and its drug-selected MDR subline. Parental sensitive cell lines were grown from acute lymphocytic leukemia (CCRF CEM), colon adenocarcinoma (LOVO 109), breast cancer (MCF7) and acute myeloid leukemia (HL60). The respective MDR sublines were selected in 300 ng/mL of vinblastine (CEM VLB), in 200 ng/mL of doxorubicin (LOVO DX and MCF7 DX) and in 400 ng/mL of daunorubicin (HL60 DNR). All cell lines were cultured at 37°C in a humidified atmosphere at 5% CO_2 and were maintained in exponential growth in RPMI 1640 (Biochem KG Seromed) supplemented with 10% heat-inactivated fetal calf serum (Biochem KG Seromed), 2 mM glutamine solution, 100 U/mL penicillin and 100 µg/mL streptomycin (Biochem KG Seromed). The expression of Pgp and of the other MDR associated proteins (lung resistance related protein and multidrug resistance associated protein) was evaluated by a flow cytometry assay using the MRK-16 (Kamiya), LRP-56 (Kamiya) and MRPm6 (Kamiya) monoclonal antibodies. Pgp was evaluated as described elsewhere.[23,24] The LRP-56 and MRPm6 monoclonal antibodies were employed according to the manufacturer's guidelines. Results were expressed as the mean fluorescence index (MFI) by calculating the ratio of the mean fluorescence intensity of cells that were incubated with MRK-16, LRP-56 or MRPm6 to the mean fluorescence intensity of the respective isotypic controls. All four MDR sublines overexpressed Pgp, as shown by their reactivity with the monoclonal antibody MRK-16 (Table 1). The multidrug resistance related protein was not expressed in any line, while the so-called lung resistance related protein was slightly expressed in the LOVO pair and in MCF7 DX (Table 1).

Drug sensitivity assay

Cell growth in the presence or absence of drugs was determined using the MTT-microcultured tetrazolium colorimetric assay as described elsewhere.[25-27] Briefly, exponentially growing cells were harvested, washed twice in RPMI 1640, checked for vitality through the trypan blue exclusion test, and plated into 96-well microtiter plates at the required concentration in complete culture medium. After 48 hours of incubation in microplates, increasing doses of F-ara (1-100 ng/mL) and ara-C (0.1-100 ng/mL) were added. Some microplates were incubated with F-ara at three different concentrations (1, 5, 10 ng/mL and after four hours increasing doses of ara-C (0.1-100 ng/mL) were added to test whether pre-incubation with F-ara could enhance ara-C cytotoxicity. Cell growth and growth inhibition were evaluated after 7 days of incubation in continuous drug exposure at 37°C in a humidified atmosphere containing 5% CO_2. MTT solution was added at 5 mg/mL, and DMSO was used as the MTT formazan-product solvent. Wells containing no drugs were used to check cell viability, and wells containing no cells were used for blanking the spectrophotometer. Optical density (OD) was read at 540 nm using a microcultured plate reader (Novapath Microplate Reader, Bio Rad). All experiments were performed at least in triplicate and every point of the dose-response curves was the mean of three independent tests. Standard deviation was always 10% or less. The inhibition dose (ID) was calculated according to Pieters *et al*.[28] with the following equation: ID = (mean OD treated wells/mean OD control wells) × 100. ID50 was defined as the drug dose that inhibited cell growth to 50% of the control.

Table 1. Four pairs of human tumor cell lines were tested. Parental sensitive cell lines were grown from acute lymphocytic leukemia (CCRF CEM), colon adenocarcinoma (LOVO 109), breast cancer (MCF7) and acute myeloid leukemia (HL60). The respective MDR sublines were selected in 300 ng/mL of vinblastine (CEM VLB), in 200 ng/mL of doxorubicin (LOVO DX and MCF7 DX) and in 400 ng/mL of daunorubicin (HL60 DNR), respectively. The table also shows the reactivity (as mean fluorescence index) of each cell line to three monoclonal antibodies: MRK-16 (Kamiya) directed against the 170 Kd glycoprotein (Pgp), MRPm6 (Kamiya) directed against the multidrug resistance related protein (MRP) and LRP-56 (Kamiya) directed against the lung resistance related protein (LRP).

	Mean fluorescence index		
Cell lines	*MRK-16 (Pgp)*	*MRPm6 (MRP)*	*LRP-56 (LRP)*
CCRF CEM	2.7	1.0	1.0
CEM VLB	28.0	1.2	1.2
LOVO 109	2.3	1.0	2.7
LOVO DX	22.0	1.0	2.9
MCF 7	2.8	1.0	1.0
MCF7 DX	18.0	1.0	3.5
HL60	3.9	1.0	1.0
HL60 DNR	22.0	1.0	1.0

Results

The dose-response curves of each line pair to F-ara and to ara-C are shown in Figures 1 and 2. With F-ara (Figure 1) the MDR subline curve was either shifted to the left of the curve of the respective non-MDR parental line (CEM VLB/CCRF CEM pair and MCF7 DX/MCF pair) or was superimposable, showing that Pgp overexpression either enhanced or did not affect cell sensitivity to F-ara. The response to ara-C was quite similar (Figure 2) since MDR cells were either more sensitive than or as sensitive as non-MDR cells.

In subsequent experiments, the MDR cells were exposed to ara-C in combination with F-ara at concentrations of 1, 5 and 10 ng/mL. At 1 and 5 ng/mL no substantial changes were detected in the ara-C dose-response curve (data not shown) but at a F-ara concentration of 10 ng/mL the dose response curve to ara-C was shifted to the left (Figure 3), showing that the drug combination was more cytotoxic than either drug alone at the same concentration. It should be noticed that F-ara alone at a concentration of 10 ng/mL was only slightly cytotoxic and inhibited 10% of LOVO DX and MCF7 DX cell growth, 26% of HL60 DNR and 31% of CEM VLB, respectively.

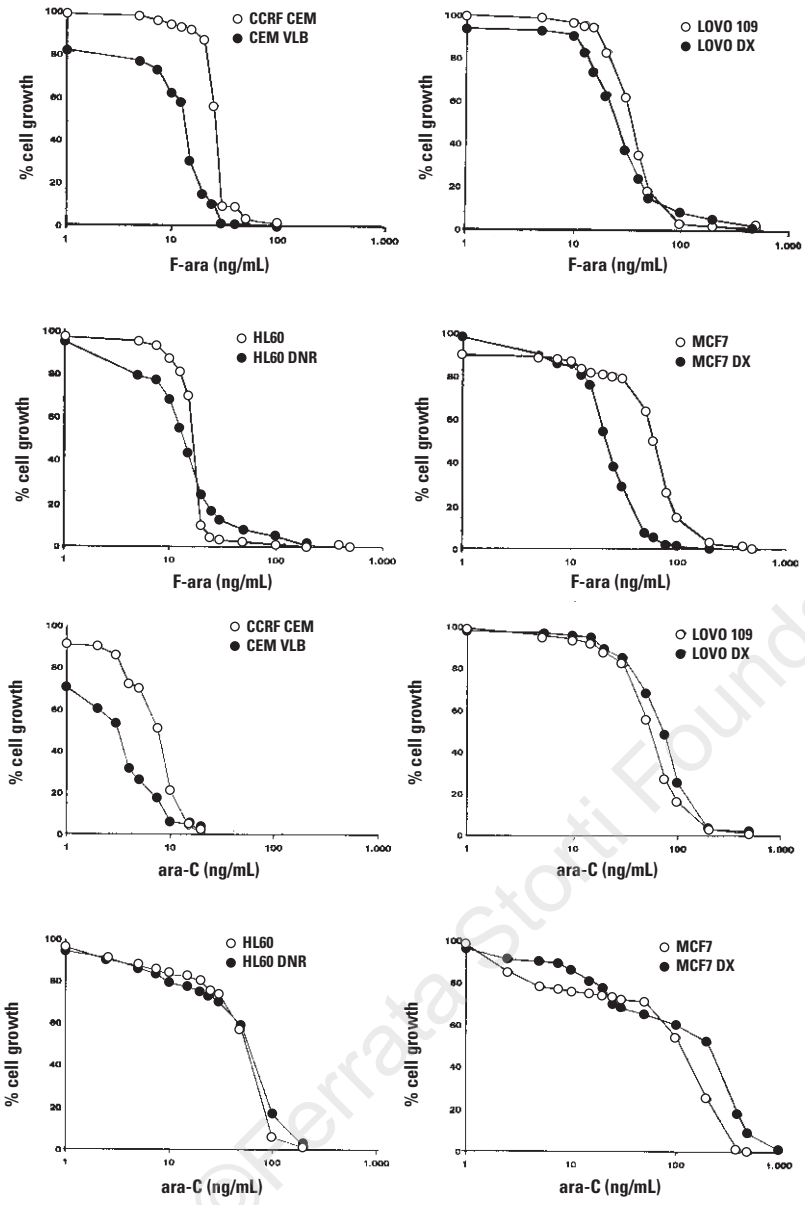

Figure 1. Growth inhibition assay (MTT test) for fludarabine (F-ara). The dose-response curve of the MDR sublines (closed symbols) is either superimposable on the dose-response curve of the non-MDR parental lines (open symbols) or is shifted to the left, indicating that Pgp did not affect cell sensitivity to F-ara.

Figure 2. Growth inhibition assay (MTT test) for arabinosylcytosine (ara-C). The dose-response curve of the MDR sublines (closed symbols) is either superimposable on the dose-response curve of the non-MDR parental lines (open symbols) or is shifted to the left, indicating that Pgp did not affect cell sensitivity to ara-C.

Discussion

Leukemic and cancer cells can overmodulate Pgp expression, which is responsible for the so-called classic MDR. Overexpression is very frequent in relapsed/resistant cases of acute non-lymphocytic leukemia (ANLL) as well as in secondary acute leukemia, but it also occurs in primary acute leukemia prior to any treatment. Although some data are discordant, the great majority of the studies in this area found that Pgp overexpression was associated with a poorer response to standard chemotherapy.[29-35] If it is clear that MDR and Pgp are important and have a significant effect on treatment results, it is much less clear what action should be taken against Pgp and how to neutralize MDR. The difficulty stems from the fact that many so-called MDR modifiers, like calcium channel blockers and cyclosporine derivatives, cannot be given to patients at doses that are likely to be effective based on *in vitro* experiments.[26,27,36-41] Moreover, effective *in vivo* neutralization of Pgp-mediated MDR could result in intolerable toxicity for the normal cells that express Pgp physiologically, such as hemopoietic stem cells, hepatocytes, renal, and intestinal epithelial cells.[4,42-48] Since anthracyclines are quickly and easily processed outside the cell by Pgp, the dominating role of anthracyclines in the treatment of ANLL is likely to be challenged, especially in cases that overexpress Pgp. Alternative approaches include the development, testing and more extended use of compounds that are not processed by Pgp, like ara-C and purine analogues.

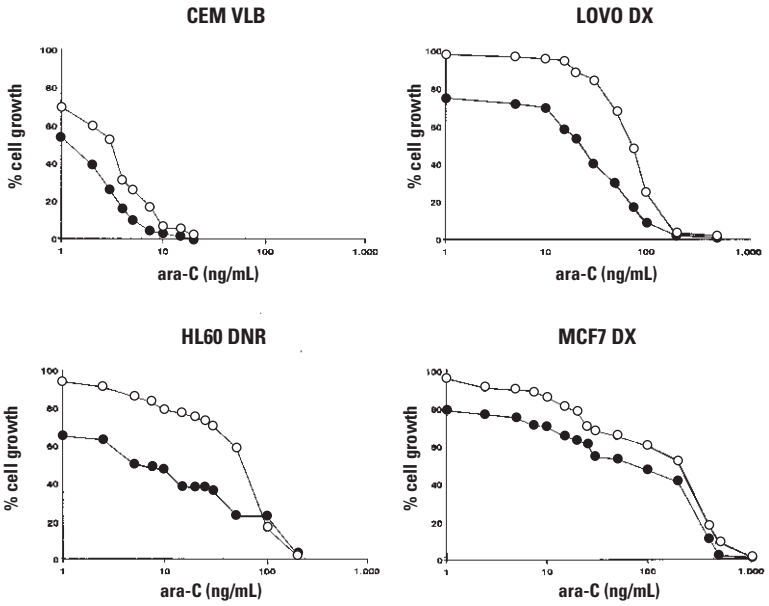

Figure 3. Growth inhibition assay (MTT test) for arabinosylcytosine (ara-C) without (open symbols) and with (closed symbols) pre-exposure to fludarabine (F-ara,10 ng/mL). In all four MDR sublines the dose-response curve to ara-C + F-ara is shifted to the left of the dose-response curve to ara-C alone.

However, the cells that develop transmembrane transport-mediated MDR can also develop other different and independent mechanisms of drug resistance.

In the present study, we confirmed that neither sensitivity to ara-C nor sensitivity to F-ara was associated with Pgp overexpression, and we showed that any combination of ara-C with a low F-ara dose (10 ng/mL) was at least as cytotoxic against all four MDR sublines as ara-C or F-ara alone at equivalent concentrations. These data were obtained in cell systems in which the only detectable mechanism of resistance was Pgp. Therefore overexpression of either LRP or MRP, as may occur in leukemia, could lead to different results.

Table 2. Resistance index of F-ara and of ara-C alone and in combination with F-ara. The resistance index was calculated by dividing the inhibition dose 50 of the MDR cell line by the inhibition dose 50 of the respective non-MDR parental cell line. The decrease in the resistance index to ara-C indicates that the combination of ara-C and F-ara was more toxic against the MDR sublines than against their respective non-MDR parental lines.

	F-ara	*ara-C*	*ara-C + F-ara 1 ng/mL*	*ara-C + F-ara 5 ng/mL*	*ara-C + F-ara 10 ng/mL*
CCRF CEM CEM VLB	0.50	0.43	0.38	0.28	0.17
LOVO 109 LOVO DX	0.73	1.27	1.30	1.25	0.81
MCF 7 MCF 7 DX	0.37	1.83	1.86	1.00	0.72
HL60 HL60 DNR	0.82	1.05	1.09	0.40	0.20

The enhancement of ara-C cytotoxicity was detected more in MDR sublines than in the non-MDR ones. This is shown in Table 2, that reports the resistance index of the four line pairs to ara-C and to the combination of ara-C and F-ara. Since the resistance index is calculated by dividing the inhibition dose 50 of the MDR subline by the inhibition dose 50 of the non-MDR parental line, a resistance index lower than one indicates that MDR cells are more sensitive to the tested drug or drug combination than non-MDR cells. As a matter of fact, the resistance index to ara-C decreased upon pre-exposure to F-ara 10 ng/mL from 0.43 to 0.17 for the CEM pair, from 1.27 to 0.81 for the LOVO pair, from 1.83 to 0.72 for the MCF7 pair, and from 1.05 to 0.20 for the HL60 pair. The reason why the combination of ara-C and F-ara was likely to be more toxic against all MDR sublines than against the parental lines could not be clarified. It was not a matter of kinetics because all cell lines were tested during the exponential phase of the growth, and because growth was faster in parental cells than in MDR cells. Whatever the explanation, these findings provide further support for the application of ara-C and F-ara to the treatment of ANLL and suggest that in relapsed/resistant and in secondary acute leukemias increasing the dose of ara-C and combining it with F-ara can be more rewarding than administering anthracyclines or other Pgp-processable compounds.

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