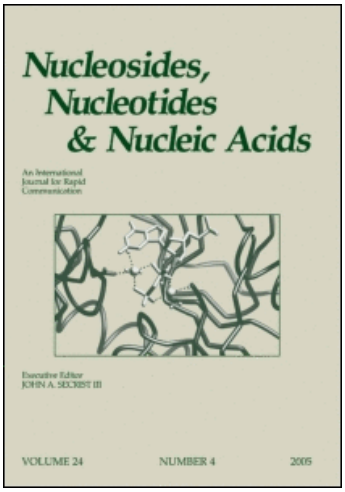

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In Vivo and in Vitro Activity And Mechanism Of Action of the Multidrug Cytarabine-L-Glycerylyl-Fluorodeoxyuridine

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IN VIVO AND IN VITRO ACTIVITY AND MECHANISM OF ACTION OF THE MULTIDRUG CYTARABINE-L-GLYCERYLYL-FLUORODEOXYURIDINE

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□ *Multidrugs have the potential to bypass resistance. We investigated the in vitro activity and resistance circumvention of the multidrug cytarabine-L-fluorodeoxyuridine (AraC-L-5FdU), linked via a glycerophospholipid linkage. Cytotoxicity was determined using sensitive (A2780, FM3A/0) and resistant (AG6000, AraC resistant, deoxycytidine kinase deficient; FM3A/TK-, 5FdU resistant, thymidine kinase deficient) cell lines. Circumvention of nucleoside transporter and activating enzymes was determined using specific inhibitors, HPLC analysis and standard radioactivity assays. AraC-L-5FdU was active (IC50: 0.03 μM in both A2780 and FM3A/0), had some activity in AG6000 (IC50: 0.28 μM), but no activity in FM3A/TK$^-$ (IC50: 18.3 μM). AraC-nucleotides were not detected in AG6000. 5FdU-nucleotides were detected in all cell lines. AraC-L-5FdU did not inhibit TS in FM3A/TK$^-$ (5%). Since phosphatase/nucleotidase-inhibition reduced cytotoxicity 7–70-fold, cleavage seems to be outside the cell, presumably to nucleotides, and then to nucleosides. The multidrug was orally active in the HT-29 colon carcinoma xenografts which are resistant toward the single drugs.*

Keywords Multidrugs; cytarabine; fluorodeoxyuridine; resistance

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INTRODUCTION

From the vast number of cytotoxic nucleoside analogs that were synthesized in the past, only a limited number of molecules, including 5-fluorouracil (5FU) and cytarabine (AraC) have reached the clinic.[1] However, the therapeutic success of these drugs is faced with the disadvantages of (severe) toxicity, the development of resistance and the dependency of a nucleoside transporter system.[2] In order to minimize these disadvantages, new multidrugs have been synthesized, which might also be administered orally. We tested the multidrug cytarabine-L-fluorodeoxyuridine (AraC-L-5FdU; Offenlegungschrift DE 19855963A1 (2000) Inventor: Schott H., Ludwig P.), in which the mononucleotide of AraC is coupled with a glycerophospholipid linkage (L) to 5FdUMP, the active metabolite of 5-fluorodeoxyurdine (5FdU)[2] (Figure 1). AraC exerts its cytotoxicity after conversion by deoxycytidine kinase (dCK) to its monophosphate AraCMP, leading to accumulation of AraCTP, which can be incorporated into the DNA and thereby inhibits DNA polymerase.[3] 5FdUMP is a potent inhibitor of thymidylate synthase (TS), leading to inhibition of DNA synthesis. Inhibition and activation of TS have been related to clinical response to 5FU.[4,5] The lipid group of AraC-L-5FdU possibly enables passive diffusion into the cell, subsequently active metabolites can be released by enzymatic cleavage, resulting in circumvention of resistance. The aim of this study was to determine the level of drug cytotoxicity in vitro, whether AraC-L-5FdU is cleaved in- or outside the cell and whether it is cleaved into nucleotides or nucleosides.

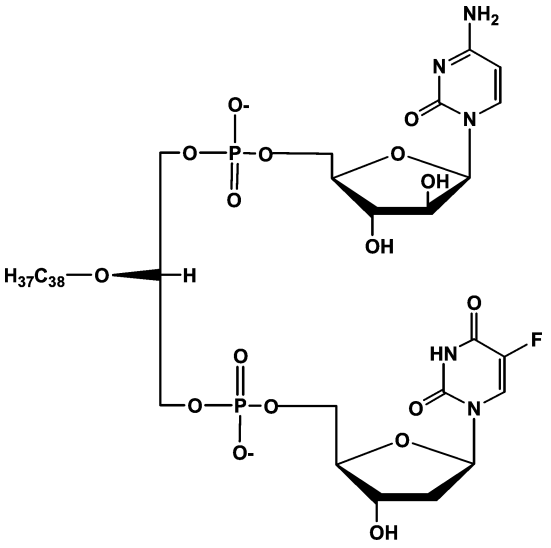

FIGURE 1 Structural formula of the multidrug AraC-L-5FdU (arabinosylcytosine-L-glycerylyl-5-fluoro-2′-deoxyuridine).

MATERIALS AND METHODS

Growth inhibition was determined using the sulphorhodamine B (SRB) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[6] IC50 values of the drugs were determined after 72 hours of drug exposure in the different cell lines by interpolating the growth inhibition curves. Circumvention of AraC resistance was studied using a dCK deficient cell line AG6000 and its parental A2780[7] and 5FdU resistance using FM3A/0 and FM3A/TK$^-$, of which the latter is deficient in thymidine kinase (TK).[8] In order to determine the potential breakdown by extracellular nucleotidases and phosphatases, cells in culture were incubated (72 hours, 37°C) with a nucleotidase inhibitor (2.5 mM α,ß-methylene-ADP) and a phosphatase inhibitor (15 mM 2-glycerol-phosphate). To determine transporter dependency, cells in culture were incubated (72 hours, 37°C) with 1 μM dipyridamole.

Nucleotide accumulation was determined with both a radioactivity assay for 5FdUMP and HPLC detection for AraCTP.[9,10] Inhibition of TS was determined by TS in situ activity.[10] In addition, we measured total nucleotide accumulation in the cells. For this purpose cells were extracted by trichloric acid, where after the intracellular nucleotides were broken down to the nucleoside by incubation with 4 units alkaline phosphatase. AraC was analyzed on RP-HPLC chromatography as described earlier for nucleoside analog determination.[11]

In vivo testing of AraC-L-5FdU was performed using 5FU resistant HT-29 colon cancer cells, which were transplanted subcutaneously to NMRI nu/nu female mice.[12] Intravenous or oral treatment (both at 150 mg/kg) of mice started 11 days after transplantation. Mice were sacrificed when tumors reached a size of more than 1 cm^3. The tumor volumes of treated tumors (T) were compared to those of controls (C), which were phosphate-buffered saline-treated.

RESULTS

The IC50 value of AraC-L-5FdU was not lower than that of the single nucleoside analogues (Table 1). Dipyridamole protected against AraC-L-5FdU in both A2780 and AG6000 cells. However, in FM3A/0 and FM3A/TK$^-$, no protection was found at all. This suggests that AraC-L-5FdU may partially bypass the nucleoside transporter. Nucleotidase and phosphatase treatment protected cells against AraC-L-5FdU cytotoxicity (Table 1).

In order to determine whether the compound would act specifically via AraC or via 5FdU, we measured the active metabolites of these compounds intracellularly, AraCTP and FdUMP, as well as total nucleotide accumulation. AraCTP accumulated after AraC-L-5FdU exposure in A2780 and to a lower extent in the FM3A cells (Table 2). Total phosphorylated AraC was

TABLE 1 Sensitivity of w.t. and resistant cell lines to AraC-L-5FdU and the parent drugs AraC and 5FdU; effect of transport and degradation inhibition

Cell line	AraC (μM)	5FdU (μM)	AraC-L-5FdU (μM)	Level of protection by dipyridamole	Level of protection nucleotidase/ phosphatase inhibition
A2780	0.027 ± 0.008	0.01 ± 0.002	0.03 ± 0.02	7.14	45
AG6000	36 ± 4	0.037 ± 0.015	0.28 ± 0.02	70	na
FM3A/0	1.48 ± 0.67	0.003 ± 0.001	0.03 ± 0.02	1	12.67
FM3A/TK$^-$	0.35 ± 0.12	4.71 ± 1.41	18.3 ± 3.8	1	1.14

Level of protection was determined by dividing the level of growth inhibition with inhibitors + drugs through the level of growth inhibition without inhibitors + drugs. Values represent the mean of three independent experiments, ± indicates standard error of the mean (SEM); na-not available.

detected in all cells but not in AG6000 cells. This concentration was at least more then 2-fold higher than that of AraCTP itself, indicating that AraC nucleotides accumulate in the cells. 5FdUMP accumulated in all tested cell lines (Table 2), including TK$^-$ cells. However, the accumulation of phosphorylated 5FdU was many fold higher than that of 5FdUMP. This means that 5FdUMP is only a minor constituent of all phosphorylated nucleosides in the cell. However, this 5FdUMP accumulation after exposure to the multidrug was high enough to inhibit TS (Figure 2). In addition, TS activity was inhibited completely in FM3A cells after treatment with 5FdU alone.

The in vivo antitumor effect of AraC-L-5FdU against HT-29 tumors was better with oral (T/C 43%) than i.v. (T/C 78%) treatment, and lower than oral 5FdU alone (200 mg/kg) (T/C 75%). AraC is ineffective against this tumor. AraC-L-5FdU did not cause hematological side effects and had only a minor influence on the body weight of the mice (7% body weight loss after i.v., no influence after oral treatment).

TABLE 2 Accumulation of intracellular AraCTP, 5FdUMP, and total phosphorylated nucleosides

	AraCTP accumulation (pmol/10^6cells)		5FdUMP accumulation (fmol/10^6cells)		Total phosphorylated nucleosides (pmol/10^6cells)	
					AraC-L-5FdU	
Cell line	AraC	AraC-L-5FdU	5FdU	AraC-L-5FdU	AraC	5FdU
A2780	229.8	255.8	ne	ne	593.5	448.5
AG6000	nd	nd	ne	ne	nd	1040
FM3A/0	11	nd	16.4	1.4	1013.5	453.5
FM3A/TK$^-$	37	nd	0.2	1.4	354	415.5

AraCTP accumulation after 24 hours of exposure to 100 μM of AraC-L-5FdU or AraC alone. 5FdUMP accumulation was determined after 24 hours of incubation with 0.05 μM of AraC-L-5FdU or 5FdU alone. Total (mono- di- and tri) phosphorylated nucleosides of AraC and 5FdU after 24 hours of exposure to 100 μM AraC-L-5FdU were detected inside the cells. Values represent means of two/three independent experiments (nd = not detected, ne = not evaluated); SEM was less than 15%.

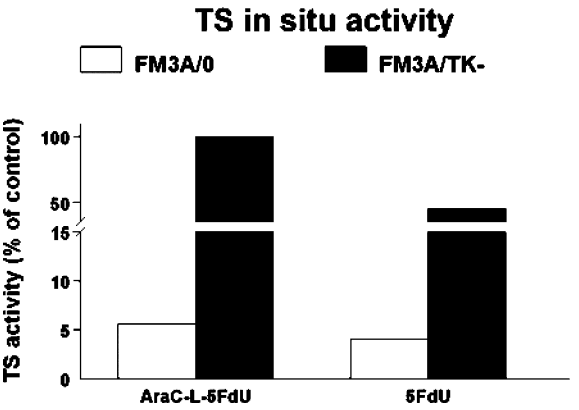

FIGURE 2 TS in situ activity after 24 hours of treatment with 0.5 μM of AraC-L-5FdU or 5FdU alone. Values represent means of three independent experiments. SEM was less than 15%.

DISCUSSION

In order to combine the advantages of two drugs, new multidrugs have been synthesized. In a previous study, multidrugs were already shown to overcome drug resistance.[13] In the present study, AraC-L-5FdU inhibited cell growth in various cell lines. Cytotoxicity was less in dCK deficient cells, in which also no phosphorylated AraC was detected. Although phosphorylated 5FdU was detected inside the cell, TS was hardly inhibited and cytotoxicity was reduced over 600-fold in TK deficient cells. This suggests that resistance due to deficiency in the activating enzymes dCK and TK was not circumvented and also that AraC-L-5FdU probably acts predominantly by its metabolite 5FdU. Inhibitors of nucleoside transporter, nucleotidase and phosphatases reduced cytotoxicity, therefore, it can be concluded that AraC-L-5FdU is probably cleaved outside the cell to nucleotides, but not completely. Otherwise IC50 values should be similar to that of one of the parent drugs. Subsequently, the nucleotide is degraded to a nucleoside after which it can enter the cell using a nucleoside transporter. AraC-L-5FdU can inhibit growth at high concentrations, explaining why dipyridamole did not protect in FM3A/TK$^-$ cells, since at this concentration the drug bypasses the nucleoside transporter.[14] Since many tumor cells express high levels of nucleotidases and phosphatases,[15] cleavage of these drugs outside the cells can be tumor specific.

Since both i.v. and oral administration of the multidrug were effective, in contrast to the single analogues which were orally inactive, this can be an advantage of the multidrug, besides circumvention of resistance. In this article, we show that combining two potent drugs may increase drug uptake and circumvent activating enzymes. Future studies should also attempt to characterize the contribution of different linkers to enhance drug uptake and prevent cleavage outside cells.[16]

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