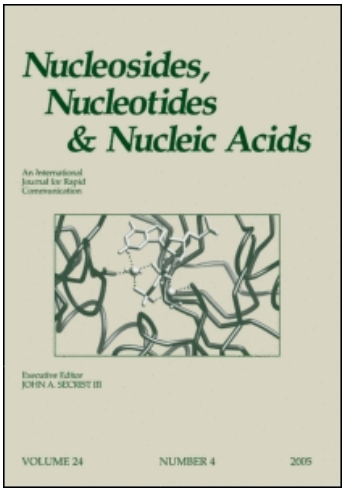

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INDUCTION OF RESISTANCE TO THE LIPOPHILIC CYTARABINE PRODRUG ELACYTARABINE (CP-4055) IN CEM LEUKEMIC CELLS

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□ *The deoxynucleoside analogs cytarabine (Ara-C) and gemcitabine (dFdC) are widely used in the treatment of cancer. Due to their hydrophilic nature they need the equilibrative (hENT) and concentrative (hCNT) nucleoside transporters to enter the cell. To bypass drug resistance due to decreased uptake, lipophilic 5′elaidic acid esters were synthesized, elacytarabine (CP-4055, from ara-C) and CP-4126 (from gemcitabine), which are currently in clinical development for solid and hematological tumors. We investigated whether resistance can be induced in vitro, and treated the CEM leukemic cell line with weekly increasing elacytarabine concentrations, up to 0.28 μM (10 times IC_{50}). The IC_{50} of the resistant CEM/CP-4055 was 35 μM, about 1,000 times that of the wildtype CEM, and comparable to that of CEM/dCK- (deoxycytidine kinase deficient) (22 μM). CEM/CP-4055 was also cross-resistant to Ara-C, gemcitabine and CP-4126 (28 and 33 μM, respectively). A low level of mRNA dCK was observed, and similar to CEM/dCK-, CEM/CP-4055 did not accumulate Ara-CTP after exposure to Ara-C or elacytarabine, which is consistent with a deficiency in dCK. In conclusion, elacytarabine induced resistance similar to Ara-C. This resistance was caused by downregulation of dCK.*

Keywords CP-4126; cytarabine; deoxycytidine; elacytarabine; gemcitabine

INTRODUCTION

The deoxynucleoside analog cytarabine (Ara-C) is commonly used in the treatment of hematological malignancies.[1] while gemcitabine is used for treatment of nonsmall cell lung cancer and pancreatic cancer. Due to their hydrophilic nature, the drugs are dependent on the equilibrative and concentrative nucleoside transporters (hENT and hCNT) to cross the cell membrane to enter the cell.[2] The subsequent rate-limiting step in the activation to their active triphosphate forms is the conversion to the intermediate monophosphate by deoxycytidine kinase (dCK).[1,3] Both drugs can be

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inactivated by deoxycytidine deaminase (dCDA). Ara-C triphosphate incorporation into DNA causes chain termination, while the active metabolite of Ara-C can inhibit DNA polymerase by competitive inhibition.. Gemcitabine can be incorporated into DNA and RNA; its incorporation into DNA leads to a masked chain termination.[3,4] In order to enhance the uptake of the drugs into the cell by making the drugs less hydrophilic, various derivatives containing a fatty acid side chain have been developed. The fatty acid side chain was coupled to the 5′ position on the sugar moiety. The derivative of cytarabine (CP-4055, elacytarabine) and of gemcitabine (CP-4126; gemcitabine-5′elaidate) contain a fatty acid with a chain length of eighteen carbon atoms and one trans-double bond (elaidic acid). Elacytarabine has shown remarkable antitumor activity in various solid cancer xenografts in which the parent drug cytarabine has no activity;[5] CP-4126 showed a similar activity as gemcitabine in various model systems, while it has oral activity as well. Other studies[6–8] have demonstrated that both elacytarabine and CP-4126 are still dependent on activation by dCK, but that the drugs have a longer cellular retention than the parent compounds, while elacytarabine has a different effect on DNA and RNA synthesis compared to Ara-C. Elacytarabine and CP-4126 both remains active in cells where the hENTs are absent or blocked.[5,13] Elacytarabine was investigated in a Phase I clinical study in solid tumors,[9] showing a favorable and predictable safety profile which was dose and schedule dependent. The drug is currently in Phase II studies. CP-4126 is in Phase I and II studies as well. We investigated whether resistance can be induced, and what the cause of this resistance was.

MATERIALS AND METHODS

Drugs

Elacytarabine and CP-4126 were provided by Clavis Pharma (Oslo, Norway), Ara-C was from Sigma-Aldrich (St. Louis, MO, USA) and gemcitabine (dFdC) was provided by Eli Lilly (Indianapolis, IN, USA).

Cell Lines

For the experiments the CCRF-CEM human leukemia cell line was used, while its dCK negative variant (CEM/dCK-) served as a reference Ara-C resistant cell line. The cell lines were cultured in RPMI medium (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum (PAA laboratories, Pasching, Austria) and HEPES buffer (BioWhittaker). Of the nucleoside influx transporters, the CEM cell line only expresses the equilibrative nucleoside transporter (hENT), but not the concentrative nucleoside transporter (hCNT).[10]

Resistant Cell Line

The resistant cell line was created by treating the CEM cell line with a weekly increasing concentration of elacytarabine (0.01 μM/week) for about 28 passages.

Chemosensitivity Assay

Sensitivity to the drugs was determined using the MTT assay as described earlier[6] with a 72-hour drug exposure. The results were expressed as percentage of control growth; the IC_{50} value was determined by interpolating at the 50% growth level.

Triphosphate Accumulation

Cells were treated with 1 and 10 μM of Ara-C; of the lipophilic analog 1 and 10 μM was added. The cells were incubated for 60 minutes. Thereafter the nucleotides were extracted with trichloroacetic acid precipitation of cellular proteins; the neutralized extract was analyzed for the triphosphates using an earlier described HPLC assay.[11]

Quantitative PCR

PCR was performed on a LightCycler (Roche Applied Science, Penzberg, Germany), using a FastStart DNA MasterPLUS SYBR Green I kit. To detect dCK previously published primers[12] were used. The amount of dCK was normalized to the expression of β-actin.

RESULTS

To create a cell line resistant to elacytarabine, the CEM cell line was treated with weekly increasing elacytarabine concentrations, up to 0.28 μM (10 times IC_{50}). The development of resistance was considered as relatively fast, since each week the concentration of elacytarabine could be increased without killing cells. However, when the cells were continuously grown at concentrations higher than 0.28 μM the cells would not grow or grow poorly, and had a poor morphology. This is possibly due to the circumstance that elacytarabine is continuously present enabling some activation However, when the elacytarabine resistant CEM cell line was tested in a short-term assay (72-hour exposure) an IC_{50} of >30 μM was found, which is about 1,000 times the IC_{50} in the wildtype CEM. This IC_{50} is comparable with the sensitivity in the CEM/dCK- cell line (35 μM). The resistant cell line was also cross-resistant with the gemcitabine analog CP-4126 (Table 1).

No Ara-CTP accumulation was detectable in the CEM/dCK- cell line and in the elacytarabine resistant cell line after 60 minutes incubation with

TABLE 1 Sensitivity of the wildtype cell line and the resistant cell lines to the parent compounds and the lipophilic analogs. Values are means (in μM) ± SEM. In the dCK- cells the highest concentration used was 20 μM; CP-4126 was not tested in CEM/dCK- cells

	Cell line		
Drug	CEM	CEM/dCK-	CEM/CP-4055
Ara-C	0.035 ± 0.010	>20	35.4 ± 4.4
Elacytarabine	0.023 ± 0.008	22	34.8 ± 1.4
Gemcitabine	0.013 ± 0.001	>20	28.3 ± 5.0
CP-4126	0.019 ± 0.006	n.d.	32.9 ± 5.2

Ara-C or elacytarabine (Figure 1). As dCK is the rate limiting step in the formation of Ara-CTP from Ara-C, the lack of Ara-CTP formation in the elacytarabine resistant CEM cell line points to a deficiency in dCK. Therefore we determined the expression of dCK in the normal and the resistant cell lines; the normalized ratio of dCK/ß-actin was 72.44 in the CEM cell line, but only 0.44 in CEM/CP-4055 cells.

DISCUSSION

Exposure to increasing concentrations of elacytarabine created a cell line which was a thousand times less sensitive to elacytarabine, and the resistant cell line was cross-resistant to other nucleoside analogs. In the wildtype

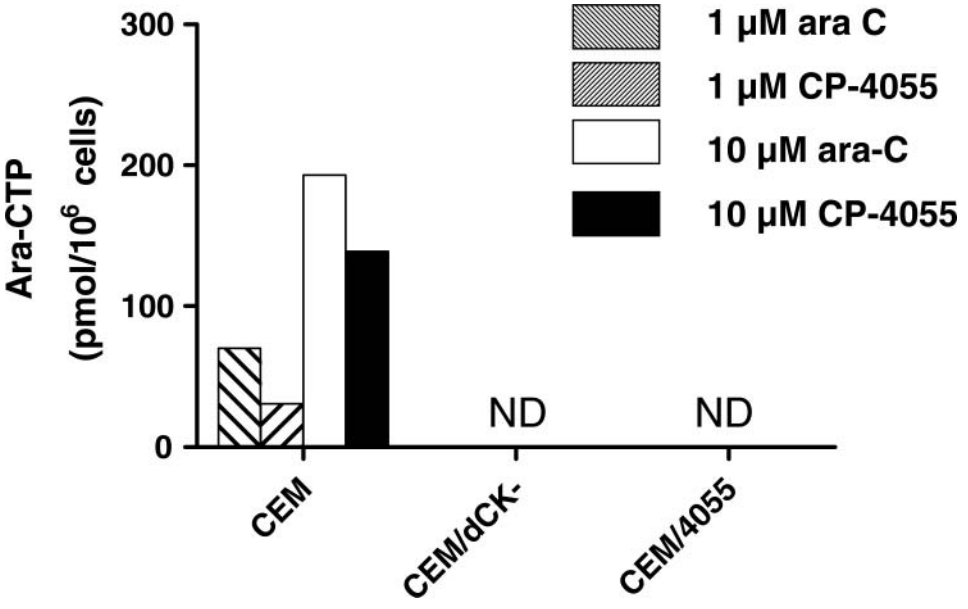

FIGURE 1 Ara-CTP accumulation in the CEM, the CEM/dCK- and the elacytarabine resistant CEM/CP-4055 cell lines. Cells were treated with 1 and 10 μM of Ara-C and elacytarabine.

cell line Ara-C and elacytarabine were converted to the active triphosphate form, while in the dCK negative and the elacytarabine resistant cell lines no tri-phosphates were detected. Deoxycytidine kinase is the rate-limiting step in the activation of nucleoside analogs, which was frequently found to be downregulated or mutated in deoxynucleoside analog resistant cell lines. This is consistent with the earlier observation that elacytarabine is also activated by dCK.[6,7] Another possible resistance mechanism could be the downregulation of the nucleoside transporters hENT and hCNT, but because of its lipophilicity the action of elacytarabine is independent on the influx transporters.[5,13] Downregulation of dCK is the most likely cause of resistance in the CEM/CP-4055 cell line. Remarkably, resistant cells could be cultured continuously only in 0.28 μM, which is much lower than the found IC_{50} value. The most likely explanation for this discrepancy is the relatively short exposure for the cytotoxicity assay (72 hours) while during maintenance the cells are continuously (weeks to months) cultured at 0.28 μM. This concentration could be increased somewhat in time, but was still far below 1 μM. Apparently there is some activation of ara-C derived from elacytarabine, during this long exposure, which is most likely mediated by the very low remaining dCK activity. Another enzyme such as thymidine kinase 2 (TK2) are unlikely to activate ara-C because of the very low substrate specificity of ara-C for TK2.

Interestingly it has not yet been possible to create resistance to CP-4126 although similar conditions were used. Apparently the more complicated mechanism of action of gemcitabine precludes an easy induction of resistance. Earlier we induced resistance to gemcitabine in A2780 ovarian cancer cells[14] which required much longer time than induction of resistance to Ara-C in CEM cells. However, resistance was related to dCK deficiency as well. Another relatively frequently observed resistance mechanism for gemcitabine includes an upregulation of ribonucleotide reductase, a target for gemcitabine.[6] However, in vivo this even took more than 18 months to be established.[15] This failure can actually be considered as a positive point, since this might preclude an easy development of resistance to CP-4126 in vivo, and possibly also in patients.

In conclusion, resistance to elacytarabine was relatively easily established in contrast to development of resistance to CP-4126. The resistance to elacytarabine was due to a deficiency of dCK.

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