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ORIGINAL PAPER

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Cytotoxicity, cell-cycle perturbations and apoptosis in human tumor cells by lipophilic N^4-alkyl-1-β-D-arabinofuranosylcytosine derivatives and the new heteronucleoside phosphate dimer arabinocytidylyl-(5′ → 5′)-N^4-octadecyl-1-β-D-arabinofuranosylcytosine

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Abstract The arabinofuranosylcytosine (AraC) derivative N^4-octadecyl-1-β-D-arabinofuranosylcytosine (NOAC) and its (5′ → 5′)-heterodinucleoside phosphate analog NOAC-AraC were compared with AraC for cytotoxicity, cell-cycle dependence, phosphorylation by deoxycytidine (dC) kinase and apoptosis induction in native, AraC- or NOAC-resistant HL-60 cells. NOAC was cytotoxic in all cells with three to seven-fold lower IC_{50} concentrations than those of NOAC-AraC or AraC. In contrast to NOAC-AraC, the lipophilic monomer NOAC overcame AraC resistance, inducing apoptosis in more than 80% of native and AraC-resistant HL-60 cells. This suggests that NOAC-AraC may be cleaved intracellularly only at very slow rates to AraC and NOAC or to the 5′-monophosphates, whereas NOAC exerts different mechanisms of action from AraC. In vitro the dimer was cleaved by phosphodiesterase or human serum to NOAC, AraC and AraC monophosphate. In contrast to AraC, N^4-alkylated AraC derivatives with alkyl chains ranging from 6–18 C atoms were not substrates for dC kinase. Furthermore, treatment of the multidrug-resistant cell lines KB-Ch^R-8-5 and KB-V1 with the N^4-hexadecyl-AraC derivative NHAC did not induce P-170 glycoprotein expression, suggesting that the N^4-alkyl-AraC derivatives are able to circumvent MDR1 multidrug resistance. The in vivo activity of liposomal NOAC in a human acute lymphatic leukemia xenograft model confirmed the antitumor activity of this representative of the N^4-alkyl-arabinofuranosylcytosines.

Key words N^4-Alkyl-AraC derivatives · NOAC-AraC dimer · Cytotoxicity · Apoptosis · Drug resistance

Abbreviations *AraC* 1-β-D-arabinofuranosylcytosine · *AraU* 1-β-D-arabinofuranosyluracil; *NHAC* N^4-hexadecyl-1-β-D-arabinofuranosylcytosine · *NOAC* N^4-octadecyl-1-β-D-arabinofuranosylcytosine · *NOAC-AraC* arabinocytidylyl-(5′ → 5′)-N^4-octadecyl-1-β-D-arabinofuranosylcytosine · *dT-dT* deoxythymidylyl-(3′ → 5′)-deoxythymidine · *BrdUrd* 5-bromo-2′-deoxyuridine · *BSA* bovine serum albumin · *FCS* fetal calf serum · *HBSS* Hanks' balanced salt solution · *PBS* phosphate buffered saline · *dC* deoxycytidine kinase · IC_{50} 50% cell-growth-inhibitory concentration · *MDR* multidrug resistance

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Introduction

1-β-D-arabinofuranosylcytosine (AraC) has an important clinical activity against human acute myelogenous leukemia (Plunkett and Ghandi 1993). However, it was recognized that the major disadvantages of the drug were the short plasma half-life and rapid degradation by deamination to the inactive metabolite 1-β-D-arabinofuranosyluracil (AraU), which also impedes the oral application of AraC. To eliminate these disadvantages a large number of 5′- and N^4-substituted derivatives of AraC have been synthesized and characterized in the

past. In a previous study, we demonstrated that N^4-acyl derivatives of AraC were active as liposomal formulations in murine L1210 leukemia and B16 melanoma at concentrations two to four times lower than AraC (Rubas et al. 1986). Nevertheless, the protection of the N^4-acyl derivatives of AraC against enzymatic deamination was only partially achieved and suggested to be still insufficient in a pilot phase I/II study (Schwendener et al. 1989). On the basis of these results, a new class of N^4-alkyl derivatives of AraC were synthesized (Schwendener and Schott 1992; Schott et al. 1994). These compounds show a typical structure/activity correlation between the length of the alkyl side-chain and their antitumor activity in the L1210 mouse model (Schwendener and Schott 1992). The most effective derivatives are N^4-hexadecyl-AraC (NHAC) and N^4-octadecyl-AraC (NOAC) (Schwendener et al. 1995a). These derivatives are extremely resistant to deamination in plasma and liver and they exert excellent antitumor activities after oral therapy as well as in human solid tumor xenografts (Horber et al. 1995a; Schwendener et al. 1995b, 1996). In contrast to the parent drug, their cellular uptake is independent of any nucleoside transporter and only about 2%–5% of NHAC was phosphorylated to AraC triphosphate in HL-60, K-562 and U-937 cells (Horber et al. 1995b). Furthermore, NHAC was found to be cytotoxic in AraC-resistant HL-60 cells (Horber et al. 1995c). We concluded therefore that the mechanisms of action of the N^4-alkyl-AraC derivatives are different from those of AraC.

The efficacy of different antitumor drugs has been associated with their ability to induce apoptosis in tumor cells. It has been shown that cells progressing through S phase were selectively susceptible to induction of apoptosis when treated with AraC (Cavazzana et al. 1988). The cell-cycle-dependent cytotoxicity of AraC is directly caused by incorporation of AraCTP into DNA and its interaction with DNA polymerase α, resulting in a block of G1 cells at the G1–S border (Fram and Kufe 1985). The cytotoxicity of NHAC, on the other hand, was shown not to be limited to S-phase cells (Horber et al. 1995c).

In the present study we investigated the cytotoxic potential of NOAC in three different HL-60 cell lines. In addition, we studied the dependence on the cell cycle of NOAC cytotoxicity and phosphorylation capacity of recombinant human dC kinase, using different N^4-alkyl-AraC derivatives. The effects of NOAC were compared to those of AraC as well as to those of the new dimer arabinocytidylyl-(5′ → 5′)-N^4-octadecyl-1-β-D-arabinofuranosylcytosine (NOAC-AraC), which is the 5′ → 5′-hetero-dinucleoside phosphate derivative of the two cytotoxic drugs AraC and NOAC. Thus, the cytotoxic effect of the dimer might be more pronounced, owing to a combination of the effects of both active molecules that can be released in the cells as monomers or the corresponding monophosphates.

Furthermore, we determined the apoptosis-inducing capability of NOAC, AraC and NOAC-AraC by terminal deoxynucleotidyltransferase and caspase-3 activity assays. In an additional set of experiments the cytotoxic effects of NHAC on sensitive and drug-resistant KB cells were investigated by analysis of drug uptake and expression of the P-170 glycoprotein. Finally, the antileukemic efficacy of NOAC against a human acute lymphatic leukemia (ALL) was evaluated in a xenotransplantation model in NOD/SCID mice.

Materials and methods

Materials

AraC, verapamil, camptothecin, 5-bromo-2′-deoxyuridine (BrdUrd), Tween 20, bovine serum albumin (BSA), propidium iodide and RNase A were purchased from Sigma Chemical Inc. (Buchs, Switzerland). Doxorubicin was a gift from Pharmacia, Milano, Italy. The cell proliferation reagent WST-1 was obtained from Boehringer Mannheim (Rotkreuz, Switzerland). Terminal deoxynucleotidyl transferase TdT and fluorescein-12-dUTP were from Promega (Madison, Wis.). Caspase-3 fluorogenic substrate (Ac-DEVD-AMC) was obtained from Pharmingen (San Diego, Calif.). Fetal calf serum (FCS), RPMI-1640 and Dulbecco's modified Eagle (DMEM) media and Hanks' balanced salt solution (HBSS) were from Gibco (Paisley, Scotland). All analytical-grade buffer salts and other chemicals were from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). NOAC and NHAC were synthesized as described before (Schwendener and Schott 1992). The anti (P-170 glycoprotein) antibody MRK16 was a gift from Dr. T. Tsuruo, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan. Fluorescein-isothiocyanato (FITC)-labeled goat $F(ab)_2$ anti-(mouse IgG) antibody was from TAGO Immunologicals. The heteronucleoside dimer NOAC-AraC was synthesized in analogy to other dimers as described before (Schott et al. 1997).

For all incubations, AraC and NOAC-AraC were dissolved in 0.9% NaCl or phosphate-buffered saline (PBS: 8 mM Na_2PO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 2.6 mM KCl). NOAC was dissolved in ethanol at 10 mM. The ethanol concentration did not exceed 1% (v/v), which had no influence on growth characterisics or toxicity to the cells. NHAC was applied as a liposomal formulation, which was prepared as described before (Horber et al. 1995a).

Cells

HL-60 human promyelocytic leukemia cells were obtained from the American Type Tissue Culture Collection (ATCC CCL 240). The AraC-resistant HL-60 cells (HL-60/AraC) were a gift from Dr. Studzinski, UMD-New Jersey Medical School, Newark, NJ (Kolla and Studzinski 1994). The multidrug-resistant KB cell lines KB-3-1, KB-Ch^R-8-5 and KB-V1 were obtained from Dr. M.M. Gottesmann, NIH, NCI, Bethesda, Md. (Horio et al. 1991). The NOAC-resistant HL-60 cell line (HL60/NOAC) was generated in our laboratory by continuous incubation of HL-60 cells with increasing amounts, from 1–20 μM, of NOAC for 28 weeks. Accordingly, the NHAC-resistant KB-NHAC cell line was obtained by incubation of KB-3-1 cells with liposomal NHAC for 18 weeks at concentrations starting at 10 μM (3 weeks), 50 μM (7 weeks), 100 μM (6 weeks) and 200 μM for the last 2 weeks. The HL-60 cells were grown in a humidified 5% CO_2 atmosphere in RPMI-1640 medium and the KB cell lines in DMEM (Glutamax medium) containing 4.5 g/l glucose and 1 mM sodium pyruvate. Both media were supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. The KB-Ch^R-8-5 were cultured in the presence of 10 ng/ml colchicine and the KB-V1 cells with 1 μg/ml vinblastine. All experiments were initiated with exponentially growing cell cultures at a density of (3–5) × 10^5 cells/ml.

Cytotoxicity assays

Cytotoxicity of the drugs was determined with the WST-1 assay. The HL-60 cells (3×10^5 cells/ml) were exposed to various concentrations (0–200 μM) of AraC, NOAC or NOAC-AraC for 24 h at 37 °C (5% CO_2). After incubation, 100 μl of each probe was transferred to 96-well plates and 10 μl WST-1 reagent was added and incubated for 2–4 h at 37 °C and 5% CO_2. The absorbance was measured against a background control without cells, in a microtiter plate reader (Dynatech MR5000, Microtec, Embrach, Switzerland) at 450 nm. IC_{50} growth-inhibitory concentrations were calculated from interpolations of the graphical data. Accordingly, the KB cell lines were treated as follows. Confluent cells (3×10^4/well) grown in DMEM/10% FCS medium were incubated (37 °C, 5% CO_2) for 96 h with different concentrations (0–500 μM) of liposomal NHAC, AraC and doxorubicin. WST-1 reagent (10 μl) was added directly to the wells and processed as described above. All experiments were repeated at least four times.

Phosphorylation of AraC derivatives by deoxycytidine kinase (dC kinase)

The phosphorylation activities of recombinant human dC kinase were investigated using different AraC derivatives as substrates. The enzyme assays were carried out as described before (Karlsson et al. 1994). The assay is based on measurements of tritiated monophosphate product bound to ion-exchange filters. Briefly, 100 μM unlabeled AraC derivatives dissolved in dimethylsulfoxide and 10–70 ng recombinant dC kinase were added to a total volume of 50 μl reaction buffer, containing 50 mM TRIS (pH 7.6), 100 mM KCl, 5 mM ATP, 5 mM $MgCl_2$, 15 mM NaF, 5 mM dithiothreitol and 0.5 mg/ml BSA. The samples were incubated at 37 °C and the amount of phosphorus transfer from α-^{32}P-labeled ATP (Amersham Int., Amersham, UK) was determined after 0, 15, 30 and 45 min to obtain a linear reaction rate.

In vitro hydrolysis of NOAC-AraC by phosphodiesterase I and human serum

The stability of NOAC-AraC against hydrolysis by phosphodiesterase I and human serum was analyzed as decribed before (Schott et al. 1999). Briefly, 100 μl NOAC-AraC (5 mg/ml water) was treated for 2, 4, 12 and 36 h at 37 °C with phosphodiesterase I (0.1 M $MgCl_2$, pH 8.1, Sigma) or human serum. The reaction mixtures were analyzed by reverse-phase HPLC with a binary gradient of 0.05 M ammonium acetate and methanol as eluent. The dimer deoxythymidylyl-(3′ → 5′)-deoxythymidine (dT-dT) was used as reference and the hydrophilic metabolites were identified by the corresponding reference compounds.

Cell-cycle distribution analysis

HL-60 cells (1.5×10^6 cells/well) were exposed to various concentrations (0–100 μM) of AraC, NOAC or NOAC-AraC for 24 h at 37 °C (5% CO_2). The cells were incubated with 10 μM BrdUrd for 30 min at 37 °C, washed in cold HBSS and resuspended in 500 μl HBSS. Cells were then injected through a fine needle into 4.5 ml cold 80% ethanol (−20 °C) for fixation. BrdUrd/propidium-iodide staining was carried out as described (Horber et al. 1995c; Preisler et al. 1992).

Quantification of the apoptotic cell fraction

HL-60 cells (1.5×10^6 cells/well) were exposed to various concentrations (0–50 μM) of AraC, NOAC or NOAC-AraC for 24 h at 37 °C (5% CO_2). Camptothecin was used as positive control at a final concentration of 0.2 μg/ml (Gong et al. 1993). After incubation, the cells were prefixed in suspension on ice for 15 min in PBS (pH 7.4) containing 1% methanol-free formaldehyde. After fixation in cold 80% ethanol (−20 °C) and rehydration in PBS, the cells were resuspended in an equilibration buffer (Promega, Madison, Wis.) for 5 min. Then the cells were centrifuged and reincubated in 50 μl equilibration buffer containing 25 units terminal deoxynucleotidyltransferase and 5 μM fluorescein-12-dUTP (Promega). After 1 h incubation at 37 °C, the reaction was stopped by adding 1 ml 20 mM EDTA; the mixture was centrifuged, washed and resuspended in 0.5 ml PBS containing 5 μg/ml propidium iodide and 250 μg of DNase-free RNase A. The cells were incubated for 30 min in the dark and analyzed by flow cytometry.

Influence of NHAC on P-170 glycoprotein expression in KB cell lines

The expression levels of P-170 on the KB cell lines was determined as follows. Trypsinized cells (2×10^6) were washed twice with PBS/1% FCS and resuspended in 0.2 ml PBS/1% FCS. MRK16 antibody (5 μl) was added and incubated for 30 min at room temperature. After two washings with cold PBS/1% FCS, 5 μl FITC-labeled goat $F(ab)_2$ anti-(mouse IgG) antibody was added and incubated for 30 min in the dark. The washed cells were fixed with 2% buffered formaldehyde and analyzed on an Epics Profile (Beckman Coulter, Fullerton, Calif.) instrument. The P-170 glycoprotein expression on the resistant lines KB-CH^R-8-5, KB-V1 and KB-NHAC was set in relation to the parent cells KB-3-1 by calculation of a fluorescence index, which was obtained from the quotient of the mean fluorescence of MRK16 staining of the resistant and of the KB-3-1 cells.

Cellular uptake of NHAC in KB cells of different P-170 glycoprotein expression

The time-dependent uptake of liposomal NHAC by the KB cell lines KB-3-1, KB-Ch^R-8-5, KB-V1 and KB-NHAC was determined as described before (Horber et al. 1995a). Briefly, subconfluent cells (2×10^5–3×10^5/well) were treated with ^{3}H-labeled liposomal NHAC (40 μM, Amersham Int., Amersham UK) for different lengths of time (0–24 h). After the given times, the cells were washed twice with PBS, trypsinized and transferred to scintillation vials. Viable cell numbers at corresponding times were determined by trypan blue staining.

In vivo antileukemic efficacy of NOAC

The acute lymphatic leukemia model ALL-SCID-3 was recently established from a bone marrow sample of a 4-year-old boy in first relapse (Fichtner et al. 1999). The leukemic cells were transplanted to highly immunodeficient NOD/SCID mice (lack of mature B, T lymphocytes, NK cells, or functioning macrophages). Leukemia develops in spleen and liver of these mice after intraperitoneal (i.p.) inoculation of 10^6 cells/mouse and solid tumor nodules and acites are formed in the peritoneum. Treatment with liposomal NOAC or the standard agents AraC and vincristine started 1 day after leukemia inoculation. Treatment schedules and doses were chosen according to previous experiments and corresponded to maximally tolerated doses. Mice were sacrificed when they became moribund. Weights of peritoneal tumors and ascites volumes were determined and compared to those of the saline-treated control group. The experiments were performed according to the German law for protection of animals (licence number of the Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit, Berlin G 0247/98).

Results

Cytotoxicity assays

The cytotoxicity of AraC, NOAC and NOAC-AraC in the three cell lines HL-60, HL-60/AraC and HL-60/

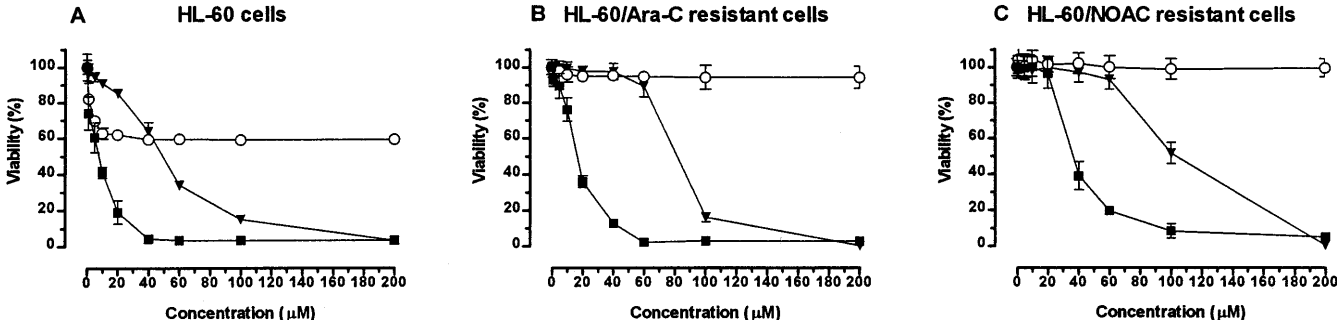

Fig. 1A–C Comparison of cytotoxicity assays after 24 h incubation of HL-60 (**A**), HL-60/AraC-resistant (**B**) and HL-60/NOAC-resistant cells (**C**) with N^4-octadecyl-1-β-D-arabinofuranosylcytosine (*NOAC*) (■), arabinofuranosylcytosine (*AraC*, ○) and NOAC-AraC (▼). The data points represent percentages and standard deviations of at least four different experiments

NOAC after continuous drug exposure for 24 h are shown in Fig. 1A–C. It should be noted that direct comparison of the three cell lines is difficult because of their different growth characteristics. The doubling time for the NOAC-resistant HL-60/NOAC cells was 41 h, whereas the HL-60 cells and the HL60/AraC-resistant cells had doubling times of 30 h and 22 h respectively. The 50% growth-inhibitory concentration (IC_{50}) for NOAC was 7.7 ± 1.8 μM in HL-60 cells, whereas the corresponding values in the resistant cell lines were 16.5 ± 2.9 μM for HL-60/AraC and 36.2 ± 4.2 μM for HL-60/NOAC cells. With AraC the IC_{50} concentrations were not reached below 200 μM drug and continuous 24 h incubation in all three cell lines. Cell viability at 200 μM AraC was 59.3% ± 2.1% in HL-60 cells, which was almost identical to the level reached at 5 μM drug concentration. The toxicity of AraC at 200 μM in the resistant cell lines was only marginal, with cell viabilities of 94% and 99% in HL-60/AraC and HL-60/NOAC cells respectively. The cytotoxic effects of NOAC-AraC were substantially lower than those of NOAC in all cells, suggesting that NOAC-AraC might not be cleaved rapidly in the cells. The IC_{50} value of NOAC-AraC in HL-60 cells was 49.3 ± 4.3 μM, in the HL-60/AraC cells 81.5 ± 5.6 μM and in the HL-60/NOAC cells 103.1 ± 6.8 μM.

Phosphorylation of N^4-alkyl-AraC derivatives by dC kinase

A phosphorus-transfer assay was used to investigate whether the different N^4-alkyl-AraC derivatives are substrates for the human dC kinase, which is known to have a broad substrate specificity for nucleosides (Karlsson et al. 1994). In Table 1 the reaction rates for the phosphorylation of AraC and N^4-alkyl-AraC derivatives are summarized. All AraC derivatives had reaction rates below 3% of those of AraC, demonstrating that the N^4-alkyl-AraC derivatives with alkyl chains of C_6–C_{18} (NOAC) are not acting as substrates of dC kinase.

Table 1 Phosphorylation of cytosine arabinoside (*AraC*) and different N^4-alkyl-AraC derivatives by recombinant human dC kinase. The assays were repeated three times with less than 20% variation. Deoxycytidine had a reaction rate of 27 900 pmol · min^{-1} mg^{-1} in the same assay

Drug	Reaction rate (pmol min^{-1} mg^{-1})	Relative reaction rate compared to AraC (%)
AraC	36 000	100
N^4-Hexyl-AraC	540	1.5
N^4-Octyl-AraC	1080	3.0
N^4-Dodecyl-AraC	900	2.5
N^4-Tetradecyl-AraC	1080	3.0
N^4-Hexadecyl-AraC	180	0.5
N^4-Octadecyl-AraC	540	1.5

Table 2 In vitro hydrolysis of arabinocytidylyl-(5′→5′)-N^4-octadecyl-1-β-D-arabinofuranosylcytosine (*NOAC-AraC*) by phosphodiesterase I and human serum. The time shown is that required for full hydrolysis. *pAraC* cytosine arabinoside monophosphate

Dimer	Phosphodiesterase I		Human serum	
	Hydrolysis (h)	Metabolites	Hydrolysis (h)	Metabolites
dT-dT	2	dT, pdT	5	dT
NOAC-AraC	4	pAraC, NOAC	8.5	AraC, NOAC

In vitro hydrolysis of NOAC-AraC by phosphodiesterase I and human serum

As summarized in Table 2 the dimer NOAC-AraC is hydrolyzed in vitro by phosphodiesterase I to AraC monophosphate and by serum to AraC. Thus, the cytotoxic compounds AraC, AraC monophosphate and NOAC can be released from the dimer. However, it remains to be investigated whether NOAC-AraC is taken up by cells in its unchanged form and whether the dimers are degraded intracellularly to yield active metabolites such as NOAC, AraC or their monophosphorylated metabolites.

Cell-cycle distribution analysis

In Table 3 the changes in cell-cycle distribution of HL-60 and HL-60/AraC cells after 24 h incubation with

Table 3 Cell-cycle distribution in HL-60 and HL-60/AraC cells after incubation with AraC, NOAC or NOAC-AraC for 24 h at increasing drug concentrations. The cell-cycle distribution was determined by the BrdUrd/propidium-iodine method. The S_0 cell fraction is described in the text. Results show the means of cell-cycle fractions (%). The standard deviation was below 10% of the mean values. *NA* not available

Drug (μM)	Distribution in HL-60 cells (%)				Distribution in HL-60/AraC cells (%)			
	G1	S	S_0	G2/M	G1	S	S_0	G2/M
AraC								
0	48.4	41.4	0.7	9.5	22.4	62.4	1.9	13.3
1	74.4	13.1	5.9	6.6	23.1	62.3	1.7	12.9
5	82.2	5.1	6.5	7.2	23.2	64.0	1.9	10.9
50	85.0	3.2	6.7	5.1	20.7	59.4	2.9	17.0
NOAC								
0	48.4	41.4	0.7	9.5	22.4	62.4	1.9	13.3
1	47.2	44.2	0.5	8.1	23.2	60.3	1.7	14.8
10	47.0	43.2	0.9	8.9	27.0	57.3	1.2	14.5
20	68.9	22.6	3.2	5.2	35.8	51.0	1.0	12.2
50	NA	NA	NA	NA	66.4	21.5	1.9	10.2
NOAC-AraC								
0	48.4	41.4	0.7	9.5	22.4	62.4	1.9	13.3
1	24.1	70.1	0.9	4.8	20.9	62.7	1.8	14.6
10	26.4	66.8	1.4	5.4	20.8	62.7	1.6	14.9
20	35.6	55.0	2.4	7.0	20.4	64.0	1.2	14.4
50	55.4	29.0	5.2	10.4	26.3	61.5	1.1	11.1
100	69.8	14.6	7.0	8.6	54.0	37.5	0.5	8.0

increasing concentrations of AraC, NOAC or NOAC-AraC are summarized. As expected, AraC led to a typical reduction of S-phase cells by inhibition of DNA polymerase α even at 1 μM drug concentration. This inhibition prevents the incorporation of BrdUrd into DNA and leads, therefore, to a shift in the cell-cycle distribution pattern from early S-phase cells to G1-phase cells, from middle S-phase cells to S_0-cells and late S-phase cells to G_2/M-phase cells. S_0-phase cells are defined as cells that, because of their DNA content, belong to the middle S-phase fraction, but do not incorporate BrdUrd into DNA owing to inhibition of DNA polymerase by AraC triphosphate (Preisler et al. 1992). NOAC did not alter cell-cycle distribution significantly at concentrations up to 10 μM, suggesting that NOAC is, as previously described for NHAC, not limited to S-phase in its cytotoxicity (Horber et al. 1995c). However, at higher concentrations, NOAC caused substantial reduction of S-phase cells in HL-60 as well as in the AraC-resistant HL-60/AraC cells, suggesting that, at higher concentrations, NOAC has an increased S-phase-specific cytotoxicity. HL-60 cells treated with NOAC-AraC showed dramatically altered cell-cycle distribution patterns. At low drug concentrations, the fraction in G1-phase decreased to 50% of the untreated control, whereas the S-phase cells increased likewise. At higher drug concentrations, however, NOAC-AraC became more S-phase-specific, similar to the effects found with NOAC. In the HL-60/AraC cells no effect on G1-phase cells was observed, whereas the increasing S-phase specificity at higher drug concentrations was preserved.

Quantification of the apoptotic cell fraction

The quantitative determination of the apoptotic cell fractions in the three different HL-60 cell lines after incubation with AraC, NOAC or NOAC-AraC are summarized in Fig. 2A–C. As a positive control for the induction of apoptosis, the topoisomerase I inhibitor camptothecin was used, which is known to be an excellent inducer of apoptosis in HL-60 cells (Gong et al. 1993). Camptothecin induced apoptosis in all three HL-60 cell lines to more than 73% after 24 h incubation. Furthermore, no cross-resistance between camptothecin and either AraC or NOAC was observed in the resistant HL-60/AraC and HL-60/NOAC cells, respectively. AraC was able to induce apoptosis in 44.9 ± 1.5% of the HL-60 cells at 5 μM; however, higher concentrations had no further effect, suggesting that the 45% of the cell population represents the S-phase cells. NOAC induced apoptosis in more than 80% of the cells at higher drug concentrations, again indicating that its mechanisms of cytotoxicty might not be limited to S-phase cells. NOAC also preserved its excellent apoptosis-inducing capability in HL-60/AraC cells, whereas in the NOAC-resistant HL-60/NOAC cell line higher NOAC concentrations were needed. The results obtained with the HL-60/AraC cells suggest that NOAC does not exert notable cross-resistance with AraC, even though the IC_{50} concentration for NOAC in the cytotoxicity assay increased from HL-60 cells to HL-60/AraC cells (cf. Fig. 1). NOAC-AraC was a weak inducer of apoptosis in HL-60 cells in the concentration range tested. In the resistant HL-60/AraC and HL-60/NOAC cells, the dimer had no detectable effect up to 50 μM (Fig. 2B, C). In addition, the effects of the drugs on the activity of caspase-3 with the different HL-60 cell lines were investigated. As with the terminal deoxynucleotidyltransferase assay, the results in the caspase-3 assay were consistent. Only NOAC was able to increase the caspase-3 activity by a factor 6 or more, whereas AraC and NOAC-AraC had only weak inducing activities. Furthermore, AraC and NOAC-AraC were not able substantially to increase the caspase-3 activities in HL-60/AraC- or HL-60/NOAC-

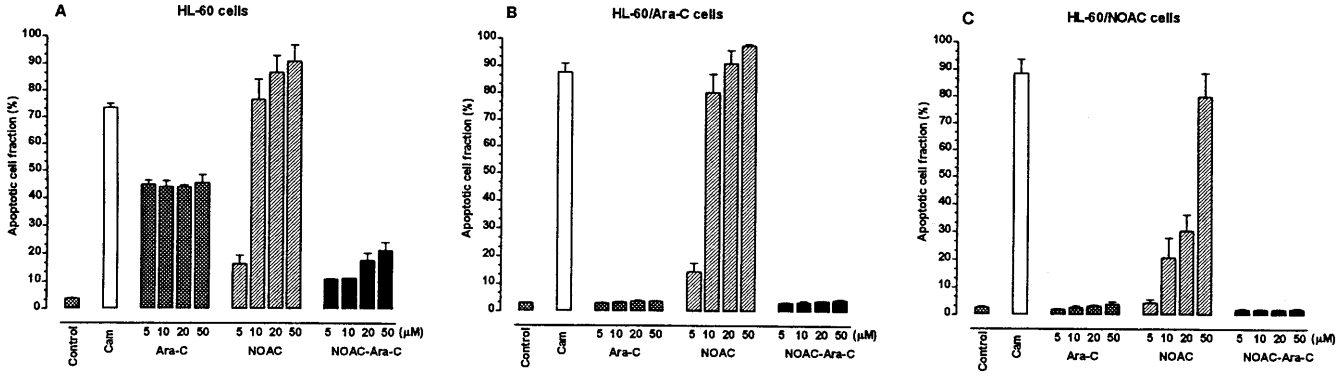

Fig. 2A–C Percentage of apoptotic cells in the terminal deoxynucleotidyltransferase assays after 24 h incubation with increasing drug concentrations (μM) of AraC, NOAC or NOAC-AraC in HL-60 (**A**), HL-60/AraC-resistant (**B**) and HL-60/NOAC-resistant cells (**C**) *Bars* represent mean and standard deviations ($n = 4$). Camptothecin (*Cam*) was used as positive control

resistant cells up to 50 μM drug, whereas NOAC preserved its effects (data not shown).

Cytotoxicity, cellular uptake and effect of NHAC on P-170 glycoprotein expression in KB cells

In an additional set of experiments, we investigated the properties of NHAC on doxorubicin-resistant epidermoid carcinoma KB cells. As summarized in Table 4, NHAC was cytotoxic in the doxorubicin-sensitive KB-3-1 and the resistant KB-Ch^R-8-5 cells, whereas neither, AraC nor any of the drugs was active in the highly resistant KB-V1 cells. NHAC was active in KB-NHAC cells, however, probably because of drug-induced resistance requiring a three-times-higher IC_{50} concentration. With increasing grade of doxorubicin resistance, less NHAC uptake was measured, resulting in reductions of 32% in KB-Ch^R-8-5 cells and 77% in KB-V1 cells. The MDR1 modulator verapamil did not significantly change the cellular concentrations of NHAC in the KB-3-1 and KB-V1 cells, suggesting that NHAC is not affected by the P-170 efflux pump. Whereas the P-170 expression levels increased in the doxorubicin-resistant KB cells (Table 5), the NHAC-resistant cells did not upregulate P-170 expression during NHAC incubation over 134 days.

The reduced levels of P-170 expression observed after 114 and 143 days in the KB-V1 cells may be due to fluctuations of the concentrations of vincristine during cell culture. Thus, these results indicate that NHAC does not induce MDR1.

Table 5 Expression levels of P-170 glycoprotein in KB cells. The fluorescence index was calculated as the mean fluorescence of MRK16-stained KB-ChR-8-5, KB-V1 and KB-NHAC cells with the parent cells KB-3-1. *ND* not determined

	Fluorescence index after culture for:			
Cells	2 days	50 days	114 days	134 days
KB-3-1	1.05	1.05	1.08	0.96
KB-Ch^R-8-5	23	22	ND	ND
KB-V1	183	ND	128	117
KB-NHAC	ND[a]	0.97[b]	1.01[c]	1.03[d]

[a–d] Long term incubation of KB-3-1 cells with [a] 10 μM NHAC, [b] 50 μM NHAC, [c] 100 μM NHAC, [d] 200 μM NHAC

In vivo antileukemic efficacy of NOAC

A newly established human ALL xenotransplanted to NOD/SCID mice was used to evaluate the potential antileukemic effect of NOAC in comparison to standard agents. As summarized in Table 6, liposomal NOAC, after i.p. or oral administration, induced the prevention of leukemia development without causing severe side-effects, whereas an i.v. application of 25 mg NOAC/kg was lethal in 50% of the animals. Thus, i.p. application of liposomal NOAC achieved the prevention of peritoneal tumors and ascites in the ALL-SCID-3 model with eightfold lower drug concentrations than needed with AraC.

Table 4 Cytotoxicity and cellular uptake of NHAC in KB cells. Uptake was measured after incubation of the cells with 40 μM ^{3}H-labeled NHAC for 24 h at 37 °C and 5% CO_2. The verapamil (10 μM) was added to the cells 10 min before NHAC

Drugs	KB-3-1	KB-Ch^R-8-5	KB-V1	KB-NHAC
IC_{50} concentration (μM)				
Doxorubicin	<1	3.8 ± 0.5	>500	<1
NHAC	157 ± 27	294 ± 34	>500	459 ± 9
AraC	>500	>500	>500	>500
Uptake (nmol/10^6 cells)				
NHAC	20.5 ± 1.8	13.9 ± 2.7	4.7 ± 0.3	16.7 ± 0.4
NHAC/verapamil	18.7 ± 1.8	18.8 ± 0.9	5.5 ± 0.1	12.5 ± 0.5

Table 6 Antileukemic efficacy of NOAC administered as a liposomal formulation in the xenotransplanted ALL-SCID-3 leukemia model. Leukemia cells (10^6/mouse) were inoculated i.p. on day 0. Treatment started on day 1. Body weight change was determined between days 1 and 4 to 7 days later. Moribund mice were sacrificed and the amounts of solid tumor nodules and ascites were determined. The average tumor burden is given as the total volume of peritoneal ascites fluid and the total mass of solid tumor nodules

Expt.	No. of mice	Substance	Route	Dose/injection		Schedule (days)	Toxic deaths	Body weight change (%)	Peritoreal tumors	Average tumor burden (ml or g/mouse)
				(mg/kg)	(μmol/kg)					
1	7	Saline	i.p.	–	–	1, 4, 7, 11	–	1	6/7	1.8 ± 1.16
	8	AraC	i.p.	100	411	1, 4, 7, 11	0	−2	0/8	0
	8	NOAC	i.p.	100	200	1, 4, 7, 11	0	−2	0/8	0
	7	Vincristine	i.p.	1	1.2	1	0	−2	0/7	0
2	8	Saline	i.p.	–	–	1, 4, 7, 11	–	4	8/8	2.92 ± 1.69
	8	NOAC	i.p.	50	100	1, 4, 7, 11	0	0	0/8	0
	8	NOAC	i.p.	25	50	1, 4, 7, 11	0	0	0/8	0
	8	NOAC	Oral	100	200	1, 4, 7, 11	0	−2	0/8	0
3	6	NOAC	i.v.	25	50	1, 4, 7	3/6	−10	0/3	0

Discussion

Previous investigations in our laboratory have shown that cytotoxic effects and mechanisms of action of the N^4-alkyl-AraC derivative NHAC are significantly different from those of AraC. In the present work, we focussed on NOAC, which was shown to exert the strongest antitumor activity of all N^4-alkyl-AraC derivatives synthesized (Schwendener et al. 1995a). Furthermore, we characterized the cytotoxicity and apoptosis-inducing capability of the new dimer NOAC-AraC. For analysis of the mechanisms of action of the N^4-alkyl-AraC derivatives and for a better characterisation of NOAC, a NOAC-resistant HL-60 cell line was established. NHAC and NOAC are highly lipophilic, because of their long alkyl side-chains. Therefore, these two AraC derivatives are practically insoluble in aqueous solutions. In our previous studies, the two compounds were used as a liposomal preparation. However, we observed that, in most in vitro assays, the lipids used to prepare the liposomes had a direct influence on the cytotoxicity of the drugs, because of altered drug distribution and uptake. To circumvent these effects in this study, NOAC was dissolved in ethanol. NOAC was found to be highly cytotoxic in the short-exposure cytotoxicity assay. Furthermore, NOAC was able to overcome AraC resistance in HL-60/AraC cells. In HL-60/NOAC-resistant cells, higher concentrations of the drug were needed, corresponding to the levels of drug-induced resistance. However, cells not responding to treatment were not observed in the HL-60/NOAC cell line, suggesting that the resistance is only qualitative and not absolute. Compared to NHAC, the IC_{50} values for NOAC were reduced by a factor of six from 47 μM for NHAC to 7.7 μM in HL-60 cells (Horber et al. 1995a). HL-60/NOAC cells were found to be cross-resistant to AraC, NOAC and NOAC-AraC. The cytotoxicity of NOAC-AraC was substantially lower in all HL-60 cell lines. Therefore we assume that, in vitro, NOAC-AraC is cleaved slowly in the HL-60 cells to release NOAC, AraC or the corresponding monophospates of the drugs (cf. Table 2).

The dC kinase assay revealed that the N^4-alkyl-AraC derivatives do not serve as substrates for the human enzyme. Furthermore, we found that NHAC was cytotoxic in dC-kinase-deficient CEM/dCK$^-$ cells (IC_{50} = 56 μM), whereas AraC was ineffective (data not shown). Thus, NHAC retains its activity in dC-kinase-deficient cells, suggesting a mechanism that is independent of the enzyme. Consequently, the N^4-alkyl-AraC derivatives NHAC and NOAC can not be phosphorylated directly and are not able to exert their cytotoxicity, as does AraC, by interaction with DNA synthesis. On the other hand, we presume that, in vitro, only small amounts of the N^4-alkyl-AraC derivatives can be cleaved to AraC, which has been shown for NHAC (Horber et al. 1995a). This indicates that the derivatives are not prodrugs of AraC, but that they exert their cytotoxicity by yet unknown mechanisms of action.

The influence of NOAC on cell-cycle distribution of the HL-60 cell lines tested revealed that, at low drug concentrations, NOAC interacts with the cells in a phase-unspecific manner, whereas at higher concentrations the drug becomes more S-phase-specific. Again, this effect can not be explained by metabolic transformation of NOAC to AraC, resulting in the latter's typical S-phase-specific cytotoxicity, because the effect was also observed in the AraC-resistant HL-60/AraC cells (cf. Table 3). Therefore, it is more likely that the S-phase-specific cytotoxicity of NOAC at higher drug concentrations is an effect of NOAC itself or of yet unidentified active metabolites.

The apoptosis-inducing capability of AraC, NOAC and NOAC-AraC in HL-60 cells was analyzed with the terminal deoxynucleotidyltransferase assay. AraC was able to induce apoptosis only in the S-phase population of the HL-60 cells, whereas it was ineffective in the resistant HL-60/AraC and HL-60/NOAC cells. NOAC preserved its apoptosis-inducing capability in the resistant cells also, but at higher drug concentrations. Since it has been shown that apoptosis can also occur in the

absence of internucleosomal DNA cleavage, the apoptosis-inducing capability of NOAC and NOAC-AraC was further tested by measurement of caspase-3 activity and the results were consistent with those of the terminal deoxynucleotidyltransferase assay, demonstrating that NOAC exerted a concentration-dependent effect on caspase-3 activation, whereas NOAC-AraC was less active. Finally, the experiments with the doxorubicin resistant KB cells, using NHAC as a representative of the N^4-alkyl-AraC derivatives, revealed that NHAC does not induce MDR1 resistance and that the lipophilic AraC derivatives seem not to be affected by the P-170 efflux mechanism.

Finally, the in vivo efficacy of NOAC, as demonstrated in the xenotransplanted human ALL-SCID-3 model, is promising and justifies additional experiments to characterize further the dependence of NOAC on dose and schedule as well as studies in AraC-resistant ALL models.

In conclusion, the N^4-alkyl-AraC derivatives, particularly NOAC and NHAC, are promising new anticancer drugs with high cytotoxicity and strong apoptosis-inducing capability. The mechanisms of action seem to be different from those of AraC, and the drugs ability to overcome AraC as well as MDR1 resistance is an important finding. Different studies on NOAC, dealing with interactions with blood components, pharmacokinetics and metabolism, further characterize the favorable properties of NOAC as a new antitumor drug for the treatment of AraC-resistant leukemias and other drug-resistant malignancies (Koller-Lucae et al. 1997, 1999; Rentsch et al. 1997). In an ongoing experimental phase I study with liposomal NOAC, the plasma elimination half-life after a 1-h i.v. infusion of liposomal NOAC into cancer patients ranged between 11 h and 16 h with peak plasma levels of 10–20 μM at 150 mg NOAC/m^2, 16–40 μM at 300 mg NOAC/m^2 and 180–240 μM at 600 mg NOAC/m^2 without untoward toxic effects, warranting further clinical development of this new drug.

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