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ORIGINAL ARTICLE

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Cellular pharmacology of N^4-hexadecyl-1-β-D-arabinofuranosylcytosine in the human leukemic cell lines K-562 and U-937

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Abstract The mechanisms of cytotoxicity, cellular drug uptake, intracellular drug distribution, cellular pharmacokinetics, formation of arabinofuranosylcytosine triphosphate (ara-CTP), and DNA incorporation of N^4-hexadecyl-1-β-D-arabinofuranosylcytosine (NHAC), a new lipophilic derivative of arabinofuranosylcytosine (ara-C) formulated in small unilamellar liposomes, were determined in vitro in the human leukemic cell lines K-562 and U-937. Furthermore, the induction of erythroid differentiation by NHAC was tested in K-562 cells. The cytotoxicity of NHAC in both cell lines was not influenced by the deoxycytidine (dCyd) concentration or the presence of the nucleoside-transport-blocking agent dipyridamole as demonstrated in coincubations with dCyd and/or dipyridamole, whereas in contrast, the cytotoxicity of ara-C was decreased additively by both drugs. As compared with ara-C, the uptake of NHAC displayed up to 16- and 5-fold increases in K-562 and U-937 cells, respectively, depending on the drug concentration. Studies of the drug distribution and pharmacokinetics of NHAC revealed a depot effect for NHAC in the cell membranes, resulting in half-lives 2.6 and 1.4 times longer than those of ara-C in the two cell lines. The ara-CTP concentrations derived from NHAC were 150- and 75-fold lower at a drug concentration of 1 μ*M* in K-562 and U-937 cells, respectively. The DNA incorporation of the drugs observed after incubation with 2 μ*M* NHAC was 60- and 30-fold lower as compared with that seen at 2 μ*M* ara-C in the two cell lines. Furthermore, NHAC was capable of inducing irreversible erythroid differentiation to a maximum of only 22% of K-562 cells, whereas ara-C induced differentiation at a drug concentration 100-fold lower in 50% of the cells. These results indicate a mechanism of cytotoxicity for NHAC that is independent of the nucleoside transport mechanism and the phosphorylation pathway and suggest that the mechanisms of action of NHAC are significantly different from those of ara-C. Therefore, NHAC might be used for the treatment of ara-C-resistant malignancies.

Key words N^4-Hexadecyl-1-β-D-arabinofuranosylcytosine, NHAC · Cellular pharmacology · Liposomes

Introduction

1-β-D-Arabinofuranosylcytosine (ara-C) is currently the most important agent for the treatment of acute myelogenous leukemia (AML) [13, 24]. However, its therapeutic spectrum is limited to leukemias and is compromised by the frequent development of resistance in tumor cells after repeated ara-C exposure [25, 35]. Thus, about 20% of patients with AML initially treated with conventional or high-dose ara-C exhibit primary resistance to the drug [8] and about one-half of previously treated but relapsed patients are resistant to induction chemotherapy regimens including ara-C [16]. As possible mechanisms for ara-C resistance, low deoxycytidine kinase activity [41, 49], increased metabolism by cytidine deaminase to inactivate ara-C to 1-β-D-arabinofuranosyluracil (ara-U) [50] and a decreased membrane transport of ara-C due to low numbers of nucleoside transport sites [54, 55], among other reasons, have been described.

Different strategies have been followed to overcome ara-C resistance or at least to modulate ara-C pharmacokinetics [39]. To inhibit the drug's rapid deamination to ara-U, combination therapies with deaminase

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inhibitors such as tetrahydrouridine [28, 33] or zebularine [12, 31] have been carried out and new, more deaminase-resistant ara-C derivatives such as 5-azacytidine [1, 15] or N^4-behenoyl-ara-C [52] have been synthesized. Furthermore, the deoxycytidine kinase activity was modulated by alteration of the natural deoxynucleotide levels. The initial phosphorylation to ara-C monophosphate (ara-CMP) by deoxycytidine (dCyd) kinase is the rate-limiting step in the activation of ara-C. DCyd is the physiological substrate of dCyd kinase but is also a competitive inhibitor of ara-C phosphorylation [11]. Therefore, different investigators have tried to decrease deoxycytidine-triphosphate (dCTP) pools by combination therapies of ara-C either with nucleotide synthetase inhibitors such as 3-deazauridine [4] or *N*-(phosphonacetyl)-L-aspartate [17] or with ribonucleotide reductase inhibitors such as hydroxyurea [44], thymidine [7], or fludarabine [14, 40].

To alter the pharmacokinetic behavior and to avoid or delay the fast deamination of ara-C in vivo, we synthesized the lipophilic N^4-alkyl-ara-C derivative N^4-hexadecyl-1-β-D-arabinofuranosylcytosine (NHAC) [46]. In a previous study we have demonstrated that NHAC is highly active in the L1210 mouse tumor model, reaching the same tumor-inhibitory effects at molar drug concentrations 16-fold lower than those of ara-C [46]. Even at single-dose schedules, NHAC exerted strong cytotoxic activity, suggesting a long-lasting drug effect. In addition, NHAC was shown to be almost fully protected against deamination in human plasma and to be significantly more stable than ara-C or corresponding N^4-acyl-ara-C derivatives against deamination in mouse liver microsomes [21, 22].

In the present study we investigated the cellular pharmacology of NHAC as compared with ara-C by studying the cytotoxicity, cellular uptake, capacity to induce differentiation in K-562 cells, intracellular drug distribution, cellular pharmacokinetics, and rate of ara-CTP formation in the human leukemic cell lines K-562 and U-937.

Materials and methods

Drugs

Ara-C, dipyridamole, and 2′-deoxycytidine (dCyd) were purchased from Sigma Chemical Inc. (Buchs, Switzerland). [5-^{3}H]-Ara-C (30 Ci/mmol) and custom-synthesized [5-^{3}H]-NHAC (5.1 Ci/mmol) were obtained from Amersham Int. (Amersham, UK). For all incubations, ara-C was dissolved in phosphate-buffered saline (PBS; 8 m*M* Na_2PO_4, 1.5 m*M* KH_2PO_4, 0.14 *M* NaCl, 2.6 m*M* KCl) with trace amounts of [5-^{3}H]-ara-C. NHAC was given in a liposomal formulation as described below. NHAC was synthesized as previously described [46]. The structural riboside analogue of NHAC, N^4-hexadecyl-1-β-D-ribofuranosylcytosine (NHRC), was synthesized as described by Schott et al. [45]. Tetrahydrouridine was a gift from the Drug Development Branch of the National Cancer Institute (Bethesda, Md., USA).

Cells

K-562 human chronic myelogenous leukemia cells and U-937 human histiocytic lymphoma cells were obtained from the American Type Tissue Culture Collection (ATCC CCL 243 and CRL 1593, respectively). Cells were grown in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS; PAA-Biologics, Linz, Austria), 100 units penicillin/ml, and 100 μg streptomycin/ml in a humidified atmosphere containing 5% CO_2.

Liposome preparation

Small unilamellar liposomes with a mean diameter of 100 ± 30 nm were prepared by filter extrusion as described elsewhere [20]. Briefly, lipid mixtures composed of soybean phosphatidylcholine (SPC), cholesterol, D,L-α-tocopherol, and NHAC or NHRC at a molar ratio of 1:0.2:0.01:0.1 were hydrated with PBS and sequentially filtrated through Nuclepore (Costar; Sterico AG, Dietikon, Switzerland) filters of decreasing pore size (1 μm, 400 nm, 100 nm). Liposomes without NHAC, termed empty liposomes, were used as controls. All preparations (20 mg SPC/ml) were sterilel filtered through 0.2-μm filters (Acrodisc; Gelman Sciences, Ann Arbor, Mich.) and stored at 4°C. Trace amounts of [5-^{3}H]-NHAC were added for detection and quantification.

Cytotoxicity assay: MTT test

Cells were counted and seeded in 96-well plates (3×10^5 cells/ml). Then the cells were exposed to various concentrations (0–400 μ*M*) of ara-C, NHAC, or empty liposomes in the presence or absence of 20 μ*M* dCyd and/or 20 μ*M* dipyridamole for 24 h at 37°C (5% CO_2). Dipyridamole and/or dCyd were added 5 min prior to drug exposure. The cytotoxicity of NHRC was tested only in K-562 cells without prior treatment. After incubation the medium was removed and the cells were washed once and resuspended in fresh, serum-free RPMI 1640 medium. The cell survival fractions were determined with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye-reduction assay as described by Mosmann [37]. All experiments were repeated at least four times.

Clonogenic assay

Cells (2×10^5 cells/well) were exposed to various concentrations (0–200 μ*M*) of ara-C or NHAC for 24 h at 37°C (5% CO_2). After being washed twice with PBS, the cells (2×10^3 cells/well) were plated in 2 ml minimal essential medium (MEM; Gibco, Paisley, Scotland) containing 20% FCS and 0.3% agar (Gibco). Cultures were incubated for 7–14 days at 37°C (5% CO_2). Colonies (≥ 50 cells) were scored using an inverted microscope at a 30 × magnification. The plating efficiency was 47% for K-562 cells and 15% for U-937 cells. All experiments were repeated four times.

Cellular drug uptake

Cells (2×10^6 cells/well) were incubated in 24-well plates with either increasing concentrations of up to 200 μ*M* [5-^{3}H]-ara-C or [5-^{3}H]-NHAC (2 μCi/sample) in the presence or absence of the nucleoside-transport-blocking agent dipyridamole (20 μ*M*) for 3 h at 37°C (5% CO_2) or for increasing periods of up to 24 h with 2 μ*M* [5-^{3}H]-ara-C or [5-^{3}H]-NHAC at 37°C (5% CO_2). Dipyridamole was added 5 min prior to drug exposure. After two washes with cold

PBS, total drug uptake was determined by scintillation counting. All experiments were performed in triplicate.

Cellular drug distribution

Cells (1.5×10^7 cells/well) were incubated in 6-well plates with 2 μM [5-^{3}H]-ara-C or [5-^{3}H]-NHAC (4 μCi/sample) for 24 h at 37°C (5% CO_2). After being washed twice with cold PBS, the cells were resuspended in 5 mM TRIS-HCl (pH 7.6). Cells were lysed by nitrogen gas cavitation at 40 bar for 10 min [10]. Fractions of the homogenates were obtained as described by Tsuruo et al. [51] with minor modifications. Briefly, homogenates were centrifuged at 1,000 g for 10 min, at 10,000 g for 15 min, at 27,000 g for 30 min, and at 105,000 g for 60 min to sediment crude nuclei and membranes, mitochondria, lysosomes, and microsomes, respectively. Each subcellular fraction was washed once. The supernatant of the 105,000-g fraction was designated as the cytosol fraction. The drug concentration in each fraction was determined by scintillation counting without further analysis of drug metabolism or distribution of drug metabolites except in the cytosol fraction obtained after NHAC incubation. These cytosol fractions were analyzed as described below. All experiments were performed in triplicate.

Induction of erythroid differentiation in K-562 cells

K-562 cells (2×10^5 cells/ml) were incubated with different concentrations (0.05–50 μM) of ara-C and NHAC for 4 days at 37°C (5% CO_2). After incubation with either drug or without drug, cells were washed once with cold PBS and resuspended in fresh medium. Cell viability was determined by trypan blue exclusion. The number of differentiated hemoglobin-containing cells was scored by benzidine staining [42]. Briefly, 5 µl of 30% hydrogen peroxide was added to 1 ml of a benzidine dihydrochloride solution (2 mg/ml in 0.9% NaCl plus 0.5% acetic acid). Equal volumes of the benzidine solution and the cell suspension were mixed and incubated for 5 min at room temperature and the cells were scored in a hemocytometer as benzidine-positive (blue) or benzidine-negative (yellow). All experiments were performed in triplicate.

Cellular pharmacokinetics

Cells (5×10^6 cells/well) were incubated with 2 μM [5-^{3}H]-ara-C or [5-^{3}H]-NHAC (2 μCi/sample) for 2 h at 37°C (5% CO_2). Cells were washed twice with cold PBS to remove unbound drug. The incubations were continued in RPMI medium and stopped after different periods of up to 210 min to determine total drug uptake and ara-CTP formation (as described). All experiments were performed in triplicate.

Cellular ara-CTP formation

Cells (5×10^6 cells/well) were incubated with increasing concentrations of [5-^{3}H]-ara-C or [5-^{3}H]-NHAC (2 μCi/sample) for 2 h at 37°C (5% CO_2). Time-dependent ara-CTP formation was determined with 2 μM drug concentration for up to 24 h. After being washed twice with cold PBS, the cells were lysed with 0.4 M perchloric acid and centrifuged after 10 min (10,000 g for 2 min). The supernatants were collected, neutralized with 10 M potassium hydroxide, and centrifuged (12,000 g for 10 min). The resulting supernatant was analyzed for ara-CTP by ion-exchange high-performance liquid chromatography (HPLC) using a Spherisorb SAX column (Phenomenex, Torrance, Calif.) and 125 mM KH_2PO_4 plus 75 mM trisodium citrate (pH 4.6) as the elution buffer at a flow rate of 0.45 ml/min. Ara-CTP-containing fractions were pooled and analyzed by scintillation counting. All experiments were performed in triplicate.

DNA incorporation

Cells (1.8×10^7 cells/well) were incubated with 2 μM [5-^{3}H]-ara-C or [5-^{3}H]-NHAC (25 μCi/sample) for different periods at 37°C (5% CO_2). After two washes with cold PBS, DNA was extracted as described by Spriggs et al. [48]. For the quantification of incorporated drug, the DNA was collected by filtration through Whatman GF/C filters (Whatman Int., Maidstone, England). The filters were extensively washed with cold ethanol and the amount of incorporated drug was quantified by scintillation counting [36].

Statistical analysis

The effects of dCyd and/or dipyridamole on the cytotoxicity of ara-C and NHAC as well as the effects of NHAC were compared statistically with those of ara-C using the two-tailed Student's t-test.

Results

Cytotoxicity in the MTT test

The cytotoxicity exerted by different concentrations of ara-C and NHAC in the MTT dye-reduction assay after continuous 24-h drug exposure in K-562 and U-937 cells is shown in Fig. 1. As a negative control, the effect of empty liposomes without NHAC was tested in both cell lines. In K-562 cells (Fig. 1A), NHAC was significantly ($P = 0.001$) more cytotoxic than ara-C in the concentration range of 1–400 μM. The concentration that inhibited cell growth by 50% (IC_{50}) was 78.5 ± 5.8 μM for NHAC, whereas for ara-C the IC_{50} was not reached within a 400-μM drug concentration and a 24-h incubation period. The cell viability at 400 μM ara-C was $69.7\% \pm 4.5\%$ as compared with the untreated control. Empty liposomes were not toxic to K-562 or U-937 cells at lipid concentrations of up to 1.6 mg SPC/ml, corresponding to a concentration of 400 μM NHAC, resulting in cell viabilities of $98.0\% \pm 2.5\%$ and $101.5\% \pm 2.4\%$, respectively. NHRC, the riboside analogue of NHAC, had no cytotoxic activity in K-562 cells (Fig. 1A), suggesting that the arabinose sugar moiety of NHAC plays an important role in the action mechanism of the drug.

In the U-937 cell line (Fig. 1B), ara-C was significantly ($P = 0.0001$) more toxic than NHAC at concentrations below 40 μM, whereas at higher concentrations, NHAC exerted superior cytotoxicity ($P = 0.0005$). The IC_{50} values were 5.9 ± 0.8 and 28.6 ± 4.0 μM for ara-C and NHAC, respectively, and the cell viabilities at 400 μM were $34.5\% \pm 1.3\%$ and $2.3\% \pm 1.2\%$, respectively.

To elucidate the mechanisms of action of NHAC, cytotoxicity assays of ara-C and NHAC in K-562 and

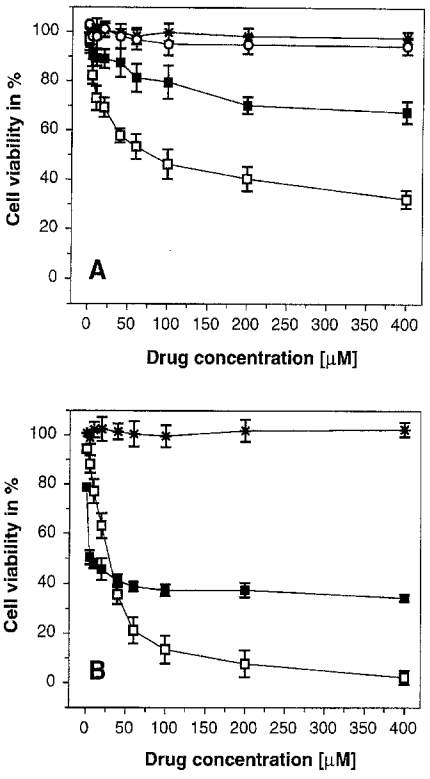

Fig. 1A, B Cytotoxicity assays by MTT dye reduction in K-562 (**A**) and U-937 (**B**) cells. Cells were incubated with ara-C (■), NHAC liposomes (□), NHRC liposomes (○), or empty liposomes (*) for 24 h at 37°C (5% CO_2) at a concentration range of 1–400 μ*M*. The viable cell fraction was quantified spectrophotometrically (540 nm) by measuring the reduction of MTT dye to a blue formazan product. Data points represent mean values ± SD for at least four separate experiments

U-937 cells in combination treatments with dCyd and/or dipyridamole were carried out (Table 1). DCyd is the natural substrate of dCyd kinase and a competitive inhibitor of ara-C phosphorylation. Dipyridamole, on the other hand, is a potent inhibitor of membrane nucleoside transport [27, 29] and is capable of partially preventing ara-C toxicity by blocking the drug's uptake. In K-562 cells the cytotoxicity of NHAC was not significantly ($P = 0.05$) affected by the addition of dCyd and/or dipyridamole. The IC_{50} value was slightly increased from 78.5 μ*M* for NHAC alone to 86.5 μ*M* for the coincubation of NHAC with dCyd plus dipyridamole. The cytotoxicity of ara-C, in contrast, was significantly decreased by dCyd ($P = 0.001$) or dipyridamole ($P = 0.001$) and completely abolished by dCyd plus dipyridamole ($P = 0.0001$) as determined by measuring the viable cell fraction. In U-937 cells, NHAC cytotoxicity was likewise not altered by the addition of dCyd and/or dipyridamole, whereas the IC_{50} values for ara-C were markedly increased from 5.9 μ*M* for ara-C alone to 54.1 μ*M* ($P = 0.0001$) and 396.2 μ*M* ($P = 0.0001$) for coincubation with dCyd and dipyridamole, respectively.

In the comparison of the cell lines, U-937 cells were found to be much more sensitive than K-562 cells to both drugs. The effects on the inhibition of cytotoxicity by dCyd and dipyridamole were additive for ara-C, whereas NHAC revealed no dependence on the dCyd concentration or on the presence of dipyridamole, indicating a mechanism of action for NHAC that is independent of both the pathway of phosphorylation by dCyd kinase as well as the nucleoside transport mechanism.

Table 1 Cytotoxicity of ara-C and NHAC in K-562 and U-937 cells as determined by MTT dye reduction dependent on the presence or absence of 20 μ*M* dCyd and/or 20 μ*M* dipyridamole[a] (*Dipyr.* Dipyridamole)

	NHAC[b]		Ara-C[c]	
	IC_{50} [μ*M*]	Viability at 400 μ*M* [%]	IC_{50} [μ*M*]	Viability at 400 μ*M* [%]
K-562 cells:				
Untreated	78.5 ± 5.8[d]	34.3 ± 3.7	NR[e]	69.7 ± 4.5
+ dCyd	78.1 ± 3.0	31.8 ± 5.1	NR	93.2 ± 3.3
+ Dipyr.	82.9 ± 4.2	32.6 ± 4.9	NR	89.6 ± 5.0
+ dCyd/Dipyr.	86.5 ± 7.6	37.5 ± 4.8	NR	102.4 ± 4.9
U-937 cells:				
Untreated	28.6 ± 4.0[d]	2.3 ± 1.2	5.9 ± 0.8	34.5 ± 1.3
+ dCyd	29.5 ± 3.2	2.1 ± 1.1	54.1 ± 3.9	41.5 ± 4.7
+ Dipyr.	30.8 ± 3.6	1.3 ± 2.2	396.2 ± 48.7	49.2 ± 5.5
+ dCyd/Dipyr.	26.1 ± 2.9	2.7 ± 2.1	NR	71.9 ± 5.1

[a]dCyd and/or dipyridamole were added 5 min prior to continuous 24-h drug exposure
[b]NHAC in liposomes
[c]Ara-C in PBS solution
[d]Mean value ± SD for four separate experiments
[e]Not reached within a drug concentration range of 1–400 μ*M*

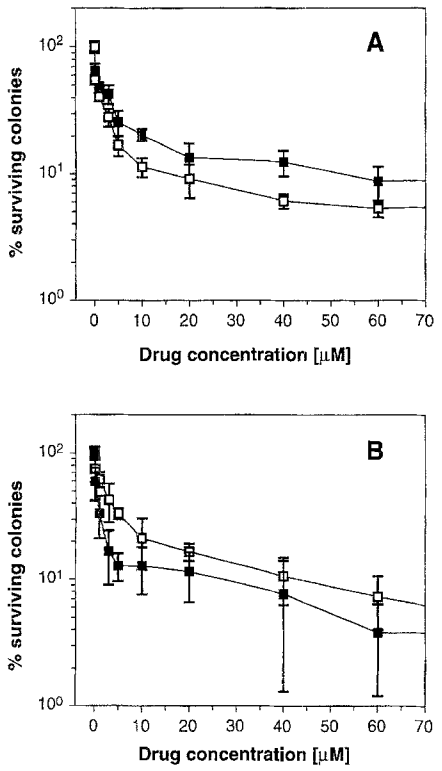

Fig. 2A, B Clonogenic assays in K-562 (**A**) and U-937 (**B**) cells. Cells were incubated with ara-C or NHAC liposomes for 24 h at 37°C (5% CO_2) at a concentration range of 1–200 μ*M*. Clonogenic growth was scored at 7–14 days after plating of 2000 cells/well in MEM medium containing 0.3% agar. Inhibition values obtained at concentrations higher than 60 μ*M* are not shown. ■, Ara-C; □, NHAC liposomes. Data points represent mean values ± SD for four separate experiments

Clonogenic assay

The results of the colony growth of K-562 and U-937 cells after incubation with ara-C or NHAC are presented in Fig. 2. In K-562 cells (Fig. 2A), NHAC was slightly more effective than ara-C in growth inhibition, resulting in an IC_{50} value of 0.40 μ*M* for NHAC as compared with 0.95 μ*M* for ara-C. In U-937 cells (Fig. 2B), in contrast, ara-C was found to be more effective, resulting in IC_{50} values of 0.42 μ*M* for ara-C and 2.21 μ*M* for NHAC.

Drug uptake

The uptake of ara-C and NHAC in K-562 and U-937 cells in the presence and absence of dipyridamole is shown in Figs. 3A and 3B. As compared with ara-C, significantly more NHAC ($P < 0.01$) was incorporated in the two cell lines, independently of the drug concentration. The uptake of NHAC was not blocked by dipyridamole, whereas the ara-C uptake decreased in the presence of 20 μ*M* dipyridamole by factors of 2–9 in

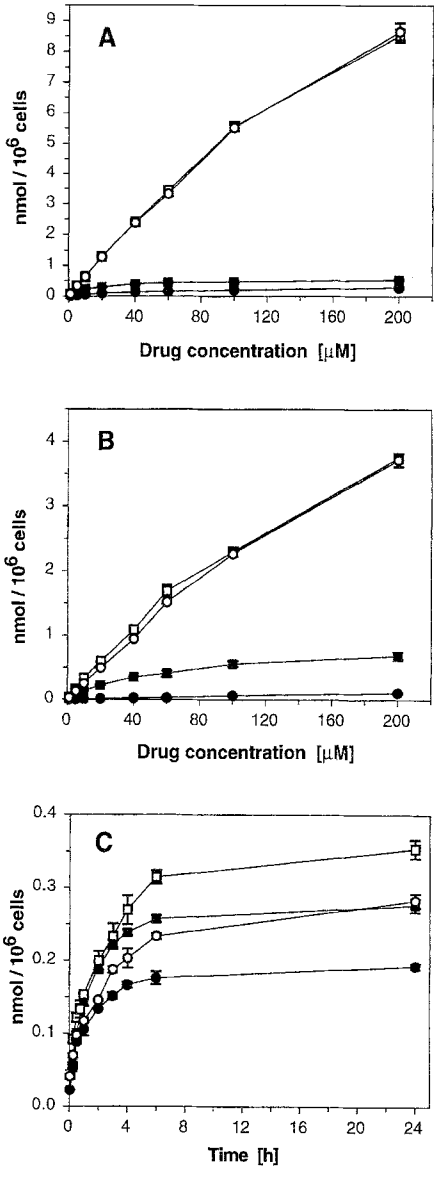

Fig. 3A–C Concentration-dependent uptake of ara-C and NHAC in K-562 (**A**) and U-937 (**B**) cells. Cells were exposed to various concentrations of [5-^{3}H]-ara-C or [5-^{3}H]-NHAC for 3 h at 37°C (5% CO_2) in the presence or absence of the nucleoside-transporter-blocking agent dipyridamole (20 μ*M*). Dipyridamole was added 5 min prior to drug exposure. The drug uptake was determined by scintillation counting. ■, Ara-C; ●, ara-C plus dipyridamole; □, NHAC; ○, NHAC plus dipyridamole. Time-dependent uptake of ara-C and NHAC in K-562 and U-937 cells (**C**). Cells were exposed for various periods of up to 24 h with 2 μ*M* [5-^{3}H]-ara-C or [5-^{3}H]-NHAC at 37°C (5% CO_2). ●, Ara-C in K-562 cells; ■, ara-C in U-937 cells; ○, NHAC in K-562 cells; □, NHAC in U-937 cells. Data points represent mean values ± SD for three separate experiments. Where no error bars are seen, they are smaller than the size of the symbols

K-562 cells (Fig. 3A) and 6–25 in U-937 cells (Fig. 3B), depending on the drug concentration. These results clearly indicate that the uptake of the lipophilic NHAC is independent of the nucleoside transport mechanism. The time-dependent uptake of ara-C or NHAC at 2 μ*M*

Table 2 Induction of erythroid differentiation of K-562 cells by ara-C and NHAC[a]

Drug	Drug concentration	Viability	Hemoglobin-positive cells
Ara-C[b]	0.05 μ*M*	39.7 ± 5.0[d,e]	49.4% ± 4.4%[e]
	0.5 μ*M*	30.0 ± 2.6	67.7% ± 1.1%
	5 μ*M*	28.5 ± 1.9	71.6% ± 1.6%
	50 μ*M*	27.9 ± 1.8	71.2% ± 1.4%
NHAC[c]	0.05 μ*M*	82.4 ± 4.7	5.4% ± 1.8%
	0.5 μ*M*	73.5 ± 3.5	8.8% ± 2.9%
	5 μ*M*	50.9 ± 5.9	22.1% ± 1.5%
	50 μ*M*	25.0 ± 4.7	6.7% ± 1.5%

[a]Cells were incubated for 4 days at 37°C (5% CO_2). Hemoglobin-positive cells were determined by benzidine staining; 1.5% of untreated cells were hemoglobin-positive
[b]Ara-C in PBS solution
[c]NHAC in liposomes
[d]Viable cells expressed as a percentage of untreated controls
[e]Mean value ± SD for three separate experiments

Table 3 Intracellular distribution in K-562 and U-937 cells after incubation with 2 μ*M* ara-C or NHAC for 24 h at 37°C (5% CO_2)[a]

Drug	Total uptake (pmol/10^6cells)	Nuclei/ Membranes (%)	Mitochondria (%)	Lysosomes (%)	Microsomes (%)	Cytosol (%)
K-562 cells:						
Ara-C[b]	302.9 ± 8.1[d]	1.4 ± 0.3	0.02 ± 0.01	0.04 ± 0.03	0.02 ± 0.01	98.5 ± 0.3
NHAC[c]	354.6 ± 9.5	93.8 ± 0.5	0.1 ± 0.06	1.3 ± 0.2	1.1 ± 0.1	3.6 ± 0.2
U-937 cells:						
Ara-C[b]	229.8 ± 19.7[d]	3.4 ± 0.2	0.07 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	96.4 ± 0.2
NHAC[c]	286.4 ± 16.5	92.1 ± 2.8	4.9 ± 2.6	0.4 ± 0.1	1.1 ± 0.3	1.5 ± 0.1

[a]Results are given as percentages of the initial radioactivity incubated with the cells
[b]Ara-C in PBS solution
[c]NHAC in liposomes
[d]Mean value ± SD for three experiments

concentration without pretreatment is shown in Fig. 3C. In contrast to that of ara-C, the NHAC uptake was not fully saturated after 6 h.

Differentiation in K-562 cells

The induction of irreversible erythroid differentiation of K-562 cells by ara-C and NHAC was investigated by benzidine staining after incubation for 4 days using different drug concentrations (Table 2). In the concentration range studied, NHAC was not capable of inducing erythroid differentiation in more than 22% of the cells, whereas ara-C led to irreversible differentiation of 50% of the cells at a concentration 100-fold lower than that of NHAC. The optimal concentration for induction of differentiation was 5 μ*M* for both drugs. Higher concentrations of NHAC led to significant toxicity (cf. Fig. 1A).

Intracellular drug distribution

In Table 3 the intracellular distribution of ara-C and NHAC in K-562 and U-937 cells after 24-h incubation is summarized. With the exception of the cytosol fractions obtained after NHAC incubation, no other fraction was further analyzed for drug metabolites. As expected, in both cell lines, ara-C was predominantly distributed in the cytosol fraction (98.5% and 96.4%). The lipophilic NHAC was mainly found in the crude nuclei-plus-membrane fraction (93.8% and 92.1%) at even 24 h after the onset of incubation. In the cytosol fraction of the NHAC incubation, ara-CTP was detected by HPLC analysis as the hydrophilic metabolite, whereas the putatively amphiphilic NHAC triphosphate could not be detected in any experiment.

Cellular pharmacokinetics

The intracellular half-lives of ara-C, NHAC, and ara-CTP derived from both drugs were determined in the tumor cells after a 2-h drug exposure followed by incubation of the cells for various periods in drug-free medium (Table 4). Both drugs followed first-order kinetics. The intracellular half-lives of total ara-C were 1.77 and 2.82 h in K-562 and U-937 cells, respectively. The half-lives of NHAC were 2.6 and 1.4 times longer than those of ara-C in these two cell lines, suggesting

Table 4 Intracellular half-lives of ara-C, NHAC, and ara-CTP in K-562 and U-937 cells

Determination	Intracellular half-lives (h)	
	Ara-C[a]	NHAC[b]
K-562 cells:		
Total drug	1.77 ± 0.19[c]	4.63 ± 0.88
Ara-CTP	1.69 ± 0.28	3.93 ± 0.34
U-937 cells:		
Total drug	2.82 ± 0.21[c]	4.03 ± 0.52
Ara-CTP	2.69 ± 0.18	7.61 ± 0.47

[a]Ara-C (2 μ*M*) in PBS solution
[b]NHAC (2 μ*M*) in liposomes
[c]Mean value ± SD for three determinations

a depot effect of the drug in the cell membranes (cf. Table 3). The intracellular half-lives of ara-CTP formed from NHAC were 2.3- and 2.8-fold longer than those of ara-CTP formed from ara-C. The longer half-lives of ara-CTP derived from NHAC might be explained by the long-lasting drug distribution of NHAC in the cell membranes, possibly combined with a very slow dealkylation of the drug to ara-C and subsequent phosphorylation to ara-CTP.

Ara-CTP formation

The concentration-dependent ara-CTP formation from ara-C and NHAC in K-562 and U-937 cells after a 2-h incubation is shown in Fig. 4A. The concentrations of ara-CTP formed from ara-C were 5.2- to 150-fold higher in the K-562 cell line and 2.5- to 75-fold higher in the U-937 cell line as compared with ara-CTP derived from NHAC. In contrast to NHAC, ara-CTP formation from ara-C revealed saturation kinetics, reaching saturation levels at 40 μ*M*. The time-dependent formation of ara-CTP originating from ara-C and NHAC was saturated after 6 h. Ara-CTP levels from ara-C at saturation were 209 ± 6 pmol/10^6 K-562 cells and 72 ± 1 pmol/10^6 U-937 cells, respectively. Ara-CTP formed from NHAC reached values of only 1.7 ± 0.01 pmol/10^6 K-562 cells and 0.7 ± 0.01 pmol/10^6 U-937 cells. Thus, at saturation, ara-CTP formation from NHAC was 100- to 120-fold lower.

Time-dependent DNA incorporation

In Fig. 4B the time-dependent DNA incorporation of the drugs is shown. The DNA incorporation of ara-CTP from ara-C was about 30- and 60-fold higher in U-937 and K-562 cells, respectively, than the incorporation after NHAC exposure. The incorporation of metabolites of ara-C and NHAC showed the same

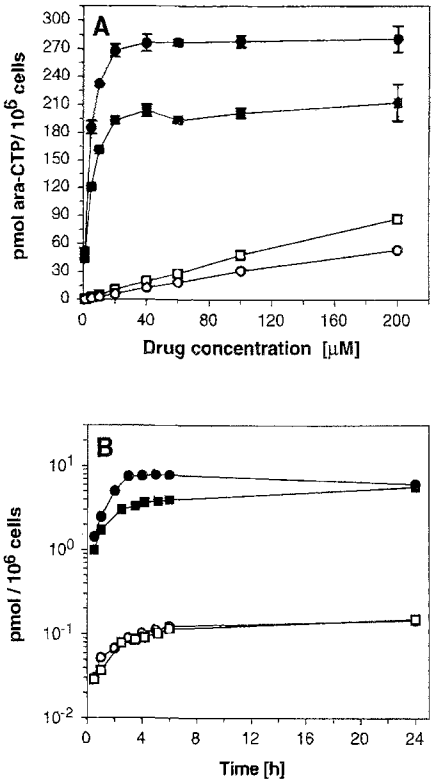

Fig. 4A, B Ara-CTP formation (**A**) in K-562 and U-937 cells after 2-h incubation of various concentrations of [5-^{3}H]-ara-C or [5-^{3}H]-NHAC at 37°C (5% CO_2). Ara-CTP formation was determined by scintillation counting after HPLC separation of perchloric acid supernatants of the cell lysates. Data points represent mean values ± SD for three separate experiments. Where no error bars are seen, they are smaller than the size of the symbols. DNA incorporation (**B**) after incubation with 2 μ*M* [5-^{3}H]-ara-C or [5-^{3}H]-NHAC for different periods at 37°C (5% CO_2) in K-562 and U-937 cells. DNA incorporation was measured by scintillation counting. Data points represent the results of a single determination. ●, Ara-C in K-562 cells; ■, ara-C in U-937 cells; ○, NHAC in K-562 cells; □, NHAC in U-937 cells

kinetics as ara-CTP formation, reaching saturation after 6 h. This result suggests that ara-CTP was the only metabolite of NHAC incorporated into the cellular DNA. Therefore, DNA incorporation does not seem to play a major role in the cytotoxicity mechanisms of NHAC.

Discussion

After the introduction of ara-C as an antineoplastic agent in the early 1960s, it was soon recognized that the major disadvantages of the drug were its very short plasma half-life and its rapid degradation by deamination to the inactive metabolite ara-U [13], which also impedes the oral application of ara-C [9]. To eliminate these disadvantages, attempts have been made at chemical derivatization of ara-C to obtain more stable compounds with improved therapeutic efficacy. The

syntheses of N^4-substituted derivatives of ara-C with short substituents, however, were not very effective [23, 53]. Lipophilic long-chain N^4-acyl derivatives were prepared by Aoshima et al. [2, 3], who found that the compounds with chain lengths of 16–24 C atoms were the most active. Among these compounds, N^4-behenoyl-1-β-D-arabinofuranosylcytosine has been investigated clinically [26]. Other derivatives were prepared by the linkage of ara-C to phospholipids [34] or to steroids [18]. We investigated the antitumor effects of various N^4-acyl-ara-C derivatives that were incorporated into the membranes of small unilamellar liposomes in the L1210 murine leukemia and B16 melanoma models [43]. In comparison with ara-C, the N^4-palmitoyl and N^4-oleyl derivatives were shown to exert superior antitumor effects, especially with delayed treatment schedules, e.g., when given on days 2 and 6 after tumor inoculation.

A later study revealed that the N^4-oleyl-ara-C derivative conferred only marginal protection against deamination [47]. Bearing the properties of the N^4-acyl derivatives in mind, we synthesized lipophilic N^4-alkyl derivatives and demonstrated their improved cytotoxic effects in the L1210 model [46]. The derivative NHAC was shown to be highly resistant against deamination in human plasma [21] and mouse liver microsomes [22], exerting greater cytotoxic effects in the L1210 mouse tumor model than did N^4-oleyl-ara-C [46]. Since NHAC is a structural derivative of ara-C containing a lipophilic alkyl chain at the N^4-position of cytosine, we expected that pharmacological effects similar to those of ara-C or N^4-oleyl-ara-C would also be responsible for the cytotoxic effects of NHAC. Our findings, however, indicate that due to the chemical modification of ara-C into a molecule that displays new physicochemical properties such as high lipophilicity and a very stable alkyl linkage, the mechanisms of action of NHAC seem to be significantly different from those of ara-C and the N^4-acyl derivatives thereof. NHAC was found to exert strong cytotoxic effects in both leukemia cell lines K-562 and U-937 (Figs. 1,2), even in the presence of dCyd and dipyridamole (Table 1) both of which are known to reduce or abolish the effects of ara-C [11, 29]. Surprisingly, the structural analogue of NHAC, NHRC, which contains a ribose sugar instead of the arabinose in NHAC, was found to be completely nontoxic (cf. Fig. 1A), indicating that the alkyl chain moiety has no cytotoxic effect and, even more importantly, that the arabinose moiety seems to be an indispensable requirement for the cytotoxic activity of NHAC. The maintenance of the cytotoxic activity of NHAC in the presence of these compounds suggests that NHAC could preserve its cytotoxic activity in ara-C-resistant tumor cells, which have low numbers of nucleoside transporting sites and whose dCyd kinase activities are weak [41, 49]. NHAC could therefore have potential for the treatment of ara-C-resistant leukemia patients.

In the clonogenic assay, NHAC was shown to exert a cytotoxic effect comparable with that of ara-C. However, as compared with the MTT assay, the clonogenic assay was significantly more sensitive with both cell lines. Thus, ara-C had much greater effects on clonogenic growth inhibition than on short-term cytotoxicity. The effects of ara-C and NHAC in short-term exposures on the cell-cycle distribution have been studied in detail by flow cytometry analysis and the results will be presented elsewhere.

The uptake studies (cf. Fig. 3) and the high concentrations of NHAC found in the cell membranes (Table 3) demonstrate that NHAC is taken up by the cells by mechanisms other than facilitated nucleoside transport and that its binding to cell membranes is nonsaturable at concentrations of up to 400 μ*M*. This renders the existence of a specific binding mechanism for NHAC, which was postulated for the lipophilic acyl derivative N^4-behenoyl-ara-C [38], highly unlikely.

Taking into account that the phosphorylation and DNA incorporation of NHAC and its possible metabolites do not seem to play a crucial role in the cytotoxicity of the drug, we conclude that the cytotoxicity of NHAC is exerted by yet unknown mechanisms. Different mechanisms of action for ara-C and NHAC could therefore explain the varying results of the two cytotoxicity assays. Because different parameters were determined in the MTT assay and the clonogenic assay as a measure of cytotoxicity, it could be assumed that drugs with different mechanisms of action show different cytotoxic effects. Nevertheless, NHAC cannot be considered a prodrug of ara-C, although small amounts of NHAC seem to follow the well-known ara-C phosphorylation pathway.

The failure of NHAC to induce erythroid differentiation in K-562 cells is another indication that NHAC exerts pharmacological effects other than DNA incorporation on the tumor cells. Although the mechanisms by which ara-C induces differentiation of K-562 cells is not fully known, it has been shown that treatment with ara-C causes aberrant DNA synthesis [30], which in turn can alter binding properties for regulatory proteins, resulting in altered gene expression [6]. However, various studies indicate that the phosphorylation of ara-C to ara-CTP and its interaction with cellular DNA are necessary steps for the induction of erythroid differentiation [19, 32]. The 100-fold higher concentration of NHAC needed to induce differentiation in 22% of the K-562 cells indicates again that DNA incorporation of NHAC or of its metabolites plays a minor role in its action mechanism (cf. Table 2).

In the present study the liposomes served only as a pharmaceutical formulation to enable the solubilization and parenteral administration of NHAC in aqueous media. In fact, we have demonstrated that NHAC is rapidly released in vivo from the liposomes and transferred to plasma proteins and erythrocytes [22].

Further studies on the mechanisms of cytotoxicity, the cell-cycle dependence, and the induction of apoptosis of NHAC in different cell lines, including dCyd kinase-deficient cells, will be helpful in confirming the findings we obtained in K-562 and U-937 cells and in clarifying the mechanisms of action of this promising new drug. Furthermore, it would be of great interest to analyze the influence of NHAC on the signal transduction pathways and on enzymes such as phosphatidylinositol-3-kinase and protein kinase C, which have been described to be influenced by synthetic alkyl ether lipids [5].

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