

# The Novel Heterodinucleoside Dimer 5-FdU-NOAC Is a Potent Cytotoxic Drug and a p53-Independent Inducer of Apoptosis in the Androgen-Independent Human Prostate Cancer Cell Lines PC-3 and DU-145

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**BACKGROUND.** We analyzed the cytotoxic properties of the new heterodinucleoside phosphate dimer 5-FdU-NOAC, which is composed of the cytotoxic drugs 5-FdU and N<sup>4</sup>-octadecyl-1-β-D-arabinofuranosylcytosine (NOAC) against human prostate tumor cells.

**METHODS.** 5-FdU-NOAC effects on cell proliferation, cell cycle distribution, thymidylate synthase activity, and apoptosis were investigated in vitro in the two human prostate carcinoma cell lines DU-145 and PC-3 and compared to cells treated with the corresponding single drugs 5-FdU and NOAC.

**RESULTS.** Treatment of the cells with 5-FdU-NOAC resulted in IC<sub>50</sub> values of 3.9–5 μM and in a complete inhibition of cell proliferation at 200 μM after 96 hr compared to 5-FdU, where 10% of the cells remained resistant. Flow cytometric analysis revealed cell cycle perturbations in S-phase only in the DU-145 cells. 5-FdU-NOAC caused 50% inhibition of thymidylate synthase after 90 min at 0.6 μM in both cell lines. Apoptotic cell fractions in DU-145 (66%) and in PC-3 (34%) cells were found after treatment with 5-FdU-NOAC for 96 hr. DNA fragmentation further confirmed the induction of apoptosis.

**CONCLUSIONS.** 5-FdU-NOAC inhibits thymidylate synthase and cell cycle progression causing proliferation arrest and apoptosis in DU-145 and PC-3 cells, suggesting a potential role of 5-FdU-NOAC for the treatment of prostate cancer. *Prostate 45:8–18, 2000.*

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**KEY WORDS:** 5-fluorodeoxyuridine; dimeric prodrug; cytotoxicity; thymidylate synthase; apoptosis

## INTRODUCTION

Prostate cancer has become the most common cancer among American men, second only to lung cancer as a cause of cancer death [1]. Despite therapy, more than 65% of the patients suffer local or systemic progression of the disease [2] and the prognosis for those with advanced prostate cancer remains dismal. A large number of cytostatic drugs have been studied in patients in whom prostate cancer has progressed despite hormonal therapy [3]. None of these agents (cisplatin, doxorubicin, mitoxantrone, cyclophosphamide, 5-fluorouracil, or combined therapies) have been

shown to affect survival significantly [4, 5]. Consequently, evaluation of the effect of new drugs or new combinations with already known ones is required.

5-FU was specifically synthesized to resemble the pyrimidine bases uracil and thymine and in the clinic,

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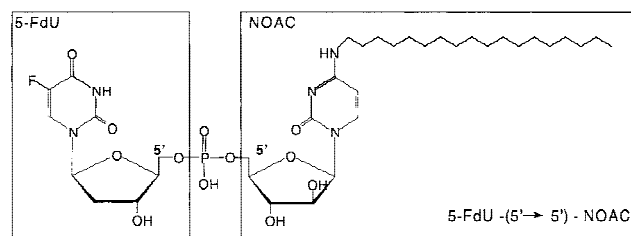
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5-FU is used for the treatment of colon, breast, and ovarian cancer [6]. To exert its cytotoxic effects 5-FU requires intracellular activation. Different mechanisms of action are responsible for the effect of 5-FU. The central mechanism of 5-FU action is the inhibition of thymidylate synthase (TS) by 5-FdU. 5-FU is converted to 5-FdU by thymidine phosphorylase and subsequent phosphorylation of 5-FdU by thymidine kinase results in formation of the active metabolite 5-fluoro-2'-deoxyuridine monophosphate (5-FdUMP). In the presence of the reduced folate 5,10-methylenetetrahydrofolate, 5-FdUMP forms a stable covalent complex with TS, inhibiting TS enzyme activity and leading to depletion of deoxythymidine triphosphate, a necessary precursor for DNA synthesis [7]. Alternatively, 5-FU may be anabolized to 5-fluorouridine monophosphate, which is further metabolized to 5-fluorouridine triphosphate. The latter can be incorporated into RNA or converted to the deoxyribonucleotide 5-FdUMP [8]. Finally, 5-FdUMP may subsequently be phosphorylated to 5-fluoro-2'-deoxyuridine-5'-triphosphate, which is incorporated into DNA [9].

The usefulness of 5-FU and 5-FdU are impaired by the frequent development of resistance in tumor cells. Resistance can develop through deletion of one of the key enzymes required for activation. The rate-limiting step in the activation is the first phosphorylation step from 5-FdU to 5-FdUMP catalyzed by the enzyme thymidine kinase. Depletion of this enzyme often is responsible for the development of resistance to 5-FU. Consequently, the introduction of a monophosphorylated 5-FdU molecule into the cell could circumvent this resistance.

*N*<sup>4</sup>-octadecyl-1- $\beta$ -D-arabinofuranosylcytosine (NOAC) [10] belongs to a new class of lipophilic 1- $\beta$ -D-arabinofuranosylcytosine (ara-C) derivatives, one of the most widely used agents for the treatment of acute myelogenous leukemia [11]. Due to the rapid deamination of ara-C to the biologically inactive metabolite 1- $\beta$ -D-arabinofuranosyluracil (ara-U) a large number of chemical modifications of ara-C were made in the past [12,13]. Not susceptible to hydrolysis are alkyl modifications of ara-C, with molecules modified with C<sub>16</sub>-C<sub>22</sub> alkyl side-chains, of which NOAC (C<sub>18</sub>-alkyl chain) was shown to be the most effective [14]. Cellular uptake, formation of ara-C-5'-triphosphate and induction of apoptosis were shown to be different from ara-C [15-17], suggesting that NOAC is a drug with new mechanisms of action. Moreover, NOAC was shown to exert strong antitumor activity in the human prostate PC-3 xenograft nude mouse model [18].

To enhance the effectiveness of 5-FU and to overcome resistance a new amphiphilic heterodinucleoside phosphate dimer of 5-FdU and NOAC was syn-



**Fig. 1.** Chemical structure of the heterodinucleoside phosphate dimer 2'-deoxy-5-fluorouridylyl-(5'  $\rightarrow$  5')-*N*<sup>4</sup>-octadecyl-1- $\beta$ -D-arabinofuranosylcytosine (5-FdU-NOAC). The masked 5-FdU and NOAC molecules are shown in the rectangles.

thesized. In this dimer a 5-FdU molecule is linked through a 5'  $\rightarrow$  5' phosphate bond to the lipophilic NOAC molecule (Fig. 1). Due to the amphiphilic/lipophilic structure of this dimer, an improved cell uptake and a different distribution in vivo can be expected. Further we anticipate introducing a monophosphorylated 5-FdU molecule directly into the cell after enzymatic cleavage in the cytoplasm. Thus, a monophosphorylated molecule would not have to pass the first phosphorylation step, which is known to be rate limiting. Prostate cancer largely represents a failure of tumor cells to undergo apoptosis. Apoptosis is a genetically programmed, active cell death, which plays an essential role in the control of normal tissue and tumor cells. Drugs that can block checkpoints that control and inhibit apoptosis might be effective for the treatment of prostate cancer. Loss of wild-type p53 function is considered the most common genetic abnormality in human cancers and a major predictor of failure to respond to chemotherapy [19]. It has been generally accepted that apoptosis is a clinically relevant mechanism of tumor response to chemotherapy and a critical determinant of tumor cell sensitivity to chemotherapeutic agents. Therefore we determined the ability of the new heterodinucleoside phosphate dimer 5-FdU-NOAC to induce apoptosis in the p53-negative human prostate carcinoma cell lines DU-145 and PC-3.

In this study, we demonstrate that the dimer 5-FdU-NOAC is a potent inhibitor of proliferation in androgen-independent prostate tumor cells and that inhibition of proliferation is caused by thymidylate synthase inhibition, cell cycle arrest, and p53-independent apoptosis of the cells.

## MATERIALS AND METHODS

### Chemicals

Bovine serum albumin (BSA), 5-bromo-2'-deoxyuridine (BrdU), propidium iodide (PI), Triton X-100, acid-washed activated charcoal, and dUMP

were purchased from Fluka Chemie (Buchs, Switzerland). SYBR green II was from Molecular Probes (Eugene, OR) and the 123-bp marker from Gibco (Paisley, UK). RPMI-1640 medium, fetal calf serum (FCS), penicillin-streptomycin, L-glutamine, and agarose were from Life Technologies (Basel, Switzerland). Trypsin-EDTA was obtained from Biochrom KG (Berlin, Germany). The WST-1 assay kit, RNase A, and proteinase K were from Boehringer Mannheim (Rotkreuz, Switzerland), Tween-20 was from Merck (Darmstadt, Germany), and T-70 dextran from Pharmacia (Dübendorf, Switzerland). Digitonin was purchased from Calbiochem (Juro Supply AG, Lucerne, Switzerland). The monoclonal antibody Apo 2.7-PE was from IL Instrumentation Laboratory AG (Zurich, Switzerland). The FITC-labeled anti-BrdU antibody and the Ac-DEVD-AMC fluorogenic substrate were from Becton-Dickinson (Basel, Switzerland). The lipophilic heteronucleoside dimer and NOAC were synthesized according to the methods described previously [20,10]. 5-FdU was obtained from Hoffman La-Roche (Basel, Switzerland). 5-FdU and 5-FdU-NOAC were dissolved in 0.9% NaCl. NOAC was formulated in small unilamellar liposomes (see below).

### Cells

The human epithelial prostate tumor cell lines PC-3 and DU-145 were obtained from the German Collection of Microorganisms and Cell Cultures, DSMZ (Braunschweig, Germany). The cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine in a humidified 5% CO<sub>2</sub> atmosphere at 37°C.

### Preparation of NOAC Liposomes

Due to the insolubility of NOAC in aqueous media, a liposomal formulation was used. Small unilamellar liposomes of 100 ± 30 nm mean diameter were prepared by filter extrusion as described [14,15]. Briefly, lipid mixtures composed of SPC, cholesterol, D,L- $\alpha$ -tocopherol, and N<sup>4</sup>-octadecyl-1- $\beta$ -D-arabinofuranosylcytosine (NOAC) at a molar ratio of 1:0.2:0.01:0.2 were hydrated with phosphate buffer (PB; 13 mM KH<sub>2</sub>PO<sub>4</sub>, 54 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) and sequentially filtered through Nuclepore (Costar, Sterico, Dietikon, Switzerland) filters of decreasing pore size (400 nm, 100 nm). Typically, the liposomes contained 5 mg NOAC per ml. Liposomes without NOAC were used as control. Liposomes were sterile filtered through 0.2 µm filters (Acrodisc, Gelman Sciences, Ann Arbor, MI), stored at 4°C, and used within 48 hr.

### Cytotoxicity Assay

To evaluate cell proliferation the WST-1 kit was used. Exponentially growing cells were seeded in sterile 96-well plates and incubated for 24 hr. Drugs were added to a final concentration of 12–200 µM. The supernatant was removed after 96 hr and 100 µl of freshly diluted WST solution were added. The plates were incubated for 30–60 min at 37°C (5% CO<sub>2</sub>). Cell viability was evaluated by measurement of the absorption at 450 nm using a Dynatech MR5000 plate reader (Microtec Produkte, Embrach, Switzerland). Fifty percent growth-inhibitory concentrations (IC<sub>50</sub>) were calculated from interpolations of the graphical data.

### Cell Cycle Distribution Analysis

Cells were seeded in 100 mm culture dishes, incubated for 48 hr, and exposed to various concentrations (0–200 µM) of 5-FdU-NOAC, 5-FdU and NOAC for 24 hr at 37°C (5% CO<sub>2</sub>) or for various time periods (0–48 hr) with 50 µM of the drugs. After the specified period the cells were incubated with 10 µM BrdU for 30 min at 37°C (5% CO<sub>2</sub>). The supernatant with dead cells and the harvested living cells were fixed in precooled (-20°C) ethanol (80%) and stored at -20°C for up to 3 days. BrdU/PI staining was carried out as described previously [21]. Briefly, after centrifugation, the cells were treated with 2 M HCl for 30 min at 20°C and resuspended in 50 µl PBS, 0.5% Tween-20, 1% BSA and incubated with FITC-labeled anti-BrdU antibody for 30 min at 20°C followed by addition of 1 ml PBS/PI (10 µg/ml). Stained cells were analyzed with an Epics Elite Analyzer (Coulter, FL). Single fluorescent samples (FITC or PI) were used to optimize instrument settings and ensure proper electronic compensation.

### Thymidylate Synthase Activity

Activity of TS was measured by the release of tritium from [5-<sup>3</sup>H]dUMP. Cells were seeded in 6-well plates and incubated for 48 hr. After exposition to the drugs for 90 min at different concentrations (0.01–100 µM) or for various time periods (0–8 hr) with 0.1 µM at 37°C (5% CO<sub>2</sub>), the cells were treated with deoxyuridine-5'-monophosphate (10 µM) trace labeled with 0.5 µCi/ml [5-<sup>3</sup>H]deoxyuridine-5'-monophosphate (Amersham Pharmacia Biotech, Dübendorf, Switzerland) [22]. After incubation at 37°C for 60 min, 0.2 ml medium were removed and added to 1 ml of a mixture of ice-cold T-70-dextran and BSA-treated charcoal to terminate the reaction. After 30 min at room temperature the probes were centrifuged (30 min, 4,400 g)

and the radioactivity of the supernatant determined in a liquid scintillation instrument (1900 TR Packard). Fifty percent inhibitory concentrations were calculated from interpolations of the graphical data.

### Quantification of the Apoptotic Cell Fraction

Cells were treated as described for cell cycle analysis. After incubation the supernatant with dead cells and the harvested living cells were pooled and permeabilized by incubation on ice for 20 min with 100  $\mu\text{g}/\text{ml}$  digitonin in PBS supplied with 2.5% FCS (v/v) and 0.01%  $\text{NaN}_3$ . After permeabilization the cells were labeled with Apo 2.7-PE for 15 min at room temperature in the dark. For flow cytometric analysis cells were resuspended in PBS supplied with 2.5% FCS (v/v) and 0.01%  $\text{NaN}_3$  and stored on ice in the dark until analysis.

### DNA Fragmentation

Cells were exposed for various time periods (0–96 hr) with 50  $\mu\text{M}$  of 5-FdU, 5-FdU-NOAC, and NOAC at 37°C (5%  $\text{CO}_2$ ). As positive control, colcemide was included (1  $\mu\text{g}/\text{ml}$ ; 24–96 hr). DNA extraction was performed with modifications as described by Kaufmann [23]. Briefly, supernatants and harvested cells were pooled, washed once with PBS, and lysed in 300  $\mu\text{l}$  lysis buffer (0.5 M Tris-HCl pH 9.0, 2 mM EDTA, 10 mM NaCl, 1% SDS, 0.33 mg/ml proteinase K). The samples were incubated at 55°C for 24 hr, extracted twice with phenol/chloroform (1:1, v/v), and once with chloroform. The probes were then incubated with 300  $\mu\text{g}/\text{ml}$  DNase free RNase A and loaded onto 1.2% (w/v) agarose gels. Staining of DNA was performed using SYBR green II dye. Gels were scanned at 488 nm on a FluorImager 595 (Molecular Dynamics, CA) using a SYBR green filter (530DF30).

### Caspase-3 Activity

Cells were treated as described for cell cycle analysis. After incubation with the drugs, dead cells in supernatant and the harvested living cells were counted and lysed with 10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM  $\text{Na}_4\text{P}_2\text{O}_7$  ( $2 \times 10^6$  cells/ml). After centrifugation (5 min, 1,400 g), 100  $\mu\text{l}$  of the cell lysate were reacted with 20  $\mu\text{M}$  Ac-DEVD-AMC fluorogenic substrate in 20 mM HEPES, pH 7.5, 10% glycerol, 2 mM dithiothreitol for 2 hr at 37°C. Released AMC from Ac-DEVD-AMC was measured using a spectrofluorometer (Kontron SFM 23/23 LC) with excitation and emission wavelengths of 380 nm and 440 nm, respectively.

### In Vitro Hydrolysis of the Dimer by Phosphodiesterase I and Human Serum

The stability of 5-FdU-NOAC against hydrolysis by phosphodiesterase I and human serum was analyzed as described before [24]. Briefly, 100  $\mu\text{l}$  of the dimer (5 mg/ml water) was treated during 2, 4, 12, and 36 hr at 37°C with phosphodiesterase I (0.1 M  $\text{MgCl}_2$ , pH 8.1) or human serum. The reaction mixtures were analyzed by reverse phase HPLC using a binary gradient of 0.05 M ammonium acetate and methanol as eluent. The dimer dT-dT was used as reference and the hydrophilic metabolites were identified by corresponding reference compounds.

## RESULTS

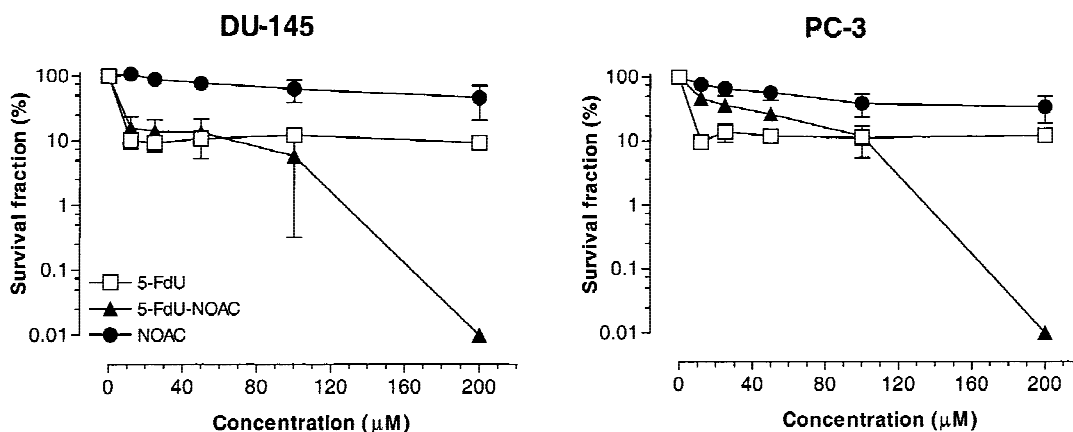
### 5-FdU-NOAC Inhibits Cell Proliferation of DU-145 and PC-3 Cells

The cytotoxicity of 5-FdU-NOAC compared to 5-FdU and to NOAC with the WST dye reduction assay after a continuous 96 hr incubation is shown in Figure 2. Untreated cells or cells treated with empty liposomes were taken as 100% viability. Empty liposomes were not toxic to the cells at a lipid concentration up to 0.8 mg/ml SPC (corresponding to liposomes with 400  $\mu\text{M}$  NOAC). 5-FdU reached 90% cell kill at 12  $\mu\text{M}$  in both cell lines. However, the further increase of 5-FdU concentration did not produce higher toxicity. NOAC did not produce a noticeable toxicity in both cell lines. In contrast, 5-FdU-NOAC treatment resulted in 100% toxicity, however, only at the rather high concentration of 200  $\mu\text{M}$ .

In DU-145 cells 5-FdU-NOAC had the same efficacy compared to 5-FdU already at low concentrations, whereas in PC-3 cells 5-FdU was more effective at low concentrations than the dinucleoside. The 50% growth inhibitory concentration ( $\text{IC}_{50}$ ) values of the drugs are given in Table I. 5-FdU-NOAC gave comparable 50% inhibitory concentrations as 5-FdU after 96 hr drug exposure, whereas the  $\text{IC}_{50}$  of liposomal NOAC was about 22-fold higher as compared to 5-FdU and the dimer.

### 5-FdU-NOAC Induces Cell Cycle Perturbations

The effects of drug exposure on cell cycle distribution of DU-145 and PC-3 cells after 8, 24, and 48 hr incubation at 50  $\mu\text{M}$  drug concentration are shown in Table II. As expected, 5-FdU induced an S-phase arrest in both cell lines. The effect was detectable after 8 hr incubation and it increased over time. In DU-145 cells 83% S-phase cells were detected after 24 hr, whereas in PC-3 70% of the cells were in S-phase after 48 hr in-



**Fig. 2.** Cytotoxicity determined by WST-1 dye reduction in DU-145 and PC-3 cells. DU-145 and PC-3 cells were treated for 96 hr at a concentration range of 12–200  $\mu\text{M}$ . Results are shown as means  $\pm$  SD of three independent experiments performed in triplicates.

**TABLE I. IC<sub>50</sub> Values in DU-145 and PC-3 Cells After Incubation with the Various Drugs for 96 h<sup>a</sup>**

Drug	IC <sub>50</sub> ( $\mu\text{M}$ )	
	DU-145 cells	PC-3 cells
5-FdU	3.35 $\pm$ 0.04 <sup>b</sup>	3.39 $\pm$ 0.01
5-FdU-NOAC	3.90 $\pm$ 0.50	5.00 $\pm$ 1.41
NOAC <sup>c</sup>	134 $\pm$ 79	110 $\pm$ 10.2

<sup>a</sup>Cytotoxicity determined using the WST-1 cell proliferation assay.

<sup>b</sup>Mean of three separate experiments performed in triplicates. SD was <10% of mean values.

<sup>c</sup>NOAC given as liposomes.

cubation. The increase in S-phase cells correlated with a decrease in G1- and G2/M-phase cells. NOAC caused a slight S-phase arrest in both cell lines. This can probably be explained with a small amount of NOAC being metabolized to ara-C [16]. The dimer 5-FdU-NOAC induced S-phase arrest comparable to 5-FdU in DU-145 cells. Surprisingly, in PC-3 cells this effect was not observed. Instead, an increase of G1-phase arrested cells after 48 hr incubation was observed (Table II). The concentration-dependent cell cycle distribution analysis at concentrations up to 200  $\mu\text{M}$  after 24 hr drug exposure confirmed the results obtained with 50  $\mu\text{M}$  (data not shown).

### 5-FdU-NOAC Inhibits Thymidylate Synthase

The central mechanism of action of 5-FdU is the inhibition of TS. To determine if 5-FdU-NOAC has the same mechanism of action we studied the effect of this drug as inhibitor of TS. Measurement of TS activity in DU-145 and PC-3 cells in situ revealed that 5-FdU-NOAC inhibited TS activity in a time- as well as in a

concentration-dependent manner (Fig. 3). Exposure of the cells to increasing concentrations of 5-FdU and 5-FdU-NOAC for 90 min diminished the catalytic activity of TS. At a concentration of 0.1  $\mu\text{M}$  5-FdU and 10  $\mu\text{M}$  5-FdU-NOAC enzyme inhibition was complete in both DU-145 and PC-3 cells. The time-dependent studies showed that 5-FdU-NOAC inhibited TS only after prolonged incubations, while with 5-FdU a complete inhibition was reached already after 30 min. The slower inhibition of TS caused by 5-FdU-NOAC compared to 5-FdU strongly suggests the prodrug nature of this drug, which requires intracellular cleavage to be activated. NOAC did not alter TS activity. The IC<sub>50</sub> values for each of the analyzed drugs are summarized in Table III.

### 5-FdU-NOAC Causes Apoptosis in Human Prostate Tumor Cells

The quantitative determination of apoptotic cell fractions was carried out with the Apo 2.7 monoclonal antibody that reacts preferentially with cells undergoing apoptosis [25]. 5-FdU and 5-FdU-NOAC induced apoptosis in both cell lines. The induction of apoptosis started after 24 hr in DU-145 and after 48 hr in PC-3 cells (Fig. 4). After 96 hr incubation, 2.4  $\pm$  1.2% DU-145 cells and 6.0  $\pm$  2.8% PC-3 cells from untreated controls were apoptotic, whereas drug treatment with 50  $\mu\text{M}$  increased the apoptotic cell fractions to 67–87% in DU-145 and to 31–34% in PC-3 cells, respectively (Fig. 4). NOAC was a weak inducer of apoptosis in DU-145 cells and in PC-3 cells this drug had no detectable effect.

These findings were further confirmed by genomic DNA fragmentation analyzed by agarose gel electrophoresis (Fig. 5). Untreated cells or cells treated with control liposomes did not show any DNA laddering

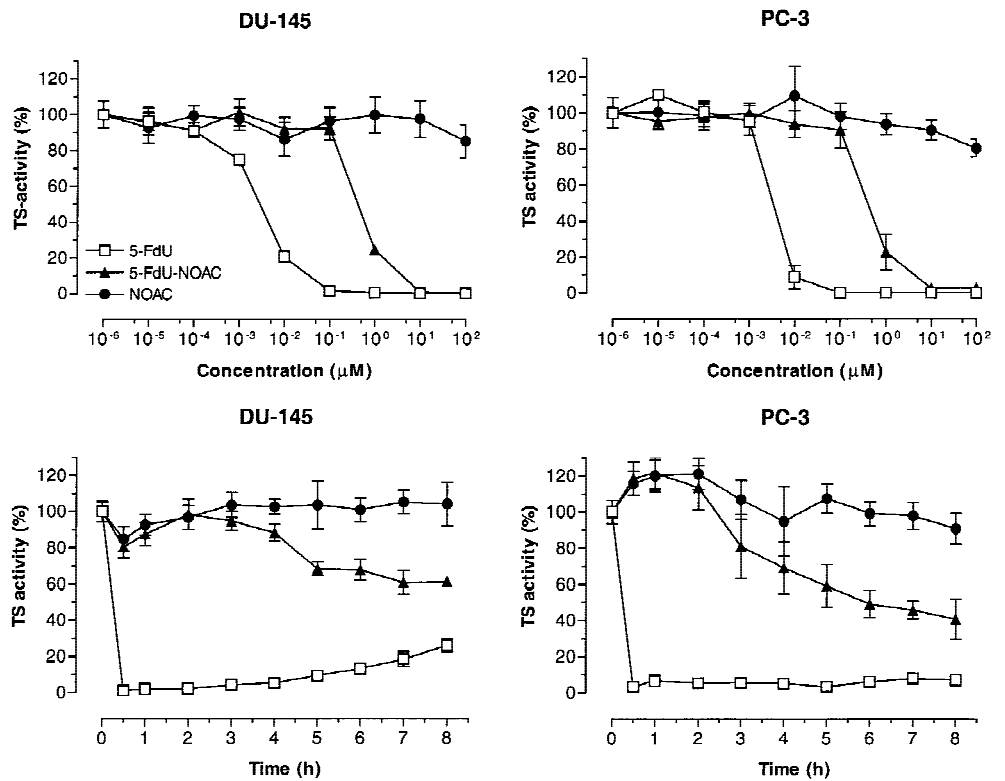
**TABLE II. Time-Dependent Cell Cycle Distribution in DU-145 and PC-3 Cells After Incubation with 50  $\mu$ M 5-FdU, 5-FdU-NOAC or NOAC\***

Drug	h	Cell cycle distribution in %					
		DU-145 cells			PC-3 cells		
		G1	S	G2/M	G1	S	G2/M
5-FdU	0	51.2 <sup>a</sup>	34.7	14.1	51.0	36.3	12.7
	8	43.4	50.9	5.7	47.4	47.3	5.4
	24	13.4	82.9	3.7	30.8	64.3	4.8
	48	39.5	56.7	3.8	25.2	70.1	4.7
5-FdU-NOAC	0	51.2	34.7	14.1	51.0	36.3	12.7
	8	50.8	42.9	6.3	60.1	34.9	5.1
	24	23.5	73.2	3.3	57.4	31.8	10.9
	48	39.1	55.2	5.7	63.0	23.6	13.4
NOAC	0	49.2	35.6	15.1	49.3	37.4	13.3
	8	49.9	41.9	8.2	49.3	46.2	4.5
	24	41.2	49.5	9.3	46.2	45.3	8.5
	48	52.7	39.0	8.3	58.8	31.6	9.6

\*Cell cycle distribution determined using the BrdU-PI method.

<sup>a</sup>Mean of cell cycle fractions (%) of two separate experiments performed in duplicates. SD was < 10% of mean values.

**Fig. 3.** TS enzyme activity of DU-145 and PC-3 cells in situ. DU-145 and PC-3 cells were exposed to the drugs for 90 min at increasing concentrations (upper graphs). Alternatively, the cells were incubated at 0.1  $\mu$ M drug for various time periods (lower graphs). TS activity values are shown as percent of untreated control. Data are means  $\pm$  SD of two independent experiments performed in duplicates.



pattern, whereas cells treated with 5-FdU and 5-FdU-NOAC induced DNA fragmentation in both DU-145 and PC-3 cells. DNA fragmentation initiated after 24 hr in DU-145 and after 48 hr in PC-3 cells (data not shown).

In additional experiments the capacity of the drugs to enhance the activity of caspase-3, a known mediator

of apoptosis, was investigated. 5-FdU was able to increase the enzyme activity up to a factor 6-11 in DU-145 and PC-3 cells after 72-96 hr incubation at 50  $\mu$ M compared to the enzyme activity in untreated cells. 5-FdU-NOAC and NOAC were not able to substantially increase the caspase-3 activity in both cell lines (data not shown).

**TABLE III. 50% TS Inhibition Values in DU-145 and PC-3 Cells After 90 min Drug Exposure**

Drug	50% TS inhibition values ( $\mu\text{M}$ )	
	DU-145 cells	PC-3 cells
5-FdU	$0.005 \pm 0.003^a$	$0.006 \pm 0.000$
5-FdU-NOAC	$0.660 \pm 0.028$	$0.640 \pm 0.156$
NOAC	nr <sup>b</sup>	nr

<sup>a</sup>Mean  $\pm$  SD of two separate experiments performed in duplicates.

<sup>b</sup>nr = not reached within the drug concentration range of 0.01 nM-100  $\mu\text{M}$ .

### In Vitro Hydrolysis of the Dimer by Phosphodiesterase I and Human Serum

As summarized in Table IV, the dimer was hydrolyzed in vitro by phosphodiesterase I and serum to the active compounds 5-FdU and 5-FdU monophosphate, demonstrating that the cytotoxic compounds 5-FdU and 5-FdUMP can be released from the dimer. The lipophilic cleavage product NOAC was not detected with the elution conditions used.

## DISCUSSION

In the present study we have evaluated the effects of the new amphiphilic heterodinucleoside phosphate dimer 5-FdU-NOAC on hormone-independent DU-145 and PC-3 cells in comparison to the monomeric anticancer drugs 5-FdU and NOAC. Due to the fact that increased proliferation and decreased cell death (apoptosis) are the two major processes contributing to the progression of tumor cell growth, agents that can inhibit cell proliferation or induce apoptosis are of important therapeutic value [26]. The findings presented here demonstrate that compared to the clinically used drugs 5-FU and 5-FdU this novel anticancer drug exerts comparable or even stronger cytotoxic effects on androgen-refractory human prostate cancer cells in vitro.

The antiproliferative effect of 5-FU has previously been associated with therapeutic benefit against different cancers including breast, gastrointestinal, head and neck, and ovarian carcinomas. However, the clinical utility of 5-FU is limited by rapid degradation and the formation of resistance. More than 80% of 5-FU administered by intravenous or intraarterial routes are inactivated by metabolic conversion by dihydropyrimidine dehydrogenase to dihydro-fluorouracil. Resistance to 5-FU and 5-FdU which is the primary metabolite of 5-FU can develop by deletion of one of the key enzymes required for its activation or by mutations in the p53 gene [27,28]. The new amphiphilic

heterodinucleoside phosphate dimer 5-FdU-NOAC strongly inhibited cell proliferation in both DU-145 and PC-3 cells in a time- and dose-dependent manner (Fig. 2). At a concentration of 12  $\mu\text{M}$ , the dimer had cytotoxic effects comparable to the parent drug 5-FdU in DU-145 cells. While higher concentrations of 5-FdU did not produce additional cytotoxicity, 5-FdU-NOAC at a concentration of 200  $\mu\text{M}$  exposed to the cells for 96 hr, was capable of inducing 100% toxicity, overcoming 5-FdU resistance in both cell lines (Fig. 2). The rather high concentration of 200  $\mu\text{M}$  required to obtain a 100% cell kill is probably necessary because of the prodrug nature of the dimer. It is conceivable that in vitro the dimer is cleaved into the active metabolites at low rates, requiring high concentrations of the intrinsically inactive prodrug.

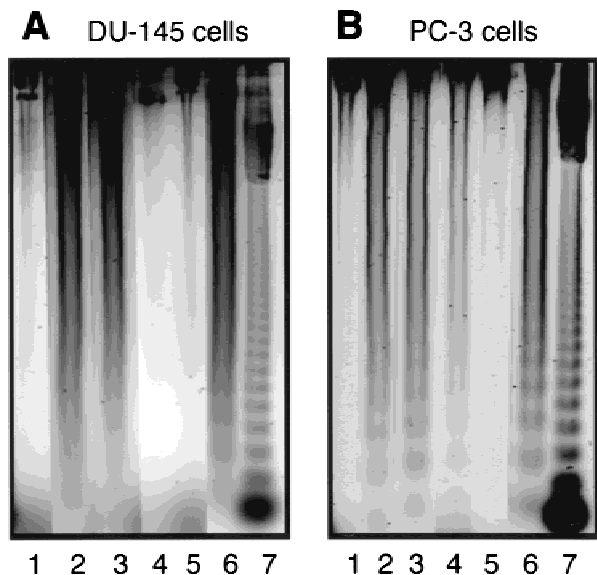
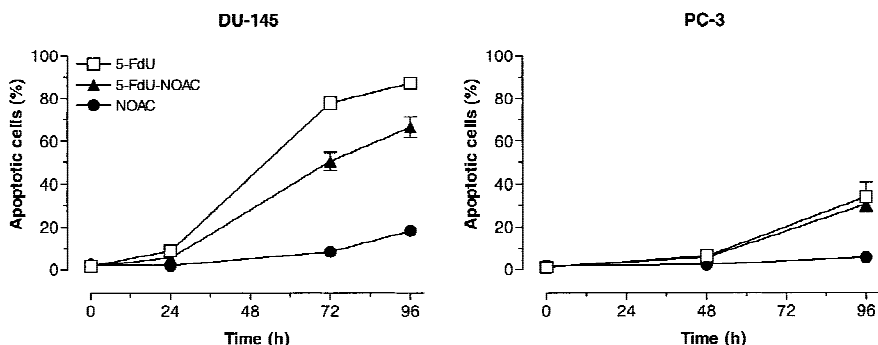
Taking into consideration that mutations in p53 and changes in the expression of this gene in advanced prostate cancer can lead to resistance to some forms of chemotherapy, the fact that 5-FdU-NOAC seems to overcome 5-FdU resistance in p53-independent cells is an important finding. To confirm its usefulness as an effective drug against prostate tumors it will be imperative to demonstrate the activity of 5-FdU-NOAC in vivo.

Previous studies had revealed an impressive efficacy of NOAC in vivo in solid tumor xenografts with PC-3 cancer cells [18]. Surprisingly, in this study NOAC did not exert strong cytotoxicity on this prostate tumor cell line eradicating only 23–66% of cells in the concentration range tested. This may be explained by a reduced uptake of NOAC containing liposomes in tumor cells in vitro compared to solid tumors in vivo.

5-FdU inhibits cell proliferation by S-phase arrest, which is caused by TS inhibition [7], single-strand breaks, and DNA fragmentation [29]. The new dimeric drug 5-FdU-NOAC caused an S-phase arrest similar to 5-FdU in DU-145 cells. In this cell line the effect of 5-FdU-NOAC and 5-FdU was seen within 8 hr incubation and it increased after 24 hr as demonstrated by flow cytometric analysis (Table II). After this time point a decrease of cells arrested in the S-phase was observed. These findings correlate with the results obtained with the Apo 2.7 binding and DNA fragmentation analysis, where in DU-145 cells an increase in the apoptotic cell population was observed after 24 hr (Fig. 4). Consequently, the decrease in S-phase cells after 24 hr was probably due to induction of apoptosis and subsequent cell fragmentation. In PC-3 cells, however, 5-FdU-NOAC caused an increase in G1-phase cells and in S-arrested cells, whereas the S-phase cell population decreased (Table II).

NOAC treatment resulted in a slight S-phase arrest in DU-145 and PC-3 cells after 24 hr at 50  $\mu\text{M}$ , prob-

**Fig. 4.** Apoptotic cell fractions determined by flow cytometry after incubation with 50  $\mu$ M drug for various incubation times in DU-145 and PC-3 cells. Results are means  $\pm$  SD of at least two separate experiments performed in duplicates.



**Fig. 5.** Endonucleolytic DNA fragmentation in DU-145 (**A**) and PC-3 cells (**B**) induced by incubation with 50  $\mu$ M 5-FdU (lane 2), 5-FdU-NOAC (lane 3), and NOAC (lane 4) for 96 hr. Agarose gel electrophoresis was used for the detection of DNA fragmentation. Untreated cells and cells treated with control liposomes are shown in lane 1, respectively, lane 5. Colcemide (1  $\mu$ g/ml, 96 hr; lane 6) was used as positive control for apoptosis. A 123-bp ladder was used as marker (lane 7).

ably caused by ara-C formed from metabolized NOAC as previously described for the  $N^4$ -hexadecyl-ara-C derivative NHAC [15]. It is known that ara-C characteristically induces cells to get arrested in the middle of the S-phase, due to the inhibition of DNA polymerase by ara-CTP.

A significant therapeutic target for the effective elimination of cancer cells is apoptosis. Many cancer malignancies have a mutated p53 gene that is associated with decreased induction of apoptosis, resulting in chemotherapeutic resistance. Mutations of this gene are seen more commonly in patients with metastatic prostate cancer than in those with primary tumors [30]. Therefore, the development of new pharmacological agents able to trigger p53-independent apoptosis may be of considerable clinical relevance [31].

Our studies demonstrate that the dimeric drug is able to induce apoptosis after incubations of 24 hr in DU-145 and after 48 hr in PC-3 cells (Fig. 4), which are both p53 negative.

Simultaneously, the activity of caspase-3 was 6–10 times higher in cells treated with 5-FdU for 72–96 hr than in untreated control cells. 5-FdU-NOAC and NOAC had no inducing activity on caspase-3. Nevertheless, 5-FdU-NOAC had the capability to induce apoptosis in DU-145 and PC-3 cells as shown by DNA laddering and Apo 2.7 antibody binding (Figs. 4, 5). Thus, the dimer seems to induce apoptosis by a caspase-independent mechanism [32].

The possible break of 5-FdU resistance observed in the cytotoxicity assay can be explained by the prodrug nature of the dimer, resulting in persisting intracellular drug concentrations of the monophosphorylated cleavage product and other active metabolites over longer time periods compared to 5-FdU. Unlike the parent compound, the active 5-FU nucleotide 5-FdUMP has a prolonged intracellular half-life. Although the decay rate varies among different tissues, its continued presence is an important determinant for duration and magnitude of drug effects. The better effect of the dimer can further be explained by the fact, that the first phosphorylation step from 5-FdU to 5-FdUMP catalyzed by the enzyme thymidine phosphorylase is a rate-limiting step. A loss in function of this enzyme is a cause of development of resistance to 5-FdU. Consequently, the introduction of a monophosphorylated 5-FdU molecule would have the advantage that the first rate-limiting and resistance-causing step could be circumvented. In addition, a conceivable reason for the improved potency of 5-FdU-NOAC over 5-FdU and NOAC could be that the molecule contains two toxic moieties that may have synergistic activities in the cell.

The hypothesis that the dimer is an effective prodrug of 5-FdU is supported by three facts. First, the dimer specifically inhibited TS activity. Inhibition of cell proliferation through 5-FdU is predominantly due to TS inhibition, followed by thymidine depletion and



**TABLE IV. In vitro Hydrolysis of the Dimer by Phosphodiesterase I and Human Serum**

Dimer	Phosphodiesterase I		Human serum	
	Hydrolysis <sup>a</sup> (h)	Metabolites	Hydrolysis <sup>a</sup> (h)	Metabolites
dT-dT	2	dT, dT-MP 5-FdU,	5	dT
5-FdU-NOAC	2.8	5-FdU-MP	7.5	5-FdU

<sup>a</sup>Time for full hydrolysis.

S-phase arrest. 5-FdUMP forms a stable covalent complex with TS, which dissociates with a half-life of 6 hr in intact cells [7]. The delayed inhibition of TS caused by the dimer (Fig. 3C, D) further sustains the hypothesis that cleavage of the dimeric drugs into the monophosphorylated molecule 5-FdUMP took place. Second, the dimer exerts its cytotoxicity by inhibiting DNA synthesis like 5-FdU and arresting cells in early S-phase in DU-145 cells. Third, the dimer can be hydrolyzed by phosphodiesterase I and human serum to the corresponding active metabolites.

The intrinsic propensity to undergo apoptosis is a general determinant for chemotherapy sensitivity and could represent a target for pharmacological modulation [33,34]. This led us to study the capability of the new amphiphilic heterodinucleoside phosphate dimer of 5-FdU and NOAC to induce apoptosis. Apoptotic cells appeared after continuous drug exposure of 24 hr in DU-145 and of 48 hr in PC-3 cells (Fig. 4). These findings were in agreement with others who also found a rather long-lasting, continuous drug exposure to be required to induce apoptosis in prostate tumor cells [35,36]. The delayed induction of apoptotic cell death can possibly be explained by these cell lines having low inclination to undergo apoptosis. A reason for this could be the absence of wild-type p53. Thus, the assumption can be made that the absence of wild-type p53 in DU-145 and PC-3 cells was a contributing factor for the long induction times. For example, thymocytes of p53 deficient mice were resistant to DNA damage-induced apoptosis by etoposide and irradiation, but they responded to glucocorticoids in the absence of p53 [37–39]. These data imply the existence of p53-dependent and -independent pathways for the induction of apoptosis. Wild-type p53 seems to be involved in setting the threshold for apoptosis induction directly or by affecting the transcription of other regulatory apoptosis genes, rather than to be effective as inducer of apoptosis [40]. According to these findings the existence of an “apostat,” a conceptual organelle-like complex in which cellular life-or-death decisions are made was proposed in a recent review [41]. Fi-

nally, the stoichiometry of pro- and anti-apoptotic molecules, perhaps integrated with survival signals, may set the threshold for survival of a particular cell type.

In conclusion, the results of this study demonstrate that the new amphiphilic heterodinucleotide phosphate dimer 5-FdU-NOAC is able to overcome 5-FdU resistance in p53 mutated and androgen-independent DU-145 and PC-3 cells. It can be assumed that the dimer is cleaved into 5-FdUMP, resulting in sustained intracellular drug concentrations over an extended period that consequently increase duration and magnitude of the cytotoxic effect. This hypothesis is supported by the fact that the new dimer specifically inhibits TS activity and exerts a cell cycle phase-dependent cytotoxicity, two mechanisms characteristic for 5-FdU. Furthermore, the dimer is able to induce apoptosis, a process often hindered or suppressed in cancer cells.

The antitumor effect of the dimer observed at higher concentrations and longer incubation periods can possibly be explained by its prodrug nature, resulting in persisting intracellular concentrations of the active metabolites. The hydrolysis of the dimer by phosphodiesterase I and human serum further indicate this prodrug nature. Thus, due to expected changes of the pharmacokinetic properties and the prodrug character of 5-FdU-NOAC, the lipophilic dimer may have more favorable in vivo properties than the individual compounds 5-FdU and NOAC. In previous studies performed with similar heterodinucleoside phosphate dimers composed of the antivirally active nucleosides azidothymidine and dideoxycytidine, significantly different pharmacokinetic properties and superior antiviral effects in comparison to the parent hydrophilic nucleosides were obtained in the murine Rauscher leukaemia virus model [42].

In summary, the results of the present study suggest the potential value of the dimer as new therapeutic agent against hormone-refractory prostate tumors, provided that the antitumor activity of the dimer can be confirmed in appropriate in vivo experiments.

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