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ORIGINAL PAPER

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Cell-cycle arrest and p53-independent induction of apoptosis in vitro by the new anticancer drugs 5-FdUrd-*P*-FdCydOct and dCydPam-*P*-FdUrd in DU-145 human prostate cancer cells

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Abstract ***Purpose:*** Current therapies have limited impact on the progression of metastatic hormone-refractory prostate cancer. Therefore, we investigated the utility of new heterodinucleoside phosphate dimers of 5-fluorodeoxyuridine (5-FdUrd) in p53-mutated and androgen-independent DU-145 human prostate tumour cells. ***Methods:*** The effects of the dimers were assessed in vitro by a cell proliferation assay for cytotoxicity, flow cytometry for cell cycle distribution, confocal laser scanning microscopy for the detection of apoptotic bodies, poly(ADP-ribose) polymerase cleavage for caspase 3 activity and by a thymidylate synthetase assay. ***Results:*** The new dimers N^4-palmitoyl-2′-deoxycytidylyl-(3′→5′)-5-fluoro-2′-deoxyuridine (dCydPam-*P*-FdUrd) and 2′-deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluoro-N^4-octadecylcytidine (5-FdUrd-*P*-FdCydOct) caused marked cytotoxicity with IC_{50} values of 3–4 μM. 5-FdUrd-*P*-FdCydOct at 200 μM was capable of eradicating 100% of tumour cells whereas 10% of the cells were resistant to 5-FdUrd. Cytotoxicity was caused by a dramatic S-phase arrest, resulting in an increase of this cell population from 34% to 85% with 5-FdUrd-*P*-FdCydOct and to 81% with dCydPam-*P*-FdUrd. S-phase arrest was followed by apoptosis, as shown by 85% of the cells staining positive for Apo 2.7 antibody, a six- to eight-fold increased caspase 3 activity and DNA fragmentation. Thymidylate synthase activity was inhibited by 50% at 0.6–0.7 μM dimer concentration. The dimers were hydrolysed in vitro by phosphodiesterase I and human serum to the corresponding nucleosides and nucleoside monophosphates. ***Conclusions:*** The new dimers dCydPam-*P*-FdUrd and 5-FdUrd-*P*-FdCydOct are effective prodrugs of 5-FdUrd and have potential value for the treatment of p53-mutated and hormone-independent human prostate carcinomas.

Key words 5-Fluorodeoxyuridine · Heterodinucleoside dimers · Prodrugs · Prostate cancer · Cytotoxicity · Cell cycle · Apoptosis

Abbreviations *5-FdUrd* 5-fluoro-2′-deoxyuridine · *dCydPam*-P-*FdUrd* N^4-palmitoyl-2′-deoxycytidylyl-(3′→5′)-5-fluoro-2′-deoxyuridine · *5-FdUrd*-P-*dCydOct* 2′-deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluoro-N^4-octadecylcytidine · *5-FUra* fluorouracil · *5-FdUrd2′*P 5-fluoro-2′-deoxyuridine monophosphate

Introduction

World-wide prostate cancer incidence and mortality rates have been increasing over the past several decades. Nearly 30% of all cancer cases and the most frequently diagnosed among men in the United States are prostate tumours. They rank second after lung cancer as the underlying cause of neoplastic death in men (Boring et al. 1994). Despite initial surgery or radiation therapy, the disease recurs in many patients. Standard androgen-ablation therapy with orchiectomy or luteinizing-hormone-releasing hormone agonists can produce significant responses, but they tend to be of short duration. Androgen-responsive tumours contain an androgen-independent population before the initiation of hormone therapy and this population emerges as a result of selection when androgen is withdrawn (Schulze

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et al. 1987; Carter and Isaacs 1988). Resistance to androgen ablation develops eventually in nearly all cases (McDonnell et al. 1992). Despite the testing of numerous drugs and drug combinations, chemotherapy has only limited success when given after the failure of androgen ablation (Kreis 1995).

Fluorouracil (5-FUra) has antitumour activity against many solid tumours, including breast, gastrointestinal, head and neck, and ovarian carcinomas. To exert their cytotoxic effects the fluoropyrimidines require intracellular activation. Three mechanisms of action are responsible for the effect of 5-FUra. First, it is converted to 5-fluoro-2′-deoxyuridine (5-FdUrd) by thymidine phosphorylase. Subsequent phosphorylation of 5-FdUrd by thymidine kinase results in formation of the active metabolite 5-fluoro-2′-deoxyuridine monophosphate (5-FdUrd2′*P*). This molecule forms a stable covalent complex with thymidylate synthase, inhibiting the enzyme activity and leading to depletion of deoxythymidine triphosphate, a precursor required for DNA synthesis. Secondly, 5-FUra may be anabolised to 5-fluorouridine monophosphate, which is further metabolised to 5-fluorouridine triphosphate. The latter can be incorporated into RNA or converted to the deoxyribonucleotide 5-FdUrd2′*P*. Thirdly, 5-FdUrd2′*P* may subsequently be phosphorylated to 5-fluoro-2′-deoxyuridine 5′-triphosphate, which is incorporated into DNA leading to strand breaks (Kinsella et al. 1997).

Most prostate cancers eventually develop resistance to hormonal therapy and chemotherapy regimens. Resistance to 5-FUra can develop by deletion of one of the key enzymes required for its activation or by mutations in the p53 gene (Aas et al. 1996; Vikhanskaya et al. 1998). In patients with metastatic prostate cancer, mutations of this gene are seen more commonly than in those with primary tumours (Navone et al. 1993). Loss of p53 function facilitates tumour cell progression through the cell cycle and renders induction of apoptosis difficult. Isaacs and co-workers (1991) demonstrated that growth of prostate cells with p53 mutations can be inhibited by introduction of the wild-type gene. However, in tumour cells that are p53-null or have a mutated p53 gene, apoptosis can occur in a p53-independent manner (Shao et al. 1996; Peled et al. 1996; Vikhanskaya et al. 1998). Loss of p53 function could cause resistance to DNA-damaging agents, as a consequence of abrogation of p53-dependent apoptosis. Thus, the identification of new agents able to trigger p53-independent apoptosis may be of clinical relevance seen the commonly occurring loss of p53 function in many tumours (Zunino et al. 1997).

N^4-Palmitoyl-2′-deoxycytidylyl-(3′→5′)-5-fluoro-2′-deoxyuridine (dCydPam-*P*-FdUrd) and 2′-deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluoro-N^4-octadecylcytidine (5-FdUrd-*P*-FdCydOct) are new dinucleoside phosphate derivatives of 5-FdUrd (Schott et al. 1997). These dimers contain 5-FdUrd2′*P* which is the primary metabolite in the phosphorylation chain of 5-FdUrd. In comparison to highly hydrophilic dinucleoside phosphate compounds, which have octanol/water partition coefficients (log *P*) around 0.01, the dimers are amphiphilic/lipophilic with coefficients ranging from 0.1 to 5, which demonstrates their high affinity to lipid membranes. Accordingly, the dimers can easily be incorporated at active concentrations into liposome membranes. We expect, that after cellular uptake, the 5′-monophosphate is released by enzymatic cleavage. Consequently, low activities of nucleoside-5′-monophosphate kinases, causing resistance of the tumours to chemotherapeutic agents, could be circumvented by these dimers, resulting in increased antitumour activities.

In the present study, we investigated the cellular pharmacology of the new anticancer drugs 5-FdUrd-*P*-FdCydOct and dCydPam-*P*-FdUrd in comparison to 5-FdUrd by studying their cytotoxic effects, cell-cycle perturbations and their capacity to induce apoptosis in vitro in the androgen-independent and p53-mutated human prostate tumour cell line DU-145.

Materials and methods

Reagents, drugs and antibodies

Bovine serum albumin (BSA), 5-bromo-2′-deoxyuridine (BrdUrd), propidium iodide, Triton X-100, acid-washed activated charcoal, dUMP, 4′,6-diamidino-2-phenylindole (DAPI), the anti-α-tubulin and the goat anti-(mouse IgG-Cy3) antibodies were purchased from Fluka Chemie (Buchs, Switzerland). Phalloidin-oregon green, SYTOX Green and SYBR Green II were from Molecular Probes (Eugene, Ore., USA) and the 123-bp marker from Gibco (Paisley, UK). RPMI-1640 medium, fetal bovine serum, penicillin/streptomycin, L-glutamine, Hank's balanced salt solution (HBSS) and 2% agar gel were from Life Technologies (Basel, Switzerland). Trypsin/EDTA was obtained from Biochrom KG (Berlin, Germany) and phosphodiesterase I from Sigma (Buchs, Switzerland). The WST-1 assay kit, RNase A and proteinase K were from Boehringer Mannheim (Rotkreuz, Switzerland), Tween 20 from Merck (Darmstadt, Germany) and T-70 dextran from Pharmacia (Dübendorf, Switzerland). Digitonin and Mowiol were purchased from Calbiochem (Juro Supply AG, Lucerne, Switzerland). The monoclonal phycoerythrin(PE)-labelled Apo 2.7 antibody was from IL Instrumentation Laboratory AG (Zurich, Switzerland). The fluorescein-isothiocyanate(FITC)-labelled anti-BrdUrd antibody and the Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-AMC(7-amino-4-methylcoumarin)) fluorogenic substrate were from Becton-Dickinson (Basel, Switzerland). The amphiphilic heteronucleoside dimers dCydPam-*P*-FdUrd and 5-FdUrd-*P*-FdCydOct (Fig. 1) were synthesised according to the methods described previously (Schott et al. 1997). 5-FdUrd was obtained from Hoffman La-Roche, Basel, Switzerland. The drugs were dissolved in 0.9% NaCl.

Cells

The human epithelial prostate tumour cell line DU-145 was obtained from the German Collection of Microorganisms and Cell Cultures, DSMZ, Braunschweig, Germany. The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in a humidified 5% CO_2 atmosphere at 37 °C.

Cytotoxicity assay

To evaluate cell proliferation the WST-1 kit was used. Exponentially growing cells were seeded in sterile 96-well plates and

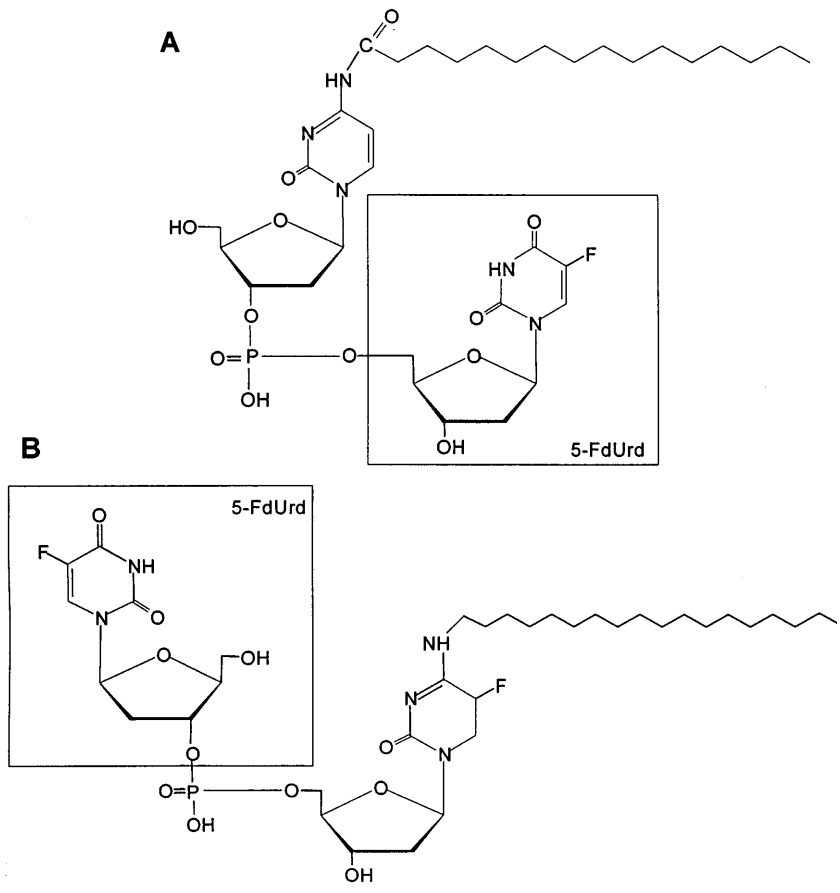

Fig. 1A, B Chemical structures of the heterodinucleoside phosphate dimers. **A** N^4-Palmitoyl-2′-deoxycytidylyl-(3′→5′)-5-fluoro-2′-deoxyuridine (dCyd-Pam-*P*-FdUrd); **B** 2′-deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluoro-N^4-octadecylcytidine (5-FdUrd-*P*-FdCydOct). The masked 5-fluoro-2′-deoxyuridine (5-FdUrd) is shown in the *rectangle*

incubated for 24 h. Drugs were added to a final concentration of 12–200 μM. The supernatant was removed after 96 h and 100 μl freshly diluted WST solution was added. The plates were incubated for 30–60 min at 37 °C in a humidified 5% CO_2 atmosphere. Cell viability was evaluated by measurement of the absorption at 450 nm in a Dynatech MR5000 plate reader (Microtec Produkte, Embrach, Switzerland); 50% growth-inhibitory concentrations (IC_{50}) were calculated from interpolations of the graphical data.

Cell-cycle distribution analysis

Cells were seeded in 100-mm culture dishes, incubated for 48 h and exposed to various concentrations (0–200 μM) of dimers and 5-FdUrd for 24 h at 37 °C (5% CO_2) or for various periods (0–48 h) with 50 μM of the drugs. After the specified period, the cells were incubated with 10 μM BrdUrd for 30 min at 37 °C (5% CO_2). The supernatant with dead cells and the harvested living cells were fixed in pre-cooled (−20 °C) ethanol (80%) and stored at −20 °C for up to 3 days. BrdUrd/propidium iodide staining was carried out as described previously (Horber et al. 1995). Briefly, after centrifugation, the cells were treated with 2 M HCl for 30 min at 20 °C and re-suspended in 50 μl phosphate buffered saline (PBS), 0.5% Tween-20, 1% BSA and incubated with FITC-labelled anti-BrdUrd antibody for 30 min at 20 °C; 1 ml PBS/propidium iodide (10 μg/ml) was then added. Stained cells were analysed with an Epics Elite Analyser (Coulter, Florida, USA). Singly fluorescent samples (FITC or propidium iodide) were used to optimise instrument settings and ensure proper electronic compensation.

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 permeabilised by incubation on ice for 20 min with 100 μg/ml digitonin in PBS supplied with 2.5% fetal bovine serum (v/v) and 0.01% NaN_3 (PBSF). After permeabilisation the cells were labelled with PE-conjugated Apo 2.7 for 15 min at room temperature in the dark. For flow-cytometric analysis cells were re-suspended in PBSF and stored on ice in the dark until analysis.

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 NaH_2PO_4, 10 mM $Na_4P_2O_7$. After centrifugation (5 min, 1400*g*) 100 μl cell lysate was reacted with 20 μM Ac-DEVD-AMC fluorogenic substrate in 20 mM HEPES, pH 7.5, 10% glycerol, 2 mM dithiothreitol for 2 h at 37 °C. AMC released from Ac-DEVD-AMC was measured by a spectrofluorometer (Kontron SFM 23/23 LC) with excitation and emission wavelengths of 380 nm and 440 nm respectively.

DNA fragmentation

Cells were exposed for various periods (0–96 h) to 50 μM 5-FdUrd, dCydPam-*P*-FdUrd and 5-FdUrd-*P*-FdCydOct at a 37 °C (5% CO_2). DNA extraction was performed with modifications as described by Kaufmann (1989). Briefly, supernatants and harvested cells were pooled, washed once with PBS and lysed in 300 μl lysis buffer (0.5 M TRIS/HCl pH 9.0, 2 mM EDTA, 10 mM NaCl, 1% sodium dodecyl sulfate, 0.33 mg/ml proteinase K). The samples were incubated at 55 °C for 24 h, extracted twice with phenol/chloroform (1:1, v/v) and once with chloroform. The probes were then incubated with 300 μg/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, Calif., USA) using a SYBR Green filter (530DF30).

Immunofluorescence labelling and confocal microscopy

Cells were incorporated in a 4:1 (v/v) mixture of rat tail collagen type I (50 000 cells/100 µl) and RPMI 10× medium supplemented with 292 mM $NaHCO_3$ and 75 mM NaOH. The collagen/cell suspensions (20 µl) were seeded on Permanox chamber slides (Life Technologies, Basel, Switzerland). After solidification of the collagen at 37 °C, the cells were incubated for 1 week in medium (37 °C, 5% CO_2). Consecutively, they were then treated with 50 µM 5-FdUrd-*P*-FdCydOct or 0.5 µg/ml colcemide for 5 days. To preserve the structural organisation of mictotubules, the cells were washed in a microtubule-protective (MT) buffer (Schliwa et al. 1981), permeabilised for 15 min with 1% Triton X-100 in MT buffer, and fixed for 30 min with 3% para-formaldehyde in MT buffer at room temperature; they were then treated with 0.1 M glycine in PBS at 4 °C for 15 min. For triple staining, cells were incubated twice with the first and second antibody at 4 °C. Antibodies were diluted in PBS containing 3% bovine serum albumin: anti-α-tubulin 1:500 (v/v) goat anti-(mouse IgG-Cy3) 1:50 (v/v). For phalloidin/oregon green the dilution was 1:10 (v/v) and for the DAPI stain 1:100 (v/v). Alternatively cell nuclei were stained with SYTOX Green overnight at 4 °C after permeabilisation. The dilution of the dye was 1:5000 (v/v) in buffer 10 mM TRIS pH 7.4, 1 mM EDTA. Cells were embedded in Mowiol and covered by a cover slip. The samples were analysed on a Zeiss LSM 410 inverted microscope.

Thymidylate synthase activity

The activity of thymidylate synthase was measured by the release of tritium from [5-^{3}H]dUMP. Cells were seeded in 6-well plates and incubated for 48 h. After exposure to the drugs for 90 min at different concentrations (0.01–100 µM) or for various times (0–8 h) with 0.1 µM at 37 °C (5% CO_2) the cells were treated with deoxyuridine 5′-monophosphate (10 µM) trace-labelled with 0.5 µCi/ml [5-^{3}H]deoxyuridine 5′-monophosphate (Amersham Pharmacia Biotech, Dübendorf, Switzerland) (Ju et al. 1998). After incubation at 37 °C for 60 min, 0.2 ml medium was removed and added to 1 ml 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, 4400*g*) and the radioactivity of the supernatant was determined in a liquid scintillation counter (1900 TR Packard). IC_{50} values were calculated from interpolations of the graphical data.

In vitro hydrolysis of the dimers by phosphodiesterase I and human serum

The stability of 5-FdUrd-*P*-FdCydOct and dCydPam-*P*-FdUrd against hydrolysis by phosphodiesterase I and human serum was analysed as described before (Schott et al. 1999). Briefly, 100 µl dimers (5 mg/ml water) were treated for 2, 4, 12 and 36 h at 37 °C with phosphodiesterase I (0.1 M $MgCl_2$, pH 8.1) or human serum. The reaction mixtures were analysed by reverse-phase HPLC using a binary gradient of 0.05 M ammonium acetate and methanol as eluent. The dimer dThd-dThd was used as reference and the hydrophilic metabolites were identified by corresponding reference compounds.

Results

Inhibition of cell growth

The DU-145 human prostate tumour cells were grown for 96 h in the presence and absence of the drugs. The antiproliferative effect of the new derivatives was determined by measuring cell viability. dCydPam-*P*-FdUrd and 5-FdUrd-*P*-FdCydOct exerted strong cytotoxic effects on DU-145 cells (Fig. 2A). The IC_{50} values of the drugs are listed in the legend to Fig 2A,B. They show that dCydPam-*P*-FdUrd and 5-FdUrd-*P*-FdCydOct have a toxicity comparable to that of 5-FdUrd. The latter reached maximal growth inhibition at a concentration of 12 µM. Higher concentrations did not-produce additional cytotoxicity. In contrast, the cytotoxicity of 5-FdUrd-*P*-FdCydOct increased continuously with higher concentrations, resulting in complete cell death at 200 µM, whereas dCydPam-*P*-FdUrd was slightly less active. The time-dependent cytotoxic activity of the various drugs at 50 µM on DU-145 cells is shown in Fig. 2B. All the drugs tested behaved in a similar way, reaching 50% growth inhibition after approximately 30 h incubation. At 200 µM and 24 h incubation a cytotoxic effect of approximately 80% was obtained with 5-FdUrd-*P*-FdCydOct, whereas the effects of 200 µM 5-FdUrd or dCydPam-*P*-FdUrd were

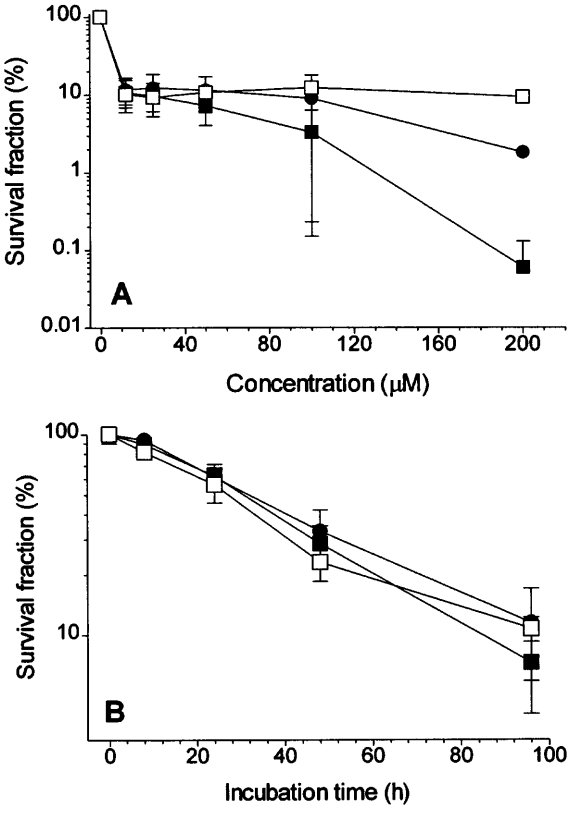

Fig. 2A, B WST-1 cell proliferation assay in DU-145 cells. **A** Cells were treated for 96 h at 12–200 µM. □ 5-FdUrd (IC_{50} = 3.35 ± 0.04 µM), ● dCydPam-*P*-FdUrd (IC_{50} = 3.59 ± 0.47 µM), ■ 5-FdUrd-*P*-FdCydOct (IC_{50} = 3.48 ± 0.32). At 12 µM all drugs produced 90% cytotoxicity. 5-FdUrd-*P*-FdCydOct reached a cytotoxicity of 100% at a concentration of 200 µM. **B** Cells were treated with 50 µM for various periods. The drugs reached 50% cytotoxicity after approximately 30 h of incubation. Results are shown as means and standard deviations of at least three independent experiments performed in triplicate

Table 1 Time-dependent effect of 5-FdUrd and the dimers (50 μM) on cell-cycle distribution. Data shown are the mean percentage cell-cycle distribution of two separate experiments performed in duplicate. SD < 10% of mean value. For structures of the drugs see Fig. 1

Drug	Incubation (h)	Cell-cycle distribution (%)			
		G1	$S+S_{arrested}$	S0	G2/M
5-FdUrd	0	51.2	34.7	13.8	14.1
	8	43.4	50.9	27.2	5.7
	24	13.4	82.9	62.3	3.7
	48	39.5	56.7	33.8	3.8
dCydPam-*P*-FdUrd	0	51.2	34.7	13.8	14.1
	8	45.2	50.8	28.3	4.0
	24	12.6	84.2	67.0	3.2
	48	36.3	57.6	41.8	6.1
5-FdUrd-*P*-FdCydOct	0	51.2	34.7	13.8	14.1
	8	46.5	48.8	29.1	4.6
	24	10.7	87.5	72.5	1.8
	48	38.7	57.7	44.9	3.6

not notably different from those with 50 μM (data not shown).

Cell-cycle arrest

Growth arrest after drug treatment occurred in early S phase (SO), as shown by cell-cycle analysis. The typical cell-cycle distribution patterns in DU-145 cells for untreated cells and for cells treated with 50 μM drug are shown in Table 1. After drug exposure for 8 h the first effects were observed and, after 24 h, 88% of the cells treated with 5-FdUrd-*P*-FdCydOct were in S phase compared to only 35% of untreated cells. Correspondingly, dCydPam-*P*-FdUrd and 5-FdUrd increased the S phase population from 35% to 84% and 83% respectively. The dramatic S-phase arrest always correlated with an increase in early S-phase cells, which was accompanied by a decreased proportion of cells in the G1 and G2/M phase. Exposure of the cells to 100 μM for 24 h increased the S-phase population to 90%–93% with all drugs (data not shown). After prolonged exposure to the drug for 48 h the S-phase cells decreased to about 50% with all drugs, probably because of the death of cells arrested in the S phase after 24 h (cf. Table 1). The results from these experiments indicate that the drugs exerted their cytotoxicity by inhibition of DNA synthesis. 5-FdUrd is known to cause DNA strand breaks after incorporation. Thus, it is conceivable that the dimers are cleaved into 5-FdUrd and the second nucleotide, leading to DNA strand breaks and/or to the inhibition of thymidylate synthase.

Induction of apoptosis

The monoclonal Apo 2.7 antibody, which reacts preferentially with cells undergoing apoptosis (Zhang et al. 1996) was used to quantify apoptotic cells. The histograms in Fig. 3A depict the time-dependent increase of apoptotic cells after treatment with dCydPam-*P*-FdUrd at 50 μM. Figure 3B summarises the time-dependent induction of apoptosis as determined by flow-cytometric analysis in untreated cells and cells treated with 5-FdUrd and the dimers. After 24 h incubation, the apoptotic cell fraction amounted to 10% after drug treatment whereas it was less than 2% in untreated control cells. Prolonged incubation for 72 h increased the fractions of apoptotic cells drastically to 70%–80% and to nearly 90% after 96 h of incubation. Control cells did not show any changes. We further analysed the

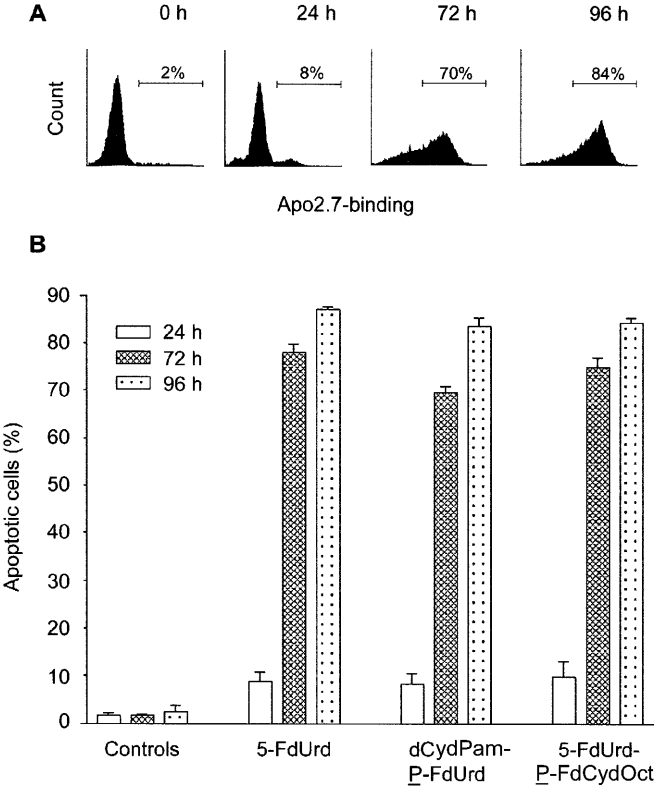

Fig. 3A, B Induction of apoptosis by 50 μM 5-FdUrd and the heterodinucleoside phosphate dimers in DU-145 cells after various incubation times. **A** Flow-cytometric detection of apoptotic cells by phycoerythrin-labelled Apo 2.7 (*Apo2.7-PE*) staining in untreated cells (control) and in cells treated for up to 96 h with 50 μM dCydPam-*P*-FdUrd. Apoptotic cell death was quantified by gating the Apo2.7-PE-positive cell population. **B** Apoptotic cell fraction after various incubation times following treatment with 50 μM drugs

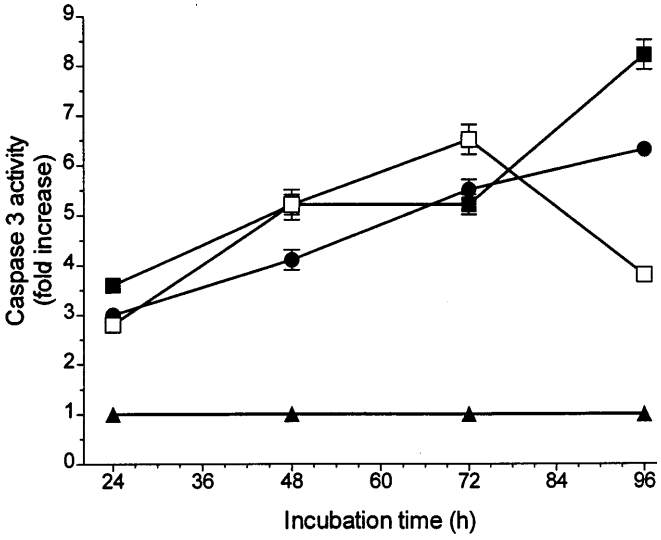

Fig. 4 Increased caspase 3 activity in DU-145 cells treated with 50 μM drugs after various incubation times. Treatment with 5-FdUrd (□), dCydPam-*P*-FdUrd (●) and 5-FdUrd-*P*-FdCydOct (■) resulted in caspase 3 activation. While caspase 3 activity induced by 5-FdUrd declined after 72 h of incubation, the dimers caused a further increase in caspase 3 activity. Results are given as increase of activity compared to untreated control cells (▲). Results are shown as means and standard deviations of one representative experiment performed in duplicate

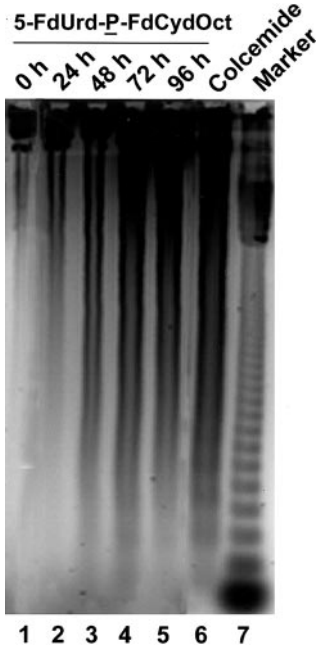

Fig. 5 Endonucleolytic DNA fragmentation in DU-145 cells induced by incubation with 50 μM 5-FdUrd-*P*-FdCydOct (*lanes 2–5*) for various periods (24–96 h). Agarose gel electrophoresis was used for the detection of DNA fragmentation. Untreated cells (*1*) did not show DNA fragmentation. Colcemide (*6*) was used as positive control for apoptosis. As marker a 123-bp ladder was used (*7*)

effects of the drugs on activation of caspase proteases. The proteolytic cleavage of the caspase substrate poly(ADP-ribose) polymerase after drug treatment at 50 μM for increasing lengths of time, is shown in Fig. 4. All the drugs induced time-dependent poly(ADP-ribose) polymerase cleavage in DU-145 cells. Treatment for 4 h resulted in a 2.8- to 3.6-fold increase of caspase 3 activity. It increased 5.2- to 6.5-fold after 72 h when 5-FdUrd reached its highest activity. With 5-FdUrd-*P*-FdCydOct and dCydPam-*P*-FdUrd a further 8.2- and 6.3-fold increase was observed after 96 h.

DNA fragmentation

To ascertain further that the new dimers induced apoptosis in DU-145 cells, DNA analysis was performed. As shown in Fig. 5, DNA fragmentation began 24 h after exposure with 5-FdUrd-*P*-FdCydOct at 50 μM and was more pronounced after longer incubation times. Untreated cells did not show an apoptotic DNA pattern. Colcemide was included as a positive control. 5-FdUrd and dCydPam-*P*-FdUrd had similar effects on DNA fragmentation (data not shown).

Disruption of the cytoskeleton and formation of apoptotic nuclei

To illustrate the morphological changes after treatment, DU-145 cells were embedded in a three-dimensional collagen I matrix and analysed by confocal laser scanning microscopy. As shown in Fig. 6, we examined the structure of the microtubules, actin filaments and nuclei of untreated control cells (Fig. 6A–D) and compared this to cells treated with 5-FdUrd-*P*-FdCydOct (50 μM, 120 h; Fig. 6E–H) and colcemide (0.5 μg/ml, 120 h; Fig. 6I–M). 5-FdUrd-*P*-FdCydOct-treated tumour cells (Fig. 6G–H) did not proliferate as untreated cells did (Fig. 6C–D). Compared with the controls (Fig. 6A), where cells had a well-developed cytoskeleton, treatment with 5-FdUrd-*P*-FdCydOct resulted in a complete disruption of actin filaments (Fig. 6E) as revealed by phalloidin/oregon green staining. Likewise the structure of the microtubules was disrupted (Fig. 6F). Fluorescence microscopy of cells stained with the DNA fluorochrome DAPI (Fig. 6G) or with SYTOX Green (Fig. 6H) revealed the presence of apoptotic nuclei with condensed and fragmented DNA in drug-treated DU-145 cells. Apoptotic nuclei were observed only sporadically in control cells (Fig. 6C). Colcemide-treated cells were included as the positive control for apoptosis, showing complete cytoskeleton disruption (Fig. 6I–K) and DNA fragmentation (Fig. 6L–M).

Inhibition of thymidylate synthase activity

The chemotherapeutic effect of 5-FdUrd is primarily due to the inhibition of thymidylate synthase by 5-FdUrd2′*P*. Measurement of thymidylate synthase activity in DU-145 cells in situ indicated that all tested compounds inhibited this activity (Fig. 7). dCydPam-*P*-FdUrd and 5-FdUrd-

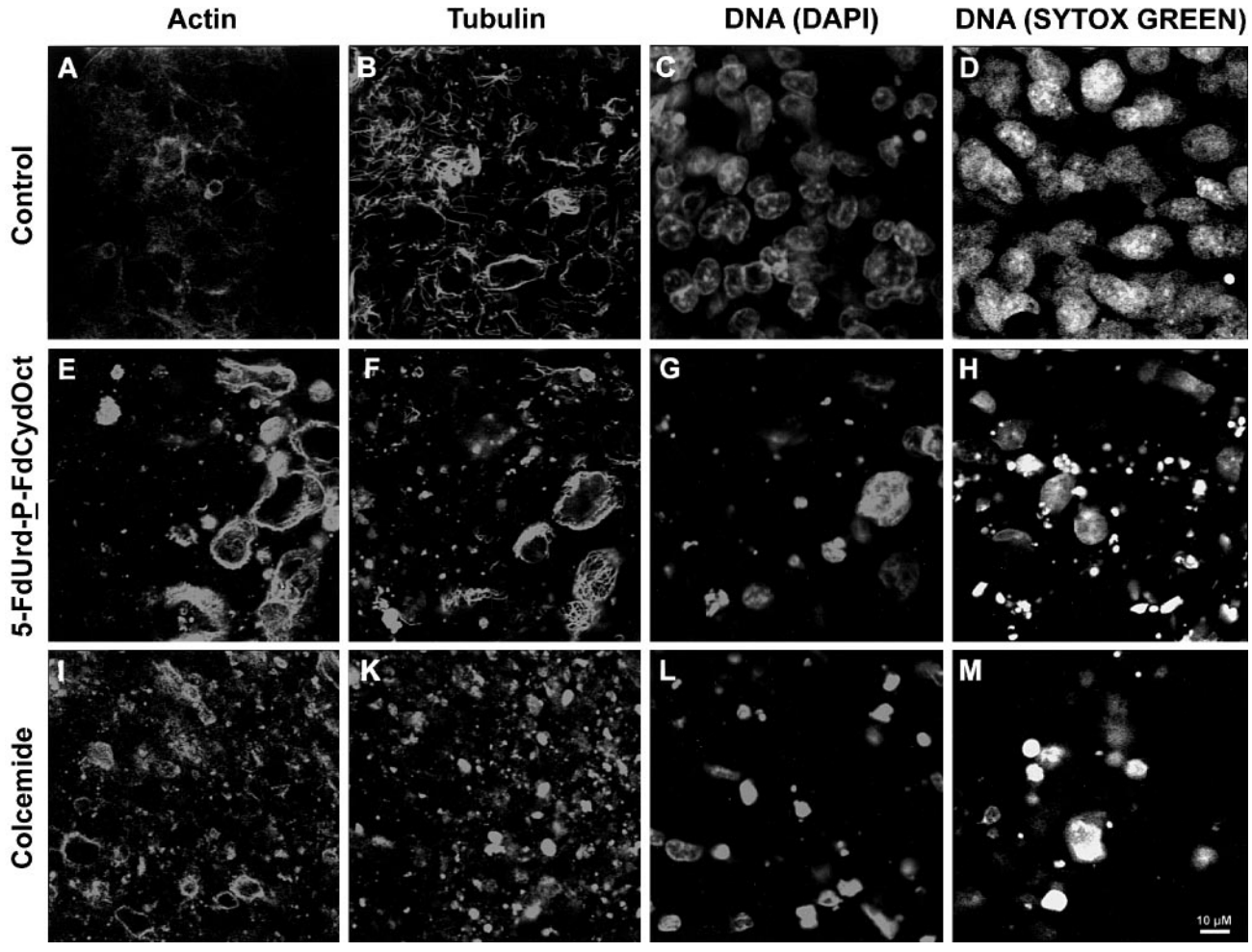

Fig. 6A–M Confocal laser scanning microscopy was used to detect structural changes after drug treatment. 5-FdUrd-*P*-FdCydOct (50 µM, 120 h; **E–H**) and colcemide (0.5 µg/ml, 120 h; **I–M**) induced apoptosis in collagen-embedded DU-145 cells. The cells were triple-stained for F-actin (**A**, **E**, **I**), α-tubulin (**B**, **F**, **K**) and cell nuclei with DAPI (**C**, **G**, **L**). Alternatively, cell nuclei were stained with SYTOX Green (**D**, **H**, **M**). Treated cells were reduced in number and displayed morphological changes, in contrast to untreated cells (**A–D**). After drug treatment the structure of actin (**E**, **I**) and tubulin (**F**, **K**) was completely disrupted. Nuclei of treated cells were fragmented into characteristic apoptotic bodies (**G–H**; **L–M**), whereas nuclei of control cells remained dense and fragmentation was seen only rarely (**C–D**). All pictures represent single optical sections

P-FdCydOct were effective as inhibitors of thymidylate synthase and complete enzyme inhibition was reached after 90 min incubation with 10 µM dimers. 5-FdUrd had the same effect at a concentration of 0.1 µM. The concentrations at which thymidylate synthase activity was inhibited by 50% are given in the legend of Fig. 7.

In vitro hydrolysis of the dimers by phosphodiesterase I and human serum

As summarised in Table 2, the dimers were hydrolysed in vitro by phosphodiesterase I and serum to the active compounds 5-FdUrd and 5-FdUrd2′*P*, demonstrating

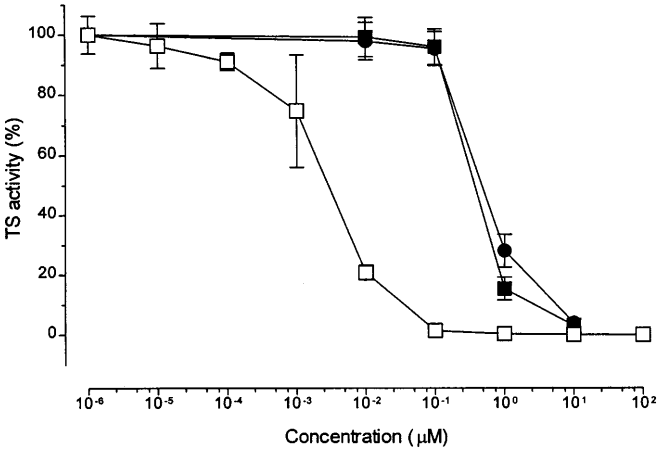

Fig. 7 Thymidylate synthetase (*TS*) enzyme activity in DU-145 cells was inhibited by the heterodinucleoside phosphate dimers in a dose-dependent manner (90 min incubation). Complete inhibition is reached with the dimers at 10 µM and with 5-FdUrd at 0.1 µM. Values are shown as percentages of the untreated control value. Data are means and SD of two independent experiments performed in duplicate. □ 5-FdUrd (IC_{50} = 0.005 ± 0.003 µM), ● dCydPam-*P*-FdUrd (IC_{50} = 0.710 ± 0.085 µM), ■ 5-FdUrd-*P*-FdCydOct (IC_{50} = 0.615 ± 0.049 µM)

Table 2 In vitro hydrolysis of the dimers by phosphodiesterase I and human serum

Dimer	Phosphodiesterase I		Human serum	
	Hydrolysis (h)	Metabolites	Hydrolysis (h)	Metabolites
dThd-dThd	2	dThd, pdThd	5	dThd
5-FdUrd-*P*-FdCydOct	6	5-FdUrd, *P*-FdCydOct	9.5	5-FdUrd, 5-FdUrdCydOct
dCydPam-*P*-FdUrd	4	5-FdUrd, 5-FdUrd2′*P*	7.5	dCd, 5-FdUrd

The time for full hydrolysis is shown

that the cytotoxic compounds 5-FdUrd and 5-FdUrd2′*P* can be released from the dimers.

Discussion

In this study we have examined new dinucleosides of 5-FdUrd for their in vitro effects on hormone-resistant and p53-negative DU-145 human prostate tumour cells. 5-FUra has previously been associated with therapeutic benefit for different cancers including breast, gastrointestinal, head and neck, and ovarian carcinomas. Specifically, 5-FUra inhibits cell proliferation predominantly through depletion of thymidine followed by S phase arrest. Accordingly, we observed a dramatic arrest of DU-145 cells in the early S phase after treatment with the two dimers. As demonstrated by flow cytometry, the effect of the dimers was time-dependent (Fig. 3A, B). Strong inhibition of cell proliferation in DU-145 cells in a time- and dose dependent manner was observed after treatment with the new dimers. The dimers had cytotoxic effects comparable to those of the parent drug 5-FdUrd; however, at the concentration of 200 μM and after 96 h exposure, 5-FdUrd-*P*-FdCydOct was capable of inducing a toxicity of 100%. Consequently, this new derivative of 5-FdUrd is also cytotoxic for the 10% of cells that seem to remain resistant to 5-FdUrd (Fig. 2A). Our results demonstrate that 5-FdUrd-*P*-FdCydOct is capable of inducing complete cell death in p53-independent DU-145 cells, though at rather high concentrations. This is an important finding, taking into consideration the fact that mutation in *p53* and changes in the expression of this gene, frequently mutated in advanced prostate cancer, can lead to resistance to some forms of chemotherapy.

The better effect of the dimers, and primarily of 5-FdUrd-*P*-FdCydOct, at higher concentrations and longer incubation periods can possibly be explained by the prodrug nature of the dimers, resulting in persisting intracellular concentrations of the active metabolites released from the dimers over longer periods as compared to 5-FdUrd. These data are supported by the finding, that the derivatives inhibited thymidylate synthase activity as 5-FdUrd does. The delayed inhibition of thymidylate synthase with the dimers suggests again that 5-FdUrd has to be released from the dimers to exert an inhibitory action on the thymidylate synthetase. The hydrolyis of the dimers by phosphodiesterase I and human serum further indicate the prodrug nature of the dimers. It remains to be investigated whether the dimers are taken up unchanged by cells and whether they are degraded intracellularly to yield active metabolites such as 5-FdUrd or their corresponding monophosphosphates.

In cancer patients the parent drug 5-FdUrd is administered at 20–80 μM/m^2 (5–20 mg/m^2) as a continuous infusion over 14–21 days with mild to moderate toxic effects. 5-FdUrd has a short plasma half-life of 20 min, which makes the continuous application schedule necessary. Hence, the amphiphilic dimers may have more favourable in vivo properties as a result of the expected changes of their pharmacokinetic properties and their prodrug nature. However, it will be important to demonstrate that, with the dimers, plasma concentrations comparable to those of the parent drug can be reached in vivo without causing untolerable toxic effects. In our previous studies, similar heterodinucleoside phosphate dimers composed of the antivirally active nucleosides azidothymidine and dideoxycytidine were found to have significantly different pharmacokinetic properties from those of the parent hydrophilic nucleosides and excellent antiviral effects in the murine Rauscher leukaemia virus model (Peghini et al. 1998; Schwendener et al. 1994).

A conceivable reason why 5-FdUrd-*P*-FdCydOct is more potent than the other two drugs could be that the molecule contains not only the masked 5-FdUrd or its monophosphate (5-FdUrd2′*P*) but also an additional molecule with a potential cytotoxic activity, namely the 5-FdCydOct moiety of the dimer. With a similar lipophilic molecule, N^4-octadecyl-1-β-D-arabinofuranosylcytosine we have identified the hydrophilic metabolites arabinosylcytosine and arabinosyluracil which were formed by metabolic cleavage of the alkyl chain from the parent molecule (Koller-Lucae et al. 1999). Thus, it might be possible that, in 5-FdUrd-*P*-FdCydOct, the alkyl chain can also be cleaved and, after an additional oxidation reaction, 5-FdUrd and 5-FdUrd2′*P* are formed.

Apoptosis has emerged as a significant therapeutic target for the effective elimination of cancer cells (Carson and Ribeiro 1993; Fisher 1994). Endonucleolytic DNA fragmentation studies showed that the drugs induced apoptosis after incubations of 24 h. Simulta-

neously the activity of caspase 3 was three to four times higher than in untreated control cells. The further exposure of the cells to the dimers resulted in a linear increase of caspase 3 activity for up to 96 h (6- to 8-fold increase), whereas 5-FdUrd induced maximal caspase 3 activity after 72 h (6.5-fold increase) followed by a decline at 96 h. Nevertheless, the apoptotic cell fraction increases after treatment with 5-FdUrd, as shown in Fig. 3B. Thus, it seems that the dimers continue to exert their cytotoxic activity on caspase while 5-FdUrd-induced caspase 3 activity is decreasing. Confocal microscopy confirmed further the induction of apoptosis after treatment with 5-FdUrd-*P*-FdCydOct, showing cells with characteristic apoptotic bodies. Not only DAPI but also the novel fluorescent dye SYTOX Green were used for the detection of apoptotic bodies by confocal microscopy. Both dyes intercalate with DNA. The use of SYTOX Green resulted in superior staining of the condensed and fragmented DNA that is present in apoptotic cells. 5-FdUrd-*P*-FdCydOct induced the formation of apoptotic bodies; probably a late effect of apoptosis meant that the actin-filaments and microtubules were disrupted.

The comparison of the time-dependent studies of apoptosis induction with cell-cycle distribution shows that the inhibition of DNA synthesis in DU-145 cells is measurable 8 h after exposure to the dimers, whereas apoptotic cells appear only after longer-lasting drug exposure. A reason for this could be the absence of wild-type p53. Lane (1993) demonstrated that the p53 tumour-suppressor gene is an essential component of the apoptotic pathway induced by genotoxic insults. Therefore, it can be assumed that, together with the supposed slow transformation of the dimers into active metabolites, the absence of wild-type p53 in DU-145 cells was a contributing factor for the long induction times. Accordingly, we found similar incubation times after treatment with the same dimers in human prostate tumour PC-3 cells, also known to lack p53 expression for induction of apoptosis (Cattaneo-Pangrazzi 2000). With other chemotherapeutic drugs similar results have been reported (Borner et al. 1995; Liang et al. 1999). Not all forms of apoptosis are p53-dependent (Clarke et al. 1993). Owing to the fact that many cancer malignancies have mutated p53, which is associated with chemotherapeutic resistance, the development of new agents able to trigger p53-independent apoptosis may be of clinical relevance (Zunino et al. 1997).

In conclusion, our findings are evidence that the new amphiphilic dimers containing 5-FdUrd are able to overcome 5-FdUrd resistance in p53- and androgen-independent DU-145 cells. The new dimers exert a cell-cycle-phase-dependent cytotoxicity and are able to induce p53-independent apoptosis through the activation of caspase 3. Therefore they may have a potential value as new therapeutic agents against hormone-refractory prostate carcinoma. The investigation of the effects of the dimers on cell-cycle regulation and p53-independent apoptotic pathways will further characterise these new compounds.

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