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Induction of Cell Cycle-Dependent Cytotoxicity and Apoptosis by New Heterodinucleoside Phosphate Dimers of 5-Fluorodeoxyuridine in PC-3 Human Prostate Cancer Cells

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ABSTRACT. Fluorodeoxyuridine (5-FdUrd) is an antineoplastic agent with clinical activity against different types of solid tumours. To enhance the effectiveness of this drug, we have synthesised new heterodinucleoside phosphate dimers of 5-FdUrd. These dimers were compared to 5-FdUrd for their cytotoxic effect and the cell cycle dependence of cytotoxicity, as well as for their capacity to induce apoptosis and inhibit thymidylate synthetase (TS) in androgen-independent human PC-3 prostate tumour cells. Incubation of the cells with the dimers N^4-palmitoyl-2′-deoxycytidylyl-(3′→5′)-5-fluoro-2′-deoxyuridine (dCpam-5-FdUrd) and 2′-deoxy-5-flourouridylyl-(3′→5′)-2′-deoxy-5-fluoro-N^4-octadecylcytidine (5-FdUrd-5-FdC18) resulted in a marked cytotoxicity with IC_{50} values of 4 μM, similar to 5-FdUrd. In contrast to 5-FdUrd, 100% toxicity was achieved with concentrations of 100–200 μM 5-FdUrd-5-FdC18. Flow cytometric analysis revealed an increase in the cell population in S-phase after treatment with 5-FdUrd, 5-FdUrd-5-FdC18, and dCpam-5-FdUrd from 36 to 63%, 50%, and 77%, respectively. dCpam-5-FdUrd was more potent than 5-FdUrd in arresting the cell cycle. Significant S-phase arrest was indicated by a decreased proportion of cells in G1- and G2/M-phases. Cell cycle arrest and inhibition of cell proliferation were followed by apoptosis, as shown by a 6- to 8-fold increased binding of Apo2.7 antibody, a 9- to 11-fold increase in caspase-3 activity, DNA fragmentation, and by cell morphology showing the appearance of apoptotic bodies. Importantly, 5-FdUrd-5-FdC18 increased the number of apoptotic cells to 160% compared to 5-FdUrd under the same conditions. As with 5-FdUrd, the two dimers also inhibited TS in a time- and concentration-dependent manner, although requiring 100-fold higher concentrations. In conclusion, dCpam-5-FdUrd and 5-FdUrd-5-FdC18 exert stronger cytotoxicity and induce more S-phase arrest and apoptosis than does 5-FdUrd in PC-3 cells, suggesting their potential role in the treatment of human prostate cancer. BIOCHEM PHARMACOL **60**;12:1887–1896, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. 5-fluorodeoxyuridine; dimeric prodrugs; prostate cancer; cytotoxicity; apoptosis; thymidylate synthetase

Prostate cancer incidence has been increasing in most developed countries. In the United States, it has become the most common cancer and is second only to lung cancer as cause of cancer deaths [1]. Most cancers are diagnosed when they are locally advanced or metastatic. The androgen-dependent cancer cells initiate an apoptotic cascade in human prostate cancer upon androgen ablation. However, the androgen-independent cells continue to proliferate [2] and are typically refractory to cytotoxic drugs. PC-3 is a commonly used cell line in human prostate research which is androgen-unresponsive and, as a result of a mutated gene, null of p53 protein. This cell line is used as representative of hormone-refractory human prostate cancer.

5-FU,‖ resembling the pyrimidine bases uracil and thymine, was specifically synthesised for clinical use. Currently, colon, breast, and ovarian cancers are treated with this drug. After rapid transporter-mediated cell uptake, the effect of 5-FU depends on the ability of tumour cells to form the active nucleoside 5-FdUrd, the nucleotides 5-FdUMP

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‖ *Abbreviations:* 5-FU, fluorouracil; dCpam-5-FdUrd, N^4-palmitoyl-2′-deoxycytidylyl-(3′→5′)-5-fluoro-2′-deoxyuridine; 5-FdUrd, 5-fluoro-2′-deoxyuridine; 5-FdUrd-5-FdC18, 2′-deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluro-N^4-octadecylcytidine; 5-FdUTP, 5-fluoro-2′-deoxyuridine-5′-triphosphate; FBS, fetal bovine serum; BrdUrd, 5-bromo-2-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; Apo, Apo2.7-PE monoclonal antibody; PE, phycoerythrin, FITC, fluorescein isothiocyanate; PARP, poly-(ADP-ribose) polymerase; Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; PI, propidium iodide; and TS, thymidylate synthestase.

and 5-FUMP, and the corresponding triphosphates 5-FdUTP and 5-FUTP. The triphosphates compete with their normal counterparts for incorporation into RNA and DNA, but the chemotherapeutic effect of 5-FdUrd is primarily due to the inhibition of TS by 5-FdUMP. This enzyme catalyses the reductive methylation of dUMP to dTMP. For DNA replication and repair, this reaction constitutes the sole intracellular *de novo* source of thymidylate [3]. Predominantly rapidly proliferating cells require TS, thereby representing an important target for cancer chemotherapy [4].

To enhance the cytotoxic activity of 5-FdUrd, we have developed a new strategy for masking nucleoside phosphates by the synthesis of amphiphilic dinucleoside phosphates [5]. These dimers contain 5-FdUMP, which is the primary metabolite in the phosphorylation chain of 5-FdUrd. The amphiphilic/lipophilic nature provides the dimers with new pharmacokinetic properties. We expect that after cellular uptake the 5′-monophosphate is released by enzymatic cleavage. Consequently, low activities of nucleoside-5′-monophosphate kinases could be circumvented by these dimers, resulting in increased antitumour activities.

In the present study, we examined two dimers of 5-FdUrd for their effect on cell survival, their cell cycle dependence on cytotoxicity, and for their capacity to induce apoptosis in PC-3 cells. Furthermore, we analysed the ability of these compounds to inhibit TS and therefore reduce or affect DNA synthesis through the depletion of thymidine. Our findings suggest that these novel dimers may be potential therapeutic agents for the treatment of hormone-refractory prostate tumours.

MATERIALS AND METHODS

Materials

BSA, BrdUrd, PI, Triton X-100, acid-washed activated charcoal, dUMP, DAPI, the anti-α-tubulin, and the goat anti-mouse immunoglobulin IgG-Cy3 antibodies were purchased from Fluka. Phalloidin-Oregon Green and SYBR Green II were from Molecular Probes. RPMI-1640 medium, FBS, penicillin–streptomycin, L-glutamine, Hanks' balanced salt solution, and agar gel 2% were from Life Technologies. Trypsin–EDTA was obtained from Biochrom KG. RNAse A (EC 3.1.27.5), proteinase K (EC 3.4.21.14), and the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,2 benzene disulphonate (WST-1) assay kit were from Boehringer Mannheim, Tween from Merck, and T-70 dextran from Pharmacia. Digitonin and Mowiol were purchased from Calbiochem. The monoclonal Apo2.7-PE antibody was from IL Instrumentation Laboratory AG. The FITC-labelled anti-BrdUrd antibody and the Ac-DEVD-AMC fluorogenic substrate were from Becton Dickinson.

The heteronucleoside dimers dCpam-5-FdUrd and 5-FdUrd-5-FdC18 (Fig. 1) were synthesised according to the methods described previously [5]. Briefly, the dimers were obtained according to the phosphotriester method, starting from 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 for lipid membranes. Accordingly, the dimers can easily be incorporated at active concentrations into liposome membranes. 5-FdUrd was obtained from Hoffman La-Roche. In this study, the drugs were dissolved in 0.9% NaCl.

Cell Culture

The human epithelial prostate tumour cell line PC-3 was obtained from the German Collection of Microorganisms and Cell Cultures, DSMZ. The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM L-glutamine in a humidified 5% CO_2 atmosphere at 37°.

Cytotoxicity Assay

Cell proliferation was evaluated by using the WST-1 cell proliferation assay kit. 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. Ninety-six hours later, the supernatant was removed and 100 μL of freshly diluted WST solution was added. The plates were incubated for 30–60 min at 37° in a humidified 5% CO_2 atmosphere. Cell viability was evaluated by measurement of the absorption at 450 nm using a Dynatech MR5000 plate reader (Microtec). IC_{50} values were calculated from interpolations of the graphical data.

Cell Cycle Analysis

Cells were seeded in 100-mm culture dishes and incubated for 48 hr. Cells were exposed to various concentrations (0–200 μM) of dimers and 5-FdUrd for 24 hr at 37° (5% CO_2) 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 BrdUrd for 30 min at 37° (5% CO_2). The supernatant with dead cells and the harvested living cells were fixed in precooled (−20°) ethanol (80%) and stored at −20° for up to 3 days. BrdUrd/PI staining was carried out as described previously [6]. Briefly, after centrifugation, the cells were treated with 2 M HCl for 30 min at 20° and re-suspended in 50 μL PBS + 0.5% Tween 20 + 1% BSA and incubated with FITC-labelled anti-BrdUrd antibody for 30 min at 20° followed by addition of 1 mL PBS + PI (10 μg/mL). Stained cells were analysed with an Epics Elite Analyzer (Coulter). Single fluorescent samples (FITC or PI) were used to optimise instrument settings and ensure proper electronic compensation.

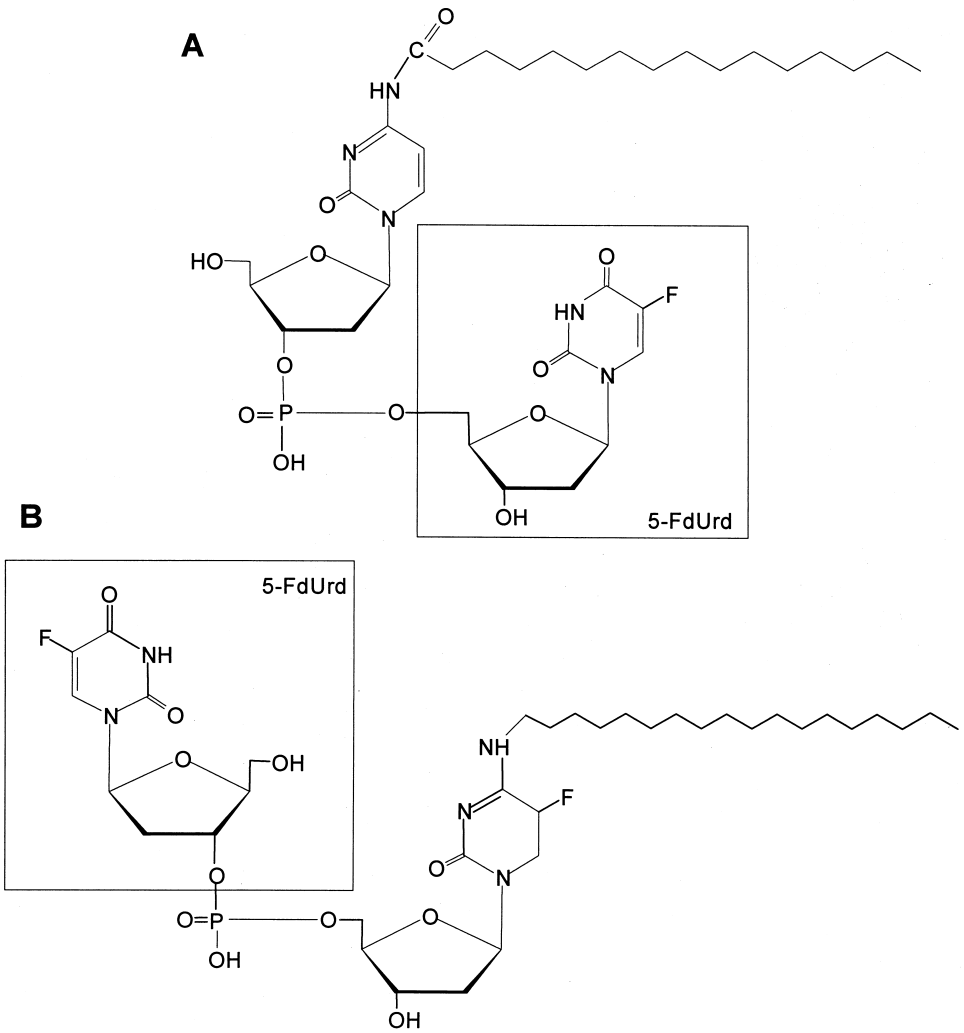

FIG. 1. Chemical structures of the heterodinucleoside phosphate dimers. (A) N^4-palmitoyl-2′-deoxycytidylyl-(3′→5′)-5-fluoro-2′-deoxyuridine (dCpam-5-FdUrd) and (B) 2′-deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluoro-N^4-octadecylcytidine (5-FdUrd-5-FdC18). The masked 5-fluoro-2′-deoxyuridine (5-FdUrd) is shown in the rectangle.

Quantification of Apoptosis

Cells were treated as described for the cell cycle analysis. After the incubation period, 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 of digitonin in PBS supplied with 2.5% FBS (v/v) and 0.01% NaN_3. After permeabilisation, the cells were labelled with Apo2.7-PE for 15 min at room temperature in the dark. For flow cytometric analysis, cells were resuspended in PBS supplied with 2.5% FBS (v/v) and 0.01% NaN_3 and stored on ice in the dark until analysis.

PARP Cleavage

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_2O7, After centrifugation (5 min, 1400 *g*), 100 μL of the cell lysate was reacted with 20 μM PARP (Ac-DEVD-AMC) fluorogenic substrate in 20 mM HEPES, pH 7.5, +10% glycerol, +2 mM dithiothreitol for 2 hr at 37°. Released AMC from Ac-DEVD-AMC was measured using a spectrofluorometer (Kontron SFM 23/23 LC) with excitation and emission wavelengths of 380 and 440 nm, respectively.

DNA Fragmentation

Cells were exposed for various time periods (0–96 hr) to 50 μM 5-FdUrd, dCpam-5-FdUrd, and 5-FdUrd-5-FdC18 at 37° (5% CO_2). Colcemide (5 μg/mL) was used as a positive control. DNA extraction was performed as described before [6]. For detection, SYBR Green II dyed gels were scanned at 488 nm on a FlourImager 595 (Molecular Dynamics) using an SYBR Green filter (530DF30).

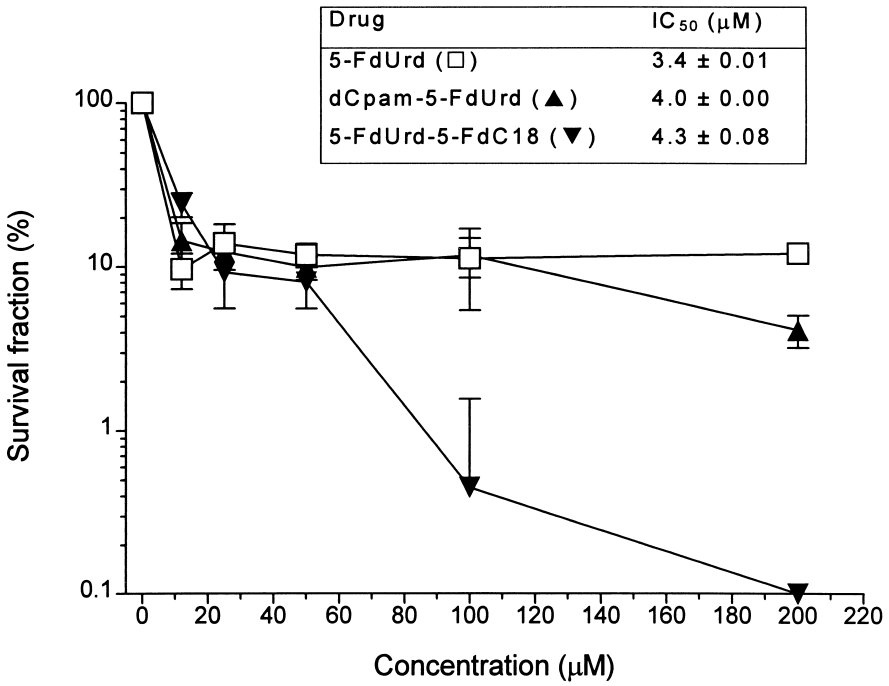

FIG. 2. Cytotoxicity of the heterodinucleoside phosphates on PC-3 cells. Cells were treated for 96 hr at 12–200 μM. At 12 μM, dCpam-5-FdUrd had 85–90% cytotoxicity compared to 5-FdUrd. In contrast, 5-FdUrd-5-FdC18 reached a cytotoxicity of 100% at a concentration of 100 and 200 μM, respectively. Results are shown as means and SD of at least three independent experiments performed in triplicate.

Confocal and Electron Microscopy

Cells were incorporated in a 4:1 (v/v) mixture of rat tail collagen (50,000 cells/100 μL) and RPMI 10× supplemented with 292 mM $NaHCO_3$ and 75 mM NaOH. The collagen–cell suspensions (20 μL) were seeded on Permanox chamber slides (Life Technologies). After solidification of the collagen at 37°, the cells were incubated for 1 week in medium (5% CO_2, 37°). Consecutively, they were treated with 50 μM 5-FdUrd-5-FdC18 for 5 days. To preserve the structural organisation of microtubules, cells were washed in a microtubule protective buffer (MT buffer) [7], permeabilised for 15 min with 1% Triton X-100 in MT buffer, fixed for 30 min with 3% paraformaldehyde in MT buffer at room temperature, followed by treatment with 0.1 M glycine in PBS at 4° for 15 min. The cells were stained with anti-α-tubulin and goat anti-mouse immunoglobulin IgG-Cy3 antibodies, with Phalloidin-Oregon Green, and with DAPI. Cells were embedded in Mowiol and analysed on a Zeiss LSM 410 inverted microscope. For electron microscopy, PC-3 cells were seeded in 100-mm culture dishes, incubated for 48 hr, and then exposed to 50 μM 5-FdUrd-5-FdC18 for 48 hr at 37° (5% CO_2). Control cells were not treated. The supernatant with dead cells and the trypsinised living cells were pooled. Cells were fixed with 2.5% glutaraldehyde and embedded in Epon. Thin sections were stained with 0.5% uranyl acetate and lead citrate. After preparation, cells were examined on a Philips CM 10 electron microscope.

TS Assay

Activity of TS was measured by the release of tritium from [5-^{3}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 nM to 100 μM) or for various time periods (0–8 hr) with 0.1 μM at 37° (5% CO_2), the cells were treated with deoxyuridine-5′-monophosphate (10 μM) trace-labelled with 0.5 μCi/mL of [5-^{3}H]deoxyuridine-5′-monophosphate (Amersham Pharmacia Biotech) [8]. After incubation at 37° for 60 min, 0.2 mL medium was 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, 4400 × *g*) and the radioactivity of the supernatant determined in a liquid scintillation instrument (1900 TR Packard).

RESULTS

Cytotoxicity of dCpam-5-FdUrd and 5-FdUrd-5-FdC18

The cytotoxic effects of the dimers compared to 5-FdUrd in PC-3 cells after a continuous 96-hr incubation are shown in Fig. 2. IC_{50} values of the drugs are listed in the inset on Fig. 2, showing that dCpam-5-FdUrd and 5-FdUrd-5-FdC18 have IC_{50} values comparable to 5-FdUrd. However, in contrast to 5-FdUrd, 100% toxicity was achieved at concentrations of 100 and 200 μM, respectively, after 96-hr incubation with 5-FdUrd-5-FdC18. The time-dependent

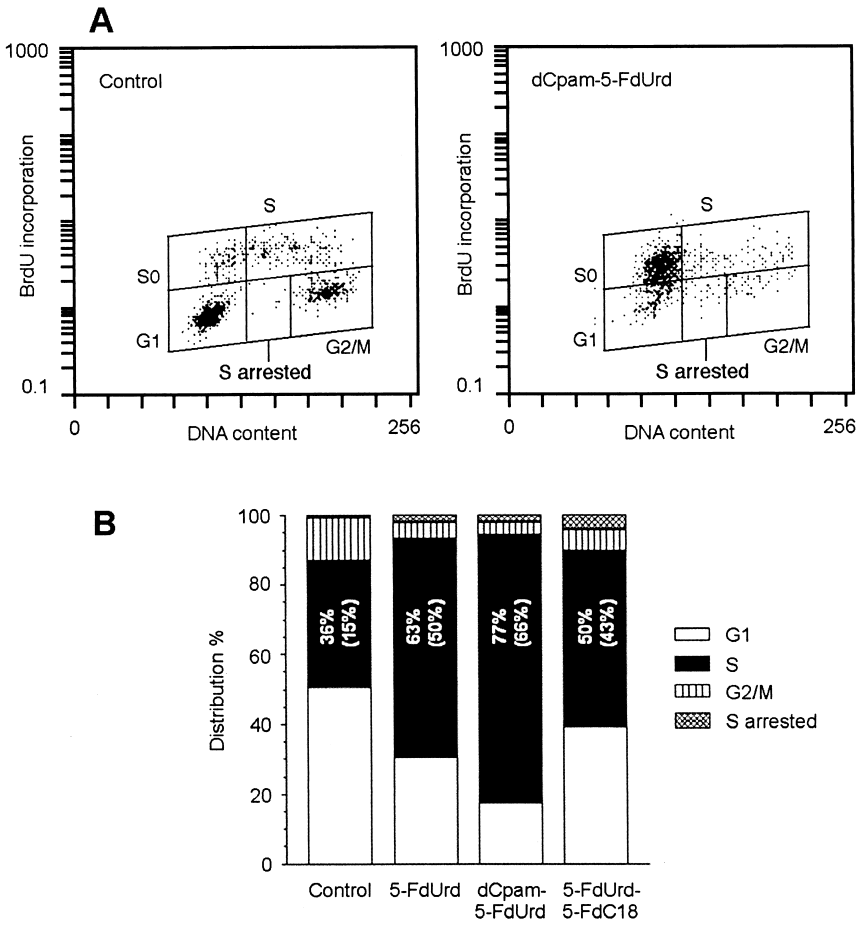

FIG. 3. The heterodinucleoside phosphate dimers cause alteration in cell cycle distribution of PC-3 cells. (A) Cell cycle distribution of untreated cells (left panel) and cells treated with 50 μM dCpam-5-FdUrd for 24 hr (right panel) showing a marked cell cycle arrest in the early S-phase (S_0). Data are shown as contour plots with DNA content on the x-axis (PI staining) and BrdUrd content on the y-axis (BrdUrd–FITC antibody). G1-, S-, and G2/M-phase distribution was quantified by gating the cell populations. (B) Cell cycle distribution after 24-hr incubation with 50 μM drugs. Like 5-FdUrd, dCpam-5-FdUrd and 5-FdUrd-5-FdC18 caused an increase in S-phase cells (total S-phase cell fraction in percent). The increase in S-phase cells always correlated with an increase in early S-phase (S_0; percent in brackets). Data are means of at least two independent experiments performed in duplicate.

cytotoxicity of the various drugs at 50 μM on PC-3 cells showed that after a short incubation of 8 hr, 5-FdUrd was more toxic than the dimers, resulting in survival fraction of 50.4 ± 2.4%. At this time point, 5-FdUrd-5-FdC18 and dCpam-5-FdUrd had a survival fraction of 67.3 ± 6.8% and 79.4 ± 3.6%. Both dimers reached 80% toxicity of 5-FdUrd after 24 hr, which increased to 90% after 48-hr incubation with the various drugs (data not shown). It is conceivable that the dimers are more effective at higher concentrations and longer incubation periods because of their prodrug nature, resulting in persisting intracellular drug concentrations over longer time periods as compared to 5-FdUrd. After 24-hr incubation, the cytotoxic effects of 200 μM 5-FdUrd or dCpam-5-FdUrd were not significantly different from those with 50 μM, whereas toxicity of 92.8 ± 5.2% was obtained with 200 μM 5-FdUrd-5-FdC18 (data not shown).

Induction of Cell Cycle Arrest with dCpam-5-FdUrd and 5-FdUrd-5-FdC18

Since the new anticancer dimers were found to inhibit cell growth, it was of interest to determine whether the dimers would interfere with cell cycle progression. The cell cycle was analysed by flow cytometry (Fig. 3A), showing the marked cell cycle arrest in the early S-phase caused by treatment with dCpam-5-FdUrd (50 μM, 24 hr). The individual changes in phase distribution after 24-hr incubation with the various drugs at a concentration of 50 μM are summarised in Fig. 3B. The dimers dCpam-5-FdUrd, 5-FdUrd-5-FdC18, and 5-FdUrd caused a marked reduction in G1- and G2/M-phase cells and an increase in S-phase cells after 24-hr incubation. The increase in S-phase cells represents a stop in early S-phase, which we designated as S_0 (Fig. 3B, values in brackets). S_0-phase cells belong to the early S-phase based on their DNA content. They incorpo-

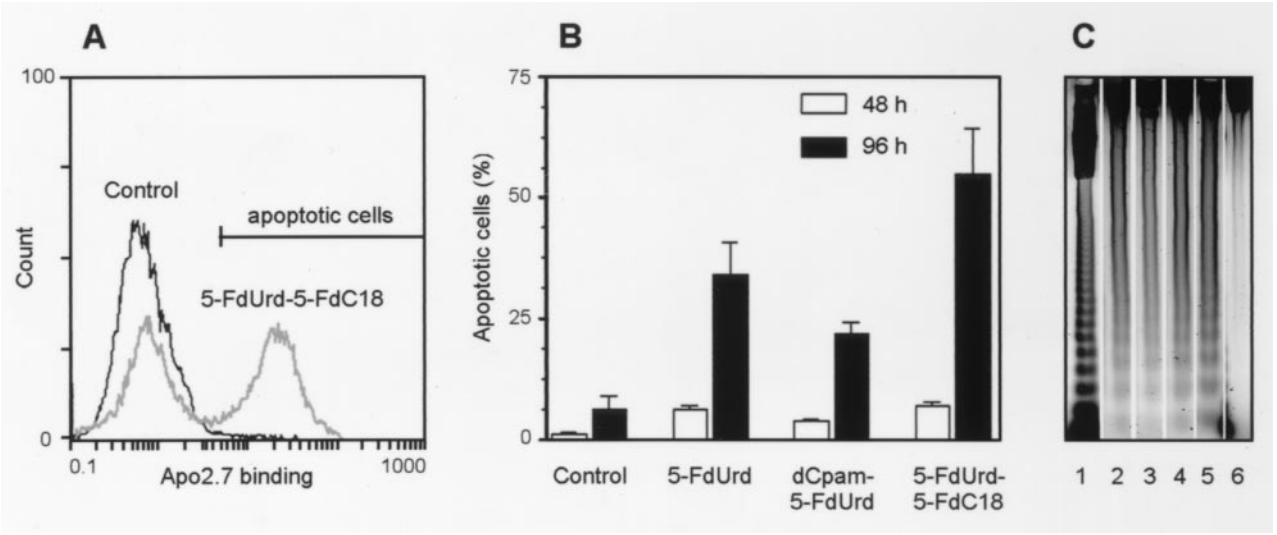

FIG. 4. Incubation with heterodinucleoside phosphate dimers induced apoptosis in PC-3 cells. (A) Flow cytometric detection of Apo2.7-PE staining in untreated cells (control) and in cells treated for 96 hr with 50 μM 5-FdUrd-5-FdC18. Apoptotic cell death was quantified by gating the Apo2.7-PE-positive cell population. (B) Apoptotic cell fraction after 48- and 96-hr drug treatment at 50 μM. (C) DNA fragmentation: 123-pb marker, lane 1; 5-FdUrd, lane 2; dCpam-5-FdUrd, lane 3; 5-FdUrd-5-FdC18, lane 4. Colcemide (lane 5) was used as positive control for apoptosis. Untreated cells (lane 6) did not shown DNA fragmentation. Results are shown as means and SD of two separate experiments performed in duplicate.

rate BrdUrd into DNA without DNA elongation, probably due to strand breaks. 5-FdUrd-5-FdC18 had strong effect, arresting cells in the S-phase (S-arrested). A linear increase in these cells, which do not incorporate BrdUrd, from 0.3 ± 0.1 to 6.1 ± 1.0% after 48 hr was observed. In comparison with 5-FdUrd and dCpam-5-FdUrd, this cell population increased by more than 200% (data not shown).

Induction of Apoptosis of dCpam-5-FdUrd and 5-FdUrd-5-FdC18

Quantification of apoptotic cells was carried out with the Apo2.7 monoclonal antibody that reacts preferentially with cells undergoing apoptosis [9]. The various effects on induction of apoptosis by the drugs are summarised in Fig. 4. Characteristic staining of apoptotic cells with the Apo2.7 antibody for untreated cells and cells treated with 50 μM 5-FdUrd-5-FdC18 are shown in Fig. 4A. After 48- or 96-hr incubation time, 1.2 ± 0.2% and 6.1 ± 2.8%, respectively, of untreated cells (control) were apoptotic, whereas drug treatment increased the apoptotic cell fraction to 3–7% after 48 hr and to 20–55% after 96 hr (Fig. 4B). 5-FdUrd-5-FdC18 was nearly twice as effective as 5-FdUrd, inducing 55% apoptotic cells in comparison to only 34% induced by 5-FdUrd. Apoptosis was confirmed by agarose gel electrophoresis as shown in Fig. 4C, demonstrating the characteristic DNA ladders after drug treatment. Colcemide-treated cells were included as a positive control for DNA fragmentation.

To determine whether the derivatives lead to the activation of caspase proteases, we studied the proteolytic cleavage of the caspase substrate PARP, which is involved in DNA repair. The drugs induce a time-dependent PARP cleavage in PC-3 cells. Treatment for 24 to 96 hr with 5-FdUrd and 5-FdUrd-5-FdC18 resulted in a linear caspase-3 activation, whereas induction with dCpam-dCpam-5-FdUrd was delayed by 24 hr. After 48 hr, the caspase-3 activity was 2- to 4-fold increased and after 72 hr 5 to 8 times. The highest cleavage activity was seen after 96 hr of incubation, resulting in a 9- to 11-fold increase in caspase-3 activity in comparison to the untreated control (data not shown).

Changes in Cellular and Nuclear Morphology

To examine the structure of the microtubules, actin filaments, and the nuclei, we compared untreated control cells (Fig. 5, A, C, and E) with 5-FdUrd-5-FdC18-treated cells (Fig. 5, B, D, and F) by confocal laser scanning microscopy. In contrast to untreated cells, drug-treated tumour cells embedded in collagen did not proliferate. Compared with the controls, where cells had a well-developed cytoskeleton of actin, PC-S cells treated for 120 hr with 50 μM 5-FdUrd-5-FdC18 had completely disrupted actin filaments (Fig. 5, C and D), as visualised by Phalloidin-Oregon Green staining. The microtubules remained unaltered. Fluorescence microscopy of cells stained with the DNA fluorochrome DAPI revealed the presence of apoptotic nuclei with condensed and fragmented DNA in PC-3 cells treated with 5-FdUrd-5-FdC18 (Fig. 5F). Apoptotic nuclei were observed only sporadically in control cells (Fig. 5E).

The morphological changes in PC-3 cells after a 48-hr exposure to 5-FdUrd-5-FdC18 at 50 μM were also assessed by electron microscopy (Fig. 5, G and H). About 10% of the analysed cells displayed the characteristic formation of

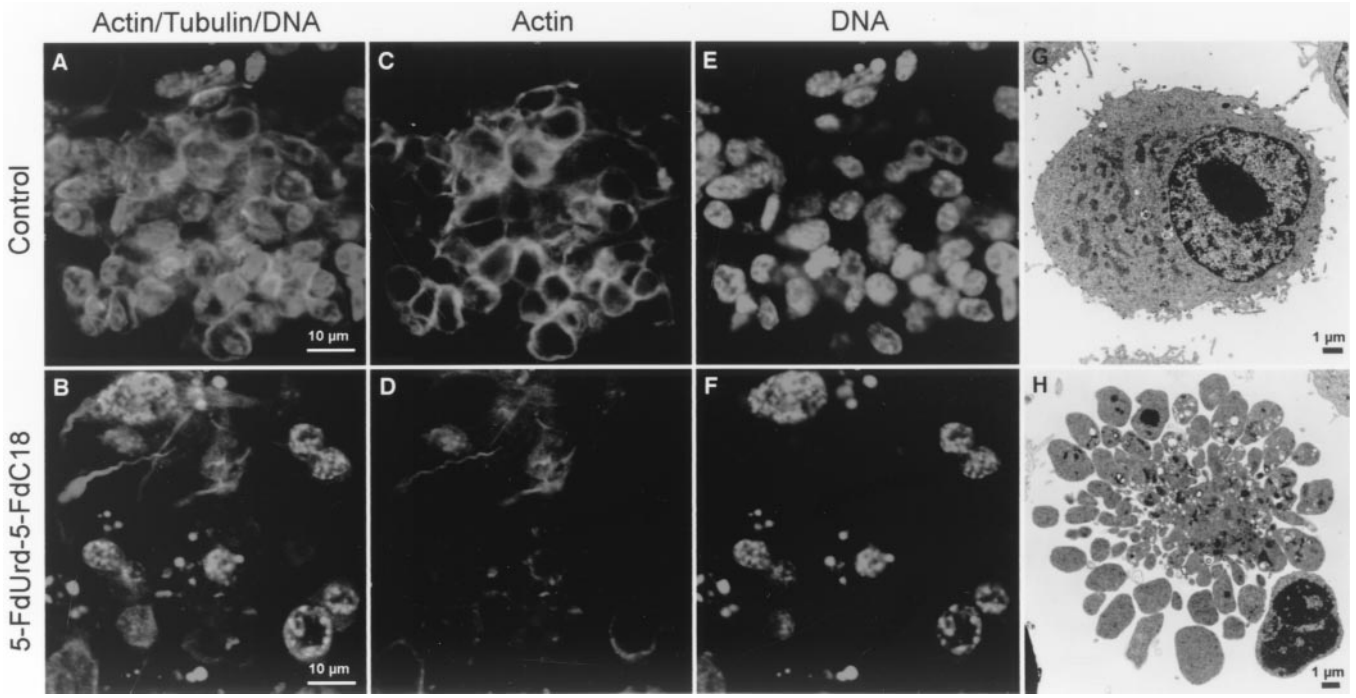

FIG. 5. Collagen-embedded PC-3 cells treated with 5-FdUrd-5-FdC18 (50 μM, 120 hr) showed apoptotic bodies in confocal microscopy. The cells were triple-stained for F-actin (green; A–D), α-tubulin (red; A and B), and cell nuclei (blue; A, B, E, F). Untreated cells are shown in panels A, C, and E and cells treated with 5-FdUrd-5-FdC18 in B, D, and F. Treated cells were reduced in numbers and displayed morphological changes. After drug treatment, the structure of actin was completely disrupted (D) in comparison to control cells (C). Nuclei of treated cells were fragmented into characteristic apoptotic bodies (F), whereas nuclei of control cells remained dense and fragmentation was seen only rarely (E). All pictures represent single optical sections. G, electron micrographs of an untreated PC-3 cell and (H) a cell treated with 50 μM of 5-FdUrd-5-FdC18 for 48 hr. The latter is fragmented into vesicles with various densities, which contain morphologically unaltered organelles such as mitochondria and endoplasmatic reticulum.

apoptotic bodies, containing condensed chromatin and intact organelles such as mitochondria and endoplasmatic reticulum. In the depicted cell in Fig. 5H, the cytoplasm was almost completely fractionated into vesicles. Control cells (Fig. 5G) did not display abnormal chromatin condensation or apoptotic bodies. Less than five percent of the control cells had disrupted membranes and organelles, the characteristic features of late apoptosis or necrosis.

TS Inhibition by dCpam-5-FdUrd and 5-FdUrd-5-FdC18

The chemotherapeutic effect of 5-FdUrd is primarily due to the inhibition of TS by 5-FdUMP. Measurement of TS activity in PC-3 cells *in situ* indicated that all tested compounds inhibited TS activity in a time- as well as in a dose-dependent manner (Fig. 6). dCpam-5-FdUrd and 5-FdUrd-5-FdC18 were effective as inhibitors of TS activity, and a nearly complete inhibition was reached after 90-min incubation with 1 μM of the dimers (Fig. 6A). 5-FdUrd had the same effect at the 100-fold lower concentration of 0.01 μM (Fig. 6A). Moreover, in the time-dependent experiments with drug concentrations of 0.1 μM, 5-FdUrd reacted rapidly, reaching 95% inhibition of TS activity already after 30 min (Fig. 6B), whereas dCpam-5-FdUrd reached 50% inhibition only after 3 hr and 5-FdUrd-5-FdC18 after 5 hr. The delayed inhibition of TS with the dimers again suggests that 5-FdUrd has to be released from the dimers to exert an inhibitory action on the thymidylate synthetase.

DISCUSSION

Increased proliferation and decreased cell death (apoptosis) are two major processes that contribute to the progression of tumour cell growth. Consequently, agents that can inhibit cell proliferation or induce apoptosis are of great therapeutic value in preventing tumour cell growth. In the present study, we have evaluated and compared the effect of two new agents on cell growth and apoptosis in hormone-independent PC-3 cells. Our results show that compared to the clinically used 5-FdUrd, these novel antitumour agents exert stronger cytotoxicity due to a drastic cell cycle arrest and apoptosis.

Specifically, 5-FU inhibits cell proliferation predominantly through thymidine depletion followed by S-phase arrest. Accordingly, we observed that dimer-treated PC-3 cells were arrested in the early S-phase, whereas untreated control cells showed no change in cell cycle distribution. The effect of the dimers was seen after 8-hr incubation and increased up to 48 hr as demonstrated by flow cytometry analysis. The new amphiphilic heterodinucleoside phosphate dimers of 5-FdUrd strongly inhibited cell prolifera-

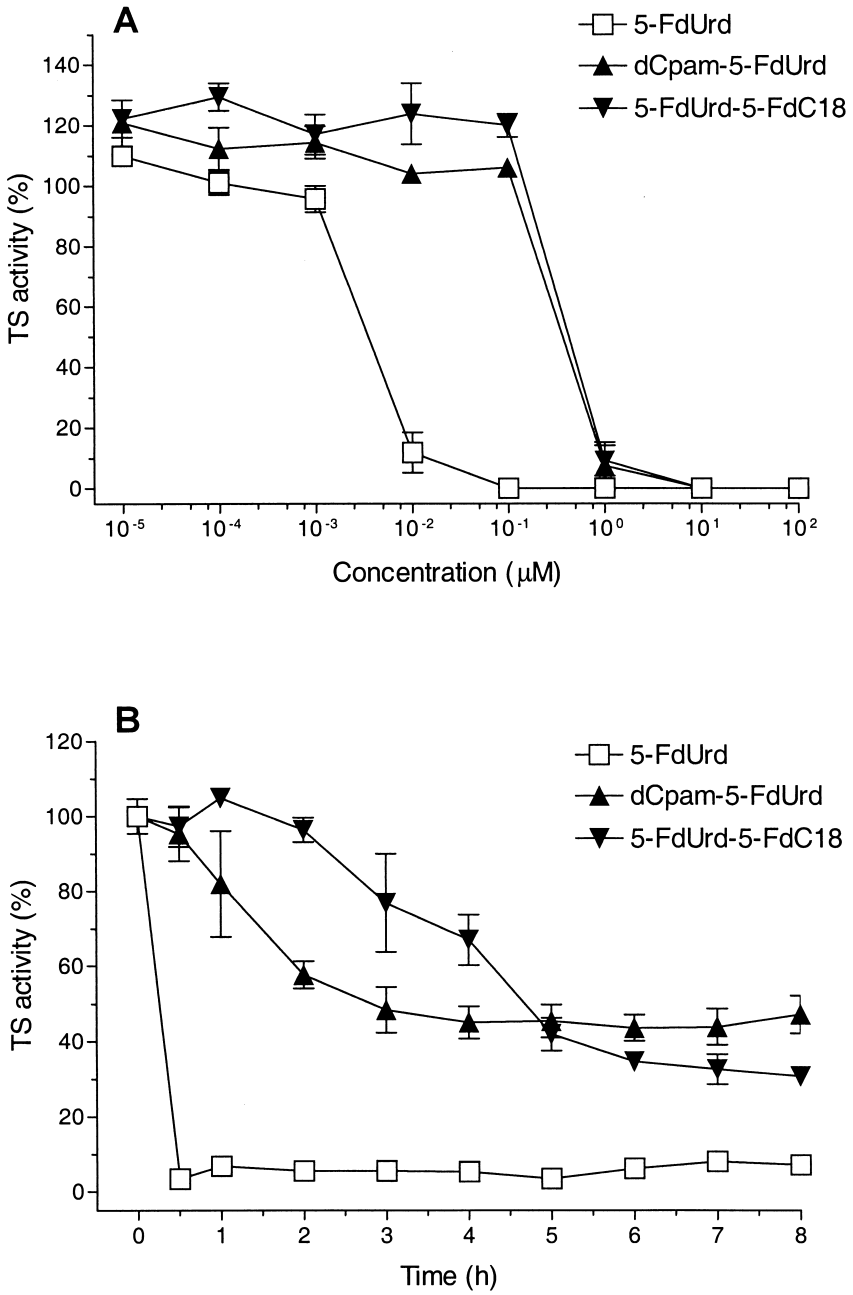

FIG. 6. TS enzyme activity in PC-3 cells was inhibited by the heterodinucleoside phosphate dimers in (A) a dose-dependent manner (90-min incubation time) and (B) a time-dependent manner (0.1-μM drug concentration). TS activity values are shown as percent of untreated control. Data are means and SD of at least two independent experiments performed in duplicate.

tion in PC-3 cells in time- and dose-dependent manner. At a low concentration of 12 μM, dCpam-FdUrd caused 90% cytotoxicity compared to 5-FdUrd. Importantly, at higher concentrations of 100 and 200 μM, the new dimers reached a toxicity of 100% after 96-hr incubation. Consequently, the new derivatives are also cytotoxic for the 10% of cells which seem to be resistant to 5-FdUrd. It is conceivable that high cytotoxic dimer concentrations are required in the *in vitro* setting because the prodrugs have to be cleaved by enzymes that may be present only at low concentrations. However, it will be important to achieve these high concentrations *in vivo* as well, without causing too strong toxic side effects.

Recently, new compounds were found to be cytotoxic for human prostate tumour cells. The nucleoside drug gemcitabine, which is a synthetic antimetabolite of the pyrimidine nucleotide metabolism, inhibited cell growth and colony formation of the androgen-sensitive prostate tumour cell line LNCaP and the androgen-sensitive cells PC-3 and DU-145 [10]. Colony formation could be suppressed, whereas cell viability did not fall below 10%. The synthetic retinoids fenretinide and CD437 induced S-phase arrest

and apoptosis in human prostate tumour cells [11, 12]. While fenretinide completely inhibited PC-3 cell growth as the dimers did, the synthetic retinoid CD437 did not reach this cytotoxicity even after 6 days continuous incubation.

Apoptosis has emerged as a significant therapeutic target for the effective elimination of cancer cells [13, 14]. In agreement with others [12, 15], we have found that a continuous drug exposure was required to induce apoptosis in PC-3 cells. Studies with Apo2.7 antibody showed that the drugs induced significant proportions of apoptotic cell fractions after prolonged incubations of 96 hr. 5-FdUrd-5-FdC18 was the most potent inducing agent at 50 μM, resulting in a nearly twice as high apoptotic cell fraction as 5-FdUrd. Interestingly, 5-FdUrd, dCpam-5-FdUrd, and 5-FdUrd-5-FdC18 exhibited comparable toxicity in PC-3 cells after 48- and 96-hr incubation, but only 5-FdUrd-5-FdC18 induced more than 50% of the cells to undergo apoptosis after 96-hr continuous incubation. This finding may be due to the fact that the other drugs induced more non-apoptotic cell deaths. Significantly, our experiments showed that exposure of the cells to the dimers resulted in the activation of caspase-3, with cleavage of PARP and subsequent internucleosomal degradation of genomic DNA. For 5-FdUrd-5-FdC18, apoptosis was confirmed by electron microscopy, where after 48-hr exposure we found about 10% of all cells with condensed chromatin and apoptotic bodies. 5-FdUrd-5-FdC18 also acts on the cytoskeleton, leading to disruption of actin filaments but leaving the microtubule structure intact.

The delayed induction of apoptotic cell death can possibly be explained by this cell line having low propensity to undergo apoptosis. The *p53* tumour suppressor gene has been shown to be an essential component of the apoptotic pathway induced by genotoxic insults [16]. Therefore, it can be assumed that the absence of wild-type *p53* in PC-3 cells was a contributing factor to the long induction times. Experiments on lymphocytes of *p53*-deficient mice showed that *p53*-null thymocytes were resistant to DNA damage-induced apoptosis by etoposide and irradiation, but that their response to glucocorticoids was unaltered by the absence of *p53* [17–19]. These data imply the existence of *p53*-dependent and -independent pathways for the induction of apoptosis. In PC-3 cells, wild-type *p53* does not seem to be an effector of induced apoptosis. Rather, it appears to be involved in setting the threshold for apoptosis induction directly or by affecting the transcription of other regulatory genes of apoptosis [14, 20]. A recent study reported that cell death of PC-3 prostate tumour cells can result from the inhibition of proteasome activity, which is also independent of *p53* and functional Bcl-2 [21].

Interestingly, dCpam-5-FdUrd and 5-FdUrd-5-FdC18 specifically repress TS, suggesting that the dimers act as prodrugs that are cleaved intracellularly into the monophosphorylated molecule 5-FdUMP, which in turn inhibits TS. This also explains the longer incubation times and the higher concentrations needed to inhibit TS activity to the same extent as 5-FdUrd.

It is possible that 5-FdUrd-5-FdC18 is more potent than the other two drugs because the molecule contains not only the masked 5-FdUrd or its monophosphate (5-FdUMP) but also an additional molecule with a potential cytotoxic activity, namely the 5-FdC18 moiety of the dimer. With the similar lipophilic molecule NOAC, we have detected the hydrophilic metabolites arabinosyl cytosine (ara-C) and arabinosyl uracil (ara-U) in mice, which were formed by metabolic cleavage of the alkyl chain from the parent molecule [22]. Thus, it might be possible that in 5-FdUrd-5-FdC18 the alkyl chain can also be cleaved and, with an additional oxidation reaction, 5-FdUrd and subsequently 5-FdUMP are formed. Further studies with the dimers are needed to verify these assumptions in *in vivo* experiments.

The development of pharmacological agents that induce cell death in p53-independent cells may prove useful in the treatment of cancer patients, given the frequency of these molecular alterations in human tumours and the association of these alterations with therapy failure. Our results suggest that the new dimers have a potential value as new therapeutic agents against androgen-insensitive prostate carcinoma through the inhibition of TS activity and induction of a p53-independent apoptotic cascade.

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