Synthesis and in vitro Antitumor Activity of 2′-Deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluoro-N^4-octadecylcytidine: A New Amphiphilic Dinucleoside Phosphate

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Received June 12, 1996

Keywords: 2′-Deoxy-5-fluorouridine derivatives / Phosphotriester method / Liposomes / Antitumor agents / MIA PaCa 2 pancreas tumor cell line

The new amphiphilic dinucleoside phosphate, 2′-deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluoro-N^4-octadecylcytidine (**4**) was synthesized on a gram scale, using the phosphotriester method, starting from the cytostatic drug 2′-deoxy-5-fluorouridine (5FdU) and 2′-deoxy-5-fluoro-N^4-octadecylcytidine (**1d**). In in vitro clonogenic growth assays using the human pancreatic adenocarcinoma cell line MIA PaCa 2, the amphiphilic dimer was significantly more effective than the parent monomeric 5FdU. The IC_{50} of the dimer was 10 μg/ml when applied as an aqueous solution and 12 μg/ml when given as a liposome dispersion, whereas with 5FdU the IC_{50} concentration was not reached within the concentration range used.

5FdU is clinically used for the treatment of gastrointestinal tumors. The cytostatic activity of 5FdU is considered to be primarily the result of the inhibition of DNA biosynthesis in the presence of 2′-deoxy-5-fluorouridine-5′-monophosphate (p5FdU)[1]. Cancer cells which lack the enzymes for the phosphorylation of 5FdU to p5FdU are resistant against 5FdU[2]. However, because of its high polarity, p5FdU is not taken up by cells in its intact form, but rather as the dephosphorylated 5FdU[3]. Attempts to mask the phosphate group in such a way that the resulting prodrugs gain a facilitated entry into the cell and release p5FdU in the cytoplasm have generally proved to be unsuccessful. Important prodrugs containing masked p5FdU are the polar dinucleoside phosphates[4], the 5′-phosphodiamidates[5], the 5′-*O*-alkyl phosphates[6], conjugates of 5FdU with cholesterol[7] and corticosteroids[8].

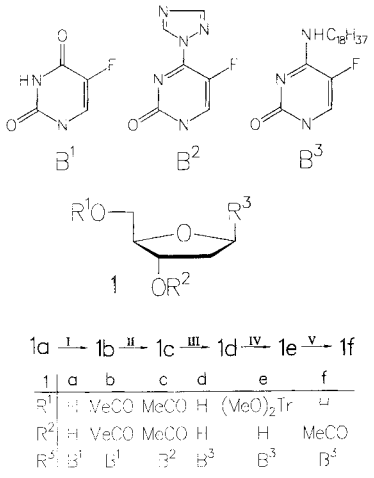
We have developed a new strategy of masking nucleoside phosphates by the synthesis of amphiphilic dinucleoside phosphates[9–11], which is based on the condensation of a lipophilic nucleoside with a nucleoside of known therapeutic activity, via a natural phosphodiester linkage. In comparison to polar nucleotides, these amphiphilic dimers possess the advantage that they can be incorporated into liposomes, forming stable dispersions. The formulation of the prodrugs in liposomes can offer additional properties. This paper concerns the derivatization of 5FdU into an amphiphilic dinucleoside phosphate, and the first results of the in vitro cytostatic activity of the amphiphilic dimer are reported.

The amphiphilic dinucleoside phosphate 2′-deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluoro-N^4-octadecylcytidine (**4**) was synthesized (Scheme 2) according to the phosphotriester method[12], starting from the hydroxyl compound 3′-*O*-acetyl-2′-deoxy-5-fluoro-N^4-octadecylcytidine (**1f**) and the protected 3′-phosphate compound, the barium salt of 2′-deoxy-5′-*O*-(4,4′-dimethoxy)trityl-5-fluorouridine-3′-(2-chlorophenyl)phosphate (**3**). The new lipophilic compound **1d** was synthesized in four steps in an analogous manner to N^4-octadecyl-1-β-D-arabinofuranosylcytosine[13], starting from 5FdU (Scheme 1). The condensation of the phosphate compound **3** with the hydroxyl compound **1f** was performed using 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) and *N*-methylimidazole as condensation agents. After chromatographing the reaction mixture on silica gel, the 2-chlorophenyl group was removed with tetrabutylammonium fluoride from the fully protected dinucleoside phosphate. The partially protected dinucleoside phosphate was detritylated with acetic acid and then deacetylated with NH_3/methanol. After chromatography on silica gel, **4** was obtained at a purity of >95% and in an overall yield of 77% based on **1f**. Remaining impurities were removed by HPLC.

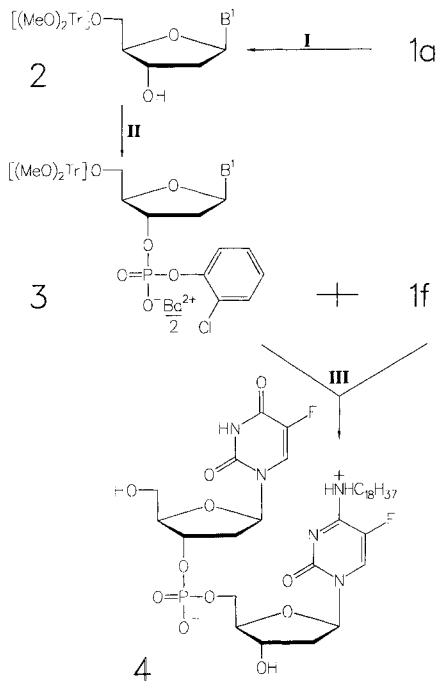
4 is water soluble but can also be incorporated using standard procedures into the lipid membranes of liposomes.

  0947–3440/97/0202–0413 $ 15.00+.25/0

Scheme 1. Synthesis of 3′-O-acetyl-2′-deoxy-5-fluoro-N^4-octadecylcytidine (**1f**) starting from 2′-deoxy-5-fluorouridine (**1a**); the reaction steps are: **I**: acetic anhydride in pyridine; **II**: phosphoryl chloride, 1*H*-1,2,4-triazole, triethylamine in acetonitrile; **III**: (1) octadecylamine in chloroform; (2) NH_3/methanol; (3) column chromatography on silica gel; **IV**: (1) 4,4′-dimethoxytrityl chloride [$(MeO)_2TrCl$] in pyridine; (2) column chromatography on silica gel; **V**: (1) acetic anhydrid in pyridine; (2) acetic acid (80%)



Scheme 2. Synthesis of the amphiphilic dinucleoside phosphate **4** according to the phosphotriester method; the reaction steps are: **I**: 4,4′-dimethoxytrityl chloride in pyridine; **II**: (1) 2-chlorophenyl dichlorophosphate in dioxane; (2) barium chloride in water; **III**: (1) 2,4,6-triisopropylbenzenesulfonyl chloride, *N*-methylimidazole in pyridine; (2) column chromatography on silica gel; (3) tetrabutylammonium fluoride in THF/water/pyridine; (4) acetic acid (80%); (5) NH_3/methanol; (6) column chromatography on silica gel



Liposomes prepared by sonication had diameters below 100 nm and were homogeneous. The liposome dispersion of **4** was stable for two weeks, when kept at 2–4 °C. In the liposome dispersions of 2′-deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluorouridine (5FdU-5FdU), however, precipitates were observed after 24 h. The stable incorporation of up to 20 mg of the dinucleoside phopshates into 1 ml of liposome dispersion was confirmed by repeated ultrafiltration through Amicon-PM-30-membranes, whereby no UV-active compounds were detected in the filtrates. Additionally, when the liposome formulations were chromatographed on ACA-Ultrogel columns, over 98% of the UV-active compounds were eluted within the liposome peak, confirming the almost quantitative incorporation of the dimers. When mixtures of empty liposomes and free dimers were chromatographed, no UV-active substances were detected during the elution of the liposomes and the dinucleoside phosphate was eluted as a separate peak.

By means of clonogenic assays, the cytostatic activities of the polar dimer 5FdU-5FdU and the amphiphilic dimer **4** were compared to the activity of 5FdU on the MIA PaCa 2 human pancreatic adenocarcinoma cells. Both dinucleoside phosphates were applied either dissolved in buffer or in the form of a liposome dispersion, while 5FdU was used as a solution in phosphate buffered saline (PBS). With 100 μg/ml 5FdU the reduction of clonogenic growth of MIA PaCa 2 cells evaluated 11 days after treatment was 27%, whereas **4** given in liposomes or as an aqueous solution resulted in inhibitions of 87 and 96%, respectively. The corresponding IC_{50} values were 12 μg/ml and 10 μg/ml, whereas with 5FdU the IC_{50} concentration was not reached within the concentration range used. 5FdU-5FdU was inactive in both application forms. Similar, but less pronounced effects were observed when the colony growth inhibition was evaluated on day 5 (data not shown). The results of these clonogenic assays demonstrate that the transformation of 5FdU into an amphiphilic dinucleoside phosphate confers a marked improvement in cytostatic activity. The dimerization of 5FdU leads to a decrease in cytostatic activity, showing that this potential method of masking p5FdU is not advantageous. 5FdU-5FdU is probably too polar to be able to cross cell membrane. Similar results with dimers have previously been reported from tests using Ehrlich ascites cells[4]. The increased cytostatic activity of **4** may be conferred by the presence of the lipophilic alkyl chain, which provides the dimer with amphiphilic properties.

The in vivo cleavage of the phosphodiester linkage of **4** can lead either to 2′-deoxy-5-fluoro-N^4-octadecylcytidine (**1d**) with a free or a phosphorylated 5′-hydroxyl group, or to 5FdU with a free or a phosphorylated 3′-hydroxyl group **1d** containing a free hydroxyl group represents a new lipophilic derivative, and with a phosphorylated hydroxyl group it is a new amphiphilic nucleotide derivative. For the evaluation of the relationship between chemical structure and therapeutic activity it is therefore important to determine whether such derivatives of **1d** can alone exert a therapeutic activity. Thus, the cytostatic activity of monomeric **1d** was tested in the clonogenic assay, and was found to be about 50% lower than that of the dimeric **4**. This result excludes **1d** as a hypothetical metabolic responsible for the observed

antitumor activity of the dimer. The question as to whether the dimer or its metabolites would be cytotoxic without being incorporated into the liposome, or whether their higher activity is attributable to an increased cellular uptake, will be the subject of future investigations.

Experimental Section

5FdU was obtained from Mack (Karlsruhe, Germany). 1*H*-1,2,4-triazole, *n*-octadecylamine (Fluka, Buchs, Switzerland), and tetrabutylammonium fluoride · 2 H_2O (Jansson, Nettetal, Germany) were used as obtained. Phosphoryl chloride was distilled before used. *N*-Methylimidazole (Aldrich, Steinheim, Germany) was dried by means of 0.4-nm molecular sieve. Triethylamine and methanol used for HPLC were freshly distilled. Acetonitrile, diethyl ether and dioxane were distilled over calcium hydride. Pyridine was distilled over KOH and stored over 0.4-nm molecular sieve. The following substances were synthesized in our laboratory according to published methods: 4,4′-dimethoxytrityl chloride [(MeO$_2$)TrCl][14]; 2,4,6-triisopropylbenzenesulfonyl chloride[15]; and 2-chlorophenyl dichlorophosphate[16]. 5FdU-5FdU was synthesized in an analogous manner to dU-dU[17], using the phosphotriester method[12] starting from 5FdU. N^2-Palmitoyl-N^6-succinyl-L-lysine was synthesized according to ref.[18]. The synthesis of **1b** was performed according to a modified method as described in ref.[19]. **2** was synthesized in the same way as **1e**, starting from 5FdU (**1a**).

Chromatography: Preparative column chromatography was carried out on silica gel 60 (0.04−0.063 mm). The reaction mixtures were applied on dry packed columns and fractionated using chloroform/methanol gradients. All solvent mixtures were prepared by volume ratios (v/v). The desired products were detected by TLC from aliquots of the eluates. TLC was carried out on precoated silica gel plates 60 F_{254} (0.25 mm, Merck, Darmstadt, Germany). The UV-active nucleoside derivatives were detected at 254 nm as black spots which turned brown after spraying with perchloric acid (60%) and heating (sugar moieties). UV-active spots that turned orange after perchloric acid treatment indicated the presence of [(MeO)$_2$Tr] groups. TLC spots of nucleoside derivatives that were protected by [(MeO)$_2$Tr] groups and contained a phosphodiester linkage turned brown after spraying with a molybdic reagent[10]. In the absence of [(MeO)$_2$Tr] groups, these derivatives formed blue molybdate complexes. Nucleoside derivatives bearing octadecyl residues formed fluorescent spots (366 nm) after spraying with 2,7-dichlorofluorescein (0.2%) in ethanol. Impurities containing amine and amide linkages were detected by treatment with chlorine gas followed by a toluidine spray reagent[20].

HPLC was performed under isocratic conditions on a 10-μm Polygosil 60−10 C_8 (25 × 0.8 cm) column (Macherey & Nagel, Düren, Germany) using the binary mixtures I = A: 25% 0.1 M ammonium acetate; B: 75% methanol (v/v) and II = A: 30% 0.1 M ammonium acetate; B: 70% methanol (v/v) as the eluents. The course of elution was automatically monitored at 254 nm.

Syntheses: Before the reactions of nucleoside derivatives were carried out in pyridine, the dissolved compounds were rendered anhydrous by triple coevaporation with dry pyridine. The reaction vessels were ventilated with dry air prior to each addition of pyridine. After the final evaporation step the amount of pyridine was reduced to the volume described for the reaction. Having added the condensation agents as rapidly as possible, the reaction vessel was immediately evacuated and sealed. Organic solvents extracted by water were dried by filtration through Na_2SO_4 before they were concentrated. We used methanol saturated with ammonia (NH_3/methanol) at room temp. for the deacylation. The course of the reactions was monitored by TLC. The intermediate products were purified until the compounds migrated on TLC plates as single spots and the observed molecular masses corresponded to the calculated values. Mass spectra (electrospray; ES) were obtained using a Sciex API III with an electronspray source. Products that contained minor impurities after fractionation on silica gel columns were purified by HPLC.

2′-Deoxy-5-fluoro-N^4-octadecylcytidine (**1d**): 10 ml (0.11 mol) of phosphoryl chloride was added dropwise to a cooled (0 °C) suspension of 33.4 g (0.48 mol) of 1*H*-1,2,4-triazole in 130 ml of acetonitrile under vigorous stirring, keeping the temp. at 30 °C. After cooling to 10 °C, 65 ml (0.88 mol) of triethylamine was added dropwise, keeping the temp. below 20 °C. Then 20 g (61 mmol) of **1b** dissolved in 50 ml of acetonitrile was added at 10 °C and the reaction mixture was stirred at room temp. for 3.5 h before 45 ml (0.61 mol) of triethylamine and 11.6 ml (0.64 mol) of water were added. After further stirring for 10 min, the mixture was concentrated and the residue was dissolved in 200 ml of chloroform, extracted three times with 20 ml of water and concentrated to a syrup. To the syrup dissolved in 150 ml of chloroform, 16.3 g (60 mmol) of octadecylamine was added. The reaction mixture was refluxed for 1.5 h, cooled to room temp., washed with 75 ml of water and concentrated to a syrup. The concentrate was dissolved in 40 ml chloroform, and 350 ml of NH_3/methanol were added to the resulting solution. The mixture was kept sealed for 12 h, then concentrated to half of the volume and cooled to −20 °C. The obtained precipitate was collected by filtration, washed with diethyl ether and dried in vacuo, yielding 24 g of 2′-deoxy-5-fluoro-N^4-octadecylcytidine (**1d**). The filtrate was concentrated, dissolved in 50 ml chloroform and chromatographed on a silica gel column (20 × 9 cm) using a three step chloroform/methanol gradient with step 1: 94:6 (4 l); step 2: 9:1 (4 l); step 3: 8:2 (2 l). The desired compound (UV- and fluorescent-positive) was eluted during steps 2 and 3, concentrated and crystallized by addition of methanol. The appropriate fractions were pooled and dried in vacuo, yielding 28.5 g (95%) of **1d** as colourless crystals (m.p. 139 °C). R_f = 0.22 (chloroform/methanol, 9:1); $C_{27}H_{48}FN_3O_4$ (497.7): calcd. C 65.16, H 9.72, N 8.44; found C 65.29, H 9.68, N 8.56; MS (IS), *m/z* 496.0 (M − H$^+$). − ^{1}H NMR ($CDCl_3$): δ = 0.87 (t, *J* = 7 Hz, 3H, CH_3), 1.25 [s, 32H, $(CH_2)_{16}$], 2.36 (m, 2H, $H_{2'}$ + $H_{2''}$), 3.71 (m, 1H, $H_{5'}$), 3.87 (m, 1H, $H_{5''}$), 3.98 (m, 1H, $H_{4'}$), 4.52 (m, 1H, $H_{3'}$), 6.08 (t, *J* = 6 Hz, 1H, $H_{1'}$), 7.77 (d, *J* = 6 Hz, 1H, H_6). − ^{13}C NMR ($CDCl_3$): δ = 14.10 (s, CH_3), 29.71 [s, $(CH_2)_{16}$], 40.78 (s, $C_{2'}$), 62.45 (s, $C_{5'}$), 76.51 (s, $C_{3'}$), 85.53 (s, $C_{1'}$), 87.28 (s, $C_{4'}$), 125.82 (s, C_5), 140.59 (s, C_6), 156.53 (s, C_2), 166.50 (s, C_4).

3′-O-Acetyl-2′-deoxy-5-fluoro-N^4-octadecyl cytidine (**1f**): To a solution of 18.8 g (37.7 mmol) of **1d** in 50 ml of pyridine, 15.3 g (45.2 mmol) of 4,4′-dimethoxytrityl chloride were quickly added. The vessel was sealed and the reaction mixture was stirred for 2 h. The precipitate was removed by filtration and washed with ether. Methanol (10 ml) was added to the filtrate and the mixture was stirred for another 10 min, then concentrated to a syrup which was twice coevaporated with toluene (20 ml). The resulting syrup was dissolved in 100 ml of chloroform and chromatographed on a silica gel column (20 × 9 cm) using a three-step gradient with step 1: chloroform (5 l); step 2: chloroform/methanol 97:3 (4 l) and step 3: chloroform/methanol 95:5 (4 l). The fractions, containing the desired product (UV- and trityl-positive), which was eluted in the third step were pooled, concentrated and dried in vacuo. The colourless foam yielded 23 g (76%) of 2′-deoxy-5′-O-(4,4′-dimethoxy)trityl-5-fluoro-N^4-octadecylcytidine (**1e**); R_f = 0.69 (chloroform/methanol, 9:1).

To a solution of 11.5 g (14.4 mmol) of **1e** in 25 ml of pyridine, 6.8 ml (72 mmol) of acetic anhydride was added. The vessel was sealed and the reaction mixture was stirred for 5 h. Methanol (6 ml) was added, the mixture was stirred for 10 min then concentrated to a syrup, which was coevaporated twice with 15 ml of toluene. To the residue 50 ml of acetic acid (80%) was added, and the mixture was stirred for 30 min. Then 100 ml of chloroform/methanol (1:1) was added and the resulting solution was extracted with three 50 ml portions of water. After concentration of the organic phase and addtion of ethyl acetate, 3′-O-acetyl-2′-deoxy-5-fluoro-N^4-octadecylcytidine (**1f**) precipitated. Recrystallization from ethyl acetate yielded 6.2 g (80%) of **1f** as colourless crystals (m.p. 82–84°C). R_f = 0.51 (chloroform/methanol 9:1); $C_{29}H_{50}FN_3O_5$ (539.7): calcd. C 64.53, H 9.34, N 7.79; found C 64.81, H 9.26, N 7.78; MS (IS), *m/z* 538.0 ($M - H^+$). – ^{1}H NMR ($CDCl_3$): δ = 0.86 (t, *J* = 7 Hz, 3H, CH_3), 1.23 [s, 32H, $(CH_2)_{16}$], 2.07 (s, 3H, $COCH_3$), 2.31 (m, 2H, $H_{2'}$ + $H_{2''}$), 3.91 (m, 2H, $H_{5'}$ + $H_{5''}$), 4.07 (m, 1H, $H_{4'}$), 5.32 (m, 1H, $H_{3'}$), 6.25 (t, *J* = 6 Hz, 1H, $H_{1'}$), 7.82 (d, *J* = 6 Hz, 1H, H_6). – ^{13}C NMR ($CDCl_3$): δ = 14.10 (s, CH_3), 29.36 [s, $(CH_2)_{16}$], 40.76 (s, $C_{2'}$), 62.59 (s, $C_{5'}$), 77.04 (s, $C_{3'}$), 85.22 (s, $C_{1'}$), 87.29 (s, $C_{4'}$), 124.46 (s, C_5), 139.00 (s, C_6), 155.86 (s, C_2), 166.41 (s, C_4).

Barium Salt of 2′-Deoxy-5′-O-(4,4′-dimethoxy)trityl-5-fluorouridine-3′-(2-chlorophenyl)phosphate (**3**): 8.4 ml (60 mmol) of triethylamine and 4.9 ml (30 mmol) of 2-chlorophenyl dichlorophosphate were added to a solution of 4.2 g (60 mmol) of 1*H*-1,2,4-triazole in 100 ml of dioxane with continuous stirring and under exclusion of moisture. After 30 min, the precipitate was removed by filtration. The filtrate was added to a solution of 5.5 g (10 mmol) of **2** in 50 ml of pyridine. The tightly sealed reaction vessel was stirred for 30 min and then the mixture was poured with stirring into 0.5 l of cold water, keeping the pH above 7.5 by addition of NaOH (0.5 N). Then the mixture was poured into 2 l of an aqueous solution of $BaCl_2$ (1%), with vigorous stirring, keeping the pH between 7 and 8. After stirring for 1 h the precipitate was collected by filtration, washed with water and dried in vacuo, yielding 6.6 g (82%) of the barium salt of 2′-deoxy-5′-O-(4,4′-dimethoxy)trityl-5-fluorouridine-3′-(2-chlorophenyl)phosphate (**3**); R_f = 0.37 (chloroform/methanol, 7:3). – ^{1}H NMR ([D_6]DMSO): δ = 0.85 (t, *J* = 7 Hz, 3H, CH_3), 1.23 [s, 32H, $(CH_2)_{16}$], 2.33 (m, 2H, $H_{2'}$ + $H_{2''}$), 3.72 (s, 6H, OCH_3), 3.85 (m, 1H, $H_{5'}$), 4.00 (m, 1H, $H_{5''}$), 4.15 (m, 1H, $H_{4'}$), 4.81 (m, 1H, $H_{3'}$), 6.13 (t, *J* = 6 Hz, 1H, $H_{1'}$), 6.81–7.61 (m, 17H, aromatic-H), 7.82 (d, *J* = 6 Hz, 1H, H_6). – ^{13}C NMR ([D_6]DMSO): δ = ca. 40.5 (obscured by DMSO signal, $C_{2'}$), 63.90 (s, $C_{5'}$), 75.07 (s, $C_{3'}$), 84.67 (s, $C_{1'}$), 85.91 (s, $C_{4'}$), 128.75 (s, C_5), 141.84 (s, C_6), 156.75 (s, C_2), 165.14 (s, C_4).

2′-Deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluoro-N^4-octadecylcytidine (**4**): For the condensation, 2.4 g (3.0 mmol) of **3** and 1.3 g (2.4 mmol) of **1f** were dissolved in 10 ml of pyridine. After the addition of 1.2 g (4.1 mmol) of TPS and 1 ml (12.3 mmol) of *N*-methylimidazole the tightly sealed reaction vessel was shaken for 40 min at room temperature. Water (2 ml) was then added, the reaction mixture was shaken for 10 min, and concentrated to a syrup which was twice coevaporated with 15 ml of toluene. The residue was dissolved in 150 ml of chloroform/*n*-heptane (1:1) and chromatographed on a silica gel column (14 × 9 cm) using a three step gradient with step 1: chloroform/n-heptane 1:1 (2 l); step 2: chloroform (4 l) and step 3: chloroform/methanol 9:1 (4 l). The fractions containing the desired compound were pooled and concentrated to a foam. The obtained foam was dissolved in 185 ml of THF/water/pyridine (8:1:1). After adding 3 g (10 mmol) of tetrabutylammonium fluoride the reaction mixture was shaken for 45 min and concentrated to a syrup which was coevaporated with 30 ml of toluene. The residue was dissolved in 60 ml of ethyl acetate, washed with 50 ml of water and concentrated to a syrup. The syrup was treated with 50 ml of acetic acid (80%) for 30 min at 50°C and concentrated again to a syrup, to which 150 ml of NH_3/methanol was added. The mixture was kept sealed for 14 h. After removal of the solvent, the residue was dissolved in chloroform/methanol (9:1) and chromatographed on a silica gel column (14 × 9 cm) using a four-step gradient with step 1: chloroform/methanol 9:1 (6 l); step 2: 8:2 (4 l); step 3: 6:4 (5 l) and step 4: chloroform/methanol/ammonia 10:10:3 (1 l). The desired compound was eluted during steps 3 and 4, concentrated and crystallized by addition of methanol. 1.5 g (77%) of 2′-deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluoro-N^4-octadecylcytidine (**4**) was obtained as colourless crystals; R_f = 0.32 (chloroform/methanol, 6:4); Aliquots (50 mg) of **4** dissolved in 0.5 ml water were purified by HPLC using the binary eluent II at a flow rate of 1.5 ml/min. The eluate, collected between 18 and 28 min, was lyophilized yielding 35 mg of analytically pure **4**; $C_{36}H_{58}F_2N_5O_{11}P$ (805.9): calcd. C 53.66, H 7.25, F 4.72, N 8.69; found C 52.83, H 7.41, F 4.80, N 8.82; MS (IS), *m/z* 804.5 ($M - H^+$). – ^{1}H NMR ([D_6]DMSO): δ = 0.85 (t, *J* = 7 Hz, 3H, CH_3), 1.22 [s, 32H, $(CH_2)_{16}$], 2.08 + 2.29 (m, 4H, $H_{2'}$ + $H_{2''}$), 3.60 + 3.84 (m, 4H, $H_{5'}$ + $H_{5''}$), 4.00 (m, 2H, $H_{4'}$), 4.23 + 4.66 (m, 2H, $H_{3'}$), 6.13 (m, 2H, $H_{1'}$), 7.91 [d, *J* = 6 Hz, 1H, H_6 (5FdC[18])], 8.20 [d, *J* = 6 Hz, 1H, H_6 (5FdU)]. – ^{13}C NMR ([D_6]DMSO): δ = 13.92 (s, CH_3), 29.01 [s, $(CH_2)_{16}$], ca. 40.5 (obscured by DMSO signal, $C_{2'}$), 61.36 + 63.90 (s, $C_{5'}$), 70.75 + 74.28 (s, $C_{3'}$), 84.48 + 85.02 (s, $C_{1'}$), 85.76 + 86.43 (s, $C_{4'}$), 124.28 + 126.33 (s, C_5), 138.25 + 141.82 (s, C_6), 153.33 + 157.17 (s, C_2), 166.20 + 167.38 (s, C_4).

Liposomes: The liposomes were prepared as described previously[21]. Briefly, to obtain 2 ml of liposome dispersion, 40 mg of the amphiphilic dinucleoside phosphate **4** or of the dimer 5FdU-5FdU, was dissolved in 1.6 ml of a stock solution (chloroform/methanol, 1:1) containing 160 mg of soy phosphatidylcholine (SPC), 16 mg of cholesterol, 1.6 mg of DL-α-tocopherol and 11.2 mg of N^2-palmitoyl-N^6-succinyl-L-lysine. This solution was concentrated to a thin film by removing the solvents. Two ml of PBS buffer (0.9% NaCl + 10 mM NaH_2PO_4, pH 7.3) was added and liposomes were obtained by sonification for 15 min with a Branson Sonifier 250 (Branson-Sonic Power, USA) using a microtip. The liposome dispersion was filtered through a sterile filter and kept at 2–4°C. Liposome diameters and polydispersity were determined using a Nicomp 370 particle sizer (Particle Sizing Systems, Sta. Barbara, CA, USA).

Clonogenic Assay: Clonogenic assays were performed as described previously[22]. Cells of the human pancreatic adenocarcinoma cell line MIA PaCa 2 (ATCC No. CRL 1420, American Type Culture Collection) were trypsinized and the viability determined by Trypan blue staining. 2.5×10^4 cells were incubated in McCoy's 5 A Medium (Gibco, Eggenstein, Germany) with 10% foetal calf serum (Biochrom, Berlin, Germany) in a final volume of 1.5 ml with concentrations of 1, 10 and 100 μg/ml of (a) 5-fluoro-2′-deoxyuridine in PBS, (b) 2′-deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluorouridine in PBS, (c) a liposome formulation of (b), (d) 2′-deoxy-5-fluorouridylyl-(3′→5′)-2′-deoxy-5-fluoro-N^4-octadecylcytidine (**4**) PBS (e) a liposome formulation of (d), and (f) a liposome formulation of 2′-deoxy-5-fluoro-N^4-octadecylvytidine (**1d**). As negative control, equal volumes of PBS were added to the cells. After 24 h incubation (37°C, 5% CO_2) the drugs were removed from the cells by two washings with medium. Then 2×10^4 cells were mixed with 1 ml of 0.3% soft agar and transferred to Petri dishes (Greiner, Frickenhausen, Germany) containing as bottom layer 1 ml of 0.5% soft agar (Bacto Agar, Difco, Augsburg, Germany). All experiments were performed in triplicate. After 5 and

11 days culture under normal conditions (37 °C, 5% CO_2) the numbers of grown colonies were determined by light microscopy.

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