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Short communication

Synthesis and anticancer activities of amphiphilic 5-fluoro-2′-deoxyuridylic acid prodrugs

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Abstract

Amphiphilic anticancer prodrugs of 5′-fluoro-2′-deoxyuridine-5′-monophosphate (5-FdUMP) were synthesized according to the hydrogen phosphonate method by coupling lipophilic cytosine derivatives or a phospholipid with 5-fluoro-2′-deoxyuridine (5-FdU). Studies within the in vitro Anticancer Screen Program of the National Cancer Institute have demonstrated high anticancer activities of the heterodinucleoside phosphates: N^4-palmitoyl-2′-deoxycytidylyl-(3′ → 5′)-3′-*O*-acetyl-5-fluoro-2′-deoxyuridine (dC^{pam}-5-FdU(Ac), N^4-palmitoyl-2′,3′-dideoxycytidylyl-(5′ → 5′)-3′-*O*-acetyl-5-fluoro-2′-deoxyuridine (ddC^{pam}-(5′ → 5′)-5-FdU(Ac), 5-fluoro-2′-deoxyuridylyl-(3′ → 5′)-5-fluoro-N^4-hexadecyl-2′-deoxycytidine (5-FdU-5-FdC^{hex}), and of the new liponucleotide 1-*O*-octadecyl-rac-glycerylyl-(3 → 5′)-5-fluoro-2′-deoxyuridine (Oct^1Gro-(3 → 5′)-5-FdU). The anticancer activities of these prodrugs are comparable to those of 5-FdU and the tumor specificities are modulated by their structures. The highest cytotoxic activity being even superior to 5-FdU was expressed by the dimer 5-FdU-5-FdC^{hex}.

Keywords: 5-Fluoro-2′-deoxyuridine derivatives; Anticancer prodrugs; Amphiphilic heterodinucleoside phosphates; Liponucleotide; Antimetabolites

1. Introduction

5-Fluorouracil (5-FU) is a widely used antimetabolite for the treatment of human solid tumors. Moreover, 5-FU and derivatives thereof have invoked interest because of their synergistic interaction with other antitumor agents, physiologic nucleosides and radiotherapy. To exert its cytotoxic activity 5-FU requires intracellular activation. Different mechanisms are responsible for the anticancer effect of 5-FU, whereby inhibition of thymidylate synthase (TS) was found to be the central mechanism. The nucleobase 5-FU is converted to the deoxynucleoside 5-FdU by thymidine phosphorylase and subsequent phosphorylation of the nucleoside by thymidine kinase results in the cytotoxic nucleotide 5-fluoro-2′-deoxyuridine-5′-monophosphate (5-FdUMP). In the presence of the reduced folate 5, 10-methylene-tetrahydrofolate, 5-FdUMP forms a complex with TS, inhibiting TS enzyme activity and leading to depletion of deoxythymidine triphosphate, a monomeric unit necessary for DNA synthesis [1]. Alternatively, 5-FU is anabolized to 5-fluorouridine-5′-triphosphate that can incorporated into RNA or converted to 5-fluoro-2′-deoxyuridine-5′-triphosphate, which, in turn can be incorporated into DNA [2,3]. The antitumor activity of 5-FU is comparable to that of 5-FdU. Due to extensive hepatic extraction 5-FdU is a useful drug for hepatic arterial chemotherapy of liver metastases [4].

The usefulness of 5-FU and 5-FdU is impaired by the frequent development of resistance in tumor cells. Resistance can occur through deletion of one of the key enzymes required for the first phosphorylation step from 5-FdU to 5-FdUMP

Abbreviations: Heterodinucleoside phosphates, dC^{pam}-5-FdU(Ac) = N^4-palmitoyl-2′-deoxycytidylyl-(3′ → 5′)-3′-*O*-acetyl-5-fluoro-2′-deoxyuridine; ddC^{pam}-(5′ → 5′)-5-FdU(Ac) = N^4-palmitoyl-2′,3′-dideoxycytidylyl-(5′ → 5′)-3′-*O*-acetyl-5-fluoro-2′-deoxyuridine; 5-FdU-5-FdC^{hex} = 5-fluoro-2′-deoxyuridylyl-(3′ → 5′)-5-fluoro-N^4-hexadecyl-2′-deoxycytidine, 5FdU-5FdC^{oct} = 5-fluoro-2′-deoxyuridylyl-(3′ → 5′)-5-fluoro-N^4-octadecyl-2′-deoxycytidine; 5FdC^{oct}-5FdU = 5-fluoro-N^4-octadecyl-2′-deoxycytidylyl-(3′ → 5′)-5-fluoro-2′-deoxyuridine, dC^{pam}-5-FdU = N^4-palmitoyl-2′-deoxycytidylyl-(3′ → 5′)-5-fluoro-2′-deoxyuridine; of the liponucleotide: Oct^1Gro-(3 → 5′)-5-FdU = 1-*O*-octadecyl-rac-glycerylyl-(3 → 5′)-5-fluoro-2′-deoxyuridine and of the monomers; p5-FdC^{oct} = 5′-fluoro-N^4-octadecyl-2′-deoxycytidine-5′-monophosphate; 5-FdC^{hex} = 5-fluoro-N^4-hexadecyl-2′-deoxycytidine.

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catalyzed by thymidine kinase. Depletion of this enzyme is often the main reason for 5-FU resistance. Consequently, the direct introduction of 5-FdUMP into cells could circumvent this resistance. However, because phosphate residues are strongly acidic and thus negatively charged at physiological pH, 5-FdUMP is not able to penetrate cell membranes. In addition, plasma and cell surface phosphohydrolases rapidly metabolize the nucleotide to the corresponding nucleoside.

In order to overcome the poor cell membrane penetration of therapeutically useful nucleotides various approaches were developed with the aim to enhance the intracellular delivery of anticancer nucleoside monophosphates [5]. Despite success and failure of different approaches many questions and issues remain to be addressed because no single concept has proven to be generally useful for all nucleotides.

To overcome these disadvantages we have developed the strategy of masking mononucleotides by their incorporation into amphiphilic dimers with the aim to improve cellular uptake of antiviral and anticancer nucleotide analogues and to extent the range of their therapeutic applications and cytotoxic potency by including them into liposome formulations [6–15]. Amphiphilic dimers can be obtained by condensation of a lipophilic nucleoside with a hydrophilic nucleotide and vice versa via phosphodiester linkage. The natural enzymatically easily cleavable phosphodiester bonds warrant that active mononucleotides are released from the dimers.

The transformation of therapeutically active mononucleotides into amphiphilic prodrugs using lipophilic nucleoside derivatives can be achieved with a large number of derivatives. In a previous work [12] we had coupled 5-FdU to the lipophilic cytidine derivative 5-fluoro-N^4-octadecyl-2′-deoxycytidine yielding the amphiphilic 5-FdUMP prodrug 5-fluoro-2′-deoxyuridylyl-(3′ → 5′)-5-fluoro-N^4-octadecyl-2′deoxycytidine (5-FdU-5-FdC^{oct}, **11a**). To investigate structure–activity relations the two dimers 5-fluoro-2′-deoxyuridylyl-(3′ → 5′)-5-fluoro-N^4-hexadecyl-2′-deoxycytidine (5-FdU-5-FdC^{hex}, **10**) and 5-fluoro-N^4-octadecyl-2′-deoxycytidylyl-(3′ → 5′)-5-fluoro-2′-deoxyuridine (5-FdC^{oct}-5-FdU, **11**) the sequence isomer to **11a** were synthesized in analogy to **11** (unpublished data). The structures of these compounds together with the monomeric cytidine derivatives are shown in Fig. 1. We describe herein two synthetic routes for the preparation of new amphiphilic prodrugs of 5-FdUMP. Such syntheses are easier than those previously published.

2. Chemistry

The synthesis of amphiphilic heterodinucleoside phosphates and liponucleotides containing 5-FdUMP started with 3′-*O*-acetyl-5-fluoro-2′-deoxyuridine (**2**) which can be obtained in analogy to a published procedure [16] from 5-fluoro-2′-deoxyuridine in two steps. According to the first synthesis route (Fig. 2) the free 5′-hydroxyl group of 3′-*O*-acetyl-5-fluoro-2′-deoxyuridine (**2**, hydroxyl compound) was linked to the phosphonate compounds N^4-palmitoyl-2′,3′-dideoxycytidine-5′-hydrogen phosphonate (**3**) or to 5′-*O*-(4-monomethoxy-trityl)-N^4-palmitoyl-2′-deoxycytidine-3′-

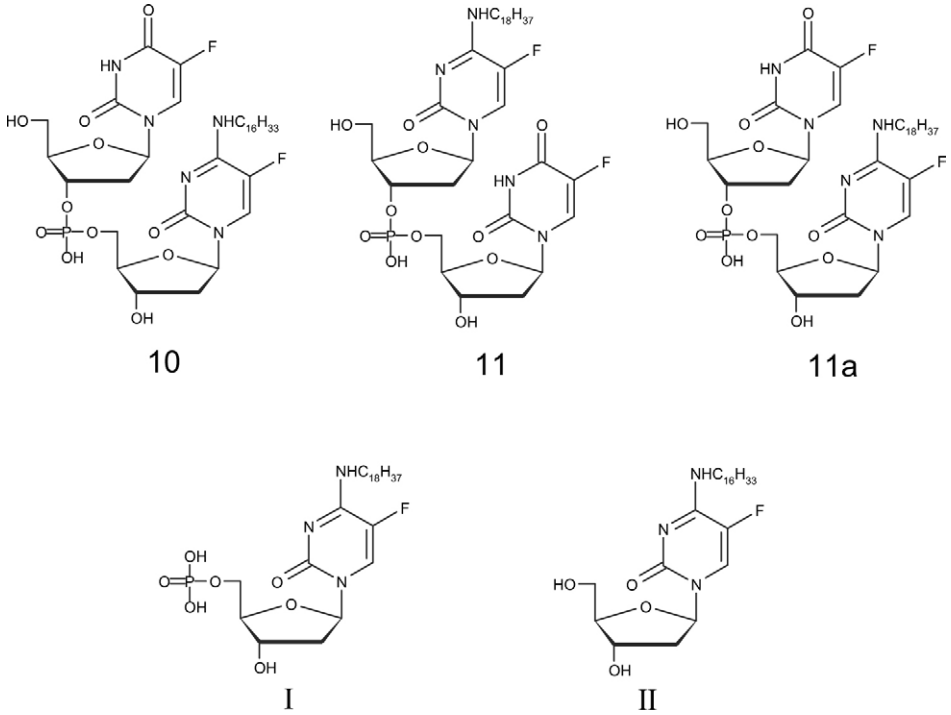

Fig. 1. Structure formulas of the amphiphilic heterodinucleoside phosphates.
10: 5-fluoro-2′-deoxyurididylyl-(3′ → 5′)-5-fluoro-N^4-hexadecyl-2′-deoxycytidine; **11**: 5-fluoro-N^4-octadecyl-2′-deoxycytidylyl-(3′ → 5′)-5-fluoro-2′-deoxyuridine; **11a**: 5-fluoro-2′-deoxy-uridylyl-(3′ → 5′)-5-fluoro-N^4-octadecyl-2′-deoxycytidine and the monomers **I**: 5-fluoro-N^4-octadecyl-2′-deoxycytidine-5′-monophosphate; **II**: 5-fluoro-N^4-hexadecyl-2′-deoxycytidine.

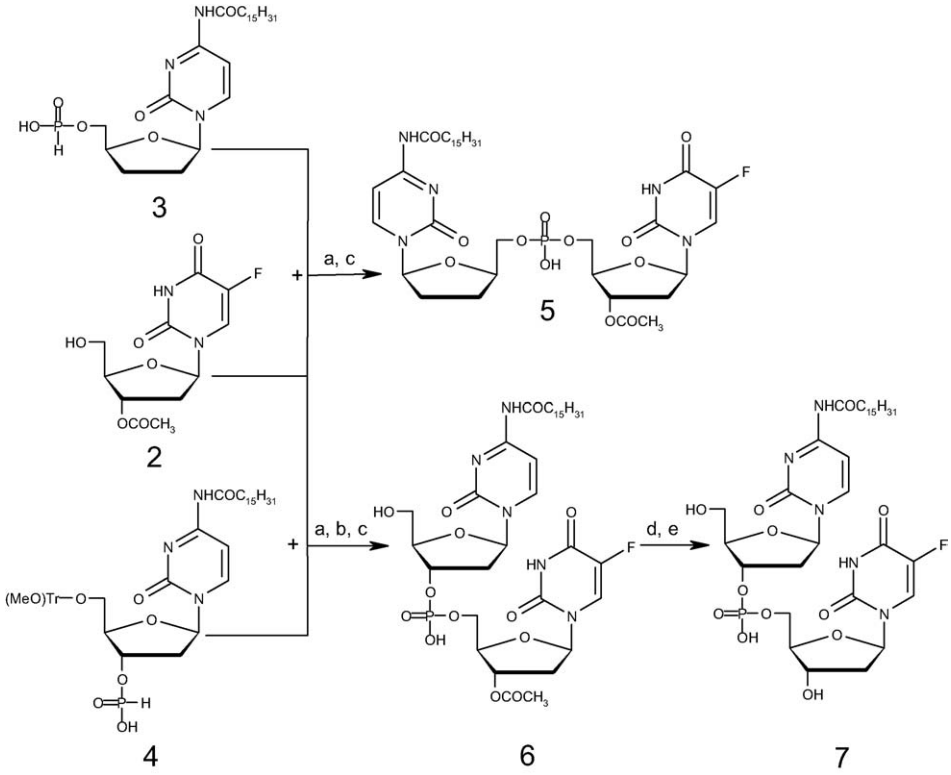

Fig. 2. Synthesis of heterodinucleoside phosphates (**5**, **6**, **7**) starting from 3′-*O*-acetyl-5-fluoro-2′-deoxyuridine (**2**) and N^4-palmitoyl-2′,3′-dideoxycytidine-3′-hydrogen phosphonate (**3**) or 5′-*O*-(4-monomethoxytrityl)-N^4-palmitoyl-2′-deoxycytidine-3′-hydrogen phosphonate (**4**).
The reaction steps are: a = 1. pivaloyl chloride in pyridine, 2. iodine (0.22 M) in THF/pyridine/water (16:1:1). b = *p*-toluenesulfonic acid (2%) in $CHCL_3$/MeOH. c = column chromatography on silica gel. d = methanol saturated with ammonia. e = column chromatography on RP_{18}.

hydrogen phosphonate (**4**) using the hydrogen phosphonate method. The internucleoside linkage of the resulting two dimers was oxidized with iodine. After chromatographic purification on silica gel the pure amphiphilic heterodinucleoside phosphate N^4-palmitoyl-2′,3′-dideoxycytidylyl-(5′ → 5′)-3′-*O*-acetyl-5-fluoro-2′-deoxyuridine (ddC^{pam}-(5′ → 5′)-5-FdU(Ac), **5**) was obtained in 68% yield. The reaction mixture obtained by condensation of **4** with **2** was concentrated to a syrup and treated with acid to remove the 4-monomethoxytrityl protecting group followed by purification on silica gel. The partially deprotected dimer dC^{pam}-5-FdU(Ac), **6** was obtained in 70% yield. By careful treatment with ammonia the 3′-acetyl group of **6** was completely cleaved whereas the equally alkali-labile N^4-palmitoyl residue was only partially lost. After partial deprotection the reaction mixture was purified using reversed phase chromatography and the pure amphiphilic heterodinucleoside phosphate N^4-palmitoyl-2′-deoxycytidylyl-(3′ → 5′)-5-fluoro-2′-deoxyuridine (**7**) resulted in 48% yield.

As illustrated in Fig. 3, instead of a second nucleoside derivative the free 5′-hydroxyl group of **2** was coupled to the lipophilic glyceryl derivative 1-*O*-octadecyl-2-*O*-acetyl-rac-glycerol-3-hydrogen phosphonate (**8**, phosphonate compound) in analogy to the synthesis described in Fig. 2. The reaction mixture was chromatographed using silica gel and the acetyl protecting groups of the fully protected liponucleotide were removed by ammonia treatment. The resulting amphiphilic liponucleotide 1-*O*-octadecyl-rac-glycerylyl-(3 → 5′)-5-fluoro-2′-deoxyuridine (Oct^{1}Gro-(3 → 5′)-5-FdU, **9**) was isolated by crystallization in a yield of 82%. The course of the synthesis and the purification steps were monitored by thin-layer chromatography (TLC). The chemical structure and the analytical purity of the products were confirmed by NMR spectroscopy and high resolution mass spectroscopy (< ± 5 ppm).

3. Anticancer activity evaluation

The anticancer activities of the dimers ddC^{pam}-(5′ → 5′)-5-FdU(Ac) (**5**), dC^{pam}-5-FdU(Ac) (**6**), Oct^{1}Gro-(3 → 5′)-5-FdU (**9**), 5-FdU-5-FdC^{hex} (**10**), 5-FdC^{oct}-5-FdU (**11**) and the monomeric 5-fluoro-2′-deoxycytidine derivatives p5-FdC^{oct} (**I**), 5-FdC^{hex} (**II**) were tested in the framework of the in vitro Anticancer Screen Program of the National Cancer Institute (USA) on a panel of 60 human cancer cell lines [17]. The anticancer activities (cf. Tables 1 and 2) were obtained from the screening data report including the data sheet, dose–response curves and the mean graphs. Mean graphs facilitate visual scanning of data for the selection of potential compounds for particular cell lines or for particular tumor subpanels with respect to a selected response parameter. The response parameter GI_{50} ($\log_{10}$ of molar sample concentration resulting in 50% growth inhibition) given in the mean

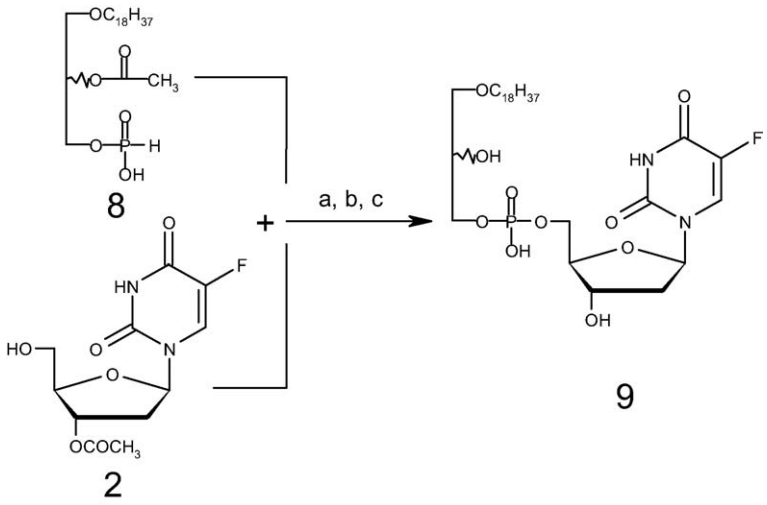

Fig. 3. Synthesis of the amphiphilic liponucleotide (**9**) containing 5-FdUMP as monomer unit starting from 3′-*O*-acetyl-5-fluoro-2′-deoxyuridine (**2**) and 1-*O*-octadecyl-2-*O*-acetyl-rac-glycerol-3-hydrogen phosphonate (**8**).
The reaction steps are: a = 1. pivaloyl chloride in pyridine, 2. iodine (0.2 M) in THF/pyridine/water (16:1:1); b, column chromatography on silica gel. c = methanol saturated with ammonia.

graphs are listed in Table 1. The average of the GI_{50}-values for all 60 cell lines is indicated by the mean graphs midpoint.

4. Results and discussion

Amphiphilic drugs are soluble in aqueous systems as well as in organic solvents and should be able to penetrate cell membranes by passive transport mechanisms. The amphiphilic nature and the corresponding intrinsic membrane permeability of a drug can be estimated with the octanol/water partition coefficient (PC). Based on previous studies with the amphiphilic [^{3}H]-labeled heterodinucleoside phosphate N^4-palmitoyl-2′-deoxycytidylyl-(3′ → 5′)-3′-azido-2′,3′-dideoxythymidine that has PC value of 0.62 [13] and penetrates through the membranes of H9 cells [11] we estimate that passive membrane diffusion of the new 5-FdUMP prodrugs should also occur because they have higher PC-values ranging from 1.38 to 8.15.

Through coupling of 5-FdUMP to a lipid the liponucleotide Oct^{1}Gro-(3 → 5′)-5-FdU (**9**) is obtained which has the highest PC value of 8.15 of all 5-FdUMP prodrugs. The dimers ddC^{pam}-(5′ → 5′)-5-FdU(Ac) (**5**) and dC^{pam}-5-FdU(Ac) (**6**) have similar PC values of 3.20 and 3.78 but they are significantly less lipophilic than liponucleotide **9**. The additional polar 5′-hydroxyl group of **6** which is absent in **5** does not seem to influence the partition properties of **6**. The dimer N^4-palmitoyl-2′-deoxycytidylyl-(3′ → 5′)-5-fluoro-2′-deoxyuridine (**7**) is obtained through cleavage of the 3′-acetyl group of **6**. Compared to **6** the deprotected **7** is significantly more hydrophilic which is reflected in the low PC value of 1.38.

The liponucleotide is significantly more lipophilic than the heterodinucleoside phosphates. However, this advantage is counteracted by a relatively complicated synthesis. The lipid derivatives necessary for the coupling reaction require multi-step syntheses that are very complicated for the preparation of an enantiomerically pure form. The hydrolytic stability of the liponucleotides can be varied by different derivatization of the hydroxyl groups of the glycerol backbone. Alkylation with long carbon chains produces alkali-stable ether lipids of high hydrolytic stability, whereas acylation with fatty acid derivatives yields alkali-labile esters that are easily cleavable by hydrolysis. The synthesis of ether liponucleotides is of particular advantage when prodrugs with slow-relase properties and high stability are desired.

Cytosine nucleosides were chosen as phosphonate components for the synthesis of the N^4-palmitoylated heterodinucleoside phosphates. The syntheses of these nucleosides are simple and the products have long storage stabilities. Although the use of the expensive 2′,3′-dideoxycytidine as alternative to the inexpensive 2′-deoxycytidine renders the synthesis easier requiring less protective groups, however it allows only 5′-coupling of the 2′,3′-dideoxycytidine derivatives. Introduction of the natural palmitoyl residue at the N^4-position of cytosine contributes not only to the amphiphilic property of the dimers but it also slows down enzymatic hydrolysis. A reduced hydrolysis rate may cause a depot effect in the cell membrane that is considerably less pronounced in analogous unprotected dimers. Upon enzymatic degradation in human serum of N^4-palmitoylated dinucleoside phosphates the palmitoyl residue is preferentially removed before the hydrolytic cleavage of the phosphodiester bond [13]. This property might increase the ratio of in vivo cell uptake of unmetabolized prodrugs.

The dimers ddC^{pam}-(5′ → 5′)-5-FdU(Ac) (**5**) and dC^{pam}-5-FdU(Ac) (**6**), where N^4-palmitoyl-2′,3′-dideoxycytidine or N^4-palmitoyl-2′-deoxycytidine are linked to 5-FdUMP are new alternative compounds to 5-FdU-5-FdC^{hex} (**10**) and 5-FdC^{oct}-5-FdU (**11**) which were synthesized before (unpublished data). Instead of the non-toxic N^4-palmitoyl cytidine moieties compounds **10** and **11** contain the cytotoxic N^4-

Table 1
In vitro anticancer activities of 5FdU, 5-fluoro-2-deoxycytidine derivatives (I, II) and 5-FdUMP-prodrugs (amphiphilic heterodinucleoside phosphates **5**, **6**, **10**, **11**; liponucleotide **9**) which were screened on a panel of 60 human cancer cell lines (panel/cell line) and expressed by the GI_{50} (log_{10} of sample concentration (molar) resulting in 50% growth inhibition; concentration below 10^{-7} M are shown in bold face)

	Growth inhibition GI_{50} (M)[a]							
	5-FdU	**I**	**II**	**5**[c]	**6**[c]	**10**	**11**	**9**[c]
Panel/cell line								
Leukemias								
CCRF-CEM	**–8.2**	–4.42	–[b]	–6.03	–5.45	**<–8.00**	–4.89	–6.52
HL-60	–6.7	–[b]	–5.26	–4.95	–6.20	–6.69	–5.29	–5.13
K-562	–6.1	>–4.00	–5.33	–5.71	–5.08	–5.97	–4.57	–5.00
MOLT-4	**–7.4**	–4.38	–[b]	–5.07	–5.00	–5.72	–4.83	–5.27
RPMI–8226	–6.1	>–4.00	–4.80	–6.23	–5.42	**–7.09**	–4.95	–5.66
SR	**–7.9**	–[b]	–5.40	–6.37	–[b]	**<–8.00**	–5.31	–6.32
Non-small cell lung cancers								
A549/ATCC	**–7.9**	–4.95	–6.03	–6.01	–6.60	**–7.64**	–5.14	–6.36
EKVX	–5.0	>–4.00	–4.84	–4.51	–4.43	–4.65	>–4.00	–4.18
HOP-62	**–7.6**	–4.09	–5.12	–5.49	–6.45	**<–8.00**	–4.82	**–7.34**
HOP-92	–6.1	–5.59	–4.99	–5.69	–6.31	–5.37	–5.27	–5.95
NCI-H226	–5.0	–4.49	–5.89	–4.49	–4.66	–5.52	–4.01	>–4.00
NCI-H23	–6.3	–4.31	–5.31	–5.11	–5.36	–6.33	–4.41	–5.50
NCI-H322 M	–6.3	>–4.00	–4.89	–4.52	–4.76	**–7.94**	–4.27	–6.76
NCI-H460	**–8.7**	–[b]	–4.97	–6.41	**–7.18**	**<–8.00**	–[b]	**–7.38**
NCI-H522	–5.6	>–4.00	–5.28	–[b]	–5.04	–5.16	–4.52	–4.90
Colon cancers								
COLO205	–5.8	>–4.00	–4.90	–4.95	–4.88	–4.97	4.68	–5.14
HCC-2998	**–9.0**	–4.82	**–7.15**	–5.13	**<–8.00**	**–8.00**	–5.22	**–7.33**
HCT-116	–6.9	–4.03	–5.08	–5.21	–5.41	–6.41	–4.33	–5.55
HCT-15	–5.7	>–4.00	–6.06	–4.66	–4.80	–4.87	–4.43	–4.94
HT29	–5.6	>–4.00	–5.01	?[b]	–5.34	–6.52	–4.78	–4.97
KM12	–5.2	>–4.00	–4.86	–4.80	–4.90	–4.56	–4.95	–4.86
SW-620	–5.0	>–4.00	–4.89	–4.61	–4.82	–4.74	–4.73	–4.92
SW-6 CNS CNS cancer								
SF-268	**–7.9**	–4.26	–5.53	–6.26	–6.51	**–8.00**	–4.90	–6.86
SF-295	**–7.4**	>–4.00	–4.78	–5.26	–6.02	**<–8.00**	–4.87	–6.37
SF-539	**–8.4**	–4.96	–6.55	–6.43	–6.86	**<–8.00**	–5.18	**–7.14**
SNB-19	–5.7	>–4.00	–4.86	–5.48	–4.77	–6.57	–4.92	–4.98
SNB-75	–6.7	>–4.00	–5.30	–4.92	–4.96	–5.80	–4.43	–6.07
U251	–6.9	>–4.00	–4.93	–4.96	–4.84	–5.84	–4.76	–5.86
Melanomas								
LOXI MVI	**–7.6**	–4.18	–4.98	–5.18	–5.89	**–7.37**	–4.58	–6.13
MALME-3 M	–5.1	–[b]	–4.97	–4.72	–[b]	–5.18	–[b]	–4.86
M14	–6.8	>–4.00	–5.07	–4.84	–5.60	–6.32	–4.71	–5.60
SK-MEL-2	–5.0	>–4.00	–4.71	–4.64	–4.69	–4.37	–4.56	–4.60
SK-MEL-28	–5.7	>–4.00	–4.90	–4.69	–4.52	–4.76	–4.57	–4.79
SK-MEL-5	–6.7	>–4.00	–4.94	–4.87	–5.15	–5.88	–4.46	–5.45
UACC-257	–5.5	>–4.00	–4.78	–4.80	–4.49	–4.88	–4.22	–4.95
UACC-62	**–7.4**	–4.21	–4.97	–4.94	–4.56	–6.53	–4.69	–6.27
Ovarian cancers								
IGROV1	–5.6	>–4.00	–4.94	–4.69	–4.71	–5.52	–4.33	–4.96
OVCAR-3	–5.6	>–4.00	–4.86	–4.53	–4.55	–5.08	–4.54	–4.82
OVCAR-4	–5.0	>–4.00	–4.80	–4.76	–4.50	–4.03	>–4.00	–4.85
OVCAR-5	–5.2	>–4.00	–4.74	–4.31	–4.76	–4.35	–4.27	–4.79
OVCAR-8	–6.9	–4.54	–5.74	–5.06	–5.41	–6.82	–4.76	–5.53
SK-OV-3	–5.7	>–4.00	–4.87	–4.55	–5.35	–4.94	–4.50	–4.89
Renal cancers								
786-0	**–7.0**	–4.15	–5.54	–5.45	–5.51	–6.85	–4.31	–6.09
A498	–5.9	–[b]	–[b]	–4.31	–4.91	**–7.90**	–4.89	–6.16
ACHN	**–7.5**	–5.20	–6.59	–5.74	–6.89	**–7.84**	–4.95	–6.40
CAKL-1	**–7.5**	–5.07	–5.68	–5.24	–6.19	–6.59	–4.58	–5.67

(continued on next page)

Table 1
(continued)

	Growth inhibition GI_{50} (M)[a]							
	5-FdU	**I**	**II**	**5**[c]	**6**[c]	**10**	**11**	**9**[c]
RXF393	–5.4	>–4.00	–4.87	–4.69	–4.77	–4.88	–4.94	–4.97
SN12C	–6.7	>–4.00	–4.90	–5.03	–5.38	–6.58	>–4.00	–5.24
TK-10	–5.3	>–4.00	–4.92	–4.45	–4.39	–4.76	–4.15	–4.99
UO-31	–6.9	–4.54	–5.85	–5.21	–6.10	–6.54	–4.87	–6.10
Prostate cancers								
PC-3	–6.5	–4.67	–5.41	–4.74	–5.10	–5.12	–4.65	–5.46
DU-145	–6.6	>–4.00	–5.00	–5.09	–5.71	–6.97	–4.21	–5.88
Breast cancers								
MCF7	**–8.2**	–5.05	–5.88	–6.30	>–4.00	**<–8.00**	–5.01	–6.90
NCI /ADR-RES	–5.9	–[b]	–5.54	–5.09	–4.94	–6.33	–4.52	–5.44
MDA-MB-231	–5.4	>–4.00	–4.66	–4.27	–4.73	–4.55	>–4.00	–4.69
HS 578T	–5.4	>–4.00	–4.76	–4.46	–4.92	–4.82	–4.45	–4.69
MDA-MB-435	–5.5	>–4.00	–4.91	–4.80	–4.76	–4.84	–4.12	–4.62
MDA-N	–5.9	>–4.00	–4.80	–4.73	–4.37	–4.86	–[b]	–4.74
BT-549	–6.0	>–4.00	–4.79	–4.87	–5.50	–4.88	–[b]	–4.89
T-47D	–5.9	–4.37	–4.93	–4.90	–5.06	–5.85	–4.51	–5.11
Mean graphs midpoint	–6.4	–4.23	–5.20	–5.09	–5.27	–6.05	–4.62	–5.51

[a] Screening data (mean graphs) obtained from the National Cancer Institute Developmental Therapeutics Program, USA.
[b] not screened.
[c] octanol/water PC of **5** = 3.20; **6** = 3.78 and **9** = 8.15.

Table 2
Cell lines showing specific sensitivities to 5-FdU, 5-FdUMP prodrugs (**5**, **6**, **9**, **10**) and 5-fluoro-2′-desoxycytidine derivatives (**I**, **II**). Compounds with GI_{50} values 10- to 100-fold below average (mean graphs midpoint) of all the tested 60 cell lines are listed under sensitivity I, GI_{50} values > 100-fold under sensitivity II

Panel/cell line	Sensitivity I compound	Sensitivity II compound
Leukemias		
CCRF-CEM	5-FdU, **9**	**10**
MOLT-4	5-FdU	
RPMI-8226	**5**, **10**	
SR	5-FdU, **5**	**10**
Non-small cell lung cancers		
A549/ATCC	5-FdU, **6**, **10**	**10**
HOP-62	5-FdU, **6**, **9**	
HOP-92	**6**, **I**	
NCI-H322M	**9**, **10**	**6**, **10**
NCI-H460	5-FdU, **5**, **9**	
Colon cancers		
HCC-2998	**9**, **II**	5-FdU, **6**, **10**
CNS cancers		
SF-268	5-FdU, **5**, **6**, **9**, **10**	
SF-295	5-FdU	**10**
SF-539	**5**, **6**, **9**, **II**	5-FdU, **10**
Melanoma		
LOXI MVI	5-FdU, **10**	
UACC-62	5-FdU	
Renal cancers		
A498	**10**	
ACHN	5-FdU, **6**, **II**, **10**	
CAKL-1	5-FdU	
Breast cancers		
MCF7	5-FdU, **5**, **9**	**10**

octadecyl(hexadecyl)-5-fluoro-2′-deoxycytidine monomer units. The synthesis of the required N^4-alkylated 5-fluorocytidine nucleosides entails more steps than the acylated compounds rendering their preparation very cumbersome [18]. The major difference between dimers **5** and **6** where 5-FdUMP is linked to an inactive nucleoside and compounds **10** and **11** is that they contain a second cytotoxic nucleoside moiety. The cytotoxic activity of the N^4-alkylated 5-fluorocytidine derivatives which was observed, e.g. with p5-FdC^{oct} (**I**) and 5-FdC^{hex} (**II**) (cf. Table 1) is probably caused by their intrinsic cytotoxic activity or by dealkylation followed by enzymatic deamination to 5-FdU.

The amphiphilic properties of the 5-FdUMP prodrugs are not only based on the concomitant presence of octadecyl- or palmitoyl residues with hydrophilic hydroxyl groups and a polar phosphodiester linkage. Rather, the structural alignment of these functional groups seems to be responsible for the amphiphilic nature of the molecules. This is supported by the different PC-values found for dimer dC^{pam}-5-FdU (**7**) (PC = 1.38) and the liponucleotide Oct^1Gro-(3 → 5′)-5-FdU (**9**) (PC = 8.15). Despite the fact that both prodrugs contain the same number of functional groups (2 OH groups, a phosphodiester link, a long chain hydrocarbon residue and a 5′-linked 5-FdUMP moiety) structural differences must be responsible for their different amphiphilic properties as reflected in the significantly different PC-values.

After successful synthesis of the 5-FdUMP prodrugs their antitumor activities were tested in vitro on a panel of 60 human tumor cell lines. Thereby it was analyzed to what extent the chemical structure would influence the antitumor activity. As a measure for the anticancer activity the GI_{50} values were used (Table 1). The values show that all tested compounds exert cytotoxic activities, however with large differences in activity and tumor type specificity. The mean overall GI_{50}

value of 5-FdU was the lowest one with –6.4, followed by dimer 5-FdU-5-FdC^{hex} (**10**) with a value of –6.05. The GI_{50} values of the other prodrugs were –5.51 (Oct^{1}Gro-(3 → 5′)-5-FdU (**9**), –5.27 (dC^{pam}-5-FdU(Ac) (**6**), –5.20 (5-FdC^{hex} (**II**), –5.09 (ddC^{pam}-(5′ → 5′)-5-FdU(Ac) (**5**), –4.62 (5-FdC^{oct}-5-FdU (**11**), –4.23 (p5-FdC^{oct} (**I**). Compounds **I** and **11** are not suited as antitumor drugs because their activity is approximately 100-fold lower than that of 5-FdU. The marked difference of the GI_{50}-values of **10** (–6.05) and **11** (–4.62) is surprising because of their high structural similarity (cf. Fig. 1). It is conceivable that the nucleoside sequence of the dimers is responsible for the low activity detected for **11** causing a very slow release of the active compounds 5-FdU and 5-FdUMP. This depot effect might have prevented the formation of the active metabolites in the time course of the cytotoxicity test, avoiding the release of enough free 5-FdU to exert antitumor effects. This example shows that by variation of the nucleoside sequence of the heterodinucleotide phosphates the rate of enzymatic degradation and thus the depot effect can be influenced. This assumption finds support in an earlier finding that prodrug 5-FdU-5-FdC^{oct} (**11a**), the sequence isomer to 5-FdC^{oct}-5-FdU (**11**), was significantly more effective than 5-FdU in an in vitro clonogenic growth assay using the human pancreatic adenocarcinoma cell line MIA/PaCa 2 [12]. Furthermore, the investigation of the antitumor activity of **11a** and dC^{pam}-5-FdU (**7**) (Fig. 2) demonstrated that both compounds have comparably high anticancer activities against DU-145 human prostate cancer cells and are effective prodrugs of 5-FdU. Dimer **11a** was capable of eradicating 100% of cancer cells, whereas 10% of the cells remained resistant to 5-FdU [14,19].

For compounds **5**, **6**, **9** the mean GI_{50} values (–5.09 to –5.51) are higher than the value of 5-FdU (–6.4). The reduced activity can also probably be explained by depot effects caused by structural differences. On the other hand, the high cytotoxic activity of 5-FdU-5-FdC^{hex} (GI_{50} –6.05) that is comparable to that of 5-FdU (GI_{50} –6.4) can be explained by the coupling of two cytotoxic nucleosides, where upon enzymatic cleavage the two active compounds 5-FdU and 5-FdC^{hex} which have high GI_{50} values (–6.4 for 5-FdU and –5.2 for (5-FdC^{hex}), are released as single compounds. The depot effect of 5-FdU-5-FdC^{hex} is probably concealed by the fast release of 5-FdC^{hex}. Conversely, it is also possible that the slow hydrolysis of compound 5-FdU-5-FdC^{hex} is prevents that a higher in vitro activity than that of 5-FdU is obtained.

The therapeutic application of the prodrugs requires not only high cytotoxic activity but also high specificity which is reflected in the differing sensitivities against the tumor cell lines investigated. In Table 2 the cell lines are listed which had sensitivities more than 10-fold below the mean GI_{50}-value of all 60 cell lines tested. Thereby, we found that 16/60 cell lines were more sensible towards prodrugs **5**, **6**, **9** and **10** as the mean of all cells. Of these 16 cell lines 12 were also highly sensible towards 5-FdU, allowing the conclusion that the 5-FdUMP-derivatives are prodrugs of 5-FdU. As shown in the following example, the chemical structure of the prodrugs does not only affect cytotoxic activity but also tumor cell specificity. Monomer 5-FdC^{hex} (GI_{50} –5.20) had a comparable mean activity as dimer dC^{pam}-5-FdU(Ac) (GI_{50} –5.27) but only three cell lines were highly sensitive against 5-FdC^{hex} as compared to eight highly sensitive cell lines with dimer dC^{pam}-5-FdU(Ac). Of all tested prodrugs the compound 5-FdU-5-FdC^{hex} (A) has the highest similarity to the cytotoxic activity of 5-FdU (B) which is reflected in the ratio of the highly sensitive cell lines to the total number of the cell lines in the panel: leukemias A: 3/6, B: 3/6; non-small cell lung cancer A: 4/9; B: 3/9; CNS cancer A: 3/6; B 3/6; renal cancer A: 2/8, B: 3/8; melanoma A: 1/8, B: 2/8; colon cancer A: 1/8, B 1/8 and breast cancer A: 1/8, B: 1/8.

The amphiphilic character of the prodrugs facilitates their cell uptake. Due to the natural phosphodiester bond that functions as potential cleavage site intracellular enzymatic degradation leads to 5-FdUMP. The frequently occurring development of drug resistance that is caused by reduced or absent 5′-phosphorylation of 5-FdU can be circumvented by the described prodrugs. However, since the panel of tumor cells in which the prodrugs were tested did not include 5-FU or 5-FdU resistant cells it will be important to analyze their activities on such cells. Moreover, as further advantage the prodrugs can be formulated in small antibody-tagged liposomes for specific delivery to corresponding targets [20]. Thus, the advantages of the 5-FdUMP prodrugs over 5-FdU warrant their further development as potential new anticancer drugs.

5. Conclusion

The coupling of lipophilic cytosine or phospholipid derivatives with 5-FdU affords amphiphilic antitumor prodrugs that contain a caged 5-FdUMP moiety. The concept presented here is not restricted to 5-FdU, it can be adapted in a general manner for the transformation of antimetabolites into amphiphilic prodrugs. The linkage of 5-FdU with N^4-palmitoylated cytidine derivatives instead of the N^4-hexadecyl- (or octadecyl-) 5-fluoro-2′-deoxycytidine derivatives simplifies the synthesis but also reduces the activity of the resulting prodrugs. Therefore, it is important to carefully evaluate properties and nucleoside sequences of the individual compounds to obtain prodrugs of high activity. The structure–activity parameters analyzed in in vivo experiments will finally determine which prodrug will have the most favorable properties in regard of low unwanted toxicity, bioavailability and metabolism. Such tests will finally determine the suitability of the prodrugs for further development.

6. Experimental protocols

6.1. Chemistry

Commercially available were: 5-fluoro-2′-deoxyuridine (Pharma-Waldhof, Düsseldorf, Germany), pivaloyl chloride,

pyridine and silica gel 60 (Merck, Darmstadt, Germany). Prepared as described were: salicylchlorophosphite [21], 4-monomethoxytrityl chloride [22], 1-*O*-octadecyl-2-*O*-acetyl-rac-glycerol [23], N^4-palmitoyl-2′,3′-dideoxycytidine-5′-hydrogen phosphonate [13], 5′-*O*-(4-monomethoxytrityl)-N^4-palmitoyl-2′-deoxycytidine [7]. Prepared after a published method [16] with major modifications were: 5′-*O*-(4-monomethoxytrityl)-5-fluoro-2′-deoxyuridine and 3′-*O*-acetyl-5-fluoro-2′-deoxyuridine. Pyridine was refluxed over potassium hydroxide, distilled and stored over molecular sieves (4 Å). Diethylether was dried over potassium hydroxide, distilled and stored over molecular sieves (4 Å).

All reactions were monitored by TLC on pre-coated silica gel 60 F_{254} plates (0.25 mm, Merck) using UV light for visualization and spray reagents as developing agents. Sugar moieties were developed by spraying the plates with perchloric acid (60%) followed by heating. Compounds protected by the 4-monomethoxytrityl group were detected as yellow spots when acid was used as spraying reagent. Compounds containing hydrogen phosphonate or phosphodiester groups were detected as blue spots on the TLC-plates when a molybdic acid spraying reagent was used for the detection. Long alkyl chains were detected as orange fluorescent spots under UV (366 nm) when ethanolic 2,7-dichlorofluorescein was used as spray reagent. Multi-step flash chromatography was carried out on dry packed silica gel 60 (0.040–0.063 mm) columns using binary solvent mixtures prepared by volume ratios (v/v) as eluent. NMR spectra were recorded with Bruker AMX 250 (250 MHz) and AMX400 (400 MHz) instruments and calibrated using DMSO-d_6 or $CDCl_3$ as solvent and internal standards. 2D-Cosy and Dept135-spectra were recorded on a Bruker AMX 400 instrument and used when necessary to determine the shifts of protons and carbons. FD mass spectra of intermediate products were recorded on a Finnigan MAT 711 A HRMS (ESI, negative mode) mass spectra of the derivatives were recorded on a Bruker APEX II. Melting points (not corrected) were determined in a Stuart Scientific SMP3 capillary melting point apparatus. PC in 1-octanol/water were determined using UV-absorbance. The UV-absorbance (A_{260}(I)) of an aqueous solution of the test compound was determined. Equal volumes of this solution and 1-octanol were mixed and vigorously shaken at room temperature for 15 min, followed by centrifugation (10,000 rpm, 20 min). The UV-absorbance (A_{260}(II)) of the aqueous layer was determined and the PC-value was calculated as the quotient: [A_{260}(I)–A_{260}(II)]/A_{260}(II).

6.1.1. General condensation procedure using the hydrogen phosphonate method

All reactions were performed at room temperature if not stated differently. The concentration of the reaction mixtures, solutions, organic layers and eluted fractions was done in vacuum at a bath temperature of 45 °C. Equimolar amounts of a hydroxyl- and a hydrogen phosphonate compound (cf. Table 3) were dissolved in dry pyridine. This solution was cooled to 0 °C before the fivefold amount of pivaloylchloride was added under the exclusion of moisture. After stirring for 3 min (7 min in case of glyceryl phosphonates) the reaction mixture was cooled to 0 °C before the reaction was stopped by the addition of water. The obtained phosphonic diester was oxidized immediately by addition of iodine in THF (0.2 M). After 1 h stirring at room temperature excess iodine was removed by addition of solid sodium hydrogen sulfite before the reaction mixture was concentrated to a syrup that was dissolved in chloroform and extracted twice with water. The organic layer was concentrated to a syrup which was co-evaporated three times with some toluene yielding the crude condensation product which was purified and isolated as described below.

*6.1.2. 5′-O-(4-Monomethoxytrityl)-5-fluoro-2′-deoxyuridine (**1**)*

To a solution of 5-fluoro-2′-deoxyuridine (10 g, 41 mmol) in dry pyridine (50 ml) 4-monomethoxytrityl chloride (14 g, 46 mmol) was added under the exclusion of moisture. The reaction vessel was sealed air tight and shaken for 5 h The reaction mixture was cooled to 0 °C and the obtained precipitate collected by filtration, washed with dry ether (20 ml) and discarded. To the filtrates methanol (20 ml) was added, followed by 10 min shaking before concentration to a syrup that was co-evaporated with toluene (50 ml). This syrup was dissolved in chloroform (150 ml) and extracted three times with a saturated aqueous solution of sodium hydrogen carbonate (50 ml). The organic layer was concentrated to a syrup that was co-evaporated twice with toluene (50 ml) before being dissolved in ether (200 ml) and chromatographed on a silica gel column (16 × 9 cm) using ether (6 l) as eluent. The desired fractions were pooled, concentrated and dried, affording 5′-(4-

Table 3
Experimental data for the synthesis of the liponucleotide (**9**) and the heterodinucleoside phosphates (**5**, **6**) using the hydrogen phosphonate method

Condensation reaction	Obtained crude condensation products		
	9	**5**	**6**
Hydroxyl compound number (g/mmol)	**2**, 3.5/12.2	**2**, 1.5/5.2	**2**, 1.5/5.2
+ Phosphonate compound number (g/mmol)	+ **8**, 5.5/12.2	+ **3**, 2.7/5.2	+ **4**, 4.0/5.2
Pyridine (ml)	90	40	40
Pivaloyl chloride (ml/mmol)	7.5/61	3.5/28.4	3.5/28.4
Water (ml)	4	2	2
Iodine in THF (ml)	55	24	24
Chloroform (ml)	200	100	100
Water (ml)	50	25	25

monomethoxytrityl)-5-fluoro-2′-deoxyuridine (**1**) as a colorless foam (19.8 g, 94%) that could be precipitated from n-pentane under sonification yielding a white powder. M.p. 90–93 °C (foam), 95–97 °C (solid). TLC (ether) $R_f = 0.15$. MS (FD) *m/z*: 541.0 ($M + Na^+$), 518.1 ($M + H^+$).

^{1}H-NMR (DMSO-d_6): δ 7.90 (d, $J = 6.82$, 1H, H_6), 6.88–7.44 (m, 14H, aromatic H of trityl), 6.16 (t, $J = 5.79$, 1H, $H_{1'}$), 5.37 (bs, 1H, $H_{3'OH}$), 4.30 (m, 1H, $H_{3'}$), 3.90 (m, 1H, $H_{4'}$), 3.74 (s, 3H, OCH_3), 3.13–3.32 (m, 2H, $H_{5'}$, $H_{5''}$), 2.08–2.32 (m, 2H, $H_{2'}$, $H_{2''}$).

^{13}C-NMR (DMSO-d_6): δ 158.28, 144.21, 144.06, 134.80, 130.06, 127.95, 126.94, 123.88, 113.28, 86.15 (trityl-group), 157.02 (d, $J = 25.91$ Hz, C_4), 148.94 (C_2), 140.00 (d, $J = 231.52$ Hz, C_5), 124.49 (d, $J = 33.90$ Hz, C_6), 85.58 ($C_{4'}$), 84.57 ($C_{1'}$), 70.06 ($C_{3'}$), 64.91, 63.75 ($C_{5'}$), 55.04 (OCH_3), 39.41 ($C_{2'}$).

*6.1.3. 3′-O-Acetyl-5-fluoro-2′-deoxyuridine (**2**)*

To a solution of **1** (9.1 g, 17.5 mmol) in dry pyridine (25 ml) acetic anhydride (5 ml, 53 mmol) was added under the exclusion of moisture. The reaction vessel was sealed and shaken for 5 h before methanol (6 ml) was added under cooling (0 °C). After 10 min of shaking the reaction mixture was concentrated to a syrup which was co-evaporated tree times with toluene (20 ml). This syrup was dissolved in acetic acid (80%, 50 ml) and refluxed for 20 min followed by another concentration step and co-evaporation with toluene (3 × 50 ml). The crude product was dissolved in hot ethyl acetate (80 ml) and crystallized at –25 °C. The obtained precipitate was collected by filtration and recrystallized form ethyl acetate (80 ml) at 0 °C yielding 3′-*O*-acetyl-5-fluoro-2′-deoxyuridine (**2**) as colorless crystals (4.2 g, 83%). M.p. 201 °C. TLC ($CHCl_3$/MeOH 9:1) $R_f = 0.44$. MS (FD) *m/z*: 289.1 ($M + H^+$).

^{1}H-NMR (DMSO-d_6): δ 8.20 (d, $J = 7.18$, 1H, H_6), 6.15 (t, $J = 7.12$, 1H, $H_{1'}$), 5.30 (bs, 1H, $H_{5'OH}$), 5.21 (m, 1H, $H_{3'}$), 4.01 (m, 1H, $H_{4'}$), 3.64 (m, 2H, $H_{5'}$,$H_{5''}$), 2.25–2.30 (m, 2H, $H_{2'}$,$H_{2''}$), 2.06 (s, 3H, CH_3).

^{13}C-NMR (DMSO-d_6): δ 170.00 (CO-acetyl), 156.97 (d, $J = 26.35$ Hz, C_4), 149.03 (C_2), 140.10 (d, $J = 230.64$ Hz, C_5), 124.42 (d, $J = 34.59$ Hz, C_6), 84.94 ($C_{4'}$), 84.46 ($C_{1'}$), 74.68 ($C_{3'}$), 36.89 ($C_{2'}$), 20.78 (CH_3-acetyl).

*6.1.4. 5′-O-(4-Monomethoxytrityl)-N^4-palmitoyl-2′-deoxycytidine-3′-hydrogen phosphonate (**4**)*

To a solution of 5′-*O*-(4-monomethoxytrityl)-N^4-palmitoyl-2′-deoxycytidine (20 g, 27 mmol) in dry pyridine (40 ml) and dry dioxane (160 ml) salicylchlorophosphite (6.6 g, 32.5 mmol) was added under the exclusion of moisture. The reaction mixture was shaken for 1 h before a saturated solution of sodium hydrogen carbonate (50 ml) was added at 0 °C, followed by additional shaking for 15 min. The reaction mixture was concentrated to a syrup which was dissolved in chloroform (200 ml) and extracted with a saturated aqueous solution of sodium hydrogen carbonate (3 × 100 ml). The organic layer was concentrated to a syrup which was dissolved in chloroform/methanol (95:5, 150 ml) and chromatographed on a silica gel column (16 × 9 cm) using a three step chloroform/methanol gradient: Step 1: 95/5 (4 l); step 2: 9/1 (4 l) and step 3: 8/2 (8 l). The fractions containing the desired product were pooled, concentrated and dried, affording 5′-*O*-(4-monomethoxytrityl)-N^4-palmitoyl-2′-deoxycytidine-3′-hydrogen phosphonate (**4**) as a colorless foam (16.3 g, 75%). M.p. 159–164 °C. TLC ($CHCl_3$/MeOH 8:2) $R_f = 0.34$.

^{1}H-NMR (DMSO-d_6, 250.134 MHz): δ 8.09 (d, $J = 7.46$ Hz, 1H, H_5), 7.22–7.39 (m, 12H, aromatic-*H*), 7.09 (d, $J = 7.42$ Hz, 1H, H_6), 6.89 (d, $J = 8.89$ Hz, 2H, aromatic-H), 6.59 (d, $J = 604.18$ Hz, 1H, P-*H*), 6.11 (t, $J = 5.79$ Hz, 1H, $H_{1'}$), 4.73 (m, 1H, $H_{3'}$), 4.15 (m, 1H, $H_{4'}$), 3.73 (s, 3H, OCH_3), 3.18–3.37 (m, 2H, $H_{5'} + H_{5''}$), 2.35–2.41 (m, 2H, $H_{2'} + H_{2''}$), 1.52 (m, 2H, $-CH_2-$), 1.22 (s, 26H, $-(CH_2)_n-$), 0.83 (t, $J = 6.56$ Hz, 3H, $-CH_3$).

^{13}C-NMR (DMSO-d_6, 100.624 MHz): δ 173.84 (NH*C*O), 162.33 (C_4), 154. 28 (C_2), 144.24 (C_6), 158.20, 143.87, 143.73, 134.63, 129.94, 127.90, 126.92, 113.23 (aromatic-*C*), 86.24 ($C(C_6H_5)_3$), 86.24 ($C_{1'}$), 86.06 ($C_{4'}$), 71.66 ($C_{3'}$), 62.77 ($C_{5'}$), 54.94 (OCH_3), 36.32 ($C_{2'}$), 31.27, 29.02, 28.99, 28.86, 28.72, 28.69, 28.43, 24.41, 22.06 ($(CH_2)_n$), 13.87 ($(CH_2)_nCH_3$). HRMS calculated for $C_{45}H_{59}N_3O_8P$ [$M - H^+$]: 800.40453, found: 800.40471.

*6.1.5. N^4-Palmitoyl-2′,3′-dideoxycytidylyl-(5′ → 5′)-3′-O-acetyl-5-fluoro-2′-deoxyuridine (**5**)*

The syrup, obtained by condensation of compound **3** with compound **2** (Table 3), was crystallized form methanol (50 ml) at –25 °C. The crude product was dissolved in $CHCl_3$/MeOH (9:1, 50 ml) and purified by flash chromatography on a silica gel column (20 × 5 cm) using a three step $CHCl_3$/MeOH gradient: step 1: 9/1 (3 l); step 2: 8/2 (3 l); and step 3: 7/3 (3 l). The fractions containing the desired product were pooled, concentrated and crystallized from methanol yielding N^4-palmitoyl-2′,3′-dideoxycytidylyl-(5′ → 5′)-3′-*O*-acetyl-5-fluoro-2′-deoxyuridine (**5**) as colorless crystals (2.9 g, 68%). M.p. 195–205 °C. TLC ($CHCl_3$/MeOH, 6:4) $R_f = 0.71$.

^{1}H-NMR (DMSO-d_6, 250.134 MHz): δ 11.78 (bs, 1H, NH-5FdU), 10.80 (bs, 1H, N*H*CO), 8.34 (d, $J = 7.50$ Hz, 1H, H_5-ddC), 8.18 (m, 1H, H_6-5FdU), 7.20 (d, $J = 7.48$, H_6-ddC), 6.16 (m, 1H, $H_{1'}$-5FdU), 5.95 (m, 1H, $H_{1'}$-ddC), 5.18–5.25 (m, 1H, $H_{3'}$-5FdU), 4.13–4.25 (m, 1H, $H_{4'}$), 3.85–4.10 (m, 5H, $H_{5'} + H_{5''} + H_{4'}$), 2.04 (s, 3H, $COCH_3$), 1.80–2.40 (m, 6H, $H_{2'}$; $H_{2''}$ (5FdU; ddC) + $H_{3'}$; $H_{3''}$(ddC)), 1.51 (m, 2H, $CH_2-(CH_2)_{13}-CH_3$), 1.23 (s, 26H $(CH_2)_{13}-CH_3$) 0.85 (t, $J = 6.81$, 3H $(CH_2)_{13}-CH_3$).

^{13}C-NMR (DMSO-d_6, 250.134 MHz): δ 173.70 (NH*C*O), 169.81 (*C*OCH_3), 162.17 (C_4-ddC), 156.90 (d, $J = 26.71$ Hz, C_4-5FdU), 154.43 (C_2-ddC), 148.98 (C_2-5FdU), 144.72 (C_6-ddC), 140.07 (d, $J = 232.70$ Hz, C_5-5FdU), 124.46 (d, $J = 36.24$ Hz, C_6-5FdU), 94.95 (C_5-ddC), 86.93, 84.44 ($C_{1'}$), 83.12, 80.64 ($C_{4'}$), 74.76 ($C_{3'}$-5FdU), 65.24, 64.65 ($C_{5'}$), 36.27, 32.47 ($C_{2'}$), 24.43 ($C_{3'}$-ddC), 31.21, 28.95, 28.62, 28.40, 27.66, 26.94, 26.02, 25.84, 22.00 (–(*C*H_2)–), 20.68 (CO*C*H_3), 13.84 ($(CH_2)_n-CH_3$).

HRMS calculated for $C_{36}H_{54}FN_2O_{12}P$ [$M - H^+$]: 798.34961, found: 798.34987.

*6.1.6. N^4-Palmitoyl-2′-deoxycytidylyl-(3′ → 5′)-3′-O-acetyl-5-fluoro-2′-deoxyuridine (**6**)*

The syrup, obtained by condensation of compound **4** with compound **2** (Table 3), was dissolved in chloroform (35 ml) and after the addition of a methanolic p-toluene-sulfonic acid solution (4.0%, 35 ml) the resulting mixture was stirred for 5 min. The reaction mixture was then poured into a half saturated aqueous solution (100 ml) of sodium hydrogen carbonate. The organic layer was separated, concentrated to a syrup which was dissolved in $CHCl_3$/MeOH (9:1, 100 ml) and purified on a silica gel column (15 × 5 cm) using a three step $CHCl_3$/MeOH gradient: Step 1: 9/1 (2 l); step 2: 8/2 (2 l) and step 3: 7/3 (3 l). The fractions containing the desired product were pooled, concentrated and dried yielding N^4-palmitoyl-2′-deoxycytidylyl-(3′ → 5′)-3′-*O*-acetyl-5-fluoro-2′-deoxyuridine (**6**) as a colorless foam (2.9 g, 70%). M.p. 185–190 °C. TLC ($CHCl_3$/MeOH, 7:3) R_f = 0.33.

^{1}H-NMR (DMSO-d_6, 400.136 MHz): δ 11.89 (bs, 1H, N*H*-5FdU), 10.83 (bs, 1H, N*H*CO), 8.29 (d, J = 7.40 Hz, 1H, H_5-dC), 8.14 (m, 1H, H_6-5FdU), 7.21 (d, J = 7.40 Hz, 1H, H_6-dC), 6.07–6.16 (m, 2H, $H_{1'}$), 5.37 (bs, 1H, 5′O*H*), 5.21 (m, 1H, $H_{3'}$), 4.71 (m, 1H, $H_{3'}$), 4.10 (m, 2H, $H_{4'}$), 3.93–3.98 (m, 2H, $H_{5'}$ + $H_{5''}$), 3.59 (m, 2H, $H_{5'}$ + $H_{5''}$), 2.33–2.37 (m, 2H, COCH_2), 2.05–2.30 (m, 4H, $H_{2'}$ + $H_{2''}$), 2.03 (s, 3H, COCH_3), 1.50 (m, 2H, COCH$_2$CH_2), 1.21 (s, 26H (CH_2)$_{12}$), 0.82 (t, J = 6.64 Hz (CH$_2$)$_n$–CH_3).

^{13}C-NMR (DMSO-d_6, 100.624 MHz): δ 173.87 (NH*C*O), 169.96 (*C*OCH$_3$), 162.35 (C_4-dC), 157.01 (d, J = 26.16 Hz, C_4-5FdU), 154.51 (C_2-dC), 149.12 (C_2-5FdU), 144.82 (C_6-dC) 140.19 (d, J = 232.24 Hz, C_5-5FdU), 124.72 (d, J = 34.31 Hz, C_6-5FdU), 95.47 (C_5-dC), 86.89 ($C_{4'}$), 86.20 ($C_{1'}$), 84.43 ($C_{1'}$), 83.18 ($C_{4'}$), 74.90 ($C_{3'}$, $C_{3'}$), 64.66 ($C_{5'}$), 61.28 ($C_{5'}$), 36.37 ($C_{2'}$), 36.20 ($C_{2'}$), 31.33, 29.09, 29.05, 28.93, 28.80, 28.75, 28.53, 24.46, 23.09, 22.12 ((CH$_2$)$_n$), 20.79 (*C*OCH$_3$), 13.94 ((CH$_2$)$_n$CH$_3$).

HRMS calculated for $C_{36}H_{54}FN_5O_{13}P$ [M – H$^+$]: 814.34453, found: 814.34350.

*6.1.7. N^4-Palmitoyl-2′-deoxycytidylyl-(3′ → 5′)-5-fluoro-2′-deoxyuridine (**7**)*

To a solution of **6** (1.5 g, 1.8 mmol) in $CHCl_3$ (20 ml) methanolic ammonia (50 ml) was added and the reaction mixture was kept for 1 h After concentration the crude product was dissolved in H_2O/MeOH (3:7, v/v, 5 ml) and purified by reversed phase chromatography on a LiChroprep RP-18 (40–63 nm) column (Merck) (310 × 25 mm) using a binary NH_4OAc (aq., 0.05 N)/MeOH-gradient (2 l) from 70% MeOH to 100% MeOH. The product containing fractions were pooled, concentrated and twice lyophilized, yielding N^4-palmitoyl-2′-deoxycytidylyl-(3′ → 5′)-5-fluoro-2′-deoxyuridine (**7**) as colorless powder (0.68 g, 48%). M.p. 180–185 °C. TLC ($CHCl_3$/MeOH, 7:3) R_f = 0.11.

^{1}H-NMR (DMSO-d_6, 250.134 MHz): δ 11.82 (bs, 1H, N*H*-5FdU), 10.81 (bs, 1H, N*H*CO), 8.30 (d, J = 7.69 Hz, 1H, H_5-dC), 8.05 (m, 1H, H_6-5FdU), 7.23 (d, J = 7.37 Hz, 1H, H_6-dC), 6.09–6.15 (m, 2H, $H_{1'}$), 4.66 (m, 1H, $H_{3'}$), 4.27 (m, 1H, $H_{3'}$), 4.08 (m, 1H, $H_{4'}$), 3.88 (m, 3H, $H_{4'}$ + $H_{5'}$ + $H_{5''}$), 3.59 (m, 2H, $H_{5'}$ + $H_{5''}$), 2.36 (t, J = 7.20 Hz, COCH_2–), 2.06–2.16 (m, 4H, $H_{2'}$ + $H_{2''}$), 1.52 (bs, 2H, CH_2(CH$_2$)$_{12}$CH$_3$), 1.23 (s, 24H, CH$_2$(CH_2)$_{12}$CH$_3$), 0.84 (t, J = 6.65 Hz, 3H, CH$_2$(CH$_2$)$_{12}$CH_3).

^{13}C-NMR (DMSO-d_6, 62.896 MHz): δ 173.87 (NH*C*O), 162.35 (C_4-dC), 156.94 (d, J = 26.05 Hz, C_4-5FdU), 154.49 (C_2-dC), 149.05 (C_2-5FdU), 144.78 (C_6-dC), 140.11 (d, J = 232.12 Hz, C_5-5FdU), 124.66 (d, J = 34.28 Hz, C_6-5FdU), 95.46 (C_5-dC), 86.81 ($C_{4'}$), 86.14 ($C_{4'}$), 84.48 ($C_{1'}$ + $C_{1'}$), 74.58 ($C_{3'}$), 70.67 ($C_{3'}$), 65.02 ($C_{5'}$), 61.22 ($C_{5'}$), 39.08 ($C_{2'}$), 36.38 ($C_{2'}$), 33.71, 31.29, 29.02, 28.88, 28.70, 28.49, 24.44, 22.08 (–(CH$_2$)$_{14}$–), 13.92 ((CH$_2$)$_{14}$*C*H$_3$).

HRMS calculated for $C_{34}H_{52}FN_5O_{12}P$ [M – H$^+$]: 772.33373, found: 772.33360.

*6.1.8. 1-O-Octadecyl-2-O-acetyl-rac-glycerol-3-hydrogen phosphonate (**8**)*

To a solution of 1-*O*-octadecyl-2-*O*-acetyl-rac-glycerol (10 g, 26 mmol) dissolved in dry pyridine (30 ml) and dry dioxane (120 ml) salicylchlorophosphite (7.9 g, 39 mmol) was added and the reaction mixture was shaken for 1 h under the exclusion of moisture. After hydrolysis with water (9 ml) the reaction mixture was concentrated to a syrup which was dissolved in chloroform (150 ml) and extracted with a saturated aqueous solution of sodium hydrogen carbonate (2 × 100 ml). The organic layer was concentrated to a syrup that was co-evaporated with toluene (3 × 50 ml) before being dissolved in hot ethyl acetate (80 ml) and crystallized at –25 °C. The obtained precipitate was collected by filtration and re-crystallized from ethyl acetate (80 ml) at 0 °C, yielding 1-*O*-octadecyl-2-*O*-acetyl-rac-glycerol-3-hydrogen phosphonate (**8**) as colorless crystals (8.9 g, 76%). M.p. 63 °C. TLC ($CHCl_3$/MeOH, 8:2) R_f = 0.22.

^{1}H-NMR ($CDCl_3$, 400.136 MHz): δ 6.74 (d, J = 629.41 Hz, 1H, P*H*), 4.13–4.48 (m, 3H, C*H*–,CH_2-glycerol), 3.41–3.56 (m, 4H, O–CH_2– +CH_2-glycerol), 2.03 (s, 3H, COCH_3), 1.50 (bs, 2H, O–CH$_2$–CH_2–), 1.22 (bs, 30H, –(CH_2)$_{15}$–), 0.84 (t, J = 6.80 Hz, 3H, –(CH$_2$)$_{15}$–CH_3).

^{13}C-NMR ($CDCl_3$, 100.624 MHz): δ 171.40 (*C*OCH$_3$), 71.26 (*C*H$_2$-glycerol), 70.24 (O–*C*H$_2$–(CH$_2$)$_{16}$), 68.63 (*C*H-glycerol), 64.21 (*C*H$_2$-glycerol), 31.85, 29.89, 29.71, 29.66, 29.61, 29.48, 29.38, 29.30, 25.96, 22.61, 21.13, 20.83 (–(*C*H$_2$)$_{16}$–), 20.77 (–CO*C*H$_3$), 14.02 (–(CH$_2$)$_{16}$–*C*H$_3$).

HRMS calculated for $C_{23}H_{46}O_6P$ [M – H$^+$]: 449.30375, found: 449.30567.

*6.1.9. 1-O-Octadecyl-rac-glycerylyl-(3 → 5′)-5-fluoro-2′-deoxyuridine (**9**)*

The syrup, obtained by condensation of compound **8** with compound **2** (Table 3), was dissolved in $CHCl_3$/MeOH (95:5, 50 ml) and purified on a silica gel column (15 × 5 cm) using a three step chloroform/methanol gradient: Step 1: 9/1 (2 l); step 2: 8/2 (2 l); step 3: 7/3 (2 l). The fractions containing the desired product were pooled and concentrated, affording the fully protected product which was dissolved in chloroform

(50 ml). To that solution methanolic ammonia (200 ml) was added and the resulting solution was kept over night, before being concentrated to a syrup which was crystallized from ethyl acetate/ethanol (8:2) at 0 °C yielding 1-*O*-octadecyl-rac-glycerylyl-(3 → 5′)-5-fluoro-2′-deoxyuridine (**9**) as colorless crystals (6.5 g, 82%). M.p. 188–193 °C. TLC ($CHCl_3$/MeOH, 6:4) $R_f = 0.28$.

^{1}H-NMR (DMSO-d_6, 400.136 MHz): δ 7.97 (d, $J = 6.46$, 1H, H_6), 6.15 (t, $J = 6.34$ Hz, 1H, $H_{1'}$), 4.30 (m, 1H, $H_{3'}$), 3.78–3.90 (m, 3H, $H_{4'}$ + CH_2-glycerol), 3.61–3.72 (m, 2H, $H_{5'} + H_{5''}$), 3.25–3.45 (m, 5H, C*H*-glycerol + CH_2-glycerol + OCH_2CH_2–), 2.06–2.12 (m, 2H, $H_{2'}$ + $H_{2''}$), 1.41 (m, 2H,OCH_2CH_2(CH_2)$_{15}$CH_3), 1.21 (s, 30H, –(CH_2)$_{15}$), 0.83 (t, $J = 6.46$ Hz,3H (CH_2)$_{15}$CH_3).

^{13}C-NMR (DMSO-d_6, 100.624 MHz): δ 157.29 (d; J=25.79 Hz; C_4), 149.33 (C_2), 140.04 (d; $J = 232.14$ Hz; C_5), 124.39 (d; $J = 34.72$ Hz; C_6), 86.15 ($C_{4'}$), 84.53 ($C_{1'}$), 71.92 (*C*H$_2$-glycerol), 70.80 ($C_{3'}$), 70.59 (O*C*H$_2$CH$_2$(CH$_2$)$_{15}$CH$_3$), 69.23 (CH-glycerol), 66.48 ($C_{5'}$), 64.71 (*C*H$_2$-glycerol), 39.25 ($C_{2'}$), 31.28, 29.68, 29.22, 29.04, 28.70, 25.63, 22.08 (–(CH$_2$)$_{15}$–), 13.90 ((CH$_2$)$_{15}$*C*H$_3$).

HRMS calculated for $C_{30}H_{53}FN_2O_{10}P$ [M – H^+]: 651.34273, found: 651.34358.

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References

[1] H. Sommer, D.V. Santi, Biochem. Biophys. Res. Commun. 57 (1974) 689–695.
[2] H.G. Mandel, Prog. Mol. Subcell. Biol. 1 (1969) 82–135.
[3] R. Kanamaru, H. Kakuta, T. Sato, C. Ishioka, A. Wakui, Cancer Chemother. Pharmacol. 17 (1986) 43–46.
[4] W.D. Enminger, J.W. Gyves, Semin. Oncol. 10 (1983) 176–182.
[5] C.R. Wagner, V.I. Vidhya, E.J. McIntee, Med. Res. Rev. 20 (2000) 417–451.
[6] R.A. Schwendener, F. Guerin, H. Schott, EP 0642527 B1, Chem. Abstr. 120 (1994) 54904n.
[7] H. Schott, M.P. Häussler, P. Gowland, D.H. Horber, R.A. Schwendener, Antiviral Chem. Chemother. 5 (1994) 387–394.
[8] R.A. Schwendener, P. Gowland, D.H. Horber, R. Zahner, A. Schertler, H. Schott, Antiviral Res. 24 (1994) 79–93.
[9] H. Schott, M.P. Häussler, R.A. Schwendener, Liebigs Ann. Chem. (1994) 277–282.
[10] H. Schott, M.P. Häussler, P. Gowland, A. Bender, H. von Briesen, R.A. Schwendener, Antiviral Chem. Chemother. 6 (1995) 320–326.
[11] P.A. Peghini, R. Zahner, H. Kuster, H. Schott, R.A. Schwendener, Antiviral Chem. Chemother. 9 (1998) 117–126.
[12] H. Schott, P.S. Ludwig, F. Gansauge, S. Gansauge, R.A. Schwendener, Liebigs Ann. Recueil (1997) 413–417.
[13] H. Schott, P.S. Ludwig, A. Immelmann, R.A. Schwendener, Eur. J. Med. Chem. 34 (1999) 343–352.
[14] R.M.C. Cattaneo-Pangrazzi, H. Schott, H. Wunderli-Allenspach, B. Rothen-Rutishausser, M. Guenthert, R.A. Schwendener, J. Cancer Res. Clin. Oncol. 126 (2000) 247–256.
[15] D.H. Horber, R.M.C. Cattaneo-Pangrazzi, P. von Ballmoos, H. Schott, P.S. Ludwig, S. Eriksson, et al., J. Cancer Res. Clin. Oncol. 126 (2000) 311–319.
[16] S.-H. An, C.R. West, C.I. Hong, Steroids 47 (1986) 413–420.
[17] R.M. Grever, S.A. Schepartz, B.A. Chabner, Semin. Oncol. 19 (1992) 622–638.
[18] H. Schott, M.P. Häussler, R.A. Schwendener, Liebigs Ann. Chem. (1994) 465–470.
[19] R.M.C. Cattaneo-Pangrazzi, H. Schott, H. Wunderli-Allenspach, B. Derighetti, R.A. Schwendener, Biochem. Pharmacol. 60 (2000) 1887–1896.
[20] C. Marty, B. Odermatt, H. Schott, K. Ballmer-Hofer, R. Klemenz, R.A. Schwendener, Br. J. Cancer 87 (2002) 106–112.
[21] R. Anschütz, W.O. Emery, Liebigs Ann. Chem. 239 (1887) 301–313.
[22] M. Gomberg, C.C. Bulcher, J. Am. Chem. Soc. 45 (1923) 217–218.
[23] G. Hirth, R. Barner, Helv. Chim. Acta 65 (1982) 1059–1084.