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Synthesis and in vitro activities of new anticancer duplex drugs linking 2′-deoxy-5-fluorouridine (5-FdU) with 3′-*C*-ethynylcytidine (ECyd) via a phosphodiester bonding

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ABSTRACT

Two isomeric cytostatic duplex drugs 2′-deoxy-5-fluorouridylyl-(3′→5′)-3′-*C*-ethynylcytidine [5-FdU(3′→5′)ECyd] and 2′-deoxy-5-fluorouridylyl-(5′→5′)-3′-*C*-ethynylcytidine [5-FdU(5′→5′)ECyd] were designed and synthesized at gram scale according to the hydrogenphosphonate method in an overall yield of about 40%. The in vitro evaluation of the anticancer effects indicated highly varying sensibilities of the panel of 60 tested tumor cell lines against the duplex drugs. 5-FdU(3′→5′)ECyd had a 50% growth inhibition ($IC_{50} \leqslant 10^{-8}$ M) in 44/58 cell lines. However, only 25/53 of those cell lines showed corresponding IC_{50} values when the isomeric 5-FdU(5′→5′)ECyd was tested. Total growth inhibition was achieved using micromolar concentrations of the duplex drugs. The 5-FdU residue of the duplex drug can cause very different effects like additive, synergistic, antagonistic as well as sequence-depending activities, which drastically changed efficiency as well as specificity of the anticancer activities of the duplex drugs, in comparison to those of the monomeric drugs.

1. Introduction

It is known that antitumor chemotherapy is unsuccessful if resistance against the administered cytostatic drug occurs. Drug monotherapy, using only one anticancer compound, often leads to drug resistance. The combination therapy as an alternative, which is based on a simultaneous or sequential application of various anticancer drugs, can optimize the therapeutic success and may prevent resistance. However, combination therapy schedules are often complex and exhausting for the patients. Another promising but not yet well-evaluated possibility of anticancer combination therapy consists in the chemical linkage of two different, clinically well-characterized cytostatic drugs into one molecule, a so called duplex drug. After application of a duplex drug as a monotherapy the molecule should be degraded into a mixture of several metabolites each possessing different cytostatic profiles with additive or synergistic properties. This concept could exploit the advantage of a combination therapy without additional burden for patients. Cytostatic drugs are suitable for the design of duplex drugs provided that they vary considerably in their anticancer mechanisms in order to cause additive or synergistic antitumor activities and, optimally, a simultaneous reduction of side effects. The chemical coupling of two single compounds to a new duplex drug must go along with a justifiable synthetic effort. Furthermore, the molecular structure of the created duplex drug should allow an in vivo metabolism resulting in multiple active compounds. Nucleoside analogues[1–3] have become a major class of successful antimetabolites in cancer therapy over many years and fulfill important preconditions for the preparation of duplex drugs. Two different nucleoside analogues can be coupled via a natural phosphodiester bonding resulting in heterodinucleoside phosphate analogues that can easily be cleaved in vivo by phosphodiesterases into the parent nucleosides.

Heterodinucleoside phosphates linking 2′-deoxy-5-fluorouridine (5-FdU) with thioinosine were among the first dimers that inhibited effectively both 6-mercaptopurine sensitive as well as resistant cancer cells in vitro.[4] However, the potential of such dimers as possible duplex drugs has not yet been recognized. The direct coupling of 5-FdU with the lipophilic 2′-deoxy-5-fluoro-N^4-octadecylcytidine resulted in an amphiphilic heterodinucleoside phosphate analogue. In in vitro clonogenic growth assays using the human pancreatic adenocarcinoma cell line MIAPaCa 2, this duplex drug was significantly more cytotoxic than 5-FdU.[5] The antitumor potential of the duplex drug evaluated in p53-mutated and androgen-independent DU-145 human prostate tumor cells showed a 100% eradication of tumor cells whereas 10% of cells were resistant to 5-FdU.[6] In PC-3 cells the duplex drug exerted

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stronger cytotoxicity and induced more S-phase arrest and apoptosis than 5-FdU.[7] The direct linkage of arabinofuranosylcytosine (araC) with N^4-octadecyl-1-β-D-arabinofuranosylcytosine, a lipophilic derivative of the antitumor drug araC resulted in a potent duplex drug.[8] An amphiphilic duplex drug can also be stably incorporated in liposomes. This offers a new opportunity for a targeted anticancer combination therapy that has been proven in mouse models.[9] The indirect linkage of 5-FdU and araC via a phospholipid backbone resulted in an amphiphilic duplex drug that might be an additional option for the treatment of 5-FdU sensitive and resistant colon cancer, human lymphoma and hematological malignancies.[10–12]

A new derivative of cytidine, 3′-*C*-ethynylcytidine (ECyd), which is meanwhile evaluated in clinical studies,[13] is highly active and suitable for the synthesis of cytostatic duplex drugs. Because the antitumor mechanism of ECyd differs from that of 5-FdU it can be expected that the synthesis of dinucleoside phosphates linking ECyd and 5-FdU, which is described here, will result in new duplex drugs with a broad spectrum of antitumor activity based on additive or synergistic effects of 5-FdU and ECyd metabolites arising from enzymatic degradation of the duplex drug. As a proof of principal to this assumption two isomeric duplex drugs were synthesized by combining 5-FdU and ECyd either via a 3′→5′or a 5′→5′phosphodiester linkage to evaluate the influence of the direction of the phosphodiester on the antitumor activity of the dinucleoside phosphate.

2. Results and discussion

2.1. Chemistry

The synthesis of the two isomeric duplex drugs summarized in Figure 1 was performed according to the phosphonate method. Partially protected ECyd was used as the 5′-hydroxyl compound

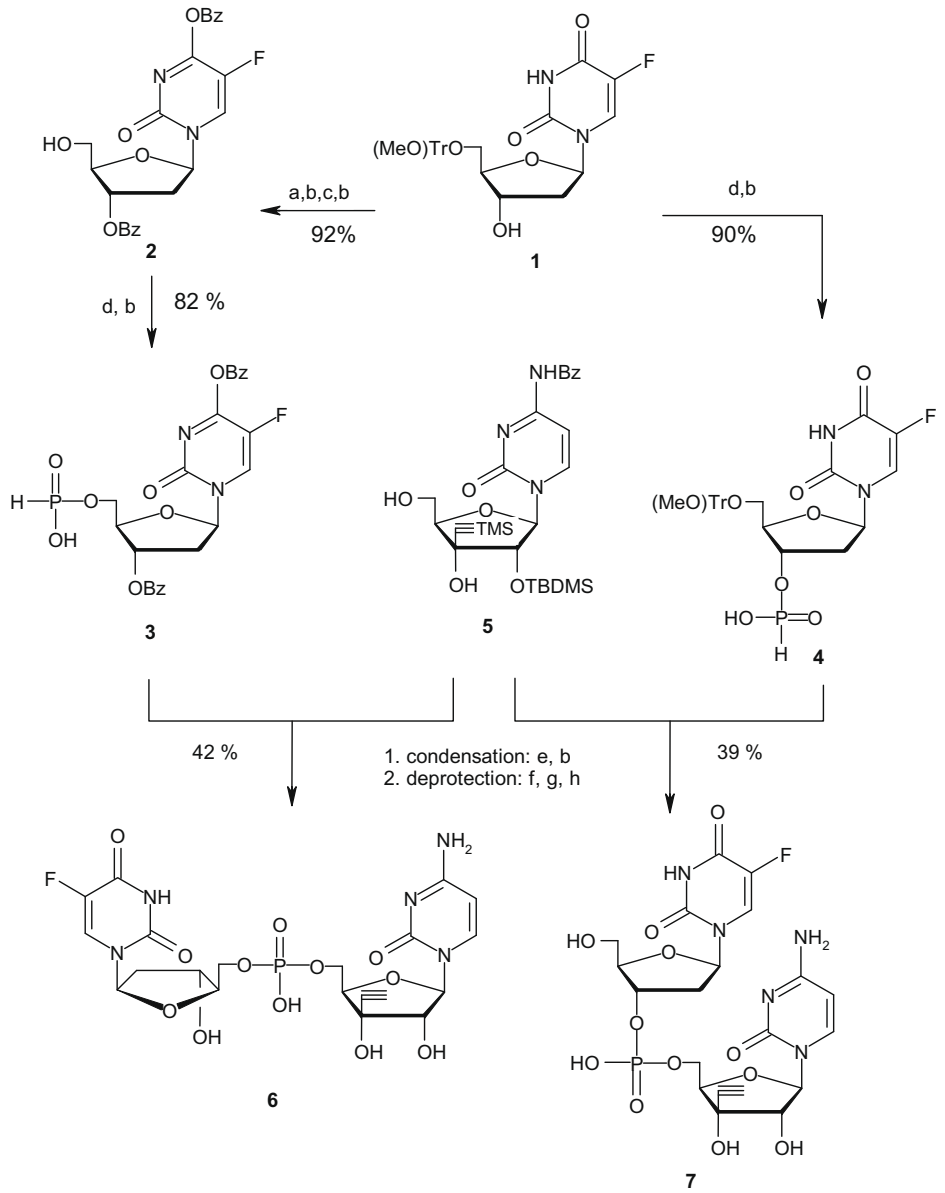

Figure 1. Synthesis of the heterodinucleoside phosphate 2′-deoxy-5-fluorouridylyl-(5′→5′)-3′-*C*-ethynylcytidine (**6**) and 2′-deoxy-5-fluorouridylyl-(3′→5′)-3′-*C*-ethynylcytidine (**7**) according to the phosphonate method starting from 5′-*O*-(4-monomethoxytrityl)-2′-deoxy-5-fluorouridine (**1**) and N^4-benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-3′-(trimethylsilylethynyl)cytidine (**5**). *Reagents and conditions*: (a) Benzoylchloride, pyridine, (b) column chromatography on silica gel, (c) *p*-toluenesulfonic acid, acetone, (d) salicylchlorophosphite, pyridine/dioxane, (e) (1) pivaloylchloride, pyridine, (2) iodine, THF/pyridine/water, (f) TBAF in THF, (g) satd NH_3/MeOH, (h) column chromatography on RP_{18}.

5 whereas two derivatives of 5-FdU were used as 5′- or 3′-phosphonate compounds **3**, **4**. The rationale for this synthesis concept is that the necessary 5′-hydroxyl compound of the partially protected ECyd derivative N^4-benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-3′-*C*-(trimethylsilylethynyl)cytidine **5** occurs as an intermediate product of the published six step synthesis of ECyd, which used cytidine as starting material.[14] According to the alternative multistep synthesis of ECyd[13,15,16] which did not use cytidine as starting compound a fully protected ECyd was obtained as an intermediate compound. The selective deprotection of the 5′-or 3′-hydroxyl function of these fully protected ECyd intermediates cannot easily be performed in order to obtain the desired partially protected hydroxyl compound for the dinucleoside phosphate synthesis. The synthesis of the sequence isomer duplex drugs with 5-FdU as the 3′ and ECyd as the 5′-terminal, which cannot be obtained according to our proposed synthesis route is expected to be more complicated. The postulated selective derivation of one of three hydroxyl groups of ECyd to obtain a 3′-hydroxyl or 3′-phosphonate compound of ECyd is more difficult as in the case of 5-FdU, which has only two hydroxyl groups.

The 5-FdU-3′-phosphonate compound **4** can be obtained from 5-FdU in two steps, whereas the synthesis of the 5-FdU-5′-phosphonate **3** needs a three step procedure. The additional steps render the synthesis of 5-FdU-(5′→5′)ECyd in respect to the preparation of the isomeric compound 5′-FdU(3′→5′)ECyd more difficult.

The synthesis of both phosphonate compounds **3**, **4** started with 5′-*O*-(4-monomethoxytrityl)-2′-deoxy-5-fluorouridine (**1**) which was obtained from 5-FdU according to published procedures.[17] The phosphonylation of the 3′-hydroxyl residue of **1** resulted with 90% yield in the desired 3′-phosphonate compound 5′-*O*-(4-monomethoxytrityl)-2′-deoxy-5-fluorouridine-3′-hydrogenphosphonate (**4**). The 5′-phosphonate compound **3** was also obtained from **1** in two steps. In the first step protection of hydroxyl groups of **1** with benzoyl residues followed by removing of the 4′-monomethoxytrityl protection from the 5′hydroxyl group resulted in 92% yield of 3′,4-di-*O*-benzoyl-2′-deoxy-5-fluorouridine (**2**) which was phosphonylated at the 5′-hydroxyl group without further purification at 82% yield resulting in the desired 5′-phosphonate compound 3′-4-di-*O*-benzoyl-2′-deoxy-5-fluorouridine-5′-hydrogenphosphonate (**3**). After coupling of the free 5′-hydroxyl group of **5** with the 5′-or 3′-phosphonate compounds **3** or **4** according to the conditions given in Table 1 the resulted heterodinucleoside phosphonates were subsequently oxidized with iodine resulting in the protected heterodinucleoside phosphates. It cannot be excluded that the unprotected free 3′-hydroxyl group of **5** may be involved in the condensation reaction. In this case undesired side reactions will occur which reduces the yield of the desired condensation product. The prevalent condensation reaction, however, should be the coupling to the 5′-hydroxyl group of **5** because this alcohol function has a sterical advantage for the condensation reaction in respect to the 3′-hydroxyl group of **5**.

Table 1
Experimental data for the synthesis according to the hydrogenphosphonate method yielding the fully protected isomeric heterodinucleoside phosphates of 2′ -deoxy-5-fluorouridylyl-(5′→5′-)-3′-*C*-ethynylcytidine (**6**) and of 2′deoxy-5-fluorouridylyl-(3′→5′)-3′-*C*-ethynylcytidine (**7**)

Experimental data for the condensation	Obtained protected crude heterodinucleoside phosphates	
	6	**7**
Hydroxyl compound number, (g/mmol)	**5**, 9.5/17	**5**, 18.4/33
Phosphonate compound number, (g/mmol)	+**3**, 11.0/21.2	+**4**, 19.2/33
Pyridine (mL)	80	100
Pivaloylchloride (mL/mmol)	13/106	24/195
H_2O (mL)	10	20
Iodine in THF (mL) + H_2O (mL)	80 + 10	160 + 20
$CHCl_3$/MeOH (9:1) (mL)	200	300
H_2O (mL)	150	250

After chromatographic purification the different protection groups of the dimers were removed using the following procedures. At first the acid labile 4-monomethoxytrityl group of the dimer formed by condensation of **4** + **5** was cleaved with acetic acid. Trimethylsilyl- and *tert*-butyldimethylsilyl protecting groups of both dimers were removed by treatment with tetrabutylammonium fluoride (TBAF) and the deprotected dinucleoside phosphate analogues were obtained as tetrabutylammonium salts. The alkali labile benzoyl residue was finally removed with ammonia. After the chromatographic purification of the deprotected dinucleoside phosphate analogues using a preparative reversed phase (RP-18) column and their lyophilization both duplex drugs 2′-deoxy-5-fluorouridylyl-(5′→5′)-3′-*C*-ethynylcytidine (**6**) and the isomeric 2′-deoxy-5-fluorouridylyl-(3′→5′)-3′-*C*-ethynylcytidine (**7**) were obtained at about 40% yield. The low yield may be partially explained by possible side reactions during the condensation as discussed above. The quantitative exchange of the tetrabutylammonium cations cannot be achieved using a cation exchanger (H^+ form). The presence of the tetrabutylammonium cation in both dinucleoside phosphates was detected by elementary analysis and mass spectrometry. The course of the synthesis and purification was controlled by thin-layer chromatography (TLC) on silica gel plates. The chemical structure and the analytical purity of the products were confirmed by NMR spectra, elementary analysis and high resolution mass spectra.

2.2. In vitro anticancer activities

The in vitro antitumor activities of the duplex drugs 5-FdU(3′→5′)ECyd, 5-FdU(5′→5′)ECyd and of the parent monomeric drugs 5-FdU and ECyd were evaluated in the framework of the anticancer screen program of the National Cancer Institute (NCI, USA). The NCI anticancer screen consists of 60 human tumor cell lines. In those 60 cell lines the drugs are tested at a minimum of five concentrations at 10-fold dilutions. A 48 h drug exposure protocol and a sulforhodamine B (SRB) protein assay were used to estimate cell viability or growth.[18] Additional details can be found at http://dtp.nci.nih.gov. The anticancer activities were obtained from the data screening report including the data sheet, dose response curves and the mean graphs. Mean graphs facilitated visual scanning of data for the selection of potential compounds for particular cell lines or for particular tumor subpanels with respect to a suspected response parameter. The response parameter GI_{50} ($\log_{10}$ of molar sample concentration resulting in 50% growth inhibition) of 5-FdU and ECyd given in the mean graphs are listed in Table 2. The average of the GI_{50} values for all 60 cell lines is indicated by the mean graphs midpoint. The comparison of the in vitro tumor cell growth inhibition activity which is based on the mean graphs midpoint of ECyd [$\log_{10} GI_{50}$ (M) = −7.6] and of 5-FdU [$\log_{10} GI_{50}$ (M) = −6.4] shows that ECyd was active against all 60 tested tumor cell lines at high nanomolar concentrations and that it was in general about 10-times more cytostatic than 5-FdU. In many cases however, the response of ECyd and 5-FdU to the same cell line significantly differs as follows. More than a 100-fold difference of the GI_{50}-values of ECyd and 5-FdU was observed in 3/9 non small cell lung; 2/8 melanoma; 2/6 ovarian; 3/8 renal; 5/7 colon and 6/8 breast cancer cell lines. These data demonstrated a pronounced drug specific antitumor activity of ECyd and 5-FdU against several cancer cell lines, predominantly in colon and breast tumor cell lines. Corresponding high differences of GI_{50} values were not observed in the 6 leukemias, 6 CNS and 2 prostate tumor cell lines included in the screen.

The response parameter GI_{50} was also used to compare the antitumor activities of ECyd and both isomeric duplex drugs. Half of

Table 2
In vitro anticancer activities resulting in 50% growth inhibition ($\log_{10}$ GI$_{50}$) of the monomeric drugs 5-FdU and ECyd and the 100% growth inhibition ($\log_{10}$ TGI) of the duplex drugs 5-FdU(3′→5′)ECyd, 5-FdU(5′→5′)ECyd and ECyd which were screened twofold on 60 human cancer cell lines (panel/cell line) and expressed by sample concentration (M)

Panel/Cell line	$\log_{10}$ GI$_{50}$ (M)		$\log_{10}$ TGI (M)		
	5-FdU	ECyd	ECyd	5-FdU(3′→5′)ECyd	5-FdU(5′→5′)ECyd
Leukemia					
CCRF-CEM	−8.2	<−8.0	−5.90	−5.65	−5.76
HL-60	−6.7	<−8.0	>−**4.00**	−**5.98**	A
K-562	−6.1	<−8.0	>−4.00	>−4.00	>−4.00
MOLT-4	−7.4	<−8.0	−**4.99**	−**4.82**	−**7.91**
RPMI-8226	−6.1	<−8.0	−6.89	[a]	−7.78
SR	−7.9	<−8.0	[a]	−**6.85**	>−**4.00**
Non-small cell lung cancer					
A549/ATCC	−7.9	<−8.0	−4.00	>−4.53	>−4.00
EKVX	−**5.0**	−**7.2**	−5.40	−5.20	−5.05
HOP-62	−7.6	<−8.0	[a]	>−**4.00**	−**6.77**
HOP-92	−6.1	−7.2	−5.36	−5.66	−4.67
NCI-H226	−**5.0**	−**7.8**	[a]	−4.89	>−4.00
NCI-H23	−6.3	−7.5	−**6.59**	−**4.84**	−**6.92**
NCI-H322M	−6.3	−7.7	−**5.94**	−**4.94**	A
NCI-H460	−8.7	<−8.0	−**7.00**	−**4.99**	−**5.34**
NCI-H522	−**5.6**	<−**8.0**	−**7.45**	−**5.94**	>−**4.00**
Colon cancer					
COLO 205	−**5.8**	<−**8.0**	−6.71	−6.06	−6.02
HCC-2998	−9.0	<−8.0	−7.40	−6.96	A
HCT-116	−6.9	<−8.0	−4.94	>−4.00	>−4.00
HCT-15	−**5.7**	<−**8.0**	>−4.00	−4.82	>−4.00
HT29	−**5.6**	<−**8.0**	[a]	>−4.00	>−4.00
KM12	−**5.2**	<−**8.0**	−6.06	−5.08	−5.25
SW-620	−**5.0**	<−**8.0**	−**5.89**	>−**4.00**	>−**4.00**
CNS cancer					
SF-268	−7.9	−7.8	−5.89	−5.11	−5.54
SF-295	−7.4	<−8.0	−6.24	−5.97	−6.06
SF-539	−8.4	−7.4	−**5.17**	−**6.78**	−**6.86**
SNB-19	−5.7	−7.2	−**5.24**	>−**4.00**	>−**4.00**
SNB-75	−6.7	−7.8	[a]	−6.05	A
U251	−6.9	-7.7	−**5.78**	>− **4.00**	>−**4.00**
Melanomas					
LOXI MVI	−7.6	<−8.0	−7.42	−6.46	A
MALME-3 M	−**5.1**	−**7.8**	−6.28	−5.89	−6.49
M14	−6.8	−7.4	−5.57	−6.54	−6.14
SK-MEL-2	−**5.0**	−**7.9**	−**6.60**	−**6.37**	−**5.24**
SK-MEL-28	−5.7	−7.3	−5.76	−5.85	−6.42
SK-MRL-5	−6.7	−8.0	−6.46	−6.80	−7.39
UACC-257	−5.5	−7.1	−5.27	−5.66	−5.27
UACC-62	−7.4	−7.5	−6.51	−5.89	−6.53
Ovarian cancers					
IGROV1	−**5.6**	−**7.9**	−4.84	−4.89	>−4.00
OVCAR-3	−5.6	−7.1	−5.37	−5.54	−6.13
OVCAR-4	−5.0	−6.8	−**5.81**	−**5.70**	>−**4.00**
OVCAR-5	−**5.2**	−**7.3**	[a]	−4.95	−4.46
OVCAR-8	−6.9	<−8.0	>−**4.00**	−**5.54**	−**5.68**
SK-OV-3	−5.7	−7.1	>−4.00	>−4.00	>−4.00
Renal cancers					
786-0	−7.0	<−8.0	[a]	>−4.00	>−4.00
A489	−**5.9**	<−**8.0**	−7.03	−6.12	−7.08
ACHN	−7.5	<−8.0	−**6.58**	−**5.82**	>−**4.00**
CAKI-1	−7.5	<−8.0	−7.53	−7.10	A
RXF 393	−**5.4**	−**7.6**	−5.68	−5.83	−6.36
SN12C	−6.7	<−8.0	[a]	>−4.00	A
TK-10	−**5.3**	−**7.8**	−**6.12**	−**4.74**	A
UO-31	−6.9	<−8.0	−**6.40**	[a]	>−**4.00**
Prostate cancers					
PC-3	−6.5	−7.5	−**6.51**	−**4.62**	−**4.32**
DU-145	−6.6	<−8.0	−**6.02**	−**6.36**	>−**4.00**

(*continued on next page*)

Table 2 (*continued*)

Panel/Cell line	Log_{10} GI_{50} (M)		Log_{10} TGI (M)		
	5-FdU	ECyd	ECyd	5-FdU(3′→5′)ECyd	5-FdU(5′→5′)ECyd
Breast cancers					
MCF7	−8.2	<−8.0	>−**4.00**	−**6.86**	>−**4.00**
NCI/ADR-RES	−**5.9**	<−**8.0**	>−**4.00**	−**5.53**	−**5.01**
MDA-MB-231	−**5.4**	−**7.4**	>−**4.00**	−**4.42**	−**6.56**
HS 578T	−**5.4**	−**7.9**	−**4.80**	−**4.90**	−**5.82**
MDA-MB-435	−**5.5**	<−**8.0**	−6.77	−6.60	−6.74
MDA-N	−**5.9**	−**7.9**	−6.76	—	—
BT-549	−6.0	−7.4	−**5.23**	−**6.64**	−**6.26**
T-47D	−**5.9**	<−**8.0**	[a]	−4.75	A
Mean graphs midpoint	−6.4	−7.6	−5.56	−5.28	−5.39

Results of one representative screen are shown. The lowest GI_{50} value of ECyd and 5-FdU being more than 100-fold different in the same cell lines and the lowest TGI values of ECyd and of the two duplex drugs being more than 10-fold different in the same cell lines are shown by bold face.

[a] TGI values are not listed when the screening resulted in more than 100-fold different values.

the tested tumor cell lines had GI_{50} values of $\leqslant 10^{-8}$ M after ECyd treatment. Correspondingly, 44/58 cell lines produced low GI_{50} values with 5-FdU(3′→5′)ECyd. However, GI_{50} values of $\leqslant 10^{-8}$ M were observed only in 25/53 cell lines when the same cell lines were treated with isomeric 5-FdU(5′→5′)ECyd. On the basis of these results it could be speculated that the antitumor activities of 5-FdU(3′→5′)ECyd linking 5-FdU with ECyd via the natural 3′→5 phosphodiester bonding is generally higher in respect to those of the 5′→5′ isomer. This assumption is in agreement with the published results that 3′→5′linked arabinofuranosylcytidine dimers were found to be more active than 5′→5′ linked dimers.[19] For the evaluation of the antitumor activities of the duplex drugs the cell growth inhibition was compared only with that of ECyd because ECyd was 10-fold more active than 5-FdU. For this comparison the too low and similar GI_{50} values of ECyd and the duplex drugs were less suitable. Therefore, instead of the GI_{50} the response parameter TGI (log_{10} of molar sample concentration resulting in 100% growth inhibition) listed in Table 2 was used to demonstrate the different sensibility of the same tumor cell lines against ECyd and the duplex drugs.

The mean graphs midpoint of ECyd [log_{10} TGI (M) = −5.56 corresponds to the values of 5-FdU-(3′→5′)ECyd [log_{10} TGI (M) = −5.28 and of 5-FdU(5′→5′)ECyd [log_{10} TGI (M) = −5.39. In respect to the similar TGI mean graphs midpoint of the three compounds it could be assumed that the cytostatic potential of ECyd and both duplex drugs would be similar. However, the TGI values of the drugs obtained after the parallel treatment of the same tumor cell line clearly demonstrated that the main part of the 60 tested cell lines showed significantly different sensibilities against ECyd, 5-FdU(3′→5′)ECyd and 5-FdU(5′→5′)ECyd because their responses against single cell lines varied in a broad range.

The following 14 cell lines showed TGI values of ECyd (see Table 2), which are more than 10-fold lower than those of one or both duplex drugs: Melanomas (SK-Mel-2); ovarian (OVAR4); colon (SW-620); CNS (SNB-19, U251); renal (ACHN, TK-10, UO-31); non small lung (NCI-H23, NCI-H322M, NCI-H460, NCI-H522) and the prostate cancer cell lines (PC-3, DU-145). On the basis of these TGI values it can be concluded that the equimolar coupling of 5′-FdU with ECyd results in an antagonistic effect, thus reducing the cytostatic activity of the duplex drug, in respect to that of ECyd.

In contrast to these results the following 9 cell lines were more than 10-times more sensitive to one or both duplex drugs in comparison to ECyd: HL-60, Molt-4 (leukemia); SF-539 (CNS); OVCAR-8 (ovarian); MCF7, NCI/ADR-RES, MDA-MB-231, HS 578T; BT-549 (breast). In these examples the linked 5-FdU acts additively or synergistically when coupled with ECyd. Of the tested cell lines 27 showed only little to unchanged growth inhibition in comparison to that of ECyd independently of treatment with 5-FdU(3′→5′)ECyd or 5-FdU(5′→5′)ECyd. The double screened TGI values of 18 tests of the 60 tested cell lines could not be evaluated because the values obtained differed more than 100-fold.

Besides the change of the cytostatic activities of ECyd, which was observed after coupling with 5-FdU it was surprising that the direction of the phosphodiester linkage sequence in which 5-FdU and ECyd were coupled has a significant influence on the antitumor activity and specificity of the synthesized duplex drugs. However, 25 of the tested 60 cell lines demonstrated a marked structure–activity relationship against the duplex drugs. The sequence dependent antitumor activity can be observed from the difference of the TGI values resulting after parallel treatment of the same cell line with 5-FdU(3′→5′)ECyd and 5-FdU(5′→5′)ECyd. One half of these cell lines were more sensitive against 5-FdU(3′→5′)ECyd whereas the other half showed higher growth inhibition with the isomeric 5-FdU(5′→5′)ECyd duplex. The following 7 cell lines showed a significant high sequence activity relationship expressed by an about 100-fold difference of the TGI values obtained with the same cell line after parallel treatment with both duplex drugs: Molt-4, SR (leukemia); HOP-62, NCI-H23 (non small cell lung cancer), DU-145 (prostate); MCF7; MDA-MB-231(breast).

The results demonstrate that the chemical coupling of 5-FdU and ECyd caused, depending on the cell lines resulted either in increased, decreased or unchanged antitumor activities of the corresponding duplex drug compared to monomeric ECyd. Activity and specificity of the antitumor effects of the duplex drugs depend on the cytostatic potential of the coupled monomeric drugs as well as on the structure of the obtained dimer, for example the direction of the phosphodiester bonding in a dinucleoside phosphate. Thus, the described transformation of cytostatic antimetabolites to dinucleoside phosphate analogues represents a valuable alternative to the intensive search for new anticancer drugs.

2.2.1. Possible reasons of the antitumor activities of the duplex drugs

The principal mechanism for the cytostatic effect of 5-FdU is the inhibition of thymidylate synthase (TS).[20] The inhibition of TS enzyme activity leads to depletion of deoxythymidine triphosphate which is necessary for DNA synthesis. ECyd can contribute to the antitumor activity through its action mechanisms which are different from those of 5-FdU.[21–25]

By coupling ECyd and 5-FdU the resulting duplex drug should be a potent inhibitor of tumor cell growth by simultaneous inhibition of both DNA and RNA synthesis. It can be supposed that the intact duplex drugs do not act as active compounds. However, the several cytostatic metabolites, which can be formed by enzymatic degradation of the duplex drugs should initiate the

antitumor effects. Because the phosphodiester cleavage can either be initiated at the 3′- as well as the 5′-terminal, a mixture of different active metabolites can be expected. The cleavage of 5-FdU (3′→5′)ECyd produces an equimolar mixture of ECyd-5′-monophosphate and 5-FdU if the phosphodiesterase cleaves at the 3′-terminal. Cleavage at the 5′-terminal results in a mixture of 5-FdU-3′-monophosphate and ECyd. The degradation of the isomeric 5-FdU(5′→5′)ECyd results in a 1:1 molar mixture of 5-FdU-5′-monophosphate and ECyd if cleavage occurs at the 5-FdU residue. An equimolar mixture of ECyd-5′-monophosphate and 5-FdU will be formed if cleavage occurs at the ECyd residue of the duplex drugs. However, certain steps of the metabolic pathway can be favored, if for example the recognition and cleavage of the ECyd will be hindered by the 3′-*C*-ethynyl group of the carbohydrate residue, whereas the unmodified carbohydrate residue of 5-FdU is more accessible to the enzyme.

The antitumor activity of ECyd depends essentially on its phosphorylation. The first phosphorylation step from ECyd to ECyd-5′-monophosphate is unnecessary if ECyd-5′-monophosphate is produced by metabolic action. In this example the nucleotide sequence of the duplex drug which favors the metabolic pathway resulting in ECyd-5′-monophosphate instead of ECyd can result in increased antitumor activity of the duplex. Under this aspect the other sequence isomeric dinucleoside phosphate ECyd(3′→5′)5-FdU with ECyd as the 5′- and 5-FdU as the 3′-terminal coupling partner is less favored to be metabolized by phosphodiesterase to ECyd-5′-monophosphate in comparison to 5-FdU(3′→5′)ECyd. This duplex drug was therefore not synthesized.

In addition to phosphodiesterase cleavage phosphohydrolases can convert the phosphorylated metabolites to the corresponding nucleosides. The main part of the duplex drug will be metabolized before reaching the cytoplasm because the negatively charged phosphodiester renders the dinucleoside phosphate too hydrophilic to penetrate the lipid rich cell membrane easily.[26] The extracellular release of active metabolites may cause a depot effect which does not occur by application of a mixture of 5-FdU and ECyd. The suspected extracellular metabolism could be a disadvantage if duplex drugs are synthesized as hydrophilic heterodinucleoside phosphate analogues.

In case that the metabolism of both isomeric duplex drugs would produce an identical mixture of active metabolites the observed structure–activity relationship would be difficult to explain. Thus, it can be speculated that the sequence dependent metabolic pathways which produce in a time dependent manner different amounts and species of cytotoxic metabolites can modulate their antitumor activity and tumor cell specificity.

The results of the in vitro cytotoxicity test screens provide a preliminary orientation of expected in vivo antitumor activities. However, a wide variety of biochemical and physiological processes can drastically influence the patterns of the antitumor effects in vivo. For example, it has been reported that a duplex drug tested on murine leukemia cells with the highest cytotoxicity in vitro exerted the lowest therapeutic effect in vivo.[12] Nevertheless, the excellent in vitro antitumor activities of the described new duplex drugs justify further evaluation in in vivo tumor models.

3. Experimental

3.1. General chemistry

3.1.1. Reagents

Pivaloyl chloride, benzoyl chloride, tetrabutylammonium fluoride trihydrate, *p*-toluenesulfonic acid monohydrate were obtained commercially. Salicylchlorophosphite[27] 5′-*O*-(4-monomethoxytrityl)-2′-deoxy-5-fluorouridine (**1**),[17] N^4-benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-3′-(trimethylsilylethynyl) cytidine (**5**)[14] were prepared as described. All solvents were of technical grade and used without further purification unless stated otherwise. Dioxane was dried with sodium, distilled and stored over 5 Å molecular sieve and pyridine was refluxed over KOH, distilled and stored over 4 Å molecular sieve. The TBAF cleaving solution was obtained by dissolving tetrabutylammonium fluoride trihydrate (157.8 g) in THF (500 mL). For the oxidation reaction a solution of iodine (25 g) in THF (500 mL) was used. All reactions were monitored by TLC on precoated Silica Gel 60 F_{254} plates (0.25 mm, Merck) using UV light for visualization and spray reagents as developing agents.[17] Multi step flash chromatography was carried out on self packed Silica Gel 60 (0.040–0.0063 mm, Merck) columns using eluent mixtures prepared by volume ratios. 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 40 °C. ^{1}H- and ^{13}C NMR spectra were obtained on a Bruker AC 250 spectrometer at 250 MHz and 62.9 MHz, respectively or on a Bruker Avance 400 spectrometer at 400 MHz and 100 MHz, respectively. DMSO-d_6 was used as solvents. Me_4Si was used as an internal standard. ^{31}P NMR spectra were obtained on a Bruker Avance 400 spectrometer at 161 MHz, using H_3PO_4 as an external standard. Mass spectra were measured on a Finnigan TSQ 70 or a MAT 95 instrument. For FAB mass-spectra, all compounds were measured in a NBA-or glycerine-matrix. HRMS were measured on a Bruker Apex II FT-ICR instrument.

3.2. General chemistry methods

3.2.1. Condensation procedure using the hydrogenphosphonate method

The corresponding hydroxyl- and hydrogenphosphonate compounds were dissolved together in dry pyridine and rigorously dried by repetitive evaporation and addition of pyridine before the condensation reaction was started by addition of pivaloyl chloride. After drying and addition of the required amount of pyridine (see Table. 1) the solution was cooled to 0 °C before pivaloyl chloride was added under exclusion of moisture. After stirring for 5 min the reaction mixture was cooled again to 0 °C before the reaction was stopped by addition of water. The obtained phosphonic diester was immediately oxidized by addition of iodine in THF and H_2O. After 1 h stirring excess iodine was reduced by addition of solid sodium hydrogensulfite before the reaction mixture was concentrated to a syrup that was dissolved in $CHCl_3$/MeOH and extracted with water. The organic layer was concentrated to a syrup, which was co-evaporated three times with toluene yielding the crude protected heterodinucleoside phosphates, which were purified and de-protected as described below.

3.3. Synthesis of 3′, 4-Di-*O*-benzoyl-2′-deoxy-5-fluorouridine-5′-hydrogenphosphonate (3)

To a solution of 5′-*O*-(4-monomethoxytrityl)-2′-deoxy-5-fluorouridine (**1**) (26 g, 50 mmol) in dry pyridine (150 ml) benzoylchloride (56 g, 400 mmol) was added under cooling. The reaction vessel was sealed airtight and shaken for 8 h at room temperature before saturated (satd) aq Na_2CO_3 (130 mL) was added under cooling. The resulting reaction mixture was concentrated to a syrup which was diluted with $CHCl_3$ (300 mL) and then extracted with satd aq Na_2CO_3 (130 mL). The $CHCl_3$ layer was concentrated to a syrup which was dissolved in a mixture of $CHCl_3$/petroleum ether (1:1, 200 mL) followed by chromatography on a silica gel column using a $CHCl_3$/petroleum ether gradient with increasing percentage of $CHCl_3$. The fractions containing the desired product were concentrated to a foam (46 g) which was dissolved in acetone (100 mL) containing *p*-toluenesulfonic acid monohydrate (16 g)

and stirred for 20 min at room temperature, before satd aq Na_2CO_3 (50 mL) was added. The resulting solution was concentrated to a syrup which was dissolved in $CHCl_3$ (500 mL) and extracted with H_2O (100 mL). The organic layer was separated and chromatographed on a silica gel column using a two step gradient with step 1; $CHCl_3$/petroleum ether with increasing percentage of $CHCl_3$ and step 2; ether. The fractions containing the desired product were concentrated, affording crude 3′,4-di-*O*-benzoyl-2′-deoxy-5-fluorouridine (**2**) as a colorless foam (21 g, 46 mmol) at 92% yield. In the following step **2** was dissolved without further purification in dry pyridine (90 mL) and diluted with dry dioxane (180 mL). To this solution dioxane (75 mL) was added in which salicylchlorophosphite (13 g, 64 mmol) was dissolved. After stirring of the reaction mixture at room temperature for 2 h satd aq $NaHCO_3$ (12 mL) was added and the solution was evaporated to a syrup which was dissolved in $CHCl_3$ (500 mL) and extracted with a mixture of H_2O/satd aq NaCl/MeOH (1:1:2) (3 × 100 mL). The separated $CHCl_3$-phase was concentrated and the resulting syrup dissolved in $CHCl_3$ (150 mL). By slow addition of the obtained solution to vigorously stirred ether (1.5 L) the desired **3** precipitated. The isolated and dried precipitate was then extracted during 70 h with ether. After drying of the remaining precipitate, **3** (20 g, 39 mmol) was obtained at 82% yield as a white powder. TLC ($CHCl_3$/MeOH 70:30) R_f = 0.46; MS (FAB$^-$) *m*/*z*: 517.1 [M−H]$^-$; ^{1}H NMR, 400 MHz, DMSO-d_6: δ = 2.44–2.75 (m, 2H, $H_{2'}$), 3.48–3.89 (m, 5H, PH, H_2O (DMSO-d_6)), 4.0–4.425 (m, 2H, $H_{5'}$), 4.34–4.47 (m, 1H, $H_{4'}$), 5.47–5.64 (m, 1H, $H_{3'}$), 6.22–6.4 (m, 1H, $H_{1'}$), 7.35–8.27 (m, 10H, $H_{aromat.}$), 8.51 (s, 1H, H_6), 12.0 (s, br, 1H, P-OH); ^{13}C NMR, 100 MHz, DMSO-d_6: δ = 36.7 ($C_{2'}$), 62.8 ($C_{5'}$), 75.5 ($C_{3'}$), 83.6 ($C_{1'}$), 85.4 ($C_{4'}$), 128.6–147.9 ($C_{aromat.}$), 155.7 (C_6), 156.0 (C_5), 165.1 (C=O), 168.0 (C=O); ^{31}P NMR, 161 MHz, DMSO-d_6: δ = 1.23 ppm(–O–PH(O)–OH). Anal. Calcd for $C_{23}H_{20}FN_2O_9P{\cdot}Na{\cdot}1/2\ H_2O$: C, 50.28; H, 3.67; N, 5.10. Found: C, 50.30; H, 4.21; N, 5.35.

3.4. Synthesis of 5′-*O*-(4-monomethoxytrityl)-2′-deoxy-5-fluorouridine-3′-hydrogenphosphonate (4)

5′-*O*-(4-Monomethoxytrityl)-2′-deoxy-5-fluorouridine (**1**) (20 g, 39 mmol) was dissolved in dry pyridine (50 mL) and the resulting solution diluted with dry dioxane (90 mL) followed by addition of salicylchlorophosphite (11 g, 54 mmol). After stirring the reaction mixture at room temperature for 1.5 h the formed precipitate was removed by filtration and washed with cold ether. To the combined filtrate and wash liquid satd aq Na_2CO_3 (50 mL) was added. The obtained mixture was concentrated to a foam which was then dissolved in a mixture of $CHCl_3$/MeOH (95:5) and chromatographed on a silica gel column using a $CHCl_3$/MeOH elution gradient with increasing percentage of MeOH. After the evaporation of the product containing fractions compound **4** was obtained as a foam (20 g, 35 mmol) at 90% yield. TLC ($CHCl_3$/MeOH 80:20) R_f = 0.17; MS (FAB$^-$) *m*/*z*: 581.2 [M−H]$^-$; 603.2 [M+Na]; ^{1}H NMR, 400 MHz, DMSO-d_6: δ = 2.20–2.65 (m, 2H, $H_{2'}$), 3.18–3.43 (m, 2H, $H_{5'}$), 3.45–3.68 (m, 3H, NH, PH, POH), 3.73 (s, 4H, OCH_3, H_2O (DMSO-d_6)), 4.21 (m, 1H, $H_{4'}$), 4.91 (m, 1H, $H_{3'}$), 6.23 (m, 1H, $H_{1'}$), 6.79–7.48 (m, 14H, $H_{aromat.}$), 7.79 (d, 1H, *J* = 6.2 Hz, H_6); ^{13}C NMR, 100 MHz, DMSO-d_6: δ = 23.4 ($C_{2'}$), 60.0 (OCH_3), 61.6, ($C_{5'}$), 68.4 ($C_{3'}$), 89.9 ($C_{4'}$), 91.8 ($C_{1'}$), 128.9 (C_6), 132.0.148.9 ($C_{aromat.}$ + C_5), 154.2 (C=O), 163.5 (C=O); ^{31}P NMR, 161 MHz, DMSO-d_6: δ = 7.3 ppm, (–O–PH(O)–OH). Anal. Calcd for $C_{29}H_{28}FN_2O_8P{\cdot}2Na{\cdot}H_2O$: C, 53.96; H, 4.53; N, 4.34. Found: C, 53.55; H, 4.44; N, 4.17.

3.5. Synthesis of 2′-deoxy-5-fluorouridylyl-(5′→5′)-3′-C-ethynylcytidine (6)

The syrup obtained after the condensation of **5** with **3** according to the experimental data of Table 1 was dissolved in $CHCl_3$ and chromatographed on a silica gel column using a $CHCl_3$/MeOH gradient with increasing percentages of MeOH. The fractions containing the desired protected heterodinucleoside phosphate were concentrated to a syrup which changed to a fine solid by vigorously shaking after addition of ether. To the isolated solid, dissolved in THF (45 mL) a solution (22 mL) of TBAF in THF was added. The sealed reaction mixture was stirred for three days whereby the trimethylsilyl- and *tert*-butyldimethylsilyl protecting groups were cleaved. After the concentration of the reaction mixture 33% aq ammonia (80 mL) was added to the resulting syrup and the sealed solution was stirred for five days to cleave the benzoyl protecting groups. When the reaction mixture was concentrated to about 250 mL, a fine solid precipitate was removed by centrifugation and the supernatant liquid concentrated and lyophilized. The resulting lyophilisate of crude **6** was dissolved in H_2O (60 mL) and chromatographed on a preparative RP-18 column using a H_2O/MeOH gradient with increasing percentage of methanol. The desired **6** eluted at 15–40% CH_3OH. The product containing fractions were pooled, the pH value adjusted to 5.8 by addition of a cation exchanger resin (H^+), that was removed before the solution was concentrated and lyophilized yielding **6** as a white powder (5.8 g, 41.8%).

HRMS calcd for $C_{20}H_{22}FN_5O_{12}P$ [M−H]$^-$: 574.09921, found: 574.09907; ^{1}H NMR (DMSO-d_6, 250 MHz): δ = 11.,8 (br, s, 1H, NH–5FdU), 8.10 (d, *J* = 6.64 Hz, 1H, H_6–5FdU), 7.85 (d, *J* = 7.25 Hz, 1H, H_6–ECyd), 6.14 (t, *J* = 6.42 Hz, 1H, H_1–5FdU), 5.85 (d, *J* = 6.19 Hz), 1H, H_1–ECyd) 5.81 (d, *J* = 7.07 Hz, 1H, H_5–ECyd), 5.64–5.75 (m, 2H, $H_{2'}$–OH, $H_{3'}$–OH–ECyd), 4.29 (m, 1H, $H_{3'}$–5FdU), 4.08 (m, 1H, $H_{2'}$–ECyd), 3.83–3.97 (m, 6H, $H_{4'}$–,$H_{5'}$–,$H_{5''}$–ECyd/5FdU), 3.46 (s, 1H, ≡CH–ECyd) 3.16 (m, 8H, NCH_2–TBA), 2.10 (m, 2H, H2′, H2″-5FdU) 1.56 (m, 8H, CH_2–C_2H_5–TBA), 1.30 (m, 8H, CH_2–CH_2–TBA), 0.92 (m, 12H, CH_3–TBA); ^{13}C NMR (DMSO-d_6, 62 MHz): δ = 165.3 (C_4–ECyd), 158.0 (C_4–5FdU), 156.9 (C_2–ECyd), 149.1 (C_2–5FdU), 142.2 (C_6–ECyd), 137.8 (C_5–5FdU), 124.6 (C_6–5FdU), 94.4 (C_5–ECyd), 87.3 ($C_{1'}$–ECyd), 86.3 ($C_{4'}$–ECyd), 84.6 ($C_{4'}$–5FdU), 82.9 (≡CH–ECyd), 78.7 ($C_{2'}$–ECyd), 77.3 (C≡–ECyd), 72.7 ($C_{3'}$–ECyd), 71.1 ($C_{3'}$–5FdU), 64.6 ($C_{5'}$–ECyd, $C_{5'}$–5FdU), 57.6 (C_1–TBA), 23.1 (C_2–TBA), 19.2 (C_3–TBA), 13.5 (C_4–TBA). Anal. Calcd for $C_{20}H_{22}FN_5O_{12}P{\cdot}C_{16}H_{36}N$: C, 52.93; H, 7.16; N, 10.29. Found: C, 52.58; H, 6.86; N, 9.56; ^{31}P NMR, 161 MHz DMSO-d_6: δ = −1.5 ppm.

3.6. Synthesis of 2′-deoxy-5-fluorouridylyl-(3′→5′)-3′-*C*-ethynylcytidine (7)

The syrup, obtained after condensation of **5** with **4** according to the experimental data of Table 1 was dissolved in $CHCl_3$ (300 mL) and chromatographed on a silica gel column using mixtures of $CHCl_3$/MeOH with increasing percentages of MeOH as the eluent. Fully protected **7** was eluted first, followed by fractions containing **7** without the monomethoxytrityl protection group. Fractions containing fully protected as well as partially deprotected **7** were pooled and concentrated to a foam which was dissolved in MeOH (60 mL). Then 80% aq acetic acid (60 mL) was added, the reaction mixture stirred for 24 h and then concentrated to a syrup. The syrup changed to a fine solid after adding ether and vigorous shaking. The solid was isolated by centrifugation, dissolved in $CHCl_3$ (250 mL) and re-chromatographed on a silica gel column as described above. After the second chromatographic purification the resulting solid of the partially protected **7** was dissolved in THF (170 mL), followed by addition of TBAF (8 mL). The sealed reaction mixture was stirred for three days and concentrated to a syrup. The obtained syrup was treated another five days with 33% aq ammonia (300 mL) and chromatographed on a RP-18 column as described above for the purification of **6** affording pure **7** (10.6 g, 39.3%).

MS (FAB$^-$) *m*/*z*: 574.0 [M−H]$^-$; 815.3 [M+$C_{16}H_{36}N$]$^-$; ^{1}H NMR (DMSO-d_6, 250 MHz):δ = 11.82 (br, s, 1H, NH–5FdU), 8.20 (d, *J* = 7.1 Hz, 1H, H_6–5FdU), 7.92 (d, *J* = 7.5 Hz, 1H, H_6–ECyd), 7.83

(d, J = 7.5 Hz, 1H, H_5–ECyd), 6.09–6.14 (m, 2H, 2×$H_{1'}$–ECyd/5FdU) 5.69–5.85 (m, 6H, OH/NH–ECyd/5FdU), 4.65 (m, 1H, $H_{3'}$–5FdU), 3.87–4.01 (m, 4H, $H_{5'}$–ECyd/$H_{3'}$–5FdU), 3.59 (m, 2H, $H_{5'}$–5FdU), 3.48 (s, 1H, C≡C–H), 3.16 (m, 8H, N–CH_2–TBA), 2.50 (m, 1H, $H_{2'}$–ECyd), 2.28–2.1 (m, 2H, $H_{2'}$–5FdU), 1.53 (m, 8H, CH_2–TBA), 1.30 (m, 8H, CH_2–TBA), 0.92 (t, J = 7.4, 12H, CH_3–TBA); ^{13}C NMR (DMSO-d_6, 62 MHz): δ = 165.0 (C_4–ECyd), 158.0 (C_4-5FdU), 157.29 (C_2–ECyd), 149.0 (C_2–5FdU), 142.2 (C_6–ECyd), 137.8 (C_5–5FdU), 124.6 (C_6-5FdU), 94.4 (C_5–ECyd), 87.5 ($C_{1'}$–ECyd), 86.5 ($C_{4'}$–ECyd), 84.5 ($C_{4'}$-5FdU), 82.9 (≡CH–ECyd), 78.7 ($C_{2'}$–ECyd), 77.4 (C≡–ECyd), 74.5 ($C_{3'}$–5FdU), 72.6 ($C_{3'}$–ECyd), 65.2 ($C_{5'}$–ECyd), 61.5 ($C_{5'}$–5FdU) 57.6 (C_1–TBA), 23.1 (C_2–TBA), 19.2 (C_3–TBA), 13.5 (C_4–TBA); ^{31}P NMR, 161 MHz, DMSO-d_6: δ = −1.85 ppm. Anal. Calcd for $C_{20}H_{22}FN_5O_{12}P{\cdot}C_{16}H_{36}N$: C, 52.93; H, 7.16; N, 10.29. Found: C, 52.62; H, 7.00; N, 9.60.

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