Eur. J. Med. Chem. 34 (1999) 343–352
© Elsevier, Paris

Original article

Synthesis and in vitro anti-HIV activities of amphiphilic heterodinucleoside phosphate derivatives containing the 2′,3′-dideoxynucleosides ddC, AZT and ddI

Herbert Schott[a]*, Peter S. Ludwig[a], Andreas Immelmann[b], Reto A. Schwendener[c]

[a]*Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, D-76076 Tübingen, Germany*
[b]*Analysis GmbH, D-60596 Frankfurt, Germany*
[c]*Department of Pathology, Division of Cancer Research, University Hospital, CH-8091 Zürich, Switzerland*

(Received 8 July 1998; revised 18 November 1998; accepted 10 December 1998)

Abstract – *N*^{4}-Palmitoyl-2′,3′-dideoxycytidine-5′-hydrogenphosphonate was condensed with 2′,3′-dideoxyinosine (ddI) and 3′-azido-2′,3′-dideoxythymidine (AZT) to the amphiphilic heterodimers 2′,3′-dideoxyinosinylyl-(5′→5′)-*N*^{4}-palmitoyl-2′,3′-dideoxycytidine (**2**) and 3′-azido-2′,3′-dideoxythymidylyl-(5′→5′)-*N*^{4}-palmitoyl-2′,3′-dideoxycytidine (**3**) which was also converted to the hydrophilic heterodimer 3′-azido-2′,3′-dideoxythymidylyl-(5′→5′)-2′,3′-dideoxycytidine (**3a**). The heterodimers exhibit strong activity against HIV wild type and an AZT-resistant virus variant. The anti-HIV activity of **3** combining AZT with p*N*^{4}-pamddC was lower than that of 2′-deoxythymidylyl-(3′→5′)-*N*^{4}-palmitoyl-2′,3′-dideoxycytidine (**4**) linking p*N*^{4}-pamddC as the only antiviral monomer unit to the inactive thymidine. It is expected that the anti-HIV activities and selectivities of ddNs can be varied by their dimerization to heterodimers offering the prospect of new therapeutic modalities. © Elsevier, Paris

amphiphilic heterodimers / dideoxynucleosides / hydrogenphosphonate method / HIV / HIV-resistance

1. Introduction

The main benefits of a combination therapy against HIV-infections, the simultaneous application of two different antiviral dideoxynucleosides (ddNs), over the monotherapy with only one ddN, lie in the retardation of the development of resistant virus strains and the reduction of toxicity. This therapeutic advantage, which is due to additive and/or synergistic mechanisms, is the basis of the synthesis of heterodinucleoside phosphates (heterodimers) in which ddNs are coupled chemically [1]. When compared on an equimolar basis with their monomers, higher anti-HIV potency and enhanced therapeutic indices were observed in vitro with such heterodimers [2, 3]. Other results, however, suggest that the anti-HIV activity of heterodimers is primarily attributed to the action of the more potent ddN of the two coupled components [4].

We have developed a new concept [5] for the synthesis of amphiphilic heterodimers of ddNs with the aim to improve the cellular uptake of the heterodimers and to extend their therapeutic potency. Amphiphilic dimers, which can be obtained by coupling a lipophilic to a hydrophilic nucleoside derivative, should more easily penetrate the lipophilic cell membrane either by a passive diffusion or by structural membrane deformation than unmodified hydrophilic heterodimers which can not enter a cell in such a manner. We have found that amphiphilic heterodimers in which only one ddN derivative was coupled with a natural nucleoside via a phosphodiester bond can produce high anti-HIV activity in vitro [6]. In the following we will focus on the question: Which effect does the transformation of two ddN derivatives to an amphiphilic heterodimer have on the anti-HIV activity? For these investigations we have selected as model systems, amphiphilic heterodimers containing the ddNs 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyinosine (ddI) and 3′-azido-2′,3′-dideoxythymidine (AZT). These ddNs were successfully used in combination therapy [7], and a synergistic inhibition of HIV replication in vitro has also been reported [8]. An additional reason for the incorporation of ddC in a heterodimer lies in the possi-

---

*Correspondence and reprints

bility to transform ddC into a lipophilic derivative by the introduction of the *N*^{4}-palmitoyl residue as a *N*^{4}-protecting group.

2. Chemistry

The syntheses of the amphiphilic dinucleoside phosphates of which the structure formulas are given in *figure 1* were performed in analogy to the previous synthesis of dT-N^4-pamddC (**4**) [9]. According to the synthesis route shown in *figure 2, N*^{4}-palmitoyl-2′,3′-dideoxycytidine-5′-hydrogenphosphonate (**1**), was coupled in the presence of pivaloyl chloride to the free 5′-hydroxyl group of AZT or ddI using the hydrogenphosphonate method. The internucleoside linkage of the resulting dimers was oxidized with iodine to obtain the two amphiphilic dinucleoside phosphates 2′,3′-dideoxyinosinylyl-(5′→5′)-*N*^{4}-palmitoyl-2′,3′-dideoxycytidine (ddI-*N*^{4}-pamddC, **2**) and 3′-azido-2′,3′-dideoxythymidylyl-(5′→5′)-*N*^{4}-palmitoyl-2′, 3′-dideoxycytidine (AZT-*N*^{4}-pamddC, **3**) in yields between 62–65%. Both condensation products were purified by chromatography on silica gel. By treatment with ammonia, **3** and **4** were deprotected to the hydrophilic 3′-azido-2′,3′-dideoxythymidylyl-(5′→5′)-2′,3′-dideoxycytidine (AZT-ddC, **3a**) and 2′-deoxythymidylyl-(3′→5′)-2′,3′-dideoxycytidine (dT-ddC, **4a**) respectively at 80% yield. Traces of impurities were removed by reversed-phase HPLC. The course of the synthesis and the purification steps were monitored by thin-layer chromatography (TLC) and/or HPLC. The structure and the analytical purity of the products were confirmed by NMR, *(tables I, II,* and *III)* mass spectroscopy and elementary analysis. The 5′→5′-phosphodiester linkage of the heterodimers (**2, 3** and **3a**) leads to a downfield shift of the 5′-carbon atoms of about 3 ppm compared to the monomer units. The three 5′-phosphorylated ddNs (**5–7**) were used at a degree of purity of at least 80% above all as references for the identification of the metabolites formed during the enzymatic hydrolyses of the heterodimers. For the determination of the relative anti-HIV activity of the lipophilized monomer pN^4-pamddC (**5**) a purity of about 80% was also sufficient because the test results show at least approximately 70–250 times lower activity of **5** compared to AZT. Further attempts to purify these compounds were not carried out. Therefore the elementary analysis and the NMR-spectra of these compounds were omitted.

Table I. $^1H(^{13}C)$ chemical shifts in ppm of AZT, ddC and ddI.

Proton (Carbon)	Compound AZT	ddC	ddI
H_2	–	–	8.03
(C_2)	(150.36)	(155.14)	(147.56)
(C_4)	(163.67)	(165.60)	(145.55)
H_5	–	5.67	–
(C_5)	(109.47)	(93.18)	(124.29)
H_6	7.66	7.89	–
(C_6)	(136.01)	(140.95)	(156.56)
H_8	–	–	8.32
(C_8)	–	–	(138.13)
$H_{1'}$	6.08	5.90–5.92	6.18–6.22
$(C_{1'})$	(83.37)	(85.69)	(84.44)
$H_{2'}$ $H_{2''}$	2.21–2.43	1.74–1.84	2.35–2.38
		2.16–2.32	2.41–2.47
$(C_{2'})$	(36.14)	(32.38)	(32.09)
$H_{3'}$ $H_{3''}$	4.36–4.43	1.74–1.84	1.98–2.06
$(C_{3'})$	(60.13)	(24.74)	(25.43)
H_4’	3.78–3.82	3.96–4.04	4.06–4.15
$(C_{4'})$	(83.95)	(81.40)	(81.99)
$H_{5'}$ $H_{5''}$	3.54–3.69	3.48–3.57	3.45–3.54
		3.62–3.71	3.59–3.65
$(C_{5'})$	(60.77)	(62.13)	(62.62)
$H_{5'OH}$	5.21	5.01	4.95

Table II. ^{13}C chemical shifts in ppm of the heterodimers **2, 3, 3a, 4a** and pN^4-pamddC (**5**).

Carbon	Compound-Nr.				
Nr.	**2**	**3**	**3a**	**5**	**4a**
C_2	154.51^a	154.57^a	155.09^a	154.46	150.48^d
	147.66^b	150.45^c	150.41^c		155.18^a
C_4	162.18^a	162.26^a	165.54^a	162.17	163.68^d
	145.56^b	163.73^c	163.69^c		165.61^a
C_5	95.01^a	95.11^a	93.58^a	95.03	109.55^d
	124.24^b	109.93^c	109.89^c		93.66^a
C_6	144.96^a	145.00^a	140.75^a	144.96	135.93^d
	156.54^b	136.12^c	136.09^c		140.80^a
C_8	–	–	–	–	–
	138.06^b	–	–		–
$C_{1'}$	86.91^a	86.97^a	85.74^a	85.75	83.74^d
	84.37^b	83.59^c	83.57^c		85.70^a
$C_{2'}$	32.57^a	32.62^a	31.96^a	32.57	DMSO
	31.75^b	36.35^c	35.91^c		31.96^a
$C_{3'}$	24.46^a	24.47^a	25.33^a	25.01	72.24^d
	26.05^b	60.96^c	60.87^c		25.40^a
$C_{4'}$	80.27	80.83	79.16	80.62	86.11^d
	80.76	82.64	82.63		79.50^a
$C_{5'}$	65.03	65.17	65.83	64.79	60.21^d
	66.26	64.50	64.66		61.47^a

^{a}Signal of ddC; ^{b}signal of ddI ; ^{c}signal of AZT ; ^{d}signal of dT.

Table III. ^{1}H chemical shifts in ppm of the heterodimers **2, 3, 3a, 4a** and pN^4-pamddC (**5**).

Proton	Compound-Nr.				
Nr.	**2**	**3**	**3a**	**5**	**4a**
H_2	8.00	–	–	–	–
H_5	5.73	5.72	5.72	5.72	–
	–	–	–		5.74
H_6	7.17	7.20	7.79	7.20	7.79
	–	7.69	7.70		7.75
H_8	8.25	–	–	–	–
$H_{1'}$	5.88–5.90	5.90–5.94	5.91–5.94	5.90–5.94	6.12–6.18
	6.16–6.20	6.08–6.13	6.10–6.13		5.92–5.96
$H_{2'}$, $H_{2''}$	1.76–1.88	1.77–1.90	1.75–1.89	1.79–1.98	1.74–1.93
	2.31–2.37	2.22–2.29	2.23–2.28	2.15–2.33	2.24–2.29
	2.37–2.42	2.37–2.48	2.37–2.46		
$H_{3'}$, $H_{3''}$	1.76–1.88	1.77–1.90	1.75–1.89	1.79–1.98	4.72
	1.92–2.07	4.43	4.44		1.74–1.93
H_4'	3.95–4.21	4.19	4.11	3.95–4.10	3.90–4.11
		3.98	3.94		
$H_{5'}$, $H_{5''}$	3.41–3.50	3.91–3.94	3.87–3.94	3.50–3.80	3.78–3.90

3. Biological evaluation

3.1. Hydrolysis of the heterodimers in the presence of phosphodiesterase or human serum

To explain the anti-HIV activity of the heterodimers it is important to determine whether these dimers can be cleaved into the anti-HIV active monomers. At first the metabolism of **3, 3a, 4** and **4a** for 2–24 h in the presence of phosphodiesterase from snake venom, which hydrolizes natural occurring oligonucleotides from the 3′ to the 5′ terminus to nucleoside-5′-monophosphates, compared to the natural dinucleoside phosphate dT-dT was evaluated. In a second experiment the degradation of these heterodimers in the presence of human serum, which contains a mixture of different nucleases was investigated. The metabolites of both experiments were separated using reversed phase HPLC and identified using the reference monomers (ddC, AZT, dT, pdT, pN^4-pamddC (**5**), pAZT (**6**), pddC (**7**)). In the case of the hydrolysis with human serum the proteins were removed by acid precipitation before the mixture was applied to the column.

3.2. Anti-HIV activity

All compounds were included in the Anti-AIDS-Drug-Discovery-Program and tested for their in vitro anti-HIV activity at the National Cancer Institute, Bethesda MD, USA using the standard in vitro T4-lymphocyte assay [10]. The anti-HIV activity was expressed by the

Table IV. In vitro anti-HIV activity and cytotoxicity of N^4-palmitoyl-2′,3′-dideoxycytidine-5′-monophosphate (**5**) and the heterodimers (**2–4** and **8**) against HIV-1 infected T4-lymphocytes (CEM-SS cells)[a].

Nr.	Compound	IC_{50}[b] (Molar)	EC_{50}[c] (Molar)	Ti[d]
3a	AZT-ddC	$> 2.00 \times 10^{-5}$	$(2.59–8.31) \times 10^{-8}$	> 241–>772
4	dT-N^4-pamddC	$(3.13–> 5.00) \times 10^{-5}$	$(6.44–8.24) \times 10^{-8}$	410–>685
4a	dT-ddC	$(3.04–4.10) \times 10^{-5}$	$(1.34–2.67) \times 10^{-7}$	102–257
3	AZT-N^4-pamddC	$(1.07–1.24) \times 10^{-4}$	$(1.50–7.10) \times 10^{-7}$	167–827
2	ddI-N^4-pamddC	$> 1.00 \times 10^{-4}$	$(6.97–10.2) \times 10^{-7}$	>98–>158
5	pN^4-pamddC	$(3.11–>5.00) \times 10^{-5}$	$(7.08–25.3) \times 10^{-7}$	19.8–>46
8	N^4-pamdC-AZT	$>1.00 \times 10^{-4}$	$(3.84–35.4) \times 10^{-7}$	28.2–>260
	AZT	1.00×10^{-6}	$(0.13–1.1) \times 10^{-8}$	90.9–769

[a] Range of four assays which were obtained from the National Cancer Institute (USA); [b] IC_{50}, concentration of dimers required to inhibit 50% cell growth compared to untreated controls; [c] EC_{50}, concentration of dimers required to protect 50% of the cells from HIV induced cell death compared to uninfected controls; [d] TI (IC/EC), therapeutic index.

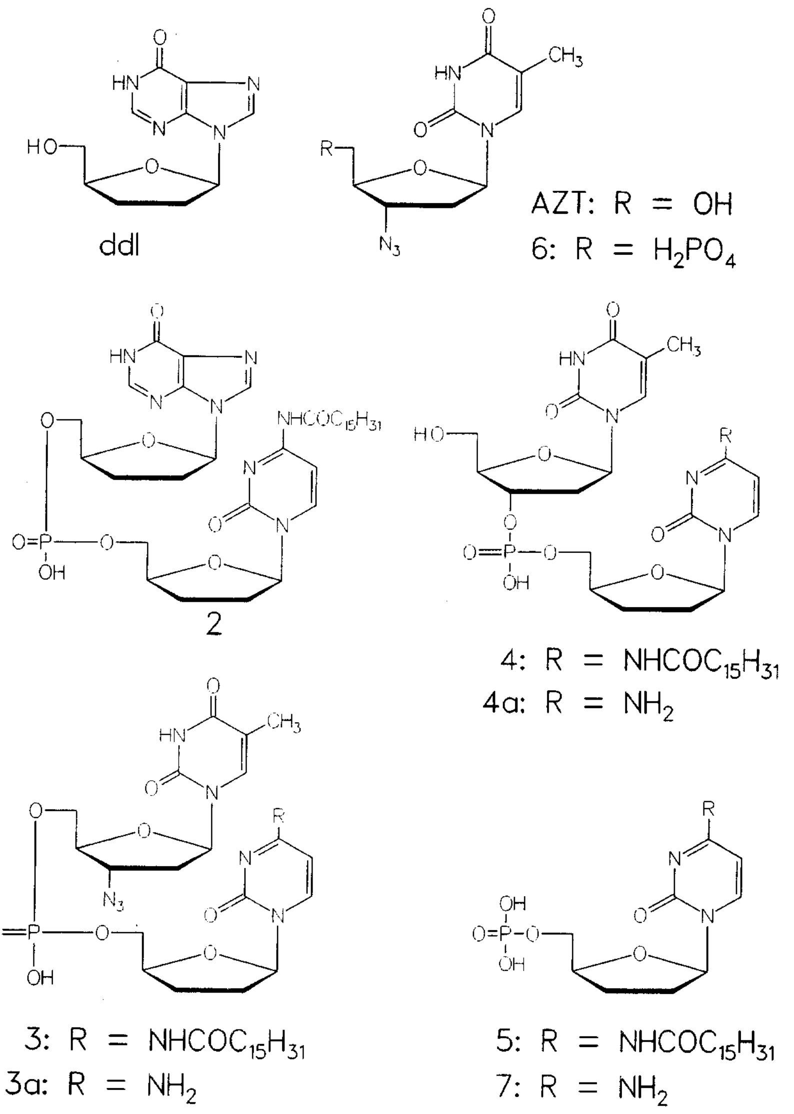

Figure 1. Structures, formulae and abbreviations of the anti-HIV active compounds. 2: 2′,3′-dideoxyinosylyl-(5′→5′)-N^4-palmitoyl-2′,3′-dideoxycytidine (ddI-N^4-pamddC); 3: 3′-azido-2′,3′-dideoxythymidylyl-(5′→5′)-N^4-palmitoyl-2′,3′-dideoxycytidine (AZT-N^4-pamddC); 3a: 3′-azido-2′,3′-dideoxythymidylyl-(5′→5′)-2′,3′-dideoxycytidine (AZT-ddC); 4: 2′-deoxythymidylyl-(3′→5′)-N^4-palmitoyl-2′,3′-dideoxycytidine (dT-N^4-pamddC); 4a: 2′-deoxythymidylyl-(3′→5′)-2′,3′-dideoxycytidine (dT-ddC); 5: N^4-palmitoyl-2′,3′-dideoxycytidine-5′-monophosphate (pN^4-pamddC); 6: 3′-azido-2′,3′-dideoxythymidine-5′-monophosphate (pAZT); 7: 2′,3′-dideoxycytidine-5′-monophosphate (pddC).

EC_{50} value, which represents the concentration of the test compound required to protect 50% of the cells from HIV induced cell death, compared to uninfected controls. Briefly, HIV-1 and a solution of the heterodimers (in Me_2SO and cell culture medium) were added to T_4-lymphocytes (CEM cell line). The resultant mixture was incubated for 6 d in 5% CO_2 atmosphere at 37 °C. Cell viability was then determined both microscopically and by using the tetrazolium (XTT) salt procedure. Toxic controls were also used to evaluate the toxicity of the heterodimers. All heterodimers and the lipophilized mononucleotide (**5**) were active against HIV *(table IV)* whereby the activity was mainly influenced by the kind of the incorporated nucleoside derivatives.

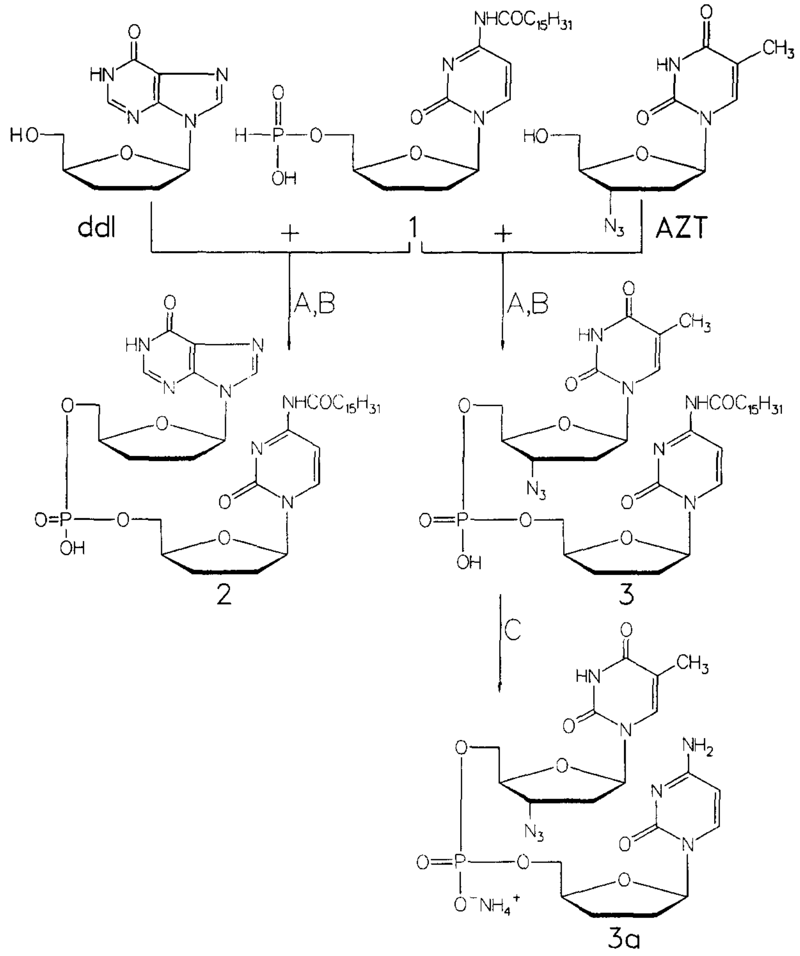

Figure 2. Synthesis of heterodinucleoside phosphates (**2**, **3** and **3a**) starting from N^4-palmitoyl-2′,3′-dideoxycytidine-5′-hydrogenphosphonate (**1**) and 2′,3′-dideoxyinosine (ddI) or 3′-azido-2′,3′-dideoxythymidine (AZT). The reaction steps are: A = pivaloyl chloride in pyridine (3 min); iodine (0.2 M) in THF/pyridine/water (16: 1: 1). B = column chromatography on silica gel. C = methanol saturated with ammonia.

4. Results and discussion

A qualitative empirical estimation of the membrane diffusion properties of the drugs was obtained from their partition coefficients (PC) in a 1-octanol/water system (*table V*). In respect to the PC value of AZT (0.97), which penetrates through membranes by passive diffusion [11], it can be assumed that passive membrane diffusion of a nucleoside analogue should be possible if it has a similar PC value to or higher than AZT. This assumption has been confirmed in the case of the [^{3}H]-labelled heterodimer N^4-pamdC-[^{3}H]AZT (PC = 0.62) that enters H9 cells in a comparable amount to AZT [6].

Table V. Partition coefficients (PC) of AZT, ddC and their derivatives in 1-octanol/water.

Compound	Nr	Partition coefficient (PC)
ddI-N^4-pamddC	**2**	7.22
AZT-N^4-pamddC	**3**	3.40
AZT		0.97
dT-N^4-pamddC	**4**	0.89
N^4-pamdC-AZT	**8**	0.62
pN^4-pamddC	**5**	0.40
AZT-ddC	**3a**	0.083
dT-ddC	**4a**	0.045
ddC		0.032
pddC	**7**	0.0045
pAZT	**6**	0.0032

All heterodimers (**2**, **3** and **4**) containing the N^4-pamddC monomer unit (PC range: 0.89–7.22) should be able to cross the cell membrane, whereas the highly hydrophilic 5′-phosphorylated monomers (**6** and **7**) (PC range: 0.0032–0.0045) and the deprotected heterodimers AZT-ddC (**3a**) (PC = 0.083) and dT-ddC (**4a**) (PC = 0.045) should not be able to enter the cell by passive diffusion. In the case of the 5′-phosphorylated amphiphilic ddC derivative pN^4-pamddC (**5**) the lipophilic N^4-palmitoyl residue increases the PC value of pddC from 0.0045 to 0.40 for **5**, where passive membrane penetration is likely to occur.

The anti-HIV activity of the heterodimers, however, was not exclusively induced by the activity of the incorporated single monomer units. The antiviral activity of the 5′-mononucleotide (pN^4-pamddC) against the prototype HIV-1 strain IIIB increased after coupling to a second nucleoside derivative, resulting in the amphiphilic heterodimers **3** and **4**. Within the group of amphiphilic heterodimers, the EC_{50} value decreased about 10-fold from $(7–10) \times 10^{-7}$ M (**2**) to $(0.6–0.8) \times 10^{-7}$ M (**4**) and the therapeutic index (TI) increased from 98–158 to 410–> 685. The anti-HIV activity of **3** was considerably lower than that of **4** in which the highly active AZT monomer unit is replaced by the natural and inactive nucleoside thymidine. This result is surprising because heterodimers that can release two different ddNs should be more effective, due to additive and/or synergistic effects, than dimers which contain only one active ddN. This unexpected result demonstrates that corresponding EC_{50} values of different heterodimers can be obtained by the coupling of one ddN to a natural and inactive nucleoside instead of a second ddN possessing anti-HIV activity. The anti-HIV activity of the heterodimers is not predominantly determined by the single antiviral activity of the corresponding monomer units, nor is the high activity principally caused by the coupling of two active ddNs. The transformation of the amphiphilic **3** into the hydrophilic **3a** by removing the lipophilic palmitoyl protecting group led to approximately a 10-fold decrease of the EC_{50} value. The EC_{50} value of **3a**, combining AZT and ddC is only about three times lower than that of **4** in which pN^4-pamddC is linked to the inactive thymidine. The removal of the lipophilic protection group of **4**, resulting in **4a**, led to an approximate fourfold increase of the EC_{50} value. The coupling of a lipophilic derivative of the natural nucleoside dC (N^4-pamdC) to AZT results in the amphiphilic heterodimer N^4-pamdC-AZT (**8**, PC = 0.62), which showed an anti-HIV activity that was 300 times lower than that of AZT and about four times lower than that of AZT-N^4-pamddC (**3**), in which the lipophilized nucleoside dC is replaced by the anti-HIV active ddC. These differences in the anti-HIV activity demonstrate that similar lipophilic monomer units cause drastic changes in the structure-activity relationship of the amphiphilic heterodimers.

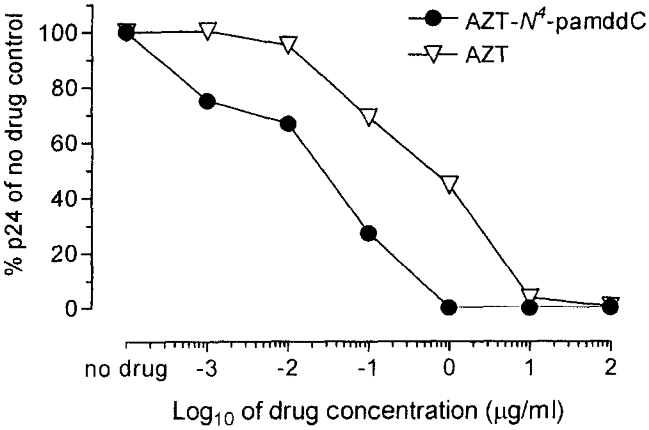

Figure 3. Anti-HIV activities determined by p24 antigen reduction of AZT (-∇-) and AZT-N^4-pamddC (**3**) (-●-) against primary human lymphocytes infected with the 500-fold AZT-resistant HIV isolate 148/89. The concentrations (EC_{50}) required to reduce the viral core protein p24 by 50% were calculated as 2.36 μM for AZT and 0.045 μM for AZT-N^4-pamddC.

A significant difference of the anti-HIV activity between AZT and **3**, in which AZT is coupled to N^4-pamddC, was observed in a test *(figure 3)* with about a 500-fold more AZT-resistant HIV isolate (148/89) which was consecutively obtained from a patient during a four year AZT-monotherapy. AZT reached a 50% inhibition of HIV replication in primary human lymphocytes infected de novo with the HIV isolate at a concentration of 2.36 μM. The dimer **3** reached 50% inhibition in this test system, at a concentration of 0.045 μM which is 10 times lower than that of ddC and about 50 times lower compared to AZT.

The explanation for the differences in the anti-HIV activity of amphiphilic heterodimers lies, in our opinion, in the influence of the palmitoyl protecting group. On one hand the lipophilic residue is necessary for the cellular uptake of the heterodimer, since only amphiphilic heterodimers with PC values higher than 0.5 should be taken up by the target cell in a sufficient amount. On the other hand this protecting group may delay the intracellular cleavage of the heterodimer into the desired active monomer units, which can be followed by the inhibited enzymatic hydrolysis with human serum and phosphodiesterase. The enzymatic hydrolysis *(table VI)* of the natural dinucleoside phosphate dT-dT with phosphodi-

Table VI. Time necessary for the total hydrolysis of the heterodimers at 37 °C in the presence of A) phosphodiesterase I and B) human serum.

		Total hydrolysis (time in h)	
Nr.	Heterodimer	A	B
	dT-dT	2	5
4a	dT-ddC	2	6
3a	AZT-ddC	6	20
4	dT-N^4-pamddC	stable (> 24)	20
3	AZT-N^4-pamddC	stable (> 24)	27
8	N^4-pamdC-AZT	stable (> 24)	38

esterase quickly led to the expected mixture of thymidine and thymidine-5′-monophosphate and only to thymidine if human serum was used. The hydrolysis of the other heterodimers was clearly delayed depending on the monomer units incorporated. If one of the two natural nucleosides in dT-dT was replaced by a ddN monomer unit the phosphodiesterase cleavage was delayed about 1.5 times. The coupling of two ddNs as in the case of AZT-ddC led to a fourfold increase in the time necessary for the hydrolysis. Unexpected was that the enzymatic cleavage of heterodimers containing 3′-terminal ddNs with phosphodiesterase led to a mixture of the two corresponding nucleotides. The presence of the lipophilic N^4-palmitoyl protecting group suppressed the enzymatic cleavage with phosphodiesterase, with human serum however, it led to a further delay comparable to the effect caused by the incorporation of one ddN-unit. No N^4-palmitoylated monomer could be detected in the hydrolysate formed during the human serum incubation of the amphiphilic heterodimers, which is shown in the HPLC-chromatogram of the hydrolysis of **3**, given in *figure 4* as an example for the hydrolysates. It can be followed that the N^4-palmitoyl residue has to be removed before the enzymatic cleavage of the phosphodiester bonding can occur, whereby the N^4-palmitoyl residue can be more easily removed from ddC than from dC.

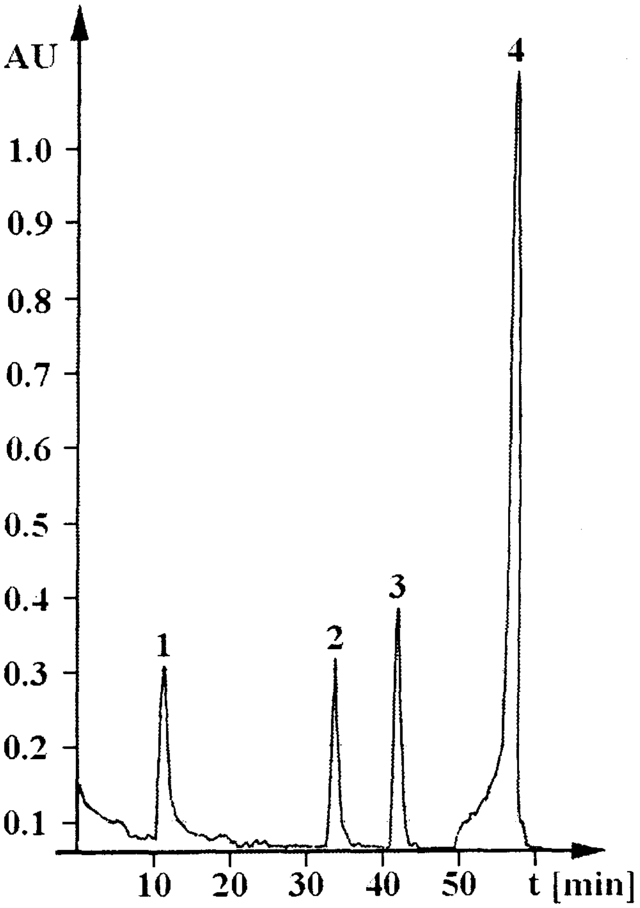

Figure 4. HPLC-chromatogram of the hydrolysated AZT-N^4-pamddC after 8 h of incubation with human serum at 37 °C. The fractionation was performed on a reverse phased column (250 × 4.6 mm) using four elution steps: 1. 0–5 min 0% B; 2. 5–30 min 0–15% B; 3. 30–50 min 30% B; 4. 50–65 min 70–100% B of a binary gradient (A: 0.05 M ammonia acetate, pH = 7.5; B: methanol). The following compounds of the hydrolysate were eluted in Peak 1 = ddC; 2 = AZT; 3 = AZT-ddC; 4 = AZT-N^4-pamddC.

5. Conclusion

It can be expected that the incorporation of one or two ddNs in heterodimers can at least improve the low compliance of the combination therapy, by simplification of the therapeutic schedule, if the resulting heterodimers release their active monomer units extracellulary. Amphiphilic heterodimers of ddNs however, which can enter the target cells and release ddN-5′-monophosphates intracellularly, eliminate (kinase by-passing) the need for the enzymatic 5′-phosphorylation which is the limiting step in the formation of the anti-HIV active ddN anabolites.

The enhanced cellular uptake of amphiphilic heterodimers will only result in an increased anti-HIV activity if they can be intracellularly metabolized to the active monomer units. In the case of dT-N^4-pamddC (**4**), this requirement is fulfilled, resulting in an increased anti-HIV activity of **4** compared to the hydrophilic deprotected dimer **4a**. In the case of the amphiphilic AZT-N^4-pamddC (**3**) the cellular uptake should also be possible based on the PC-value. The intracellular metabolism, however, seems to be strongly delayed, resulting in a lower concentration of the monomer units (AZT + ddC) in the cell, resulting in a lower anti-HIV activity of this heterodimer in respect to the deprotected hydrophilic AZT-ddC (**3a**) which should not be able to cross the cell membrane, but has an increased extracellular metabolism compared to **3**. The released ddNs are taken up by the cell in sufficient amounts and act as an equimolar mixture (ddC + AZT) resulting in additive and/or synergistic effects, that should be able to reduce the undesired side effects and prevent them from resistance towards a single ddN.

The high activity of **3** against the AZT resistant virus variant cannot be explained by a difference in the mechanism of hydrolysis. It can not be excluded that amphiphilic heterodimers can exert their anti-HIV activity by unknown mechanisms, which have to be the aim of further investigations.

6. Experimental protocols

6.1. Chemistry

The following substances were synthesized according to published methods: 2′-deoxythymidylyl-(5′→3′)-2′-deoxythymidine (dT-dT), 2′-deoxythymidylyl-(3′→5′)-N^4-palmitoyl-2′,3′-dideoxycytidine (dT-N^4-pamddC) (**4**) [9], N^4-palmitoyl-2′,3′-dideoxycytidine, N^4-palmitoyl-2′-deoxycytidylyl-(3′→5′)-3′-azido-2′,3′-dideoxythymidin (N^4-pamdC-AZT) (**8**) [12]. AZT and ddI were obtained in our laboratory by recycling expired pharmaceutical formulations (capsules, tablets) using extraction procedures and silica gel column chromatography. The following materials were commercially available: Trimethylphosphate (Aldrich, Germany), Sephadex-G10 (Pharmacia Fine Chemicals, Sweden), Partisil-10 SAX (Whatman, USA).

NMR spectra *(tables I, II,* and *III)* were recorded using: ^{1}H-NMR: Bruker AMX 250 at 250 MHz or Bruker AMX 400 at 400 MHz (DMSO as internal standard). ^{13}C-NMR: Bruker AMX 250 at 63 MHz or Bruker AMX 400 at 100 MHz (DMSO as internal standard). Mass spectrometry: ionspray MS: Sciex API III, negative mode, $CHCl_3/CH_3OH$ (3:7) as solvent. HPLC: Analytical HPLC of the synthesized compounds: Nucleosil 7 C_{18} column (250 × 4.6 mm) (Macherey & Nagel, Düren, Germany), binary mixtures of 0.1 M ammonium acetate (A) and methanol (B), isocratic conditions, flow rate 0.9 mL /min., UV detection at 254 nm. TLC: Analytical thin-layer chromatography was performed on Merck pre-coated silica gel plates 60 F_{254} (0.25 mm) using chloroform/methanol mixtures as mobile phases. The nucleoside derivatives and impurities were detected as described [13]. Column chromatography: Merck silica gel 60 (0.04–0.063 mm). Chromatography was carried out on dry packed columns using chloroform/methanol gradients. All solvent mixtures were prepared by volume ratios (v/v). The concentration of solutions was performed in vacuo. Partition coefficients: PC values in 1-octanol/water were determined using UV absorbance. The compound (10 mg) was dissolved in water (1 mL) and the A_{260} values (I) of the resulting solutions were determined. 1-octanol (1 mL) was added, the mixture vigorously shaken at room temperature for 15 min, followed by centrifugation (10 000 rpm, 20 min). The A_{260} values (II) of the aqueous layer were measured and the partition coefficient (PC) calculated as the quotient:

$$PC = [A_{260}(I) - A_{260}(II)] \,/\, A_{260}(II).$$

6.1.1. N^4-Palmitoyl-2′,3′-dideoxycytidine-5′-hydrogenphosphonate **1**

To a cold solution (10 °C) of N^4-palmitoyl-2′,3′-dideoxycytidine (6.5 g, 14.5 mmol) in a mixture of pyridine (50 mL) and dry dioxane (100 mL) salicylchlorophosphite (3.5 g, 17.4 mmol) was added quickly. The reaction mixture was shaken at room temperature for 1 h. After cooling to 0 °C and addition of water (4 mL) the mixture was shaken at room temperature for another 15 min, concentrated to a syrup which was dissolved in chloroform (200 mL) and washed three times with saturated aqueous $NaHCO_3$/methanol (1:1, 80 mL). The organic layer was concentrated to dryness and co-evaporated three times with toluene (50 mL). The obtained syrup was dissolved in chloroform/methanol (9:1, 120 mL) and chromatographed on a silica gel column (11 × 9 cm) using a three step gradient [steps 1–3: chloroform/methanol 9:1 (4 L); 4:1 (2 L) and 7:3 (4 L)]. The desired compound was eluted during the third step. The pooled fractions (UV-, molybdic reagent- and fluoresceine-positive) were concentrated, dissolved in hot ethyl acetate and crystallized at –25 °C yielding N^4-palmitoyl-2′, 3′-dideoxycytidine-5′-hydrogenphosphonate (**1**) (6.5 g, 87%) as colourless crystals, mp 248 °C. TLC ($CHCl_3/CH_3OH$, 7:3) R_f 0.50. HPLC (78% B) R_t 11.3 min; MS (IS); m/z: 512.5 [M – H^+]. Anal $C_{25}H_{43}N_3NaO_6P \times H_2O$ (531.54): calcd. C 54.24, H 8.19, N 7.59; found C 54.09, H 8.05, N 7.47.

6.1.2. 2′,3′-Dideoxyinosylyl-(5′→5′)-N^4-palmitoyl-2′,3′-dideoxycytidine **2** *and 3′-azido-2′,3′-dideoxythymidylyl-(5′→5′)-N^4-palmitoyl-2′,3′-dideoxycytidine* **3**

To a cooled solution (10 °C) of (a) **1** (4.6 g, 9 mmol) and ddI (2.1 g, 9 mmol) dissolved in dry pyridine

(90 mL) or (b) **1** (3.1 g, 6 mmol) and AZT (1.6 g, 6 mmol) dissolved in dry pyridine (60 mL) pivaloyl chloride (for reaction (a) 5.5 mL, 45 mmol; for reaction (b) 3.7 mL, 30 mmol) was added and the reaction mixture was shaken at room temperature for 3 min. After cooling to 0 °C, water ((a): 3 mL; (b): 2 mL) was added. Into the stirred mixture, iodine (0.2 M in dry THF) was dropped until the solution retained a dark red colour. The excess of iodine was removed by adding sodium hydrogen sulfite before the reaction mixture was concentrated to a syrup which was dissolved in chloroform (200 mL) and then extracted three times with a mixture of saturated aqueous NaCl/water/methanol (1:1:2, 80 mL). The organic layer was concentrated to a syrup which was co-evaporated twice with toluene (60 mL) before dissolving in chloroform/methanol (9:1). The obtained solutions were chromatographed on a silica gel column ((a): 12 × 9 cm; (b): 14 × 9 cm) using a chloroform/methanol gradient [(a): 9:1 (4 L); 4:1 (4 L); 7:3 (3 L); 1:1 (1 L); (b): 9:1 (4 L); 4:1 (4 L); 7:3 (2 L)]. The desired products were eluted during the last two steps. The pooled fractions (UV-, molybdic reagent- and fluoresceine-positive) were concentrated to dryness and (a) crystallized from methanol (20 mL) or (b) dissolved in water and lyophilized. (a) 2′,3′-dideoxyinosylyl-(5′→5′)-N^4-palmitoyl-2′,3′-dideoxycytidine (**2**) (4.2 g, 62%) was obtained as a colourless solid, m.p. > 240 °C. TLC ($CHCl_3/CH_3OH$, 6:4) R_f 0.23. HPLC (80% B) R_t 7.4 min. MS(IS); m/z: 746.5 [M – H $^+$]. Anal $C_{35}H_{54}N_7O_9P$ (747.83): calcd. C 56.21, H 7.28, N 13.11; found: C 56.08, H 7.15, N 12.97; (b) 3′-azido-2′,3′-dideoxythymidylyl-(5′→5′)-N^4-palmitoyl-2′,3′-dideoxycytidine (**3**) (3.1 g, 65%) was obtained as white powder, m.p. 195 °C. TLC ($CHCl_3/CH_3OH$, 7:3) R_f 0.37. HPLC (77% B) R_t 9.3 min. MS(IS); m/z: 777.5 [M – H^+]. Anal $C_{35}H_{55}N_8O_{10}P \times H_2O$ (796.87): calcd. C 52.76, H 7.21, N 14.06; found C 52.59, H 7.20, N 13.88.

6.1.3. 3′-Azido-2′,3′-dideoxythymidylyl-(5′→5′)-2′, 3′-dideoxycytidine **3a** *and 2′-deoxythymidylyl-(3′→5′)-2′-3′-dideoxycytidine* **4a**

To (a) **3** (2 g, 2.6 mmol) or (b) **4** (1 g, 1.3 mmol) dissolved in chloroform/methanol (9:1, 10 mL), methanol saturated with ammonia at room temperature (150 mL) was added. The reaction vessel was sealed and kept at room temperature for 3 d. The reaction mixture was concentrated to a syrup which was dissolved in water (50 mL) and extracted three times with chloroform (30 mL). The aqueous layer was concentrated. The residue was crystallized from ether yielding (a) 3′-azido-2′,3′-dideoxythymidylyl-(5′→5′) - 2′,3′ -dideoxycytidine (**3a**) (1.1 g, 80%) as white powder, decp 225 °C. TLC ($CHCl_3/CH_3OH/NH_3$, 10:10:3) R_f 0.60. HPLC (22% B) R_t 10.3 min. MS (IS); m/z: 540.0 [M – H^+]. Anal $C_{19}H_{25}N_8O_9P \times NH_3$ (557.46): calcd. C 40.94, H 5.06, N 22.61; found C 40.78, H 4.94, N 22.48 or (b) 2′-deoxythymidylyl-(3′→5′)-2′,3′-dideoxycytidine (**4a**) 0.52 g (76%) as white powder, decp 235–238 °C. HPLC (15% B) R_t 7.1 min. MS (IS); m/z 514.5 [M – H^+]. Anal $C_{19}H_{26}N_5O_{10}P \times NH_3$ (532.45): calcd. C 42.86, H 5.49, N 15.78; found C 42.80, H 5.38, N 15.64.

6.1.4. N^4-Palmitoyl-2′,3′-dideoxycytidine-5′-monophosphate **5**, *3′-azido-2′,3′-dideoxythymidine-5′-monophosphate* **6**, *2′,3′-dideoxycytidine-5′-monophosphate* **7**

To a cooled solution (0 °C) of freshly distilled phosphoroxy chloride (0.5 mL, 5.6 mmol) in trimethylphosphate (9 mL) a) N^4-palmitoyl-2′,3′-dideoxycytidine (1.2 g, 2.6 mmol), or b) 3′-azido-2′,3′-dideoxythymidine (0.7 g, 2.6 mmol) or c) 2′,3′-dideoxycytidine (0.55 g, 2.6 mmol) was added in portions within 15 min. The reaction mixture (0 °C) was stirred for 2 h, then neutralized under cooling with NaOH (0.5 M). The obtained precipitate was centrifuged and crystallized twice from ethanol yielding N^4-palmitoyl-2′,3′-dideoxycytidine-5′-monophosphate (**5**) (0.9 g, 65%) as colourless crystals, m.p. 165 °C. TLC ($CHCl_3/CH_3OH/NH_3$, 10:10:3) R_f 0.28. MS (IS); m/z: 528.5 [M – H^+]. b) and c): The reaction mixtures were concentrated, dissolved in water and chromatographed on a Sephadex-G_{10} column. The product containing fractions were pooled, concentrated to a syrup, which was dissolved in water and chromatographed on a Partisil column (250 × 8 mm) using a gradient from water to 0.3 M ammonium acetate within 30 min. The product containing fractions were pooled, concentrated, desalted on a Sephadex-G_{10} column and lyophilized, yielding b) 3′-azido-2′,3′-dideoxythymidine-5′-monophosphate (**6**) (0.45 g, 50%) as a white powder, decp 190 °C. TLC ($CHCl_3$/ CH_3OH/ NH_3, 10:10:3) R_f 0.36. MS(IS); m/z: 346.5 [M – H^+], 692.5 [2M – H^+] and c) 2′,3′-dideoxycytidine-5′-monophosphate (**7**) (0.36 g, 48%) as a white powder, decp 165 °C. TLC ($CHCl_3/CH_3OH/NH_3$ = 10:10:3) R_f 0.24. MS (IS); m/z: 290.5 [M – H^+], 581.0 [2M – H^+].

6.2. Biological evaluation

6.2.1. In vitro activity against cells infected with the AZT resistant HIV isolate 148/89

Peripheral blood mononuclear cells were isolated from regular blood donations by density gradient centrifugation. The isolated cells were resuspended in cell culture medium (RPMI 1640, supplemented with 5% foetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin) and stimulated with phytohaemagglutinin at 7.5 μg/mL for 2 d. Subsequently, the stimulated cells were infected de novo with the AZT-resistant HIV isolate 148/89, derived from a patient under AZT therapy for three years, at a multiplicity of infection of 0.001 for 60 min at 37 °C. After washing off the inoculum the infected cells were resuspended in cell culture medium supplemented with 10 U/mL of recombinant IL-2. The cells were seeded at a density of 3×10^6 cells/mL in 96 well microtitre plates. AZT, ddC or AZT-N^4-pamddC (**3**) were added at various concentrations to the infected cells. Three to five days post infection the cell culture supernatants were tested for virus replication by determination of the viral core protein p24 using a sandwich ELISA [14]. The concentration of p24 was calibrated with a standardized preparation of recombinant p24.

6.2.2. In vitro stability of the heterodimers against phosphodiesterase I and human serum

For the enzymatic hydrolysis, a stock solution for each heterodimer was prepared containing 5 mg of dimer dissolved in 0.5 mL of water. For the treatment with phosphodiesterase I (Boehringer, Mannheim, Germany) 100 μL of the dissolved enzymes were added to a mixture of 300 μL of the stock solution, 75 μL 0.1 M $MgCl_2$ and 75 μL of 1 M Tris-HCl (pH 8.1). This mixture was stored at 37 °C. After 2, 4, 12, and 36 h, 100 μL of the reaction mixture were applied onto an analytical reversed phase HPLC column (Nucleosil 7 C_{18}, 250×46 mm) and chromatographed using a binary gradient (A: 0.05 M ammonium acetate, pH 7.5 and B: methanol) at a flow rate of 0.9 mL/min. The fractionation of the reaction mixture was performed within the following steps: 1. 0–5 min: 0% B; 2. 5–30 min: 0–15% B; 3. 30–50 min: 30% B in case of the hydrophilic dT-dT, **3a** and **4a**. A fourth step was added: 4. 50–65 min: 70–100% B in the case of the amphiphilic **3**, **4** and **8.** The course of the elution was automatically monitored at 280 nm and the eluted metabolites were identified by the reference substances.

For the treatment with human serum, 0.5 mL of the serum were added to 20 μL of the stock solution and the mixture was kept sealed at 37 °C. After 2, 4, 12 and 36 h, 100 μL of the reaction mixture and 200 μL of perchloric acid (1%) were added. After 10 min the aliquots were neuturalized with 2 M KOH, filtered through a sterile filter (0.45 μm) and 100 μL of the obtained filtrate were fractionated by means of analytical HPLC as described above. The time necessary for the total hydrolysis of the heterodimers *(table VI)* is obtained from the HPLC-chromatograms using the decreasing peak area of the uncleaved heterodimer.

Acknowledgements

The authors thank the National Cancer Institute (Bethesda, MD, USA) for testing the heterodimers and the Fonds der Chemischen Industrie (Germany) for partial financial support.

References

[1] Krise J.P., Stella V.J., Adv. Drug Delivery Rev. 19 (1996) 287–310.

[2] Busso M., Mian A.M., Hahn E.F., Resnick L., AIDS Res/Human Retroviruses 4 (1988) 449–455.

[3] Schinazi R.F., Sommadossi J.P., Saalmann V., Cannon D.L., Xie M-Y., Hart G.C., Smith G.A., Hahn E.F., Antimicrob. Agents Chemother. 34 (1990) 1061–1067.

[4] Ijichi K., Fujiwara M., Mori K., Morozumi M., Machida H., Shigeta S., Konno K., Yokota T., Baba M., Antiviral Res. 31 (1996) 115–120.

[5] Schwendener R.A., Guerin F., Schott H., EP 0642527 B1; Chem. Abstr. 120 (1994) 54904n.

[6] Peghini P.A., Zahner R., Kuster H., Schott H., Schwendener R.A., Antiviral Chem. Chemother. 9 (1998) 117–126.

[7] Delta Coordinating Committee, Lancet 348 (1996) 283–291.

[8] Eron J.J., Johnson V.A., Merrill D.P., Chou T.C., Hirsch M.S., Antimicrob. Agents Chemother. 36 (1992) 1559–1562.

[9] Schott H., Häussler M.P., Gowland P., Bender A., von Briesen H., Schwendener R.A., Antiviral Chem. Chemother. 6 (1995) 320–326.

[10] Sharma A.P., Ollapally A.P., Lee H.J., Antiviral Chem. Chemother. 4 (1993) 93–96.

[11] Zimmerman T.P., Mahony W.B., Prus K.L., J. Biol. Chem. 262 (1987) 5748–5754.

[12] Schott H., Häussler M.P., Gowland P., Horber D.H., Schwendener R.A., Antiviral Chem. Chemother. 5 (1994) 387–394.

[13] Schott H., Ludwig P.S., Gansauge F., Gansauge S., Schwendener R.A., Liebigs Ann-Recl. (1997) 413–417.

[14] Müller C.H., Moritz R., Prigge G., Weitzel D., Kunze R., Kage A., Köttgen E., Fresenius Z., Anal. Chem. 330 (1988) 352–353.