[Frontiers in Bioscience 9, 873-890, January 1, 2004]

*CYCLO*SAL-PRONUCLEOTIDES – DEVELOPMENT OF FIRST AND SECOND GENERATION CHEMICAL TROJAN HORSES FOR ANTIVIRAL CHEMOTHERAPY

Chris Meier [1], Astrid Meerbach [2] and Jan Balzarini [3]

[1] Institute of Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6; D-20146 Hamburg, Germany, [2] Institute for Virology and Antiviral Therapy, Friedrich-Schiller-University Jena, Winzerlaer Straße 10, D-07745 Jena, Germany, [3] Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

TABLE OF CONTENTS

1. ABSTRACT

Pronucleotides represent a promising alternative to improve the biological activity of nucleoside analogs against different viral diseases. Moreover, pronucleotides are valuable tools for studies concerning the nucleoside/nucleotide metabolism. The basic idea is to achieve nucleotide delivery into cells, bypassing limitations with intracellular formation of nucleotides from their nucleoside precursors. The *cyclo*Sal-concept is one of several pronucleotide systems reported so far but is the only approach in which a pronucleotide is cleaved successfully by a simple but selective chemical hydrolysis. Beside others, for the nucleoside analog d4T the application of the *cyclo*Sal-approach improved antiviral potency. In the first part, the basic concept, the chemistry, different structural modifications and their effects on the antiviral potency of the *cyclo*Sal-d4TMP triesters have been discussed in this review. In the second part, first results of a conceptional extension of the original *cyclo*Sal-approach will be summarized. Once the pronucleotides have passed the membrane, the aim is to trap the *cyclo*Sal-phosphate triesters inside the cells. Therefore, enzyme-cleavable groups have been attached via a linker to the *cyclo*Sal-moiety.

2. INTRODUCTION

Since the discovery of 3'-azido-3'-deoxythymidine (AZT) as the first nucleoside drug for the treatment of AIDS (1), considerable efforts have been made to develop new nucleoside analogs that would be more active and less toxic inhibitors of HIV reverse transcriptase (RT) (2). These analogs differ from the natural nucleosides with regard to modifications of the glycon and/or the aglycon residue (3). Today, synthetic nucleoside mimetics represent a highly valuable source of antiviral compounds that contribute significantly to the arsenal of agents for the treatment of viral diseases, e.g. HIV, herpes and hepatitis virus infections. The general mode of action of nucleoside analogs is through the inhibition of DNA polymerases, including reverse transcriptase, by acting as competitive inhibitors and/or as DNA chain terminators. To act as DNA chain termination agents/polymerase inhibitors, intracellular conversion of the nucleoside analogs into their 5'-mono-, 5'-di- and finally 5'-triphosphates is a prerequisite after cell penetration (2,4). However, the efficient anabolism to the corresponding nucleoside analog triphosphates often is a major hurdle due to limited anabolic phosphorylation or catabolic processes as deamination of the aglycon or cleavage of the glycosidic bond. Therefore, their eventual therapeutic efficacy is compromised (2,5). For example, the first phosphorylation step of the anti-HIV active dideoxynucleoside analog 2',3'-dideoxy-2',3'-didehydrothymidine (d4T) **1** (6-8) (Figure 1) into d4T 5'-monophosphate (d4TMP) catalyzed by thymidine kinase (TK) is the critical rate-limiting step in human cells (9).

Despite the example given above, the intracellular fate of the majority of nucleoside analogs has not been studied in detail. These compounds are often exclusively tested as nucleosides and discarded if found inactive. As they are rarely studied against the target

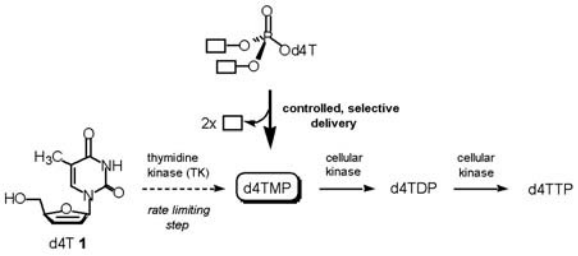

Figure 1. Bioactivation of 2',3'-dideoxy-2',3'-didehydrothymidine d4T. **1** and the principle of the pronucleotide approach for the nucleotide d4TMP.

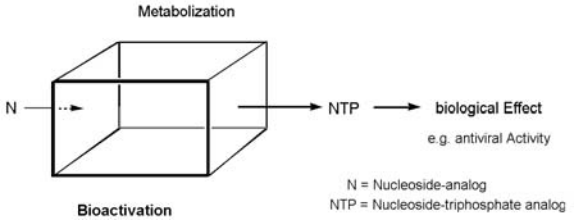

Figure 2. "Black-box"-metabolism of nucleoside analogs into the antivirally active nucleoside triphosphate.

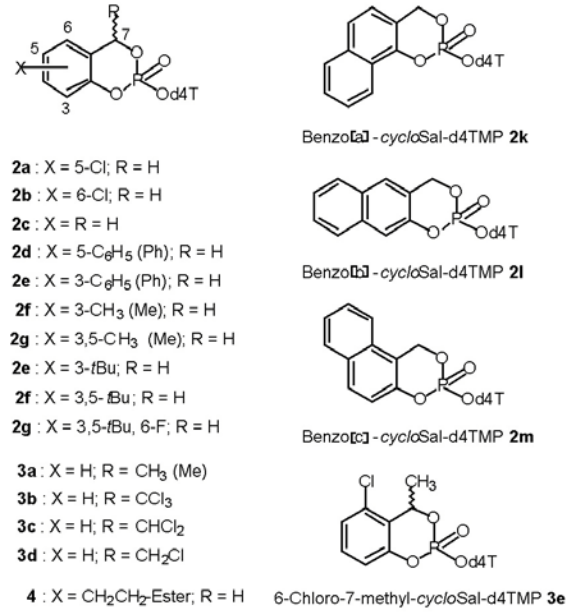

Figure 3. Prototype *cyclo*Sal-d4TMP triesters **2**, *cyclo*Sal-d4TMP triesters **3** and *cyclo*Sal-d4TMP triesters **4** bearing functionalized X-groups.

polymerases as triphosphates and thus ends in a black-box metabolism (Figure 2).

However, a lack of uncovering where the metabolic blockade exists prevents further successful development. On the other hand, knowing the limitations upon phosphorylation of a nucleoside may offer a chance to develop derivatives with improved biological potential. In principle, the direct administration of nucleotides like d4TMP should bypass the limiting step in the thymidine kinase-based anabolism of some nucleosides and thus improve their biological activity. Unfortunately, nucleotides are very polar molecules and do not easily pass cellular membranes. However, this difficulty can be surmounted by linking suitably degradable lipophilic carrier groups to the phosphate moiety that lead to neutral, membrane-permeable nucleotide delivery systems (*pronucleotide approach*; Figure 1) (10-14).

Two masking groups are necessary in order to obtain a neutral, lipophilic phosphate ester due to the presence of at least one negatively charged phosphate oxygen under physiological conditions. The pK_A1 of a phosphate monoester is ~1.6 and pK_A2 is ~6.6, which means there is an equilibrium between the mono- and the dianion in neutral solution. Moreover, the efficient intracellular delivery of nucleotides from a pronucleotide requires the design of a specific delivery mechanism. Several strategies using different nucleotide delivery mechanisms have been developed to achieve this goal (10-14). Among these, simple systems like dialkyl-, diphenyl- and dibenzyl phosphate triesters based on pure chemical hydrolysis proved to be unsuccessful because after the first hydrolysis of the neutral phosphate triester, the resulting phosphate diester is often far too stable to undergo further chemical hydrolysis (10,11,13,14). More recent pronucleotide approaches are based on the principle of selective enzymatic or chemical activation of the masking group, which leads to a second, spontaneous reaction (tripartate prodrug system (15)). These approaches utilize and exploit carboxyesterase activity and pH. The concepts working via an enzymatic trigger mechanism are the [bis(pivaloyloxymethyl) (POM)], [bis(*iso*propyloxycarbonyloxymethyl) (POC)], [bis(*S*-glycosylthioethyl) (SGTE)], [bis(4'-acylbenzyl (AB)], the aryloxyphosphoramidate (APA), the phosphoramidate monoester and a (modified) [bis(*S*-acylthioethyl) (SATE)] concept. The delivery mechanisms of these enzyme-cleavable compounds have been summarized recently (11-13). All these enzyme-triggered approaches have demonstrated that the successful intracellular delivery of nucleotides is indeed possible. However, the only successful, pH-driven nucleotide delivery strategy by chemical means is the *cyclo*Sal-approach (16). This approach, which also belongs to the group of tripartate prodrug delivery systems, has been developed in our laboratories and will be the topic of this review. The effect of differently modified *cyclo*Sal-triesters **2** and **3** of the nucleoside analog d4T will be described. Three groups of derivatives will be discussed. The so-called prototypes (**2**) bearing no additional substituent at the benzyl carbon atom. A second series of compounds (**3**) bearing alkyl residues in the benzyl position (7-position) and finally a series of triesters but having an ester-functionalized group as the X substituent but are unsubstituted in the 7-position (**4**) (Figure 3).

The first two compound series will be discussed concerning their hydrolytic behavior. These first generation compounds will offer valuable mechanistic insights into the designed cascade cleavage mechanism as well as their antiviral potential. The third series will be discussed in the last chapters of this review. These compounds may act as second generation *cyclo*Sal-pronucleotides.

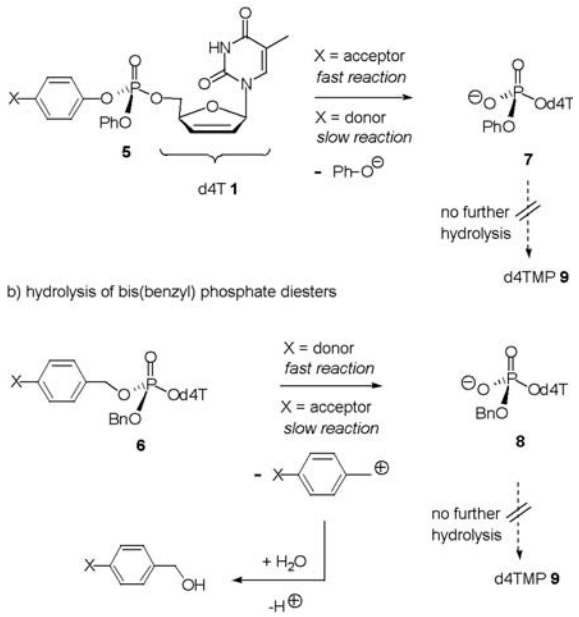

Figure 4. Hydrolysis of bisphenyl.- and bisbenzyl. phosphate triesters **5,6.**

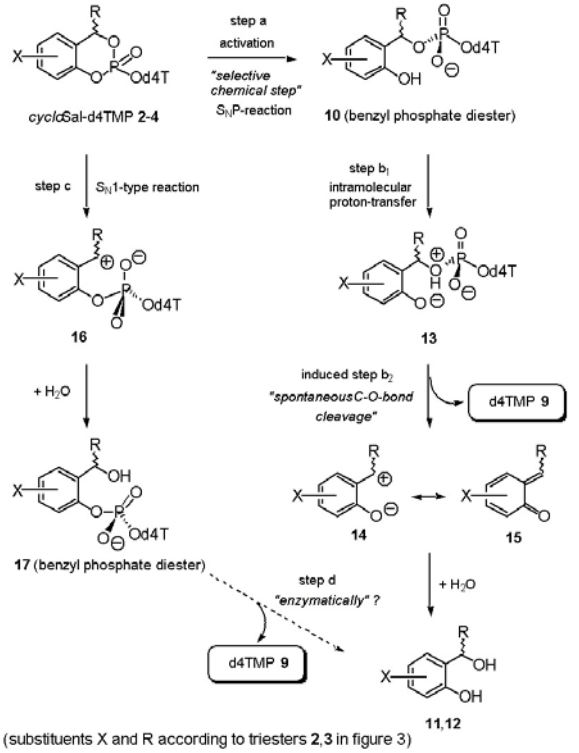

Figure 5. Two possible hydrolysis pathways of the *cyclo*Sal-d4TMP triesters **2 -4.**

3. THE *CYCLO*SALIGENYL-PRONUCLEOTIDE APPROACH: THE FIRST GENERATION

3.1. *Cyclo*Saligenyl-nucleotides (*cyclo*Sal-NMPs) – The Design of a Concept

In contrast to the approaches mentioned previously, our aim was the development of a selective delivery mechanism that is based on an exclusively pH-dependent, chemically-induced cascade mechanism (16,17). However, the chemically driven release of the free nucleotide from a lipophilic precursor is not as easy as it seems (18-21). By contrast to the enzymatically triggered pronucleotides (11), the *cyclo*Sal-strategy requires only *one* activation step to deliver the nucleotide and due to the bifunctional character of the *cyclo*Sal group the ratio of the masking unit per nucleotide molecule is 1:1. By contrast, other pronucleotide concepts employ ratios up to 4:1 (22-25). As summarized in Figure 4, simple bis(phenyl)- **5** or bis(benzyl) nucleotide triesters **6** are unable to deliver the nucleotide (26-28). Hydrolysis always stops at the phosphate diesters **7** and **8**, respectively, without formation of d4TMP **9**.

Interestingly, the influence of substituents in the aromatic rings of **5** or **6** are just the opposite: while acceptors in bis(phenyl) esters **5** cause a fast hydrolysis, donors in bis(benzyl) esters 6 cause a fast cleavage to yield the diester and finally benzyl alcohol (Figure 4). However, a combination of both may form the basis of a suitable pronucleotide approach. Thus, the basis of the *cyclo*Sal concept consists of a combination of two ester bond types as part of a cyclic bifunctional group (masking unit). Salicyl alcohols have been attached via a phenyl- and a benzyl ester bond. Additionally, the nucleoside analog is attached through an alkyl ester bond. Only the introduction of these three ester bonds would allow sufficient discrimination between the different phosphate ester bonds. The designed chemically-induced coupled process (tandem or cascade mechanism) is the following (17,29,30): The phenyl ester bond in the *cyclo*Sal-triester structure should be the most labile one after nucleophilic attack of hydroxide to phosphorus (S_NP-reaction). The developing negative charge can be delocalized by the aromatic system which makes the phenolate the best leaving group in the triester. Cleavage yields a 2-hydroxy*benzyl*phosphate diester 10 (Figure 5, step a). As a consequence of the initial step, the *ortho*-substituent to the benzyl ester is changed from a very weak electron-donating group (phosphate ester) to a strong electron-donating group (hydroxyl). This effect of the 2-substituent intrinsically activates the remaining masking group and this induces a spontaneous rupture of diester **10** to yield the nucleotide and salicyl alcohols **11** (R = H) and **12** (R = alkyl) (cascade reaction; step b_1 and b_2). This rupture proceeds presumably after intramolecular proton transfer (intermediate 13) via zwitter-ion **14** or 2-quinone methide **15**. By this pathway, a cleavage mechanism is achieved that takes place within the masking group only, and so prevents a pseudorotation process (18) that may partly lead to nucleoside liberation instead of the nucleotide. This is the preferred pathway via the benzyl phosphate diester intermediate **10**.

Although unfavored, a cleavage of the benzyl ester bond should also be taken into account (step c). From literature it is known that benzyl esters are cleaved via S_N1-type C_{benzyl}-O bond rupture which leads to the formation of a stabilized benzyl cation and an anionic phosphate ester group (intermediate **16**). The cation **16** is rapidly trapped by water to yield the phenyl phosphate diester of type **17** (Figure 5). However, no further hydrolysis can be expected

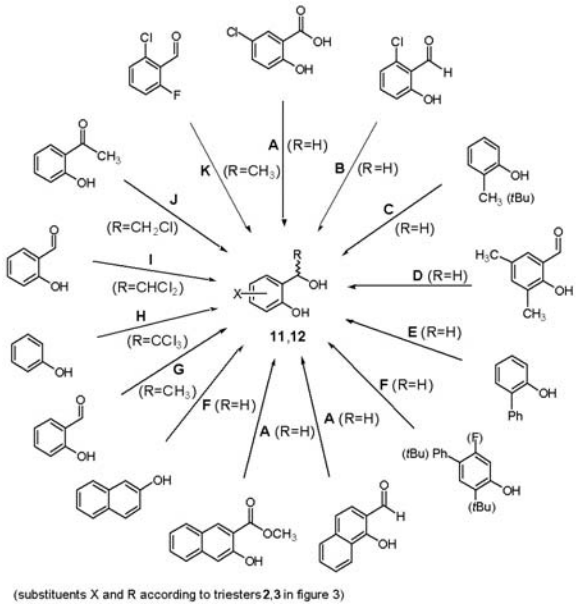

Figure 6. Synthetic pathways to the salicyl alcohols **11**,**12.** Reaction conditions. Method **A**: $LiAlH_4$ or $NaBH_4/I_2$, THF, rt, 5h; Method **B**: $NaBH_4$, EtOH, rt, 4h; Method **C**: i. $(HCHO)_n$, $SnCl_4$, 2,6-lutidine, toluene, 110°C, 16h; ii. $NaBH_4$, 2-propanol, rt, 5h; Method **D**: $NaBH_4$, 2-propanol, rt, 5h; Method **E**: i. $(HCHO)_n$, $PhB(OH)_2$, propionic acid cat., toluene, reflux, 10h; ii. 30% H_2O_2, THF, 0°C, 35 min; Method **F**: 37% $HCHO/H_2O$, KOH, rt, 24h; Method **G**: methyllithium, Et_2O, 0°C, 1.5h; Method **H**: i. CCl_3CHO, $PhB(OH)_2$, propionic acid cat., toluene, reflux, 10h; ii. 30% H_2O_2, THF, 0°C, 35min; Method **I**: CH_2Cl_2, methyllithium, Et_2O, -78°C, 1.5h; Method **J**: i. 2,3,4,5,6,6-hexachloro-2,4-cyclohexadien-1-one, EtOH, reflux, 7h; ii. 2-propanol, rt, 5h; Method **K**: i. NaOH, 75°C, 45h; ii. methyllithium, Et_2O, 0°C, 1.5h.

from this diester, thus hindering nucleotide release. The reason is the formation of a negative charge at the phosphorus atom. This prevents a second nucleophilic attack at the phosphorus atom in phenyl phosphate diesters. It also would decrease the leaving group properties of the 5'-nucleoside phosphate fragment in benzyl phosphate diesters (19-21). Consequently, this C_{benzyl}-O bond rupture would lead to a dead end (Figure 5) by formation of the phenyl phosphate diester **17**.

Salicyl alcohols **11**,**12** used as masking units were tested for their biological potency but showed neither antiviral activity nor cytotoxicity (29,30). Further *in-vivo* studies in mice did not show toxic side effects (feeding of mice with 250 mg/kg of 3-methyl-salicyl alcohol does not cause any visible toxic effects (unpublished results). It should be added that salicyl alcohol (saligenin) is used as part of the antirheumatic and analgetic drug Salicin (2-(hydroxymethyl)phenyl-β-D-glucopyranoside; Assalix®) (31). β-Glucosidases hydrolyze Salicin to D-glucose and saligenin and the latter is then slowly oxidized by cytochrome P450 to salicylic acid in the blood and in the liver.

3.2. Chemistry

For the synthesis of the prototype *cyclo*Sal-d4TMP triesters the salicyl alcohols **11** had to be prepared first from the corresponding salicylic aldehydes, -acids or -esters (**32**) by standard reduction protocols ($NaBH_4$ or $LiAlH_4$ (**33**); Figure 6) in high yields. In most cases, the aldehydes/acids/esters were not commercially available. Diols **11** (for triesters **2**) then have been synthesized starting with appropriately substituted phenols. Selective *ortho*-formylations have been achieved according to Casiraghi- (34) or the Rieche-formylation protocols (35). Both methods led to salicyl aldehydes, which then could be reduced to the corresponding diols 11. Alternatives are the direct hydroxymethylation according to Nagata (36) or a direct hydroxymethylation using formaldehyde in aqueous basic medium (37). The latter methods are the mildest of the procedures above (Figure 6).

These generally highly efficient methods are suitable for the synthesis of the prototype *cyclo*Sal-derivatives without substituent in the benzyl position. For the second series of derivatives, 7-methylated diols **12** (for triesters **3**) were prepared by alkylation of salicyl aldehyde with methyllithium (38) or dichloromethyllithium (39) to give diols **12a** and **12c**. 7-Chloromethylsalicyl alcohol **12d** was prepared by chlorination of *ortho*-hydroxy acetophenone using 2,3,4,5,6,6-hexachloro-2,4-cyclohexadiene-1-one (40) to give the ketone intermediate which was reduced to give the alcohol. The 7-trichloromethyl derivative **12b** was synthesized by the Nagata protocol using chloral instead of formaldehyde, while double 6,7-modified salicyl alcohol **12e** was prepared by nucleophilic substitution of the fluorine in 2-chloro-6-fluoro-benzaldehyde by hydroxide (41) and subsequent aldol-type addition as mentioned above.

The synthesis of the *cyclo*Sal-pronucleotides has been carried out using reactive phosphorus(III)-reagents (Figure 7). Therefore, diols **11**,**12** were reacted with phosphorus trichloride to give the cyclic chlorophosphites **18**. Phosphites **18** were reacted directly with the nucleoside analog, e.g. d4T, in the presence of diisopropylethylamine (DIPEA; Hünig`s base) to yield the cyclic phosphite triesters which were oxidized in a one-pot-reaction using *t*-butylhydroperoxide (TBHP) or dimethyldioxirane. The phosphate triesters **2**-**4** were obtained in reasonable yields (50-73%) as mixtures of stereoisomers (29,30).

Alternatively, chlorophosphites **18** were treated with diisopropylamine to yield the phosphoramidites **19**. The coupling with the nucleoside analog was carried out in acetonitrile in the presence of pyridinium chloride or imidazolium triflate as coupling activator. In some cases the latter procedure resulted in yields of > 90% using imidazolium triflate as activator (42).

3.3. Proof-of-Principle

3.3.1. D4TMP-Release from *cyclo*Sal-d4TMP Pronucleotides

Extensive studies have been performed in order to investigate the designed delivery mechanism of d4TMP from the *cyclo*Sal-triesters (30,43). Chemical hydrolysis

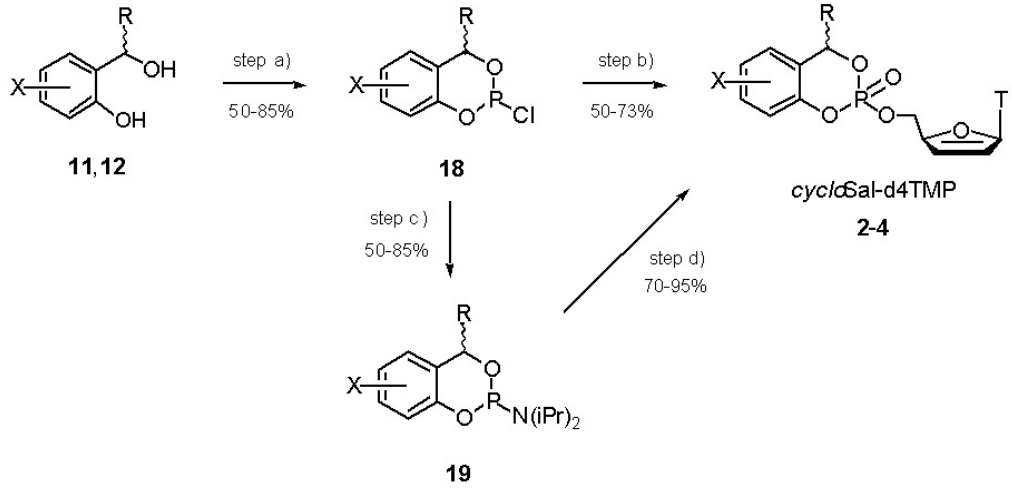

Figure 7. Synthesis of the *cyclo*Sal-d4TMP triesters **2-4.** Reaction conditions. a: PCl_3, pyridine, Et_2O, -10°C, 2h; b: i. d4T **1**, DIPEA, CH_3CN, -20°C to rt, 1h; ii. TBHP, CH_3CN, -20°C to rt, 1h; c: diisopropylamine 2eq.., Et_2O, 0°C, 30 min; d: i. d4T **1**, pyridinium chloride, tetrazole or imidazolium triflate, CH_3CN, 0°C, 30 min; ii. TBHP, CH_3CN, rt, 1h.

studies in different buffer solutions at different pH values proved that all prototype compounds released selectively d4TMP in a pH dependent manner. As second product, the salicyl alcohols **11** have been detected also by means of HPLC analysis. D4TMP has been identified undoubtedly by using ion-pairing eluents and the coelution technique. The half-lives of the triesters have been determined from these studies and are summarized in (Table 1). Within the prototype compound series, the following interesting effects were observed: As expected, the half-lives depend on the substitution pattern of the aromatic ring (11,16,30). In general, acceptor substituents like the 5- or 6-chloro atom cause a decrease in hydrolytic stability while donor-substituents like 3-methyl-, 3,5-dimethyl- and particular 3-*t*-butyl or 3,5-di-*t*-butyl group(s) lead to an increased stability with respect to the unsubstituted prototype triester **2c**.

However, the phenyl ring substituent in the 3- (**2e**) and 5-position (**2d**), respectively, showed a stabilizing effect for the first ($t_{1/2}$ = 5.1 h) and a destabilizing effect for the latter ($t_{1/2}$ = 3.1 h) compared to a $t_{1/2}$ of 4.4 hours for the unsubstituted prototype triester **2c**. It should be mentioned that these differences are out of the experimental error (±0.2 h) and are relevant. Our interpretation is that the phenyl ring in the case of 5-phenyl-*cyclo*Sal-d4TMP **2d** is involved in the delocalization of the negative charge appearing after the initial cleavage of the phenyl ester bond to give the benzyl phosphate diester 10 (Figure 5). Thus, the accelerating effect dominates due to a (–)M-effect of the phenyl group and the stability of the triesters decreases. In contrast, the rotation of the phenyl-aryl bond in 3-phenyl-*cyclo*Sal-d4TMP **2e** is hindered due to the bulky dioxaphosphorine-2-oxide ring and the delocalization of the negative charge in diester **10** is less effective. Thus a weak donor (+)I-effect dominates and the stability increases. However, this donating effect is considerably weaker as compared to the strong (+)I-effect of a methyl group, as reflected by the longer half-life of 3-methyl-*cyclo*Sal-d4TMP **2f**. Another contribution of the 3-phenyl ring may be hindering the nucleophilic attack of water or hydroxide to the P-atom due to its lipophilicity by making the trajectory less available. By contrast, the presence of t-butyl groups has an unexpectedly big influence to the hydrolysis stability. Although the electron-donating property of a *t*-butyl group differs only slightly from the +I-effect of a methyl group, triesters **2h** (3-*t*Bu) and **2i** (3,5-*t*Bu) showed considerably higher half-lives as compared to the methyl counterparts **2f** and **2g**. We attribute this to differences in the lipophilicity and therefore in the accessibility of the phosphate group.

It is interesting to note that the compounds bearing an extended aromatic system **2k-m** (43) all showed a decreased hydrolytic stability as compared to the unsubstituted prototype triester **2c**. Half-lives ranged between 2.8 h and 1.4 h for compound benzo[a]- (**2k**), benzo[b]- (**2l**) and benzo[c]-*cyclo*Sal-d4TMP **2m**. This may be explained by the increasing stabilization of the negative charge in the extended aromatic ring system particularly in the case of the benzo[b]-derivative **2l** as compared to the monocyclic prototype counterparts.

In addition to these HPLC-based studies we were able to study the delivery mechanism by a ^{31}P-NMR-experiment. The reason for using this technique was that the detection limit judged by integration of the resonance signals of possible reaction side products in the NMR technique using the ^{31}P-nucleus as a probe is much lower (1%) as compared with the HPLC method (~5%). However, phosphate buffer is not an appropriate buffer system for this experiment. Compounds were dissolved therefore in an imidazole/HCl buffer adjusted to pH 7.3. A similar NMR experiment using only a DMSO/water mixture has been done earlier in our lab for the hydrolysis of 5-nitro-*cyclo*Sal-d4TMP (30). In that case d4TMP has been detected as the only hydrolysis product pointing to an entirely selective delivery reaction.

Table 1. Half-lives, product ratio and antiviral data of the prototype *cyclo*Sal-d4TMP triesters **2** and the 7-modified *cyclo*Sal-d4TMPs **3**

Compound	Modification	$t_{1/2}$; 37°C[1]	Product ratio	EC_{50} (μM)[4]			CC_{50} (μM)[5]
	X and/or R	pH 7.3[2] [h]	d4TMP : 17[3]	CEM/O HIV-1	CEM/O HIV-2	CEM/TK$^-$ HIV-2	
2a	5-Cl	1.1	100 : 0	0.42	1.40	2.67	49
2b	6-Cl	0.9	100 : 0	0.087	0.15	0.8	36
2c	5-H	4.4	99 : 1	0.20	0.22	0.15	50
2d	5-C_6H_5	3.1	100 : 0	0.40	0.47	2	54
2e	3-C_6H_5	5.1	97 : 3	0.13	0.27	0.15	22
2f	3-CH_3	17.5	94 : 6	0.057	0.07	0.048	32
2g	3,5-CH_3	29	92 : 8	0.09	0.17	0.08	21
2h	3-tBu	96	92 : 8	0.18	0.65	0.33	35
2i	3,5-tBu	73	66 : 34	1.1	1.2	2.0	27
2j	3,5-tBu; 6-F	6.2	100 : 0	1.23	0.73	0.6	26
2k	benzo[a]	2.8	91 : 9	0.14	0.12	0.6	53
2l	benzo[b]	1.4	97 : 3	0.41	0.50	4	132
2m	benzo[c]	2.8	88 : 12	0.09	0.17	0.8	> 50
3a	7-CH_3	0.25	17 : 83	0.22	0.34	35	152
3b	7-CCl_3	0.9	85 : 15[6]:	0.19	0.60	42	54
3c	7-$CHCl_2$	1.4	97 : 3	0.16	0.55	27	67
3d	7-CH_2Cl	2.8	100 : 0	0.19	0.35	22	56
3e	6-Cl,7-CH_3	2.2	100 : 0	0.19	0.25	7	42
d4T 1	--	--	--	0.18	0.55	28	35

[1] hydrolysis half-lives in hours; [2] 25 mM sodium phosphate buffer; [3] ratio of d4TMP: phenyl phosphate diester **17** determined by ^{31}P-NMR; [4] antiviral activity: 50% effective concentration; [5] cytotoxic concentration: 50% cytostatic/toxic activity; [6] benzyl diester 4 : phenyl diester **17.**

The prototype *cyclo*Sal-d4TMP **2c** led to 99% formation of d4TMP in the imidazole/HCl buffer, pH 7.3. Moreover, 1% of phenyl phosphate diester of type **17** (Figure 5) was detected (Table 1). Diester **17** was entirely stable for several weeks in the NMR tube at 37°C. This diester of the S_N1-type reaction has also been found in other cases in amounts of 3% (3-phenyl derivative **2e**) until up to 8% for the 3,5-dimethyl triester **2g** and 3-*t*-butyl triester **2h** (Table 1). The formation of the phenyl phosphate diesters could not been followed in the HPLC studies due to the lower detection sensitivity. The benzo-annulated benzo[a]- and benzo[c]-*cyclo*Sal-triesters **2k**, **2m** formed 9% and 12% of the phenyl phosphate diester under the same reaction conditions, respectively. The difference between the above benzo-annulated triesters **2k**, **2m** and benzo[b]-*cyclo*Sal-d4TMP **2l** (3% diester **17**) results from a better mesomeric stabilization of the cation intermediate of the first two compounds. This additional stabilization increases the rate of the S_N1-type bond rupture. By contrast, the worst case was found for the introduction of two *t*-butyl groups in the 3 and 5 position (compound **2i**) led to the formation of 34% of the phenyl phosphate diester. Here, the concurrent S_N1-type reaction plays a considerable role in the degradation of triester **2i**. In contrast, 5-chloro- (**2a**), 6-chloro- (**2b**) and 5-phenyl-*cyclo*Sal-d4TMPs **2d** turned out to deliver d4TMP exclusively. No side products were observed in the NMR experiment.

In conclusion, the S_NP-type hydrolysis of the prototype *cyclo*Sal-triesters showed a strongly favored delivery of d4TMP following the designed cascade mechanism. However, a small amount of the phenyl phosphate diester **17** was also formed via an S_N1-type reaction. Hydrolysis half-lives may be controlled by introduction of appropriate substituents in the aryl moiety. Benzo-annulated compounds do not show favorable properties with respect to the half-life as well as delivery mechanism. It should be added that an additional hydrolysis study has been done in ^{18}O-labeled water. This experiment clearly proved the spontaneous second step involving a zwitter-ion or a quinone methide (16,30).

Very interesting effects have been observed for the second series of compounds. First, 7-methyl-*cyclo*Sal-d4TMP **3a** has been investigated. Surprisingly, an enormous decrease in chemical stability was measured. The half-life dropped to 0.25 h (compared to 4.4 h for prototype triester **2c**) and even product distribution has changed dramatically. NMR studies confirmed that the major product was the phenyl phosphate diester **17** (83%; Table 1). Obviously, in this case the S_N1-type reaction is preferred. This may be due to the formation of a secondary benzyl cation intermediate as compared to a primary benzyl cation in the prototype cases. The additional alkyl residue stabilizes the cation by a (+)I-effect. This interpretation has been further confirmed by density functional theory (DFT)-calculations on the B3LYP/6-311G(d,p) level on model compounds of the prodrug systems (44). The activation energy barrier for the initial S_N1 reaction is considerably lower for 7-methyl-*cyclo*Sal-methylMP compared to the prototype triester analog (unpublished results). On the other

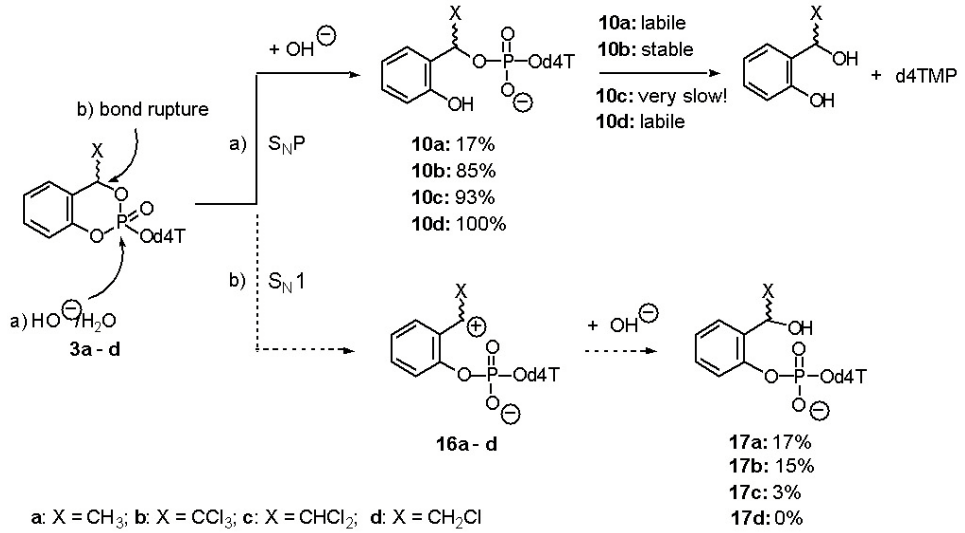

Figure 8. Hydrolysis of the 7-alkyl-modified *cyclo*Sal-d4TMP triesters **3.**

hand, this predominant S_N1-type reaction should be avoided by reduction of the methyl (+)I-effect or by introduction of a strong electron-withdrawing substituent like a chloro-atom in the 6-position of the aromatic ring. Both should decrease the stability of the intermediate benzyl cation and therefore should favor again the phenyl ester bond cleavage. Hence, the corresponding *cyclo*Sal-triesters **3b**-**3e** bearing a 7-trichloromethyl, a 7-dichloromethyl and a 7-chloromethyl and a 6-chloro,7-methyl group instead of a 7-methyl group (**3a**) were prepared. The half-lives of these compounds are listed in (Table 1). NMR studies proved in all cases that the favored degradation pathway is again the phenyl ester bond cleavage giving the benzyl phosphate diesters **10a-d** (Figure 8). Unexpectedly, the intermediate benzyl diester bearing a 7-trichloromethyl group proved to be entirely stable in phosphate buffer, pH 7.3 as well as in imidazole/HCl, pH 7.3. No d4TMP formation was observed. By introduction of three chloro atoms, the second, spontaneous step leading to the formation of d4TMP is prevented completely (Figure 8). By introducing only two chloro atoms, 7-dichloromethyl-*cyclo*Sal-d4TMP **3c** gave the diester **10** in 93% which subsequently delivers d4TMP. However, the reaction proceeds very slowly. 7-chloromethyl-*cyclo*Sal-d4TMP **3d** led to an entirely selective cleavage of the triester to give the benzyl phosphate diester **10** that then delivered d4TMP as known for the prototype triesters **2**. Thus, a single chloro atom in the 7-methyl group completely turns the delivery mechanism from a predominant S_N1-type reaction into the desired highly selective S_NP-reaction (Figure 8)!

For 6-chloro-7-methyl-*cyclo*Sal-d4TMP **3e** similar results were obtained: Again a highly selective d4TMP delivery was observed. Obviously, the introduction of the destabilizing 6-chloro atom overcompensates the stabilizing effect of the 7-methyl group efficiently. It should be mentioned that also the prototype 6-chloro-*cyclo*Sal-d4TMP **2b** gave an exclusive d4TMP delivery as discussed above. The additional 7-methyl group did not interfere although it results in an increase in chemical stability (0.9 h (**2b**) vs. 2.2 h (**3e**)). Finally, the tremendous effect of a halogen atom in the 6-position leading only to the formation of d4TMP **9** has been transferred to the worst-case 3,5-*t*-butyl *cyclo*Sal-triester **2i**. Our aim was to avoid or at least to minimize the formation of the hydrolytically stabile phenyl phosphate diester. The introduction of a fluorine atom in the 6-position in addition to two *t*-butyl groups in the 3- and 5-position in triester **2j**, the concurrent S_N1-type reaction should be limited and, moreover, this fluorine atom should have an impact on the chemical stability at the same time (45). Triester **2j** showed as expected a pronounced decrease in chemical stability (half-life 6.2 h instead of 73 h for **2i**) and moreover, to our surprise, this compound delivers d4TMP **9** highly selectively (0% for **2j** instead of 34% for **2i**)! Thus, again the concurrent S_N1-type cleavage of the triesters was entirely excluded.

In conclusion, it became apparent that besides the d4TMP formation the prototype triesters lead to a small amount of the phenyl phosphate diesters **17**, too. However, this can be efficiently avoided by introduction of an electron-withdrawing 6-substituent or by a mono-acceptor-substituted methyl group in the 7-benzyl position.

No evidence of an enzymatic degradation in RPMI-1640 medium containing 10% fetal calf serum (pH 7.3) has been observed (data not shown). Studies in CEM-cell extracts showed that the hydrolysis half-lives slightly decreased only as compared to the buffer hydrolyses (unpublished results). Further studies of the prototype triesters in human serum (10% serum in phosphate buffer) exhibited no difference in stability as compared to the buffer hydrolysis studies. Again no enzymatic contribution could be detected and thus confirmed the initial idea of a delivery mechanism independent to enzymatic activation. All data obtained from hydrolysis and NMR studies are in perfect agreement with the designed degradation cascade-

reaction mechanism and showed convincingly that the mechanism may be controlled efficiently by structural modification of the *cyclo*Sal-moiety (Figure 5).

3.3.2. Antiviral Activity

The antiviral potency of the *cyclo*Sal-nucleotides against HIV-1 and -2 in CEM cells was assessed (16,30). It became apparent that the unsubstituted prototype **2c**, 3-phenyl- (**2e**), 3-methyl- (**2f**) as well as 3,5-dimethyl-*cyclo*Sal-d4TMP **2g** showed comparable or even higher antiviral potency (0.087 μM) in a wild-type T-lymphocytic cell line (CEM/O) compared to d4T **1** (0.18 μM, Table 1). Moreover, particularly striking is the complete retention of the antiviral potency in mutant thymidine kinase-deficient cells (CEM/TK$^-$) of the unsubstituted (**2c**), 3-phenyl- (**2e**), 3-methyl- (**2f**), 3,5-dimethyl- (**2g**), 3-*t*-butyl- (**2h**), and 3,5-t-butyl-6-fluoro- (**2j**) substituted triesters. The antiviral data and the hydrolysis half-lives clearly point to the fact that certain stability is needed, but beyond this point no further improvement of activity could be observed.

By contrast, the short half-life of the 5-chloro-triester **2a** ($t_{1/2}$ = 1.1 h) seems to be responsible for a considerable loss of antiviral activity in the CEM/TK$^-$ cell assay although the antiviral activity in the TK$^-$ competent cells (CEM/O cells) was comparable to that of d4T. The compound hydrolyzes extracellularly to yield d4TMP that can not be taken up by the cells. After extracellular dephosphorylation, d4T is taken up inside the cells and can be converted into the triphosphate. This is only possible in the TK$^-$ competent cells. Similar results were obtained for the 6-chloro derivative and all the triesters bearing the extended aromatic ring system. All of them are losing activity in the CEM/TK$^-$ cell assay. An interesting observation has been made in the case of the unsubstituted, the 5-phenyl-, and the 3-phenyl prototype triesters **2c**, **2d**, **2e**, respectively. While the 3-phenyl and the unsubstituted triester retained all antiviral potency in the CEM/TK$^-$ cells, 5-phenyl-*cyclo*Sal-d4TMP **2d** loses activity (4-fold). Half-lives were found to be 5.1 h, 4.4 h and 3.1 h. This may point to a threshold of about 4 h of hydrolytic stability needed in order to get a biologically active compound in the CEM/TK$^-$ cell assay. Having this in mind it is also reasonable that all compounds of the second series bearing the additional group in the 7-position and 3,5-*t*-butyl-*cyclo*Sal-triester **2i** lack antiviral activity in the CEM/ TK$^-$ cells. Even the most stable derivative 7-chloromethyl-*cyclo*Sal-d4TMP **3d** lost all antiviral potency in the TK$^-$ deficient cell line (Table 1). Moreover, also a loss in activity has been found for some of the compounds in the CEM/O cells. This can be attributed to the fact that predominantly the phenyl phosphate diester **17** was formed or a stable benzyl phosphate diester **10** was formed that did not release d4TMP efficiently in the time scale of the *in-vitro* assay. It should be added that from experiments using an isolated recombinant RT/RNA/DNA template primer it became clear that the *cyclo*Sal-triesters themselves have no inhibitory effect on DNA-synthesis, which is consistent with a mechanism of action for the *cyclo*Sal-triesters that relies on the formation of free d4TTP. Taken together, these results confirm i) the cellular uptake of the compounds, ii) the highly selective intracellular delivery of d4TMP and iii) the independence of the biological activity on cellular thymidine kinase activation for some of the described *cyclo*Sal-phosphate triesters.

Nevertheless, the *in vitro* anti-HIV assays give only an indirect proof of the intracellular delivery of d4TMP. Therefore, a series of incubation experiments with wild-type CEM/O and CEM/TK$^-$ cells and radiolabeled 3-methyl-*cyclo*Sal-d4TMP 2f (tritium-label in the methyl group of thymine) was conducted (46). The amount of d4TMP in CEM/O cells was considerably higher (15-fold, 6 h incubations) as compared to the amount of d4TMP resulting from the metabolism of d4T in the same cell line. In addition, an increase in the concentration of d4TTP was observed (16-fold, 6 h incubation), which may explain the higher activity of the prototype *cyclo*Sal-d4TMPs in wild-type CEM cells compared to d4T. These results are consistent with a mechanism of the *cyclo*Sal-d4TMPs that successfully bypasses thymidine kinase and releases d4TMP inside the cells.

Furthermore, the *cyclo*Sal-d4TMP triesters demonstrated significant antiviral activity in AZT-resistant H9^{r}AZT^{250} cells (47). The resistance is concomitant with a five-fold lower expression of the TK gene in comparison to parental H9 cells. The consequence is that also d4T showed reduced antiviral potency due to insufficient phosphorylation. In contrast, the prototype *cyclo*Sal-d4TMP **2c** proved to be equipotent in parental and in H9^{r}AZT^{250} cells (EC_{50} 0.3 μM and 0.5 μM) proving again the entire independence of the expressed TK levels.

Finally, it should be mentioned that, beside to d4T **1**, the *cyclo*Sal-approach also has been applied to the following nucleoside analogs: 2',3'-dideoxyadenosine **20** and 2',3'-dideoxy-2',3'-didehydroadenosine **21** (48), 2'-*ara*-fluoro-2',3'-dideoxyadenosine **22** and 2'-*ribo*-fluoro-2',3'-dideoxyadenosine **23** (49), 3'-azidothymidine (AZT) **24** (50), 5-fluoro-2'-deoxyuridine (5F-dU) **25** (51), carbovir **26** and abacavir **27** (52), acyclovir (ACV) **28** (53,54) and most recently very successful to 5-(*E*)-bromovinyl-2'-deoxyuridine (BVdU) 29 (55,56) (Figure 9). Chemical hydrolysis studies showed in all cases the delivery of the corresponding nucleotides. In most of the cases the antiviral evaluation revealed the improvement of the antiviral potency except the cycloSal-AZTMP and cycloSal-FdUMP derivatives. The reason for the failure in those cases is a intracellular dephosphorylation of the released nucleotides to give the nucleosides. The involved enzyme seems to be 3',5'-(deoxy)nucleotidase (57).

4. APPLICATION OF THE *CYCLO*SAL-APPROACH TO THE ANTI-HERPES DRUG BVDU

As mentioned above, the *cyclo*Sal-concept has been applied to the nucleoside analogue 5-[(*E*)-2-bromovinyl]-2'-deoxyuridine (BVdU or Brivudin **29**, Figure 9) which is a very potent and highly selective inhibitor of the replication of HSV-1 and particularly VZV (58). By contrast, BVdU is not markedly active against HSV-2 and EBV. Again, selectivity as inhibitor primarily depends upon a specific activation by HSV-encoded

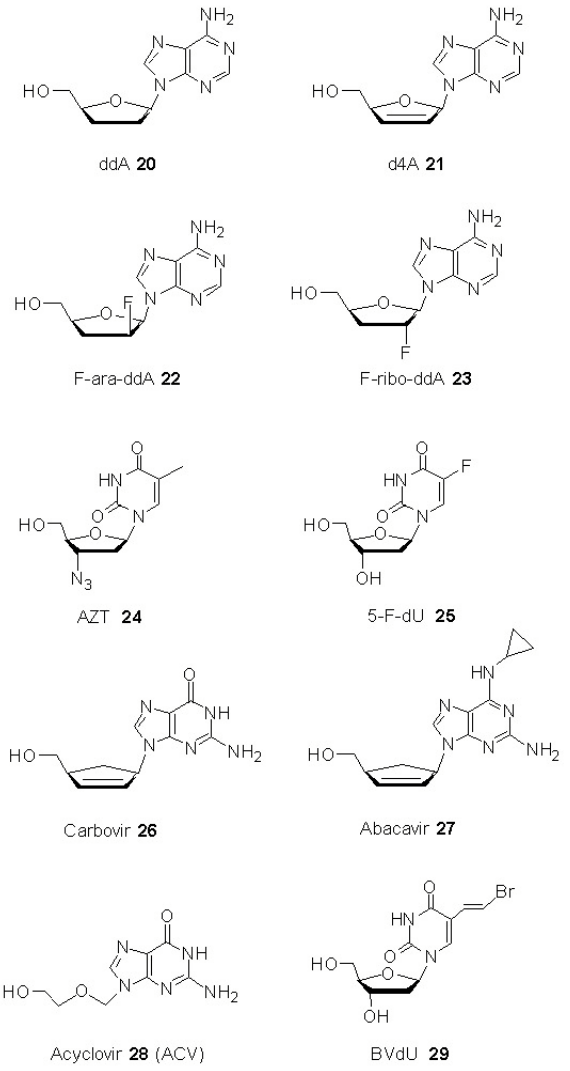

Figure 9. Nucleoside analogs use with the *cyclo*Sal-approach.

thymidine-kinase (TK) to the mono- and diphosphate and finally to the triphosphate by cellular enzymes (59). BVdU-triphosphate (BVdUTP) can act either as an inhibitor of the cellular DNA polymerase or alternate substrate that would render the DNA more prone to degradation when incorporated in DNA (60). Some limitations for the use of BVdU are known: lack of activity during virus latency because of missing viral TK; drug resistant virus strains are known and BVdU will be enzymatically degraded to the nucleobase 5-[(*E*)-2-bromovinyl]uracil within 2–3 h from the bloodstream (60). Moreover, it has been shown that EBV does not express a HSV-like thymidine kinase and this may be the reason why BVdU is inactive to inhibit EBV-replication (Table 2). Obviously, cytosolic TK are unable to activate BVdU. The aim was to prove if the *cyclo*Sal-concept is able to broaden the application of BVdU against Epstein-Barr-virus (EBV) caused infections (61). Such infections play a significant role as secondary infection in e.g. AIDS patients. It should be mentioned that two reports on pronucleotides of BVDU have been published before but both were unsuccessful (62,63). Two different series of *cyclo*Sal-BVdUMP triesters were synthesized: *cyclo*Sal-BVDUMP **30** having different substituents in the aromatic residue and a series of 3'-*O*-esterified 3-methyl-*cyclo*Sal-derivatives **31** and **32** (55,56). As 3'-*O*-modification different lipophilic carboxylic acids (**31**) as well as α-amino acids (**32**) have been used (Figure 10).

The compounds were prepared using 3'-*O*-levulinylated BVdU or 3'-*O*-esterified BVdU derived from *N*-Boc-α-aminoacids like L-/D-alanine, L-phenylalanine as well as carboxylic acids. These compounds were phosphorylated using the phosphoramidite/oxidation method (42). The levulinyl protection group was removed by treatment of the triester with hydrazine hydrate. The *N*-Boc protecting group was removed by treatment with trifluoroacetic acid (55,56).

First, chemical hydrolysis studies proved clearly the selective delivery of BVdUMP **33** as sole product without formation of 3',5'-cyclicBVdUMP **34** (Figure 11). By contrast, *cyclo*Sal-BVdUMP triesters modified by esterification with a carboxylic acid (**31**) lead to the formation of the 3'-esterified BVdUMP **35**. However, the 3'-aminoacyl-esterified compounds **32** showed shorter half-lives as compared to the others but to our surprise the main product was 3-methyl-*cyclo*Sal-BVdUMP **30c** and not the corresponding 3'-aminoacyl-esterified BVdUMP **35**. 3-Methyl-*cyclo*Sal-BVdUMP **30c** then hydrolyzed as before to yield BVdUMP. Consequently, triesters **30** and **32** should act as sources for BVdUMP **33**.

First, 3-methyl-*cyclo*Sal-BVdUMP **30c** as well as the 3'-*O*-acetyl derivative **31a** were tested for inhibition of VZV replication. BVdU **29** prove to be highly active against VZV/TK$^+$ with EC_{50}s of 0.033 μM and 0.010 μM using the YS and the OKA strain, respectively. As expected, this activity was completely lost when VZV/ TK$^-$ (YS/R and O7/1 strain; > 200 μM) was used. Interestingly, both 3-methyl-*cyclo*Sal-BVdUMP triesters **30c**, **31a** showed comparable anti-VZV activity as compared to the parent. However, both are also loosing all the antiviral activity against the VZV/ TK$^-$ strains (EC_{50} > 50 μM). This led to the conclusion that only the VZV/TK associated viral thymidylate kinase activity is involved in the intracellular formation of BVdUDP. Thus, cellular enzymes are unable to phosphorylate BVdUMP.

Much more interesting were the results obtained in the assays against inhibition of EBV replication in P3HR-1 cells (56). BVdU itself was found to be entirely inactive (EC_{50} > 100 μM in the EBV DNA synthesis assay as well as the EB-VCA expression assay) (54). It has been described that EBV does not possess a HSV-1-like thymidine kinase. The lack of activity of BVdU clearly indicates that cellular kinases are unable to activate BVdU into its monophosphate BVdUMP. Strikingly, some of the *cyclo*Sal-BVdUMP triesters exhibited pronounced anti-EBV activity. The most active compound was the prototype

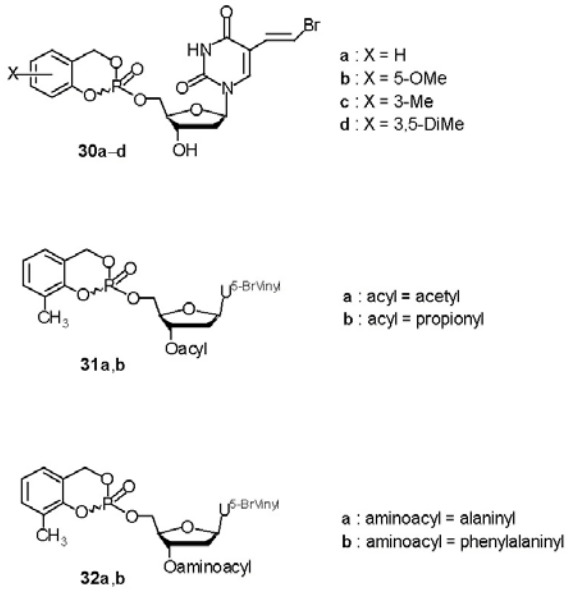

Figure 10. Structure of the *cyclo*Sal-BVDUMP triesters **30**-**32.**

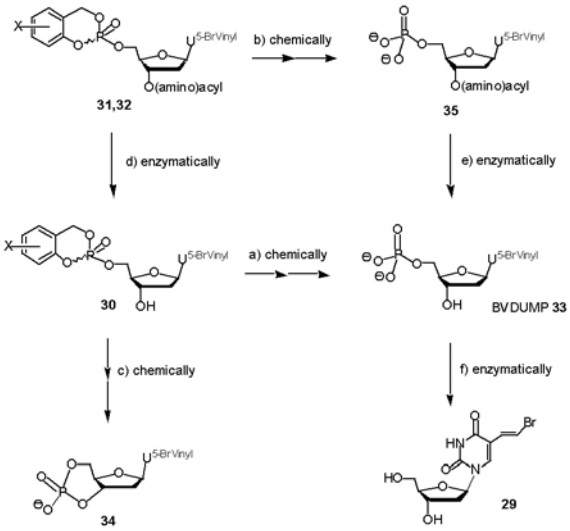

Figure 11. Degradation pathways of the prototype *cyclo*Sal-BVDUMPs **30** and the 3'-modified derivatives **31**,**32.**

5-methoxy-*cyclo*Sal-BVDUMP **30b** (Table 2). As compared to the inactive BVdU **29**, triester **30b** was >166-fold more active and about 4-fold more active than the reference compound acyclovir (ACV).

The 3'-alanine *cyclo*Sal-triesters showed antiviral activity that was 5- (D-**32a**) and 12-fold (L-**32a**) lower as compared to **30c** but both were still significantly more potent than BVdU. It was interesting to realize that in both cases the attachment of L-amino acids led to lower antiviral activity as compared to the unnatural D-amino acids. Surprisingly, both derivatives **31** esterified with carboxylic acids devoid of any antiviral activity (Table 2). Obviously, BVdUMP **33** released from the *cyclo*Sal-pronucleotide led to antiviral activity and thus the phosphorylation of BVdUMP into the ultimate metabolite BVdUTP seems to be achieved by cellular enzymes. Such insights of biosynthetic pathways are only possible since *cyclo*Sal-pronucleotides led to an intracellular delivery of the corresponding nucleotides. Thus, this is a good example that *cyclo*Sal-triesters may also be used as biochemical tools to study nucleoside metabolism.

In order to understand the different behavior of the mentioned 3-methyl-*cyclo*Sal-BVdUMP triesters studies using isolated carboxyesterase (64) and cell extracts from CEM/O cells as well as P3HR-1 cells (that were used for the antiviral evaluation) were performed (56).

In studies concerning a possible enzymatic cleavage caused by intracellular (carboxy)esterases, the triesters **31a**,**b** and D/L-**32a**, L-**32b** were treated with 50 units of pig liver esterase (PLE) in phosphate buffer, pH 7.3 as a model for enzymatic cleavage. The half-lives were found to be markedly lower as compared to previous studies in phosphate buffer at the same pH (Table 2). More importantly, the product was in all cases the 3'-*O*-deesterified 3-methyl-*cyclo*Sal-BVdUMP **30c** which clearly proves an efficient enzymatic cleavage that also explains the shorter half-lives found in the study (given half-lives represent only the disappearance of the *cyclo*Sal-triester). After enzymatic deesterification, 3-methyl-*cyclo*Sal-BVdUMP released BVdUMP as in the chemical hydrolysis studies. In these incubation studies 3'-*O*-esterified BVdUMP was not detected. It is worth mentioning that the 3'-*O*-α-amino acid containing triesters **32** were cleaved to the same extent as the 3'-*O*-carboxylic acid bearing derivatives **31**. Hence, the reason for the significant differences in antiviral activity remains unclear (Table 2).

A few striking differences have been observed in P3HR-1 cell extracts. Triester **30c** was hydrolyzed to BVdUMP with a half-life comparable to that observed in chemical hydrolysis studies ($t_{1/2}$ = 8.9 h; Table 3). Thus, the degradation is chemically driven and not enzymatically. Again, no cBVdUMP **34** was detected (65). In contrast to the chemical hydrolyses, BVdU **29** was observed also to a minor extend after 4 h (5%) and 8 h (22%) which is due to an enzymatic dephosphorylation of BVdUMP by phosphatases/nucleotidases (path f, Figure 11). In separate studies, BVdUMP was converted to an extent of 13% to BVdU within 4 h.

The hydrolyses of the 3'-*O*-acyl derivatives **31** exhibited a clear difference with respect to the attached acid. For the 3'-*O*-Ac-derivative **31a** enzymatic deesterification by carboxyesterases yielded the prototype **30c** as the major product (32%) but only 9% of BVdUMP was found. By contrast, the 3'-*O*-Prop derivative **31b** yielded the 3'-*O*-esterified BVdUMP derivatives **35c** as the major hydrolysis products due to the chemically driven cleavage of the *cyclo*Sal-mask (path b, Figure 11) (Table 3).

The situation was significantly different for the α-aminoacid modified 3-methyl-*cyclo*Sal-BVdUMP triesters **32a**,**b**. All four compounds were rapidly

Table 2. Hydrolysis data in buffer, with pig liver esterase (PLE) and antiviral activity of the *cyclo*Sal-BVdUMP triesters **30-32** as compared to the parent nucleoside BVdU **29** and acyclovir (ACV) **28**

Comp.	**Hydrolysis in aqueous $t_{1/2}$ (h) and product (%)**	**Incubation with PLE $t_{1/2}$ (h) and product (%)**	**Antiviral Activity EC_{50} [μM][1]**	**Cytotoxicity CC_{50} [μM][2]**
	PBS buffer ; pH 7.3	**PBS buffer ; pH 7.3**	**DNA synthesis**	
30a	1.5; BVdUMP (100)	n.a.[3]	6.0	92
30b	2.3; BVdUMP (100)	n.a.[3]	1.8	137
30c	6.7, BVdUMP (100)	n.a.[3]	4.1	122
30d	8.6; BVdUMP (100)	n.a.[3]	11	143
31a	5.8, **35a** (100)	3.6; **30c** (100)	>85	110
31b	6.3, **35b** (100)	2.8; **30c** (100)	>150	>300
D-32a	1.39, BVdUMP (100)	0.3; **30c** (100)	9.5	83
L-32a	1.40; BVdUMP (100)	0.6; **30c** (100)	22	140
D-32b	1.68, BVdUMP (100)	n.a.[3]; **30c** (100)	7.6	66
L-32b	1.18, BVdUMP (100)	1.2; **30c** (100)	>100	78
ACV **28**	--	--	7.2	422
BVdU **29**	--	--	>300	225

[1] EC_{50}: concentration required to reduce EBV DNA synthesis by 50%; [2] CC_{50}: concentration required to reduce the growth of exponentially growing P3HR-1 cells by 50%; [3] not available.

Table 3. Incubation studies of the *cyclo*Sal-BVdUMP triesters **30-32** in CEM and P3HR-1 cell extracts

Comp.	**Hydrolysis in cell extracts[1] $t_{1/2}$ (h) and [products (%)]**	
	CEM cell extract	**P3HR1 cell extract**
30a	2.3 [BVdUMP 82, **29** 18]	2.3 [BVdUMP 51, **29** 49]
30b	3.0 [BVdUMP 58, **29** 10]	3.2 [BVdUMP 42, **29** 24]
30c	4.8 [BVdUMP 50, **29** 8]	8.9 [BVdUMP 31, **29** 18]
30d	9.8 [BVdUMP 38, **29** 10]	10.9 [BVdUMP 22, **29** 15]
31a	4.1 [BVdUMP 22, **30c** 24, **35a** 17, **29** 4]	2.2 [BVdUMP 16, **30c** 29, **35a** 16, **29** 10]
31b	4.7 [BVdUMP 3, **30c** 3, **35b** 18, **29** <1]	4.5 [BVdUMP 3, **30c** 4, **35b** 13, **29** 2]
D-32a	0.8 [BVdUMP 52, **30c** 38, **29** 10]	0.5 [BVdUMP 33, **30c** 43, **29** 24]
L-32a	0.08 [BVdUMP 53, **30c** 37, **29** 10]	0.12 [BVdUMP 30, **30c** 44, **29** 26]
D-32b	n.d.[2] [BVdUMP 53, **30c** 35, **29** 12]	n.d.[2] [BVdUMP 35, **30c** 40, **29** 25]
L-32b	0.2 [BVdUMP 47, **30c** 39, **29** 14]	1.1 [BVDU BVdUMP MP 29, **30c** 46, **29** 25]

[1] Results shown in the table are at the end of an 8 h-incubation; missing percentage to 100% is remaining *cyclo*Sal-phosphate triester; [2] not determined.

deesterified to yield the prototype triester **30c** as major product (40-44%). This result also differs considerably to the PLE studies described above. The half-lives were dependent on the stereochemistry and the type of the α-amino acid (Table 3). As for 3-methyl-*cyclo*Sal-BVdUMP **30c**, 33 % BVdUMP has been formed starting from amino acid esters D/L-**53a**,**b** after incubation for 8 hours (Table 3).

Comparable data were obtained in the CEM/O cell extract incubation. The major difference was that the triesters yielded higher amounts of BVdUMP after 8 h as in the P3HR-1 extracts. Again, the triesters modified at C3' with a carboxylic acid exhibited considerable lower amounts of BVdUMP (1-22%, Table 3). Moreover, dephosphorylation in CEM/O cell extracts BVdUMP proceeded to a lower extend compared to P3HR-1 cell extracts (13% vs. 26%, respectively).

Taking these data together, the experiments with both extracts result in a much higher formation of BVdUMP for triesters **30** and aminoacid-modified triesters **32** as compared to the carboxylic acid-modified derivatives. Extrapolated to a cellular situation, this would lead to higher BVdUMP concentrations and a higher degree of forward phosphorylation to the ultimate metabolite BVdUTP.

The promising anti-EBV data of some *cyclo*Sal-BVdUMP triesters (e.g. **30b**) proved that by applying the *cyclo*Sal-approach, the inactive BVdU can be converted into a anti-EBV active agent that is even more active than the reference acyclovir.

5. SECOND GENERATION *CYCLO*SAL-PHOSPHATE TRIESTERS

5.1. "Lock-in"- *cyclo*Sal-d4TMP Triesters – A conceptional Extension of the trojan Horse Concept

The compounds described so far belong to the first generation compounds of the *cyclo*Sal-concept.

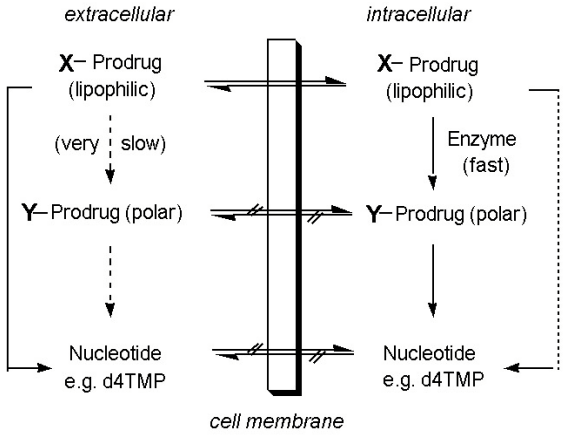

Figure 12. "Lock-in" principle with the *cyclo*Sal-approach.

Although these first generation *cyclo*Sal-triesters led to convincing antiviral results, the use of a chemical hydrolysis mechanism may also have some limitations. We have clearly shown for several *cyclo*Sal-triesters that an enzymatic contribution to the hydrolysis could not be found neither in cell extracts nor in human serum. Moreover, we have clearly proven that the lipophilic *cyclo*Sal-triesters are able to penetrate the cell membranes and deliver nucleotides *inside* the cell. However, it can not be excluded that the chemical hydrolysis also takes place outside the cells. In addition, although the compounds are lipophilic enough to migrate inside the cells we can not exclude that they also can diffuse in the opposite direction through the membrane. This would lead to the formation of an equilibrium. In order to avoid such a back-diffusion we planned to convert the triesters inside the cell into a much more polar compound by an enzymatic reaction, thus preventing the efflux ("lock-in" mechanism; Figure 12).

Therefore, we used an (carboxy)esterase reaction on a carboxylic ester attached to the *cyclo*Sal-aromatic ring via a linker. As a linker, a C2-alkyl chain was introduced. The ethylene spacer should separate the ester group efficiently from the aromatic ring in order to avoid an electronic effect on the hydrolysis of the phosphate triester moiety. Due to results obtained from the first generation *cyclo*Sal-phosphate triesters, two positions in the *cyclo*Sal-moiety have been selected for the introduction of the ester-spacer residue: the 3- and the 5-position. As an ester group, two possibilities were used: i) esterification of a *cyclo*Sal-acid (**36**,**37**) with an alcohol and ii) esterification of a *cyclo*Sal-alcohol (**38**,**39**) with a carboxylic acid leading to compound series **40**,**41** and **42**,**43**, respectively (Figure 13).

After enzymatic cleavage, the former triesters **40**,**41** should lead to the formation of a free carboxylic acid residue that should be deprotonated under physiological pH-conditions while the latter triesters **42**,**43** would lead to a free alcohol group. Different esters bearing linear or branched alkyl groups were introduced and the new concept first has been applied to the nucleoside analog d4T.

5.2. Chemistry

The synthesis of the *cyclo*Sal-pronucleotides has been done as for the prototype compounds **2** and **3**, respectively, using the above mentioned reactive phosphorus(III)-reagents (Figure 14). Again, the phosphate triesters **40**-**43** were obtained in reasonable yields as diastereomeric mixtures.

Originally, salicyl alcohols **11** have been prepared from the corresponding salicylic aldehydes or -acids by standard reduction protocols. However, the aldehydes/acids that were used here were not commercially available. Then, diols **11** have been synthesized from the phenols **44**. Selective *ortho*-hydroxymethylation was possible according to Nagata (36). This method is the mildest procedure used leading to the diols **11** without any side reaction in the ester moiety. Unfortunately, there is no generally applicable method for the preparation of the substituted phenols **44** bearing the ester-spacer residue.
The 3-(2-hydroxyphenyl)alkylpropionates were prepared from dihydrocoumarin **45** by transesterification with methanol or 2-propanol in the presence of H_2SO_4 in 96% and 73% yield (step A). The methyl ester of 4-hydroxyphenylpropionate **46** was formed by refluxing the acid, methanol and sulfuric acid in CH_2Cl_2 (step C, 93% yield). The *t*-butyl esters of 2- (**47**) and 3-(4-hydroxyphenyl)propionic acid **46** can be isolated in 80% yield using DMF-dineopentylacetale and *t*-butanol (step B) (66). 3-(4-Hydroxyphenyl)propionic acid **46** was also the starting material for the *i*-propyl ester that has been formed in 2-propanol and with HCl-gas in 62% yield (step D) (67). The acetyl esters 2-(hydroxyphenyl)ethanols **48**,**49** were prepared by transesterification from ethylacetate catalyzed by SiO_2•$NaHSO_4$ in 95% and 60% yield (step E) (68). Both, 2-(2-hydroxyphenyl)ethylpivalate and 2-(4-hydroxyphenyl)ethylpivalate were prepared from 2-(hydroxyphenyl)ethanols using the "twisted"-amide method in toluene in 80% and 51% yield (step F) (69), respectively. These phenol derivatives were then converted into the corresponding *cyclo*Sal-phosphate triesters using the known procedures (steps G-I). As reference compounds, 3- and 5-*cyclo*Sal-acids **36**,**37** were prepared from *t*-butyl esters **40c**,**41c** by treatment with trifluoroacetic acid in 85% yield (step K). 3-*Cyclo*Sal-alcohol **38** was synthesized from the corresponding levulinyl (Lev) ester **42b**. The Lev-group has been cleaved from the triester by hydrazine-hydrate treatment in 25% yield (step J) (70). The Lev-ester of the phenol was prepared as the acetyl esters (step E). Unfortunately, the acetyl esters could not be cleaved chemically when incorporated into the *cyclo*Sal-phosphate triester structure. All triesters obtained were then studied concerning their properties to liberate the polar group by the enzymatic reaction as well as their chemical hydrolysis to yield d4TMP.

5.3. Proof of the "lock-in" Concept

First, chemical hydrolysis studies were conducted. The results are summarized in (Table 4). As expected, all new triesters were cleaved to yield d4TMP at pH 7.3 in 25 mM phosphate buffer. Half-lives were found to be between 7.3 and 13.5 hours in the case of the 3-

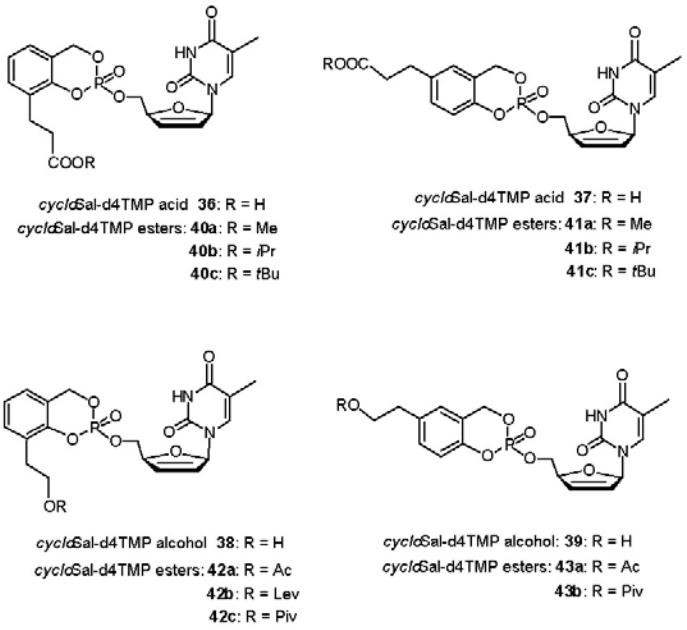

Figure 13. Target *cyclo*Sal-phosphate triesters for the"lock-in" approach.

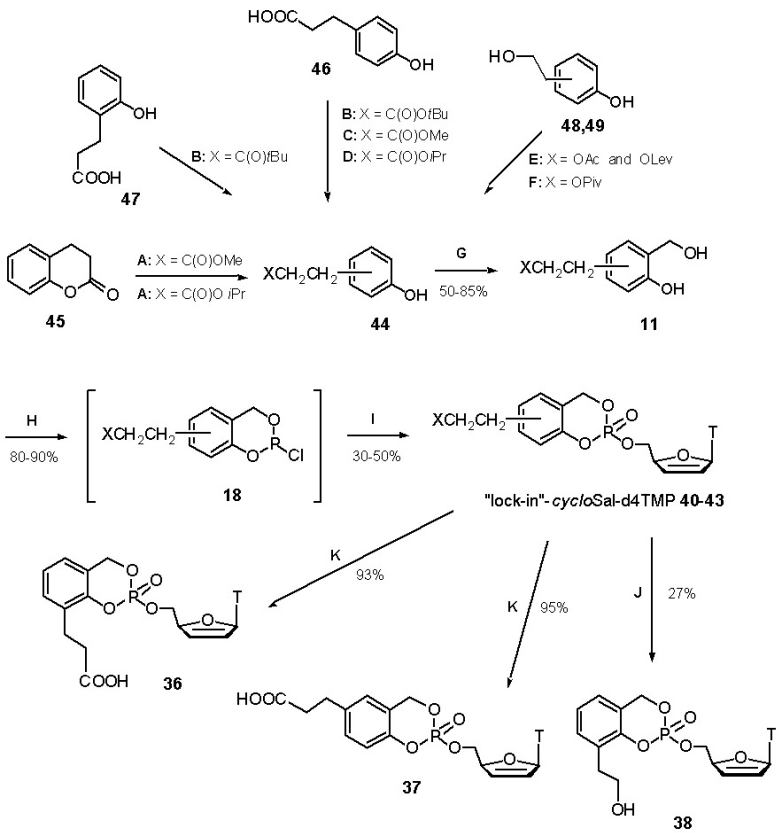

Figure 14. Synthesis of the target *cyclo*Sal-phosphate triesters for the"lock-in" approach. Reaction conditions. Method **A**: alcohol, H_2SO_4, reflux, 5-8 h; method **B**: $(CH_3)_2NCH(OCH_2tBu.)_2$, toluene, reflux, 5 h; method **C**: methanol, CH_2Cl_2, H_2SO_4, reflux, 5 h; method **D**: 2-propanol, HCl-gas, rt, 16 h; method **E**: ethylacylate, *n*-hexane, SiO_2•$NaHSO_4$, 67°C, 6-18 h; method **F**: 3-pivaloyl-1,3-thiazolidine-2-thion, toluene, 65°C, 48 h; method **G**: i. $PhB(OH)_2$, propionic acid cat., $(HCHO)_n$, toluene, reflux, 6-8 h; ii. H_2O_2, THF, 0°C, 30 min; method **H**: PCl_3, pyridine, diethylether, 0-21°C, 12 h; method **I**: i. d4T **1**, AcCN, DIPEA, 0-20°C; ii. *t*BuOOH, AcCN, rt, 30 min; method **J**: hydrazine•hydrate, pyridine/acetic acid 3:2, pyridine, 0°C, 10 min; method **K**: TFA 10 equiv., CH_2Cl_2, rt, 1h.

Table 4. Antiviral data of *cyclo*Sal-d4TMP triesters **36-38** and **40-43**

Compound	Substituent	Hydrolysis ($t_{1/2}$) at 37°C				EC_{50} (μM)[5]		CC_{50} (μM)[6]
	X	pH 7.3[1] [h][4]	PLE[2] [h][4]	CE[3] [h][4]	CEM/O HIV-1	CEM/O HIV-2	CEM/TK$^-$ HIV-2	
40a	3-MePr[7]	7.3	7.6	7.2	0.09	0.25	0.40	57
40b	3-iPrPr	12.5	9.3	10.1	0.14	0.80	1.50	54
40c	3-tBuPr	13.5	10.9	9.0	0.33	0.50	1.14	43
36	3-HPr	22.9	22.6	20.4	0.19	1.4	20	100
42a	3-AcEt[8]	13.6	8.3	1.9	0.16	0.33	0.15	40
42b	3-LevEt	12.5	n.d.[9]	1.9	0.13	0.15	0.33	58
42c	3-PivEt	13.1	8.2	6.6	0.16	0.70	0.40	55
38	3-HOEt	12.6	n.d.[9]	14.9	0.24	0.25	0.49	96
41a	5-MePr	7.0	4.0	5.7	0.33	1.05	1.20	58
41b	5-iPrPr	7.3	4.7	5.7	0.17	0.90	3.00	59
41c	5-tBuPr	7.1	5.5	5.3	0.18	2.40	4.00	42
37	5-HPr	12.5	9.1	11.4	0.14	0.80	50	76
43a	5-AcEt	6.3	5.8	2.6	0.15	0.80	0.55	44
43b	5-PivEt	5.4	4.8	5.5	0.23	0.90	0.60	22
d4T **1**	--	n.a.[10]	n.a.[10]	n.a.[10]	0.25	0.15	50	56

[1] 25 mM sodium phosphate buffer; [2] 25 mM phosphate buffer + 50 units pig liver esterase (PLE); [3] CEM cell extracts; [4] half-lives in hours; [5] antiviral activity: 50% effective concentration; [6] cytotoxicity: 50% cytostatic concentration; [7] X=3-MeOC(O)CH_2CH_2; [8] X=3-MeC(O)OCH_2CH_2; [9] not determined; [10] not available

modified triesters **37**,**39** and between 5.4 h and 7.3 h for their 5-modified counterparts **41**,**43**. A comparison with the 3- (2f; $t_{1/2}$ = 17.5 h) and 5-methyl-*cyclo*Sal-d4TMP triesters ($t_{1/2}$ = 8.1 h) proved that the ethylene-spacer separates the electron-withdrawing ester group and the *cyclo*Sal-aromatic ring sufficiently. Interestingly, both free acid-*cyclo*Sal-d4TMP triesters **36**,**37** showed up to two-fold higher half-lives as compared to the neutral ester-modified *cyclo*Sal-triesters. A possible explanation for this effect may be the presence of an overall negative charge on the molecule due to the formed carboxylate at pH 7.3, which slows down the nucleophilic reaction necessary for the initial cleavage step.

As expected, 3-*cyclo*Sal-triester **38** having the hydroxyl group in the side chain did not show such an increase in the half-lives ($t_{1/2}$ = 12.6 h; Table 4). This value is very close to the esters **42a**,**c** and the levulinyl-ester **42b** ($t_{1/2}$ = 12.5-13.6 h). In addition, the hydrolysis of 3-MePr-*cyclo*Sal-d4TMP **40a** followed by ^{31}P-NMR showed that also the phenyl phosphate diester was formed in a minor extend (~2%). However, this amount is considerably lower as compared to the situation found for 3-methyl-*cyclo*Sal-d4TMP **2f** (5.5%). In both experimental set-ups for chemical hydrolysis, no cleavage of the carboxylic ester group was observed.

Next, studies in 25 mM phosphate buffer (pH 7.3) containing 50 units of pig liver esterase (PLE) were carried out as a model for the enzymatic cleavage of the carboxylic esters (Table 2). It was observed that the half-lives of the 3-modified *cyclo*Sal-triesters **40** (methyl-, *i*-propyl and *t*-butyl-esters) were slightly lowered as compared to the situation in pure phosphate buffer. However, no trace of the expected *cyclo*Sal-triester acid **36** could be detected. Thus, no enzymatic cleavage took place. In contrast, the acetyl and the pivaloyl triester **42a**,**c** showed a two-fold decrease in the half-lives, and alcohol **38** was observed in the HPL-chromatograms. Interestingly, the situation was different for the 5-modified *cyclo*Sal-4TMPs. Here, none of the studied esters of the *cyclo*Sal-d4TMP acid **37** and the *cyclo*Sal-d4TMP alcohol **39** were hydrolyzed. In conclusion, the outcome of these cleavage studies using PLE was disappointing. It should be added that first experiments using the methyl ester of the 3-propionate-*cyclo*Sal-mask showed an extremely fast deesterification under the same conditions. Therefore, we also expected a fast ester hydrolysis in most of the cases of the *cyclo*Sal-triesters **40**. Nevertheless, d4TMP **9** was formed in all cases as a result of a chemical hydrolysis of the phosphate triester entity.

Further studies were done in CEM/O cell extracts. Triesters were incubated for 10 hours at 37°C (Table 4). The acetyl- (**42a**) and the levulinyl ester of the *cyclo*Sal-d4TMP alcohol **42b** showed the most impressive result. These triesters were degraded 6- to 7-fold faster as compared to the buffer incubations, and the intermediate alcohol **38** was clearly detected in the chromatogram. So, the cleavage capacity of the extracts was markedly higher as compared to the isolated enzyme PLE. However, the enzyme responsible for the ester hydrolysis in the extracts is not known. The pivaloyl ester **42c** was also cleaved (half-life dropped two-fold) while all the other esters were again not cleaved. In the 5-ester-modified *cyclo*Sal-d4TMP series **41**,**43**, only the acetyl ester **43a** showed a two-fold decrease in stability. In conclusion, the acetyl esters were proven to be good substrates for the human esterases while all alkyl esters were not cleaved by the extracts. However, the final products of the complete chemical hydrolysis of all triesters were d4TMP and the salicyl alcohols **11** again.

The reason for the insusceptibility of the alkyl esters is surprising because alkyl esters are often used in prodrug strategies and at least the phosphoramidate approach developed by the McGuigan group is based on an initial cleavage of such an ester group (71). However, esters of natural α-amino acids are used in their case.

It was interesting to note that in studies of the corresponding BVdUMP triesters also the benzyl esters

were cleaved in P3HR-1 cell extracts in addition to the acetyl ester (data not shown). So, obviously the cleavage is also dependent on the nature of the nucleoside analog. However, we can not exclude that the extracts from P3HR-1 cells contain different esterases or different concentrations of esterases as CEM cell extracts.

5.4. Antiviral Activity

Finally, the triesters were tested for their antiviral potency in CEM/O cells infected with HIV-1 and HIV-2 as well as in HIV-2-infected CEM/ TK$^-$ cells. The results are summarized in (Table 4).

All *cyclo*Sal-triesters proved to be active in the wild-type cell line against both virus types. Only the 5-*t*Bu-ester **41c** was found to be 5-fold less active against HIV-2 as compared to the reference compound d4T. More interesting are the results obtained in the thymidine kinase deficient CEM cells (CEM/ TK$^-$). First, both *cyclo*Sal-triesters **36** and **37** having the unesterified acid functionality in the side chain lost all their antiviral activity in the mutant cell line. The reason is the charge at the carboxylate which prevents an efficient membrane penetration. However, this result shows that in the case of a liberation of the carboxylate inside the cell by an enzymatic cleavage, the resulting polar product would stay trapped inside the cell. This is the first hint that the planned lock-in mechanism should work. Moreover, and in contrast to the parent d4T, all *cyclo*Sal-triesters bearing alkyl esters in the 5- or 3-position of the *cyclo*Sal-aromatic ring retained their antiviral activity in the CEM/ TK$^-$ cells (EC_{50} 1-2 μM) and thus proving at least the TK-bypass envisaged by these pronucleotides. Taking into account the results of the cell extract studies, no additional effect of the lock-in could be expected. However, *cyclo*Sal-triesters **42a** and **43a** that were enzymatically cleaved in the extracts showed lower EC_{50} values in the CEM/ TK$^-$ cells. Although these triesters do not liberate a charged carboxylate but a more polar neutral alcohol group (**38** and **39**, respectively), it appears that these compounds show first evidence for a successful trapping inside the cells.

6. CONCLUSION

Summarizing, the *cyclo*Sal approach convincingly demonstrated the intracellular delivery of active nucleotides by a non-enzymatically induced cascade reaction. Compared to other pronucleotide systems, one advantage of *cyclo*Sal prodrugs is their easy synthesis and their reasonable solubility in aqueous media. Moreover, the drug/masking group ratio for *cyclo*Sal prodrugs is 1:1 only whereas almost all enzymatically triggered nucleotide delivery systems show a ratio of 1:2 or 1:4. The 1:1 ratio may be favorable in terms of reducing potential toxicity. As reported earlier, the *cyclo*Sal-pronucleotide system is further an ideal tool to study biochemical pathways in nucleoside metabolism. Modification of the *cyclo*Sal-moiety and particularly of the benzyl position led to considerable differences in the hydrolysis pathway. This allowed further insights into the mechanism of degradation and at the same time gave clues to improve the d4TMP delivery. It has considerably improved the antiviral activity of certain nucleoside analogs using the first generation *cyclo*Sal-triesters. First attempts have been made to influence the equilibrium formed by a lipophilic phosphate triester through the membrane resulting in the development of second generation *cyclo*Sal-triesters having an ester bearing side chain in the *cyclo*Sal-aromatic ring. So far, only acetyl- and (partly) pivaloyl-esters were found to be substrates for cellular esterases. Nevertheless, the liberation of a negatively charged carboxylate would be advantageous in order to achieve an efficient intracellular trapping ("lock-in") of the triester. Work in order to achieve this goal is currently in progress in our laboratories.

7. ACKNOWLEDGEMENT

The authors would like to acknowledge the tremendous efforts of the following Ph.D. students for reported work: Martina Lorey, Andreas Lomp, Jürgen Renze, Christian Ducho, Manuel Ruppel and Dalibor Vukadinovic. Financial support by the Deutsche Forschungsgemeinschaft, Bonn, Germany, the Fonds der Chemischen Industrie, Frankfurt/Main, Germany, the Adolf-Messer-Stiftung, Frankfurt/Main, Germany and the René Descartes Prize 2001 of the European Commission is gratefully acknowledged.

8. REFERENCES

1 Mitsuya H, K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. Nusinoff-Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry & S. Broder: 3'-Azidothymidine (BWA509U); an antiviral agent that inhibits the infectivity and cytopathic effect of human t-lymphotropic virus type III/lymphadenopathy-associated virus in-vitro. *Proc Natl Acad Sci, USA* 82, 7096-7100 (1985)

2 Balzarini J: Metabolism and mechanism of antiretroviral action of purine and pyrimidine derivates. *Pharm World Sci* 16, 113-126 (1994)

3 Herdewijn P, J. Balzarini & E. De Clercq: 2',3'-Dideoxynucleoside analogues as anti-HIV agents. In: advances in antiviral drug design. Eds: De Clercq E, JAI Press, Greenwich, Conneticut, London, England, 1, 233-318 (1993)

4 Furman P A, J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrmann, D. P. Bolognesi & S. Broder: Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the triphosphate with human immunodeficiency virus reverse transcriptase. *Proc Natl Acad Sci, USA* 83, 8333-8337 (1986)

5 Hao Z, D. A. Cooney, N. R. Hartman, C. F. Perno, A. Fridland, A. L. DeVico, M. G. Sarngadharan, S. Broder & D. G. Johns: Factors determining the activity of 2',3'-dideoxynucleosides in suppressing human immunodeficiency virus in vitro. *Mol Pharmacol* 34, 431-435 (1988)

6 Riddler S A, R. E. Anderson & J. W. Mellors: Antiretroviral activity of stavudine (2',3'-didehydro-2',3'-dideoxythymidine d4T). *Antiviral Res* 27, 189-203 (1995)

7 Hitchcock M J M: 2',3'-Didehydro-2',3'-dideoxythymidine (d4T), an antiviral agent. *Antiviral Chem Chemother* 2, 125-132 (1991)

8 Sommadossi J P: Comparison of metabolism and in vitro antiviral activity of stavudine versus other2',3'-dideoxynucleoside analogues. *Infect Dis* 171 (Suppl.2), 88-92 (1995)
9 Balzarini J, P. Herdewijn & E. De Clercq: Differential patterns of intracellular metabolism of 2',3'-didehydro-2',3'-dideoxythymidine and 3'-azido-2',3'-dideoxythymidine. Two potent anti-human immunodeficiency virus compounds. *J Biol Chem* 264, 6127-6133 (1989)
10 Jones R J & N. Bischofberger: Nucleotide prodrugs. *Antiviral Research* 27, 1-17 (1995)
11 Freeman S & K. C. Ross: Prodrug design for phosphates and phosphonates. In: Progress in medicinal chemistry. Eds: Ellis G P, Luscombe D K, Elsevier Science B. V., Amsterdam 34, 111-147 (1997)
12 Meier C: Pronucleotides – recent advances in the design of efficient tools for the delivery of biologically active nucleoside monophosphates. *Synlett* 233-242 (1998)
13 Wagner C R, V. V. Iyer & E. J. McIntee: Pronucleotides: Toward the in vivo delivery of antiviral and anticancer nucleotides. *Med Res Rev* 20, 417-451 (2000)
14 Périgaud C, G. Gosselin & J. L. Imbach: Anti-HIV phosphotriester nucleotides. Basis for the rational design of biolabile phosphate protection. In: Biomedical chemistry/applying chemical principles to the understanding and treatment of disease. Eds: Torrence P F, John Wiley & Sons Inc., New York 55, 115-141 (2000)
15 Carl P L, P. K. Chakravarty & J. A. Katzenellenbogen: A novel connector linkage applicable in prodrug design. *J Med Chem* 24,479-480 (1981)
16 Meier C: *Cyclo*sal-pronucleotides – design of chemical trojan horses. *Minirev Med Chem* 2, 219-234 (2002)
17 Meier C: 2-Nucleos-5'-*O*-yl-4H-1,3,2-benzodioxaphosphinin-2-oxide – a new concept for lipophilic, potential prodrugs of biologically active nucleoside monophosphates. *Angew Chem* 108, 77-79 (1996); *Angew Chem Int Ed Engl* 35, 70-72 (1996)
18 Westheimer F H: Pseudorotation in the hydrolysis of phosphate esters. *Acc Chem Res* 1, 70-78 (1968)
19 Buchwald S L, D. H. Pliura & J. R. Knowles: Stereochemical evidence for pseudorotation in the reaction of a phosphoric monoester. *J Am Chem Soc* 106, 4916-4922 (1984)
20 Bunton C A, M. M. Mhala, K. G. Oldham & C. A. Vernon: The reaction of organic phosphates. Part III. The hydrolysis of dimethylphosphate. *J Chem Soc* 3293-3301 (1960)
21 Kirby A J & M. Younas: The reactivity of phosphate esters. Diester hydrolysis. *J Chem Soc (B)* 510-513 (1970)
22 Routledge A, I. Walker, S. Freeman, A. Hay & N. Mahmood: Synthesis, bioactivation and anti-HIV activity of 4-acyloxybenzyl bis(nucleosid-5'-yl) phosphates. *Nucleosides & Nucleotides* 14, 1545-1558 (1995)
23 Briggs A D, M. Camplo, S. Freeman, J. Lundström & B. G. Pring: acyloxymethyl and4-acyloxybenzyl diester prodrugs of phosphonoformate. *Tetrahedron* 47, 14937-14950 (1996)
24 Shafiee M, S. M. Deferme, A. L. Villard, D. Egron, G. Gosselin, J. L. Imbach, T. Lioux, A. Pompon, S. Varray, A. M. Aubertin, G. V. Mooter, R. Kinget, C. Périgaud & P. Augustijns: New bis (SATE) prodrug of AZT 5'-monophosphate: in *vitro* anti-HIV activity, stability and potential oral absorption. *J Pharm Sci* 90, 448-463 (2001)
25 Saboulard D, L. Naesens, D. Cahard, A. Salgado, R. Pathirana, S. Velázquez, C. McGuigan, E. De Clercq & J. Balzarini: Characterization of the activation pathway of phosphoramidate triester prodrugs of stavudine and zidovudine. *Mol Pharmacol* 56, 693-704 (1999)
26 Meier C, U. Muus, J. Renze, L. Naesens, E. De Clercq & J. Balzarini: Comparitive study of bis(benzyl)phosphate triesters of2',3'-Dideoxy-2',3'-didehydrothymidine (d4T) and *cyclo*sal-d4TMP – hydrolysis, mechanistic insights and anti-HIV activity. *Antiviral Chem & Chemother* 13, 101-114 (2002)
27 Farrow S. N, A. S. Jones, A. Kumar, R. T. Walker, J. Balzarini & E. De Clercq: Synthesis and biological properties of novel phosphotriesters: a new approach to the introduction of biologically active nucleotides into cells. *J Med Chem* 33, 1400-1406 (1990)
28 Shimizu S I, J. Balzarini, E. De Clercq & R. T. Walker: The synthesis and biological properties of some aryl bis(nucleotid-5'-yl) phosphates using nucleosides with proven anti-HIV activity. *Nucleosides & Nucleotides* 11, 583-594 (1992)
29 Meier C, M. Lorey, E. De Clercq & J. Balzarini: *Cyclo*sal-2',3'-dideoxy-2',3'-didehydrothymidine mono-phosphate (*cyclo*sal-d4TMP): synthesis and antiviral evaluation of a new d4TMP delivery system. *J Med Chem* 41, 1417-1427 (1998)
30 Meier C, M. Lorey, E. De Clercq & J. Balzarini: Cyclic saligenyl phosphotriesters of 2',3'-dideoxy-2',3'-didehydrothymidine (d4T) – a new approach. *Bioorg Med Chem Lett* 7, 99-104 (1997)
31 Glaser R: Aspirin. An ab initio quantum-mechanical study of conformational preferences and of neighboring group interactions. *J Org Chem* 66, 771-779 (2001)
32 Li R, X. Chen, B. Gong, P. M. Selzer, Z. Li, E. Davidson, G. Kurzban, R. E. Miller, E. O. Nuzum, J. H. McKerrow, R. J. Fletterick, S. A. Gillmor, C. S. Craik, I. D. Kuntz, F. E. Cohen & G. L. Kenyon: Structure-based design of parasitic protease inhibitors. *Bioorg Med Chem* 9, 1421-1427 (1996)
33 Georghiou P E, M. Ashram, H. J. Clase & J. N. Bridson: spirodienone and bis(spirodienone) derivatives of calix4)naphthalenes. *J Org Chem* 63, 1819-1826 (1989)
34 Casiraghi G, G. Casnati, G. Puglia, G. Satori & G. Terenghi: Selective reactions between phenols and formaldehyd. A novel route to salicylaldehyds. *J Chem Soc, Perkin Trans I* 1862-1865 (1980)
35 Gross H, H. Rieche & G. Matthey: Neue verfahren zur darstellung von phenolaldehyden. *Chem Berichte* 96, 308-313 (1963)
36 Nagata W, K. Okada & T. Aoki: *Ortho*-specific α-hydroxyalkylation of phenols with aldehydes. An efficient synthesis of saligenol derivatives. *Synthesis* 365-368 (1979)
37 No K H & C. D. Gutsche: Calixarenes. 8. Short, stepwise synthesis of *p*-phenylcalix4)arene, *p*-phenyl-*p*-*tert*-butylcalix4)arene, and derived products. *J Org Chem* 47, 2713-2719 (1982)
38 Podraza K F: Synthetic studies toward 4*H*-1,3-benzodioxin-2-ones. *J Heterocyclic Chem* 24, 801-804 (1987)

39 Wang Z, J. Yin, S. Campagna, J. A. Pesti & J. M. Fortunak: An alternative approach for the conversion of aldehydes to terminal alkynes. *J Org Chem* 64, 6918-6920 (1999)
40 Guy A, M. Lemaire & J.-P. Guetté: Selective α-chlorination of alkyl aryl ketones. *Synthesis* 1018-1020 (1982)
41 Anelli P L, S. Banfi, S. Legramandi, F. Momtanari, G. Pozzi & S. Quici: Tailed Mn^{III}-tetraarylporphyrins bearing an axial ligand and/or a carboxylic group. Self-consistent catalysts for H_2O_2 or NaOCl alkene epoxidation. *J Chem Soc Perkin I* 1345-1358 (1993)
42 Mugnier F & C. Meier: Phosphoramidite chemistry for the synthesis of *cyclo*sal-pronucleotides. *Nucleosides & Nucleotides* 18, 941-942 (1999)
43 Ducho C, J. Balzarini & C. Meier: Aryl-substituted and benzo-annelated *cyclo*sal-derivatives of 2',3'-dideoxy-2',3'-didehydrothymidine monophosphate (d4TMP) – correlation of structure, hydrolysis properties and anti-HIV activity. *Antiviral Chem & Chemother* 13, 129-141 (2002)
44 Meier C, J. T. Renze, J. Balzarini & E. De Clercq: D4TMP delivery from 7-substituted *cyclo*sal-d4TMPs. *Nucleosides, Nucleotides & Nucleic Acids* 22, 825-827 (2003)
45 Ducho C, S. Wendicke, U. Görbig, J. Balzarini & C. Meier: 3,5-t-Butyl-6-fluoro-*cyclo*sal-d4TMP – a pronucleotide with a highly optimized masking group. *Eur J Org Chem*, in press (2004)
46 Balzarini J, S. Aquaro, T. Knispel, C. Rampazzo, V. Bianchi, C. F. Perno, E. De Clercq & C. Meier: Cyclosaligenyl-2',3'-didehydro-2',3'-dideoxythymidine monophosphate: efficient intracellular delivery of d4TMP. *Mol Pharmacol* 58, 928-935 (2000)
47 Gröschel B, C. Meier, R. Zehner, J. Cinatl, H. W. Doerr & J. Cinatl Jr.: Effects of *cyclo*sal-d4TMP derivatives in H9 cells with induced AZT resistance phenotype. *Nucleosides & Nucleotides* 18, 933-936 (1999)
48 Meier C, T. Knispel, E. De Clercq & J. Balzarini: *Cyclo*sal-pro-nucleotides (*cyclo*sal-NMP) of 2',3'-dideoxyadenosine (ddA) and 2',3'-dideoxy-2',3'-didehydroadenosine (d4A): synthesis and antiviral evaluation of a highly efficient nucleotide delivery system. *J Med Chem* 42, 1604-1614 (1999)
49 Meier C, T. Knispel, V. E. Marquez, M. A. Siddiqui, E. De Clercq & J. Balzarini: *Cyclo*sal-pro-nucleotides of 2'-fluoro-ara- and 2'-fluoro-ribo-2',3'-dideoxyadenosine (F-ara- and F-ribo-ddA) as a strategy to bypass a metabolic blockade. *J Med Chem 4*2, 1615-1624 (1999)
50 Meier C, E. De Clercq & J. Balzarini: *Cyclo*sal-3'-azido-2',3'-dideoxythymidine monophosphate (*cyclo*sal-AZTMP) – an unexpected failure of nucleotide delivery from a proven pro-nucleotide system. *Eur J Org* 837-846 (1998)
51 Lorey M, C. Meier, E. De Clercq & J. Balzarini: New synthesis and antitumor activity of *cyclo*sal-derivatives of 5-fluoro-2'-deoxyuridinemonophosphate. *Nucleosides & Nucleotides* 16, 789-792 (1997); b) Lorey M, C. Meier, C. E. De Clercq & J. Balzarini: *Cyclo*saligenyl-5-fluoro-2'-deoxyuridinemonophosphate (*cyclo*sal-FdUMP) – a new prodrug approach for FdUMP. *Nucleosides & Nucleotides* 16, 1307-1310 (1997)
52 Balzarini J, F. Haller-Meier, E. De Clercq & C. Meier: Antiviral activity of cyclosaligenyl prodrugs of acyclovir, carbovir and abacavir. *Antiviral Chem Chemother* 12, 301-306 (2002)
53 Meier C, L. Habel, F. Haller-Meier, A. Lomp, M. Herderich, R. Klöcking, A. Meerbach & P. Wutzler: Chemistry and anti-herpes simplex virus type1 evaluation of *cyclo*sal-nucleotides of acyclic nucleoside analogues. *Antiviral Chem Chemother* 9, 389-402 (1998)
54 Meerbach A, R. Klöcking, C. Meier, A. Lomp, B. Helbig & P. Wutzler: Inhibitory effect of *cyclo*saligenyl-nucleoside monophosphates (*cyclo*sal-NMP) of acyclic nucleoside analogues on HSV-1 and EBV. *Antiviral Res* 45, 69-77 (2000)
55 Meier C, A. Lomp, A. Meerbach & P. Wutzler: *cyclo*saligenyl-5-[(E)-2-bromovinyl]-2'-deoxyuridine monophosphate (*cyclo*sal-BVDUMP) pronucleotides active against epstein–barr virus. *ChemBioChem* 4, 283-285 (2001)
56 Meier C, A. Lomp, A. Meerbach & P. Wutzler: *Cyclo*sal-BVdUMP pronucleotides: how to convert an antiviral-inactive nucleoside analogue into a bioactive compound against EBV. *J Med Chem* 45, 5157-5172 (2002)
57 Mazzon C, C. Rampazzo, M. C. Scain, L. Gallinar, A. Karlsson, C. Meier, J. Balzarini, P. Reichard & V. Bianchi: Cytosolic and mitochondrial deoxyribonucleotidases: activity wit substrate analogs, inhibitors and implications for therapy. *Biochem Pharmacol* 66, 471-479 (2003)
58 De Clercq E, J. Descamps, P. De Somer, P. J. Barr, A. S. Jones & R. T. Walker: (*E*)-5-(2-bromovinyl)-2'-deoxyuridine: a potent and selective anti-herpes agent. *Proc Natl Acad Sci, USA* 76, 2947-2951 (1979)
59. Wutzler P: Antiviral therapy of herpes simplex and varicella-zoster virus infections. *Intervirology 4*0, 343-356 (1997)
60 De Clercq E: In search of selective antiviral chemotherapy. *Clinical Microbiol Rev* 10, 674-693 (1997)
61 Anagnostopoulos I & M. Hummel: Epstein-Barr virus in tumours. *Histo-Pathol* 29, 297-315 (1996)
62 Farrow S N, A. S. Jones, A. Kumar, R. T. Walker, J. Balzarini & E. De Clercq: Synthesis and biological properties of novel phosphortriester: a new approach to the introduction of biologically active nucleotides into cells. *J Med Chem* 33, 1400-1406 (1990)
63 Herdewijn P, R. Charubala, E. De Clercq & W. Pfleiderer: Synthesis of 2'-5' connected oligonucleotides. Prodrugs for antiviral and antitumoral nucleosides. *Helv Chim Acta* 72, 1739-1748 (1989)
64 Meier C, A. Lomp, A. Meerbach & P. Wutzler: Synthesis, hydrolysis and anti-EBV activity of a series of 3'-modified *cyclo*sal-BVdUMP pronucleotides. *Nucleosides, Nucleotides & Nucleic Acids* 20, 307-314 (2001)
65 Lomp A, C. Meier, M. Herderich & P. Wutzler: Evidence for cyclophosphate formation during hydrolysis of 3-methyl-*cyclo*sal-PCVMP. *Nucleosides & Nucleotides* 18, 943-944 (1999)
66 Brechbühler H, H. Büchi, E. Hatz, J. Schreiber & A. Eschenmoser: Die reaktion von carbonsäuren mit acetalen des N,N-dimethylformamids: eine veresterungsmethode. *Helv Chim Acta* 48, 1746-1771 (1965)
67 Garber S B, J. S. Kingsbury, B. L. Gray & A. H. Hoveyda: Efficient and recyclable monomeric and dentritic

ru-based metathesis catalysts. *J Am Chem Soc 1*22, 8168-8179 (2000)
68 Breton G W: Selective monoacetylation of unsymmetric diols catalyzed by silica gel-supported sodium hydrogen sulfate. *J Org Chem* 62, 8952-8954 (1997)
69 Yamada S, T. Sugaki & K. Matsuzaki: Twisted amides as selective acylating agents for hydroxyl groups under neutral conditions: models for activated peptides during enzymatic acyl transfer reaction. *J Org Chem* 61, 5932-5938 (1996)
70 Van Boom J H & P. M. J. Burgers: Use of levulinic acid in the protection of oligonucleotides via the modified phosphotriester method: synthesis of decaribonucleotide U-A-U-A-U-A-U-A. *Tetrahedron Lett* 52, 4875-4878 (1976)
71 Saboulard D, L. Naesens, D. Cahard, A. Salgado, R. Pathirana, S. Velazquez, C. McGuigan, E. De Clercq & J. Balzarini: Characterisation of the activation pathway of phosphoramidate triester prodrugs of stavudine and zidovudine. *Mol Pharmacol* 56, 693-704 (1999)

Key Words: Virus, Treatment, Therapy, Drug, Nucleoside Analogs, Prodrug, Pronucleotide, *Cyclo*Sal-Pronucleotides, Antiviral Chemotherapy, Review

Send correspondence to: Chris Meier, Institute of Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6; D-20146 Hamburg, Germany; Tel: +49-40-42838-4324, Fax: +49-40-42838-2495, E-mail: chris.meier@chemie.uni-hamburg.de