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

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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 cycloSal-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. Besides others, for the nucleoside analog d4T the application of the cycloSal-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 cycloSal-d4TMP triesters have been discussed in this review. In the second part, first results of a conceptional extension of the original cycloSal-approach will be summarized. Once the pronucleotides have passed the membrane, the aim is to trap the cycloSal-phosphate triesters inside the cells. Therefore, enzyme-cleavable groups have been attached via a linker to the cycloSal-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
cycloSal-pronucleotides

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 $pK_a$ of a phosphate monoester is $\sim1.6$ and $pK_a$ of $2$ is $\sim6.6$, 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 \textit{bis(pivaloyloxymethyl) (POM)}, \textit{bis(isoproxyloxycarbonyloxymethyl) (POC)}, \textit{bis(S-glycosylthioethyl) (SGTE)}, \textit{bis(4'-acylbenzyl (AB)}, the arylphosphoramidate (APA), the phosphoramidate monoester and a modified \textit{bis(4'-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 \textit{cycloSal}-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 \textit{cycloSal}-triesters \textit{2} and \textit{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 \textit{cycloSal}-pronucleotides.

![Figure 1](image1.png)

**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](image2.png)

**Figure 2.** “Black-box”-metabolism of nucleoside analogs into the antivirally active nucleoside triphosphate.

![Figure 3](image3.png)

**Figure 3.** Prototype \textit{cycloSal}-d4TMP triesters 2, \textit{cycloSal}-d4TMP triesters 3 and \textit{cycloSal}-d4TMP triesters 4 bearing functionalized X-groups.

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).
3. THE CYCLOSALIGENYL-PRONUCLEOTIDE APPROACH: THE FIRST GENERATION

3.1. Cyclosaligenyl-nucleotides (cycloSal-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 cycloSal-strategy requires only one activation step to deliver the nucleotide and due to the bifunctional character of the cycloSal 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 cycloSal 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 cycloSal-triester structure should be the most labile one after nucleophilic attack of hydroxide to phosphorus (SN1-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-hydroxybenzylphosphate 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 b1 and b2). This rupture proceeds presumably after intramolecular proton transfer (intermediate 13) via zwitterion 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 SN1-type Cbenzyl-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.
3.2. Chemistry

For the synthesis of the prototype cycloSal-d4TMP triesters the salicyl alcohols 11 had to be prepared first from the corresponding salicylic aldehydes, -acids or -esters (32) by standard reduction protocols (NaBH₄ or LiAlH₄ (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 cycloSal-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 methylithium (38) or dichloromethylithium (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 fluorne in 2-chloro-6-fluoro-benzaldehyde by hydroxide (41) and subsequent aldol-type addition as mentioned above.

The synthesis of the cycloSal-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 phosphate triesters which were oxidized in a one-pot-reaction using tert-butyldihydroperoxide (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 disopropylamine to yield the phosphoramidites 19. The coupling with the nucleoside analog was carried out in acetonitrile in the presence of pyridinium chloride or imidazolium trflate as coupling activator. In some cases the latter procedure resulted in yields of > 90% using imidazolium trflate as activator (42).

3.3. Proof-of-Principle
3.3.1. D4TMP-Release from cycloSal-d4TMP Pronucleotides

Extensive studies have been performed in order to investigate the designed delivery mechanism of d4TMP from the cycloSal-triesters (30,43). Chemical hydrolysis
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 in general, acceptor substituents like the 5- or 6-chloro group on the substitution pattern of the aromatic ring (11,16,30). In contrast, the rotation of the phenyl-aryl bond in 3-phenyl-cycloSal-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-tBu) and 2i (3,5-tBu) 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]-cycloSal-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 31P-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 31P-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-cycloSal-d4TMP (30). In that case d4TMP has been detected as the only hydrolysis product pointing to an entirely selective delivery reaction.
**cycloSal-pronucleotides**

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Modification</th>
<th>t1/2; 37°C</th>
<th>Product ratio</th>
<th>EC50 (µM)</th>
<th>CC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X and/or R</td>
<td>pH 7.3</td>
<td>d4TMP : 17</td>
<td>CEM/O HIV-1</td>
<td>CEM/O HIV-2</td>
</tr>
<tr>
<td>2a</td>
<td>5-Cl</td>
<td>1.1</td>
<td>100 : 0</td>
<td>0.42</td>
<td>1.40</td>
</tr>
<tr>
<td>2b</td>
<td>6-Cl</td>
<td>0.9</td>
<td>100 : 0</td>
<td>0.087</td>
<td>0.15</td>
</tr>
<tr>
<td>2c</td>
<td>5-H</td>
<td>4.4</td>
<td>99 : 1</td>
<td>0.20</td>
<td>0.22</td>
</tr>
<tr>
<td>2d</td>
<td>5-C6H5</td>
<td>3.1</td>
<td>100 : 0</td>
<td>0.40</td>
<td>0.47</td>
</tr>
<tr>
<td>2e</td>
<td>3-C6H5</td>
<td>5.1</td>
<td>97 : 3</td>
<td>0.13</td>
<td>0.27</td>
</tr>
<tr>
<td>2f</td>
<td>3-CH3</td>
<td>17.5</td>
<td>94 : 6</td>
<td>0.057</td>
<td>0.07</td>
</tr>
<tr>
<td>2g</td>
<td>3,5-CH3</td>
<td>29</td>
<td>92 : 8</td>
<td>0.09</td>
<td>0.17</td>
</tr>
<tr>
<td>2h</td>
<td>3-tBu</td>
<td>96</td>
<td>92 : 8</td>
<td>0.18</td>
<td>0.65</td>
</tr>
<tr>
<td>2i</td>
<td>3,5-tBu; 6-F</td>
<td>73</td>
<td>66 : 34</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>2j</td>
<td>3,5-tBu; 6-F</td>
<td>6.2</td>
<td>100 : 0</td>
<td>1.23</td>
<td>0.73</td>
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<td>2k</td>
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<td>2.8</td>
<td>91 : 9</td>
<td>0.14</td>
<td>0.12</td>
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<tr>
<td>2l</td>
<td>benzo[b]</td>
<td>1.4</td>
<td>97 : 3</td>
<td>0.41</td>
<td>0.50</td>
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<tr>
<td>2m</td>
<td>benzo[c]</td>
<td>2.8</td>
<td>88 : 12</td>
<td>0.09</td>
<td>0.17</td>
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<tr>
<td>3a</td>
<td>7-CH3</td>
<td>0.25</td>
<td>17 : 83</td>
<td>0.22</td>
<td>0.34</td>
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<tr>
<td>3b</td>
<td>7-C6H5</td>
<td>0.9</td>
<td>85 : 156</td>
<td>0.19</td>
<td>0.60</td>
</tr>
<tr>
<td>3c</td>
<td>7-CHCl2</td>
<td>1.4</td>
<td>97 : 3</td>
<td>0.16</td>
<td>0.55</td>
</tr>
<tr>
<td>3d</td>
<td>7-CH2Cl</td>
<td>2.8</td>
<td>100 : 0</td>
<td>0.19</td>
<td>0.35</td>
</tr>
<tr>
<td>3e</td>
<td>6-Cl,7-CH3</td>
<td>2.2</td>
<td>100 : 0</td>
<td>0.19</td>
<td>0.25</td>
</tr>
<tr>
<td>d4T 1</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.18</td>
<td>0.55</td>
</tr>
</tbody>
</table>

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

The prototype cycloSal-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 SN1-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-tert-butyl triester 2h (Table 1). The formation of the phenyl phosphate diesters could not be followed in the HPLC studies due to the lower detection sensitivity. The benzo-annulated benzo[a]- and benzo[c]-cycloSal-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]-cycloSal-d4TMP 2i (3% diester 17) results from a better mesomorphic stabilization of the cation intermediate of the first two compounds. This additional stabilization increases the rate of the SN1-type bond rupture. By contrast, the worst case was found for the introduction of two tert-butyl groups in the 3 and 5 position (compound 2i) led to the formation of 34% of the phenyl phosphate diester. Here, the concurrent SN1-type reaction plays a considerable role in the degradation of triester 2i. In contrast, 5-chloro- (2a), 6-chloro- (2b) and 5-phenyl-cycloSal-d4TMPs 2d turned out to deliver d4TMP exclusively. No side products were observed in the NMR experiment.

In conclusion, the SN1-type hydrolysis of the prototype cycloSal-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 SN1-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 18O-labeled water. This experiment clearly proves 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-cycloSal-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 SN1-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 SN1 reaction is considerably lower for 7-methyl-cycloSal-methylMP compared to the prototype triester analog (unpublished results). On the other
hand, this predominant S_N1-type reaction should be avoided by reduction of the methyl (+)-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 cycloSal-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). 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 highly selectively (0% for 2j instead of 34% for 2i)!

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-

For 6-chloro-7-methyl-cycloSal-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-cycloSal-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 cycloSal-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 highly selectively (0% for 2j instead of 34% for 2i)!

Figure 8. Hydrolysis of the 7-alkyl-modified cycloSal-d4TMP triesters 3.
reaction mechanism and showed convincingly that the mechanism may be controlled efficiently by structural modification of the cycloSal-moiety (Figure 5).

### 3.3.2. Antiviral Activity

The antiviral potency of the cycloSal-nucleotides against HIV-1 and -2 in CEM cells was assessed (16,30). It became apparent that the unsubstituted prototype 2e, 3-phenyl- (2e), 3-methyl- (2f) as well as 3,5-dimethyl-cycloSal-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 (2e), 3-phenyl- (2e), 3-methyl- (2f), 3,5-dimethyl- (2g), 3-<i>t</i>-butyl- (2h), and 3,5-t-buty1-6-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-chlorotriester 2a (<i>t</i>/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 3-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-cycloSal-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-cycloSal-triester 2i lack antiviral activity in the CEM/TK cells. Even the most stable derivative 7-chloromethyl-cycloSal-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 cycloSal-triesters themselves have no inhibitory effect on DNA-synthesis, which is consistent with a mechanism of action for the cycloSal-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 cycloSal-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-cycloSal-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 cycloSal-d4TMPs in wild-type CEM cells compared to d4T. These results are consistent with a mechanism of the cycloSal-d4TMPs that successfully bypasses thymidine kinase and releases d4TMP inside the cells.

Furthermore, the cycloSal-d4TMP triesters demonstrated significant antiviral activity in AZT-resistant H9/AZT250 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 cycloSal-d4TMP 2e proved to be equipotent in parental and in H9/AZT250 cells (EC50 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 cycloSal-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), 6-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-AZTPMP 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 CYCLOSal-APPROACH TO THE ANTI-HERPES DRUG BVDU

As mentioned above, the cycloSal-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
cycloSal-pronucleotides

![CycloSal-pronucleotides](image)

**Figure 9.** Nucleoside analogs use with the cycloSal-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 cycloSal-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 cycloSal-BVdUMP triesters were synthesized: cycloSal-BVdUMP 30 having different substituents in the aromatic residue and a series of 3'-O-esterified 3-methyl-cycloSal-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, cycloSal-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-cycloSal-BVdUMP 30c and not the corresponding 3'-aminoacyl-esterified BVdUMP 35. 3-Methyl-cycloSal-BVdUMP 30c then hydrolyzed as before to yield BVdUMP. Consequently, triesters 30 and 32 should act as sources for BVdUMP 33.

First, 3-methyl-cycloSal-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 EC50s 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-cycloSal-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 (EC50 > 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 (EC50 > 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 cycloSal-BVdUMP triesters exhibited pronounced anti-EBV activity. The most active compound was the prototype...
cycloSal-pronucleotides

![Figure 10. Structure of the cycloSal-BVDUMP triesters 30-32.](image)

![Figure 11. Degradation pathways of the prototype cycloSal-BVDUMP 30 and the 3’-modified derivatives 31, 32.](image)

5-methoxy-cycloSal-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 cycloSal-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 cycloSal-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 cycloSal-pronucleotides led to an intracellular delivery of the corresponding nucleotides. Thus, this is a good example that cycloSal-triesters may also be used as biochemical tools to study nucleoside metabolism.

In order to understand the different behavior of the mentioned 3-methyl-cycloSal-BVdUMP triesters studies using isolated carboxysterases, 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-cycloSal-BVdUMP 30e 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 cycloSal-triester). After enzymatic deesterification, 3-methyl-cycloSal-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 (t1/2 = 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 carboxysterases 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 cycloSal-mask (path b, Figure 11) (Table 3).

The situation was significantly different for the α-aminoacid modified 3-methyl-cycloSal-BVdUMP triesters 32a, b. All four compounds were rapidly
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 amino acid (Table 3). As for 3-methyl-cycloSal-BVdUMP 30c, 33% BVdUMP has been formed starting from amino acid esters D/L-32a,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 extent 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 amino acid-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 cycloSal-BVdUMP triesters (e.g. 30b) proved that by applying the cycloSal-approach, the inactive BVdU can be converted into an anti-EBV active agent that is even more active than the reference acyclovir.

5. SECOND GENERATION CYCLOSal-PHOSPHATE TRIESTERS

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

The compounds described so far belong to the first generation compounds of the cycloSal-concept.
5.2. Chemistry

The synthesis of the cycloSal-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 H₂SO₄ 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 CHCl₃ (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-dimethylacetate 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 SiO₂•NaHSO₄ 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 cycloSal-phosphate triesters using the known procedures (steps G-I). As reference compounds, 3- and 5-cycloSal-acids 36,37 were prepared from i-butyl esters 40c,41c by treatment with trifluoroacetic acid in 85% yield (step K). 3-CycloSal-alcohol 38 was synthesized from the corresponding levulinyl (Lev) ester 42b. The Lev-group has been cleaved from the triester by hydrazine-hydrazide 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 cycloSal-phosphate triester structure. All triesters obtained were then studied concerning their properties to migrate inside the cells we cannot 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 estriesters 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 cycloSal-aromatic ring via a linker. As a linker, a C2-alkyl chain was introduced. The ethylene spacer should separate the ester group 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 cycloSal-phosphate triesters, two positions in the cycloSal-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 cycloSal-acid (36,37) with an alcohol and ii) esterification of a cycloSal-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.
**cycloSal-pronucleotides**

**Figure 13.** Target cycloSal-phosphate triesters for the “lock-in” approach.

**Figure 14.** Synthesis of the target cycloSal-phosphate triesters for the “lock-in” approach. Reaction conditions. Method A: alcohol, H<sub>2</sub>SO<sub>4</sub>, reflux, 5-8 h; method B: (CH<sub>3</sub>)<sub>2</sub>NCH(OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>)<sub>2</sub>, toluene, reflux, 5 h; method C: methanol, CH<sub>2</sub>Cl<sub>2</sub>, H<sub>2</sub>SO<sub>4</sub>, reflux, 5 h; method D: 2-propanol, HCl-gas, rt, 16 h; method E: ethylacrylate, n-hexane, SiO<sub>2</sub>|NaHSO<sub>4</sub>, 67°C, 6-18 h; method F: 3-pivaloyl-1,3-thiazolidine-2-thion, toluene, 65°C, 48 h; method G: i. PhB(OH)<sub>2</sub>, propionic acid cat., (HCHO)<sub>n</sub>, toluene, reflux, 6-8 h; ii. H<sub>2</sub>O<sub>2</sub>, THF, 0°C, 30 min; method H: PCl<sub>3</sub>, pyridine, diethylether, 0-21°C, 12 h; method I: i. d4T 1, AcCN, DIPEA, 0-20°C; ii. tBuOOH, AcCN, rt, 30 min; method J: hydrazine•hydrate, pyridine/acetic acid 3:2, pyridine, 0°C, 10 min; method K: TFA 10 equiv., CH<sub>2</sub>Cl<sub>2</sub>, rt, 1 h.
Table 4. Antiviral data of cycloSal-d4TMP triesters 36-38 and 40-43

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<th>Compound</th>
<th>X</th>
<th>pH 7.3</th>
<th>PLE</th>
<th>CE</th>
<th>CEM/O HIV-1</th>
<th>CEM/O HIV-2</th>
<th>CEM/TK HIV-2</th>
<th>EC50 (µM)</th>
<th>CC50 (µM)</th>
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<td>0.15</td>
<td>50</td>
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</table>

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)CH2CH2; 8 X=3-MeOC(O)CH2CH2; 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; t1/2 = 17.5 h) and 5-methyl-cycloSal-d4TMP triesters (t1/2 = 8.1 h) proved that the ethylene-spacer separates the electron-withdrawing ester group and the cycloSal-aromatic ring sufficiently. Interestingly, both free acid-cycloSal-d4TMP triesters 36,37 showed up to two-fold higher half-lives as compared to the neutral ester-modified cycloSal-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-cycloSal-triester 38 having the hydroxyl group in the side chain did not show such an increase in the half-lives (t1/2 = 12.6 h; Table 4). This value is very close to the esters 42a,c and the levulinyl-ester 42b (t1/2 = 12.5-13.6 h). In addition, the hydrolysis of 3-MePr-cycloSal-d4TMP 40a followed by 31P-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-cycloSal-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 cycloSal-triesters 40 (methyl-, i-propyl and i-buty1-esters) were slightly lowered as compared to the situation in pure phosphate buffer. However, no trace of the expected cycloSal-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 cycloSal-4TPMs. Here, none of the studied esters of the cycloSal-d4TMP acid 37 and the cycloSal-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-cycloSal-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 cycloSal-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 cycloSal-d4TMP alcohol 42b showed the most impressive result. These triesters were deacylated 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 cycloSal-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 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 cycloSal-triesters proved to be active in the wild-type cell line against both virus types. Only the 5-<i>dBu</i>-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 cycloSal-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 cycloSal-triesters bearing alkyl esters in the 5- or 3-position of the cycloSal-aromatic ring retained their antiviral activity in the CEM/TK cells (EC<sub>50</sub> 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, cycloSal-triesters 42a and 43a that were enzymatically cleaved in the extracts showed lower EC<sub>50</sub> 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 cycloSal approach convincingly demonstrated the intracellular delivery of active nucleotides by a non-enzymatically induced cascade reaction. Compared to other pronucleotide systems, one advantage of cycloSal prodrugs is their easy synthesis and their reasonable solubility in aqueous media. Moreover, the drug/masking group ratio for cycloSal 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 cycloSal-triester system is further an ideal tool to study biochemical pathways in nucleoside metabolism. Modification of the cycloSal-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 cycloSal-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 cycloSal-triesters having an ester bearing side chain in the cycloSal-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.

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**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