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Mini-review

Application of the *cyclo*Sal-prodrug approach for improving the biological potential of phosphorylated biomolecules

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Dedicated to Prof. Erik De Clercq on the occasion of reaching the status of Emeritus-Professor at the Katholieke Universiteit Leuven in September 2006.

Abstract

Pronucleotides represent a promising tool to improve the biological activity of nucleoside analogs in antiviral and cancer chemotherapy. The *cyclo*Sal-approach is one of several conceptually different pronucleotide systems. This approach can be applied to various nucleoside analogs. A salicyl alcohol as a cyclic bifunctional masking unit is used, and shown to afford a chemically driven release of the particular nucleotide from the lipophilic phosphate triester precursor molecule. A conceptual extension of the *cyclo*Sal-approach results in the design of "lock-in"-*cyclo*Sal-derivatives. The *cyclo*Sal-approach is not restricted to the delivery of bioactive nucleotides but also useful for the intracellular delivery of hexose-1-phosphates.

Keywords: Nucleoside analogs; Antiviral agent; *cyclo*Sal-pronucleotides; Carbohydrate drugs

Contents

1. Introduction

The development of nucleoside analogs as potential bioactive agents is an attractive field of research (De Clercq, 2002). Today, synthetic nucleoside mimetics represent a valuable source of compounds that contribute significantly to the arsenal of chemotherapeutic agents against viruses and cancer. The mode of action of most of the nucleoside analogs occurs through the inhibition of DNA polymerases. To act as DNA chain termination agents/polymerase inhibitors, intracellular conversion of the nucleoside analogs into their 5′-triphosphates is a prerequisite. However, efficient anabolism to the 5′-triphosphates is often the

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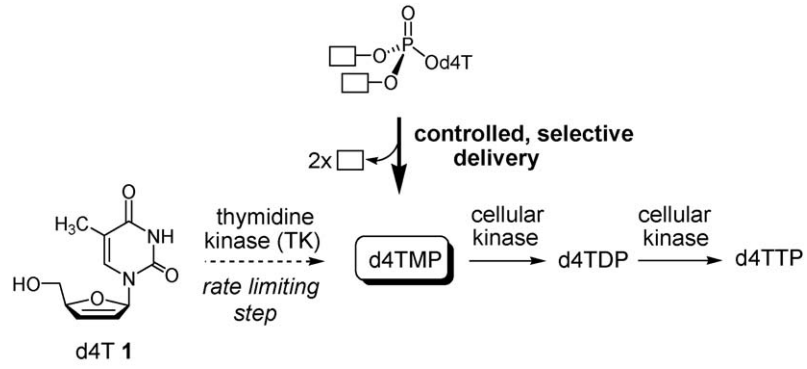

Fig. 1. Phosphorylation pattern of d4T **1** and the idea of the pronucleotide approach.

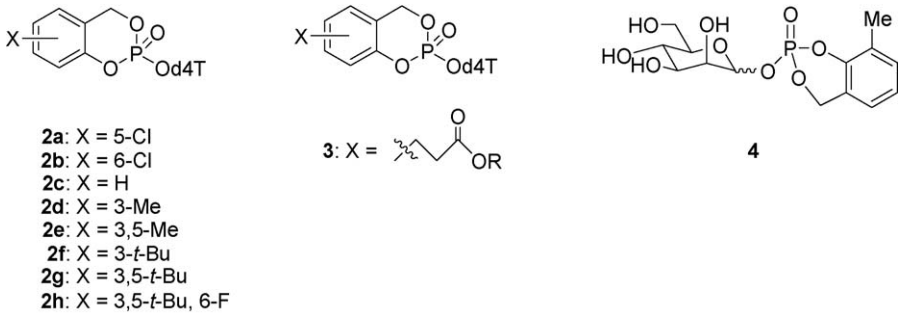

Fig. 2. *Cyclo*Sal-d4TMP triesters **2**, "lock-in"-*cyclo*Sal-d4TMP triesters **3** and *cyclo*Sal-mannosyl-1-phosphate **4**.

major hurdle due to limited anabolic phosphorylation or efficient catabolic processes. For example, the first phosphorylation step of the anti-HIV active 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) **1** (Fig. 1) into d4T 5′-monophosphate (d4TMP) catalyzed by thymidine kinase (TK) is a critical rate-limiting step in human cells. Because both an effective anabolism and a poor catabolism cannot always be predicted, the rational design of nucleoside analogs is impossible.

The intracellular fate of the majority of nucleoside analogs is often unknown. Therefore, these compounds are usually exclusively tested in the nucleoside form and discarded if found inactive. On the other hand, understanding the limitations of phosphorylation of a nucleoside analogue offers a chance to develop derivatives with improved biological potential.

Administration of the nucleotide d4TMP could bypass the metabolic bottleneck and thus should improve the biological activity. However, nucleotides are charged molecules under physiological conditions and do not efficiently penetrate cell membranes. This difficulty can be surmounted by attaching suitably degradable lipophilic groups to the phosphate moiety that leads to neutral, membrane-permeable masked nucleotides (pronucleotide approach; Fig. 1) (Meier, 1998a).

For efficient intracellular nucleotide delivery from such pronucleotides, a specific delivery mechanism is required. Several strategies using different delivery mechanisms have been developed in the past years (Cahard et al., 2004; Peyrottes et al., 2004; Meier et al., 2004a; Drontle and Wagner, 2004). Recent pronucleotide approaches are based on selective enzymatic or chemical activation of the masking group. The delivery mechanisms of the enzyme-cleavable compounds have been summarized (Wagner et al., 2000). In our laboratories, a unique successful, pH-driven nucleotide delivery strategy was developed: the *cyclo*Sal-approach (Meier, 2002a). Here, three aspects of the *cyclo*Sal-approach will be summarized: the design of the prototype *cyclo*Sal-nucleotides **2**; the development of the original concept to new molecules **3** with functionalized side chains ("lock-in"-concept) and the transfer of the technique to the delivery of mannose-1-phosphate (sugar-phosphate prodrugs **4**) (Fig. 2).

2. Design of a chemical trojan horse

The original aim of our approach was to develop a selective nucleotide delivery mechanism based on an exclusively chemically induced cascade mechanism. However, the chemically driven release of the nucleotide from a lipophilic phosphate triester precursor is not as easy as it seems because intermediately a charged phosphate diester is formed after the first ester cleavage which is extremely resistant to further chemical hydrolysis or even to enzymatic degradation.

We used salicyl alcohol as a cyclic bifunctional masking unit in our pronucleotide approach. Salicyl alcohols were attached via the phenyl- and the benzyl ester bond while the nucleoside analog is attached through an alkyl ester bond. The introduction of these three different ester bonds allows sufficient discrimination in the hydrolysis process. In a chemically induced coupled process (tandem or cascade mechanism) first the phenolate is displaced preferentially at the phosphate group leading to 2-

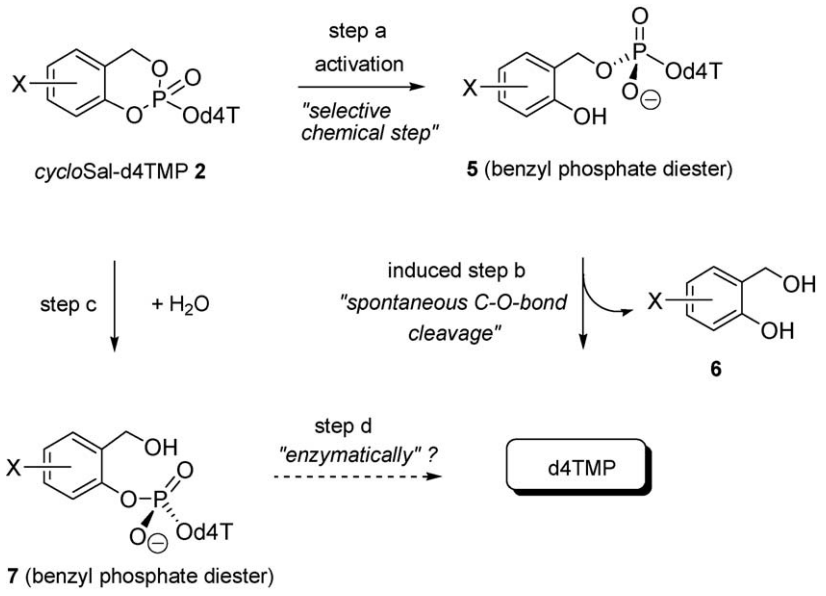

Fig. 3. Hydrolysis pathways of the *cyclo*Sal-d4TMP triesters **2**.

hydroxybenzylphosphate diester **5** (Fig. 3, step a) (Meier et al., 1998b). Due to this initial cleavage, the remaining masking group is activated and a spontaneous breakdown of diester **5** to the nucleotide and salicyl alcohol **6** (cascade reaction; step b) is induced.

Although unfavored, it has been observed that a cleavage of the benzyl ester bond to give diester **7** also occurs under specific circumstances (step c; Fig. 3). However, no further chemical hydrolysis of the resulting phosphate diester takes place at physiologic pH.

An important concern in prodrug design is the non-toxicity of the masking group that will be released. However, salicyl alcohols **6** were tested for their biological potency but showed neither antiviral activity nor cytotoxicity (Meier et al., 1998b). Additionally, feeding of mice with 250 mg/kg of 3-methyl salicyl alcohol did not cause any visible toxic side effect. It should be added that salicyl alcohol is used as part of the analgestic drug salicin (2-[hydroxymethyl]phenyl-β-D-glucopyranoside; Assalix®). Salicin is cleaved by β-glucosidases to D-glucose and salicyl alcohol, which is then slowly oxidized by cytochrome P450 to salicylic acid in blood and liver.

In summary, the *cyclo*Sal-approach needs only one bifunctional masking unit per equivalent nucleotide molecule. In contrast, enzymatically triggered pronucleotides developed by others need mask: nucleotide ratios of up to 4:1.

3. Synthesis

The synthesis of the *cyclo*Sal-pronucleotides has mostly been achieved by using chlorophosphites **8** or phosphoramidites **9** (Fig. 4). Generally, diols **6** were reacted with PCl_3 to give chlorophosphites **8** that were reacted with the nucleoside analog in the presence of diisopropylethylamine to yield the cyclic phosphite triesters that were oxidized in a one-pot-reaction using *t*-butylhydroperoxide. Phosphate triesters **2** were obtained in reasonable yields (50–73%) as mixtures of stereoisomers. The salicyl alcohols needed were prepared previously by different methods. These have been reported and gave the materials in good chemical yields (Meier, 2002a).

Alternatively, phosphoramidites **9** were used for triester synthesis. The coupling with the nucleoside analog was carried out in acetonitrile in the presence of a weak acid. Yields up to >90% were obtained in some cases (Mugnier and Meier, 1999). Recently, we obtained also high yields (85–93%) by using the corresponding phosphorochloridates **10** (2006, data not published yet).

4. Proof-of-principle—D4TMP-release from cycloSal-pronucleotides

Extensive studies have been performed in order to prove the delivery mechanism of d4TMP from *cyclo*Sal-triesters (Meier, 2002a). Chemical hydrolysis studies in different buffer solutions at different pH values proved that all prototype compounds released d4TMP and salicyl alcohols **6** in a pH dependent manner (Meier et al., 1998b).

As expected, half-lives depend on the substitution pattern of the aromatic ring: acceptor substituents cause a decrease in hydrolytic stability while donor-substituents (methyl- and particular *t*-butyl group(s)) led to an increased stability with respect to prototype triester **2c** (Table 1).

The presence of *t*-butyl groups has a surprisingly pronounced effect on the hydrolysis stability. Although, the electron-donating property of a *t*-butyl group differs only marginally from that of a methyl group, triesters **2f** (3-*t*Bu) and **2g** (3,5-*t*Bu) showed considerably higher half-lives as compared to the methyl counterparts **2d** and **2e**. We attribute this to differences in lipophilicity and steric reasons during nucleophilic attack at the phosphate group.

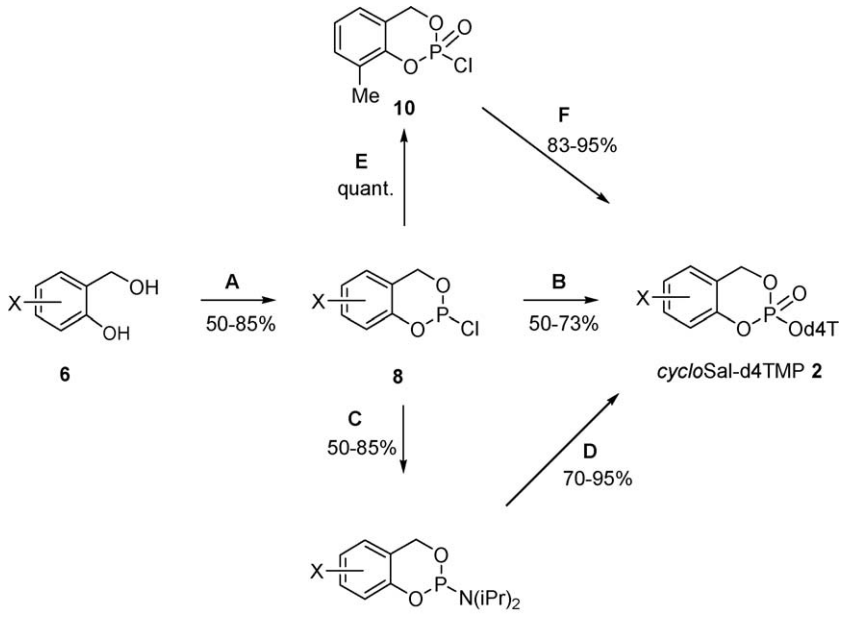

Fig. 4. Synthesis of *cyclo*Sal-d4TMP triesters **2**. Method A: PCl_3, pyridine, Et_2O, −10 °C, 2 h; Method B: (i) d4T **1**, DIPEA, CH_3CN, −20 °C to rt, 1 h; (ii) TBHP, CH_3CN, −20 °C to rt, 1 h; Method C: diisopropylamine (2 eq.), Et_2O, 0 °C, 30 min; Method D: (i) d4T **1**, pyridinium chloride, tetrazole or imidazolium triflate, CH_3CN, 0 °C, 30 min; (ii) TBHP, CH_3CN, rt, 1 h; Method E: O_2, toluene, rt, 16 h; Method F: d4T **1**, pyridine, −50 °C, 4 h.

In addition to these HPLC-based studies we followed the nucleotide delivery by ^{31}P NMR-spectroscopy in imidazole/HCl buffer, pH 7.3 (Ducho et al., 2002). *Cyclo*Sal-d4TMP **2c** led to d4TMP in 99% in imidazole/HCl buffer. Moreover, 1% of phenyl phosphate diester **7** (Fig. 3) was detected. As expected, diester **7** proved to be entirely stable for several weeks in the NMR tube at 37 °C. This diester was also found in the cases of 3,5-dimethyl triester **2e** or 3-*t*-butyl triester **2f**. However, 34% of diester **7** was found for compound **2g** having *t*-butyl groups in the 3- and 5-position. In contrast, 5-chloro- (**2a**), 6-chloro- (**2b**) and 6-fluoro (data not shown) turned out to exclusively deliver d4TMP.

No evidence of an enzymatic degradation in RPMI-1640 medium containing 10% fetal calf serum (pH 7.3) has been observed. Studies in CEM cell extracts showed that hydrolysis half-lives were slightly decreased compared to hydrolysis in buffer. Further studies of the triesters in human serum (10% serum in phosphate buffer) exhibited no difference in stability. Again, no enzymatic contribution could be detected and thus confirmed the initial idea of a purely chemical delivery mechanism independent of 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 shown in Fig. 3 can be efficiently fine-tuned by structural modification of the *cyclo*Sal-moiety. Later we have shown that the discussed hydrolysis pathway is independent on the nucleoside analog attached to the phosphate moiety.

Table 1
Half-lives, product ratio and antiviral data of *cyclo*Sal-d4TMP triesters **2**

Compound	Modification	$t_{1/2}$; 37 °C[a]	Product ratio	EC_{50} (μM)[d]			CC_{50} (μM)[e]
	X and/or R	pH 7.3[b] [h]	d4TMP:**7**[c]	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	0.15	0.13	0.30	50
2d	3-Me	17.5	94:6	0.11	0.08	0.08	32
2e	3,5-Me	29	92:8	0.09	0.17	0.08	21
2f	3-*t*Bu	96	92:8	0.18	0.65	0.33	35
2g	3,5-*t*Bu	73	66:34	1.1	1.2	2.0	27
2h	3,5-*t*Bu,6-F	6.2	100:0	0.12	0.33	0.6	41
d4T **1**	–	–	–	0.18	0.55	28	35

[a] Hydrolysis half-lives in hours.
[b] 25 mM sodium phosphate buffer.
[c] Ratio of d4TMP: phenyl phosphate diester **7** determined by ^{31}P NMR.
[d] Antiviral activity: 50% effective concentration.
[e] Cytotoxic concentration: 50% cytostatic/toxic activity.

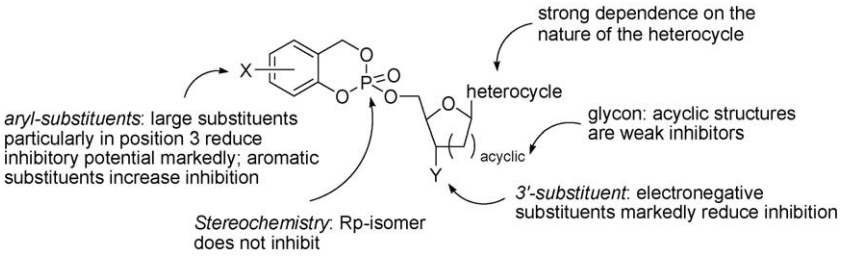

Fig. 5. Structure activity relationship of *cyclo*Sal-triesters and BChE.

5. Antiviral evaluation

The in vitro antiviral potency of the *cyclo*Sal-nucleotides against HIV-1 and HIV-2 in CEM cells was assessed. It became apparent that most of the *cyclo*Sal-derivatives **2** showed comparable or even higher antiviral potency in a wild-type T-lymphocytic cell line (CEM/O) compared to d4T **1** (Table 1). Particularly striking was the complete retention of the antiviral potency in mutant thymidine kinase-deficient cells (CEM/TK−) of triesters bearing alkyl substituents in the 3- and/or 5-position of the *cyclo*Sal-ring. The antiviral data in relation to hydrolysis half-lives clearly showed that certain stability of the prodrug is needed, but beyond this point no further improvement of activity could be observed. Short chemical hydrolysis half-lives seem 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) was comparable to that of d4T. It should be added that *cyclo*Sal-triesters as such have no inhibitory effect on DNA-synthesis using an isolated recombinant RT/RNA/DNA template primer, which is consistent with a mechanism of action that obligatorily relies on the formation of d4TTP.

Several conclusions can be drawn from the above results: (i) the prodrug compounds have to be taken up into the cells, (ii) they need to deliver d4TMP intracellularly and (iii) they act independent of the presence or absence of cellular thymidine kinase. These conclusions were confirmed by incubation experiments of wild-type CEM/O and CEM/TK− cells with tritium-labeled 3-Me-*cyclo*Sal-d4TMP **2d** (Balzarini et al., 2000).

Beside the results in CEM/TK- cells, *cyclo*Sal-d4TMP triesters proved also active in AZT-resistant $H9^{r}AZT^{250}$ cells (Gröschel et al., 1999). The AZT resistance is due to a five-fold lower expression of the TK gene in comparison to parental H9 cells. Again this observation confirms the independence of the intracellular TK concentrations in the eventual antiviral activity of the prodrugs.

6. Interaction of *cyclo*Sal-compounds and human acetyl- (AChE) and butyrylcholinesterase (BChE)

Because *cyclo*Sal-phosphate triesters are reactive organophosphates, they may act as potential inhibitors of human acetylcholine esterase (AChE). Numerous *cyclo*Sal-nucleotide triesters bearing different nucleoside analogs and substitution patterns in the aromatic ring have been studied concerning their ability to inhibit cholinesterases of different origins. It was shown that the tested triesters were not inhibitory against human acetylcholinesterase (AChE; isolated enzyme) as well as against AChE from beef erythrocytes, calf serum and electric eel (*Electrophorus electricus*) (Ducho et al., 2003a).

In contrast, inhibition of butyrylcholinesterase (BChE) has been observed in a few cases in human and mouse serum (Meier et al., 2004b). The physiological significance of this enzyme is not known but it is not related to nerve signal transfer. The *cyclo*Sal pronucleotides showed competitive inhibition with respect to the substrate acetylcholine chloride (K_i/K_m: $\sim 2 \times 10^{-5}$) and acted by irreversible inhibition of human BChE. Detailed studies demonstrated that the inhibitory effect against BChE is highly dependent on the nucleoside analog, the substitution pattern in the *cyclo*Sal-moiety and particularly, on the stereochemistry at the phosphorus atom (Fig. 5).

Moreover, an interesting correlation between the inhibitory potency against BChE and the antiviral activity was observed: The *R*p diastereomer of 3-Me-*cyclo*Sal-d4TMP **2d** being not inhibitory to human BChE was five-fold more anti-HIV active (0.087 μM) compared to the *S*p diastereomer. In contrast, the *S*p diastereomer was quite inhibitory against BChE (0.24 μM).

As mentioned above, the inhibitory potential of the *cyclo*Sal-triesters was dependent on the substitution pattern of the aromatic ring where bulky alkyl substituents in the 3- and 5-position led to a considerable reduction of the inhibitory potential.

In this context, a new masking group was synthesized: 3,5-*t*-butyl-6-fluoro-*cyclo*Sal-d4TMP **2h** proved to be non-inhibitory to AChE *and* BChE (Ducho et al., 2003b) while selectively releasing d4TMP, having reasonable chemical stability parameters and achieving the TK-bypass. 3,5-Di-*t*-butyl-6-fluoro salicyl alcohol **6h** was also introduced to ACVMP, ddAMP and d4AMP with the consequence of a considerable reduction in BChE inhibition.

7. *Cyclo*Sal-pronucleotides of different nucleoside analogs

The *cyclo*Sal-approach has been applied to 2′,3′-dideoxyadenosine (ddA) **11** and 2′,3′-dideoxy-2′,3′-didehydroadenosine (d4A) **12** (Meier et al., 1999a) (Fig. 6).

Both nucleosides suffer from rapid deamination by adenosine deaminase (ADA) and thus poor conversion into their monophosphates. The anti-HIV activity has been increased by 100-fold compared to the parent nucleoside analog ddA **11** while

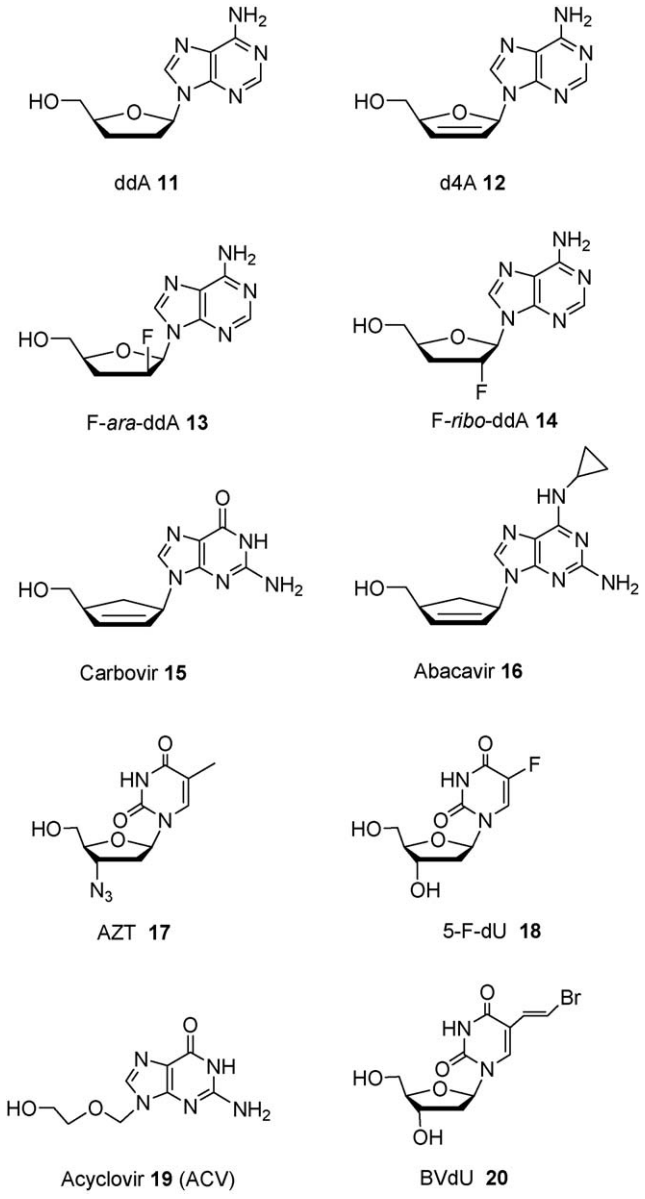

Fig. 6. Nucleoside analogs use with the *cyclo*Sal-approach.

the activity of d4A **12** has been increased by a 600-fold after *cyclo*Sal derivatisation. Two 2-fluorinated ddA derivatives **13**,**14** have also been used (Meier et al., 1999b). The activity of 2′-*ara*-F-ddA **13** was improved by 10-fold but, more interestingly, the entirely inactive nucleoside 2′-*ribo*-F-ddA **14** was converted into an anti-HIV active compound after modification with the 3-methyl-*cyclo*Sal-moiety. This was the first example for the conversion of an inactive nucleoside into a bioactive derivative by the *cyclo*Sal-approach.

The *cyclo*Sal-technology was also applied to the purine nucleoside analogs carbovir **15** and abacavir **16** were used. For carbovir no improvement against HIV and HSV-1/2 was observed for the prodrug compared to the parent nucleoside. However, the anti-HIV potency of abacavir has been improved after *cyclo*Sal-modification and anti-HSV1/2 activity has been observed while abacavir itself was non-inhibitory (Balzarini et al., 2002).

In two cases the *cyclo*Sal-approach failed to improve the biological activity of the nucleoside or to achieve the TK-bypass. One case is the nucleoside analog 3′-azidothymidine **17** (AZT). The *cyclo*Sal-triesters proved to be very active in the wild-type CEM cells. Although, they were still much more active as compared to AZT, they lost some of their activity in the TK-deficient CEM cells (Meier et al., 1998c). The reason for that behavior might be related to rapid dephosphorylation of AZTMP inside the cells by 3′,5′-(deoxy)nucleotidase (Balzarini et al., 1999; Mazzon et al., 2003).

The second case is the antitumor-active 5-fluoro-2′-deoxyuridine **18** (5-FdU) (Lorey et al., 1997). The bioactive metabolite is 5-fluoro-2′-deoxyuridine monophosphate (FdUMP), which is an inhibitor of thymidylate synthase.

Although, chemical hydrolysis studies confirmed FdUMP release, *cyclo*Sal-FdUMP triesters lost nearly all their activity in different TK-deficient tumor cell lines. One reason for the low antitumor activity might be a limited cellular uptake of the triesters. Evidence for this may be derived from a thymidylate synthase assay with permeabilized L1210/0 cells. IC_{50} values obtained were 2.5 μM (FdU), 0.15 μM (FdUMP) and 0.3 μM (5-Cl-*cyclo*Sal-FdUMP), respectively. In addition, an intracellular dephosphorylation of FdUMP as found for AZTMP cannot be excluded. In fact FdUMP is indeed a very good substrate for 3′,5′-(deoxy)nucleotidase.

This may point to an important factor if pronucleotides are used in order to bypass metabolic limitations: although, a selective release of the nucleotide occurs, this alone may not be sufficient to overcome a limitation if intensive catabolic processes are also operative.

DNA viruses are also attractive targets for the application of pronucleotides. Some of the known antivirals against DNA viruses are not monophosphorylated by a cellular kinase but a virus-encoded thymidine kinase (De Clercq, 1984). Often, drug resistant virus strains are selected in vivo. One reason for this drug resistance is associated with a down-regulation of the expression of viral thymidine kinase. The *cyclo*Sal-approach has been applied to the anti-herpetic acyclic purine nucleoside acyclovir (ACV) **19** and the pyrimidine-modified nucleoside 5-[(*E*)-2-bromovinyl]-2′-deoxyuridine (BVDU or Brivudin) **20** (Fig. 6). Although, the selectivity for herpes viruses will be lost for the *cyclo*Sal-triesters of ACV and BVdU, the advantage may be an overcome on resistance profiles or a broadening of the application. Both aims can be reached with the *cyclo*Sal-approach, which will be summarized below.

After monophosphorylation by a viral thymidine kinase, the triphosphate of ACV acts as a DNA chain terminator and/or as an inhibitor of the HSV DNA polymerase. However, the most common mutations related to resistance against ACV appear in the virus-encoded TK.

While the parent nucleoside ACV was active against herpes simplex virus type-1 (HSV-1/TK^+) in Vero cells but inactive against ACV-resistant HSV-1/TK^-, 3-methyl-*cyclo*Sal-ACVMP **21** (Fig. 7) showed identical activity values of ∼0.5 μM against both virus strains (HSV-1 TK^+ and TK^-) without increasing the toxicity (Meier et al., 1998d; Meerbach et al., 2000).

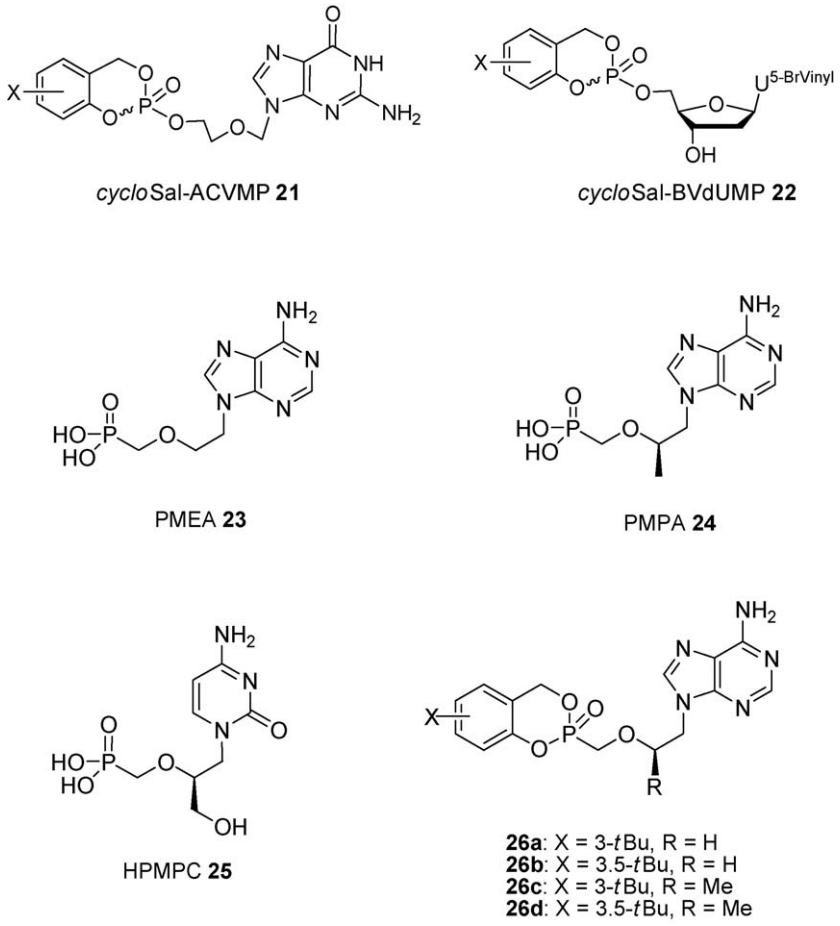

Fig. 7. Chemical formulae of *cyclo*Sal-ACVMP, *cyclo*Sal-BVDUMPs **36** and different nucleoside phosphonates.

Comparable results were obtained for the antiviral activity against varicella zoster virus (VZV). Again, no toxicity was found (MCC >200 μM). In addition, 3-Me-*cyclo*Sal-ACVMP proved to be antivirally active at 10 μM against cytomegalovirus (CMV) while ACV itself was entirely inactive. Recently we have shown that 3-Me-*cyclo*Sal-ACVMP was active against cow pox virus (Sauerbrei et al., 2005) and 3,5-di-*t*-Bu-6-fluor-*cyclo*Sal-ACVMP was active against vaccinia virus. In both cases the parent nucleoside was entirely inactive. These examples clearly show the potential of the *cyclo*Sal-phosphate triester approach: it can broaden the spectrum of antiviral activity for some (inactive) nucleoside analogues.

After phosphorylation of BVdU **20**, BVdU-triphosphate (BVdUTP) act either as an inhibitor of the cellular DNA polymerase or as an alternative substrate that leads to formation of nonsense-DNA or renders DNA more prone to degradation after incorporation. In contrast, BVdU is not active against HSV-2 and Epstein-Barr virus (EBV) because unlike HSV-1 TK and VZV-TK, the thymidine kinase encoded by HSV-2 and EBV cannot convert BVdUMP to its diphosphate. Here, the *cyclo*Sal-approach was used to broaden the application of BVdU to Epstein-Barr virus caused infections. EBV infections play a significant role as secondary infection in, e.g. AIDS patients.

First, chemical hydrolysis studies of *cyclo*Sal-BVdUMP triesters **22** (Fig. 7) demonstrated the selective delivery of BVdUMP without formation of 3′,5′-cyclicBVdUMP **32** (Meier et al., 2005b). 3-Me-*cyclo*Sal-BVdUMP was hydrolyzed in P3HR-1 cell extracts to BVdUMP with a half-life comparable to that found in chemical hydrolysis studies (Lomp et al., 1999; Meier et al., 2002b). However, beside BVdUMP BVdU was also observed to a minor extent (5%) after 4 h obviously is due to an enzymatic dephosphorylation of BVdUMP by phosphatases/nucleotidases.

Antiviral testing against EBV in P3HR-1 cells confirmed the non-activity of the parent compound (EC50 > 100 μM in the EBV DNA synthesis assay). In contrast, some *cyclo*Sal-BVdUMP triesters exhibited pronounced anti-EBV activity. The most active compound was 5-methoxy-*cyclo*Sal-BVDUMP (EC_{50} 1.8 μM) which was even four-fold more active than the reference compound ACV (EC_{50} 7.2 μM). Obviously, after BVdUMP release from the pronucleotide, phosphorylation of BVdUMP into BVdUTP seems to be achieved by cellular enzymes. Again, the *cyclo*Sal-approach converts the inactive nucleoside analog into a bioactive agent.

8. Application of the cycloSal pronucleotide approach to acyclic nucleoside phosphonates

Acyclic nucleoside phosphonates represent another class of interesting antivirals. In 1986, De Clercq and Holý reported on

the antiviral activity of such compounds against DNA viruses, which started a tremendous effort in order to find highly potent derivatives of this class of compounds (De Clercq et al., 1986). Often these acyclic nucleoside phosphonates exhibited a broad antiviral activity spectrum. The most prominent compounds are 9-(2-phosphonylmethoxyethyl)adenine **23** (PMEA, adefovir), (*R*)-9-(2-phosphonylmethoxypropyl)adenine **24** (PMPA, tenofovir) and (*S*)-9-(3-hydroxyphosphonylmethoxypropyl)cytosine **25** (HPMPC, cidofovir) (Fig. 8).

However, the compounds are highly polar because of the charged phosphonate group and therefore, the bioavailability and cellular uptake is limited.

The application of the *cyclo*Sal-approach on these molecules (**26**) leads to a surprising result. In studies regarding the hydrolysis properties of the *cyclo*Sal-PMEA and -PMPA derivatives, the half-lives of compounds **26** were found to be markedly lower compared to the corresponding *cyclo*Sal-d4TMP triesters (3,5-*t*-butyl-*cyclo*Sal-PMPA: $t_{1/2} = 6$ h and 3,5-*t*-butyl-*cyclo*Sal-d4TMP: $t_{1/2} = 73$ h; phosphate buffer, pH 7.3) (Meier et al., 2005a) but the hydrolysis proceeded highly selective to PMPA. Similar as for the ACVMP-derivatives the *cyclo*Sal-nucleoside phosphonates were entirely non-inhibitory to human BChE and human AChE. *Cyclo*Sal-nucleoside phosphonates showed EC_{50} values against HIV-1 and HIV-2 in wild-type CEM cells that

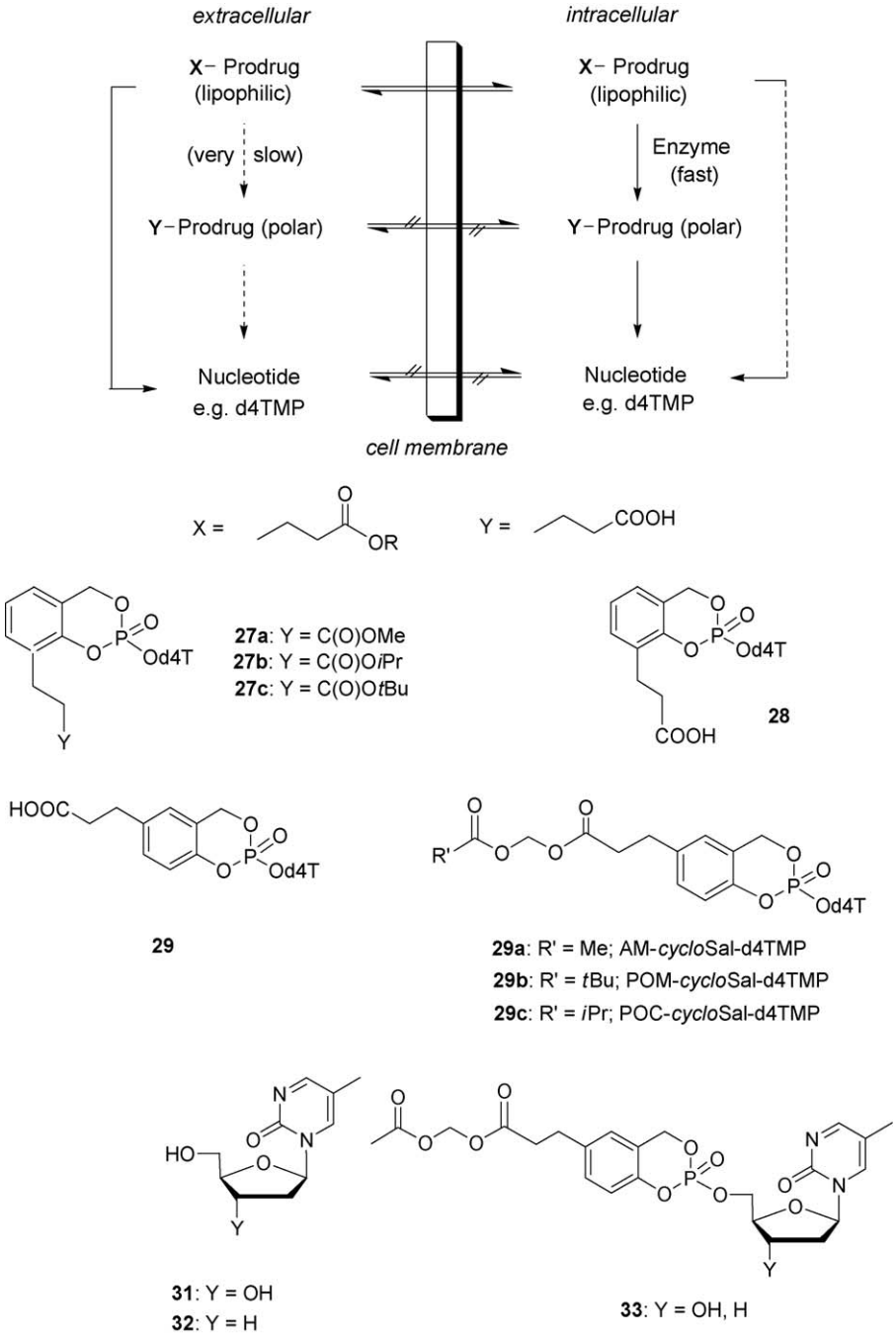

Fig. 8. "Lock-in" principle with the *cyclo*Sal-approach and the target *cyclo*Sal-phosphate trimesters.

were two- to three-fold better as compared to the parent nucleoside phosphonates PMEA and PMPA.

9. "Lock-in"-*cyclo*Sal-d4TMP triesters (second generation cycloSal-triesters)

The compounds described so far belong to the first generation compounds of the *cyclo*Sal-concept. Although, these first generation *cyclo*Sal-triesters led to convincing antiviral results, the use of a pure chemical hydrolysis mechanism may also have some limitations. Due to similar pH values, chemical hydrolysis also takes place extracellularly. In addition, it is not unlikely that lipophilic *cyclo*Sal-triesters can again be exported from the cells, leading to the formation of an equilibrium between both sides of the cell membrane. To avoid such equilibrium formation, the triesters should be designed to be converted inside the cell into a more polar compound by an enzymatic reaction ("lock-in" or trapping mechanism; Fig. 8).

To study such an intracellular trapping, a(n) (carboxy)esterase reaction on a carboxylic ester attached to the *cyclo*Sal-aromatic ring via a linker was investigated.

Under physiological pH-conditions, alkylpropionate *cyclo*Sal-triesters **27a-c** would lead to the formation of the deprotonated propionate-*cyclo*Sal-d4TMP after enzymatic cleavage. All triesters were cleaved chemically to d4TMP at pH 7.3 in 25 mM phosphate buffer without cleavage of the carboxylic ester. Interestingly, propionate-*cyclo*Sal-d4TMP triester **28** showed a two-fold higher half-life compared to the neutral ester-modified *cyclo*Sal-triesters. Disappointingly, in studies with CEM/0 cell extracts no trace of *cyclo*Sal-triester propionate **28** was detected and consequently an enzymatic ester cleavage did not take place (Meier et al., 2004c).

Nevertheless, the triesters were tested for their anti-HIV potency against HIV-1 and HIV-2. All *cyclo*Sal-triesters proved to be active in wild-type CEM cells against both viruses and they retained their antiviral activity in the CEM/TK$^-$ cells. In contrast, the charged propionate-*cyclo*Sal-triester **28** lost all its antiviral activity in the TK-deficient cell line due to low membrane permeability.

Next, acyloxymethyl groups were introduced to the carboxylates in the *cyclo*Sal-side chain (Meier et al., 2006). Three different groups were studied: (i) acetoxymethyl (AM, **29a**), (ii) pivaloyloxymethyl (POM, **29b**) and (iii) isopropyloxycarbonyloxymethyl (POC, **29c**) (Fig. 8).

As in the case of simple ester-modified *cyclo*Sal-triesters, the chemical hydrolysis stability (pH 7.3) of the acylal-*cyclo*Sal-nucleotides was found to be more or less identical to those observed for alkylated *cyclo*Sal-triesters. As before, only chemical cleavage of *cyclo*Sal-triesters to d4TMP was detected while the acylal group proved to be stable at pH 7.3. However, in CEM cell extracts and liver extracts but not in human serum the acylal group was efficiently cleaved to give the free propionate **30**, e.g. the acylal in 5-AM-*cyclo*Sal-d4TMP **29a** was cleaved enzymatically with a half-life of 15 min to yield the charged propionate-*cyclo*Sal-derivative. There was a clear correlation between the stability and the nature of the acylal structure. The AM-acylal is the least stable compound while the POC-acylal (**29c**) was the most stable group ($t_{1/2}$ = 0.9 h). Thus, the stability difference between the chemical hydrolysis of the *cyclo*Sal-ester and the acylal cleavage in the cell extracts was up to 16-fold. Interestingly, in anti-HIV tests the AM-acylal lost its activity in the mutant CEM/TK$^-$ cell line while in the POM- and the POC-derivatives retained their antiviral activity. Later, we could show that the AM-acylal was stable in RPMI culture medium but labile in the mixture with FCS. In contrast, the POM- and the POC-acylals were found to be stable in the FCS-containing medium. For further exploration of the "lock-in"-concept, we intrinsically fluorescent nucleoside analogs as analytical tools to study membrane uptake processes were recently introduced.

A fluorescent analog was needed which is structurally as close as possible to the nucleosides used before. As a surrogate for the pyrimidine-type nucleoside analogs, e.g. ddT, ddC or d4T the highly intrinsic fluorescent 5-methylpyrimidin-2-one nucleoside **31** (m^5 K; Fig. 8) was used (Jessen et al., 2006). Meanwhile we prepared also the dideoxy analog dm^5 K **32**. Both nucleosides were converted into the 5-acetoxymethyl-(AM) propionyl-*cyclo*Sal-nucleotides **33**. The detection limit of these compounds in cell extract hydrolysis studies was 500–1000-fold lower compared to UV detection. In cell extracts 5-AM-*cyclo*Sal-triesters **33** were rapidly cleaved enzymatically at the acylal site delivering propionate-*cyclo*Sal-m^5KMP and subsequently the nucleotide m^5KMP by chemical hydrolysis.

In model studies for cellular uptake using fluorescence spectroscopy the migration of the triesters from an aqueous donor phase to an aqueous acceptor phase via an organic phase (CH_2Cl_2) was monitored. Three different experimental set-ups were used: (i) in both aqueous phases the pH was set to 6.8, (ii) the pH in the donor phase was set to pH 6.8 while that of the acceptor phase was pH 8.7 and (iii) the donor phase was phosphate buffer, pH 6.8, but the acceptor phase was PLE-containing phosphate buffer, pH 7.3. In the first experiment, the expected formation of an equilibrium between both phases was detected. However, in the second set-up an accumulation in the acceptor phase was observed because the triester was hydrolysed rapidly to give the nucleotide. In the third experiment, an accumulation of all fluorescent material in the acceptor phase was detected. Compounds found in the acceptor phase were 5-propionate-*cyclo*Sal-m^5KMP and its hydrolysis product m^5KMP. No 5-AM-*cyclo*Sal-triester was detected due to its high susceptibility to enzyme degradation. This experiment clearly supports the idea of a possible intracellular "lock-in" of the *cyclo*Sal-phosphate triesters.

10. Delivery of mannosyl-1-phosphates from cycloSal-prodrugs

The congenital disorders of glycosylation (CDG) syndrome are autosomal recessively inherited disorders first described by Jaeken et al. (1980). CDG are classified into two types corresponding to the type and intracellular localization of the glycosylation pathways. Protein glycosylation plays an important role in the metabolism, function and structure of glycoconjugates. The hypoglycosylation of several lipids or proteins in CDG has severe consequences for the patients. The far most

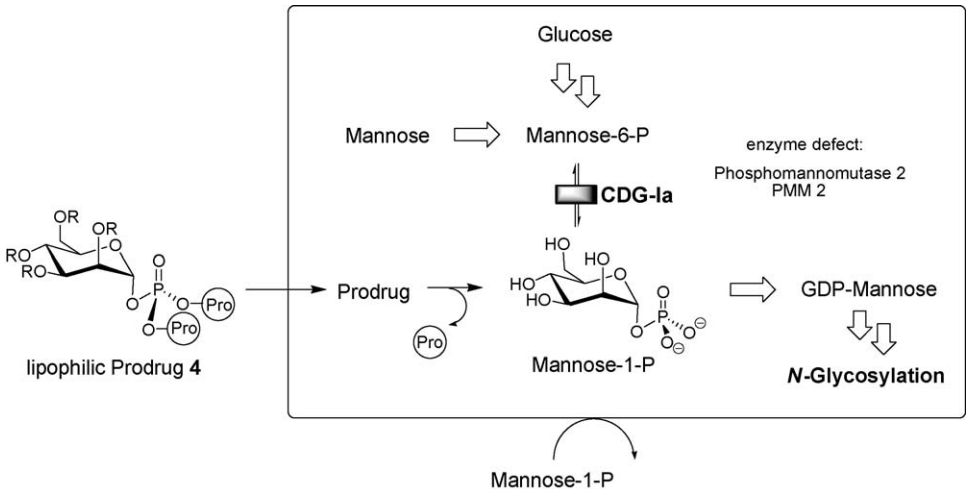

Fig. 9. Carbohydrate prodrugs based on the *cyclo*Sal-approach.

frequent CDG-type is CDG-Ia. This defect leads to a significant reduction in the concentration of phosphomannomutase 2 (PMM 2), thus preventing the conversion of mannose-6-phosphate to mannose-1-phosphate. As a consequence, the concentration of GDP-mannose decreases resulting in a general hypoglycosylation of many glycoconjugates. A therapeutic approach may be the intracellular delivery of mannose-1-phosphate. However, as for nucleotides, mannose-1-phosphate does not penetrate cellular membranes and rapid dephosphorylation occurs in the blood. In 2004, we reported on the synthesis of acetylated *cyclo*Sal-mannose-1-phosphates **4** leading to successful "carbohydrate monophosphate prodrugs" (Fig. 9) (Muus et al., 2004).

In hydrolysis assays, *cyclo*Sal-mannose-1-phosphates delivered mannose-1-phosphate. However, the selectivity was found to be much lower compared to the nucleotide derivatives. Finally, the biological activity of 3-methyl-*cyclo*Sal-mannose-1-phosphate **4** was tested in vitro in fibroblasts. Healthy fibroblasts assemble complete oligosaccharide chains having a $Glc_3Man_9GlcNAc_2$-structure. In contrast, PMM 2-deficient fibroblasts synthesize only truncated oligosaccharide chains with $Man_4GlcNAc_2$- and $Man_3GlcNAc_2$-structures.

The 3-Me-*cyclo*Sal-compound **4** showed a total correction of the hypoglycosylation pattern proving the successful intracellular mannose-1-phosphate delivery. This was the first example that the *cyclo*Sal-approach can be efficiently transferred to other phosphorylated bioactive metabolites different from nucleotides.

11. Conclusion

The *cyclo*Sal-approach convincingly demonstrated the intracellular delivery of nucleotides by a non-enzymatically induced cascade reaction. These compounds can improve the antiviral activity of several nucleoside analogs considerably. In addition, the *cyclo*Sal-pronucleotide system may be used as biochemical tools to study nucleoside metabolism and allows to obtain new insights in biosynthetic pathways.

Finally, the promising results obtained regarding the total correction of the hypoglycosylation shows that the delivery of biologically active phosphorylated compounds from *cyclo*Sal-phosphate triesters generally may lead to a strong improvement in the bioactivity.

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