*cyclo*Saligenyl-5-[(*E*)-2-bromovinyl]-2′-deoxyuridine Monophosphate (*cyclo*Sal-BVDUMP) Pronucleotides Active against Epstein – Barr Virus

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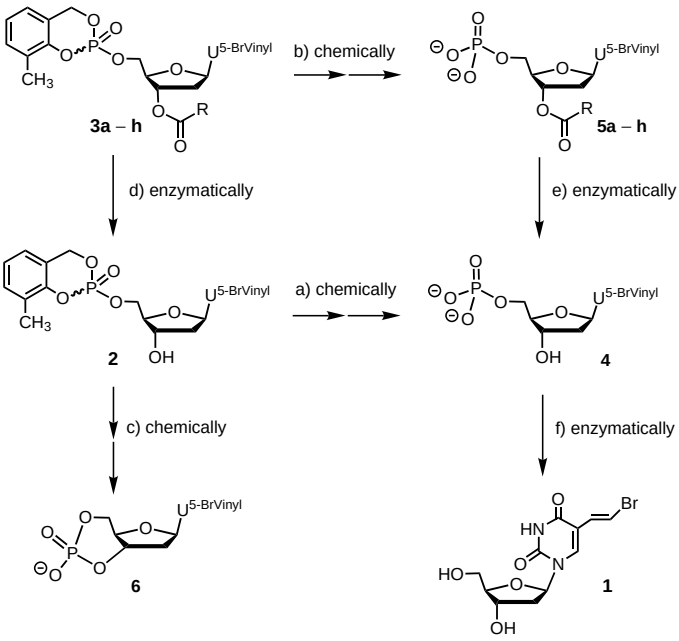
KEYWORDS:

antiviral agents · Epstein – Barr virus · nucleosides · nucleotides · prodrugs

5-[(*E*)-2-Bromovinyl]-2′-deoxyuridine (BVDU or Brivudin, **1**)[1] is a potent and highly selective nucleoside analogue-type inhibitor[2] of the replication of several herpes viruses (e.g. HSV-1 and VZV).[3] The selectivity of the inhibitory action primarily depends upon a specific activation to the corresponding mono- and diphosphate by the HSV-encoded viral thymidine kinase (TK) and, finally, to the triphosphate by cellular enzymes. BVDU triphosphate (BVDUTP) can act either as an inhibitor of the cellular DNA polymerase or as an alternate substrate; incorporation of BVDUTP into DNA would render the latter more prone to degradation.[4] Some limitations to the use of BVDU are known: lack of activity during virus latency because of the absence of 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.[3] To overcome some of these limitations the pronucleotide concept has been developed.[5] The basic idea is to use a lipophilic precursor of the nucleoside monophosphate (nucleotide) that is capable of entering the cells and efficiently and selectively releasing the nucleotide. In this context we developed the *cyclo*Sal-pronucleotides,[6] which have been successfully applied to the intracellular delivery of a number of anti-HIV-active nucleotides[7] and the anti-HSV-active acyclovir (ACV).[8] Here we report on the application of the *cyclo*Sal method to BVDU (**1**). Our aim was to prove if the *cyclo*Sal concept can be used to broaden the application of **1** against infections caused by Epstein – Barr virus (EBV).[9] Two reports on pronucleotides of BVDU (**1**) have been published before, but both were unsuccessful.[10]

We synthesized 3-methyl-*cyclo*Sal-BVDUMP (**2**) and a series of 3′-*O*-modified derivatives (**3**, Scheme 1). As 3′-*O*-modification, different lipophilic carboxylic acids (**3a** – **e**) as well as α-amino acids (**3f** – **h**) were used. The 3-methyl substituent in the masking unit was introduced because *cyclo*Sal nucleotides bearing this substituent showed the best antiviral activities so far.[7]



a: R = CH_3; **b**: R = CH_3CH_2; **c**: R = *n*-C_5H_{11}; **d**: R = *n*-C_9H_{19}; **e**: R = *t*Bu; **f**: R = L-$CH(CH_3)NH_2$; **g**: R = D-$CH(CH_3)NH_2$; **h**: R = L-$CH(CH_2Ph)NH_2$

Scheme 1. *Possible degradation pathways of **2** and **3a** – **h** leading to 3′-O-acyl-BVDUMPs **5**, BVDUMP (**4**), BVDU (**1**), and cBVDUMP (**6**). 5-BrVinyl = 5-[(E)-2-bromovinyl].*

The preparation of prototype compound **2** was carried out from BVDU (**1**) by direct phosphitylation and oxidation in DMF/THF (2:1) in 56% yield as described before.[7b,c] 3′-Esterified compounds **3** were prepared starting from BVDU (**1**), which was first 5′-*O*-silylated (TBDMS) in pyridine (84%) and then esterified using DCC/DMAP and carboxylic acids or *N*-Boc-protected amino acids. Desilylation (2% TBAF in THF) yielded 3′-modified BVDU derivatives (90 – 97% over both steps). Again, 5′-*O*-phosphitylation and oxidation gave triesters **3a** – **e** as well as the *N*-Boc-protected precursors of triesters **3f** – **h** (50 – 60%).[7a,d] Finally, the *N*-Boc group was cleaved off by acid treatment (5% TFA, CH_2Cl_2/MeOH (7:3); 50 – 52%) to give triesters **3f** – **h** (Scheme 1). Characterization of the triesters **3** was achieved by multinuclear NMR spectroscopy as well as electrospray ionization mass spectrometry (ESI-MS). The purity was checked by high-resolution mass spectrometry (HR-MS) and HPLC analysis.

Next, different hydrolysis studies were carried out to prove the selective delivery of BVDUMP (**4**) from the title triesters **2**, **3** (paths a, b in Scheme 1). In contrast to our previously published work on *cyclo*Sal triesters,[7, 8] an intramolecular attack of the 3′-hydroxy group at the phosphorus center may occur in 3-methyl-*cyclo*Sal-BVDUMP (**2**), which would lead to the formation of 3′,5′-cyclic BVDUMP (cBVDUMP, **6**; path c in Scheme 1). We observed such formation of a cyclic phosphate diester previously for the acyclic antiherpetic compound penciclovir.[11] This may also occur if first a fast deesterification of triesters **3** to **2** by cellular (carboxy)esterases takes place (path d, then path c in Scheme 1).

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Alternatively, in case of a slow deesterification the cleavage of the *cyclo*Sal moiety may occur as the major process to give the 3′-esterified BVDUMPs **5** and further deesterification may lead to BVDUMP (**4**) (path b, then path e in Scheme 1).

Hydrolysis in phosphate buffer at pH 7.3 showed selective cleavage of the *cyclo*Sal masking group to yield solely BVDUMP (**4**) or 3′-*O*-acyl-BVDUMP (**5**) from triesters **2** or **3**, respectively. No formation of cBVDUMP (**6**) was observed, and therefore path c can be excluded. 3-Methyl-*cyclo*Sal-BVDUMP (**2**) showed a half-life ($t_{1/2}$) of 6.7 h, whereas triesters **3a**–**e** showed half-lives in the range of 6–48 h. However, the α-amino acid derivatives **3f**–**h** showed markedly lower $t_{1/2}$ values (data not shown).

Next, we used P3HR-1 cell extracts because this cell type (derived from Burkitt's lymphoma) was used to test the antiviral activity of **2** and **3** against EBV. A few striking differences were observed. The prototype **2** was degraded to BVDUMP (**4**) with a half-life of 8.9 h (Table 1). This value is comparable to that found in the chemical hydrolysis studies. Hence, the degradation is purely chemically driven and not enzymatically. No cBVDUMP (**6**) was detected. Besides BVDUMP (**4**), BVDU (**1**) was observed after 4 h of incubation to a minor extent (3%), which is due to an enzymatic dephosphorylation of **4** by phosphatases/nucleotidases (path f in Scheme 1). In independent studies, BVDUMP (**4**) was shown to be converted to BVDU (**1**) to an extent of 13% within 4 h.

Table 1. *Hydrolysis data and antiviral activity of the title triesters* ***2*** *and* ***3a–h****.*

Compd	$t_{1/2}$ [h]	Hydrolysis data[a] products (%)[b]	Antiviral activity EC_{50} [μM][c]	CC_{50} [μM][d]
2	8.9	**4** (30), **1** (3)	4.1	122
3a	3.4	**2** (26), **5a** (14), **4** (9), **1** (3)	>85	110
3b	4.5	**2** (5), **5b** (15), **4** (12), **1** (3)	>150	>300
3c	7.7	**2** (5), **4** (10), **1** (3)	>150	>300
3d	19.5	**2** (7), **4** (3), **1** (<1)	>150	>300
3e	7.0	**2** (4), **5e** (13), **4** (3)	>85	57
3f	0.12	**2** (77), **4** (20), **1** (1)	21.8	140
3g	0.5	**2** (79), **4** (20), **1** (1)	9.5	83
3h	1.1	**2** (94), **4** (5), **1** (1)	40	20
ACV	–	–	7.2	422
BVDU	–	–	>300	225

[a] Measured in P3HR-1 cell extracts. [b] Products obtained after 4 h of incubation; difference to 100% is the amount of remaining starting phosphate triester. [c] EC_{50} = concentration required to reduce EBV DNA synthesis by 50%. [d] CC_{50} = concentration required to reduce the growth of exponentially growing P3HR-1 cells by 50%.

The hydrolyses of the 3′-*O*-acyl derivatives **3** exhibited a clear difference with respect to the attached acid moiety. For the 3′-OAc derivative **3a**, enzymatic deesterification by carboxyesterases yielded the prototype **2** as the major product (32%), but only 9% of BVDUMP (**4**) was found. In contrast to that, compounds **3d** (3′-*O*-decanoyl-substituted) gave only 3% of BVDUMP (**4**) and 7% of triester **2**. The 3′-*O*-propionyl derivative **3b** and the 3′-*O*-pivaloyl compound **3e** yielded the 3′-*O*-esterified BVDUMP derivatives **5b** and **e**, respectively, as the major hydrolysis products due the chemically driven cleavage of the *cyclo*Sal mask (path b in Scheme 1) (Table 1). It is noteworthy that for compounds **3a**–**c** only about 10% conversion into BVDUMP (**4**) was observed. The situation was significantly different for the triesters **3f**–**h**. All three compounds were rapidly deesterified to yield triester **2** as the major product (77–94%). The half-lives were dependent on the configuration and the type of the α-amino acid (Table 1). Moreover, in 4-h incubations a total of 20% BVDUMP was formed starting from both alanine esters **3f** and **g**. At the same time point, 30% BVDUMP was formed starting directly from triester **2**. However, only 5% BVDUMP was detected for the conversion of phenylalanine ester **3h**.

Finally, the *cyclo*Sal-BVDUMP triesters were tested for their antiviral potency against EBV in P3HR-1 cells (Table 1). The most active compound was 3-methyl-*cyclo*Sal-BVDUMP (**2**). As compared to the completely inactive BVDU (**1**), triester **2** was >73-fold more active, and about twofold more active than the reference compound acyclovir (ACV). The 3′-alanyl-substituted *cyclo*Sal triesters showed antiviral activity that was 5- (**3f**) and 2.5-fold lower (**3g**) as compared to that of **2**, but both compounds were still significantly more potent than **1**. Surprisingly, all derivatives bearing carboxylic acid moieties (**3a**–**e**) were devoid of any antiviral activity. In contrast, with the alanine-bearing triesters, formation of BVDUMP in the extracts (and therefore also inside the cells) was observed in considerable amounts and this seems to result in a high degree of forward phosphorylation to the ultimate metabolite BVDUTP. The antiviral evaluation proved that esterification of the 3′-hydroxy group with simple carboxylic acids abolishes all biological activity for unknown reasons, whereas the use of α-amino acid esters retains the activity. The advantage of the latter compounds is their higher solubility in aqueous media even as compared to that of prototype **2**. Further structure–activity relationship (SAR) studies with α-amino acid esters will be carried out in our laboratories.

The promising antiviral data of some of the reported *cyclo*Sal-BVDUMP triesters proved that with the *cyclo*Sal approach it is possible to convert the anti-EBV-inactive compound BVDU (**1**) into a bioactive agent. Finally, to the best of our knowledge the work reported here represents the first example of the application of a pronucleotide approach to a nucleoside analogue possessing a 3′-hydroxy group resulting in a considerable improvement of antiviral activity.

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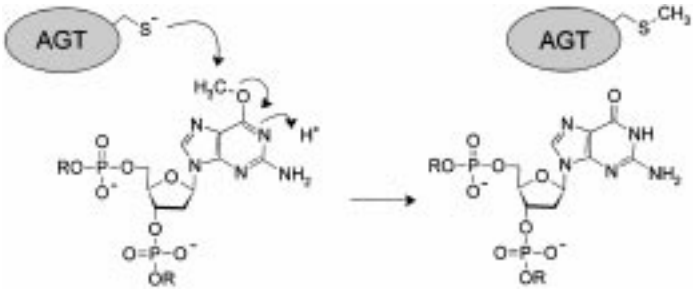

Scheme 1. *Schematic representation of the DNA repair reaction catalyzed by AGT (see text for details).*

Synthesis and Applications of Chemical Probes for Human *O*^{6}-Alkylguanine-DNA Alkyltransferase

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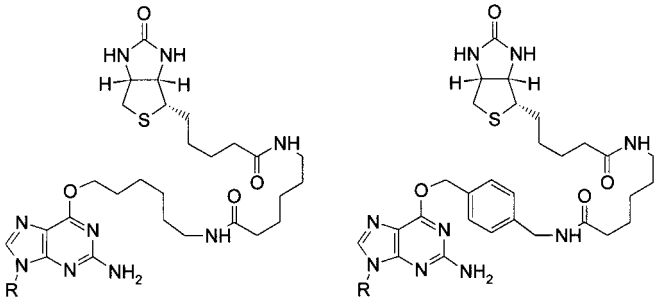
O^6-alkylguanine-DNA alkyltransferase · DNA repair · nucleobases · phage display · transferases

The design and synthesis of chemical probes to study and to elucidate complex biological problems is becoming an increasingly important field in chemistry. We are interested in the repair of *O*^{6}-alkylated guanines in DNA, a DNA lesion that results from alkylation by *S*-adenosylmethionine or exogenous toxins and which has been shown to be highly mutagenic and carcinogenic.[1] The DNA repair protein *O*^{6}-alkylguanine-DNA alkyltransferase (AGT; EC 2.1.1.63) reverses this alkylation by transferring the alkyl group to a reactive cysteine residue in the protein, leading to repaired DNA and an irreversibly alkylated protein (Scheme 1).[2] The expression level of human AGT (hAGT) in tumor cells is also crucial for their sensitivity to chemotherapeutic agents that alkylate DNA, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and temozolomide.[2] Consequently, hAGT has become a target in cancer chemotherapy, as its inhibition would increase the efficiency of currently used DNA-alkylating drugs.[3] A simple and reliable assay to measure the activity of hAGT in cell extracts would be of great importance for research on the role of hAGT in the chemotherapy of tumors, as currently used assays rely on radioactively labeled substrates and a subsequent HPLC separation. We describe here the synthesis of oligonucleotides containing *O*^{6}-alkylated guanine derivatives of the type **1** and **2** that serve as affinity labels for hAGT and their use in a highly specific assay for this alkyltransferase. In addition, we introduce a novel system for the directed molecular evolution of hAGT,[4] which relies on the display of active hAGT on phage λ and on oligonucleotides containing *O*^{6}-alkylated guanine derivatives of the type **1** and **2**.



1 **2**

To incorporate nucleosides of the type **1** and **2** into oligonucleotides, the corresponding phosphoramidites **3** and **4** were synthesized starting from the readily available nucleoside **5** (Figure 1 A, B).[5] The phosphoramidites **3** and **4** were then incorporated into oligonucleotides with coupling yields of about 93 % (Figure 1). After synthesis and complete deprotection of the oligonucleotides, the amino group of the *O*^{6}-alkylguanine moiety was biotinylated with *N*-(+)-biotinyl-6-aminocaproic acid *N*-succinimidyl ester.[5] The incorporation of **1** and **2**, their complete deprotection and biotinylation as well as the base composition of **7** and **8** were verified by digesting the oligonucleotides with snake venom phosphodiesterase and calf intestinal alkaline phosphatase followed by HPLC analysis.[6] The nucleosides **1** and **2** were identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI) of the corresponding HPLC peak eluates.[5]

To demonstrate the use of the *O*^{6}-alkylated oligonucleotides **7** and **8** (Figure 1 C) as sensitive probes for active hAGT, the double-stranded oligonucleotides were incubated with recombinant hAGT[5, 7] and the reaction mixture transferred into streptavidin-coated microtiter plate wells, leading to immobilization of biotinylated hAGT. After washing of the wells, immobilized hAGT was detected by an enzyme-linked immunosorbent assay (ELISA) based on an anti-hAGT antibody and an

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