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COMMENTARY

BIOCHEMICAL ASPECTS OF THE SELECTIVE ANTIHERPES ACTIVITY OF NUCLEOSIDE ANALOGUES*

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In recent years several new antiviral compounds have been developed which offer great promise for the specific treatment of herpesvirus infections of both medical importance, i.e. infections caused by herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein–Barr virus (EBV), and veterinary importance, i.e. infections caused by pseudorabies virus (suid herpesvirus type 1, SHV-1) and infectious bovine rhinotracheitis virus (bovid herpesvirus type 1, BHV-1). Among the most promising of these new antiherpes agents are 9-(2-hydroxyethoxymethyl) guanine (acyclovir, *ACV*), 9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]guanine or (9-[(1,3-dihydroxy-2-propoxy)methyl]guanine, *DHPG*, also referred to as BW-759, BIOLF-62, 2′-nor-2′-deoxyguanosine or 2′NDG), (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (bromovinyldeoxyuridine, *BVdU*), (*E*)-5-(2-bromovinyl)-2′-deoxycytidine (bromovinyldeoxycytidine, *BVdC*), (*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil (bromovinylarabinosyluracil, *BVaraU*), 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-iodocytosine (fluoroiodoarabinosylcytosine, *FIAC*) and 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil (fluoromethylarabinosyluracil, *FMAU*). The structural formulae of these and some related compounds are presented in Fig. 1. After a brief description of their antiviral spectrum *in vitro* and their therapeutic potentials in the clinic, I will address the biochemical bases for the selective antiviral activity of these nucleoside analogues.

ANTIVIRAL SPECTRUM *IN VITRO*

ACV is particularly effective against HSV-1 and HSV-2 [1, 2]. To a lesser extent, it is also inhibitory to VZV [3], CVM [4–9] and EBV [8, 10–12]. DHPG is as effective as ACV in inhibition of HSV-1, HSV-2 and VZV [6–8, 13] but, according to Field *et al.* [8], 10-fold more potent than ACV in inhibition of CMV and EBV replication. With a minimum inhibitory concentration of approximately 0.005 μM BVdU supersedes both ACV and DHPG in potency against HSV-1 [14–20] and VZV [21, 22], but, quite surprisingly, inhibits HSV-2 at a 100- to 1000-fold higher concentration than that required for inhibition of HSV-1 [15, 23]. BVdU is also effective against CMV and EBV, but only so at a relatively high concentration (10 μM) [5, 9, 11, 12]. BVdC follows the same pattern as BVdU but is about 10-fold less potent [22, 24]. BVaraU is less potent than BVdU against HSV-1 and HSV-2 [16, 26] but slightly more potent against VZV [22, 25]. FIAC and FMAU are potent inhibitors of all herpesvirus types, including CMV and EBV [9, 11, 25, 27–29].

Since all these studies have been conducted by different investigators using different virus strains, cell culture systems and assay methods, it is rather difficult to assess the relative potencies of the antiherpes drugs. If the lowest representative inhibitory dose-50 (ID_{50}) values reported in the literature for each individual compound are compared (Table 1), BVdU, FIAC, FMAU emerge as the most potent against HSV-1, FIAC and FMAU as the most potent against HSV-2, BVaraU and BVdU as the most potent against VZV, and, again, FIAC and FMAU as the most potent against CMV. For EBV, ID_{50} values cannot be directly compared because of the diversity of parameters used to monitor EBV replication.

Of course, ID_{50} values for virus replication are only meaningful if compared with the cytotoxic doses evaluated under the same experimental conditions, and in this respect all compounds exhibit marked selectivity in their antiviral action. The greatest selectivity indexes, as determined by the TD_{50}/ID_{50} ratio (Table 1), are achieved by BVdU and BVaraU in their inhibitory effects on HSV-1 and VZV replication (67,000 and 50,000, respectively). As a rule, the nucleoside analogues display greater selectivity in their anti-HSV and anti-VZV activities than for CMV or EBV inhibition. With a selectivity index of approximately 100 towards CMV, DHPG, FIAC and FMAU may be considered as potentially promising anti-CMV agents.

THERAPEUTIC POTENTIALS IN THE CLINIC

Of all compounds listed in Fig. 1, ACV has been most extensively pursued for its clinical efficacy. According to a large number of placebo-controlled double-blind clinical studies, ACV would be efficacious in (*i*) the topical treatment (at 3% in an eye ointment) of herpetic keratitis [30]; (*ii*) the topical treatment (at 5% in polyethylene glycol) of primary genital herpes [31] and, to a lesser extent, recurrent

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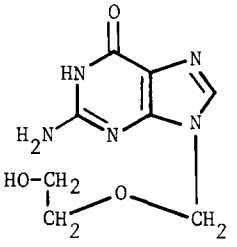
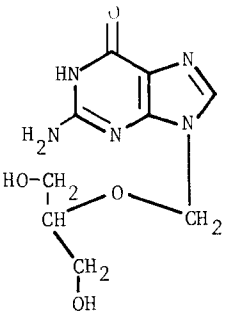
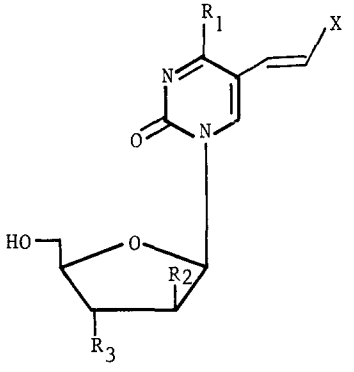
1) Guanine acyclic nucleosides



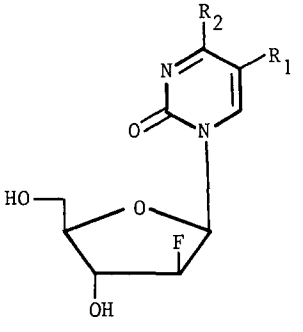


2) Bromovinyldeoxyuridine and related compounds



X	R_1	R_2	R_3	Compound
Br	OH	H	OH	BVdU
I	OH	H	OH	IVdU
CH_3	OH	H	OH	PedU
CF_3	OH	H	OH	F_3PedU
Br	NH_2	H	OH	BVdC
Br	OH	OH	OH	BVaraU
Br	OH	H	NH_2	BVamU

3) 2'-Fluoro-substituted pyrimidine arabinosides



R_1	R_2	Compound
I	NH_2	FIAC
I	OH	FIAU
CH_3	OH	FMAU
H	OH	FAU
H	NH_2	FAC

Fig. 1. Nucleoside analogues with selective antiherpes activity.

genital herpes [31, 32]; (*iii*) the systemic treatment (3 × 5 mg/kg/day for 5 days intravenously, or 5 × 200 mg/day for 5 or 10 days perorally) of primary and recurrent genital herpes [33–35]; (*iv*) the topical treatment of recurrent herpes labialis, if applied as a 5% ACV cream [36] (which should be superior to the 5% ACV polyethylene glycol ointment [37]); (*v*) the systemic treatment (3 × 250 mg/m^2/day for 7 days intravenously) of mucocutaneous HSV infections in immunocompromised patients, i.e. kidney, bone-marrow or heart allograft recipients [38–40]; and (*vi*) the systemic treatment (3 × 500 mg/m^2/day for 5–7 days intravenously) of acute herpes zoster (VZV infection) in either immunocompetent or immunocompromised patients [41–43]. When given prophylactically, i.e. at 3 × 250 mg/m^2/day intravenously or 4 × 200 mg/day orally, ACV protects against recurrent HSV infection in bone-marrow

Table 1. Antiviral activities of ACV, DHPG, BVdU, BVdC, BVaraU, FIAC and FMAU*

Compound	ID_{50} (μM) HSV-1	HSV-2	VZV	CMV	EBV	TD_{50} (μM)	References
ACV	0.1	0.1	4	2–100†	1–100†	15–300‡	[2–9, 15]
DHPG	0.2	0.3	5	≥1	10	150	[6–8]
BVdU	0.003	3	0.007	10	10	200	[5, 9, 11, 12, 14–23]
BVdC	0.2	30	0.07	ND	ND	200	[22, 24]
BVaraU	0.1	100	0.004	ND	ND	200	[16, 22, 23, 25, 26]
FIAC	0.01	0.01	0.01	0.3	5	10–25	[9, 11, 27–29]
FMAU	0.01	0.01	0.01	0.1	<5	1–10	[9, 11, 25, 28]

ND, not determined.

* Based on the lowest representative ID_{50} and TD_{50} values reported in each case. ID_{50} (or inhibitory dose-50) stands for the drug concentration required to inhibit virus replication (generally cytopathogenicity or plaque formation) by 50%. TD_{50} (or toxic dose-50) stands for the drug concentration required to inhibit host cell growth or metabolism (DNA synthesis) by 50%.

† The ID_{50} values of ACV for CMV vary considerably from one study to another [4–9], and so do the ID_{50} values reported for EBV [8, 10–12].

‡ Again, highly varying ID_{50} values have been reported for ACV depending on the procedure followed to evaluate host cell toxicity [1, 6, 9, 13–15].

transplant recipients [44, 45]. Moreover, oral ACV treatment may prevent the recurrences of genital herpes, at least as long as ACV is administered.

Hence, there is ample evidence for the clinical efficacy of ACV in both therapy and prophylaxis of HSV and VZV infections in humans. As compared to ACV, DHPG may even have greater promise as an antiviral drug since it is significantly more efficacious in the treatment of HSV-1 and HSV-2 infections in mice [46, 47]. Additionally, DHPG is less cytotoxic and markedly more active than ACV against CMV and EBV [6, 8, 47]. Clinical studies with DHPG are in abeyance.

With BVdU a series of phase I clinical trials have been undertaken to evaluate its safety and efficacy in the topical and systemic treatment of HSV-1 and VZV infections. From these studies, BVdU appears to be highly efficacious in (*i*) the topical treatment (as 0.1% eye drops) of herpetic keratitis [48]; (*ii*) the systemic treatment (3×2.5 mg/kg/day for 5 days orally) of patients with ophthalmic zoster [49]; (*iii*) the systemic treatment (3×2.5 mg/kg/day for 5 days orally) of cancer patients with severe localized or disseminated herpes zoster [50, 51]; and (*iv*) the systemic treatment (3×5 mg/kg/day for 5 days orally) of leukemic children with an intercurrent VZV infection [52]. In the majority of the patients, progress of the VZV episode was arrested within 1 or 2 days after BVdU therapy was started, and in no case it appeared necessary to prolong treatment for more than 5 days. For the BVdU derivatives BVdC or BVaraU no clinical studies are presently envisaged.

Of the 2′-fluoro-substituted pyrimidine arabinosides, only FIAC has been the subject of phase I clinical trials. FIAC was found to stabilize cutaneous lesions within 2 to 3 days when administered intravenously at 120 mg/m^2/day to immunocompromised patients with an intercurrent VZV infection [53]. However, toxic side effects (nausea, myelosuppression) were noted from a dosage of 400 mg/m^2/day. This points to a rather narrow safety margin of FIAC *in vivo*.

METABOLIC CONVERSIONS

The nucleoside analogues are subject to a number of metabolic conversions: i.e. phosphorylation to the mono-, di- and triphosphate derivatives (Fig. 2). The active form of all nucleoside analogues would in fact correspond to the triphosphate, and in this form, the nucleoside analogues could interfere with several enzymes, in particular DNA polymerase. Additionally or alternatively, the nucleoside triphosphates may also act as substrates for the DNA polymerase, and hence be incorporated into DNA. Also, nucleoside triphosphates could interact allosterically with several enzymes involved in nucleotide metabolism, such as ribonucleoside diphosphate reductase or deoxythymidine (dThd) kinase. Deoxycytidine (dCyd) analogues (i.e. BVdC and FIAC) are prone to deamination, i.e. by dCyd deaminase; and dThd analogues (i.e. BVdU) are susceptible to phosphorolytic cleavage of the *N*-glycosydic linkage. Furthermore, pyrimidine nucleoside triphosphates may be degraded to the corresponding monophosphates by a deoxyribopyrimidine triphosphatase. All these enzymatic conversions are of obvious importance for the antiviral action of the compounds and will now be discussed in more detail.

DEOXYTHYMIDINE (dThd) KINASE

The selectivity of the antiherpes compounds depends primarily upon a specific phosphorylation by the virus-encoded dThd kinase. The dThd kinase specified by HSV-1, HSV-2 and VZV is also endowed with dCyd kinase activity and can, therefore, be designated as dThd(dCyd)kinase. Other virus-induced dThd kinases, however, i.e. those encoded by BHV-1 [54] and herpesvirus saimiri [55] and possibly SHV-1 [56], are devoid of dCyd kinase activity. The nucleoside analogues ACV [57], DHPG [8], BVdU [58], BVaraU [59], FIAC [59] and FMAU [59] have a much greater affinity for the HSV-1-encoded dThd(dCyd)kinase than for the cellular (cytosol) dThd kinase. This is a general phenomenon

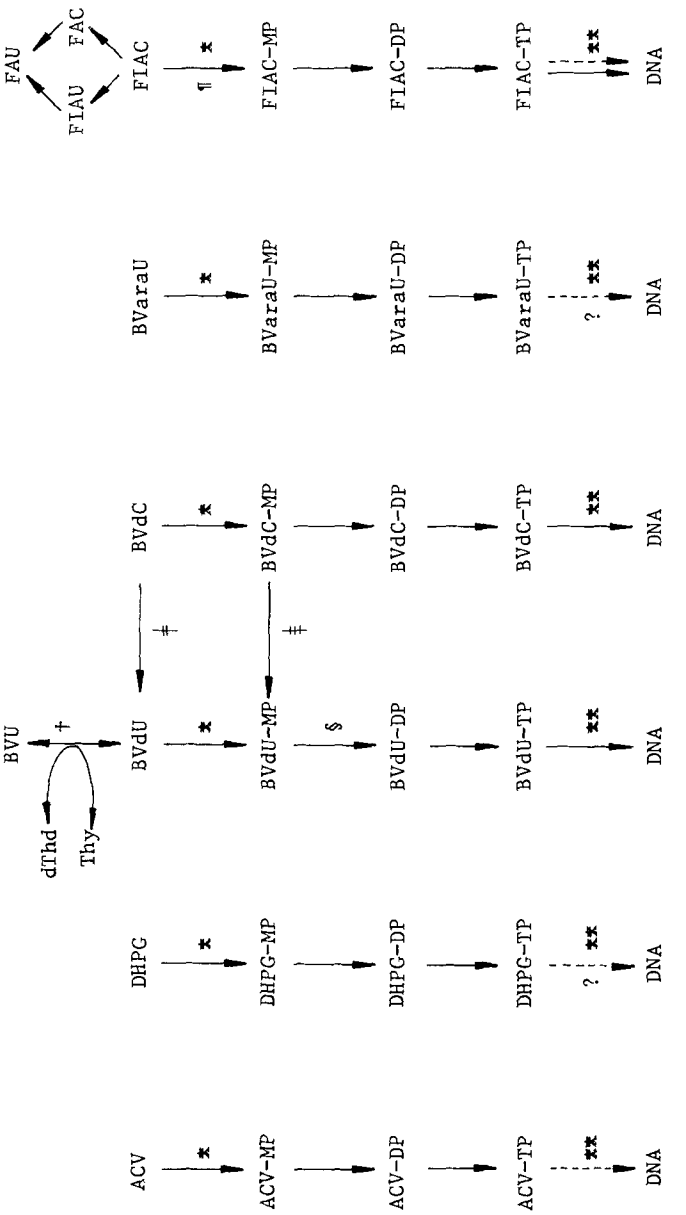


Fig. 2. Metabolic conversions of the nucleoside analogues ACV, DHPG, BVdU, BVdC, BVaraU, FIAC and FMAU. For the structural formulae of these nucleoside analogues, see Fig. 1. The abbreviations MP, DP and TP refer to the monophosphate, diphosphate and triphosphate derivatives, respectively. *Catalyzed by the virus-encoded dThd(dCyd)kinase (TK). †Completely reversible reaction catalyzed by dThd phosphorylase. ‡Catalyzed by Cyd(dCyd) deaminase, an enzyme that is blocked by tetrahydrouridine and 2′-deoxytetrahydrouridine. ‡Catalyzed by dCMP deaminase, an enzyme that is blocked by 2′-deoxytetrahydrouridine. §Catalyzed by the virus-encoded dThd(dCyd)kinase-associated dTMP kinase. ¶In addition to FIAC, its metabolites FIAU, FAC and FAU could also be phosphorylated and eventually be incorporated into DNA. **Once they have been converted to their triphosphate form, all nucleoside analogues can be incorporated into DNA. For ACV-TP, this incorporation would lead to chain termination, since ACV is incorporated at the 3′-end only. BVdU-TP is incorporated internally, whereas FIAC-TP and FMAU-TP may be incorporated within the interior (of some DNA strands) as well as at the 3′-terminal (of other DNA strands). It is not clearly established to what extent DHPG-TP and BVaraU-TP are incorporated into DNA.

that has been observed for all selective inhibitors of HSV-1, HSV-2 and VZV replication, including several compounds that are structurally related to ACV [60] or BVdU (i.e. PedU [61, 62] and BVamU [63]). As has been established with a series of ACV analogues, there is a close correlation between antiherpetic activity and the degree of phosphorylation by HSV-1 dThd kinase [60]. The enhanced antiviral activity of DHPG over ACV *in vivo* may also result at least in part from a more efficient phosphorylation by HSV-1 dThd kinase (V_{max}/K_m for DHPG is 30-fold higher than for ACV) [8].

Thus, ACV, DHPG, BVdU and their congeners act as preferential substrates for the HSV-encoded dThd(dCyd)kinase and, consequently, their phosphorylation and further action will be confined to the virus-infected cell. BVdU also serves as substrate for mitochondrial dThd kinase [58, 59], but the biological relevance of this finding is not clear.

The phosphorylation of ACV, DHPG, BVdU, BVdC, BVaraU, FIAC and FMAU to their mono-, di- and triphosphate derivatives, as shown in Fig. 2, is thus restricted to virus-infected cells; in the uninfected cell, these phosphorylations do not occur or only to a very limited, and often undetectable, extent [64–66]. Obviously, the greatly increased phosphorylation of the compounds by the virus-infected *versus* uninfected cells probably accounts, at least in part, for the selectivity of their antiviral action against HSV-1, HSV-2 and VZV. But, ACV, DHPG, BVdU, FIAC and FMAU are also active against EBV and CMV, two viruses which do not code for their own dThd(dCyd)kinase (Table 1), and BVdU, FIAC and FMAU are even effective against EBV for up to 52 days after removal of the drug from the cell cultures [11]. Apparently, BVdU, FIAC and the other nucleoside analogues can also be phosphorylated by nucleoside kinases other than specific virus-induced kinases, and these host cell kinases may somehow be activated during CMV or EBV infection. In fact, ACV is phosphorylated to its mono-, di- and triphosphate derivatives in EBV-infected cells [67].

Another curious observation which suggests that the virus-induced dThd(dCyd)kinase can be bypassed is the potent activity of ACV against murine CMV (ID_{50}: 0.23 μM) [68]. Murine CMV, like human CMV, is unable to express an own dThd kinase activity. Equally intriguing is the unexpectedly high sensitivity of the TK^- (dThd kinase deficient) murine LM and L1210 cell lines (both selected in the presence of 5-bromo-dUrd) to the growth-inhibitory effects of BVdU (ID_{50} for $LMTK^-$: 0.3 μM) [69, 70]. Possibly, the selection of TK^- cells by passage in 5-bromo-dUrd may have coselected for pathways that facilitate the phosphorylation of BVdU. To what extent BVdU is phosphorylated in these TK^- cells remains to be determined.

Of particular interest are the growth-inhibitory effects of FIAC and FMAU on human tumor cell lines (ID_{50}: 0.5–0.1 μM) and the cytotoxicity of FMAU for murine leukemic cells which are resistant to ara-C (cytosine arabinoside) [71, 72]. Apart from their implications in cancer chemotherapy, these findings, again, suggest that FIAC and FMAU can be phosphorylated by nucleoside kinases other than the virus-specific dThd(dCyd)kinase(s). In addition, FIAC and FMAU may also interfere with these nucleoside kinases, either directly or indirectly, i.e. through feedback inhibition.

Irrespective of the nature of the enzymes by which nucleoside analogues could be phosphorylated under varying conditions, it is clear that their activity against HSV and VZV is primarily dependent on a specific phosphorylation by the virus-encoded dThd(dCyd)kinase. Hence, virus mutants which are resistant to one or more nucleoside analogues may arise by mutations within the dThd(dCyd)kinase locus [73–75]. A variety of such HSV-1 mutants have been described, and they either show a reduction or total loss in their ability to induce dThd(dCyd)kinase or express a dThd(dCyd)kinase with altered substrate specificity [76–81]. HSV mutants which are completely deficient in inducing dThd(dCyd)kinase, the so-called TK^- strains, are likely to be resistant to all nucleoside analogues which require the viral dThd kinase for antiviral activity. Those HSV mutants, however, that express dThd kinase activity at a lower rate or with altered substrate specificity, may show resistance to some but not all nucleoside analogues. This means that, depending on the specific affinity of the nucleoside analogue for the mutant viral dThd kinase, compound x may still be effective against HSV mutants which are resistant to compounds y and z. Such a situation may occur with HSV mutants arising *in vivo*, i.e. in patients treated with ACV [82, 83].

DEOXYTHYMIDYLATE (dTMP) KINASE

The dThd(dCyd)kinase encoded by HSV-1 and VZV, but not the HSV-2 dThd(dCyd)kinase, has a concomitant dTMP kinase activity [84]. Thus, the multifunctional dThd(dCyd)-dTMP kinase phosphorylates dThd and close analogues of dThd, such as BVdU [85] and 5′-amino-5-iodo-2′,5′-dideoxyuridine [86], successively to their 5′-mono- and 5′-diphosphate derivatives. Whether the dThd(dCyd)-dTMP kinase also recognizes araUMP analogues, i.e. BVaraU-MP and FMAU-MP, and dCMP analogues, i.e. BVdC-MP and FIAC-MP, as substrates, is an interesting question that cannot be answered yet. In any case, ACV-MP does not serve as substrate for this virus-encoded dTMP kinase; instead, ACV-MP is phosphorylated by a GMP kinase of cellular origin [87]. As compared to ACV-MP, DHPG-MP is even a more efficient substrate for GMP kinase (V_{max}/K_m for DHPG-MP is 492-fold higher than that for ACV-MP) [8].

The phosphorylation of BVdU-MP to BVdU-DP may be a crucial and rate-limiting step in the sensitivity of HSV and VZV strains to the drug. BVdU-resistant mutants might be affected in the dTMP kinase, rather than the dThd kinase activity, of the multifunctional enzyme [88]. In particular, the low sensitivity of HSV-2 strains to BVdU could be accounted for by the failure of the HSV-2-encoded dThd kinase to phosphorylate BVdU-MP to its diphosphate [65, 85]. Indeed, BVdU and its structural analogues, IVdU, PedU, F_3PedU, BVdC, BVaraU, BVamU [15, 23, 24, 26, 63, 89], are 100- to 1000-fold less inhibitory for HSV-2 than for HSV-1,

and, based on this discriminative behavior, BVdU and its congeners could be advocated as probes for the identification of HSV-1 and HSV-2 strains in clinical isolates [15, 90–92]. Due to the lack of phosphorylation of BVdU-MP in cells infected with HSV-2, BVdU-MP may accumulate in these cells, and subsequently be converted by deoxythymidylate (dTMP) synthetase to a series of putatively inert metabolites [93]. Such sequence of events may obviously contribute to the relatively low activity of BVdU against HSV-2.

DEOXYTHYMIDYLATE (dTMP) SYNTHETASE

dTMP synthetase can be considered as the prime target for the antitumor activity of dThd analogues such as 5-fluoro-dUrd, 5-trifluoromethyl-dUrd, 5-nitro-dUrd, 5-ethynyl-dUrd, 5-(1-chlorovinyl)-dUrd, 5-formyl-dUrd and 5-oxime of 5-formyl-dUrd [94]. These compounds are far more inhibitory to dUrd incorporation into host cell DNA than to dThd incorporation, and their inhibitory effects on tumor cell proliferation are more readily reversed by dThd than by dUrd. From such conduct one may infer that 5-fluoro-dUrd and the other dThd analogues, following phosphorylation to their 5′-monophosphate derivatives, interact at the dTMP synthetase level. The dTMP synthetase reaction converts dUMP to dTMP. Inhibition of this enzyme leads to a differential availability of dThd and dUrd for final incorporation into DNA [95]. Direct measurements have confirmed that 5-fluoro-dUMP, 5-nitro-dUMP and 5-formyl-dUMP are indeed potent inhibitors of dTMP synthetase (K_i/K_m: ~0.01) [96].

Unlike 5-fluoro-dUrd, 5-nitro-dUrd, etc., BVdU is a rather poor inhibitor of tumor cell proliferation (ID_{50}: ~0.1 mM [94]) and dTMP synthetase (K_i/K_m for BVdU-MP: ~1 [96]). Moreover, the K_i of BVdU-MP for dTMP synthetase is approximately 1.2 μM [96], that is much higher than the ID_{50} of BVdU for the replication of HSV-1 and VZV (0.003 and 0.007 μM, respectively) (Table 1). Furthermore, BVdU inhibits dUrd and dThd incorporation into host cell DNA to a similar extent [14]. Taken together, these observations argue against a specific action of BVdU at the dTMP synthetase level. However, these observations concern uninfected cells only. The possibility that BVdU may interfere with dTMP synthesis in the virus-infected cell has not been ruled out, however. Neither have the other antiviral compounds (ACV, DHPG, BVaraU, FIAC, FMAU) been assessed for their potential effects on dTMP synthetase.

RIBONUCLEOTIDE REDUCTASE

Ribonucleotide reductase, the enzyme that converts the ribonucleoside diphosphates to their 2′-deoxyribonucleoside counterparts, is allosterically regulated by both ribo- and 2′-deoxyribonucleoside triphosphates. The triphosphates of BVdU, PedU and FMAU inhibit the ribonucleotide reductase from both uninfected and HSV-infected cells [97], but only so at very high concentrations ($\geq$50 μM), thus much higher than the concentrations required for inhibition of HSV and VZV replication. It is unlikely, therefore, that the antiviral activities of BVdU and the other nucleoside analogues are mediated to any significant extent through the inhibition of ribonucleotide reductase.

DEOXYTHYMIDINE (dThd) PHOSPHORYLASE

dThd and its analogues, i.e. BVdU, IVdU, PedU, F_3PedU, etc., are excellent substrates for dThd phosphorylase [98, 99]. The enzyme is normally present in the cytosol of mammalian cells, and, in particular, blood platelets, liver and spleen are very rich sources of dThd phosphorylase [100, 101]. dThd phosphorylase cleaves the N-glycosydic linkage in dThd, as follows: dThd + Pi $\rightleftharpoons$ Thy + dR-1-P. This phosphorolytic cleavage may be responsible for the rapid degradation of dThd and its analogues *in vivo*. As a consequence of the interaction of dThd phosphorylase, BVdU is cleared from the bloodstream within 2–3 hr, whereas its degradation product, BVU [(*E*)-5-(2-bromovinyl)uracil], remains in circulation for at least 24 h [102].

The rapid elimination of BVdU may obviously affect its antiviral efficacy, but, provided the degradation to BVU occurs after the compound has had the opportunity to reach its target organ(s), elimination of excessive compound may be desirable so as to prevent possible toxic side effects. On the other hand, blood levels of BVdU may be increased by using dThd phosphorylase inhibitors, such as 6-aminothymine, which counteract the phosphorolytic cleavage of BVdU to BVU [100–102]. Of great interest is the finding [102] that BVdU can be regenerated *in vivo* from BVU upon administration of dThd (or dThd analogues such as 5-iodo-dUrd, 5-fluoro-dUrd or 5-trifluoromethyl-dUrd), following the reaction: BVU + dThd $\rightleftharpoons$ BVdU + Thy. BVdU can even be generated *de novo*, starting from BVU and dThd, after both these compounds have been administered to rats [102]. This is a unique example of (re)generation of an active drug from its inactive metabolite *in vivo*. The site of this (re)generation is most probably the liver.

Since most, if not all, selective antiherpes compounds take advantage of the viral dThd kinase for their initial phosphorylation (Fig. 2), it is generally surmised that high endogenous levels of dThd would defeat the antiviral activity of these compounds by competing with the initial phosphorylation step. This assumption does not necessarily hold for BVdU, as in the presence of high dThd levels the reaction BVU + dThd $\rightleftharpoons$ BVdU + Thy may be shifted towards the formation of BVdU, and this may lead to an enhancement, rather than diminution, of the antiviral activity of BVdU.

DEOXYCYTIDINE (dCyd) DEAMINASE AND dCMP DEAMINASE

Normally, dCyd is rapidly deaminated at either the nucleoside level by dCyd deaminase (actually Cyd deaminase) or the nucleotide level by dCMP deaminase. These deaminations would also occur with dCyd analogues such as 5-bromo-dCyd, 5-iodo-dCyd [103], BVdC [24] and FIAC [104–106]. As a consequence, these dCyd analogues may eventually

be incorporated into DNA, not as such but as the corresponding dThd analogues (5-bromo-dUrd, 5-iodo-dUrd, BVdU or FIAU). In addition, FIAC and FIAU may also be deiodinated, i.e. FIAU (in its 5′-monophosphate form) by dTMP synthetase and FIAC by a yet undefined enzyme [105], and the deiodinated products, FAU and FAC, could also be incorporated into DNA.

If, however, deamination is blocked, i.e. by tetrahydrouridine (H_4Urd, a potent inhibitor of dCyd deaminase) or 2′-deoxytetrahydrouridine (H_4dUrd, a potent inhibitor of dCyd deaminase and dCMP deaminase), dCyd and its analogues may be incorporated as such into DNA. This has been directly demonstrated for dCyd [107] and the 5-halogenated derivatives of dCyd [108], and it is likely that in the presence of these deaminase inhibitors BVdC and FIAC follow a similar course. The combination of dCyd analogues, such as BVdC, with deaminase inhibitors should be worth pursuing from a therapeutic viewpoint: (*i*) like their dUrd counterparts, the dCyd analogues are recognized as substrate by the virus-encoded dThd(dCyd)kinase, which will restrict their phosphorylation to the virus-infected cell; (*ii*) unlike their dUrd counterparts, the dCyd analogues are not susceptible to phosphorolytic cleavage by dThd phosphorylase; (*iii*) as the triphosphate, the dCyd analogues would not inhibit the host ribonucleotide reductase, as do the triphosphates of some dUrd analogues (i.e. PedU [97]); and (*iv*) as the monophosphate, the dCyd analogues cannot serve as either substrate for dTMP synthetase (which may otherwise degrade the compound to some inactive metabolites, as shown for BVdU-MP [93]) or inhibitor of dTMP synthetase (which may otherwise lead to non-specific toxicity for the host cell). Following these premises [108], it would be interesting to evaluate combinations of BVdC (or FIAC) with H_4dUrd or H_4Urd in the treatment of herpesvirus infections. Based on preliminary results reported for the combination 5-bromo-dCyd plus H_4Urd in mice infected intracerebrally with HSV-1 [109], such approach may seem particularly promising.

DEOXYRIBOPYRIMIDINE (dPyTPase) TRIPHOSPHATASE

dPyTPase degrades deoxyribopyrimidine nucleoside 5′-triphosphates to the corresponding 5′-monophosphates. A virus-specific dPyTPase has been detected in the nuclei of cells infected with HSV-1 [110]. A related enzyme, dUTP nucleotidohydrolase (dUTPase), which catalyzes the hydrolysis of dUTP to dUMP, arises in both HSV-1- and HSV-2-infected cells [111]. There is a complete correlation between the ability of HSV-1 strains to induce dPyTPase activity and their susceptibility to inhibition by BVdU [112]. The significance of this correlation is at present unclear. It has not been determined to what extent BVdU-TP acts as substrate or inhibitor of dPyTPase or dUTPase. If dPyTPase (or dUTPase) recognize BVdU-TP as substrate, they should contribute to the degradation of BVdU-TP *via* BVdU-MP, but, if, on the other hand, dPyTPase (or dUTPase) are inhibited by BVdU-TP, an increase in the intracellular levels of BVdU-TP and dUTP may ensue. This should potentiate the action of BVdU at the DNA polymerase level and augment its chances of being incorporated into DNA.

DNA POLYMERASE

A primary target for the antiviral action of the nucleoside analogues is the viral DNA polymerase. To interact at the DNA polymerase level, all nucleoside analogues should first be phosphorylated to the corresponding triphosphate derivatives (Fig. 2). The inhibitory effects of these triphosphates on DNA polymerase are competitive with regard to the natural substrates; thus ACV-TP competes with dGTP, BVdU-TP with dTTP, and FIAC-TP with dCTP. As a rule, the triphosphates of the nucleoside analogues are more inhibitory to viral (HSV-1 and HSV-2) DNA polymerases than to cellular (α, β or γ) DNA polymerases [113–118]. For example, the K_i of HSV-1 DNA polymerase for ACV-TP is 0.03 μM (K_m for dGTP: 0.15 μM) as compared to 0.15 μM for cellular DNA polymerase α (K_m for dGTP: 1.11 μM) and 11.9 μM for cellular DNA polymerase β (K_m for dGTP: 7.8 μM) [118]. For BVdU-TP, the K_i values of HSV-1 DNA polymerase, cellular DNA polymerase α and cellular DNA polymerase β are 0.25, 3.6 and 16.4 μM, respectively (K_m values for dTTP: 0.66, 5.3 and 17.8, respectively) [114]. For FIAC-TP, the K_i values of HSV-1 DNA polymerase, cellular DNA polymerase α and cellular DNA polymerase β are 0.26, 2.7 and 7.9 μM, respectively (K_m values for dCTP: 0.4, 4.3 and 6.2, respectively) [117]. The nucleoside analogue triphosphates are equally inhibitory to HSV-1 and HSV-2 DNA polymerases [116]. This is also the case for BVdU-TP and BVaraU-TP, which excludes the viral DNA polymerase as the site of action for the differential inhibitory effects of BVdU and BVaraU on the multiplication of HSV-1 and HSV-2 strains. While ACV-TP, BVdU-TP and FIAC-TP are strongly inhibitory to HSV-1 and HSV-2 DNA polymerase, they are much less so to EBV DNA polymerase (K_i for ACV-TP, BVdU-TP and FIAC-TP: 9.8, 16.1 and 32.2 μM, respectively [114, 117, 118]).

Thus, the assumption that the viral DNA polymerase is a primary target for the antiviral action of ACV, BVdU and FIAC may hold for HSV-1, HSV-2 (and several other herpesviruses), but not for EBV. That the anti-HSV activity of ACV, BVdU and the other nucleoside analogues may be targeted at the viral DNA polymerase is further supported by the existence of drug-resistant HSV mutants with mutations located within the DNA polymerase gene [73–75, 78, 119]. These HSV mutants express an altered DNA polymerase that is no longer inhibited by ACV-TP or BVdU-TP. The region specifying ACV- and BVdU-resistance within the DNA polymerase locus has actually been defined: ACV-resistance is located within map units 40.2–41.8, whereas BVdU-resistance is located within map units 41.0–42.8 [75, 119]. The DNA polymerase locus is clearly distinct from the HSV dThd kinase locus which is found within the map limits 0.27–0.35. Since the regions conferring resistance to BVdU and ACV (within the DNA polymerase locus) overlap only partially, resistance to one drug may not necessarily

implicate cross-resistance to other drugs. This situation is similar to that described above for the dThd kinase-based mutants.

INCORPORATION INTO DNA

While the nucleoside analogue triphosphates act as competitive inhibitors of DNA polymerase in the presence of the natural substrates (i.e. dGTP, dTTP, dCTP), they are able to support DNA synthesis in the absence of competing substrates. Thus, BVdU-TP and its structural analogues IVdU-TP and PedU-TP serve as excellent alternate substrates for DNA polymerase (irrespective of the source of the latter: viral, mammalian or bacterial [116, 120, 121]). Also, 5-*n*-alkyl-dUrd triphosphates, such as 5-ethyl-dUTP, 5-propyl-dUTP, 5-butyl-dUTP, 5-pentyl-dUTP and 5-hexyl-dUTP, and 5-iso-alkyl-dUrd triphosphates, such as 5-isopropyl-dUrd and 5-*tert*-butyl-dUTP, are efficient substrates for viral, mammalian and bacterial DNA polymerases [122, 123]. In marked contrast, 5-substituted araUTP analogues, such as 5-ethyl-araUTP, 5-propyl-araUTP, 5-butyl-araUTP, 5-fluoro-araUTP, 5-chloro-araUTP, 5-bromo-araUTP and 5-iodo-araUTP, do not replace dTTP as a substrate but inhibit DNA polymerase activity [124, 125]. Neither does BVaraU-TP act as an alternate substrate for DNA elongation [26, 116]. FMAU-TP supports DNA synthesis very little (not more than 5% over background [116]), and FIAC-TP could be utilized to a limited extent (<50%) only by viral DNA polymerase [116, 117]. ACV-TP can also be used as substrate instead of dGTP, and, like FIAC-TP, ACV-TP is a much more efficient substrate for viral than for cellular DNA polymerase [113].

Thus, ACV-TP, BVdU-TP, FIAC-TP and, to a lesser extent, FMAU-TP can serve as alternate substrate for DNA polymerase and hence be incorporated into DNA. Since the initial phosphorylation of ACV, BVdU, FIAC and FMAU is confined to the virus-infected cell, the eventual incorporation into DNA will also be restricted to DNA of the virus-infected cell. If used at supraoptimal concentrations (30 μM), BVdU is incorporated into both viral and cellular DNA of the HSV-1-infected cell [121], but if used at optimal concentrations (0.05–0.5 μM), BVdU is incorporated into viral DNA only [126]. Since FIAC-TP and ACV-TP are preferentially utilized as alternate substrate by the viral DNA polymerase [113, 116, 117], they may also be incorporated exclusively into viral DNA if present at the appropriate concentrations.

BVdU is incorporated *via* an internucleotide linkage, whereas ACV has to be incorporated at the 3′-terminal of DNA, since it does not offer the necessary 3′-hydroxyl group for further chain elongation. FIAC and FMAU may be incorporated internally in some DNA strands as well as at the 3′-terminals of other DNA strands [127]. Not being a substrate for DNA polymerase, BVaraU-TP will not be incorporated into the growing DNA chain; however, it may be incorporated at the 3′-end. To what extent DHPG is incorporated has not been determined yet. Hypothetically, DHPG offers the required 3′-hydroxyl group for further chain elongation. It is questionable, however, whether this hydroxyl group is in the right configuration to allow a phosphodiester linkage with the next nucleotide. Like ACV, DHPG may be expected to be incorporated at the 3′-terminal.

The incorporation of the nucleoside analogues into DNA will have varying consequences, depending on the nature of the drug which has become incorporated. For ACV, which is incorporated at the 3′-end (and not excised again by the DNA polymerase-associated 3′,5′-exonuclease [115]), the incorporation prevents further chain elongation; thus, ACV acts as a chain terminator and this leads to the accumulation of short DNA fragments [128]. FIAC and FMAU, after incorporation, may either act as chain terminators or allow chain elongation to proceed. When incorporated in the polynucleotide chains, they would lead to an increased susceptibility of the fraudulent DNA towards degradation by nucleases, *viz.* viral-specified DNase [127]. Incorporation of BVdU into viral DNA makes the DNA more labile, as is suggested by a dose-dependent increase in single strand breaks [126]; furthermore, the substitution of BVdU for dThd impairs the template activity for RNA synthesis [129]. This means that BVdU-substituted DNA may be more prone to degradation and less functioning for transcription. Anyhow, the incorporation of BVdU into HSV-1 DNA is crucial for its antiviral activity, since the extent of virus yield reduction is closely correlated with the amount of BVdU substituted for dThd [126]. Likewise, the antiviral action of ACV, FIAC and the other selective antiherpes drugs may be attributed at least in part to their incorporation into viral DNA. This generalization may be extended to herpesviruses other than HSV, i.e. EBV and CMV.

The incorporation into host cell DNA could be considered as an undesirable feature of an antiviral drug, since it implicates an array of toxic side effects such as chromosomal damage, mutagenesis, teratogenesis, carcinogenesis, induction of oncogenic viruses, inhibition of differentiation, etc. These toxic side effects have been well documented for the 5-halogenated deoxyuridines, 5-iodo-dUrd and 5-bromo-dUrd [130]. These are two dThd analogues which are readily incorporated into DNA of uninfected cells. However, the selective antiherpes drugs reviewed here, and in particular ACV and BVdU, are not incorporated to a significant extent into DNA of normal uninfected cells. Their incorporation is essentially limited to viral DNA of virus-infected cells. Hence, these compounds should be devoid of any of the above mentioned side effects if used at therapeutically effective doses. Indeed, BVdU has proven not to be mutagenic or teratogenic. It does not trigger the release of oncogenic RNA virus particles under conditions where 5-iodo-dUrd acts as a proficient inducer of such particles [131], and it does not cause chromosomal damage (as monitored by the rate of sister chromatid exchange) except at a concentration (>100 μM) which is far in excess of the minimal antiviral concentration (0.003 μM: see Table 1) [132, 133]. Other dThd analogues (i.e. 5-trifluoro-dThd) increase the rate of sister chromatid exchange at concentrations that coincide with their minimal antiviral concentrations.

CONCLUSION

At the time of Prusoff's previous commentary on "Nucleoside analogs with antiviral activity" [134], we were just on the eve of a new era in the search for antiviral drugs. Quite fittingly, Prusoff and Ward gave a painstaking list of all the characteristics of the so-called "ideal" antiviral drug, thereby emphasizing the need for compounds with little or no host toxicity. Eight years later, we have at our disposal not one but several antiviral compounds which are perhaps not "ideal" but come close to it. This new generation of nucleoside analogues includes ACV, DHPG, BVdU, BVdC, BVaraU, FIAC and FMAU. They are all directed towards herpesviruses (HSV-1, HSV-2, VZV, CMV and EBV) and in their antiviral action they surpass the "classical" antiviral drugs such as 5-iodo-dUrd and 5-trifluoro-dThd in both potency and selectivity.

The mechanism of action of these new antiherpes agents against CMV and EBV is not fully understood. However, the basis for their selective activity against HSV-1, HSV-2 and VZV has been unraveled to a considerable extent. Of prime importance for the selectivity of these agents is a specific phosphorylation by the virus-induced dThd kinase, which restricts the further action of the nucleoside analogues to the virus-infected cell. After they have been converted to the triphosphate derivatives, the compounds may interact with viral DNA polymerase as either inhibitor or substrate. If acting as substrate, they will be incorporated into DNA. Both events would lead to a fundamental disturbance of viral DNA replication.

From the survey of all the enzymes which could serve as targets for the antiviral action of the nucleoside analogues, it is clear that, to exert a selective antiherpes activity, the nucleoside analogues should interact with some well-defined enzymes, i.e. viral dThd kinase, viral (or cellular) dTMP kinase and dTDP kinase, and viral DNA polymerase, while avoiding others, i.e. nucleoside phosphorylase, dTMP synthetase, and the dThd kinase and DNA polymerase of cellular origin. The antiviral potency and selectivity of the drugs will, to a large extent, depend on their capacity to distinguish between these different enzymes.

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