Efficacy of antiherpetic drugs in combined gene/chemotherapy of cancer is not affected by a specific nuclear or cytoplasmic compartmentation of herpes thymidine kinases

Research Article

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Abbreviations

AA, amino acid; **ACV**, 9-(2-hydroxyethoxymethyl)guanine (acyclovir); **araT**, 1- -D-(arabinofuranosyl)thymine; **BCV**, (*R*)-9-[(3,4-dihydroxybutyl)guanine] (buciclovir); **BVDC**, (*E*)-5-(2-bromovinyl)-2'-deoxycytidine; **BVaraU**, (*E*)-5-(2-bromovinyl)-1- -D-arabinofuranosyluracil; **BVDU**, (*E*)-5-(2-bromovinyl)-2'-deoxyuridine; **FIAC**, 1-(2'-fluoro-2'-deoxy- -D-arabinofuranosyl)-5-iodocytosine; **FMAU**, 1-(2'-fluoro-2'-deoxy-1- -D-arabinofuranosyl)-5-methyluracil; **GCV**, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (ganciclovir); **GFP**, green fluorescent protein; **HSV-1 and HSV-2**, herpes simplex virus type 1 and herpes simplex virus type 2; **LBV**, (*R*)-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine (lobucavir, cyclobut-G, BMS180194); **NLS**, nuclear localization signal; **PCV**, 9-[4-hydroxy-3-(hydroxymethyl)but-1-yl]guanine (penciclovir); **S-BVDU**, (*E*)-5-(2-bromovinyl)-2'-deoxy-4'-thiouridine; **VZV**, varicella-zoster virus.

Summary

Introduction of the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) gene in tumor cells, followed by treatment of the transfected tumor cells with an antiherpes drug has shown promise in the treatment of solid tumors. We have recently shown that the HSV-1 TK fused to green fluorescent protein (GFP) was localized almost exclusively in the nuclei of HSV-1 TK-GFP fusion gene-transfected human osteosarcoma cells, due to the presence of a nuclear localization signal (NLS) at the N-terminus of the HSV-1 TK. A deletion mutant, lacking the N-terminal 34 amino acids [Δ(AA1-34)HSV-1 TK-GFP], was distributed throughout the cytoplasm and nucleus of transfected tumor cells. In addition, varicella-zoster virus (VZV) TK-GFP, which lacks the NLS and which is uniformly distributed in the nucleus and cytoplasm of the VZV TK-GFP gene-transfected tumor cells, could be specifically targeted to the nucleus by ligating the HSV-1 TK nuclear localization signal to the VZV TK-GFP sequence. Two pairs of osteosarcoma cell lines stably expressing HSV-1 TK-GFP or VZV TK-GFP either in the nucleus or throughout the cell were established and compared for their sensitivity to the cytostatic effects of a variety of antiherpetic nucleoside analogues. In addition, the efficacy of nucleoside analogues in contributing to the bystander effect (i.e., the killing of non-transfected tumor cells by neighbouring TK gene-transfected cells after gap junctional transfer of phosphorylated nucleoside metabolites), was evaluated using the HSV-1 TK-GFP and Δ(AA1-34)HSV-1 TK-GFP gene constructs. From our experiments it is inferred that there is no difference in cytostatic activity of the antiherpetic

nucleoside analogues against TK gene-transfected cells, whether the TK activity is solely localized in the nucleus or spread over the nucleus and cytosol. Also, the bystander killing effect of the antiviral compounds was independent of the nature of the intracellular compartment in which the HSV-1 TK-GFP fusion protein was expressed.

I. Introduction

The broad substrate specificity of the thymidine kinase (TK) of most herpes viruses, including herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2) and varicella-zoster virus (VZV), can be exploited in the treatment of herpesvirus infections (De Clercq, 1993; 1995). Exquisitely potent antiherpetic nucleoside analogues including (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) and ganciclovir (GCV) have been developed, which owe their selective antiviral activity to their specific phosphorylation by the herpetic TK but not by the mammalian TK (**Figure 1**). This concept of the herpetic TK-dependent cytostatic effect of otherwise non-toxic nucleoside analogues was later introduced in the field of anticancer research. Balzarini and coworkers reported on the highly selective cytostatic activity of the antiherpetic drugs GCV and BVDU, and various structurally related derivatives thereof, against murine mammary carcinoma (FM3A) cells transfected with the HSV-1 or HSV-2 TK gene (Balzarini et al., 1985, 1987, 1993, 1994). Differences were found in the cytostatic potency of the drugs depending on the nature of the suicide gene (i.e. HSV-1 or HSV-2 TK gene). In 1992, Culver and coworkers showed complete regression of established brain tumors in rats after *in situ* transduction with the HSV-1 TK gene and subsequent treatment with GCV (Culver et al., 1992). Several clinical trials, all utilizing the HSV-1 TK/GCV system, are underway to assess the safety and efficacy of this combined gene/chemotherapy treatment for cancer (Oldfield et al., 1993; Culver et al., 1994; Freeman et al., 1995; Kun et al., 1995; Raffel et al., 1994). Recently, we demonstrated that VZV TK and a variety of pyrimidine nucleoside analogues represent appropriate alternatives for the HSV-1 TK/GCV combination therapy (Degrève et al., 1997).

We also recently studied the intracellular localization of HSV-1, HSV-2 and VZV TK (Degrève et al., 1998). The herpetic TKs were expressed as fusion proteins with the green fluorescent protein (GFP) (Chalfie et al., 1994; Rizzuto et al., 1995; Youvan et al., 1996) in human OstTK⁻ cells and their intracellular localization was examined using a fluorescence microscope. HSV-1 TK fused with GFP was almost exclusively localized in the nuclei of HSV-1 TK-GFP gene-transfected tumor cells. In contrast, introduction of the HSV-2 TK-GFP fusion gene gave rise to predominant cytosolic fluorescence. VZV TK-GFP showed a uniformly distributed fluorescence pattern. When the N-terminal 34 amino acids (AAs) were deleted

from the HSV-1 TK-GFP construct, the resulting mutant fusion protein lost its specific nuclear localization. We proved that this 34 amino acid stretch was also capable of targeting VZV TK-GFP and GFP to the nucleus of gene-transfected OstTK⁻ cells, indicating that a nuclear localization signal (NLS) was present in this N-terminal part of HSV-1 TK. By site-directed mutagenesis of each of the positively charged amino acids at the N-terminus of HSV-1 TK, we were able to identify a nonapeptide, [25]R-R-T-A-L-R-P-R-R[33], which is strictly required for specific nuclear localization of HSV-1 TK (Degrève et al., 1998).
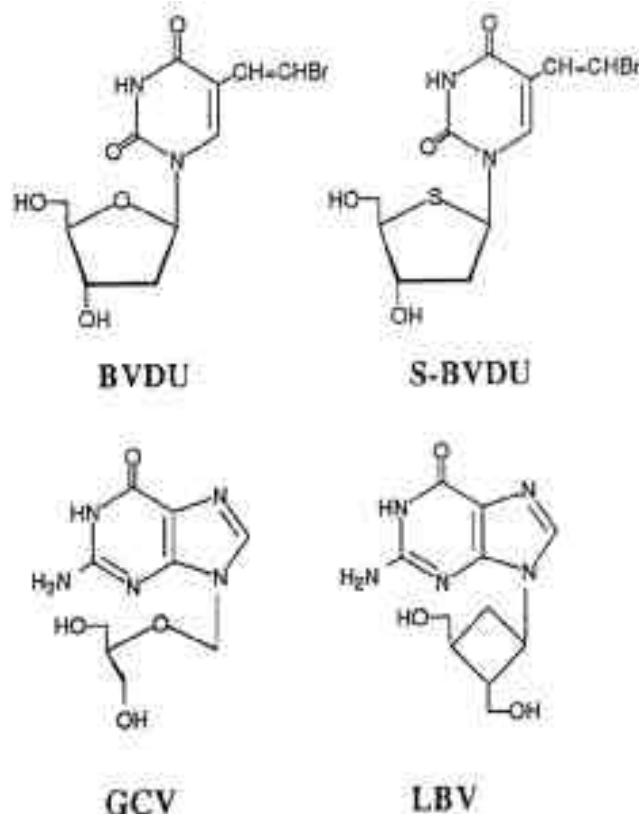

Figure 1. Structural formulae of 4 representative test compounds.

The expression of HSV-1, HSV-2 and VZV TK in different intracellular localizations prompted us to investigate whether the intracellular localization of a particular TK would influence the cytostatic effects of antiherpetic nucleoside analogues. Therefore, a series of antiherpetic pyrimidine and purine nucleoside analogues were evaluated for their inhibitory activity on the proliferation of OstTK⁻ cells expressing either the nucleus-targeted (wild-type) HSV-1 TK fused to GFP, the uniformly distributed (AA1-34)HSV-1 TK-GFP (lacking the first 34 amino acids that contain the nuclear targeting signal nonapeptide), the wild-type uniformly distributed VZV TK-GFP and the nucleus-targeted NLS-VZV TK-GFP (containing the HSV-1 TK AA1-34 NLS). We have also recently explored the ability of a variety of purine and pyrimidine nucleoside analogues to exert a bystander killing effect in mixed tumor cell populations (Degrève et al., 1999), i.e. the potency of the compounds to kill TK⁻ tumor cells that are neighbouring HSV-1 TK-GFP gene-transfected cells upon gap junctional transfer of the phosphorylated compounds. We showed that purine nucleoside analogues (represented by GCV) have a far more pronounced bystander killer effect than pyrimidine nucleoside analogues (represented by BVDU), regardless of their potent inhibitory potential against the HSV-1 TK-GFP gene-transfected tumor cells. We have now evaluated the impact of intracellular compartmentation (ie nucleus or cytoplasm) of HSV-1 TK-GFP on the bystander effect of purine and pyrimidine nucleoside analogues.

Our experimental data revealed that the intracellular localization of HSV-1 TK-GFP or VZV TK-GFP expression has no significant influence on either the cytostatic effect or the bystander effect of antiviral nucleoside analogues. These findings argue against the compartmentation of nucleotide pools in mammalian cells and suggest that phosphorylated nucleoside anabolites can rapidly equilibrate between the nuclear and cytosolic compartments of the cell.

II. Results
A. Intracellular targeting of HSV-1 TK-GFP and VZV TK-GFP constructs

The HSV-1 TK-GFP and (AA1-34)HSV-1 TK-GFP gene constructs were stably introduced in OstTK⁻ cells and the fluorescence pattern was subsequently visualized under a fluorescence microscope. The OstTK⁻/HSV-1 TK-GFP⁺ cell line, as described earlier by Degrève et al. (1998), expresses the wild-type HSV-1 TK-GFP fusion protein, which is targeted to the nucleus of the transfected cells (**Figure 2**, panel A). In contrast, OstTK⁻/ (AA1-34)HSV-1 TK-GFP⁺ cells express an N-truncated form of HSV-1 TK-GFP in both the nucleus and cytosol (panel B). Transfection of the VZV TK-GFP gene construct (panel C)

gave rise to a uniformly distributed fluorescence pattern. Finally, ligation of the HSV-1 TK nuclear localization signal to the VZV TK-GFP construct resulted in a nuclear fluorescence pattern (panel D).

B. Effect of intracellular localization of HSV-1 TK-GFP and VZV TK-GFP on the cytostatic activity of antiviral compounds

The cytostatic activity of a series of the antiherpetic pyrimidine and purine nucleoside analogues was evaluated against OstTK⁻ cells stably expressing either HSV-1 TK-GFP, (AA1-34)HSV-1 TK-GFP, VZV TK-GFP or NLS-VZV TK-GFP. Non-transfected OstTK⁻ cells were included as a control. The selection of the compounds was based on previous studies on HSV TK and VZV TK gene-transfected tumor cells in our laboratory (i.e. the protoype antiherpetic pyrimidine nucleoside analogue (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) and its closely related derivatives S-BVDU, BVaraU and BVDC, the antiherpetic thymidine and cytidine analogues araT, FMAU and FIAC, the acyclic guanosine analogue 9-(1,3-dihydroxy-2-propoxymethyl) guanine (ganciclovir, GCV) and its derivatives ACV, BCV, LBV and PCV (Balzarini et al., 1985, 1987, 1993, 1994; Degrève et al., 1997). The structural formulae of 4 representative antiherpes nucleoside analogues are shown in **Figure 1**. Results are summarized in **Table 1**. The pyrimidine nucleosides BVDU, S-BVDU and BVaraU inhibited non-transfected OstTK⁻ cell proliferation only at concentrations that exceeded 850μM. BVDC and araT showed 50% inhibitory concentrations (IC_{50}) still above 200 μM, while FIAC and FMAU were more inhibitory to the proliferation of OstTK⁻ cells (IC_{50} values of 9 and 17 μM, respectively). In sharp contrast, the pyrimidine nucleoside analogues became exquisitely inhibitory after transfection of the osteosarcoma cells with the HSV-1 TK-GFP, (AA1-34)HSV-1 TK-GFP, VZV TK-GFP and NLS-VZV TK-GFP genes. The IC_{50} values for the individual compounds were essentially comparable for the two HSV-1 TK-GFP constructs and the two VZV TK-GFP constructs, except for FIAC which displayed a six-fold lower inhibitory effect against OstTK⁻/ (AA1-34)HSV-1 TK-GFP⁺ cells than against OstTK⁻/HSV-1 TK-GFP⁺ cells. BVDU and BVDC (IC_{50} values for TK-GFP gene-transfected cells ranging from 0.035 to 0.36 μM) exhibited 50% inhibitory concentrations that were approximately 10-fold higher than those for the other pyrimidine nucleoside analogues, which were in the lower nanomolar concentration range. The highest selectivity indices (i.e. the ratio of the IC_{50} value for non-transfected cells *versus* the IC_{50} value for TK-GFP gene-transfected cells) were observed for BVaraU (up to 250,000), S-BVDU (up to 150,000) and AraT (up to 100,000). BVDU was intermediate (selectivity index of 20,000), while BVDC, FIAC and FMAU were 1,000 to 6,000-fold more

cytostatic to the various HSV-1 TK-GFP fusion gene-transfected cells than to non-transfected OstTK⁻ cells.

The purine nucleoside analogues that were included in our study exhibited 50% inhibitory concentrations for the growth of non-transfected OstTK⁻ cells ranging from 18 μM (LBV) to 231 μM (PCV) (**Table 1**). GCV, BCV and PCV showed IC_{50} values in the nanomolar concentration range for OstTK⁻/HSV-1 TK-GFP⁺ and OstTK⁻/ (AA1-34)HSV-1 TK-GFP⁺ cells, that is at concentrations that were 15,000 to 47,000-fold lower than the concentrations required to inhibit the proliferation of the wild-type OstTK⁻ cells. ACV, which displayed the highest IC_{50} value among all antiherpetic nucleoside analogues (up to 0.14 μM) and LBV (due to its stronger inhibitory effect against non-transfected OstTK⁻ cells) ranked among the compounds with the lowest selectivity index (1,000 and 2,000, respectively). As proved to be the case with the pyrimidine
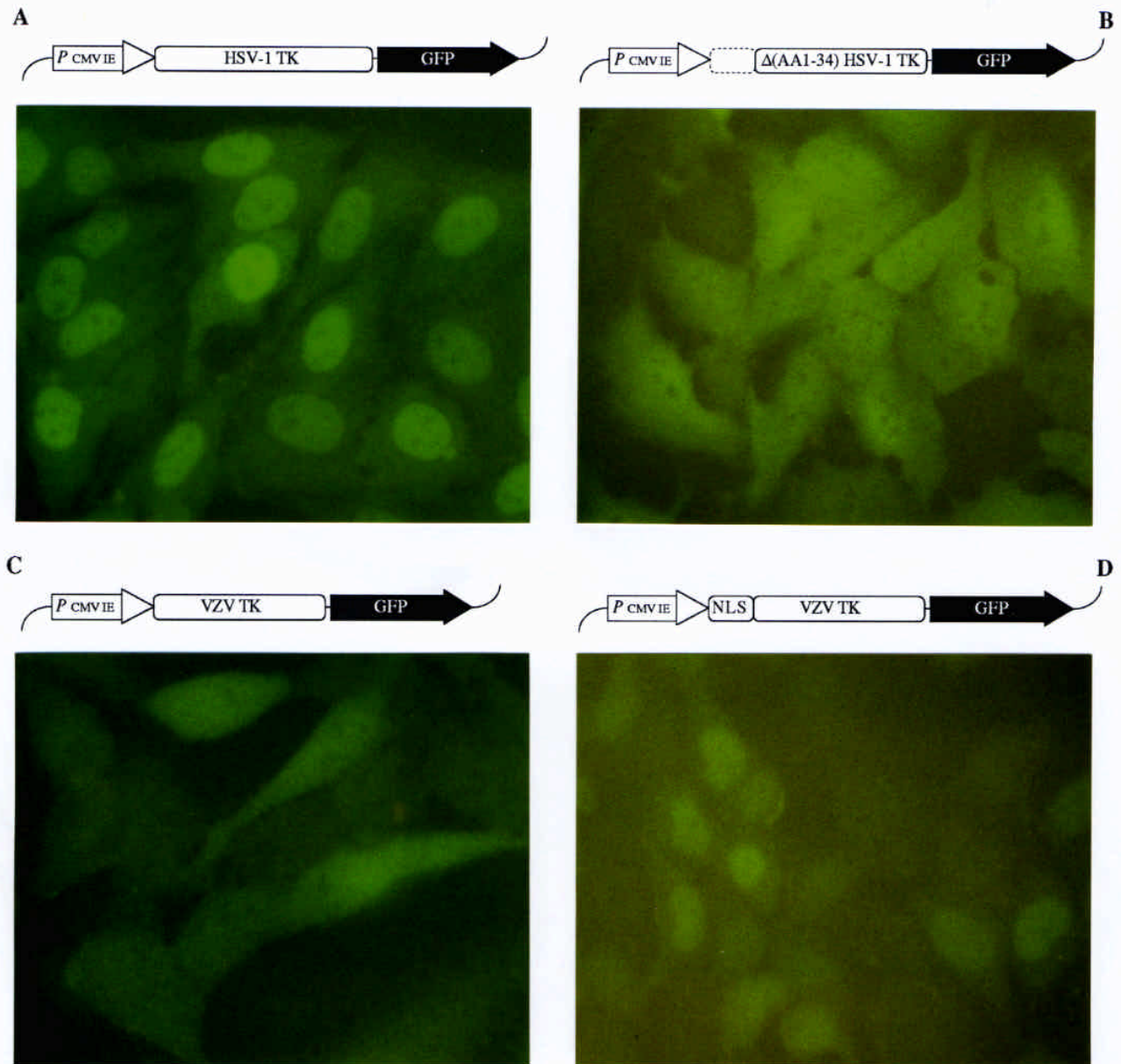

Figure 2. The HSV-1 TK-GFP and VZV TK-GFP fusion constructs (shown on top of each picture) were transfected into OstTK⁻ cells. After selection of stable transfectants, the fluorescence pattern was evaluated using a FITC filter-equipped fluorescence microscope. (A) HSV-1 TK-GFP; (B)   (AA1-34)HSV-1 TK-GFP; (C) VZV TK-GFP; (D) NLS-VZV TK-GFP.

Table 1. Cytostatic activity of nucleoside analogues against wild-type (OstTK⁻) and TK-GFP gene-transfected OstTK⁻ cells

	IC$_{50}$ (μM)[a]				
	OstTK⁻	OstTK⁻/HSV-1 TK-GFP⁺	OstTK⁻/ (AA1-34) HSV-1 TK-GFP⁺	OstTK⁻/VZV TK-GFP⁺	OstTK⁻/NLS-VZV TK-GFP⁺
BVDU	862 ± 192 [b]	0.035 ± 0.006 [b]	0.038 ± 0.022	0.091 ± 0.055	0.36 ± 0.13
S-BVDU	911 ± 105 [b]	0.008 ± 0.004 [b]	0.006 ± 0.001	0.007 ± 0.000	0.028 ± 0.026
BVaraU	942 ± 47 [b]	0.004 ± 0.001 [b]	0.004 ± 0.002	0.009 ± 0.017	0.029 ± 0.024
BVDC	209 ± 35 [b]	0.059 ± 0.019 [b]	0.10 ± 0.00	1.6 ± 0.7[c,d]	-
araT	231 ± 27 [b]	0.004 ± 0.0006 [b]	0.002 ± 0.000	0.78 ± 0.41 [c,d]	-
FIAC	9.1 ± 6.7	0.002 ± 0.0001	0.012 ± 0.001	-	-
FMAU	17 ± 0.5	0.006 ± 0.0001	0.004 ± 0.002	-	-
GCV	44 ± 22 [b]	0.001 ± 0.0005 [b]	0.003 ± 0.002	6.3 ± 7.3	14 ± 4
ACV	73 ± 29 [b]	0.059 ± 0.015 [b]	0.14 ± 0.04	48 ± 12 [d]	-
BCV	173 ± 67 [b]	0.006 ± 0.0000 [b]	0.004 ± 0.001	57 ± 10 [d]	-
LBV	18 ± 0.4 [b]	0.008 ± 0.0008 [b]	0.008 ± 0.0002	4.4 ± 1.0 [d]	-
PCV	231 ± 13 [b]	0.013 ± 0.0022 [b]	0.009 ± 0.001	27 ± 4 [d]	-

[a] The IC$_{50}$ was defined as the drug concentration required to inhibit cell proliferation by 50%. Data are the mean value (± SD) for at least 3 independent experiments.

[b] Data taken from Degrève et al. (1999).

[c] Data taken from Degrève et al. (1997), where non-fused VZV TK gene-transfected OstTK⁻ cells were evaluated.

nucleoside analogues, the IC$_{50}$ values of the purine nucleoside analogues did not depend on the intracellular compartment in which the TK was localized (**Table 1**). The poor cytostatic effect of ganciclovir against OstTK⁻ cells expressing VZV TK-GFP and NLS-VZV TK-GFP was not unexpected, since this drug has poor, if any, affinity for VZV TK (Degrève et al., 1997).

C. Bystander effect

The bystander effect of two pyrimidine (BVDU and S-BVDU) and two purine (GCV and LBV) nucleoside analogues was evaluated. We have recently demonstrated the superior bystander effect of purine *versus* pyrimidine nucleoside analogues in mixed OstTK⁻ and OstTK⁻/HSV-1 TK-GFP⁺ tumor cell populations (Degrève et al., 1999). Mixed tumor cell populations were cultured in the presence of 5-fold dilutions of the test compounds, after which the viable cell number was assessed using a colorimetric assay, as described in Materials and methods (**Figure 3**). The thick line in each graph represents the theoretically predicted values, in case no bystander effect is active (for

example, 25% non-transfected cells in the mixed tumor cell culture should result in 25% cell viability at the end of the 3-day incubation period in the presence of a lethal concentration of the nucleoside analogue). As shown in **Figure 3**, the inefficient bystander effect exerted by BVDU and S-BVDU was not enhanced by changing the intracellular HSV-1 TK-GFP localization. The very weak bystander effect of BVDU in OstTK⁻/HSV-1 TK-GFP⁺ cells was even completely absent in OstTK⁻/ (AA1-34)HSV-1 TK-GFP⁺ cells. For S-BVDU, the cell viability curves, obtained using a colorimetric assay, exactly reflected the percentages of OstTK⁻ and HSV-1 TK-GFP gene-transfected tumor cells (**Figure 3**). In sharp contrast with the pyrimidine nucleosides, the guanosine nucleoside analogues GCV and LBV exhibited a pronounced bystander effect which was dose-dependent. LBV was not tested at 50μM because of profound inhibition of OstTK⁻ cell growth at this concentration (IC$_{50}$ value, 18μM). Even at the lowest concentration tested (2 μM), bystander killing was still observed with GCV and LBV. At a concentration
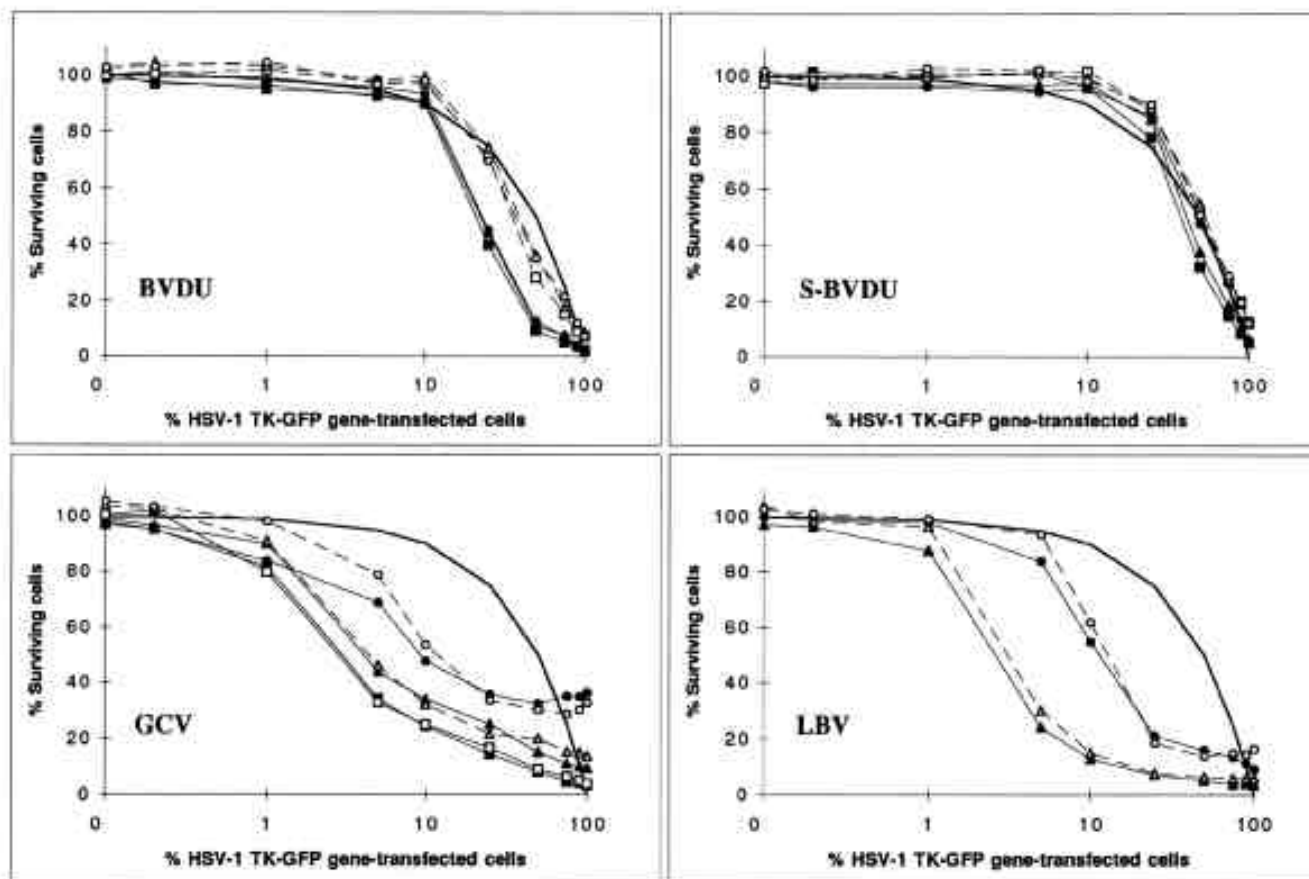
Figure 3. Bystander effect of nucleoside analogues in mixed cell cultures. The thick line in each graph represents the theoretically predicted values, in case no bystander effect was noted. Concentrations tested, 50 μM (squares), 10 μM (triangles), 2μM (circles). OstTK⁻ were mixed with OstTK⁻/HSV-1 TK-GFP⁺ cells (black symbols, data taken from Degrève et al., 1999) or OstTK⁻/ (AA1-34)HSV-1 TK-GFP⁺cells (open symbols).

of 10μM, the bystander effect of LBV was 2- to 3-fold more pronounced than that of the prototype compound GCV. Abolishing the specific nuclear localization of HSV-1 TK-GFP by deleting the N-terminal NLS, had no significant impact on the bystander effect of GCV and LBV, that was observed in the tumor cell cultures that expressed the HSV-1 TK-GFP solely in the nucleus (**Figure 3**).

III. Discussion

We recently reported the differential intracellular localization of the TKs of three herpesviruses, i.e. HSV-1, HSV-2 and VZV in TK-GFP fusion gene-transfected osteosarcoma cells (Degrève et al., 1998). The TK-GFP fusion proteins were localized in the nucleus of HSV-1 TK-GFP gene-transfected tumor cells, in the cytosol of HSV-2 TK-GFP gene-transfected tumor cells and in both the nucleus and the cytosol of VZV TK-GFP gene-transfected tumor cells. The N-terminal 34 amino acids of HSV-1 TK, the deletion of which resulted in the loss of specific nuclear localization of HSV-1 TK-GFP, were also sufficient to target the otherwise uniformly distributed VZV TK-GFP to the nucleus of gene-transfected cells. In the experiments described in this report, we evaluated whether the intracellular localization of HSV-1 TK-GFP or VZV TK-GFP would influence the cytostatic potential and bystander effect of the antiherpetic nucleoside analogues in TK-GFP gene-transfected osteosarcoma cells. As shown in **Table 1**, all evaluated nucleoside analogues showed exquisite cytostatic properties against HSV-1 TK-GFP and

VZV TK-GFP expressing tumor cells, the lowest 50% inhibitory concentrations being in the lower nanomolar range. The pronounced cytostatic effect of pyrimidine nucleosides like S-BVDU and BVaraU makes them promising candidate compounds for the combined gene/chemotherapy treatment of cancer, with selectivity indices markedly higher than that of GCV, the current drug of choice for HSV-1 TK gene-mediated tumor cell killing. Moreover, S-BVDU and BVaraU are resistant to glycosidic bond cleavage by mammalian dThd phosphorylases, a major advantage compared to the cleavage-susceptible parent compound BVDU.

However, one should also take the bystander effect into account. The bystander effect was described as the ability of a drug to kill non-transfected tumor cells that were in close contact with HSV-1 TK gene-transfected cells in mixed tumor cell populations. Complete tumor eradication has been demonstrated with GCV even when as few as 10% of the tumor cell inoculum was transfected with the HSV-1 TK gene (Culver et al., 1992; Ram et al., 1993; Freeman et al., 1993). The succes of the combined herpesviral TK gene/chemotherapeutic approach seems to depend on the bystander effect, since current gene therapy vectors are not capable of introducing the viral thymidine kinase gene in 100% of the cells of a particular tumor. Instead, getting 1% of the tumor cells transfected is a more realistic goal. Moreover, the low fraction of herpesviral TK gene-transfected tumor cells should be uniformly distributed in the tumor to yield an optimal bystander effect, which is virtually impossible to achieve. We recently demonstrated that the *in vitro* bystander effect of purine nucleoside analogues was superior to that of pyrimidine nucleoside analogues in mixed OstTK⁻ and OstTK⁻/HSV-1 TK-GFP⁺ cell cultures. The bystander effect exerted by pyrimidine nucleoside analogues (i.e., BVDU and derivatives) proved to be very ineffective or even absent in most cases. In contrast, most of the evaluated purine nucleoside analogues (in particular GCV and LBV) displayed potent bystander killing potencies. Therefore, the lower selectivity index of purine nucleoside analogues like GCV and LBV is well compensated by their superior bystander killing as compared to pyrimidine nucleoside analogues.

It is clear from **Table 1** that the cytostatic activities of the evaluated compounds were generally independent of the compartmentation of the HSV-1 TK-GFP in the cell. We could also conclude from our experiments that the N-terminal 34 amino acids of the HSV-1 TK are not important for enzyme activity, since the (AA1-34)HSV-1 TK-GFP fusion protein was fully catalytically active in gene-transfected tumor cells. These findings corroborate the observation of Halpern and Smiley (1984) that the N-terminal 45 amino acids are not required for the catalytic activity of HSV-1 TK. The slightly higher IC_{50} values (at

most 4-fold) obtained for OstTK⁻/NLS-VZV TK-GFP⁺ cells compared with OstTK⁻/VZV TK-GFP⁺ cells could be attributed to differences in the expression level of the VZV TK-GFP and the NLS-VZV TK-GFP fusion proteins, rather than to the different localization of VZV TK-GFP in the tumor cells. Indeed, the lower expression of NLS-VZV TK-GFP is in agreement with a weaker fluorescence signal (**Figure 2**, panel C and D). Thus, the intracellular localization of the VZV TK-GFP fusion protein is not a determining factor in the cytostatic potency of the antiviral nucleoside analogues.

These findings are in full agreement with the observations of Johansson et al. (1997) on the cytostatic activities of nucleoside analogues against 2'-deoxycytidine kinase (dCK) expressing tumor cell lines. Until recently, it had generally been assumed that enzymes required for nucleic acid synthesis (i.e., nucleoside kinases) are localized in the cytosol (dCK and TK1) or in the mitochondria [i.e., 2'-deoxyguanosine kinase (dGK) and TK2] (Arnér and Eriksson, 1995). However, dCK, that shows substrate specificity for 2'-deoxycytidine, 2'-deoxyadenosine and several clinically important nucleoside analogues, has now been found to be predominantly localized in the nuclear compartment (Johansson et al., 1997). Moreover, Johansson and collaborators identified a nuclear targeting signal in the primary structure of human dCK and showed that this signal was required for nuclear import of the protein. Irrespective of the intracellular localization of dCK, no marked differences in the cytostatic activity of 1--D-arabinofuranosylcytosine (araC), 2',3'-dideoxy-2',3'-difluorocytidine (dFdC, gemcitabine) and 2-chloro-2'-deoxyadenosine (CdA) were noted. These data indicate that the nucleus and the cytosol do not have separate deoxynucleotide pools, and that phosphorylated nucleoside analogues are rapidly equilibrated between the nuclear and cytosolic compartments of the cell. Since the localization of nucleoside kinases in the cell does not seem to have any determining role as to their function, it is currently unclear why certain nucleoside kinases are localized in the nucleus (dCK, HSV-1 TK), others in the cytosol (HSV-2 TK, mammalian cytosolic TK1) and still others spread over the nucleus and cytosol (VZV TK).

In conclusion, we found that the inhibitory effects of antiviral nucleoside analogues against herpes TK-GFP gene-transfected cells were not significantly altered by changing the intracellular localization (either nucleus or throughout the cell) of the HSV-1 TK-GFP or VZV TK-GFP fusion protein. Also, the bystander effect of the antiviral nucleoside analogues was not affected by the intracellular targeting of HSV-1 TK-GFP. Our experimental data indicate that phosphorylated nucleoside analogues can rapidly equilibrate between the nuclear and cytosolic compartments of the cell before exerting their potent cytostatic effect.

IV. Materials and methods

A. Compounds

BVDU and BVDC were synthesized by P. Herdewijn and A. Van Aerschot at the Rega Institute for Medical Research (Katholieke Universiteit Leuven, Leuven, Belgium). S-BVDU was provided by the late R.T. Walker (University of Birmingham, Birmingham, U.K.). BVaraU was a kind gift of H. Machida (Yamasa Shoyu Co., Choshi, Japan). AraT was from Sigma Chemical Co. (St. Louis, MO), and also a kind gift from M. Sandvold and F. Myhren (Norsk Hydro, Porsgrunn, Norway). FIAC and FMAU were a kind gift of J.J. Fox (Sloan-Kettering Institute, New York). GCV was from Syntex (Palo Alto, CA), ACV from the former Wellcome Research Laboratories (Research Triangle Park, NC), BCV from Astra Läkemedel (Sodertälje, Sweden) and LBV from Bristol-Myers Squibb (Princeton, NJ). PCV was obtained from I. Winkler (Hoechst, Frankfurt, Germany).

B. Cell Culture

Adherent human osteosarcoma cells deficient in cytosol TK (OstTK⁻, ATCC CRL-8303) and all TK-GFP gene-transfected OstTK⁻ cells were maintained at 37°C in a humidified CO_2-controlled atmosphere, in MEM culture medium (Gibco, Paisley, U.K.), supplemented with 10% heat-inactivated fetal calf serum (Biochrom KG, Berlin, Germany), 2mM L-glutamine (Gibco), 0.075% (w/v) $NaHCO_3$ (Gibco), 0.5µl/ml geomycine (Gentamycin , 40mg/ml, Schering-Plough, Madison, NJ) and 0.5µl/ml Amphotericin B (Fungizone , 5mg/ml, Bristol-Myers Squibb).

C. Plasmid construction

The construction of the HSV-1 TK-GFP, (AA1-34)HSV-1 TK-GFP, VZV TK-GFP and NLS-VZV TK-GFP expression vectors has been described elsewhere (Degrève et al., 1998). Briefly, the coding sequence for the full-length and N-truncated HSV-1 TK (lacking the first 34 amino acids) were amplified by PCR from the pMCTK plasmid kindly provided by Dr. D. Ayusawa (Yokohama City University, Japan), and cloned in the multiple cloning site of the pEGFP-N1 N-Terminal Protein Fusion Vector (CLONTECH, Palo Alto, CA). The VZV TK coding sequence, PCR-amplified from the pRc/CMV/VZV TK plasmid (kindly provided by Dr. J. Piette, University of Liège, Belgium) was ligated with (NLS-VZV TK-GFP) or without (VZV TK-GFP) the PCR-amplified sequence encoding for AA1-34 of HSV-1 TK in the pEGFP-N1 vector.

D. Stable transfection of tumor cells

The construction of the OstTK⁻/HSV-1 TK-GFP⁺, OstTK/⁻ (AA1-34)HSV-1 TK-GFP⁺, OstTK⁻/VZV TK-GFP⁺ and OstTK⁻/NLS-VZV TK-GFP⁺ cell lines has been described elsewhere (Degrève et al., 1998). Briefly, the herpes virus TK-GFP fusion constructs were introduced into OstTK⁻ cells via membrane fusion-mediated transfer using plasmid/ liposome complexes (LipofectAMINE Reagent, Gibco), as described by the supplier. Stable fusion gene transfectants were isolated by maintaining the cell cultures in the presence of HAT medium (i.e. normal growth medium, supplemented with 100µM hypoxanthine, 0.4µM aminopterin and 16µM thymidine). Monoclonal transfected cell lines were obtained by plating the cells at clonal density in tissue culture plates (Corning, N.Y.), after which single colonies were isolated and expanded. A standard FITC filter-equipped fluorescence microscope was used to evaluate gene expression and GFP fusion protein localization.

E. Inhibition of tumor cell proliferation by antiherpetic compounds

The cytostatic activity of antiviral nucleoside analogues against wild-type and herpes TK-GFP-expressing cells was evaluated as follows. 10^4 OstTK⁻, OstTK⁻/HSV-1 TK-GFP⁺, OstTK⁻/ (AA1-34)HSV-1 TK-GFP⁺, OstTK⁻/VZV TK-GFP⁺ or OstTK⁻/NLS-VZV TK-GFP⁺ cells/well were plated in 96-well microtiter plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) and subsequently incubated at 37°C, in a humidified CO_2-controlled atmosphere, in the presence of 5-fold dilutions (in normal growth medium) of the compounds. After 3 days, the number of cells was evaluated in a Coulter Counter (Coulter Electronics Ltd., Harpenden Hertz, U.K.). The IC_{50} was defined as the drug concentration required to inhibit tumor cell proliferation by 50%.

F. Bystander effect

The procedure to evaluate the bystander effect of the compounds was as described elsewhere (Degrève et al., 1998). Briefly, OstTK⁻ cells were mixed with HSV-1 TK-GFP gene-transfected cells in percentages ranging from 0 to 100% (0, 0.2, 1, 5, 10, 25, 50, 75, 90 and 100%) transfected cells, and subsequently incubated in the presence of 5-fold dilutions (in 2% FCS-containing medium) of the compounds. After 3 days, i.e. the time needed by untreated cell cultures to reach confluency, cell viability was determined using the Cell Titer 96 Aqueous Non-radioactive MTT Cell Proliferation Assay (Promega, Madison, WI). Untreated cultures served as controls.

Acknowledgments

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