



BRIEF COMMUNICATION

Varicella-zoster virus thymidine kinase gene and antiherpetic pyrimidine nucleoside analogues in a combined gene/chemotherapy treatment for cancer

B Degrevè¹, G Andrei¹, M Izquierdo², J Piette³, K Morin⁴, EE Knaus⁴, LI Wiebe⁴, I Basrah⁵, RT Walker⁵, E De Clercq¹ and J Balzarini¹

¹Laboratory of Virology and Chemotherapy, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Belgium; ²Centro de Biología Molecular Severo Ochoa (UAM-CSIC), Departamento de Biología Molecular, Universidad Autónoma de Madrid, Facultad de Ciencias, Cantoblanco, Madrid, Spain; ³Laboratory of Virology, University of Liège, Sart Tilman, Belgium; ⁴Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada; and ⁵Department of Chemistry, University of Birmingham, UK

Ten pyrimidine nucleoside analogues, including (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) and closely related analogues, were evaluated for their cytostatic activity against human osteosarcoma cells transfected with the varicella-zoster virus (VZV) thymidine kinase (tk) (ATP:thymidine 5' phosphotransferase, EC 2.7.2.21) gene. (E)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil (BVaraU), (E)-5-(2-iodovinyl)-2'-deoxy-2'-fluoro-1-β-D-arabinofuranosyluracil (IVFAU) and (E)-5-(2-bromovinyl)-2'-deoxy-4'-thiouridine (S-BVDU) were among the most potent inhibitors of VZVtk gene-transfected cell proliferation. They displayed an inhibitory activity at drug concentrations that were up to four orders of magnitude lower than those required to inhibit the corresponding nontransfected tumor cells.

Inhibition of cellular DNA polymerase and/or incorporation of the drugs into cellular DNA may be a likely target for the cytostatic activity of the BVDU derivatives against the VZVtk gene-transfected tumor cells. These compounds were approximately 40- to 80-fold more potent cytostatic agents in VZVtk gene-transfected cells than the anti-VZV compound 6-methoxy-9-β-D-arabinofuranosylpurine (araM), and at least five- to 50-fold more cytostatic than ganciclovir in HSV-1tk gene-transfected murine mammary carcinoma FM3A cells. In addition, the intrinsic resistance of BVaraU, IVFAU and S-BVDU to glycosidic bond cleavage by mammalian dThd phosphorylases makes them promising candidate compounds for the treatment of VZVtk gene-transfected tumors *in vivo*.

Keywords: varicella-zoster virus; thymidine kinase; gene therapy; cancer

In the past decade, traditional anticancer therapies (ie surgical resection, radiotherapy and chemotherapy) have not resulted in major breakthroughs on therapeutic improvements. Current antineoplastic drugs are struggling with the limited ability to distinguish neoplastic cells from normal cells on the basis of proliferative functions. Also, because tumor cells, in general, use the same metabolic pathways as nonmalignant cycling cells, the lack of significant biochemical differences between normal and tumor cells prevent a marked improvement of the selectivity of most chemotherapeutics. However, advances in gene technology offer new possibilities for the treatment of malignancies, including the artificial creation of exploitable biochemical differences between neoplastic and non-neoplastic cells. Following the insertion in tumor cells of genes encoding for specific enzymes not normally present in mammalian cells, metabolic pathways can be altered in order to make these tumor cells

selectively sensitive to chemotherapeutic agents. These susceptibility genes, often of viral or prokaryotic origin, are designated 'suicide genes', because they trigger the transfected cell to commit metabolic suicide by converting the chemotherapeutic agent into a highly toxic metabolite that is able to kill the transfected cell.¹⁻³ If the suicide gene can be targeted specifically to tumor cells (eg by the use of retroviral vectors only integrating in actively dividing cells, or by the insertion of tissue- or tumor-specific promoter/enhancer sequences), selective killing of the tumor cells may be achieved without significant toxicity to adjacent tissues.⁴⁻⁶

The most intensively studied suicide gene/prodrug system is represented by the herpes simplex virus type 1 (HSV-1) thymidine kinase (*tk*) gene in combination with the antiherpetic drug ganciclovir (*Cymevene*; Gilead Sciences, CA, USA).^{2,3,7,8} This acyclic guanosine derivative needs to be monophosphorylated by the viral *tk*, and is subsequently metabolized by cellular enzymes to the triphosphate form in order to exert its deleterious effect on DNA polymerization, resulting in cell killing. The first *in vivo* demonstration of the feasibility of this novel approach dates from 1992, when Culver and co-workers⁹

showed complete regression of established brain tumors in rats after *in situ* transduction with the HSV-1*tk* gene and subsequent treatment with ganciclovir. Several clinical trials, all utilizing the HSV-1*tk*/ganciclovir system, are underway to assess the safety and clinical applicability of this combined gene/chemotherapy treatment for cancer.^{10–14}

Balzarini and co-workers^{7,15–18} reported on the highly selective cytostatic activity of ganciclovir, (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) and various structurally related derivatives thereof against murine mammary carcinoma (FM3A) cells transfected with the HSV-1 and HSV-2*tk* gene. Differences were found in the cytostatic potency of the drugs depending on the nature of the suicide gene (ie HSV-1 or HSV-2*tk* gene). In the present report, we focus on the *tk* gene of varicella-zoster virus (VZV), which has received little attention as a suicide gene so far. Huber *et al*¹⁹ introduced the VZV*tk* gene as a candidate suicide gene, demonstrating the selective activation by VZV*tk* gene-transfected cells of the nontoxic prodrug 6-methoxy-9-β-D-arabinofuranosylpurine (araM) to the DNA polymerase inhibitor 9-β-D-arabinofuranosyladenine-5'-triphosphate (araATP). Our choice of VZV*tk* as a prodrug-activating enzyme originated from the findings that several pyrimidine nucleoside analogues (eg BVDU, (E)-5-(2-iodovinyl)-2'-deoxyuridine (IVDU), and (E)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil (BVaraU) are five- to 80-fold more potent inhibitors of VZV replication than of HSV-1 replication *in vitro*.^{18,20}

Indeed, susceptibility testing of the VZV*tk*⁺ (Oka and YS) and thymidine kinase deficient (*tk*⁻)(YS-R and 07-1) strains to a variety of selected antiherpetic compounds (including BVDU and its closely related derivatives IVDU, CVDU, S-BVDU and BVDC, the 2'-fluoro-substituted IVDU derivatives IVFAU and IVFRU, and the arabinosynucleosides BVaraU, araT and araU (Figure 1)) revealed that BVaraU showed an exquisitely high inhibitory activity against *tk*⁺ VZV replication *in vitro*, displaying a mean IC₅₀ value of 0.049 nM, ie more than five orders of magnitude lower than the concentration required to inhibit *tk*⁻ VZV replication (Table 1). The compounds IVFAU, IVDU, BVDU, S-BVDU and IVFRU exhibited IC₅₀ values in the 1–10 nM range. CVDU, BVDC and araT were 350 to 1200-fold less active than BVaraU (IC₅₀:0.024–0.29 μM). AraU did not exert any effect on *tk*⁺ or *tk*⁻ VZV replication (Table 1). None of the compounds were markedly inhibitory to *tk*⁻ VZV replication, pointing to the dependence of the test compounds on the viral *tk* to become eventually antivirally effective.

To introduce the VZV*tk* gene into human osteosarcoma cells, deficient in cytosol *tk* (Ost*tk*⁻), plasmid-liposome complexes were used. The VZV*tk* gene construct is shown in Figure 2. VZV*tk* gene-transfected cells were selected, based upon the inability of *tk*⁻ cells to grow in HAT medium. The transfected cell lines were selected under independent experimental conditions and designated Ost*tk*⁻/VZV*tk*⁺-a and Ost*tk*⁻/VZV*tk*⁺-b. The growth rate of the VZV*tk* gene-transfected Ost*tk*⁻ cells was only slightly decreased when compared with the growth rate of untransfected Ost*tk*⁻ cells (data not shown). Successful VZV*tk* gene transfection was not only indicated by the dThd phosphorylating capacity of Ost*tk*⁻/VZV*tk*⁺-a and Ost*tk*⁻/VZV*tk*⁺-b cell extracts (ie 66 and 31 pmol methyl-³H-dThd converted per milligram of protein per minute) but also by the sensitivity of Ost*tk*⁻/VZV*tk*⁺-a and

Ost*tk*⁻/VZV*tk*⁺-b cells to various antiherpetic drugs, as compared to Ost*tk*⁻ cells, and by the marked incorporation of radiolabeled methyl-³H-dThd and 2'-³H-BVDU in trichloroacetic acid (TCA)-insoluble material from Ost*tk*⁻/VZV*tk*⁺ cells but not parental Ost*tk*⁻ cells (see below). The inhibitory effect of the compounds studied for anti-VZV activity in HEI cell cultures (Table 1) was also evaluated on VZV*tk*-catalyzed methyl-³H-dThd phosphorylation in Ost*tk*⁻/VZV*tk*⁺-a and Ost*tk*⁻/VZV*tk*⁺-b cell extracts. No substantial differences were observed between the two VZV*tk* gene-transfected cell lines (Table 2). BVDU, IVDU, CVDU and S-BVDU were almost equally inhibitory to thymidine phosphorylation, with 50% inhibitory concentrations ranking in the 1–3 μM range. The fluorinated IVDU derivatives IVFRU and IVFAU, and BVaraU were approximately four-fold less effective (IC₅₀: 4.4–8.3 μM), followed by BVDC, which inhibited thymidine phosphorylation at 31 μM in both cell extracts. The arabinosynucleosides araT and araU were devoid of pronounced inhibition of the thymidine kinase activity at concentrations ≤100 μM (Table 2).

The pyrimidine nucleoside analogues were then evaluated for their inhibitory effects on the proliferation of human osteosarcoma cells deficient in cytosol *tk* (Ost*tk*⁻) and the two VZV*tk* gene-transfected osteosarcoma cell lines derived from Ost*tk*⁻. As shown in Table 3, none of the compounds were inhibitory to the growth of Ost*tk*⁻ cells at 250 μM except for IVDU, which exhibited a 50% inhibitory concentration of 204 μM. In contrast, Ost*tk*⁻/VZV*tk*⁺-a and Ost*tk*⁻/VZV*tk*⁺-b cells were markedly inhibited in their growth by all compounds, except for araU, which showed an IC₅₀-value of approximately 50 μM for both cell lines. The two VZV*tk* expressing Ost*tk*⁻/VZV*tk*⁺ cell lines behaved very similarly with regard to their susceptibility to the cytostatic activity of the test compounds. Of the 2'-deoxyribosyl derivatives, BVDU, IVDU and CVDU showed a pronounced cytostatic activity against the VZV*tk* gene-transfected cells, with IC₅₀-values ranging between 0.15 and 0.81 μM, that is at concentrations approximately 500-fold (BVDU and CVDU) to 1000-fold (IVDU) lower than the concentrations required to inhibit Ost*tk*⁻ cell proliferation. Also, BVDC and the ribonucleoside IVFRU were 100-fold more inhibitory to the VZV*tk* gene-transfected cells than to the Ost*tk*⁻ cells (Table 3).

S-BVDU (IC₅₀:0.044 and 0.062 μM for Ost*tk*⁻/VZV*tk*⁺-a and Ost*tk*⁻/VZV*tk*⁺-b cells, respectively) and IVFAU (IC₅₀:0.020 and 0.038 μM for Ost*tk*⁻/VZV*tk*⁺-a and Ost*tk*⁻/VZV*tk*⁺-b cells, respectively) were identified as the most potent inhibitors of VZV*tk* gene-transfected tumor cell proliferation. S-BVDU and IVFAU inhibited the growth of the VZV*tk* gene-transfected Ost*tk*⁻ cells at concentrations that proved >5000- to >10000-fold lower than those required to inhibit the parental Ost*tk*⁻ cells (Table 3). Their cytostatic activity against the VZV*tk* gene-transfected tumor cells was even five- to 20-fold more pronounced than that of the prototype compounds BVDU and IVDU. In sharp contrast with araU, the arabinosyl nucleosides araT and BVaraU were 500-fold (araT) and 1500-fold (BVaraU) more potent in their cytostatic activity against Ost*tk*⁻/VZV*tk*⁺-a and Ost*tk*⁻/VZV*tk*⁺-b cells than against the nontransfected Ost*tk*⁻ cells (Table 3). In our studies, 6-methoxy-9-β-D-arabinofuranosylpurine (araM, provided by B Kozalsky (Glaxo-Wellcome, NC, USA)), previously described as a selective chemothera-

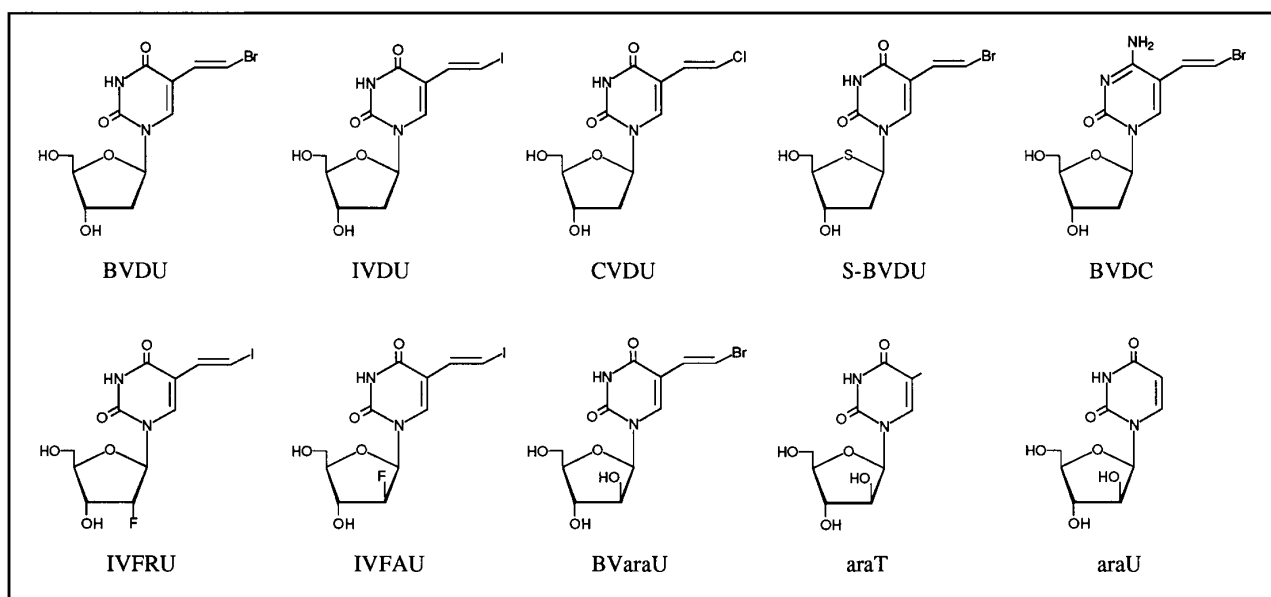


Figure 1 Chemical structures of BVDU and related compounds. BVDU ((E)-5-(2-bromovinyl)-2'-deoxyuridine), IVDU ((E)-5-(2-iodovinyl)-2'-deoxyuridine) and BVDC ((E)-5-(2-bromovinyl)-2'-deoxycytidine) were synthesized by P Herdewijn and A Van Aerschot at the Rega Institute for Medical Research (Katholieke Universiteit Leuven, Belgium). BVaraU ((E)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil) was provided by H Machida (Yamasa Shoyu Co, Choshi, Japan). S-BVDU ((E)-5-(2-bromovinyl)-2'-deoxy-4'-thiouridine) and CVDU ((E)-5-(2-chlorovinyl)-2'-deoxyuridine) were synthesized by I Basrah and RT Walker (University of Birmingham, UK). IVFRU ((E)-5-(2-iodovinyl)-2'-deoxy-2'-fluoro-1-β-D-ribofuranosyluracil) and IVFAU ((E)-5-(2-iodovinyl)-2'-deoxy-2'-fluoro-1-β-D-arabinofuranosyluracil) were synthesized by K Morin, E Knaus and L Wiebe (University of Alberta, Canada), whereas araU (1-β-D-arabinofuranosyluracil) and araT (1-β-D-arabinofuranosylthymine) were from Sigma Chemical Co. (St Louis, MO, USA).

Table 1 Inhibitory effect of BVDU and related compounds on *tk*⁺ and *tk*⁻ varicella-zoster virus plaque formation in human embryonic lung (HEL) cells

Compound	<i>IC</i> ₅₀ (μM)			
	<i>tk</i> ⁺ VZV		<i>tk</i> ⁻ VZV	
	<i>Oka strain</i>	<i>YS strain</i>	<i>07/1 strain</i>	<i>YS/R strain</i>
BVDU ²¹	0.0028	0.0045	91.9	114.7
IVDU	0.0023	0.0010	10	>50
CVDU ²¹	0.024	0.030	>138	>138
S-BVDU	0.0030	0.0060	ND	>50
BVDC	0.029	0.086	ND	>50
IVFRU	0.0090	0.01	>50	>50
IVFAU	0.0016	0.0015	10	>50
BVaraU ²¹	0.000074	0.000024	>57	>57
araT ²¹	0.15	0.29	114.3	19.8
araU	>50	>50	ND	>50

Confluent HEL cells grown in 96-well microtiter plates (Falcon; Beckton Dickinson, Grenoble, France) were infected with different strains of VZV, ie two reference strains of VZV expressing viral thymidine kinase (*tk*⁺) (YS and Oka) or two reference strains of VZV lacking the viral thymidine kinase (*tk*⁻) (YS/R and 07/1) at 20 plaque forming units.²¹ After a 2-h incubation period, residual virus was removed and the infected cells were further incubated (in duplicate) with MEM supplemented with 2% inactivated fetal calf serum (Integro, Zaandam, The Netherlands), 2 mM L-glutamine (Gibco, Paisley, UK) and 0.045% (w/v) NaHCO₃ (Gibco) and containing serial dilutions of the test compounds. After 5 days of incubation at 37°C in a humidified CO₂-controlled atmosphere, virus plaque formation was determined.

*IC*₅₀, 50% inhibitory concentration, or concentration required to reduce virus plaque formation by 50%; ND, not determined.

peutic drug for the *tk* gene-mediated elimination of hepatocellular carcinoma cells by Huber *et al*¹⁹ was used as a positive control. It displayed an *IC*₅₀ value in the lower micromolar range for VZV*tk* gene-transfected osteosarcoma cells, and thus was 40 to 80 times less potent than S-BVDU and IVFAU (data not shown).

We have previously shown that thymidylate synthase

(TS) is the principal target for the cytostatic activity of BVDU and related compounds against HSV*tk* gene-transfected murine mammary carcinoma FM3A cells.^{15,17} Thymidylate synthase, which catalyzes the reductive methylation of dUMP to dTMP, is a crucial enzyme in the *de novo* pathway of dTMP synthesis in the cell. It is considered as the principal target enzyme for the anticancer

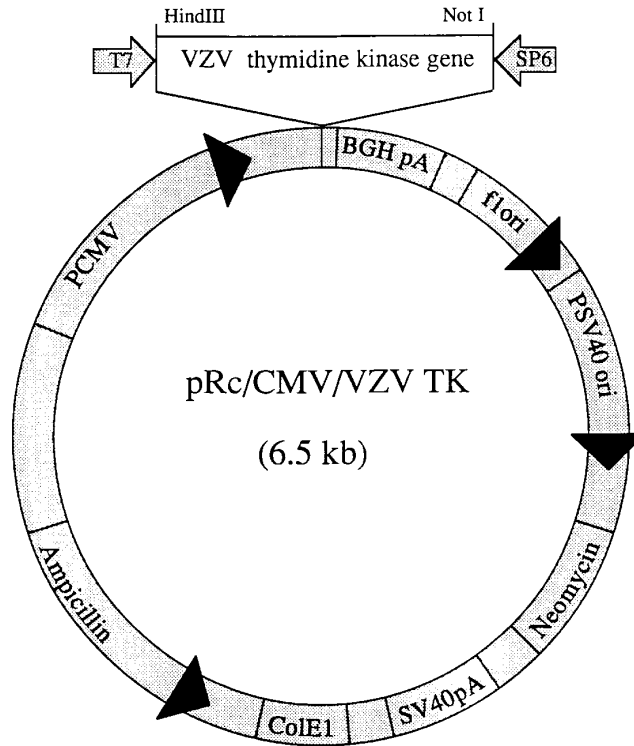


Figure 2 Map of pRc/CMV/VZVtk, containing the ampicillin and neomycin resistance genes for selection in prokaryotes and eukaryotes respectively, and the VZVtk gene (approximately 1.1 kb) under transcriptional control of the constitutive immediate-early cytomegalovirus (CMV) enhancer/promoter sequences was introduced into *Osttk⁻* cells via membrane fusion-mediated transfer using plasmid-liposome complexes. The pRc/CMV/VZVtk plasmid *Osttk⁻* cells were seeded into six-well plates (Nunc, Roskilde, Denmark) at 250 000 cells per well. The transfection was carried out (in duplicate) 24 h later. Two micrograms of plasmid DNA was diluted in 200 μ l Opti-MEM I reduced serum medium (Gibco). In another tube, 10 μ l of Lipofectin Reagent (Gibco) was diluted in 200 μ l Opti-MEM I reduced serum medium. The two solutions were combined, gently mixed, and incubated at room temperature for 15 min. Then, 1.6 ml of Opti-Mem I reduced serum medium was added and 1 ml of the resulting mixture was brought into each well after being washed with serum-free growth medium without antibacterial agents. A control culture was treated with Lipofectin, but no plasmid DNA. Five hours later, 4 ml of normal growth medium was added to each well, and the cultures were left in the incubator for 3 days. Then, cell cultures were trypsinized and further maintained in the presence of HAT medium (ie normal growth medium, supplemented with 100 μ M hypoxanthine, 0.4 μ M aminopterin and 16 μ M thymidine). The medium was changed twice a week. On the 18th day of selection, two colonies of HAT medium-resistant osteosarcoma cells (designated *Osttk⁻/VZVtk⁺-a* and *Osttk⁻/VZVtk⁺-b*, a and b representing two independently obtained osteosarcoma cell clones) were isolated and further cultured in normal growth medium.

activity of some nucleoside analogues. Tritium release from 5-³H-dCyd in intact cells has been considered as a valuable parameter for TS activity *in vivo*. After 5-³H-dCyd is converted to 5-³H-dUMP (via deamination to 5-³H-dUrd and subsequent phosphorylation or alternatively via phosphorylation to 5-³H-dCMP and subsequent deamination), the C-5 tritium atom of the pyrimidine base is released during the TS reaction. In intact *Osttk⁻* cells, none of the test compounds was able to inhibit tritium release from 5-³H-dCyd at concentrations less than 100 μ M (Table 4). However, in *Osttk⁻/VZVtk⁺-a* and *Osttk⁻/VZVtk⁺-b* cells, BVDU and IVDU exhibited a pronounced effect on tritium release, with comparable 50% inhibitory concentrations that fell within the 0.65–1.5 μ M range, and thus at more than 65- to 150-fold lower concentrations than those required to inhibit tritium release in *Osttk⁻* cells. Surprisingly, IVFRU and S-BVDU were markedly less inhibitory to tritium release from 5-³H-dCyd in the intact VZVtk gene-transfected tumor cells than the parent compounds IVDU and BVDU. In fact, IVFRU even exerted a stimulatory effect on TS in *Osttk⁻/VZVtk⁺-a* and *Osttk⁻/VZVtk⁺-b* cells, resulting in an increased tritium release from 5-³H-dCyd up to 140% of control at 100 μ M (data not shown). Except for IVFAU,

IVDU and BVDU, all other compounds studied (including CVDU, BVaraU, araT and araU) were devoid of any inhibitory effect on tritium release in VZVtk gene-transfected cells at concentrations \leq 100 μ M (Table 4).

Osttk⁻, *Osttk⁻/VZVtk⁺-a* and *Osttk⁻/VZVtk⁺-b* cell cultures were also incubated in the presence of 1 μ M 2'-³H-BVDU or 2 μ M methyl-³H-dThd to measure the incorporation of radioactivity into TCA-insoluble material after 8, 16 and 24 h. As depicted in Figure 3a, marked incorporation of methyl-³H-dThd into the TCA-insoluble fraction of osteosarcoma cell extracts was highly dependent on the presence and expression of the inserted VZVtk gene. Both VZVtk gene-transfected cell lines incorporated methyl-³H-dThd to a 12-fold higher extent than the non-transfected *Osttk⁻* cells after a 24-h incubation period. Moreover, when the cell lines were incubated for 24 h in the presence of 2'-³H-BVDU, a 17-fold higher incorporation of drug-derived radioactivity was observed in *Osttk⁻/VZVtk⁺-a* and *Osttk⁻/VZVtk⁺-b* cells as compared to *Osttk⁻* cells (Figure 3b). These data clearly point again to a marked and efficient expression of VZVtk in both VZVtk gene-transfected osteosarcoma cell lines.

There are major differences in the cytostatic potential of the pyrimidine nucleoside analogues against VZVtk

Table 2 Inhibitory effect of BVDU and related compounds on *tk* activity from *Osttk⁻/VZVtk⁺-a* and *Osttk⁻/VZVtk⁺-b* cell extracts

Compound	IC ₅₀ (μM)	
	<i>Osttk⁻/VZVtk⁺-a</i>	<i>Osttk⁻/VZVtk⁺-b</i>
BVDU	1.3 ± 0.78	1.5 ± 0.75
IVDU	1.8 ± 0.35	2.5 ± 0.24
CVDU	2.7 ± 1.4	3.0 ± 0.80
S-BVDU	1.4 ± 0.33	2.5 ± 1.9
BVDC	31 ± 4.5	31 ± 9.8
IVFRU	4.9 ± 2.2	7.3 ± 1.1
IVFAU	8.3 ± 4.0	7.7 ± 3.0
BVaraU	4.4 ± 0.0	6.2 ± 3.7
araT	≥100	≥100
araU	≥100	≥100

Osttk⁻/VZVtk⁺-a and *Osttk⁻/VZVtk⁺-b* are two different, independently obtained *VZVtk* gene-transfected *Osttk⁻* cell lines. Specific *tk* activity was 0.066 and 0.031 nmol methyl-³H-dThd converted per mg of protein per min for *Osttk⁻/VZVtk⁺-a* and *Osttk⁻/VZVtk⁺-b* cell-derived *tk*, respectively. To prepare *VZVtk*, *tk* gene-transfected *Osttk⁻* cells were grown in 175-cm² tissue culture flasks (Falcon). Extract preparations were performed at 4°C. Confluent monolayers were washed twice with PBS and trypsinized. After centrifugation (300 g, 8 min), the cell pellet was washed once with PBS and once with suspension buffer (0.1 M Tris-HCl, pH 8.0, supplemented with 0.02 M β-mercaptoethanol). Finally, cells were resuspended in suspension buffer at a concentration of 10⁸ cells/ml. Suspensions were sonicated three times for 10 s, followed by ultracentrifugation (100 000 g, 45 min). Extracts were assayed for protein content by use of Bradford Reagent (Sigma), stored in aliquots at -70°C and assayed for dThd kinase activity as described before.²⁶ Briefly, the standard reaction mixture contained 2.5 mM MgCl₂, 10 mM dithiothreitol, 1 mg/ml bovine serum albumin, 2.5 mM ATP, 10 mM NaF, 10 μM methyl-³H-dThd (1.2 μCi), an appropriate amount of inhibitor, and 5 μl cell extract in a total reaction mixture of 50 μl of 50 mM Tris-HCl, pH 8.0. The reaction mixture was incubated at 37°C for 30 min, and the reaction terminated by spotting an aliquot of 30 μl on to DE-81 discs (Whatman, Maidstone, UK). The discs were instantly immersed and thoroughly washed in ethanol (70%). Finally, filters were dried and assayed for radioactivity in a toluene-based scintillant. IC₅₀, 50% inhibitory concentration; mean value (±s.d.) for two independent experiments.

and *HSVtk* gene-transfected tumor cells. In contrast with BVDU, the arabinosyl nucleoside BVaraU has previously been found not to be inhibitory against the *HSVtk* gene-transfected murine mammary carcinoma *FM3Atk⁻/HSV-1tk⁺* and *FM3Atk⁻/HSV-2tk⁺* cells.⁷ These findings were interpreted to mean that TS is not a target for the cytostatic action of the drug. This conclusion has been corroborated by previous reports that arabinofuranosylnucleotides usually have poor affinity for TS.²³ Therefore, it was surprising to find that BVaraU proved exquisitely cytostatic against *Osttk⁻/VZVtk⁺* cells. However, examination of tritium release from 5-³H-dCyd revealed that TS does not recognize BVaraUMP as a substrate or inhibitor. Why BVaraU exerts a pronounced cytostatic activity against *Osttk⁻/VZVtk⁺* but not *FM3Atk⁻/HSVtk⁺* cells is presently unclear. One possibility is that BVaraU, upon phosphorylation by the viral *tk*, is not metabolized to its triphosphate derivative in *FM3Atk⁻/HSV-1tk⁺* and *FM3Atk⁻/HSV-2tk⁺* cells, whereas it is converted to its triphosphate derivative in *Osttk⁻/VZVtk⁺* cells and inhibits

the cellular DNA polymerase(s). Alternatively, BVaraU may be equally well converted to its 5'-triphosphate in both *tk* gene-transfected FM3A and *tk* gene-transfected osteosarcoma cell lines, but the DNA polymerases in the human osteosarcoma cells have much higher affinity for BVaraUTP than the murine FM3A cell DNA polymerases. This phenomenon may then also be extended to other arabinosyl nucleoside derivatives since it is probably no coincidence that another arabinosyl derivative, ie IVFAU, is also exquisitely inhibitory to the *Osttk⁻/VZVtk⁺* cells. A second surprising finding is the fact that CVDU, although equally inhibitory to the proliferation of *Osttk⁻/VZVtk⁺* cells as BVDU and IVDU, was devoid of any measurable inhibitory activity against TS in the intact cells, whereas BVDU and IVDU clearly acted on TS as the principal target enzyme for their cytostatic action. Thus, small structural changes in the 5-substituent of the pyrimidine ring (ie chlorovinyl *versus* bromovinyl and iodovinyl) may have a marked impact on their intracellular mechanism of action.

Whereas S-BVDU and IVFAU were found to be the most potent inhibitors of *Osttk⁻/VZVtk⁺* cell proliferation among the panel of pyrimidine nucleoside analogues included in our study, their inhibitory action against thymidylate synthase in the intact tumor cells is much less pronounced (approximately 100-fold for IVFAU to approximately 1000-fold for S-BVDU, as compared to their cytostatic activity). These findings are in sharp contrast with our previous reports on *FM3Atk⁻/HSVtk⁺* cells, where a close correlation was found between inhibition of thymidylate synthase and cell proliferation for BVDU, IVDU, S-BVDU and IVFAU. This means that the target for the cytostatic activity of the BVDU analogues differs from the *HSVtk* to the *VZVtk* gene-transfected tumor cells. It is presently unclear whether this difference is due to the nature of the viral *tk* (either herpes simplex virus or varicella-zoster virus) or to the nature of the *tk* gene-transfected cells (murine mammary carcinoma or human osteosarcoma cells). Our findings that BVDU is markedly incorporated into the TCA-insoluble material of *Osttk⁻/VZVtk⁺* cells, but not in *Osttk⁻* cells may point to the incorporation of the drug into cellular DNA and/or inhibition of DNA polymerase as the molecular basis of the cytostatic activity of BVDU and related compounds against *VZVtk* gene-transfected *Osttk⁻* cells.

An interesting feature of the BVDU and IVDU derivatives that are most inhibitory against *Osttk⁻/VZVtk⁺* cell proliferation (ie BVaraU, S-BVDU and IVFAU) is their intrinsic property to resist phosphorolytic cleavage by mammalian dThd phosphorylase. Indeed, BVaraU, S-BVDU and IVFAU are known to have poor, if any, susceptibility to glycosidic hydrolysis compared with BVDU and IVDU.^{24,25} This property may give the compounds described in this study a therapeutic edge over the phosphorolytic cleavage-susceptible compounds BVDU and IVDU in an *in vivo* setting.

It is noteworthy to mention that ganciclovir, the current drug of choice for treatment of *HSV-1tk* gene-transfected tumors is devoid of any marked activity against *VZVtk* gene-transfected osteosarcoma cells. Ganciclovir has poor, if any, affinity for *VZVtk*, and thus a pronounced antiproliferative activity of this drug against *VZVtk* gene-transfected tumor cells would not be expected. It is noteworthy that BVaraU, S-BVDU and IVFAU were at least five- to 50-fold more inhibitory to

Table 3 Cytostatic activity of BVDU and related compounds against *Osttk*⁻, *Osttk*⁻/*VZVtk*^{+a} and *Osttk*⁻/*VZVtk*^{+b} cells

Compound	<i>IC</i> ₅₀ (μM)		
	<i>Osttk</i> ⁻	<i>Osttk</i> ⁻ / <i>VZVtk</i> ^{+a}	<i>Osttk</i> ⁻ / <i>VZVtk</i> ^{+b}
BVDU	≥250	0.60 ± 0.33	0.35 ± 0.15
IVDU	204 ± 14	0.35 ± 0.14	0.15 ± 0.03
CVDU	≥250	0.38 ± 0.24	0.81 ± 0.12
S-BVDU	≥250	0.044 ± 0.036	0.062 ± 0.043
BVDC	≥250	1.6 ± 0.74	1.6 ± 1.1
IVFRU	≥250	4.6 ± 2.1	2.0 ± 0.82
IVFAU	≥250	0.020 ± 0.013	0.038 ± 0.006
BVaraU	≥250	0.15 ± 0.09	0.20 ± 0.09
araT	≥500	0.78 ± 0.41	1.2 ± 0.25
araU	>250	49 ± 20	≥118

Osttk⁻/*VZVtk*^{+a} and *Osttk*⁻/*VZVtk*^{+b} are two different, independently obtained, *VZVtk* gene-transfected *Osttk*⁻ cell lines. 10⁴ *Osttk*⁻, *Osttk*⁻/*VZVtk*^{+a} and *Osttk*⁻/*VZVtk*^{+b} cells per well were plated in 96-well microtiter plates (Falcon) and allowed to adhere. Cells were subsequently incubated at 37°C in a humidified CO₂-controlled atmosphere, in the presence of five-fold dilutions (in normal growth medium) of the compounds. After 4 days, the number of cells was evaluated in a Coulter Counter (Coulter Electronics, Harpenden, UK). *IC*₅₀, 50% inhibitory concentration, or the drug concentration required to inhibit cell proliferation by 50%; mean value (± s.d.) for three to five independent experiments.

Table 4 Inhibitory effect of BVDU and related compounds on the release of tritium from 5-³H-dCyd in intact *Osttk*⁻, *Osttk*⁻/*VZVtk*^{+a} and *Osttk*⁻/*VZVtk*^{+b} cells

Compound	<i>IC</i> ₅₀ (μM)		
	<i>Osttk</i> ⁻	<i>Osttk</i> ⁻ / <i>VZVtk</i> ^{+a}	<i>Osttk</i> ⁻ / <i>VZVtk</i> ^{+b}
BVDU	≥100	1.4 ± 0.35	1.0 ± 0.28
IVDU	>100	1.5 ± 0.26	0.65 ± 0.06
CVDU	>100	≥100	≥100
S-BVDV	>100	52 ± 16	19 ± 8.8
BVDC	>100	9.3 ± 4.9	9.8 ± 2.7
IVFRU	>100	≥100	≥100
IVFAU	≥100	3.9 ± 1.9	2.7 ± 0.69
BVaraU	>100	≥100	≥100
araT	>100	≥100	≥100
araU	>100	≥100	≥100

Osttk⁻/*VZVtk*^{+a} and *Osttk*⁻/*VZVtk*^{+b} are two different, independently obtained, *VZVtk* gene-transfected *Osttk*⁻ cell lines. Activity of thymidylate synthase (TS) in intact *Osttk*⁻, *Osttk*⁻/*VZVtk*^{+a} and *Osttk*⁻/*VZVtk*^{+b} cells was measured by estimation of tritium release from 5-³H-dUMP (formed in the cells from 5-³H-dCyd) in the reaction catalyzed by TS. This method has been described previously²² but was modified as follows. Cells were seeded in 48-well plates at a density of 5 × 10⁴ cells per well. After 48 h, medium was aspirated and 200 μl of growth medium containing an appropriate amount of inhibitor was added to each well for 3 h. Then, 200 μl of growth medium, supplemented with 0.2 μM 5-³H-dCyd (1 μCi) (specific radioactivity, 18.1 or 24 Ci/mmol) (Radiochemical Centre, Amersham, UK) was added to each well and the cells were further incubated at 37°C. One hour later, 500 μl of a cold suspension (0.1 g/ml) of carbon black (UCB, Leuven, Belgium) in 5% TCA was added and the resulting mixture was centrifuged at 1100 g for 10 min. Supernatants were analyzed for radioactivity in a toluene-based scintillant.

*IC*₅₀, 50% inhibitory concentration, or the drug concentration required to inhibit the release of tritium by 50%; mean value (±s.d.) for three to five independent experiments.

the growth of *VZVtk* gene-transfected tumor cells (ie *IC*₅₀: 0.02–0.2 μM) (Table 3) than ganciclovir to HSV-1*tk* gene-transfected tumor cells (ie *IC*₅₀: 1 μM).^{18,26} These observations suggest that *VZVtk* gene transfection may be a useful approach for the combined gene/chemotherapy of tumors.

In conclusion, we reported on the feasibility of using the *VZVtk* gene as a suicide gene for combined gene/chemotherapy of tumor cells. We revealed several pyrimidine nucleoside analogues that showed cytostatic activities against *VZVtk* gene-transfected tumor cells that were one to two orders of magnitude more pronounced

than the cytostatic activity of ganciclovir against HSV-1*tk* gene-transfected tumor cells. In addition, the principal intracellular target of the most potent compounds did not prove to be thymidylate synthase, and thus, clearly differs from the target of these pyrimidine nucleoside analogues in HSV*tk* gene-transfected cells.

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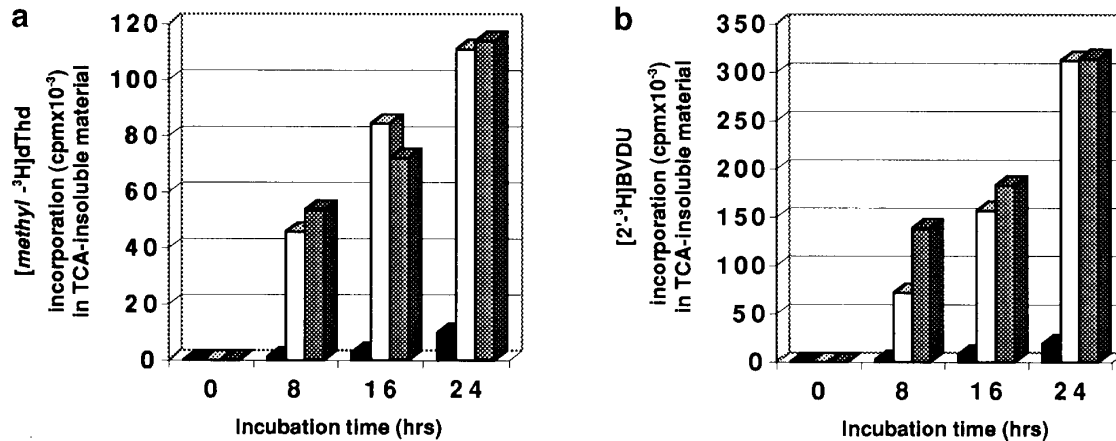


Figure 3 Incorporation of methyl-³H-dThd (a) and 2'-³H-BVDU (b) into TCA-insoluble material of Osttk⁻, Osttk⁻/VZVtk⁺-a and Osttk⁻/VZVtk⁺-b cells. Cells were seeded in 6-cm Petri dishes (Falcon) at a density of 1.25 × 10⁶ cells per 2.5 ml per dish. Ten percent fetal calf serum, dialyzed overnight against PBS at 4°C, was used in the growth medium. After 24 h incubation at 37°C, growth medium was renewed and supplemented with 2 μM methyl-³H-dThd (1 μCi/ml) (specific radioactivity, 72 Ci/mmol) (Radiochemical Centre) or 1 μM 2'-³H-BVDU (1 μCi/ml) (specific radioactivity, 1 Ci/mmol) (Moravsek Biochemicals, Brea, CA, USA). At 0, 8, 16 and 24 h, monolayers were washed twice with PBS and detached with 800 μl trypsin solution (Gibco). Cell suspensions were collected and 200 μl of ice-cold 50% TCA was added. After centrifugation at 10 000 g for 10 min at 4°C, the TCA precipitate (containing nucleic acids) was further washed twice with ethanol 70% and assayed for radioactivity. ■, Osttk⁻; □, Osttk⁻/VZVtk⁺-a; ▣, Osttk⁻/VZVtk⁺-b.

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