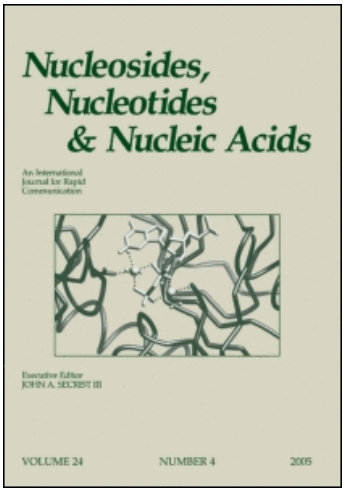

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Herpes Simplex Virus Thymidine Kinase Gene-Transfected Tumor Cells; Sensitivity to Antiherpetic Drugs

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REVIEW

HERPES SIMPLEX VIRUS THYMIDINE KINASE GENE-TRANSFECTED TUMOR CELLS: SENSITIVITY TO ANTIHERPETIC DRUGS[1,2]

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ABSTRACT. A series of antiherpetic 5-substituted 2'-deoxyuridine derivatives (i.e. BVDU) and guanine derivatives (i.e. ganciclovir) have been evaluated for their cytostatic activity against murine mammary carcinoma FM3A cell lines that are deficient in cytosol thymidine kinase, but transfected by the herpes simplex virus type 1 (HSV-1)- or type 2 (HSV-2)-specified thymidine kinase gene. Most compounds were endowed with a markedly higher cytostatic activity against the HSV TK gene-transfected tumor cells than against wild-type tumor cells. The principal target for cytostatic activity of the BVDU derivatives proved thymidylate synthase, whereas the guanine derivatives inhibited HSV TK gene-transfected tumor cell proliferation by competing with cellular DNA polymerase(s) and subsequent incorporation into the cellular genome.

INTRODUCTION

By insertion of certain genes into neoplastic cells, artificial biochemical differences are created between the non-transfected and the transfected tumor cell lines. Indeed, expression of an enzyme that normally does not exist in tumor cells may enable the neoplastic cells to convert relatively non-toxic drugs to highly toxic metabolites that are then able to arrest tumor cell proliferation and/or kill the transfected tumor cells. The normal (surrounding) cells that are not transfected by the particular gene, will not be able to generate the toxic metabolite, and thus, will escape the deleterious effects of the particular drug.

To investigate the feasibility of this approach, we have focussed on thymidine kinase (TK) from herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). These virus-encoded kinases differ from their mammalian counterpart in several aspects, including substrate spectrum for a number of nucleoside analogues (1-4). In this paper, we would like to review the cytostatic

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activities and mechanism of cytostatic action of the most promising antiherpetic compounds that are endowed with a pronounced cytostatic activity against HSV TK gene-transfected tumor cells.

ANTIHERPETIC ACTIVITY OF 5-SUBSTITUTED 2'-DEOXYURIDINE ANALOGUES AND GUANINE DERIVATIVES

A series of 5-substituted 2'-deoxyuridine derivatives, structurally related to (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) (Fig. 1) proved to be potent inhibitors of HSV-1-induced cytopathicity in cell culture (Table 1 and references 5-11). BVDU, IVDU, 4'-thio-BVDU (S-BVDU) and the 2'-fluoro-substituted IVDU derivatives IVFRU and IVFAU had a comparable anti-HSV-1 activity (EC_{50}: 0.01-0.06 μM). The carbocyclic analogues of BVDU, IVDU and BVDC, and the arabinosyl derivative of BVDU (BVAU) had EC_{50} values ranging between 0.1 and 0.5 μM, whereas the 5-(thien-2-yl)- and 5-(furan-2-yl)-substituted 2'-dUrd analogues had EC_{50} values between 0.5 and 1.0 μM (Table 1). The anti-HSV-2 activity of the 5-substituted 2'-dUrd derivatives was usually markedly less pronounced than their anti-HSV-1 activity (100-, to 10,000-fold). The anti-HSV-1 activity of the guanine derivatives (Fig. 2) ranged between 0.02 and 0.09 μM. In contrast with the BVDU derivatives, the anti-HSV-1 activity of the guanine derivatives corresponded closely to their anti-HSV-2 activity (EC_{50} ranging between 0.04 and 0.31 μM) (Table 1 and references 16-20). Thus, the 5-substituted 2'-dUrd analogues discriminate markedly more between their anti-HSV-1 and -HSV-2 activity than the guanine derivatives. This difference in antiviral selectivity is obviously due to the fact that the guanine derivatives are further metabolised (phosphorylated) by cellular enzymes from the purine nucleotide metabolism pathways (i.e. guanylate kinase) after they have been converted to their respective monophosphate derivatives by HSV-1 and HSV-2 thymidine kinase (18). In contrast, the BVDU derivatives - after being converted to their 5'-monophosphates by HSV-1 and HSV-2 thymidine kinase - are efficiently converted to their 5'-diphosphate derivatives by the HSV-1 thymidylate kinase but not by HSV-2-specified thymidylate kinase or other cellular enzymes (21). Therefore, the BVDU derivatives reach markedly higher triphosphate levels in HSV-1-infected cells than in HSV-2-infected cells.

CYTOSTATIC ACTIVITY OF 5-SUBSTITUTED 2'-DEOXYURIDINE ANALOGUES AGAINST HSV TK GENE-TRANSFECTED TUMOR CELLS. PROTOTYPE COMPOUND: (*E*)-5-(2-BROMOVINYL)-2'-DEOXYURIDINE (BVDU)

A large number of BVDU derivatives have been evaluated against a series of murine mammary carcinoma FM3A cell lines, including wild-type FM3A/0 cells, FM3A TK^- cells being deficient in cytosolic TK, FM3A TK^-/HSV-1 TK^+ cells being deficient in cytosolic TK but transfected by the TK gene of HSV-1, and FM3A TK^-/HSV-2 TK^+ cells being deficient in

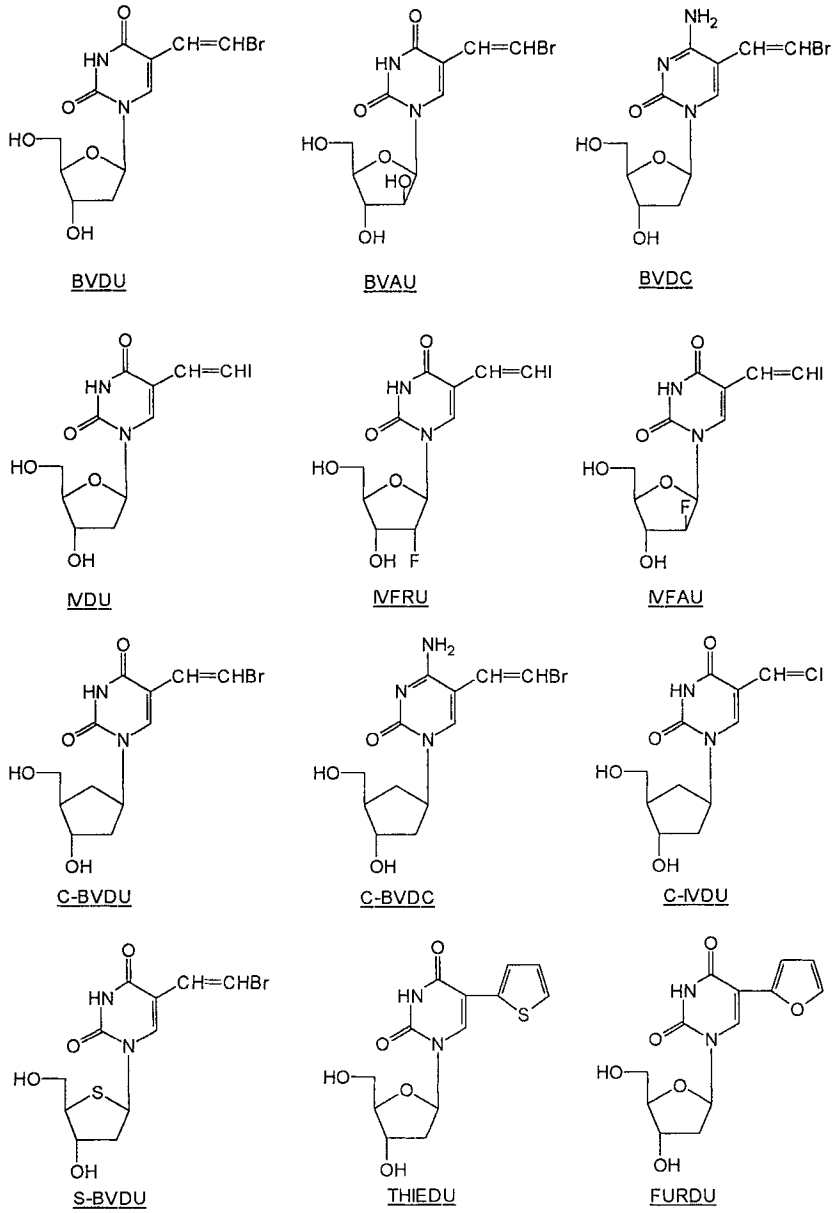

FIG. 1.

Structural formulae of antiherpetic 5-substituted 2'-deoxyuridine derivatives

TABLE 1

Antiherpetic activity and cytostatic activity of 5-substituted 2'-deoxyuridine derivatives against herpes thymidine kinase gene-transfected tumor cells

Compound	EC_{50}[a] (μM)		IC_{50}[b] (μM)				Refs.
	HSV-1	HSV-2	FM3A/0	FM3A/TK$^-$	FM3A TK$^-$/ HSV-1 TK$^+$	FM3A TK$^-$/ HSV-2 TK$^+$	
BVDU	0.01-0.06	300	14-48	0.19-0.51	0.004-0.006	0.001-0.002	12,13
C-BVDU	0.1	45	> 1,000	930	0.64	12	12,14
BVAU	0.3	100	> 500	> 500	> 500	> 500	12,14
BVDC	0.2	24	33-49	1.1-1.4	0.002-0.004	0.001	12,14
C-BVDC	0.5	60	> 1000	> 1000	2.3	1.9	14
IVDU	0.02	70	18	0.40	0.005	0.002	8,12
C-IVDU	0.21	26	884	848	3.62	-	12,14
IVFRU	0.02	≥ 40	143	155	0.027	0.34	8
IVFAU	0.01	2	222	152	0.024	0.53	8
S-BVDU	0.01	0.8	55	4.6	0.007	0.047	13
THIEDU	0.80	> 100	> 200	140	0.99	3.1	15
FURDU	0.67	> 200	123	> 40	0.51	3.6	15

[a]50% Effective concentration or compound concentration required to reduce virus-induced cytopathicity in E_6SM cell cultures by 50%.
[b]50% Inhibitory concentration or compound concentration required to inhibit cell proliferation by 50%.

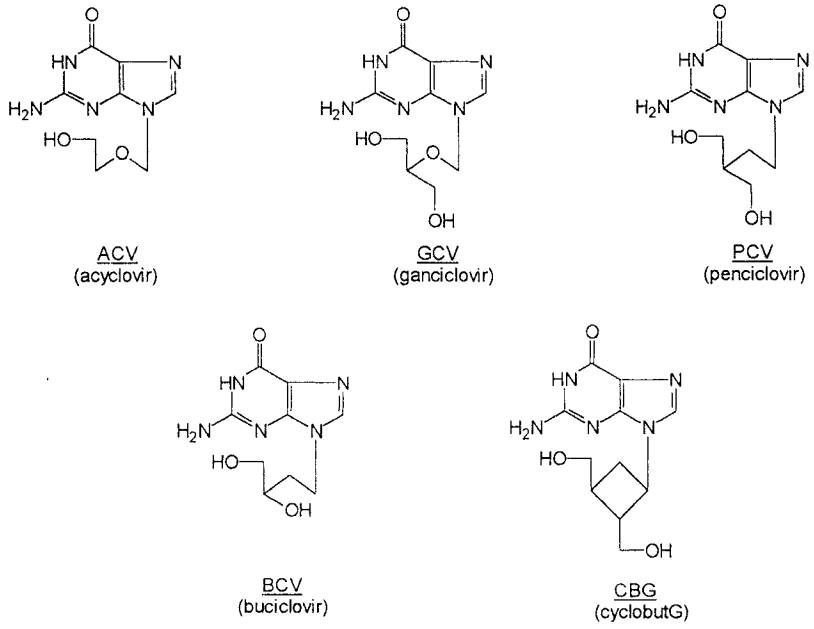

FIG. 2

Structural formulae of antiherpetic guanine derivatives

cytosolic TK but transfected by the TK gene of HSV-2 (22-25). The latter four cell lines represent a useful and convenient tool in rapidly discriminating the cytostatic potential of antiherpetic drugs in the HSV TK gene-transfected tumor cell lines *versus* the corresponding parental wild-type FM3A/0 and FM3A TK$^-$ cells.

When the 5-substituted 2'-deoxyuridine derivatives were evaluated for their cytostatic activity against wild-type FM3A/0 cells, none of them proved markedly inhibitory to the proliferation of these tumor cells (Table 1). Their cytostatic activity ranged from 14 μM to > 1,000 μM, depending the nature of the compound evaluated (Table 1). Carbocyclic BVDU (C-BVDU) and C-BVDC, BVAU and 5-(thien-2-yl)dUrd (THIEDU) were devoid of any cytostatic activity at 200 μM, whereas BVDU, BVDC, IVDU and S-BVDU were the most inhibitory to FM3A/0 cell proliferation (IC_{50}: 14-55 μM).

In contrast, all compounds, except BVAU, were endowed with a markedly increased cytostatic potential against HSV-1 and HSV-2 TK gene-transfected FM3A cells. BVDU, IVDU and BVDC showed a 10,000- to 50,000-fold increased cytostatic activity to these cell lines than to wild-type FM3A/0 tumor cells. The 2'-fluoro-substituted IVDU derivatives (IVFRU and IVFAU) and S-BVDU proved also exquisitely cytostatic to FM3A TK$^-$/HSV-1 TK$^+$ cells (2,000- to 9,000-fold increased inhibition) and FM3A TK$^-$/HSV-2 TK$^+$ cells (400- to 1,000-fold increased inhibition).

Carbocyclic BVDU (C-BVDU), C-IVDU and C-BVDC, and 5-(furan-2-yl)dUrd (FURDU) and 5-(thien-2-yl)dUrd (THIEDU) had IC_{50} values close to 1 μM against FM3A TK$^-$/HSV-1 TK$^+$ cells. BVAU completely lacked cytostatic activity at a concentration as high as 500 μM (Table 1).

Intriguingly, among the closely related BVDU derivatives, only BVDU, BVDC, IVDU and S-BVDU also show an increased inhibitory effect against FM3A TK$^-$ cell proliferation, a phenomenon that has so far not been well understood (26).

CYTOSTATIC ACTIVITY OF GUANINE DERIVATIVES AGAINST HSV TK GENE-TRANSFECTED TUMOR CELLS. PROTOTYPE COMPOUND: GANCICLOVIR

The closely related antiherpetic guanine derivatives acyclovir, ganciclovir, penciclovir, buciclovir and cyclobut G (Fig. 2) were evaluated against the panel of FM3A cell lines (Table 2). None of the compounds were markedly toxic to wild-type FM3A/0 and FM3A TK$^-$ cells. The IC_{50} ranged between 72 and 694 μM. However, when exposed to HSV-1 TK gene-transfected tumor cells, the cytostatic activity of the test compounds increased by 3- to 6-fold for cyclo-butG and acyclovir, but 130- to 400-fold for penciclovir and ganciclovir. Interestingly, the inhibitory effect of the test compounds to HSV-2 TK gene-transfected tumor cells further increased 4- to

TABLE 2

Antiherpetic activity and cytostatic activity of guanine derivatives against herpes thymidine kinase gene-transfected tumor cells

Compound[a]	EC_{50}[b] (μM)		IC_{50}[c] (μM)			
	HSV-1	HSV-2	FM3A/0	FM3A/TK$^-$	FM3A TK$^-$/ HSV-1 TK$^+$	FM3A TK$^-$/ HSV-2 TK$^+$
Acyclovir	0.09	0.31	199	148	62	4.5
Ganciclovir	0.02	0.16	321	188	1.0	0.26
Penciclovir	0.04	0.04	694	315	5.1	0.88
Buciclovir	0.03	0.04	515	304	4.0	1.1
Cyclobut G	-	-	98	72	31	2.5

[a]Data taken from ref. 13.
[b]50% Effective concentration or compound concentration required to reduce virus-induced cytopathicity in E_6SM cell cultures by 50%.
[c]50% Inhibitory concentration or compound concentration required to inhibit cell proliferation by 50%.

15-fold when compared with HSV-1 TK gene-transfected tumor cells. However, none of these compounds reached the inhibitory potential of BVDU and its congeners against the HSV TK gene-transfected cells. The latter compounds were approximately 200-fold more effective than the most potent guanine derivative (i.e. ganciclovir and penciclovir) (Tables 1 and 2).

MECHANISM OF CYTOSTATIC ACTIVITY OF THE 5-SUBSTITUTED 2'-DEOXYURIDINE AND GUANINE DERIVATIVES AGAINST HSV TK GENE-TRANSFECTED TUMOR CELLS

We observed that IVDU was extensively metabolized in HSV-1 TK gene-transfected tumor cells, but not in wild-type FM3A/0 and FM3A TK$^-$ cells to its 5'-mono-, 5'-di- and 5'-triphosphate derivatives (27,28). However, surprisingly, IVDU was only metabolized to its 5'-monophosphate derivative in FM3A TK$^-$/HSV-2 TK$^+$ cells. Since IVDU was even more cytostatic to FM3A TK$^-$/HSV-2 TK$^+$ cells than to FM3A TK$^-$/HSV-1 TK$^+$ cells, these findings let us to conclude that the principal metabolites of IVDU responsible for the cytostatic activity against the FM3A TK$^-$/HSV TK$^+$ tumor cell lines must have been most likely IVDU-MP. This conclusion let us to evaluate the potential role of thymidylate synthase (TS) in the cytostatic activity of the BVDU and IVDU derivatives (27,28).

The parameters for evaluation of the inhibitory effect of the test compounds against TS in the intact mutant tumor cell lines included measurement of the cytostatic action of the test

compounds in the presence of high concentrations of dUrd and dThd, differential inhibitory effects of the test compounds against incorporation of radiolabelled dUrd *versus* dThd into DNA, and measurement of tritium released from [5-^{3}H]dCyd in the tumor cells treated with the test compounds (29,30). In Table 3, the inhibitory effect of the test compounds against tritium release from [5-^{3}H]dCyd in the intact tumor cells is shown. A close correlation was found between the inhibiton of thymidylate synthase in the intact tumor cells (presumably by the 5'-monophosphate of the 5-substituted 2'-dUrd derivatives) and the cytostatic activity of these test compounds against FM3A TK$^-$/HSV-1 TK$^+$ cells (Table 3). Again, BVDU and IVDU were among the most potent inhibitors of tritium release from [5-^{3}H]dCyd, followed by IVFAU, BVDC, S-BVDU, IVFRU, THIEDU and FURDU. The 100-fold lower activity of the carbocyclic derivative of BVDU, and the lack of any inhibitory activity of BVAU is in full agreement with our findings that TS is the intracellular target for the cytostatic action of the BVDU derivatives, since it is well known that nucleotide analogues containing an unsubstituted arabinose or carbocyclic pentose as their sugar moiety, markedly loose their affinity for TS (31,32). This is obviously the reason why BVAU lacks marked cytostatic activity against the HSV TK gene-transfected tumor cells. It is of particular interest to notice that inhibition of TS by BVDU has been observed by Yokota *et al.* (33) in varicella zoster virus (VZV)-infected cells. Thus, inhibition of this enzyme can also contribute to the antiviral activity of BVDU analogues in VZV- (33), but also HSV-1- and HSV-2-infected cells (34).

In contrast with the 5-substituted 2'-dUrd derivatives, the cytostatic activity of the guanine derivative (i.e. ganciclovir) seems to be correlated with its conversion to the triphosphate form and subsequent incorporation into the DNA of HSV TK gene-transfected FM3A cells (35). Indeed, phosphorylation studies of [^{3}H]ganciclovir in FM3A/0, FM3A TK$^-$/HSV-1 TK$^+$ and FM3A TK$^-$/HSV-2 TK$^+$ cells revealed a 30- to 50-fold, and a 100- to 200-fold higher extent of phosphorylation in FM3A TK$^-$/HSV-1 TK$^+$ and FM3A TK$^-$/HSV-2 TK$^+$ cells than in FM3A/0 cells (35). Also, [^{3}H]ganciclovir was incorporated to a 2- to 3-fold greater extent into FM3A TK$^-$/HSV-2 TK$^+$ cell nucleic acids (DNA/RNA) than in FM3A TK$^-$/HSV-1 TK$^+$ cell nucleic acids. Thus, a close correlation was found between conversion of ganciclovir to its 5'-triphosphate derivative, its incorporation into DNA/RNA, and its cytostatic activity against the three cell lines studied (35). None of the test compounds markedly inhibited tritium release from [5-^{3}H]dCyd in the intact tumor cells (13).

IN VIVO ACTIVITY OF 5-SUBSTITUTED 2'-DURD AND GUANINE DERIVATIVES AGAINST HSV TK GENE-TRANSFECTED TUMORS

Recently, Culver and coworkers reported that rat brain tumors transfected *in vivo* by the HSV-1 thymidine kinase gene regressed upon treatment with ganciclovir (36). Similar

TABLE 3

Inhibitory effect of 5-substituted 2'-deoxyuridine derivatives on tritium release from [5-^{3}H]deoxycytidine in herpes thymidine kinase gene-transfected tumor cells

Compound	IC_{50}[a] (μM)				References
	FM3A/0	FM3A/TK$^-$	FM3A TK$^-$/HSV-1 TK$^+$	FM3A TK$^-$/HSV-2 TK$^+$	
BVDU	3-6.2	3.6-7.8	0.004-0.008	0.007-0.008	13,14,27
C-BVDU	> 100	> 100	> 100	-	-
BVDC	11	7.6	0.066	0.018	13
IVDU	8.8	18	0.005	0.007	8,14
IVFRU	109	196	0.087	0.61	8
IVFAU	135	78	0.060	0.50	8
S-BVDU	63	68	0.084	0.033	13
THIEDU	> 20	-	0.16	1.08	15
FURDU	> 20	-	0.29	1.57	15

[a]50% Inhibitory concentration or compound concentration required to inhibit tritium release from [5-^{3}H]dCyd in the intact tumor cells.

observations were also reported by Takamiya and coworkers who treated nude mice, inoculated with HSV-1 TK gene-transfected rat glioma cells, with ganciclovir (37). Based on these encouraging results, a clinical trial has been initiated to investigate the usefulness of the combined gene/chemotherapy approach in the treatment of brain tumors in humans (38). The study of Culver and coworkers (36) also revealed that, in addition to killing the tumor cells that had incorporated the HSV-1 TK gene, ganciclovir also killed other tumor cells in their vicinity. Exactly how this 'bystander effect' operates is not clear, but it has been suggested that ganciclovir 5'-triphosphate formed in the ganciclovir-treated transfected cells may be channelled to other neighbouring non-transfected tumor cells, thereby killing these tumor cells as well. Alternatively, immunologic responses to the tumor cell killing can also be involved to explain this phenomenon.

A potential drawback *in vivo* for compounds such as BVDU, to be used as cytostatic agents against HSV TK gene-transfected tumor cells, is their high susceptibility to hydrolysis by pyrimidine nucleoside phosphorylases (39). However, BVDC, S-BVDU, 5-(thien-2-yl)dUrd, 5-(furan-2-yl)dUrd, the 2'-fluoro-substituted IVDU derivatives IVFRU and IVFAU, as well as the carbocyclic derivatives of BVDU, IVDU and BVDC, are not hydrolyzed by human dThd phosphorylase. BVDC can, however, be deaminated to BVDU, and thus may become eventually vulnerable to hydrolysis by dThd phosphorylase. Thus, S-BVDU, C-BVDU, C-IVDU, C-BVDC,

IVFRU and IVFAU are not catabolized by such phosphorolysis. These compounds should, therefore, be considered as promising candidate compounds for further *in vivo* studies as cytostatic agents in the treatment of HSV TK gene-transfected tumors.

CONCLUSION

There is still a long way to go to optimize the combined gene/chemotherapeutic approach to eradicate tumor cells from the body. However, the promising results obtained from the *in vitro* experiments, and the initial encouraging data obtained in animal models should urge for a continued and more intensified effort to optimize HSV TK gene transfection of tumor cells *in vivo* and subsequent appropriate treatment modalities.

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