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Biochemical and Biophysical Research Communications 324 (2004) 8–14
BBRC
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HDACi phenylbutyrate increases bystander killing of HSV-tk transfected glioma cells

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Received 30 August 2004
Available online 21 September 2004

Abstract

Malignant glioma patients have a dismal prognosis with an urgent need of new treatment modalities. Previously developed gene therapies for brain tumors showed promising results in experimental animal models, but failed in clinical trials due to low transfection rates and insufficient expression of the transgene in tumor cells, as well as low bystander killing effects. We have previously shown that the histone deacetylase inhibitor 4-phenylbutyrate (4-PB) enhances gap junction communication between glioma cells in culture. In this study, we demonstrate an activation of recombinant HSV-tk gene expression, and a dramatic enhancement of gap junction-mediated bystander killing effect by administration of the HSV-tk prodrug ganciclovir together with 4-PB. These findings that 4-PB potentiates "suicide gene" expression as well as enhances gap junctional communication and bystander killing of tumor cells justify further testing of this paradigm as an adjunct to suicide gene therapy of malignant gliomas.

Keywords: HSV-tk; Bystander killing; HDAC inhibitor; 4-Phenylbutyrate; Ganciclovir; Glioma; Gap junction

One of the main obstacles of cancer therapy is the heterogeneity of tumor cells within a solid tumor, making them respond differently to treatment and to subsequently develop resistant clones. In an attempt to circumvent the problem, gene therapy employing prodrug suicide gene technology is a promising concept. However, there are many obstacles related to the delivery of recombinant DNA, targeting of tumor cells, and efficiency of cell killing. The use of *Herpes simplex* virus thymidine kinase (HSV-tk) gene expression in conjunction with the nucleoside analogue ganciclovir as a prodrug has been thoroughly explored [1] although many limiting factors remain. The difficulty of reaching target cells and expressing the HSV-tk gene product has been addressed by using various viral vectors for delivery of suicide genes, but still only a fraction of tumor cells are being transfected with very limited cell killing efficiency [2]. A second problem is that both the conversion of the prodrug into the cytotoxic compound and its delivery to neighboring tumor cells not expressing the suicide gene suffer from low efficiency.

One way of subjecting neighboring cells to the bystander killing effect is via gap junctions. Gap junctions are protein channels consisting of two hemi-channels, connexons, each belonging to one of the two plasma membranes of the cells participating in the intercellular contact [3]. Gap junctions permit small water-soluble substances, with a molecular weight of less than approximately 1000 Da, such as ions, metabolites, and second

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.09.016

messengers, to be exchanged between cells without secretion into the extracellular space [4]. Gap junction communication (GJC) is an integrated part of intercellular transfer of molecules, important for proliferation and differentiation pathways.

The number of gap junction channels has been found to be inversely proportional to the proliferation capacity of many cells [5]. Highly proliferative tumor cells have relatively few gap junctions, while more differentiated cells possess a greater number.

Tumor cells are maintained in more or less dedifferentiated states of the respective mature cell type in part by epigenetic mechanisms. Under normal circumstances, the epigenetic mechanisms block dedifferentiation. Thus, altered epigenetic patterns of the genome will change the repertoire of genes expressed and/or their level of expression. These changes can be potential targets for cancer therapy [6–8].

Histone deacetylases (HDACs) are enzymes which participate in modulating the epigenetic pattern and gene expression by providing a chromatin structure repressive for initiating gene transcription [9]. Inhibitors of these enzymes (HDACi:s) are currently being investigated as potential therapeutic agents [10–16] since they activate a subset of genes important for normal regulation of the cell cycle and/or apoptosis. Treatment of cancer cells with HDACi:s frequently results in cell differentiation as well as in cell death—apoptosis or necrosis—depending on concentration and time of treatment.

It was previously reported that the HDACi, 4-phenylbutyrate (4-PB), induces apoptosis and differentiation of tumor cells in vitro, as well as in xenografts [17,18]. Furthermore, we have reported that 4-PB induces increased expression levels of the gap junction component connexin 43 (Cx43), and enhances gap junction communication between glioblastoma cells as measured by dye transfer [19].

In this study, we describe the potential of 4-PB to increase the efficiency of the bystander killing effect by enhancing GJC in glioblastoma cells, using a HSV-tk suicide gene/ganciclovir technology. We conclude that 4-PB indeed enhances the killing effect by phosphorylated ganciclovir, and that this effect in part is due to increased gap junction communication.

Materials and methods

Cloning of enhanced green fluorescent protein/thymidine kinase in sense/antisense and establishment of stable cell clones. The cloning of pBudCE4.1 EGFP/HSV-tk sense and pBudCE4.1 EGFP/HSV-tk antisense carrying an enhanced green fluorescent protein (EGFP) gene driven by an EF-1α promoter, and a herpes simplex virus (HSV)-thymidine kinase gene in either sense or antisense orientation driven by a cytomegalovirus (CMV) promoter, has been described [20]. pDsRed2-C1 (red fluorescent protein, RFP) was used as provided (Clontech). Rat glioma C6 cells were transfected with either pBudCE4.1 EGFP/tk sense or pBudCE4.1 EGFP/tk antisense or pDsRed2-C1 using Lipofectamin Plus reagent (Invitrogen) according to the manufacturer's instruction. The selection of positive clones was started after 60 h by either adding Zeocin (Invitrogen; 400 μg/ml) to pBudCE4.1 EGFP/tk sense and pBudCE4.1 EGFP/tk antisense transfected cells, or Geneticin (Invitrogen) to a final concentration of 500 μg/ml to pDsRed2-C1 transfected cells. The derived cell clones carrying the pBudCE4.1 EGFP/tk sense, pBudCE4.1 EGFP/tk antisense or pDsRed2-C1 were collected by pipetting and transferred to 24-well plates. Expression of EGFP and RFP was assessed by fluorescence microscopy (Leica RM, Leica Microsystems) while the mRNA levels of HSV-tk in antisense and in sense orientation were assessed by RT-PCR. Recombinant cells were tested for sensitivity to ganciclovir (GCV) (data not shown). The cell clones "TK(S)" (HSV-tk sense, EGFP), "TK(AS)" (HSV-tk antisense, EGFP), and a pool of RFP expressing clones (RFP; weak expression) were selected for further analysis. The TK(AS) cell clone acted as a thymidine kinase negative control in all experiments.

Cell culture. Wild type C6 rat glioma cells (*Rattus norvegicus*, glial tumor) and recombinant C6 clones were grown at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 μg/ml streptomycin, and 100 μg/ml penicillin. Cells were treated with 4-phenylbutyrate (4-PB) [2 mM; Triple Crown America; dissolved in phosphate-buffered saline (PBS)], AGA (30 μM; 18α-glycyrrhetinic acid; Sigma–Aldrich; dissolved in DMSO), and Ganciclovir (GCV, 40 μM; Cymevene, Roche AB) as indicated.

FACS. After one wash with PBS, cells were trypsinized, pelleted, and adjusted to $1–5 \times 10^6$ cells/ml in PBS containing 10% FCS. Cell cytometry was performed using a FACScan cell analyzer (Becton–Dickinson Bioscience, San Jose, CA, USA). EGFP detection was conducted at 530/30 nm. Fluorescence >650 nm was measured for internal reference. WinMDI2.8 (http://facs.scripps.edu) was used for analyzing FACS data.

MTT assay. Viability of cells was determined by the MTT assay as described earlier [20,21]. Briefly, cell culture media were carefully removed and 500 μl MTT solution (0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in serum-free culture medium) was added to each well. The reaction was stopped after 1 h incubation at 37 °C, by removing the MTT solution and adding 500 μl isopropanol (Sigma–Aldrich). Aliquots of 100 μl were transferred into 96-well microplates. The absorbance at 570 nm was determined using an ELISA plate reader (Anthos Labtec H3T).

RT-PCR. For RT-PCR, RNA from 1×10^7 cells was isolated using the "Micro-to-Midi Total RNA Purification-System" (Invitrogen, Carlsbad, USA) following the supplier's instruction. For reverse transcription, 3 μg total RNA, 1 μg oligo-dT$(pd(T))_{12–18}$ (Amersham–Pharmacia Biotech, Freiburg, Germany), and 0.5 mM dNTPs (each) were heated to 65 °C for 5 min and chilled on ice. After adding 0.2 volumes 5× first-strand buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgSO_4$, and 0.1 mM DTT) and DTT to a final concentration of 10 mM, the setup was incubated for 2 min at 37 °C. Finally, 200 U M-MLV reverse transcriptase (LifeTechnologies) was added and the reaction mix was incubated for 50 min at 37 °C followed by an inactivation step for 10 min at 75 °C. RT-PCR was performed using HSV-tk (tk forward: 5′-cgatgacttactggcaggtg; tk reverse: 5′-gcggtcgaagatgagggtg) and glucose 6-phosphate dehydrogenase specific primers (G6PD forward: 5′-acgtgatgcagaaccacctactg; G6PD reverse: 5′-acgacggctgcaaaagtggcg) in combination with iQ SYBR Green Supermix (Bio-Rad, Munich, Germany) according to the following PCR-protocol: 95 °C, 3 min; 45 times (94 °C, 30 s; 58 °C, 45 s, and 72 °C, 1 min); and 72 °C, 15 min. PCR products were separated by agarose gel electrophoresis.

Western blot analysis of connexin 43. Cells were grown to 75% confluency, cultured in the presence or absence of 4-PB, and processed as previously described [20]. Briefly, cells were cultured in the presence of

4-PB for 48 h before harvesting and lysis. Cell extracts containing equal amount of protein (30 μg/lane) were loaded on 10% isocratic polyacrylamide gels for SDS–PAGE, followed by electro-transfer onto nitrocellulose membranes. Membranes were incubated with a rabbit polyclonal antibody to Cx43 diluted 1:8000 (Sigma) overnight at 4 °C. Immunodetection was performed with the enhanced chemiluminescence (ECL) detection kit (Amersham). The membranes were exposed to Hyperfilm-ECL (Amersham) for 1–5 min. For quantification, films were scanned and analyzed with Image Gauge (version 3.12, Fuji Photo Film). Data analysis was performed using the program Sigma Plot for Windows (version 6, Jandel). All results are expressed as means ± SE for the indicated number of experiments. As an internal control for equal loading on the gel, the nitrocellulose membranes were stained with Ponceau S solution (0.2% Ponceau S, 3% trichloroacetic acid, and 3% sulfosalicylic acid) for 5 min. As the bands of protein became visible, the membranes were washed several times and relative amount of loaded protein was calculated using computerized densitometric analysis.

Analysis of bystander killing effect. To study the bystander killing effect in the presence of ganciclovir and 4-phenylbutyrate, TK(S)-cells (30%), and RFP-cells (70%) were mixed. Where applicable, AGA was added to a final concentration of 30 μM and the mixed cells were seeded onto 24-well plates (12 wells with approximately 1×10^5 cells/well), one for each drug or drug combination tested (see Supplemental data Fig. S1). After 3 h, GCV and/or 4-PB was added as indicated and cells were split after 48 h and replated in 12-well plates. After 96, 120, and 168 h, MTT assays were performed with cells from three wells of each drug combination tested, to measure cell viability. Cells from the fourth well were used for FACS analysis to determine the composition of the cell mix and the relative cell death of tk-expressing cells compared to tk-negative cells. Cells from the last quadruplicate of wells of every plate (the last time point) were split and replated 144 h after starting the experiment, and these cells were analyzed after a total of 168 h (see Supplemental data Fig. S1). The same experimental setups were carried out with a mixture of 30% TK(AS)- and 70% RFP-cells. These experiments acted as a control to evaluate unspecific cytotoxic effects of the drugs tested and to calculate the specific effects of a drug or drug combination on tk-positive cells. This also allowed for a clear visualization of the bystander killing effect.

Results

Potentiated bystander-mediated cell killing of glioma cells by combined treatment with 4-phenylbutyrate and ganciclovir

Cultures of glioma cells either expressing herpes simplex virus thymidine kinase in sense (TK(S)) or in antisense (TK(AS)) were mixed with control cells (RFP) in a proportion of 30:70. These mixes are denoted TK(S) + RFP and TK(AS) + RFP, respectively. The co-cultures were treated with either 4-phenylbutyrate (4-PB), 18α-glycyrrhetinic acid (AGA), and ganciclovir (GCV) separately, by a combination of 4-PB and GCV, or by a combination of 4-PB, GCV, and AGA. PBS treated cells acted as control. The co-cultures were assayed for cell viability at the indicated time points using the MTT-assay. To read out the specific effects of each drug alone and in combination, the resulting optical density in the MTT-assay from the TK(AS) + RFP cell mixes was divided by the optical density from the TK(S) + RFP cell mixes.

The combination of 4-PB with GCV resulted in a dramatic time dependent increase in cell killing of co-cultures containing HSV-tk-positive cells (TK(S) + RFP) relative to mixtures containing cells carrying the HSV-tk gene in antisense orientation (TK(AS) + RFP), (Fig. 1A). The reduction of cell killing by the addition of AGA indicates that a large proportion of the cells were killed by a gap junction-mediated bystander killing effect. Thus, gap junction formation was essential for killing the majority of the tk-negative cells (RFP) in the

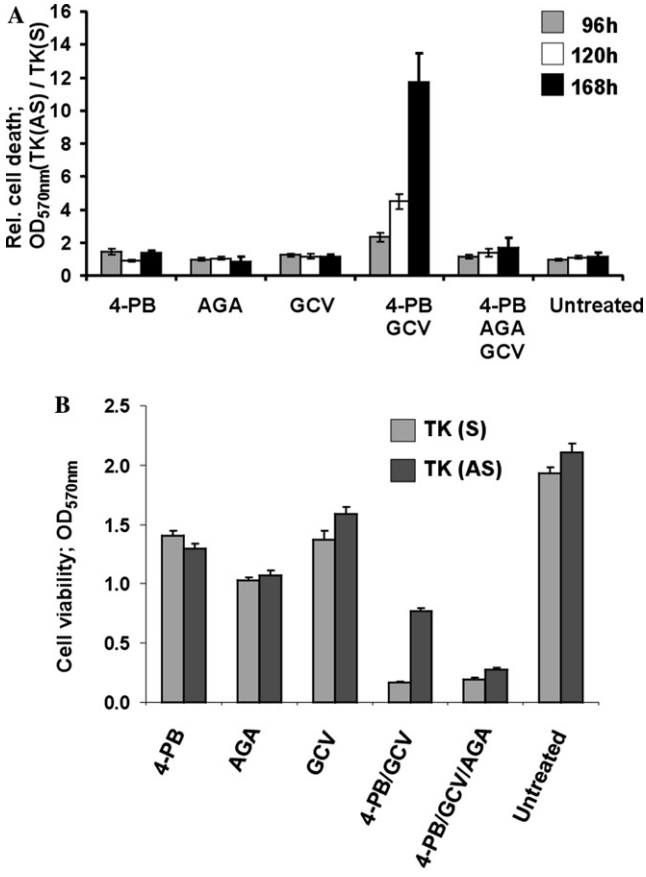

Fig. 1. The ganciclovir/thymidine kinase related glioma cell death is time dependent. (A) Glioma cell co-cultures were treated with drugs and drug combinations as indicated. MTT tests were performed on triplicate cell samples at 96 h (grey bar), 120 h (white bar), and at 168 h (black bar). The ratios of the MTT tests from thymidine kinase negative over thymidine kinase positive mixtures (TK(AS) + RFP/TK(S) + RFP) are shown. The bars indicate the mean of three independent MTT-assays, each measured three times. The error bars were calculated based on the standard deviation according to Doerffel (http://barolo.ipc.uni-tuebingen.de/pharma/2/2.7/doerffel.html (german)). (B) A representative analysis of glioma cells treated with various drugs for 120 h and subjected to MTT-test. The bars represent true values from the MTT-test for the HSV-tk positive TK(S) and negative TK(AS) cells, rather than the relative calculation given in Fig. 2A. The bars indicate the mean of three independent MTT-assays, each measured three times. The error bars indicate the standard error of the mean, SEM, of these measurements. 4-PB, 4-phenylbutyrate; AGA, 18α-glycyrrhetinic acid; and GCV, ganciclovir.

TK(S) + RFP co-culture. A representative cell viability assay using the actual results from a MTT-test rather than the relative is shown in Fig. 1B. The treatment time by the indicated drugs presented is 120 h. This also shows a tk-independent cytotoxic effect of the drugs when added simultaneously. The administration of the three drugs individually did not result in any significant difference in cell death between the TK(S) + RFP co-cultures compared to the TK(AS) + RFP co-cultures within the time and concentration frames selected for these experiments. As expected however, administration of GCV as single drug to the TK(S) + RFP co-culture led to increased cell killing after longer exposure times to the drug (data not shown).

To determine the number of tk-positive cells in the co-cultures after drug administration, the cell cultures were subjected to FACS analysis. Because of the EGFP expression in TK(S)- and TK(AS)-cells, the relative number of cells carrying either the HSV-tk gene in sense orientation in TK(S) + RFP-, or in antisense orientation in TK(AS) + RFP- co-cultures, could be calculated (number of TK(S) divided by total cells, divided by number of TK(AS) divided by total cells: (TK(S)/total)/(TK(AS)/total)). The ratios of these results demonstrated a time dependent increase in the death of HSV-tk expressing cells in the presence of 4-PB and GCV, but also in the presence of 4-PB, GCV, and AGA (Fig. 2). Thus, the composition of the cell populations shifted to a reduction of HSV-tk-positive (EGFP-positive) cells in the presence of ganciclovir and 4-phenylbutyrate, independent of the presence of AGA. As expected, AGA did not prevent tk-positive cells from death. However, in all the experiments performed ($n = 5$ independent experiments), the relative cell death of tk-expressing cells (TK(S)) was slightly reduced after addition of AGA.

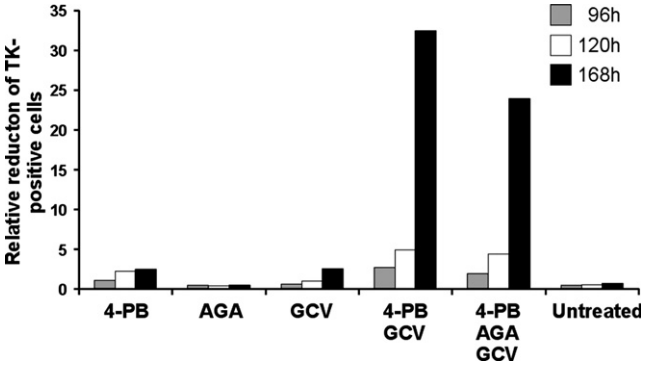

Fig. 2. Effects of drug treatment on death of thymidine kinase-expressing cells in co-cultures with control cells. FACS analysis was performed on co-cultures containing EGFP/thymidine kinase positive (TK(S) + RFP) and EGFP/thymidine kinase negative (TK(AS) + RFP) cells after 96 h (grey bar), 120 h (white bar), and 168 h (black bar) of drug treatment, and the ratios of EGFP-negative and positive cells (TK(AS) + RFP)/(TK(S) + RFP) were calculated for each combination. The figure shows representative results from three individual experiments. 4-PB, 4-phenylbutyrate; AGA, 18α-glycyrrhetinic acid; and GCV, ganciclovir.

Gap junction communication is enhanced by 4-phenylbutyrate and blocked by α-glycyrrhetinic acid

Expression of connexin 43 (Cx43) protein, a major structural component of gap junctions in glioma cells, was increased three- to fourfold in the presence of 2 mM 4-PB as demonstrated by Western blot analysis (Fig. 3). To prove the existence of gap junction-mediated intercellular transfer we performed fluorescent dye transfer experiments. The presence of intercellular dye transfer between adjacent TK(S)- and RFP-cells and its sensitivity to AGA treatment could be demonstrated (Supplemental data, Fig. S2).

Taken together, and with evidence from previous work [19], these data demonstrate that 4-PB induces connexin 43 expression and that the increased communication between cell populations occurs through enhanced gap junction coupling.

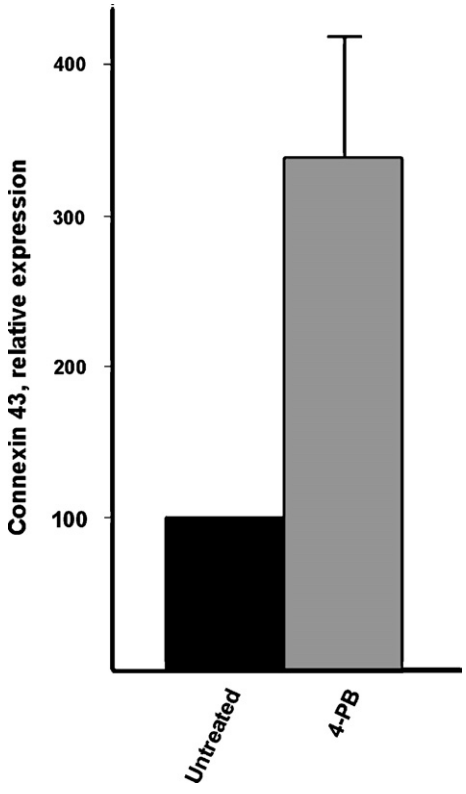

Fig. 3. Connexin 43 expression in glioma cells after 4-phenylbutyrate treatment. Administration of 4-PB induces expression of connexin 43 (Cx43) in TK(S)-cells. Protein from TK(S)-cells treated with 4-phenylbutyrate (4-PB) for 48 h was extracted and Western blot analysis was performed. The Cx43 specific signal was analyzed by Image Gauge, and the activation was calculated. TK(S)-cells not treated with 4-PB acted as control.

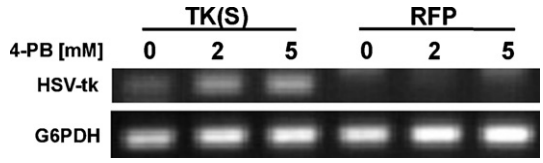

Fig. 4. 4-Phenylbutyrate induces thymidine kinase expression in tk-positive cells. Separately plated TK(S)- and RFP-cells were incubated with the indicated concentrations of 4-phenylbutyrate for 48 h. RNA was isolated and a RT-PCR analysis with herpes simplex virus thymidine kinase and glucose-6-phosphate dehydrogenase (control) specific primers was performed. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. HSV-tk, herpes simplex virus thymidine kinase; G6PDH, glucose-6-phosphate dehydrogenase.

4-Phenylbutyrate induces thymidine kinase expression in stably transfected glioma cells

The MTT-assay showed an overall protection of GCV/4-PB-dependent cell death by AGA in the co-culture experiments (Fig. 1). By using FACS analysis, it was possible to monitor the EGFP positive TK(S) cells separately, within the co-culture with the RFP-cells. This analysis revealed that in the TK(S) + RFP co-cultures, treated with a combination of GCV, 4-PB, and AGA, cell death of the TK(S)-cells specifically was more extensive compared to the same co-cultures treated with GCV alone (Fig. 2). This massive increase in TK(S)-cell death is likely based on an increase of tk-expression mediated by 4-PB. To address this, RT-PCR was performed (Fig. 4). While expression of endogenous glucose 6-phosphate dehydrogenase was not altered by 4-PB, the CMV-promoter driven HSV-tk-expression was increased. This elevated tk expression might have led to an enhanced effect of the ganciclovir treatment by an increased GCV phosphorylation rate. Thus, the enhanced bystander killing effect caused by the combined treatment with 4-PB and GCV could be inhibited by AGA, but it could not prevent the gap junction independent death of the tk-positive TK(S)-cells.

Discussion

We demonstrate here that the combined treatment with ganciclovir (GCV) and the HDAC-inhibitor 4-phenylbutyrate synergistically enhances glioma cell killing by (a) enhancing gap junction communication, which correlates to the observed increase in endogenous Cx43 protein, and (b) increasing the expression of a CMV-promoter driven transgenic HSV-tk in transfected cells. Although GCV administration has a cytotoxic effect on HSV-tk-expressing cells, induction of GJC by 4-PB increased the bystander killing effects dramatically. Thus, the cytotoxic effect of the simultaneous treatment with GCV and 4-PB substantially exceeded the effects of single drug treatment.

Glioblastoma multiforme is the most malignant of all gliomas and represents nearly 30% of all primary brain tumors. It is a devastating disease with very poor prognosis. The median life expectancy for patients with this disease is less than one year [22]. Palliative treatment includes surgery, chemotherapy, and radiotherapy which may prolong survival by a matter of months. However, no disease-modifying therapy has been developed.

In order to develop a new treatment modality, several suicide gene therapy models have been designed [1,2], primarily utilizing the HSV-tk/GCV system [23–32]. The antiviral agent ganciclovir [33] is harmless to cells not expressing HSV-tk. In HSV-tk transfected cells however, the viral thymidine kinase phosphorylates GCV to become ganciclovir-monophosphate. This is a substrate for cellular kinases that convert the metabolite into its toxic triphosphorylated form. It is incorporated into the DNA during S-phase without inhibition of the cell cycle [34] and leads to DNA strand breaks and subsequent cell death.

To date, several clinical studies utilizing HSV-tk suicide gene technology have been performed with varying responses. In some trials, a clinical response has been reported in some patients [27,30], whereas in other trials the results were negative [23,28,31]. The major reason for these treatment failures may have been that the targeting of the vector carrying the suicide gene to cancer cells was inefficient and HSV-tk expression too low.

Another obstacle is the paucity of functional gap junctions between glioblastoma cells, as a consequence of their anaplastic differentiation state [35].

Bystander killing effects, i.e., the killing of HSV-tk negative cells by adjacent HSV-tk-positive cells, depend mainly upon gap junction formation [36–38]. We have previously shown that the expression of the gap junction protein Cx43 [5] is induced by 4-PB [19], and this induction is correlated with enhanced intercellular fluorescent dye transfer.

We now demonstrate that 4-PB acts synergistically with GCV to amplify a bystander killing effect. In combination with GCV and in clinically achievable concentrations, 4-PB caused a substantial increase in the bystander killing effect. Even with GCV concentrations that by themselves only led to marginal effects on tumor cell proliferation, a dramatic decrease in cell number was seen in cultures treated with both drugs simultaneously. The GJC inhibitor AGA did not substantially prevent death of HSV-tk expressing cells, according to FACS analysis, but protected HSV-tk negative cells. Therefore, we conclude that GJC is essential for the bystander killing.

Interestingly, we noted that the effect of GCV on the relative number of HSV-tk-positive cells was reduced in the presence of AGA, while rather an increase of TK(S)-cell death in the presence of AGA was

anticipated. We had hypothesized that the inhibition of GJC "shuts the door," leading to a faster accumulation of the toxic GCV metabolite in these HSV-tk-positive cells. However, various mechanisms might prevent this. First, phosphorylated GCV primarily affects dividing cells, and the proliferation rate might be altered by the combined treatments. Thus, because of a decreased proliferation rate, it might take longer to eliminate nearly all HSV-tk expressing cells. Alternatively or additionally, it is likely that TK(S)-cells may express differential levels of the HSV-tk gene. Simultaneous administration of GCV and 4-PB enhances HSV-tk-expression in TK(S)-cells and induces gap junction formation in both TK(S)- and RFP-cells. The toxic metabolite of GCV can pass through gap junctions from TK(S)-cells with high HSV-tk expression to TK(S)-cells with weak HSV-tk expression, and to RFP-cells as well (Supplemental data, Fig. S3). Thus, nearly the whole cell population will be killed. The TK(S)-cells are killed first however, since the concentration of the toxic GCV-metabolite will reach the critical concentration sooner. In this scenario TK(S)-cells weakly expressing HSV-tk might also be poisoned by adjacent cells that strongly express the gene. Thus, the entire population of TK(S)-cells might survive longer in the presence of AGA than without the drug.

A GJC independent bystander killing effect has been previously reported [39]. Since 4-PB induces HSV-tk expression from the transfected plasmid, GJC independent killing by GCV will also be increased. We demonstrate here that 4-PB increases the bystander killing effect and that both Cx43 expression and induction of the expression of the transgene in tumor cells may be involved. Interestingly, in a current study the authors failed to induce GJC by Trichostatin A while *n*-butyrate succeeded [40]. In the same study, neither *n*-butyrate nor TSA seems to have induced the expression of the retrovirally introduced HSV-tk gene. However, in a mouse model it could be demonstrated that another HDAC-inhibitor, FR901228, improved HSV-tk/GCV therapy by inducing thymidine kinase expression [18]. Besides the fact that Robe et al. [40] used a different drug, a different construct for gene transfer or the integration locus into the genomic DNA might contribute to the varying results of these studies. However, both *n*-butyrate and 4-PB increase the bystander effect and gap junction communication in glioma cells. In contrast to *n*-butyrate which did not influence connexin 43 expression in several cell lines [40], we show here an induction of connexin 43 expression by 4-PB. Since 4-PB is an already FDA approved drug with weak or no side effects at the concentrations tested, the combined treatment of 4-PB and GCV could be beneficial for patients undergoing HSV-tk based gene therapy. Because of the increase in the bystander killing effect and the induction of suicide gene expression, a therapeutic anti-tumor effect may be obtained with a relatively low number of transfected cells. One great obstacle in suicide gene therapy, namely the efficient transfer of the suicide gene to its targets, may then be overcome.

Acknowledgments

This work was financially supported by grants from Marie Curie Fellowship (HPMF-CT-2000-00530); The Swedish Cancer Society; The Children's Cancer Foundation of Sweden; David and Astrid Hagelén's foundation; Knut and Alice Wallenberg's foundation (Wallenberg Consortium North, KAW 2003.0043); and Funds from the Karolinska Institutet.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2004.09.016.

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