



RESEARCH ARTICLE

Death receptor-independent cytochrome c release and caspase activation mediate thymidine kinase plus ganciclovir-mediated cytotoxicity in LN-18 and LN-229 human malignant glioma cells

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Suicide gene therapy using viral transfer of herpes simplex virus type I (HSV-1) thymidine kinase (TK) and subsequent ganciclovir (GCV) chemotherapy was the first approach used in clinical trials of somatic gene therapy for glioblastoma. The molecular pathways mediating TK/GCV-induced cell death remain to be elucidated. Here, we report that adenoviral (Ad)-TK/GCV-induced death is p53-independent and does not involve altered CD95 or CD95L expression. Ectopic expression of the preferential caspase 8 inhibitor, crm-A, inhibits Ad-CD95L-induced cell death but has no effect on TK/GCV cytotoxicity. LN-18 glioma cells selected for resistance to death receptor-mediated cell death do not acquire cross-resistance to TK/GCV. TK/GCV triggers mitochondrial cytochrome c release and activation of caspases 3, 7, 8 and 9 in a death receptor-independent manner. These events are associated with the loss of BCL-X_L. Forced expression of a BCL-X_L transgene, or co-exposure to a

pseudosubstrate caspase inhibitor, zVAD-fmk, inhibit TK/GCV cytotoxicity. Double-transfected cell lines expressing crm-A and enhanced green fluorescent protein (eGFP) show that the bystander effect in vitro is also death receptor- and caspase 8-independent. TK/GCV therapy does not kill glioma cells in synergy with cancer chemotherapy drugs, including lomustine, temozolomide and topotecan. In contrast, there is strong synergy of TK/GCV and CD95L. Thus, TK/GCV-induced cell death involves a mitochondria-dependent loop of caspase activation that can be synergistically enhanced by death receptor agonists such as CD95L. TK/GCV-mediated sensitization of glioma cells to CD95L expressed on immune effector cells or parenchymal brain cells might account for the immune system's and bystander effects of TK/GCV therapy observed in rodent glioma models in vivo. Gene Therapy (2001) 8, 469–476.

Keywords: glioma; gene therapy; thymidine kinase; ganciclovir; adenovirus

Introduction

Glioblastoma is a highly lethal neoplasm with a median survival of no more than 1 year even with aggressive multimodality treatment. Morbidity and death are a result of local invasiveness and brain tissue destruction but not of distant metastasis. Therefore, glioblastoma has become a major candidate tumor for the development of locally administered somatic gene therapy for cancer, notably suicide gene therapy. Suicide gene therapy is based on the transduction of tumor cells by vectors carrying foreign genes which encode enzymes capable of activating nontoxic prodrugs to cytotoxic metabolites.¹ Numerous studies have confirmed antiglioma effects of TK gene transfer followed by GCV treatment in laboratory animals.² The therapeutic effect of TK/GCV in rodent glioma models includes, in addition to the direct cytotoxic effect on TK-transduced cells which activate GCV,

the bystander effect, the phenomenon of cytotoxicity to proximate, nontransduced cells, which has been linked to gap junction trafficking and the gap junction protein, connexin 43, as well as presumably immune-mediated effects.^{3,4} However, the clinical experiences with TK/GCV therapy of glioblastoma have not fulfilled the great expectations that had been built on the experimental data.^{5–7} At present, gene delivery issues are probably the major obstacle to successful suicide gene therapy for glioblastoma. Furthermore, despite the plethora of laboratory studies confirming the efficacy of TK/GCV therapy in rodent glioma models, the molecular events underlying TK/GCV cytotoxicity and the bystander effect have not been clarified. Here, we report that TK/GCV cytotoxicity is p53- and death receptor-independent but involves the mitochondrial death pathway of cytochrome c release and caspase activation which is controlled by the level of anti-apoptotic BCL-2 family protein expression.

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Results

TK gene transfer sensitizes human malignant glioma cells to GCV cytotoxicity

The first experiments were designed to establish TK/GCV-induced cell death in two previously characterized glioma cell lines, LN-18 and LN-229.⁸ These cell lines were resistant to GCV in the absence of TK but became sensitive to GCV after TK gene transfer in a concentration- and time-dependent manner (Figure 1a, b). Similar findings were obtained in the rat CNS-1 glioma model *in vitro* (Figure 1c).

Since LN-229 cells retain wild-type p53 activity whereas LN-18 cells do not⁹ and since LN-229 cells were more sensitive to Ad-TK plus GCV (Figure 1), we next examined whether this was a consequence of the different p53 status of these cell lines. Neither cell line exhibited accumulation of p53, or of the major p53 response gene product, p21, in response to Ad-TK/GCV (Figure 2a), indicating that Ad-TK/GCV does not activate the p53 system in LN-229 cells. Exposure to camptothecin (2 μM /24 h) served as a positive control to illustrate that LN-229 cells accumulate p53 and p21 in response to genotoxic stress¹⁰ (data not shown). Moreover, expression of the dominant-negative p53 mutant, p53^{V143A}, which abrogates camptothecin-induced p21 accumulation,¹⁰ had no effect on Ad-TK/GCV cytotoxicity in LN-229 cells (Figure 2b).

TK/GCV-mediated cytotoxicity is independent of endogenous death ligand/interactions

Flow cytometry showed that TK/GCV cytotoxicity was not associated with changes in the expression of CD95L or CD95 at the cell surface. For these studies, LN-18 and LN-229 cells were infected with Ad-TK and treated with increasing concentrations of GCV for 48 h. Representative flow cytometry profiles for LN-229 cells are shown in Figure 3a. Furthermore, a two-fold approach was employed to examine a possible role for death ligand/receptor interactions in TK/GCV-mediated cell death. TK/GCV cytotoxicity was examined in LN-18 and LN-229 sublines engineered to express the viral caspase inhibitor, crm-A, which inhibits death ligand-induced cell death¹¹ and in a LN-18 subline selected for resistance to cytotoxic cytokines including CD95L, LN-18-R.⁸ As expected, cell death induced by Ad-CD95L was abrogated by crm-A (Figure 3b). In contrast, crm-A had no effect on TK/GCV-induced cytotoxicity (Figure 3c, d). Moreover, LN-18-R cells were as susceptible to TK/GCV-induced cell death as were parental LN-18 cells (Figure 3e). These data exclude a role for CD95/CD95L interactions in TK/GCV-induced cell death in glioma cells.

TK/GCV-mediated cytotoxicity involves cytochrome c release and caspase activation and is inhibited by BCL-X_L gene transfer

The next series of experiments was designed to examine whether TK/GCV induced death receptor-independent caspase activation via the mitochondrial pathway. Figure 4a shows that Ad-TK/GCV promoted the release of cytochrome c from mitochondria in a concentration-dependent manner. Moreover, immunoblot analysis revealed that caspases 3, 7, 8 and 9 were processed in both cell lines in response to TK/GCV (Figure 4b). Caspases 9 and 3 were activated at lower concentrations of GCV com-

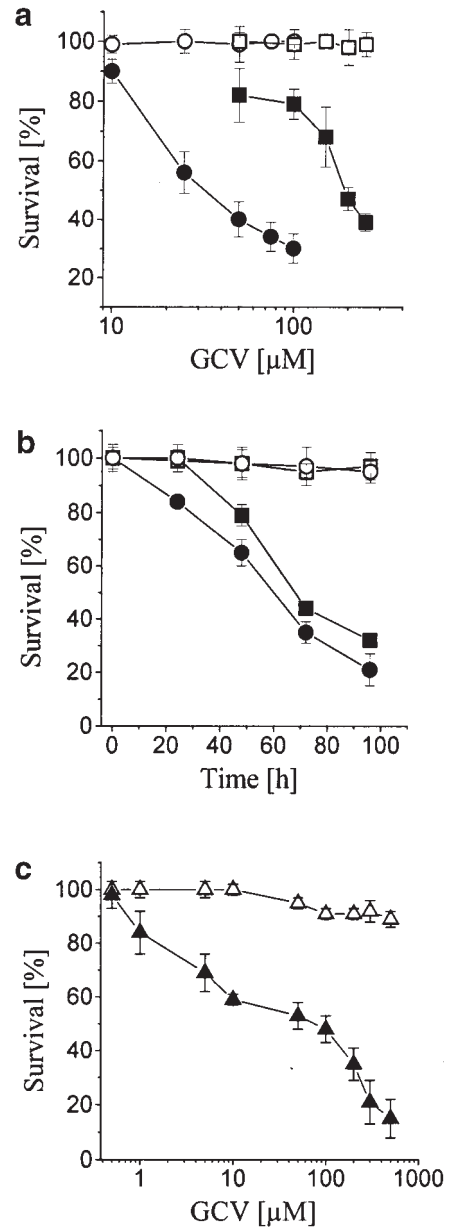


Figure 1 TK/GCV-induced cytotoxicity of human malignant glioma cell lines. LN-18 (squares) or LN-229 (circles) cells were not infected (open symbols) or infected with Ad-TK (100 MOI, 48 h, filled symbols) and then treated with GCV at increasing concentrations (a) or at 20 μM (LN-18) or 50 μM (LN-229) (b) for various lengths of time. (c) CNS-1 cells were not infected (open triangles) or infected with Ad-TK (100 MOI, 48 h, filled triangles) and treated with GCV at increasing concentrations. Survival was assessed by crystal violet staining. Data are expressed as mean percentages of survival and s.e.m. ($n = 3$). In a and c, the data are expressed relative to cells cultured in the absence of GCV, and Ad-TK alone reduced viability to 4% in LN-18, 9% in LN-229 cells and 11% in CNS-1 cells.

pared with caspase 8, consistent with a cytochrome c-triggered activation of the caspase cascade. In contrast, death receptor-dependent triggering of the caspase cascade, eg in response to CD95L, evolves with a fully synchronous pattern of caspase 3, 7, 8 and 9 activation (data not shown). Caspase activation was causally related to cell death since zVAD-fmk, a broad-spectrum caspase inhibitor, attenuated TK/GCV cytotoxicity in both cell lines

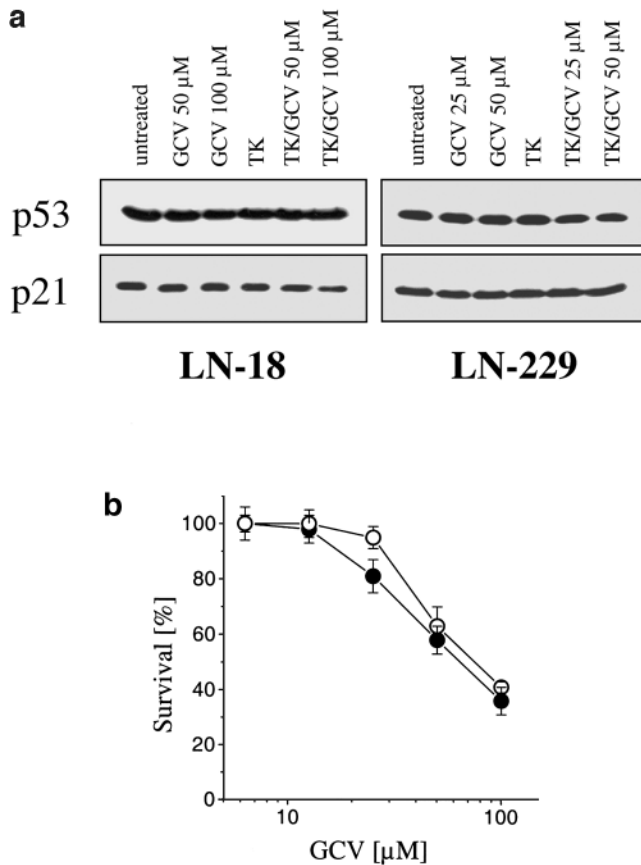


Figure 2 TK/GCV cytotoxicity of glioma cells is p53-independent. (a) LN-18 or LN-229 cells were treated with GCV alone (left) or Ad-TK-infected (100 MOI, 48 h) and then treated with GCV (right). Soluble protein lysates were harvested at 48 h and analysed for p53 and p21 levels by immunoblot. (b) LN-229 neo (filled circles) or p53^{V143A}-transfected cells (open circles) were infected with Ad-TK (100 MOI) for 48 h and then treated with increasing concentrations of GCV for an additional 48 h. Survival was assessed by crystal violet staining. Data are expressed as mean percentages of survival and s.e.m. ($n = 3$).

(Figure 4c). Since TK/GCV-induced cell death appeared to involve mitochondrial cytochrome c release (Figure 4a) and caspase activation (Figure 4b), we speculated that cell death might involve changes in BCL-2 family protein expression and would be amenable to inhibition by anti-apoptotic BCL-2 family proteins. Immunoblot analysis showed that TK/GCV induced a prominent loss of BCL-X_L protein whereas the levels of BCL-2 or BAX were unaffected (Figure 5a). Furthermore, LN-18 and LN-229 cells were transfected with a human BCL-X_L expression plasmid. Transgene expression was confirmed by immunoblot analysis (data not shown). Ectopically expressed BCL-X_L protein conferred strong protection from TK/GCV-induced cell death (Figure 5b).

The bystander effect: no inhibition by crm-A

To assess a possible role of death ligand/receptor interactions and caspase 8 in triggering the killing of bystander cells, we established an *in vitro* model of the bystander effect and took advantage of crm-A-expressing cells as targets. These cells have been shown here (Figure 3b) and previously¹¹ to resist death receptor-mediated apoptosis. Puro control and crm-A-transfected cells were cotrans-

duced with a plasmid encoding eGFP. Ad-TK-infected LN-18 or LN-229 cells were cocultured with crm-A/GFP- or puro/GFP-transfected cells and incubated with GCV for 48 h. The cytotoxicity of GCV in non-fluorescent (TK-positive) cells and GFP-expressing (TK-negative) cells was determined by propidium iodide (PI) staining. As shown in Figure 6 for LN-229, crm-A/GFP cells were as susceptible as puro/GFP cells to the bystander killing by TK-expressing cells. Similar effects were observed in LN-18 cells (data not shown). These data illustrate that not only the direct cytotoxic effects of TK/GCV but also the bystander killing *in vitro* are independent of death ligand/receptor interactions and do not require caspase 8.

Synergistic glioma cell killing by Ad-TK/GCV and CD95L

The final series of experiments sought to identify death stimuli which kill glioma cells in synergy with TK/GCV. The cells were cotreated with TK/GCV and different cancer chemotherapy drugs, including lomustine, temozolomide and topotecan, or the cytotoxic cytokine, CD95L. Synergy was assessed by isobologram analysis. The simultaneous treatment with TK/GCV and the chemotherapeutic drugs resulted in approximately additive killing, as indicated by values around the straight line in the isobologram, representatively shown for topotecan (Figure 7a). In contrast, strong synergy was observed in both cell lines when TK/GCV was combined with CD95L (Figure 7b). Synergy was not observed with non-infected cells that were treated with GCV plus CD95L alone (data not shown).

Discussion

Suicide gene therapy using transfer of the HSV-1-TK gene followed by systemic GCV is the most widely used approach of somatic gene therapy for human glioblastoma.⁵⁻⁷ The present study elucidated some of the molecular pathways of TK/GCV-induced cell death in two human malignant glioma cell lines, LN-18 and LN-229. As expected, exposure to GCV was not toxic when administered alone but resulted in time- and concentration-dependent killing of glioma cells transduced with TK (Figure 1). Conversely, Ad-TK gene transfer was largely nontoxic in the absence of GCV (Figures 1a, 3c, e). Wild-type p53 status has been hypothesized to be protective for TK/GCV cytotoxicity in the human MCF-7 breast cancer cell line.¹² In contrast, p53 has been proposed to mediate CD95 aggregation and caspase activation in TK/GCV-treated neuroblastoma cells.¹³ We find that TK/GCV therapy does not trigger the p53 pathway in p53 wild-type human glioma cells, similarly to what was described in rat C6 glioma cells,¹⁴ and that abrogation of endogenous wild-type p53 function does not alter the response to TK/GCV (Figure 2). Furthermore, death ligand/receptor interactions are not necessary for TK/GCV cytotoxicity of human glioma cells (Figure 3) even though a role for CD95L/CD95 in TK/GCV toxicity of nonglial murine tumors has been proposed.¹⁵ TK/GCV activates the mitochondrial death pathway in human glioma cells, as evidenced by the detection of mitochondrial cytochrome c release and the processing of multiple caspases. These observations confirm and extend a recently published, independent study in a neuroblastoma cell line.¹⁶ Of note, the caspase most proximate to death

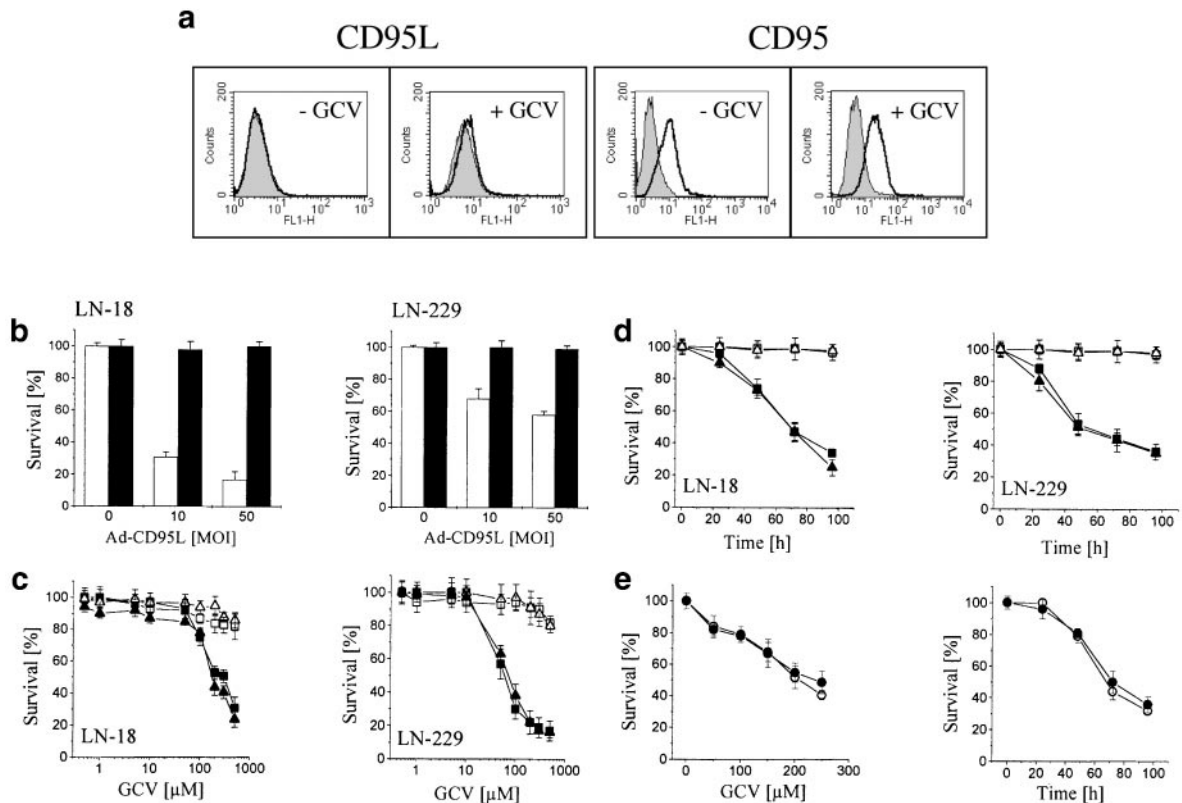


Figure 3 TK/GCV-induced cell death is not mediated by CD95/CD95L interactions. (a) LN-229 cells were infected with Ad-TK (100 MOI, 48 h), subsequently treated with GCV (25 μ M) for 48 h and analysed for CD95L and CD95 expression by flow cytometry. (b) Puro control (open bars) or *crm-A*-transfected (filled bars) LN-18 or LN-229 cells were treated with Ad-CD95L at increasing MOI. Puro control (squares) or *crm-A*-transfected (triangles) cells were not infected (open symbols) or infected with Ad-TK (100 MOI, 48 h, filled symbols) and then treated with GCV at different concentrations for 48 h (c) or at 50 μ M (LN-18) or 20 μ M (LN-229) for different lengths of time (d). (e) Parental LN-18 (open circles) or CD95L-resistant LN-18-R (filled circles) cells were infected with Ad-TK and then treated with increasing concentrations of GCV for 48 h (left) or with GCV at 50 μ M for various lengths of time (right). In b–e, survival was assessed by crystal violet staining (mean percentages of survival and s.e.m., $n = 3$). In c and in e (left panel), the data are expressed relative to cells cultured in the absence of GCV, and Ad-TK alone reduced viability to 6% in LN-18 and 8% in LN-229 cells.

receptors, caspase 8, plays no important role in the killing pathway of TK/GCV since the preferential caspase 8 inhibitor, *crm-A*, was not protective (Figure 3c, d). In fact, prominent and early caspase 9 processing is compatible with the mitochondrial triggering of the caspase cascade.¹⁷ The critical role of mitochondria is also underlined by the observations that a mitochondria integrity-preserving protein, BCL-X_L, is lost, presumably degraded, during TK/GCV-induced cell death (Figure 5a) and that BCL-X_L gene transfer protects from cell death (Figure 5b). In contrast, rat C6 glioma cells exhibited increased BAX levels, which were unaltered here (Figure 5a), but unaltered BCL-X_L levels in response to TK/GCV.¹⁴ The discrepancies between some of the data obtained in rodent models and the data obtained with human cell lines, representing naturally occurring gliomas, in the present study highlight the necessity to verify data with potential clinical implications in the human system.

The bystander effect as modeled *in vitro* here (Figure 6) did not require CD95L/CD95 interactions and was caspase 8-independent. Moreover, the lack of changes in CD95L expression does not support the suggestion that TK/GCV therapy may induce tumor cell fratricide via up-regulated CD95L in TK-expressing cells acting on CD95 in non-infected cells.¹⁵ However, the strong syn-

ergy of TK/GCV and exogenous CD95L (Figure 7) suggests that TK/GCV might not only sensitize human glioma cells to soluble CD95L *in vitro* but to CD95L expressed by immune effector cells or other normal brain parenchymal cells *in vivo*.

Although the molecular pathways of TK/GCV-induced cell death delineated here for glioma cells differ from those described in neuroblastoma cells¹³ with regard to the role of p53 and CD95 accumulation, TK/GCV effects are synergistically enhanced by cytotoxic cytokines such as CD95L both in neuroblastoma¹³ and in glioma cells (Figure 7). In contrast, there was no synergy of TK/GCV and other cytotoxic drugs, including topotecan which has been reported to act in synergy with TK/GCV in colon carcinoma cells.¹⁸

Materials and methods

Chemicals and cell lines

CD95L was obtained from murine CD95L-transfected murine N2A neuroblastoma cells. The human malignant glioma cell lines LN-18 and LN-229 were kindly provided by Dr N de Tribolet (Lausanne, Switzerland). The cells were maintained in DMEM containing 10% fetal calf serum and penicillin (100 IU/ml)/streptomycin (100

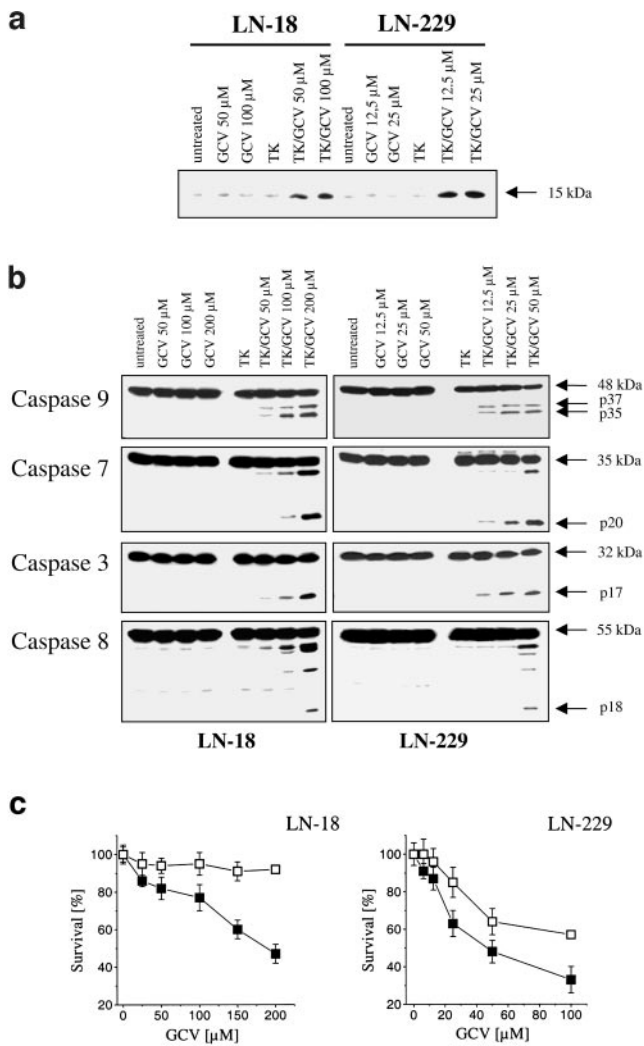


Figure 4 TK/GCV cytotoxicity involves cytochrome c release and caspase activation. Non-infected or Ad-TK-infected (100 MOI, 48 h) LN-18 or LN-229 cells were treated with increasing concentrations of GCV for 48 h and analysed for cytoplasmic cytochrome c (a) or processing of caspases 9, 7, 3 or 8 (b). Activation is reflected by the formation of p37/35/caspase 9, p20/caspase 7, p17/caspase 3 and p18/caspase 8. (c) The cells were infected with Ad-TK (100 MOI, 48 h) and treated with different concentrations of GCV in the absence (filled squares) or presence (open squares) of zVAD-fmk (100 μ M). Survival was assessed by crystal violet staining (mean percentages of survival and s.e.m, $n = 3$).

μ g/ml). The generation of glioma cell sublines expressing crm-A has been described.¹¹ Glioma cell sublines engineered to express human BCL-X_L were obtained by electroporation (Biorad Gene Pulser, 250 V, 950 μ F; Biorad, Munich, Germany) using the pSFFV-BCL-X_L plasmid, kindly provided by Dr CB Thompson (Chicago, IL, USA) or the empty neo plasmid as a control. Transgene expression was assessed by immunoblot analysis. The generation of LN-229 cell clones expression human p53^{V143A} has been described.¹⁰ The broad-spectrum caspase inhibitor, zVAD-fmk, was from Bachem (Heidelberg, Germany). GCV (Cymeven; Hoffmann-La Roche, Grenzach-Wyhlen, Germany) was dissolved in sterile phosphate-buffered saline (PBS) (0.2 M) and stored at -20°C . Dilutions were made with culture medium at the time of treatment. PI was purchased from Sigma (St Louis, MO, USA). Topotecan was obtained from Smith-

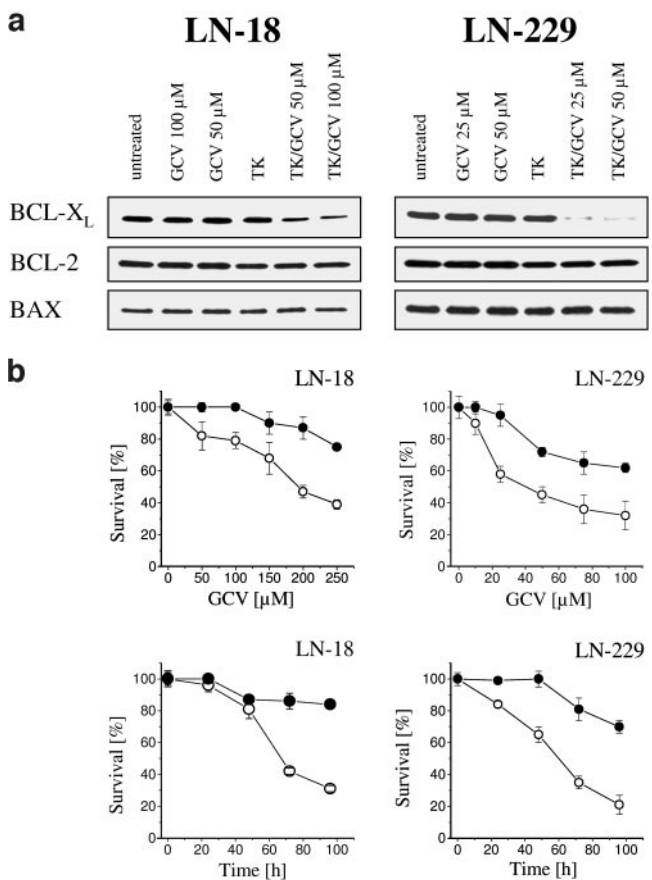


Figure 5 TK/GCV cytotoxicity in human glioma cells: role of BCL-X_L. (a) Soluble lysates from LN-18 or LN-229 cells treated with GCV alone, or infected with Ad-TK (100 MOI, 48 h) and then treated with GCV for 48 h, were analysed for the levels of BCL-X_L, BCL-2 and BAX proteins. (b) Neo control (open circles) or BCL-X_L-transfected (filled symbols) LN-18 or LN-229 cells were infected with Ad-TK (100 MOI, 48 h) and then treated with GCV at increasing concentrations (upper panels) or at 50 μ M (LN-18) or 25 μ M (LN-229) for various lengths of time (lower panels). Data are expressed as in Figure 1.

Kline Beecham Pharmaceuticals (King of Prussia, PA, USA), temozolomide was from Schering Plough (Kenilworth, NJ, USA), lomustine was from Bristol-Myers Squibb (Syracuse, NY, USA).

Infection of human glioma cells with recombinant adenoviruses and viability assays

The recombinant adenoviruses (RAd) expressing HSV-1-TK (RAd128), CD95L or the reporter gene, β -galactosidase (RAd35), were generated as described previously.^{19–21} The cells were seeded at 8×10^3 cells per well in 96-well plates and infected 24 h later with Ad-TK (100 MOI). The cells were exposed to the infectious viral particles in medium without serum for 30 min. Then, complete medium was added for 48 h. The medium was removed. GCV was added at different concentrations for another 48 h or at fixed concentrations for different lengths of times. Infection efficacy was verified by parallel infections with Ad expressing the *E. coli* β -galactosidase gene, visualized by staining with the substrate X-gal. The cytotoxic effect of GCV was determined by colorimetric quantification of viable cells by crystal violet staining. Briefly, the supernatant was removed, and the

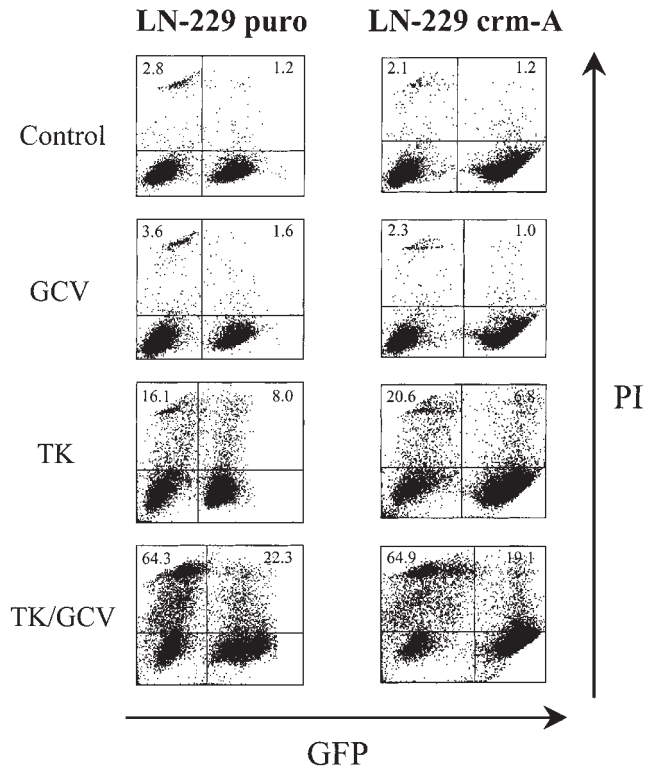


Figure 6 The bystander effect does not require CD95/CD95L interactions or caspase 8. LN-229 cells were infected with Ad-TK (100 MOI, 48 h) and then cocultured with non-infected puro/GFP- or crm-A/GFP-transfected cells in a 1:1 ratio. After 24 h, GCV (25 μ M) was added for 48 h. The cytotoxic effects of GCV to non-fluorescent (TK-positive) and GFP-expressing bystander cells were assessed by PI staining. The intensity of PI staining is visualized on the y-axis, the absence or presence of GFP is shown on the x-axis. Accordingly, eg, killed non-GFP-expressing cells are found in the left upper quadrant, and viable GFP-expressing cells in the right lower quadrant, of each panel.

cells were incubated for 10 min in crystal violet solution (2%) in 20% methanol. The plates were washed in running tap water and air-dried for 24 h. After addition of 0.1 M sodium citrate buffer, optical density (OD) values were read at 550 nm on a Dynatech plate photometer (Denkendorf, Germany).

Immunoblot analysis

Immunoblot studies were performed according to standard procedures.²² The following primary mouse monoclonal antibodies were used: anti-human caspase 3 (1:1000; Transduction Laboratories; Lexington, KY, USA), anti-human caspase 8 (C15; 1:5; kindly provided by Dr PH Krammer, Heidelberg, Germany), anti-human caspase 7 and caspase 9 (1:1000; kindly provided by Y Lazebnik, Cold Spring Harbor, NY, USA), anti-bovine cytochrome c oxidase subunit IV (1 μ g/ml; Molecular Probes, Eugene, OR, USA), anti-human p53 (2 μ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human BCL-2, BCL-X_L, BAX and cytochrome c (2 μ g/ml; PharMingen, San Diego, CA, USA). Specific antibody binding was detected by horseradish peroxidase-conjugated anti-mouse IgG (1:3000; Amersham, Braunschweig, Germany). Enhanced chemiluminescence (ECL) reagents (Amersham) were used for detection.

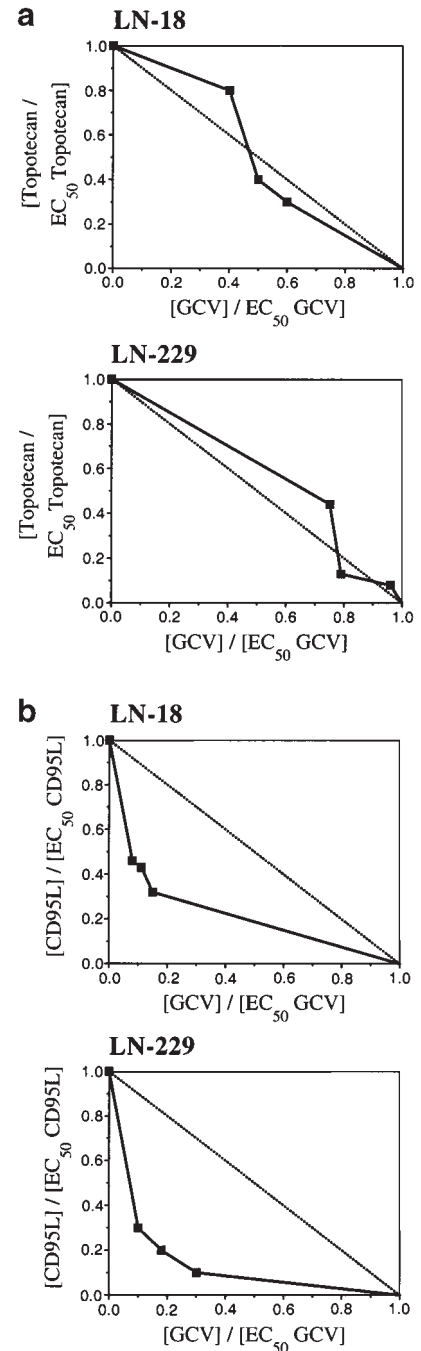


Figure 7 Synergy of TK/GCV and CD95L. LN-18 or LN-229 cells were infected with Ad-TK (100 MOI, 48 h) and treated with serial dilutions of GCV, topotecan or both (a) or GCV, CD95L or both (b) for an additional 48 h. Synergy was assessed by isobologram analysis.²³

Measurement of cytochrome c release

The cells were treated as indicated, washed with PBS and lysed for 5 min at 4°C in 210 mM D-mannitol, 70 mM sucrose, 10 mM HEPES, 200 μ M EGTA, 5 mM succinate, 0.15% BSA and 40 μ g/ml digitonin (MSH buffer). After lysis, the supernatant (cytosolic fraction) was centrifuged for 10 min at 13 000 r.p.m. The supernatant was incubated with an equal volume of 10% trichloroacetic acid. The samples were kept at -20°C for at least 30 min and then centrifuged for 10 min at 13 000 r.p.m. The pellets

were resuspended in Laemmli buffer and analysed for cytochrome c content by immunoblot analysis. The samples were also analysed for contamination due to mitochondria by immunoblot analysis for cytochrome c oxidase.

Investigation of the bystander effect

To analyse the bystander effect, LN-18 or LN-229 cells stably expressing crm-A, or the puromycin resistance gene only, were cotransfected with a plasmid encoding eGFP (pIRES1-EGFP) (Clontech, Palo Alto, CA, USA). Non-GFP-transfected glioma cells were infected with Ad-TK (100 MOI) for 48 h, harvested by trypsination and then cocultured with non-Ad-TK-infected puro/GFP or crmA/GFP cells at a 1:1 ratio. GCV was added 24 h later for an additional 48 h. PI staining was used for the determination of the loss of membrane integrity, to assess loss of viability. Floating cells were collected and pooled with trypsinised adherent cells, washed twice with PBS and resuspended in PBS containing PI (20 µg/ml). PI staining and GFP expression were analysed by a Becton Dickinson FACScalibur cytometer (Becton Dickinson, Heidelberg, Germany). The percentage of PI-positive, non-GFP-expressing cells (TK-transduced) and of PI-positive, GFP-expressing cells (bystander effect-killed cells) was quantified by CELLQuest software (Becton Dickinson).

Flow cytometry

CD95 and CD95L expression were detected by flow cytometry as described.⁸ The cells were stained with mouse anti-human CD95 (UB2; Immunotech, Krefeld, Germany) or mouse anti-human CD95L (Nok-1; PharMingen, USA) followed by sheep anti-mouse IgG-FITC (Sigma). Mouse IgG_{1κ} (Sigma) was used as an isotype control. The specific fluorescence index (SFI) was calculated as the ratio of the mean fluorescence values obtained with the specific antibody and the isotype control antibody.

Statistical analysis

All data are representative of experiments performed at least three times with similar results. Synergy was determined by isobologram analysis,²³ as described previously.²⁴ The non-infected and TK-infected cells were treated with different concentrations of each agent (GCV or cytotoxic drug) alone or with two agents in combination. The relative survival was assessed and the EC₅₀ values (median effect dosis) for each drug administered alone, or in combination with a fixed concentration of the second agent, were established from the concentration-effect curves. The EC₅₀ values of co-treatment were divided by the EC₅₀ value of each drug in the absence of the other drug. In a graphical presentation, the straight line connecting the EC₅₀ values of the two agents when applied alone corresponds to additivity, or independent effects of both agents. Values below this line indicate synergy, values above this line indicate antagonism.

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