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Up-regulation of Mismatch Repair Pathway by Suicide Gene Therapy: Implications on the use of Temozolomide Treatment in Malignant Glioma

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Abstract

Objective: The dependency of the efficacy of temozolomide (TMZ) on cellular DNA repair activities makes it therapeutically effective in approximately half of malignant glioma (MG) patient population with a dysfunctional DNA repair system. Adenovirus-mediated Herpes simplex virus thymidine kinase and ganciclovir *(AdHSV-tk/GCV)* suicide gene therapy is effective in those as well as in MG patients with a functional DNA repair system. When administered together, these two therapies show evidence of synergistic cytotoxicity. However, the validity of such claims has been questioned as the exact mechanism has been unknown.

Methods: The underlying mechanism was studied in rat and human MG cell lines and in an immunocompetent, orthotopic, syngeneic rat MG model.

Results: The results, for the first time, revealed an up-regulation of mismatch repair *(MMR)* pathway in MG cells by *AdHSV-tk/GCV* therapy, an adjunct effect to *AdHSV-tk/GCV's* pro-apoptotic therapeutic mode of action that enhanced the cytotoxicity of TMZ. When combined with *AdHSV-tk/GCV* therapy, initially resistant MG cells were sensitized to TMZ treatment. The enhancement of TMZ's efficacy was also seen *in vivo* as a significant increase in survival and a reduction in tumor growth rate, without affecting the adverse effect profile.

Conclusion: This study demonstrates a synergistic outcome of *AdHSV-tk/GCV* and TMZ treatment combination, underlying mechanism for the synergy and a possible improved therapeutic protocol for enhanced efficacy. The findings may have an impact on future clinical use of this treatment combination, as well as benefit other chemotherapies, which depend on *MMR* pathway for action.

Keywords: Malignant glioma; Suicide gene therapy; Temozolomide; Mismatch repair; Synergy; Adenovirus; DNA repair

Introduction

MGs are aggressive brain tumors carrying a dismal prognosis. In spite of the current standard-of-care, which includes surgical resection followed by radiotherapy with concomitant and adjuvant chemotherapy [1], the outcome of patients still remains grim. This unmet clinical need has led to extensive research efforts into more effective treatments for this cancer.

Over the last two decades, gene therapy has emerged as a novel experimental therapeutic option for MG [2,3]. Based principally on the pro-drug activation, HSV-tk enzyme converts non-toxic compounds, such as GCV, into toxic metabolites [4], leading to cell destruction in a cell cycle-dependent manner, affecting only the dividing cells. Cytotoxicity of HSV-tk/GCV is further facilitated by a bystander-effect [5,6] and local and systemic immune responses [7]. To-date, it is the most widely studied suicide gene therapy approach with proven efficacy in several clinical trials [8-10].

TMZ, which is a chemotherapy approved for the treatment of glioblastoma multiforme (GBM) [1,11], is an orally available, alkylating agent that crosses the blood brain barrier [12]. The primary toxic effect of TMZ is gained by methylation of guanine nucleotides in DNA at O6 position [13]. However, the efficacy of TMZ is dependent on the function of different DNA repair pathways, namely: Methyl guanine methyl transferase (MGMT) repair, MMR and base excision repair [14,15]. For example, a functional MGMT repair pathway, which is active in approximately 50% of the GBM patients, leads to TMZ resistance [16]. Since *AdHSV-tk/GCV's* main therapeutic mode of action is not affected by this mechanism, it is effective in such patients. Previous studies have combined TMZ with other chemotherapeutics [17], radiotherapy [1] and gene therapy [18,19]. Synergism between suicide gene therapy and TMZ was demonstrated by us and by others [18,19], but the underlying mechanism has remained elusive.

In this study, effectiveness of each treatment alone and the synergistic effect of *AdHSV-tk/GCV* and TMZ combination treatment was studied both *in vitro*, in human and rat MG cell lines, and *in vivo*, in an immunocompetent, orthotopic, syngeneic rat MG model, where the efficacy of treatment combination was evaluated by the effects on

tumor volume and animal survival. Our results elucidate for the first time the underlying mechanism for the synergism between *AdHSV-tk/GCV* and TMZ. Furthermore, we have delineated a potential treatment protocol to combine these two therapies in the expectation of maximizing the therapeutic outcome. Potential adverse effects of the treatment combination were also evaluated.

Materials and Methods

Adenovirus vector

AdHSV-tk (Cerepro®); a replication-deficient, first-generation, E1-E3 deleted, serotype-5 adenovirus vector carrying HSV-tk cDNA, driven by a cytomegalovirus promoter, manufactured in HEK-293 cells and purified by density gradient centrifugation [8,20], was provided by Ark Therapeutics (Kuopio, Finland). The virus stock, tested to be free of replication-competent viruses, lipopolysaccharide and other biological contaminants, had an OD260 titer of 1.7 × 1012 viral particles/ml.

Cell lines and pharmaceutical compounds

Rat BT4C [21] (generous gift from University of Bergen, Norway), and human U87MG (ATCC, HTB-14) MG cells were grown in Dulbecco's modified Eagles' medium and human T98G MG cells (ATCC, CRL-1690) (Borås, Sweden) were grown in minimal essential medium, containing L-glutamine and glucose, supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA). All cell lines were adherent and grown at 37°C in the presence of 5% CO2. TMZ (Temodal®, Schering-Plough-Merck, Whitehouse Station, NJ, USA), 100 mg capsules were dissolved in 10 ml of 10% dimethyl sulphoxide-saline, daily before the injections and the intravenous TMZ (100 mg) was prepared according to manufacturer's instructions to create 2.5 mg/ml stock solution. GCV (Cymevene® 500 mg, Roche, Espoo, Finland) was diluted in 10 ml of water for injection to achieve 50 mg/ml stock solution.

In vitro effectiveness and synergism experiments

To determine the *in vitro* effectiveness of the individual treatments and synergism of *AdHSV-tk/GCV* and TMZ, 4000 BT4C, U87MG and T98G cells per well were plated onto 96 well plates on day 3, after transduction with *AdHSV-tk* at MOIs, 10 and 25 (BT4C and T98G) and, 1 and 10 (U87MG) in 6-well plates on day 2. Lower *AdHSV-tk* MOIs were used with U87MG cells because of their extreme sensitivity to this therapy. Cells were treated with 1 µg/ml GCV and/or TMZ at a dose of 10, 100 or 1000 µmol/l on day 4, for 24 h. Untreated cells were used as controls. Cell viability was measured 144 h after treatment using a 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega CellTiter 96® Aqueous One Solution Cell Proliferation Assay), according to manufacturer's instructions. Absorbance was detected at 490 nm using a spectrophotometer. Means of the triplicate values are presented.

RT-PCR for MGMT, MSH-2 and MLH-1

To evaluate the effect of *AdHSV-tk/GCV* and TMZ on MGMT and *MMR* pathways, 50000 BT4C cells were plated per well onto 6-well plates on day 1, transduced with *AdHSV-tk* at MOI 5 on day 2 and treated with 1 µg/ml GCV and 100 µmol/l TMZ on day 4. The samples for RT-PCR were collected 24 h and 96 h after starting the treatment. Untreated cells were used as controls. Total RNA was extracted from cells by TriReagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA was reverse transcribed to cDNA by M-MuLV reverse transcriptase (MBI Fermentas, Vantaa, Finland). Target gene mRNA levels were measured by real-time PCR (StepOnePlus Real-Time PCR system, Applied Biosystems, Carlsbad, CA) using specific Taqman® gene expression assays (Applied Biosystems) for rat mutL homolog (MLH-1) (Rn00579159_m1), mutS homolog (MSH-2) (Rn00579198_m1), MGMT (Rn00563462_m1) and *beta-actin* (4352931E) for normalization. Mean of triplicate values are presented. All *in vitro* experiments were repeated at least twice.

To show *MMR* upregulation in a human MG cell line, 100 000 T98G cells per well were plated onto 6-well plates on day 1, transduced with *AdHSV-tk* at MOI 5 on day 2 and treated with 1 µg/ml GCV on day 4. The samples for RT-PCR were collected 120 h after starting the treatment. Untreated cells were used as controls. Total RNA was extracted from cells by RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA by RevertAid First Strand cDNA Synthesis Kit #1622 (Thermo Fisher Scientific Inc., Waltham MA, USA). Target gene mRNA levels were measured by real-time PCR (StepOnePlus Real-Time PCR system, Applied Biosystems, Carlsbad, CA) using specific PrimePCR SYBR Green Assays (Bio-Rad Laboratories, CA, USA) for human MLH-1 (qHsaCED0042382), MSH-2 (qHsaCID0012204) and beta-actin (qHsaCED0036269) for normalization.

In vivo experimental model

Immuno-competent, inbred, male BDIX rats (Charles Rivers Laboratories, France) (175-200 g) bearing BT4C rat MGs were created as described elsewhere [6,19,20,22]. Briefly, 10,000 BT4C cells were injected into the anaesthetized rat brain, 1 mm posterior and 2 mm to the right of bregma, at a depth of 2.5 mm from dural surface, using a 27 G, 25 µl Hamilton-syringe (Hamilton, Bonaduz Ab, Switzerland) placed on a stereotactic device. All animal experiments were approved by the Ethical Committee of the University of Eastern Finland under the ethical permit number ESLH-2008-03818/Ym-23.

Magnetic Resonance Imaging (MRI)

The presence of tumors was verified by MRI on post-tumor inoculation (p.i.) days 12 or 13. Follow-up MRIs were performed on p.i. days 28 and 42 as described elsewhere [23-46]. For MRI, anesthesia was induced with 5% and maintained at 1.5% isoflurane, in a gas chamber having a 70:30% mixture of N2:O2. MRI was done using a 4.7 T small animal MRI scanner (Magnex Scientific Ltd, Abington, UK) interfaced to Varian Unity Inova (Palo Alto, CA, USA) console. Non-contrast enhanced, T2-weighted spin-echo sequence were used to generate 17 coronal images of 1 mm thickness with a field of view of 40 × 40 mm, having no gaps between the slices, to cover the tumor area. Tumor volume was calculated by delineating the lesion area in all images with Matlab 7.1b (MathWorks Inc., Natick, MA, USA).

Study groups

After the 1st MRI animals were randomized into different study groups (Table 1). One objective of the study was to have a treatment group that "mimics" the *AdHSV-tk/GCV* and TMZ combination protocol in clinical trials, where 14-day GCV administration, starting 5 days after gene transfer, followed by commencement of TMZ therapy after variable time-gaps in-between [10]. Study group 4 (combination

group A) closely represents this clinical trial protocol, except for the reduction of GCV therapy to 7 days, in order to complete both therapies before the median survival (35 days) of this aggressive model is reached. In study group 5 (combination group B), the gaps between treatments were omitted to test our hypothesis of up-regulation of *MMR* pathway by *AdHSV-tk/GCV* enhancing the efficacy of TMZ.

Cell lines	**Viability with individual drug**					**Predicted viability for additive effect (γ1*γ2)**				**Observed viability-drug combination (γ1,2)**			
BT4C	**TK**	**GCV**	**Viability**	**TMZ**	**Viability**	**GCV 1 +**		**TMZ µmol/l**		**GCV 1 +**		**TMZ µmol/l**	
	MOI	µg/ml	γ1	µmol/l	γ2	TK MOI	10	100	1000	TK MOI	10	100	1000
144 h	0	1	0.9	10	0.92	0	0.83	0.81	0.59	0	0.82	0.82	0.68
	10	1	0.65	100	0.9	10	0.6	0.59	0.42	10	0.46	0.18	0.2
	25	1	0.32	1000	0.65	25	0.29	0.29	0.21	25	0.18	0.14	0.15
U87MG	**TK**	**GCV**	**Viability**	**TMZ**	**Viability**	**GCV 1 +**		**TMZ µmol/l**		**GCV 1 +**		**TMZ µmol/l**	
	MOI	µg/ml	γ1	µmol/l	γ2	TK MOI	10	100	1000	TK MOI	10	100	1000
	0	1	0.95	10	0.89	0	0.85	0.87	0.63	0	0.91	0.87	0.63
	1	1	0.6	100	0.92	1	0.53	0.55	0.4	1	0.51	0.4	0.34
	10	1	0.26	1000	0.66	10	0.23	0.24	0.17	10	0.25	0.2	0.2
T98G	**TK**	**GCV**	**Viability**	**TMZ**	**Viability**	**GCV 1 +**		**TMZ µmol/l**		**GCV 1 +**		**TMZ µmol/l**	
	MOI	µg/ml	γ1	µmol/l	γ2	TK MOI	10	100	1000	TK MOI	10	100	1000
144 h	0	1	0.97	10	0.9	0	0.87	0.79	0.76	0	0.76	0.71	0.67
	10	1	0.96	100	0.81	10	0.86	0.78	0.75	10	0.94	0.73	0.58
	25	1	0.72	1000	0.78	25	0.65	0.58	0.56	25	0.69	0.55	0.5

The synergism for the combination of AdHSV-tk/GCV and TMZ was derived by fractional product method of Webb [27], where γ1 and γ2 are cell viabilities after treatment with AdHSV-tk/GCV and TMZ, respectively, γ1*γ2 is the predicted fractional product cell viability for additive effect and γ1,2 is the observed cell viability after drug combination. γ1*γ2 = γ1,2 is additive effect, γ1*γ2 > γ1,2 is synergy (in bold) and γ1*γ2 <γ1,2 is antagonism. AdHSV-tk MOIs 0, 10 and 25 for BT4C and T98G cells, and 0, 1 and 10 for U87MG cells with 1 µg/ml GCV was combined with 10, 100 and 1000 µmol/l TMZ and the viability determined 144h later. GCV: Ganciclovir, H: Hours, MOI: Multiplicity of Infection, TK: Thymidine Kinase (Herpes Simplex Virus), TMZ: Temozolomide.

Table 1: Synergy determination by fractional product method of Webb [27].

Treatment

The *AdHSV-tk* gene transfers were conducted on p.i. days 14 and 15. A total vector volume of 20 µl/day was administered using a Hamilton syringe placed on a stereotactic device. A volume of 10 l per location was injected at vertical depths of 2.0 mm and 2.5 mm from bregma level. The treatment protocol for different groups is summarized in Table 1. Intra-peritoneal (IP) GCV was commenced the day after last gene transfer (study group 5) or 5 days after gene transfer (Study groups 3 and 4), at a total dose of 50 mg/kg/day per rat, given in two equal doses, 12 h apart. This dose for 14 consecutive days was proven effective against MG in several pre-clinical [6,19,20,22] and clinical studies [8-10]. IP injections of TMZ as a single dose of 60 mg/kg/day, for 5 consecutive days, were carried out as described in Table 1. TMZ dose and the duration were determined based on literature [23].

Survival

Survival was calculated from the day of tumor cell inoculation to the day of euthanasia or death of an animal. During the experiment, the animals were observed daily with regular body weights measurement, and sacrificed if the criteria for euthanasia, defined by the University of Eastern Finland Animal Ethics and Welfare Committee, were met.

Analyses of blood and serum samples

Blood and serum samples were collected after completing treatment for full blood count (FBC) and clinical chemistry analyses for bilirubin, creatinine, alkaline phosphatase (AP) and alanine aminotransferase (ALT) at Kuopio University Hospital Laboratory. The CD3, CD4 and CD8 cell counts were analysed by flow cytometer (FACScanto II, BD Biosciences, San Jose, CA) using rat leucocyte antibodies (APC Mouse anti-rat CD3, PE Mouse anti-rat CD4 and FITC Mouse Anti-Rat CD8, BD Biosciences).

Statistical analyses

All statistical data were analyzed using GraphPad Prism Version 5.01 statistical software (GraphPad Software Inc. La Jolla, CA, USA). *In vitro* cell viability and RT-PCR grouped analyses for BT4C cells were

done using two-way ANOVA and Bonferroni post-hoc test. Survival data were analyzed using Kaplan-Meier survival plots and the curves were compared using the Mantel-Cox Log-rank test. The FBC, serum chemical, T-cell, tumor volume and RT-PCR (T98G) data were analyzed (column analyses) using one-way ANOVA 10 or Kruskal-Wallis test. Values were compared to the controls or to one another using either Bonferroni or Dunn's multiple comparison post-hoc tests. In FBC, serum and T-cell analyses, only the statistical significances compared to the controls were reported.

Results and Discussion

In vitro effectiveness and synergism

The efficacy of treatment combination was evaluated in rat BT4C and human U87MG (MGMT-negative) [24] and T98G (MGMT-positive) [25] MG cell lines. As a single therapy, both *AdHSV-tk/GCV* and TMZ demonstrated almost similar efficacy in BT4C and U87MG cell lines (Figure 1 and Table 1).

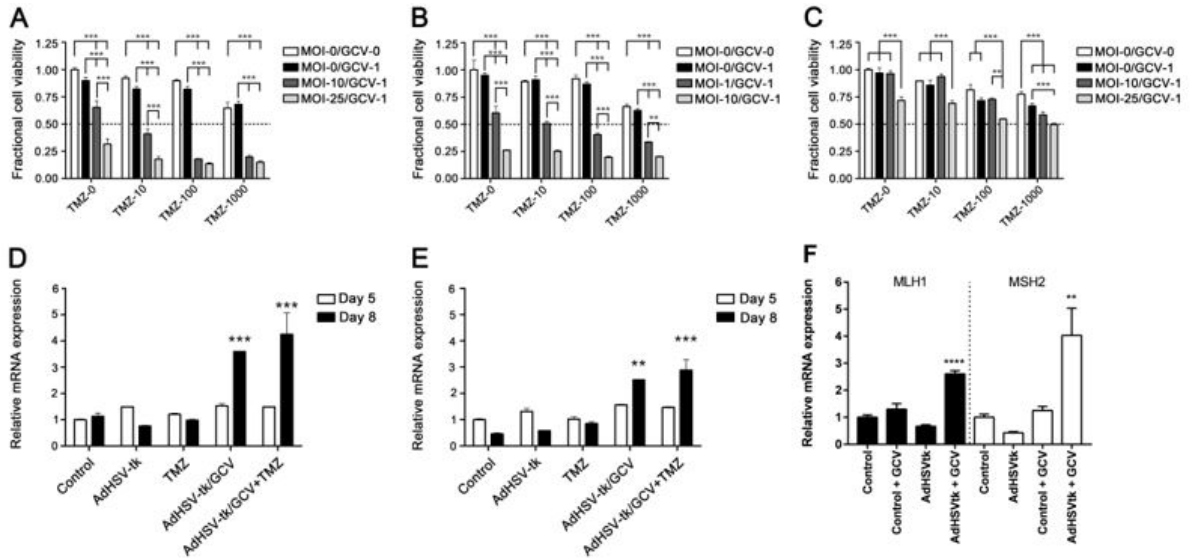

Figure 1: *In vitro* viability of BT4C (A), U87MG (B) and T98G (C) cells, 144 h after treatment with *AdHSV-tk/GCV* and TMZ combination. *AdHSV-tk* MOIs were 10 and 25 for BT4C and T98G cells, and 1 and 10 for U87MG cells. GCV 1 µg/ml and TMZ 10, 100 or 1000 µmol/l. The triplicate averages normalized to untreated control values are presented. *** and ** indicate p<0.001 and p<0.01, respectively. Horizontal line denotes ED50 value. Relative mRNA expression by *MMR* genes MLH-1 (D) and MSH-2 (E) by BT4C cells, 96 h after treatment with *AdHSV-tk/GCV* and TMZ. Relative mRNA expression by MMR genes MLH-and MSH-2 in T98G cells, 120 h after treatment with *AdHSV-tk/GCV* (F). Significances compared to the control value are indicated by **** (p<0.0001), *** (p<0.001) and ** (p<0.01). Beta-actin was used for normalization. Error-bars are standard error of mean (SEM).

However, in combination, the two therapies showed significant efficacy in all the cell lines, including T98G, with a clear dose-response in BT4C and U87MG cell lines (Figures 1A and 1B). In T98G cells a clear dose-response was evident only with 1000 µmol/l TMZ dose (Figure 1C). Compared to TMZ alone, no significant decrease in the cell viability was observed when TMZ was combined with GCV alone without *AdHSV-tk*, in any of the cell lines, indicating that the enhanced efficacy is dependent on both *AdHSV-tk* and GCV. The synergism of the therapeutic combination was derived by the combination index (CI) method, using CompuSyn software (ComboSyn, Inc. Paramus, NJ) [26]. Pre-determined CI values for synergism revealed that in BT4C cells both *AdHSV-tk* MOIs 10 and 25, with GCV were synergistic with all three doses of TMZ, whereas without *AdHSV-tk*, synergy was observed only with 10 µmol/l TMZ dose (Table 2).

In U87MG cell line, synergism was seen when *AdHSV-tk* MOI 1 with GCV was combined with all TMZ doses but without *AdHSV-tk*, it was seen only with 1000 µmol/l TMZ dose. T98G cells demonstrated synergism with all combinations, except with *AdHSV-tk* MOI 10 with GCV and 10 µmol/l TMZ dose (Table 2). The normalized isobolograms for non-constant combination of *AdHSV-tk/GCV* and TMZ in the three cell lines and the synergy determination by fractional product method of Webb [27] are given in Figure 2, respectively. The dose reduction index (DRI) revealed multi-fold reductions in the doses of each drug when combined, to achieve the same effect, compared to the individual drugs alone.

Up-regulation of MMR pathway

The mechanism behind this synergism between TMZ and *AdHSV-tk/GCV* was further studied *in vitro* in BT4C cells. It is known that the status of different DNA repair pathways plays a crucial role in therapeutic responses during cancer treatment [14,28-30]. Both MGMT and the *MMR* pathways are known to directly affect the efficacy of TMZ [30]. However, since MGMT-positive T98G and MGMT-negative U87MG cells both demonstrated synergism with this combination, it was hypothesized that the synergism could be related to MMR pathway, which is vital for the success of TMZ therapy.

Cell lines	Combination			Dose-individual drug for same Fa		DRI (fold)	
	TK MOI	TMZ	Effect	TK MOI	TMZ	TK MOI	TMZ
	(+GCV 1 µg/ml)	µmol/l	(Fa)	(+GCV 1 µg/ml)	µmol/l	(+GCV1µg/ml)	
BT4C	10	10	0.54	20.73	12549.2	2.07	1254.92
	10	100	0.82	5536.55	385504	553.66	3855.04
	10	1000	0.8	3239.67	277570	323.97	277.57
	25	10	0.82	5536.55	385504	221.46	38550.4
	25	100	0.86	18976.5	820241	759.06	8202.41
	25	1000	0.85	13608.7	669011	544.35	669.01
U87MG	1	10	0.46	2.04	24615.8	2.04	2461.58
	1	100	0.56	3.23	55335	3.23	553.35
	1	1000	0.64	4.74	108597	4.74	108.6
	10	10	0.73	7.66	253021	0.77	25302.1
	10	100	0.79	11.18	492672	1.12	4926.72
	10	1000	0.78	10.45	437156	1.04	437.16
T98G	10	10	0.06	0.16	0.41	0.02	0.04
	10	100	0.27	8542.81	2401.63	854.28	24.02
	10	1000	0.42	545040	66516.1	54504	66.52
	25	10	0.31	28451.6	6281.94	1138.06	628.19
	25	100	0.45	1159952	121639	46398.1	1216.39
	25	1000	0.5	4013407	328021	160536	328.02

DRIs derived from the CompuSyn analyses [22] 144 h after treatment reveals a multi-fold reduction of HSV-tk MOI and TMZ concentration required to achieve the same effect in most instances (in bold) when the two treatments are in combination compared to either drug alone in BT4C, U87MG and T98G cells. *AdHSV-tk*: Adenovirus-Mediated Herpes Simplex Virus Thymidine Kinase, DRI: Dose Reduction Index, Fa: Fraction Affected, GCV: Ganciclovir, MOI: Multiplicity of Infection, TK: Thymidine Kinase (Herpes Simplex Virus), TMZ: Temozolomide

Table 2: Dose reduction index (DRI) from the CompuSyn [26] analyses for the non-constant combination of *AdHSV-tk/GCV* with TMZ.

The expression of MLH-1 and MSH-2 mRNA was significantly elevated in all groups that received *AdHSV-tk/GCV* (Figures 1D and 1E). More than 3.5- and 4-fold increases in the MLH-1 mRNA levels were observed 96 h after starting GCV therapy (Figure 1D) in *AdHSV-tk/GCV* and combination groups, respectively. For MSH-2 these increases were >2.5- and 3-fold for the same groups, respectively (Figure 1E). However, no significant increases were observed in mRNA levels in the untreated control and *AdHSV-tk* and TMZ alone groups at this time point or in any of the groups 24 h after starting the treatment. Results indicate that the up-regulation of *MMR* pathway is seen after a time-lag of 48-96 h when both *AdHSV-tk* and GCV are present. It is possible that the up-regulation persists for even longer periods but this could not be confirmed since sufficient numbers of treated cells did not survive beyond this point. Similar upregulation of MLH1 and MSH2 mRNA levels were observed in human T98G MG cells 120 h after *AdHSV-tk/GCV* treatment (Figure 1F).

Tumor growth

In vivo effects of the treatment combination were studied in orthotopic, syngeneic BT4C [21] rat MGs in immunocompetent BDIX rats [31]; a model that has earlier proven to be highly predictive in assessing the efficacy of *AdHSV-tk/GCV* therapy [20]. The second MRI (Figure 3A) revealed that TMZ therapy alone (study group 2) had no impact on the tumor growth, whereas *AdHSV-tk/GCV* alone (study group 3) had a trend towards a lower mean tumor volume when compared to group 2. Study groups 4 and 5 had significantly lower ($p<0.05$) mean tumor volumes when compared to groups 1 and 2, in spite of study group 4 not receiving TMZ yet. At the third MRI (Figure 3B), combination treatment groups 4 and 5 had significantly lower mean tumor volumes compared to group 3, whereas no comparisons were possible with study groups 1 and 2 due to insufficient number of surviving animals. MRIs representative of the average tumor volumes for each group from p.i. days 28 (upper) and 42 (lower) are presented in Figure 3C.

The mean tumor volumes of untreated control group (group 1) increased by >30-fold between the 1st and 2nd MRIs and in group 2 this increase was >15-fold, whereas in study groups 3 and 4 the increase was >8-fold. However, in study group 5, which completed both therapies during this period, the increase in mean tumor volume was <2-fold. Between 2nd and 3rd MRIs, the increase in the mean tumor volume in group 5 was less than 2%, suggesting that this protocol was able to maintain a stable disease during this study period.

Survival

A summary of survival data is given in Figures 2D and 2E. Study groups 1, 2 and 3 had a median survival of approximately 35 days. The median survival was increased by 20% (up to 42 days) and 48% (up to 52 days) in study groups 4 and 5, respectively. Neither TMZ, nor *AdHSV-tk/GCV* alone was able to significantly enhance survival compared to the controls. The survival improvement in study group 4 was significant ($p<0.05$) when compared to controls, TMZ only and *AdHSV-tk/GCV* only groups. Study group 5 demonstrated an even better survival compared to these three groups. In spite of showing a 24% improvement in survival in study group 5 compared to group 4, the difference did not reach statistical significance (Figure 3D). This is probably due to the low sample number in this group.

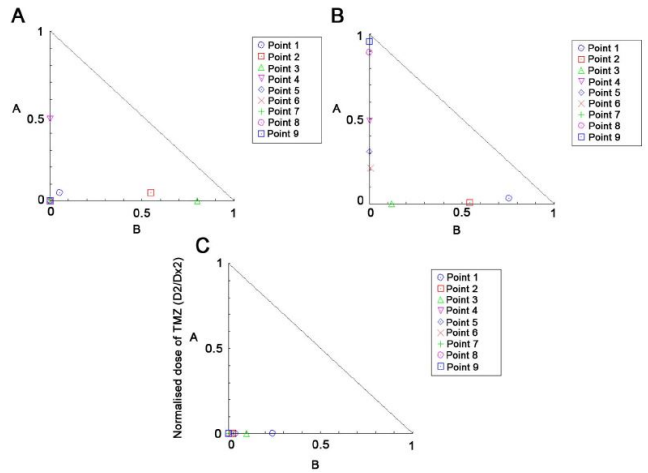

Figure 2: Normalized isobolograms for the non-constant combination of *AdHSV-tk/GCV* with TMZ, 144h after treatment, in BT4C (A), U87MG (B) and T98G (C) cells. *AdHSV-tk* MOIs 10 and 25 for BT4C and T98G, and 1 and 10 for U87MG with 1 µg/ml of GCV was combined with 10, 100 and 1000 µmol/l TMZ. X- and Y- axes indicate normalized dose of drug 1 (*AdHSV-tk* MOI with 1 µg/ml GCV) as 1/()1 and drug 2 (TMZ µmol/l) as 2/()2, respectively. D1 and D2 are respectively, the doses of drug 1and 2 in combination with each other that would inhibit x %. (Dx)1 and (Dx)2 are the doses of drug 1 and 2 alone, that would inhibit x %, respectively. Drug combination data points (1-9) are given in table 2 under "effect of drug combinations" columns.

Serum and blood analyses

Since, both TMZ and GCV are known to suppress bone marrow, FBC and serum chemical analyses were performed to evaluate the systemic toxicities (Figure 4). As expected, all groups that received TMZ had thrombocytopenia (Figure 4A) and leukocytopenia (Figure 4B). Interestingly, *AdHSV-tk/GCV* therapy alone did not show this adverse effect.

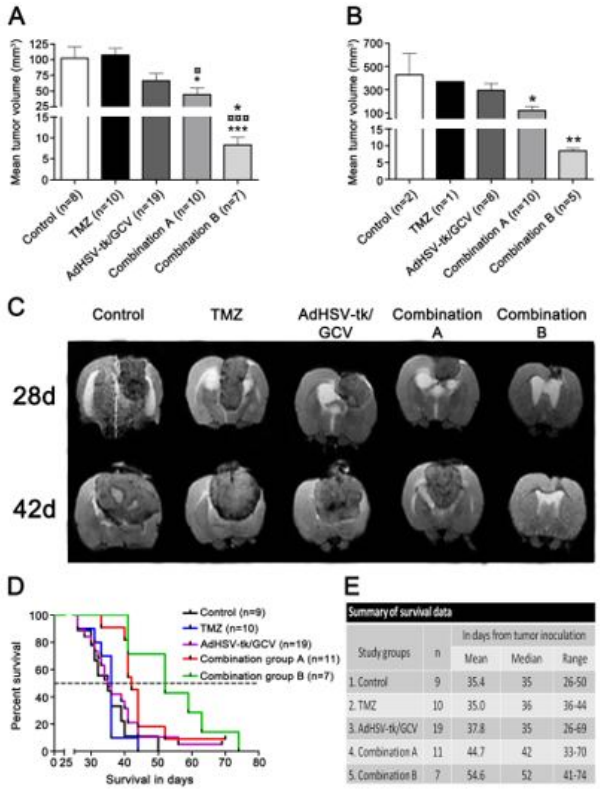

Figure 3: Mean tumor volumes measured by MRI on p.i. 28d (A) and 42d (B). The combination group B had a significantly lower mean tumor volume compared to control *** ($p<0.001$), TMZ ¤¤¤ ($p<0.001$) and *AdHSV-tk/GCV** ($p<0.05$) groups, while in combination group A, it was significantly ($p<0.05$) lower compared to control * and TMZ ¤ groups (A), on 28d. On 42d, the mean tumor volumes of combination groups A * ($p<0.05$) and B ** ($p<0.01$) were significantly lower compared to *AdHSV-tk/GCV* group (B). Error bars are SEM. Magnetic resonance images representative of the average tumor volumes of each group from p.i. 28d (upper) and 42d (lower) are given (C). Kaplan-Meier survival plot for the groups is presented (D), where the dotted line denotes median (50 %) survival. A summary of the survival data is also presented (E).

The analyses of serum samples indicated that all parameters were within normal range (Figures 4C-4F), except for a slight elevation of ALT in *AdHSV-tk/GCV* group (Figure 4C) that has been previously reported to be transient with this therapy [9]. The evaluation of treatment effect on different T-cell populations; CD3 (T-cell), CD4 (helper T-cell) and CD8 (cytotoxic T-cell) revealed a significant reduction of CD3-positive cells ($^{*}p<0.05$) (Figure 4G) with TMZ but not with *AdHSV-tk /GCV* therapy. The CD4:CD3 ratio did not differ significantly among the 15 groups (Figure 4H). However, CD8:CD3 ratio was significantly lower ($^{**}p<0.01$) in the TMZ group when compared to controls (Figure 4I). These results indicate that *AdHSV-tk/GCV* alone is a safer alternative to TMZ.

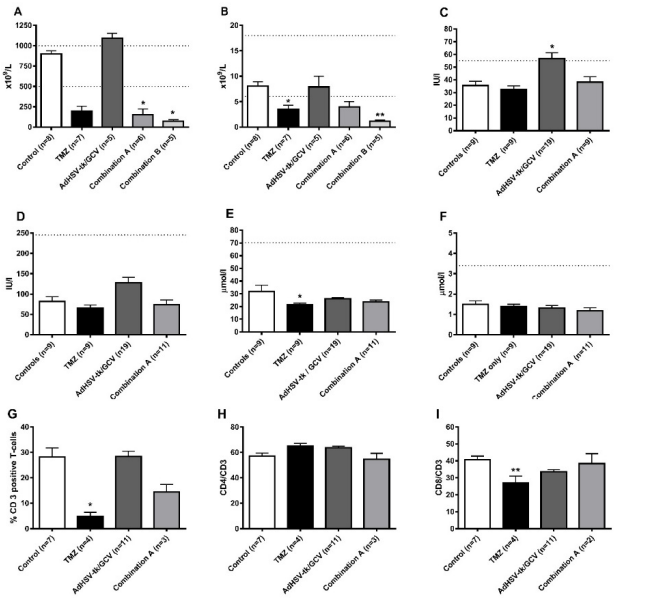

Figure 4: The evaluation of systemic toxicities of treatment combination. Platelet count (A), white blood cell count (B), alanine aminotransferase (ALT) (C), alkaline phosphatase (AP) (D), creatinine (E) and bilirubin (F) are presented. Horizontal lines denote the range/upper limit of normality for rats. The proportion of CD3+ cells out of total lymphocyte population (G), CD4:CD3 (H) and CD8:CD3 population ratios (I) are given. The significances compared to control values * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) are indicated. Error bars are SEM.

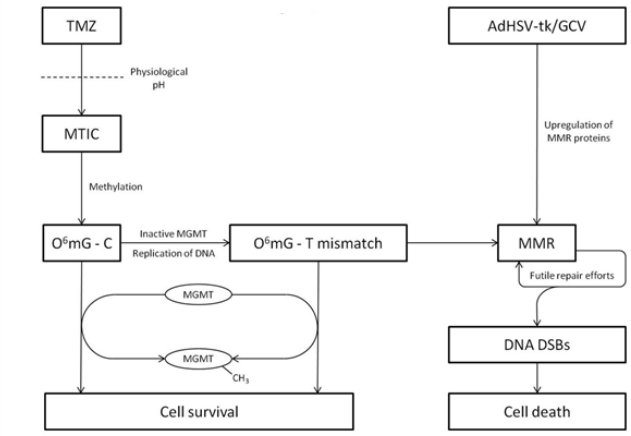

Figure 5: A schematic presentation of TMZ-mediated cytotoxicity and the proposed mechanism of synergism between *AdHSV-tk/GCV* and TMZ. *AdHSVtk/GCV*=Adenovirus-mediated Herpes simplex virus thymidine kinase and ganciclovir treatment, C=Cytosine, DSB=Double stranded break, MGMT=Methyl guanine methyl transferase, *MMR*=Mismatch repair, MTIC=3-Methyl-(triazen-1-yl) imidazole-4 carboxyamide, O6mG=Methylation at O6 of guanine, T=Thiamine, TMZ=Temozolomide.

Discussion

The lack of effective treatment options is largely attributed to the poor outcome of MG patients. TMZ is the latest addition to chemo-therapeutic armamentarium against GBM [11]. However, the efficacy of TMZ is largely dependent on DNA repair activities [30], with almost half of the patients' refractory to the treatment [32]. Even in initially responsive patients, acquired resistance due to selection pressure can lead to treatment failure and recurrences [33]. With proven safety and efficacy in several clinical trials [8-10], *AdHSV-tk/GCV* suicide gene therapy is a clear candidate to treat MG patients and could supplement TMZ treatment regimens. The advantage of gene therapy is that it provides a strong local therapeutic effect without major systemic adverse effects, as opposed to chemo- or radiotherapy [2]. According to the current practice of approving novel therapeutics, their efficacy needs to be tested along with the current standard of care. Hence, it is imperative to combine these two therapies in pre-clinical settings to test their efficacy and safety, as well as to delineate optimal treatment protocols for any possible combination therapy. In this study, the synergistic effect of *AdHSV-tk/GCV* and TMZ was studied focusing on mechanistic, safety and efficacy aspects of therapy. The primary mode of cytotoxicity of TMZ is by addition of a methyl group at O6- position of guanine nucleotides (O6-mG) [13] that is corrected by a functional MGMT repair pathway leading to resistance (Figure 5).

Promoter methylation is a known mechanism by which MGMT pathway is inactivated epigenetically and this happens in about 50% of GBM patients [32,34]. Results from this study reveal that the efficacy of TMZ in the MG patient population is limited by the cellular MGMT status whereas *AdHSV-tk/GCV* alone and the TMZ-*AdHSV-tk/GCV* combination will be effective, or synergistic, independent of the cellular MGMT status (Figures 1 and Table 2). Thus, suicide gene therapy alone is an effective alternative for MGMT expressing MGs, which do not respond to TMZ alone and a future combination of both could be a new treatment protocol for the disease. Apart from MGMT expression, the cellular p53 status also seems to play a role in TMZ sensitivity, where a mutant p53 increased the cytotoxicity of TMZ [24], while the wild-type p53 was more sensitive to the treatment [35]. Our finding that T98G cells with the mutated p53 [25] are less sensitive to TMZ than U87MG cells with the wild-type p53 [24] supports the notion that cellular sensitivity to TMZ is ultimately determined by several factors [25,36].

We were unable to demonstrate the presence of MGMT mRNA by RT-PCR in BT4C cells, suggesting a lack of functional MGMT repair pathway. Hence, theoretically, the cell line should be sensitive to TMZ therapy. However, both *in vitro* and *in vivo* experiments revealed little effect with TMZ therapy alone (Figures 1 and 2) at the doses we used, implicating other mechanisms of resistance in BT4C cells. Accepted mechanism of TMZ-mediated cytotoxicity suggests a critical role for *MMR* pathway [14,28,34,37]. Moreover, cells with reduced levels of *MMR* proteins MSH-2 and MLH-1, in spite of functional *MMR*, failed to activate cell cycle checkpoints or undergo apoptosis in repose to alkylating agents [38]. Based on this mechanism, it is apparent that a functional *MMR* pathway, whilst having little effect on the efficacy of *AdHSV-tk/GCV* suicide gene therapy, is vital to sensitize the cancer cells to TMZ. Furthermore, defects in the *MMR* pathway can lead to almost 100-fold increases in the resistance to alkylating agents [30,39].

Previous studies have demonstrated regulation of *MMR* pathway by post-translational modifications, such as nuclear translocation of MSH-2 and MSH-6 in response to TMZ therapy [40] but not at the transcriptional level.

We demonstrate, for the first time, the enhancement of *MMR* pathway at transcriptional level by *AdHSV-tk/GCV* gene therapy that this explains the synergistic effect when in combination with TMZ. Our results confirm that *AdHSV-tk/GCV* up-regulates mRNA transcription of MSH-2 and MLH-1, which are essential for MMR function and TMZ induced cytotoxicity [41-44]. A significant up-regulation was detected 4-5 days after starting GCV therapy. Based on these results, for optimal efficacy TMZ treatment should be commenced as early as possible after completing GCV therapy, while the up-regulation of *MMR* pathway is still in place or even concomitantly in the latter part of GCV therapy during the optimal induction of *MMR* pathway. This was confirmed by the *in vivo* studies using different treatment protocols (groups 4 vs. 5), where the group that received TMZ immediately after GCV (group 5) demonstrated the best control of tumor growth and survival compared to the group (group 4) that had a time-gap between these two therapies. TMZ or *AdHSV-tk* alone did not up-regulate *MMR* pathway, confirming that the up-regulation was a function of *AdHSV-tk* and GCV combination [45-47]. In *AdHSV-tk/GCV* clinical trials TMZ was started many days after completing GCV therapy [10], probably resulting in a reduced synergy. However, our results suggest that the outcome of this treatment combination would be further enhanced by concomitant administration of GCV and TMZ or by starting TMZ immediately after GCV therapy.

Since MMR pathway plays a significant role in the cytotoxicity of TMZ, there is a selective pressure on tumor cells to lose MMR function, resulting in acquired resistance to TMZ and hypermutation phenotype recurrences [33]. Since others have speculated that AdHSV-tk/GCV would be more effective against cancer cells lacking MMR function [41], it would be interesting to study whether there is a reduction or a delay in the occurrence of such treatment resistant recurrences when these two therapies are combined. Moreover, recurrences after TMZ therapy may be more sensitive to *AdHSV-tk/GCV* due to the same reason.

Impairment of *MMR* pathway is known to make cancer cells resistant to many chemotherapeutic agents, such as procarbazine [42-44], cisplatin [45], doxorubicin [44], 5-FU [46] and 6-thioguanine [47]. Up-regulation of *MMR* pathway by *AdHSV-tk/GCV* could make it an ideal combination partner to these chemotherapies by increasing their cytotoxicity, without affecting the adverse effect profile and possibly preventing or delaying the emergence of treatment resistance.

Conclusion

Myelo-suppressive adverse effects of TMZ and GCV could lead to cumulative toxicities, when combined together. Results from this study also reiterate that TMZ has profound adverse effects on WBC and platelet counts. GCV alone or in combination with *AdHSV-tk* did not reduce these values significantly indicating a generally safer treatment approach. In the groups where TMZ and GCV were combined, the reductions in WBC and platelet counts were not significantly lower compared to the groups that received only TMZ. According to the proposed mechanism of synergism, the cytotoxicity of TMZ is enhanced only within the tumor, where the gene therapy is effective and not in the bone marrow, where the TMZ-induced adverse effects occur. Hence, it is possible that the proposed gene therapy-based enhancement of TMZ-induced cytotoxicity also increases the therapeutic index of TMZ.

In conclusion, we have demonstrated a novel molecular mechanism by which *AdHSV-tk/GCV* gene therapy enhances the cytotoxicity of TMZ, leading to therapeutic synergism, without affecting the adverse effect profile. Furthermore, the significant improvements in the final outcome, gained by subtle changes in treatment protocol emphasises the need to re-consider any clinical treatment protocol where these two therapies may be combined, to maximise a synergistic therapeutic effect.

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