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1 *Article*

2 # Efficient prodrug activator gene therapy by retroviral replicating vectors prolongs survival in an immune-competent intracerebral glioma model

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25 **Abstract:** Prodrug activator gene therapy mediated by murine leukemia virus (MLV)-based
26 retroviral replicating vectors (RRV) was previously shown to be highly effective in killing glioma
27 cells both in culture and *in vivo.* To avoid receptor interference and enable dual vector co-infection
28 with MLV-RRV, we have developed another RRV based on gibbon ape leukemia virus (GALV) that
29 also shows robust replicative spread in a wide variety of tumor cells. We evaluated the potential of
30 GALV-based RRV as a cancer therapeutic agent by incorporating yeast cytosine deaminase (CD)
31 and *E. coli* nitroreductase (NTR) prodrug activator genes into the vector. The expression of CD and
32 NTR genes from GALV-RRV achieved highly efficient delivery of these prodrug activator genes to
33 RG-2 glioma cells, resulting in enhanced cytotoxicity after administering their respective prodrugs
34 5-fluorocytosine and CB1954 *in vitro.* In an immune-competent intracerebral RG-2 glioma model,
35 GALV-mediated CD and NTR gene therapy both significantly suppressed tumor growth with
36 CB1954 administration after a single injection of vector supernatant. However, NTR showed greater
37 potency than CD, with control animals receiving GALV-NTR vector alone (i.e., without CB1954
38 prodrug) showing extensive tumor growth with a median survival time of 17.5 days, while animals
39 receiving GALV-NTR and CB1954 showed significantly prolonged survival with a median survival
40 time of 30 days. In conclusion, GALV-RRV enabled high-efficiency gene transfer and persistent
41 expression of NTR, resulting in efficient cell killing, suppression of tumor growth, and prolonged
42 survival upon CB1954 administration. This validates the use of therapeutic strategies employing
43 this prodrug activator gene to arm GALV-RRV, and opens the door to the possibility of future
44 combination gene therapy with CD-armed MLV-RRV, as the latter vector is currently being
45 evaluated in clinical trials.

46

1. Introduction

Glioblastoma multiforme (GBM) is the most frequent form of primary malignant brain tumor in adults [1], and it tends to aggressively invade the surrounding brain tissue so as to make complete surgical resection virtually impossible. Clinical trials of GBM gene therapies using a conventional replication-defective retroviral vector encoding Herpes simplex virus thymidine kinase with subsequent administration of the prodrug ganciclovir did not achieve any improvement in patient survival [2], which was attributed to extremely low levels of tumor transduction. Replication-competent viral vectors enable enhanced tumor transduction levels, since these viral vectors can replicate and multiply after the initial infection event, and each infected tumor cell in effect becomes a viral vector producer cell [3-8]. We and others have previously shown that amphotropic murine leukemia virus (MLV)-based retroviral replicating vectors (RRV) achieve highly efficient and tumor-selective gene transfer to glioma cells both in culture and *in vivo* [9-11]. Furthermore, intracerebral or intravenous injection of MLV-RRV resulted in little or no detectable infection in the normal brain or extracerebral tissues [12-14]. Early phase clinical studies of RRV-mediated prodrug activator gene therapy have shown highly promising results in recurrent high-grade glioma patients [15,16], and an international phase III trial is currently on-going.

Highly efficient and tumor-selective gene transfer achieved by RRV enables efficacious prodrug activator gene therapy, which involves expression of enzymes that can convert precursor prodrugs into active chemotherapeutic drugs within the transduced tumor cells. This essentially forces the tumor to self-generate the chemotherapy drug from within. However, due to the relatively restricted payload capacity of RRV, which can only accommodate approximately 1.3-kb of additional transgene sequence inserted into the full-length replication-competent viral genome, it has generally been difficult to insert more than a single therapeutic gene per vector. For prodrug activator gene therapy, this is the equivalent of single-agent chemotherapy generated directly within the tumor.

Analogously, since combination chemotherapy with multiple drugs is generally more efficacious than single-agent chemotherapy, there may be potential to achieve greater efficacy if multiple RRV encoding different prodrug activator genes could be combined. However, in this case the retrovirus envelope sequence in different vectors may need to be varied, so that these vectors do not compete for the same cell surface receptor, a phenomenon which in retrovirology is classically described as "receptor interference". In particular, MLV-RRV currently in clinical development expresses the MLV 4070A strain amphotropic envelope, which binds the ubiquitous inorganic phosphate transporter, PiT-2 [17]. In contrast, gibbon ape leukemia virus (GALV) envelope enables cellular entry through another member of the same phosphate transporter family, PiT-1, and accordingly we have developed GALV-based RRV which also appears to show robust replicative spread in a wide variety of cancer cell lines [18-22].

To evaluate the potential of GALV-RRV as a therapeutic agent for glioma therapy, we incorporated the yeast cytosine deaminase (CD) [23] or *E. coli* nitroreductase (NTR) [24,25] prodrug activator genes into this type of vector. The CD enzyme converts 5-fluorocytosine (5-FC) to the highly toxic metabolite 5-fluorouracil [26], which is a potent antineoplastic agent routinely used in cancer therapy. The NTR enzyme converts 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) to a 4-hydroxylamino derivative that is subsequently converted into a potent cytotoxic bifunctional alkylating agent [27].

To explore the use of GALV-RRV for prodrug activator gene therapy of glioma, in these studies we used an immune-competent rodent model of intracerebral glioma to examine tumor growth as well as survival after intracerebral inoculation of GALV-RRV carrying CD and NTR genes, followed by treatment with their respective prodrugs. We evaluated whether the high transduction level and persistent gene expression achieved by GALV-RRV has the potential to enhance the efficacy of prodrug activator gene therapy.

2. Results

2.1. GALV-RRV achieves highly efficient transgene delivery to glioma cells.

As previously described, the GS4-GFP vector contains a wild-type GALV provirus into which an internal ribosome entry site (IRES)-green fluorescent protein (GFP) gene expression cassette has been inserted precisely between GALV *env* and the 3' untranslated region (UTR) [28] (Figure 1). To examine the ability of GS4-GFP to replicate in glioma cells in culture, we infected human (U-87) and rat (RG-2, CNS-1) glioma cells with the viral vector at low multiplicities of infection (MOI) and confirmed by flow-cytometric analysis that GS4-GFP could transduce each glioma cell line. We further studied the replication kinetics of GS4-GFP in each glioma cell line by initially mixing 5% infected glioma cells with 95% uninfected glioma cells, and examining horizontal transmission of GFP expression over time. Flow-cytometric analysis showed that GS4-GFP could efficiently transduce each glioma cell line, and spread throughout the entire cell population within 8 days in the case of human U-87 cells, and within 14 days in the case of rat RG-2 and CNS-1 cells (Figure 2A).
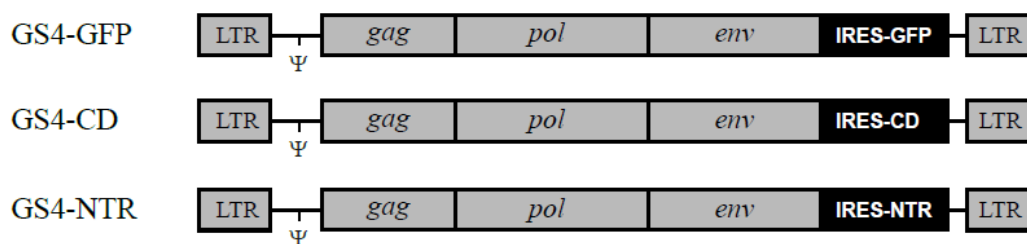
Figure 1. Structure of GALV-RRV carrying transgenes. Each GALV-RRV contains an IRES-GFP, IRES-CD or IRES-NTR gene expression cassette inserted between GALV *env* and the 3' UTR. Ψ, packaging signal. LTR, long terminal repeat.
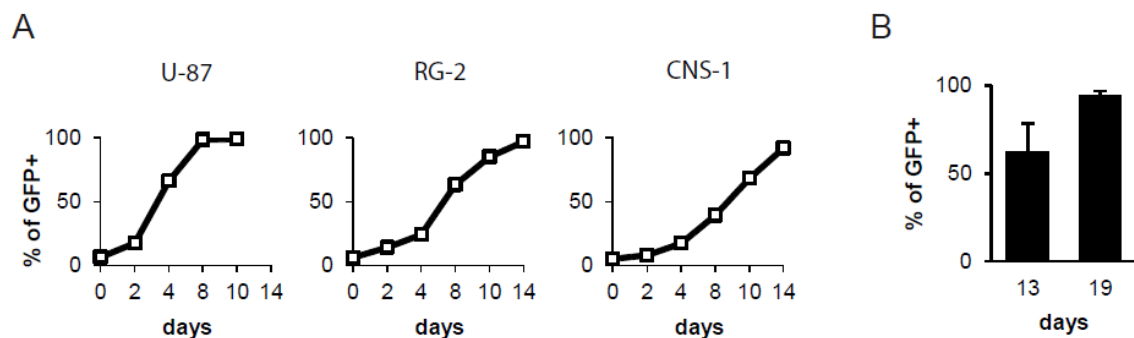
Figure 2. Replicative spread of GS4-GFP in glioma cells in culture and in intracerebral gliomas *in vivo*. (**A**) GS4-GFP-transduced RG-2 cells (5%) and uninfected RG-2 cells (95%) were mixed and seeded onto culture plates. At various time points after cell mixing, the cell populations were analyzed for GFP expression. X-axis: days after cell mixture. Y-axis: % of cells expressing GFP. (**B**) GS4-GFP (2×10^4 TU) was injected into a pre-established intracerebral RG-2 tumor model in Fischer 344 rats. The spread of GS4-GFP in tumors was examined by quantification of GFP expression at 13 (*n*=6) and 19 days (*n*=3) after viral vector inoculation.

2.2. Efficient and progressive spread of GALV-RRV in an intracerebral glioma model.

We evaluated the ability of GALV-based RRV to achieve a high level of transduction in gliomas *in vivo* by testing the GS4-GFP vector in an intracerebral RG-2 tumor model. A single dose of 2×10^4 transducing units (TU) of GS4-GFP was injected into pre-established intracerebral RG-2 tumors in

128 syngeneic Fischer 344 rats. At two time points after vector injection, intratumoral spread of GS4-GFP
129 was evaluated by flow-cytometric analysis of excised intracerebral tumors. On day 13 post-vector
130 injection, the percentage of GFP-positive RG-2 tumor cells averaged 62.0±16.4%, and by day 19 post-
131 vector injection, this had increased to 94.2±2.8% (*P*<0.01), demonstrating that GS4-GFP spread
132 efficiently and progressively in orthotopic intracerebral rat gliomas (Figure 2B).

133 *2.3. No detectable spread of GALV-RRV to extratumoral tissues.*

134 To detect any possible GALV-RRV spread to extratumoral tissues, real-time PCR assay of
135 genomic DNA extracted from various extratumoral organs was performed using primers specific for
136 the GALV *env* (Table 1). This assay could detect down to 50 copies of the GALV provirus per 5×10^4
137 cell genomes, representing a transduction level of approximately 0.1%. As expected, GALV sequence
138 could be readily detected in GS4-GFP-transduced glioma tissues (*n*=3; 19 days after viral vector
139 injection). However, no detectable spread was observed in any of the extratumoral organs examined
140 from the same GS4-GFP-injected animals.

141 **Table 1.** Biodistribution of GS4-GFP after intratumoral injection of the vector in immune-competent
142 intracerebral glioma model.

|	Copies of GALV *env* per cell
tumor	1.46 ± 0.23
contralateral normal brain	−
bone marrow	−
spleen	−
intestine	−
liver	−

143 Genomic DNA extracted from intracerebral glioma and extratumoral organs of GS4-
144 GFP-infected rats was analyzed by real-time PCR assay. The numbers of GALV *env*
145 copies per cell are presented as means ± standard deviations. −, not detectable
146 (detection limit was 0.001 copy per cell).

147

148 *2.4. Dose-dependent cytotoxicity of RRV-transduced cells after prodrug administration.*

149 To examine the ability of RRV to deliver prodrug activator genes, GS4-CD- and GS4-NTR-
150 transduced RG-2 cells were first treated with their respective prodrugs, 5-FC and CB1954, at various
151 concentrations. To distinguish the cytotoxic effects of prodrug activator gene function from any
152 potential nonspecific toxicity due to the prodrugs themselves, GS4-GFP-transduced cells were also
153 treated with each prodrug at the corresponding concentrations as experimental controls. The 5-FC
154 and CB1954 prodrugs showed negligible cytotoxicity at concentrations of ≤1 mM and ≤0.25 μM,
155 respectively.
156 Infection with GS4-CD or GS4-NTR resulted in potent killing of RG-2 cells exposed to the
157 corresponding prodrug at all concentrations tested, and the degree of cytotoxicity was highly
158 prodrug concentration-dependent (Figure 3). Exposing GS4-CD-transduced cells to 5-FC at 0.04 mM
159 led to a dramatic drop in cell viability to ~30% relative to the control group. Similarly, exposing GS4-
160 NTR-transduced cells to CB1954 at 0.1 μM induced the same degree of cytotoxicity, indicating that
161 the NTR/CB1954 enzyme prodrug system might also be useful if delivered in the context of RRV to
162 inhibit tumors and prolong survival.
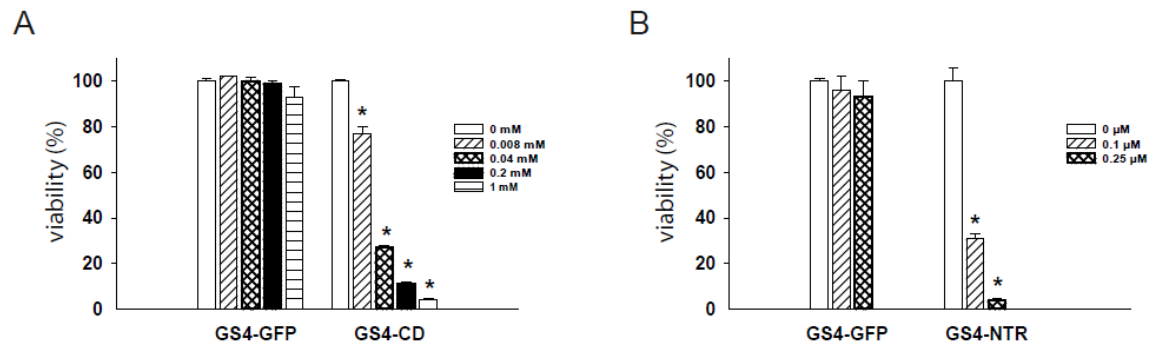
Figure 3. *In vitro* cytotoxicity achieved by GALV-based RRV plus prodrug treatment. (**A**) GS4-CD- and GS4-GFP-transduced RG-2 cells were exposed to 5-FC ranging from 0 to 1 mM, and cell viability was determined 3 days later by MTS assay. (**B**) GS4-NTR- and GS4-GFP-transduced RG-2 cells were exposed to CB1954 ranging from 0 to 0.25 μM, and cell viability was determined 3 days later by MTS assay. *, $P<0.005$.

2.5. RRV-mediated prodrug activator gene delivery significantly improves survival of immune-competent rats bearing gliomas.

We determined whether the high *in vivo* tumor transduction levels achieved by GALV-RRV has the potential to improve survival by evaluating prodrug activator gene therapy using GS4-CD and GS4-NTR in the intracerebral RG-2 glioma model. After establishing intracerebral RG-2 gliomas by stereotactic implantation, the GS4-CD or GS4-NTR vector was inoculated via intratumoral injection, as above. Treatment with the GS4-CD vector, followed by a single cycle of 5-FC administered by intraperitoneal injection, resulted in a survival advantage with a median survival time of 22 days, as compared to a median survival time of 19 days in the control group treated with the GS4-GFP vector and 5-FC ($P<0.05$) (Figure 4A). Treatment with the GS4-NTR vector followed by two doses of CB1954 administration also resulted in a significant survival advantage, with a median survival time of 30 days, compared to the median survival time of 17.5 days in the control group ($P<0.05$) (Figure 4B).
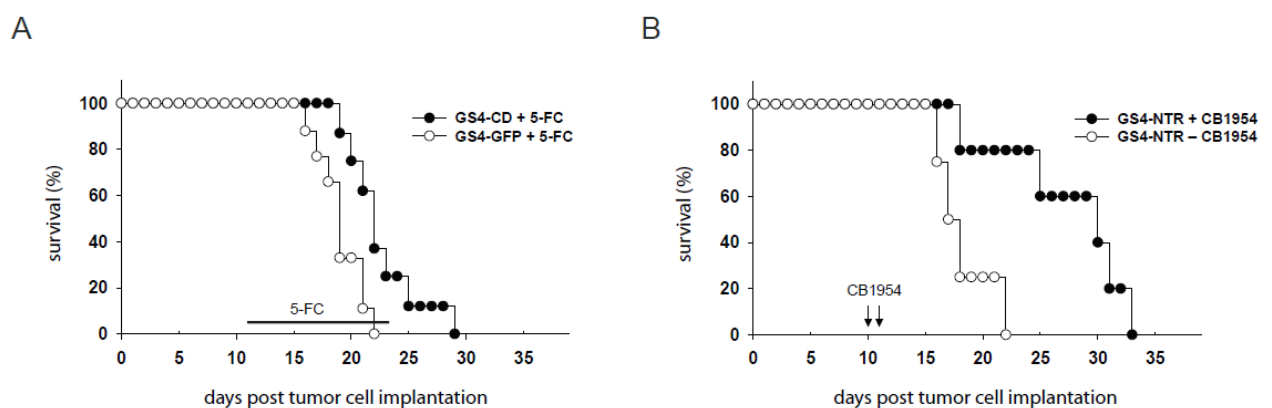

Figure 4. Survival analysis of immune-competent rats bearing intracerebral RG-2 gliomas. (**A**) GS4-CD or GS4-GFP was stereotactically injected into pre-established intracerebral RG-2 tumors three days after tumor inoculation. Eight days after viral vector inoculation, the rats received intraperitoneal injections of 5-FC (100 mg/kg), once every other day, for a total of 7 treatments. Survival curves were constructed for two treatment groups: GS4-CD plus 5-FC, and GS4-GFP plus 5-FC. (**B**) GS4-NTR was stereotactically injected into pre-established intracerebral RG-2 tumors three days after tumor inoculation. Seven and eight days after viral vector inoculation, the rats received daily intraperitoneal injections of CB1954 (2.5 mg/kg) or PBS. Survival curves were constructed for two treatment groups GS4-NTR plus CB1954 and GS4-NTR plus PBS.

191   *2.6. MicroPET imaging of RG-2 glioma-bearing rats after prodrug activator gene therapy.*

192   Our previous study reported that O-2-[18F]fluoroethyl-L-tyrosine (L-[18F]FET) microPET was
193   superior to [18F]FDG microPET for the monitoring of brain tumor due to the low uptake of L-[18F]FET
194   in normal brain [29]. MicroPET imaging of glioma-bearing rats using L-[18F]FET revealed sizable RG-
195   2 gliomas at the tumor implantation site in the group treated with GS4-NTR alone (Figure 5B). A clear
196   difference between tumor-implanted and normal brains was observed. In contrast, rats treated with
197   GS4-NTR plus CB1954 showed smaller tumors remaining at the tumor implantation site,
198   demonstrating the therapeutic benefit achieved by GALV-based RRV-mediated prodrug activator
199   gene therapy (Figure 5C).
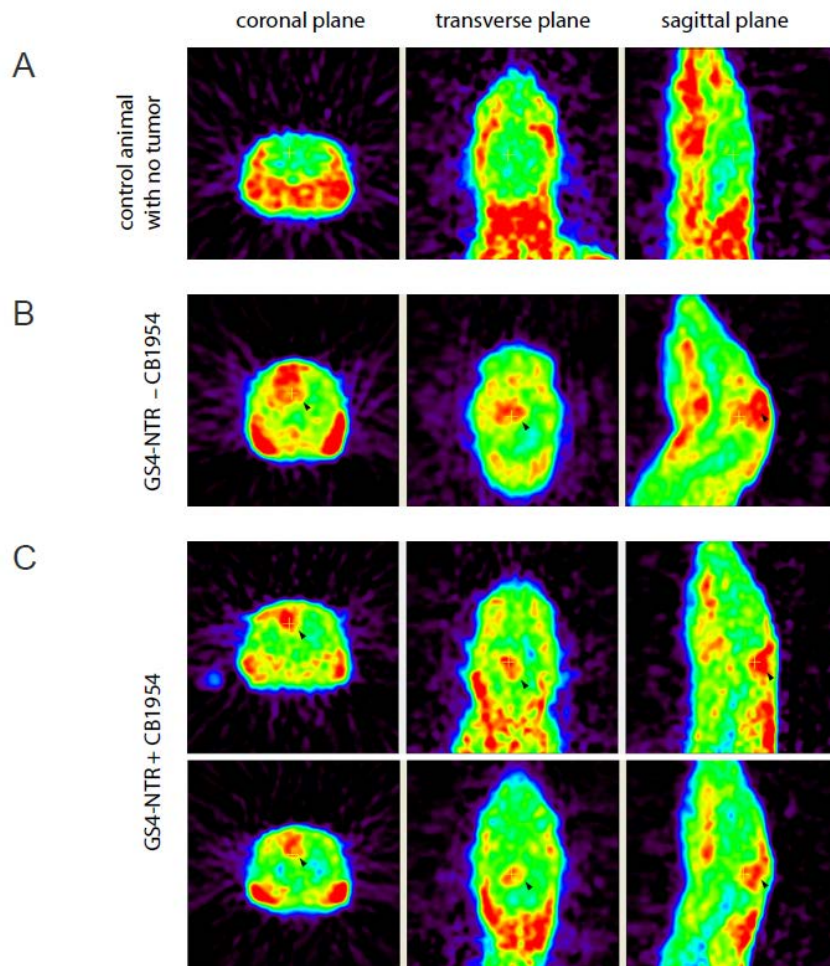
200

201   **Figure 5.** MicroPET imaging of intracerebral RG-2 glioma-bearing rats. MicroPET imaging of L-
202   [18F]FET in rats was performed using the R4 system, 20 days after intracerebral RG-2 glioma
203   implantation. Representative examples comparing microPET imaging results from non-tumor-
204   bearing rats (**A**), tumor-bearing rats treated with GS4-NTR but without prodrug administration (**B**),
205   or tumor-bearing rats with GS4-NTR and CB1954 prodrug treatments (**C**) are shown, and tumor
206   regions are indicated by arrowheads. The tumor uptake of L-[18F]FET in the GS4-NTR − CB1954 group
207   relative to the GS4-NTR + CB1954 group was 2.015-fold ($P<0.01$).

3. Discussion

209   Persistent nonlytic infection of tumor cells by RRV facilitates the widespread seeding of prodrug
210   activator genes, thereby allowing synchronized cell killing triggered by prodrug administration.
211   Using GALV-based RRV expressing the CD and NTR suicide genes followed by the administration
212   of the prodrugs 5-FC and CB1954, we have achieved highly efficient killing of glioma cells both in
213   culture and *in vivo*, resulting in significantly prolonged survival in an immune-competent
214   intracerebral glioma model.

215 In addition to the significant survival improvement achieved by GALV-based RRV-mediated
216 CD prodrug activation therapy, here we also demonstrate a significant tumor inhibitory effect on
217 intracerebral RG-2 tumors after GS4-NTR-mediated prodrug activation therapy. Of note, the *E. coli*
218 NTR isoform B (NfsB) gene cloned into the GS4-NTR vector expresses an enzyme that actively
219 converts CB1954 to a 4-hydroxylamino derivative that is subsequently converted into a potent
220 cytotoxic bifunctional alkylating agent capable of cross-linking DNA and therefore achieving cell-
221 cycle-independent killing of both actively proliferating and non-proliferating cells [30].

222 However, despite significant increases in survival, all animals did succumb to tumor burden.
223 Factors which may have contributed to the lack of eradication of tumor burden and potential avenues
224 for improvement include the vector dose and/or the schedule of prodrug delivery. *In vivo* vector
225 replication and spread was assessed with approximately 60% of tumor cells positive for GFP
226 expression (vector) 13 days post vector administration. In this study the animals were treated with
227 prodrug beginning day 7 (NTR) or day 8 (CD), where the number of tumor cells transduced would
228 be less than 60%. Increasing the vector dose and hence the number of cells transduced at the time of
229 prodrug administration may increase the survival benefit.

230 Here we have validated both CD and NTR as effective prodrug activator genes in the context of
231 GALV-RRV. As noted, the different cytocidal effects caused by CD/5-FC and NTR/CB1954 would
232 allow us to employ combination prodrug activation therapy, analogous to combination
233 chemotherapy, but generated directly within the tumor itself, thereby avoiding adverse effects of
234 systemic chemotherapy. However, for optimal combined gene therapy with both prodrug activator
235 genes, these genes must be delivered using RRV with different envelopes in order to avoid receptor
236 interference. Since MLV-RRV and GALV-RRV utilize different cellular receptors (PiT-2 and PiT-1
237 phosphate transporters, respectively) for viral entry [17], co-infection of glioma cells with both
238 vectors supplied with different prodrug activator genes may be employed to achieve synergistic
239 cytotoxic effects, thus augmenting the efficacy of gene therapy [18,22]. Now that the present studies
240 have validated the use of GALV-RRV expressing NTR as a single-agent prodrug activator vector in
241 itself, in future studies we can proceed to evaluate combination prodrug activator gene therapy
242 together with the current clinical vector, CD-armed MLV-RRV.

243 Real-time PCR analysis demonstrated that the replication of GALV-RRV is highly restricted to
244 the tumor itself, with no spread to ectopic sites such as bone marrow and spleen detectable in
245 immune-competent glioma models. This result is consistent with our previous study showing that
246 MLV-RRV delivered to intracerebral RG-2 tumors in immune-competent syngeneic hosts showed no
247 detectable spread to all normal tissues examined [13]. Our inability to detect RRV in extratumoral
248 tissues suggests that, although impaired innate and adaptive immunity in cancer cells enable
249 progressive replicative spread of the virus within tumors even in an immune-competent host [31,32],
250 these mechanisms remain intact in normal tissues and prevent systemic RRV dissemination. Of
251 course, it is possible that low levels of systemic RRV dissemination might occur below the detection
252 limit of our PCR assay, and the potential for retroviral vectors to cause insertional mutagenesis that
253 can contribute to the development of malignancies remains a concern [33-35]. However, when
254 considering the use of RRV as an agent for cancer therapy, this concern is alleviated, as incorporation
255 of prodrug activator genes not only arms the vector against cancer cells, but also helps to eliminate
256 inadvertently transduced normal cells that might become transformed. Furthermore, as an additional
257 safety mechanism, various antiretroviral drugs such as 3'-azido-3'-deoxythymidine (AZT) could be
258 used to effectively block retroviral replication and dissemination. It should be noted however that, to
259 date, more than 300 patients with recurrent high-grade glioma have been treated with CD-armed
260 MLV-RRV in multiple Phase I dose escalation trials (NCT01156584, NCT01470794, NCT01985256)
261 and an international Phase III trial (NCT02414165), and there have been no such severe adverse effects
262 related to malignant transformation. Certainly, clinical development of GALV-RRV will require
263 further confirmation of preclinical safety, biodistribution and therapeutic efficacy. Nonetheless, given
264 the extremely poor prognosis of patients with GBM, the use of RRV may represent a promising
265 treatment strategy, particularly if the therapeutic benefits outweigh the potential risks, as indicated
266 by our current results.

267

268   **4. Materials and methods**

269   *4.1. Viral vectors and cell lines.*

270   As described previously, plasmid pGS4-GFP [28] encodes a replication-competent GALV vector,
271   in which an IRES-GFP gene cassette has been inserted between GALV *env* and the 3' UTR. The CD
272   and NTR genes were amplified from plasmid pACE-CD [12] and *E. coli* genomic DNA by PCR, and
273   used to replace the GFP sequence in pGS4-GFP, generating plasmids pGS4-CD and pGS4-NTR,
274   respectively. The primer sequences used for PCR amplification of the *E. coli* NTR gene (isoform B)
275   are 5'-atggatatcatttctgtcgcct-3' and 5'-ttacacttcggttaaggtgatgtt-3' [36]. The transformed human
276   embryonic kidney cell line 293T [37], U-87 human glioma cells (obtained from the American Type Culture
277   Collection), and RG-2 and CNS-1 rat glioma cells were grown in Dulbecco's modified Eagle's medium
278   (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen). Viral vectors
279   were produced by transient transfection of 293T cells with plasmid pGS4-GFP, pGS4-CD or pGS4-
280   NTR using Lipofectamine 2000 Reagent (Invitrogen). For *in vitro* transduction experiments, 4 μg/ml
281   polybrene (Sigma, St. Louis, MO) was added to the culture medium at the time of infection. Titer
282   determination was performed on target cells in the presence of AZT (Sigma) to prevent secondary
283   vector replication, as described previously [38]. Virus titers were represented as transducing units
284   (TU)/ml.

285   *4.2. Viral vector replication assays in glioma cells.*

286   Glioma cells at 20-25% confluency in six-well plates were grown in fresh medium containing
287   GS4-GFP virus stock at low MOI. At various time points post-infection, the cells were analyzed for
288   GFP expression by flow cytometry. This procedure was performed to ensure that the entire cell
289   population exhibited GFP fluorescence. Infection of GS4-CD and GS4-NTR to RG-2 cells was
290   performed in parallel until full transduction was achieved. In a separate experiment, GS4-GFP-
291   transduced glioma cells were mixed with uninfected glioma cells at a proportion of 5% of the total
292   cell population and seeded onto six-well plates. At various time points post-infection, the cell
293   populations were analyzed for GFP expression.

294   *4.3. Viral vector replication assay in intracerebral glioma model.*

295   Intracerebral RG-2 gliomas were established by stereotactic injection of 5×10^4 RG-2 cells into the
296   right frontal lobe in Fischer 344 rats (National Laboratory Animal Center, Taipei, Taiwan) as
297   described previously [13]. Three days later, the rats were stereotactically injected with 2×10^4 TU of
298   GS4-GFP at the tumor implantation site. At various time points after viral vector transduction, the
299   rats were sacrificed and the tumors were excised and digested with collagenase (Invitrogen). The
300   dissociated cells were filtered through 100-μm cell strainers, pelleted by centrifugation, resuspended
301   in culture medium containing 50 μM AZT, and plated onto culture plates. After overnight culture,
302   the cells were trypsinized and immediately subjected to flow cytometry for GFP expression analysis.

303   *4.4. Real-time PCR analysis.*

304   To detect any integrated GALV-RRV sequence in tissue genomes, real-time PCR was performed
305   as described previously [19,39] using an ABI Prism 7700 sequence detector. The primers 5'-
306   cctattactcctccttctgttg-3' and 5'-gggcctgatattttgtctaag-3' were designed to target GALV *env*.
307   Apolipoprotein B gene for precise amounts of input genomic DNA were also quantified as an internal
308   control (primers: 5'-cacgtgggctccagcatt-3' and 5'-tcaccagtcatttctgcctttg-3'). Real-time PCR was done
309   in 25 μl of reaction mixture containing genomic DNA, 12.5 μl of 2X SYBR green real-time PCR master
310   mix (Toyobo, Osaka, Japan) and 300 nM of each primer. Products were amplified by 35 cycles of
311   successive incubation at 95°C for 15 sec and at 60°C for 1 min. A standard curve for GS4-GFP copy
312   number was generated by amplification of serially diluted GS4-GFP plasmid template at specific copy
313   numbers mixed into genomic DNA from uninfected rat cells.

314

4.5. In vitro cytotoxicity assay.

GS4-GFP-, GS4-CD- and GS4-NTR-transduced RG-2 cells were seeded onto replicate 96-well plates (2000 cells/well), cultured overnight, and exposed to 5-FC (Sigma) or CB1954 (Sigma) at various concentrations. Cell viability was determined 3 days later by MTS assay using the CellTiter Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI).

4.6. Survival assay using intracerebral glioma models.

The GS4-CD and GS4-NTR viral vectors (2×10^4 TU) was injected 3 days after intracerebral tumor implantation (5×10^4 RG-2 cells) in Fischer 344 rats. Eight days after GS4-CD vector injection, intraperitoneal injections of 5-FC (100 mg/kg) were performed, once every other day, for a total of 7 treatments. For the GS4-NTR group, CB1954 (2.5 mg/kg) was injected intraperitoneally 7 and 8 days after vector injection.

4.7. MicroPET imaging.

L-[^{18}F]FET microPET imaging of tumor-bearing rats was performed 20 days after RG-2 implantation using the R4 system (Concorde Microsystems, Knoxville, TN) as described previously [29]. Static images were acquired 60 min after the intravenous injection of 3.7 MBq of L-[^{18}F]FET in the rats.

4.8. Statistical analysis.

Student's *t*-test was used for statistical analysis of *in vitro* cytotoxicity results. Kaplan-Meier analysis and log-rank tests were used to evaluate survival. All analyses were conducted using SAS software (SAS Institute, Cary, NC).

Author contributions: S.H.C. and J.M.S. performed the laboratory experiments and drafted the manuscript. B.M.C., S.C.L. and H.F.C. contributed in the laboratory works. S.C. and N.K. revised the manuscript. D.C., W.W. and T.C.C. participated in the project design. Y.C.L. helped to draft the manuscript. S.F.W. partially supervised the *in vivo* study. H.E.W. and C.K.T. conceived and coordinated the overall study and revised the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: N.K. has consulted for Tocagen Inc. All other authors declare no conflict of interest.

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