

SHORT COMMUNICATION

Controlled propagation of replication-competent Sindbis viral vector using suicide gene strategy

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A major concern of using viral gene therapy is the potential for uncontrolled vector propagation and infection that might result in serious deleterious effects. To enhance the safety, several viral vectors, including vectors based on Sindbis virus, were engineered to lose their capability to replicate and spread after transduction of target cells. Such designs, however, could dramatically reduce the therapeutic potency of the viral vectors, resulting in the need for multiple dosages to achieve treatment goals. Earlier, we showed that a replication-defective (RD) Sindbis vector achieved specific tumor targeting without any adverse effects *in vivo*. Here, we present a replication-competent Sindbis viral vector that has an *hsvtk*

suicide gene incorporated into *ns3*, an indispensable non-structural gene for viral survival. The capability of viral propagation significantly increases tumor-specific infection and enhances growth suppression of tumor compared with the conventional RD vectors. Furthermore, in the presence of the prodrug ganciclovir, the *hsvtk* suicide gene serves as a safety mechanism to prevent uncontrolled vector propagation. In addition to suppressing vector propagation, toxic metabolites, generated by prodrug activation, could spread to neighboring uninfected tumor cells to further enhance tumor killing. Gene Therapy advance online publication, 25 September 2008; doi:10.1038/gt.2008.153

Keywords: Sindbis virus; viral vector; suicide gene; prodrug; molecular imaging

Introduction

We have reported earlier a viral vector system for detecting tumor cells and monitoring cancer therapy.^{1,2} The method is based on the ability of the Sindbis virus to infect human cancer cells that express higher levels of 37/67-kDa laminin receptor compared with normal, non-cancerous cells.³ Higher levels of unoccupied laminin receptor on the cell surface serve as receptors for the virus to recognize and selectively enter tumor cells, sparing normal cells from infection.⁴

For the purposes of gene therapy, various Sindbis viral vectors were designed, on the basis of the wild-type RNA genome. The wild-type Sindbis RNA genome contains two major segments (Figure 1a). The first, located on the 5' side of the genome, carries all non-structural genes (*ns1*, *ns2*, *ns3* and *ns4*) involved in virus replication. On the other hand, the second segment is located at the 3' end and contains the structural genes (capsid, *e3*, *e2*, *6k* and *e1*). The junction region between structural and non-structural genes contains a viral-specific subgenomic promoter that is necessary for the expression of structural genes. At an early stage of infection, the non-structural genes are translated as a polyprotein by a cap-dependent scanning mechanism. The polyprotein matures to form a replicase complex

through several cleavages catalyzed by viral and host proteases. The Sindbis replicase has two major functions. The first is the amplification of genomic RNA and the second is the expression of structural genes for virus formation. For the latter function, Sindbis replicase specifically recognizes the subgenomic promoter at the junction region to drive the expression of structural proteins. The capsid structural protein specifically recognizes the packaging signal in the *ns1* gene forming nucleocapsid, which later interacts with the remaining structural proteins to generate virus particles.

The goal of viral gene therapy systems is to achieve efficient gene transfer while avoiding possible toxicity associated with virus propagation. Figure 1b depicts a conventional dual-component vector design that meets these requirements by taking advantage of the locations of both the package signal and the subgenomic promoter. This system requires two RNAs, replicon and helper, to produce infectious vector particles. The replicon RNA contains all non-structural genes, the package signal and subgenomic promoter. However, the structural genes are replaced with the therapeutic gene of interest. In contrast, the helper RNA has a truncated non-structural gene but contains a functional subgenomic promoter and genes that encode structural proteins *in trans* for packaging replicon RNA. Most importantly, the helper RNA lacks a packaging signal. This feature would ensure that the produced vector particles carry only replicon RNA with the gene of desire. These vector particles, without an RNA genome encoding structural proteins, cannot propagate after the initial infection.

One major advantage of such conventional dual-component vector systems is that the produced vector

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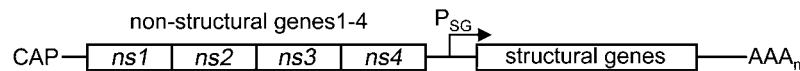
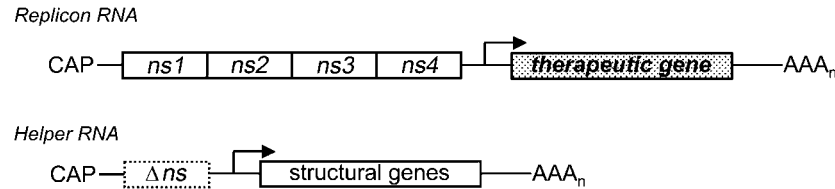
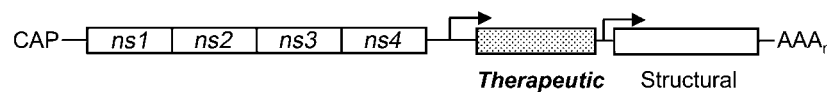
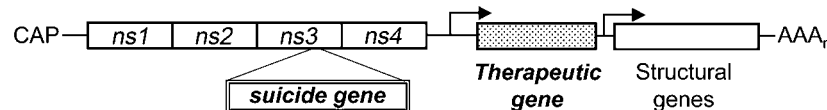
a Wild-type Sindbis virus genomic RNA: single component**b Replication-defective (RD) Sindbis viral vector system: dual components****c Replication-capable (RC) Sindbis viral vector system: single components****d Replication-capable (RC) Sindbis viral vector system with safety feature: single components**

Figure 1 Various Sindbis vector systems. (a) Wild-type Sindbis virus RNA genome has two major groups of genes: non-structural genes on the 5' side and structural genes on the 3' side. (b) The conventional two-component replication-defective (RD) system contains a replicon RNA for therapeutic gene expression and a helper RNA to provide structural genes for vector production. (c) A simple replication-capable (RC) vector system with integrated structural genes. (d) An RC vector system with a suicide gene fused in-frame with the *ns3* gene to achieve 'controlled' vector propagation and replication in tumor. *ns3* gene encodes a protein critical for viral replication and survival.

particle should be replication defective (RD), which renders it safer for clinical use. However, such an advantage could become a significant drawback for cancer gene therapy. The goal of cancer gene therapy is to infect the majority of tumor cells and deliver the therapeutic genes for tumor detection or eradication. Achieving this goal using an RD system would require repetitive treatment regimens and higher doses of vectors. In some cases, such high doses might not be easily obtained using an RD system.

A Sindbis vector system that is capable of 'controlled' replication and propagation should be of great interest for cancer gene therapy. Such an ideal replication-capable (RC) system should be single component to ensure efficient and selective propagation in tumor cells; the system requires no helper component for replication. One major benefit of such a system is that fewer treatments are required and a lower dose should possibly suffice to achieve successful therapeutic outcomes while retaining the same tumor-targeting capability as RD vectors.

In light of our discovery that the RD Sindbis vector can target tumors in living animals,² we hypothesized that a single-component RC Sindbis vector should perform better than RD vectors for cancer gene therapy. Figure 1c illustrates a simple RC system design that does not require a helper RNA, in which a second subgenomic promoter was also incorporated along with the structural genes to ensure efficient expression of structural proteins for vector propagation as well as genes of interest. This

simple design renders the vector system very similar to wild-type virus, and therefore it has been used to study the function of the subgenomic promoter in mammalian cells,^{5,6} to deliver antigen genes for vaccination purposes,⁷⁻⁹ and to study the Sindbis virus infection and propagation in the mosquito.¹⁰

Similarity with the wild-type virus, however, does raise concerns about safety and toxicity. To significantly reduce the risk of unwanted toxicity associated with possible vector propagation in normal tissue cells, a 'safe' mechanism should be implemented to control the propagation of the RC vector system. One ideal way is to incorporate a 'suicide gene' into one of the non-structural genes that are essential for viral propagation and survival. Figure 1d depicts the concept of such a design. A suicide gene that encodes an enzyme capable of activating inert prodrugs into cytotoxic metabolites can be fused in-frame with one of the non-structural genes to ensure coexpression with viral replicase. Therefore, by this design, the RC vector incorporates a safety mechanism that can be activated by a prodrug that would shut off vector propagation during or after the treatment regimen.

Results and discussion

We first tested whether a simple single-component RC system performs better than the conventional dual-component RD system for cancer gene therapy using a

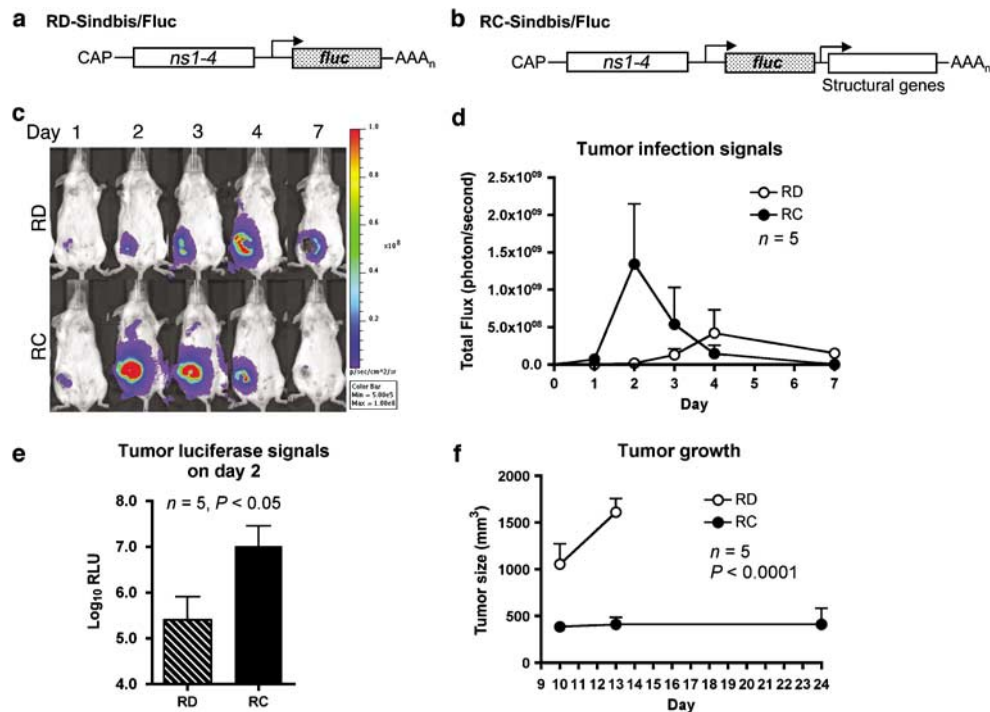


Figure 2 Replication-capable (RC) Sindbis vector shows superior tumor targeting and killing over conventional replication-defective (RD) system. Design of (a) a conventional RD-Sindbis/Fluc that was constructed using pSinRep5/Fluc plasmid as the backbone^{1,2} and (b) a prototype RC-Sindbis/Fluc both carrying firefly luciferase gene as reporter. To make the vector capable of self-propagation, a DNA segment containing a subgenomic promoter and Sindbis viral structural genes was excised from the ptRNA-DHBB plasmid (Invitrogen Corp., Carlsbad, CA, USA) using *NsiI* and *BamHI* enzymes and then inserted into pSinRep5/Fluc at the *StuI* site. Vectors were prepared using the *in vitro* transcription/electroporation method as described earlier (Tseng *et al.*^{1,2}). (c) Bioluminescent imaging of subcutaneous BHK tumor-bearing mice after daily treatments of RD- or RC-Sindbis/Fluc. BHK tumors were induced in female SCID mice (Taconic, Germantown, NY, USA) on the right hindlimb by subcutaneous injection of 2 million BHK cells on day -10. Started on day 0 when tumor is ~500 mm³, tumor-bearing mice were intravenously injected with RD or RC vector particles (~10⁶ vector particles) for 2 consecutive days (days 0 and 1). Bioluminescent IVIS imaging (Caliper LifeSciences, Hopkinton, MA, USA) of indicated days shows strong vector infection and propagation in RC-treated tumors. D-luciferin (0.3 ml of 15 mg ml⁻¹; Promega, Madison, WI, USA) was intraperitoneally injected into mice 5 min before imaging to generate bioluminescent signals. (d) Quantitative representation of tumor luminescent signals. (e) Tumor luciferase signals in log scale on day 2. *P*-values are calculated using *t*-test. (f) Tumor volume measured on days 10, 13 and 24 using the formula: $\pi/6 \times \text{length} \times \text{width} \times \text{height}$. *P*-values are calculated using two-way ANOVA. ANOVA, analysis of variance; SCID, severe combined immunodeficient.

previously established subcutaneous BHK tumor model.² BHK tumor cells were subcutaneously inoculated on the right hind limb of severe combined immunodeficient (SCID) mice. To monitor vector delivery and propagation in tumor cells, both RD (Figure 2a) and RC (Figure 2b) vectors carried a firefly luciferase (*fluc*) gene as reporter whose expression can be easily visualized using the IVIS imaging system (Figure 2c). After 10 days of inoculation, the tumors reached ~500 mm³ in size and the mice were treated with intravenous injections of RD or RC vector (10⁶ PFU) for 2 consecutive days (days 0 and 1). No further treatment was administered. Bioluminescent images were taken on days 1, 2, 3, 4 and 7 (Figure 2c). The RC vector shows significant enhancement of vector infection and propagation as indicated by luciferase activity (Figures 2d and e). The data indicate that a simple RC vector system has an ~30-fold more increase in infectivity than the conventional RD vector system. Higher infectivity also reflects on enhanced tumor killing as evident in tumor-size reduction on day 10 (Figure 2f).

These data provide proof-of-concept results and support the use of the RC Sindbis vector system for cancer gene therapy. As we used the same structural gene sequences to produce both vector particles (Figure 1),

the enhanced tumor targeting is largely contributed by the replication capability of the RC vector and is less likely due to a difference in initial infectivity (primary infection stage) or a difference in degradation or excretion. The physical differences (biochemical composition) in RD and RC vector particles are solely attributed to the RNA sequences they carry. The capability of the RC vector to propagate and spread within the tumor dramatically enhances the ability of the Sindbis vector to target and kill cancer cells. The data also suggest that the same level of tumor detection could be achieved using a lower range of effective doses of the RC vector compared with conventional RD vectors.

It is important to note that toxicity was not observed with this prototype RC vector administered at the 10⁶ level, and bioluminescent signals, except in residual tumors, were not observed in SCID mice (which do not have adaptive immunity) up to 24 days after vector injections. These observations suggest that using the same level of the RC vector, as high as 10⁶ per dose, should not cause deleterious effects in immune-competent animals. High dosage is desirable not only for detecting small lesions but also for better tumor-killing therapeutic effects. As humans have ~3000 times the

body weight of mice and ~300 times the body surface area (average human weight: 65 kg, body surface area: 1.71 m²; average mouse weight: 0.02 kg, body surface area: 0.007 m²), the range of effective dosage for humans is expected to be 10⁸–10⁹ particle per dose or more. Nevertheless, as the capability to propagate within tumors is the major advantage of such simple RC vector systems, the potential for uncontrolled replication of wild-type virus still raises safety concerns. Although we did not see serious deleterious effects associated in SCID mice treated with simple RC vectors, as no infection was observed in other normal tissues (Figure 2c) and tumor infection signals gradually declined by day 7 because of tumor eradication (Figure 2f), a safety mechanism would be a significant improvement for the goal of using such a viral system for the treatment of human diseases.

A safety mechanism should ideally be directly linked with the viral replication machinery to tightly control its replication and propagation. We hypothesized that direct in-frame fusion of a suicide gene with one of the non-structural protein (nsp) genes should be able to control vector propagation using prodrugs. An alternative choice is to use viral subgenomic promoters to drive the expression of suicide genes. However, the recombination of Sindbis RNAs at the junction region between non-structural and structural genes, during replication, may generate an RC vector with a disabled suicide gene rendering the safety mechanism defective.^{11,12} As the nsps are encoded in a long open reading frame, the direct in-frame fusion of a suicide gene in this region could dramatically reduce the likelihood of generating 'escaped' RC vectors.

A particular region of *ns3* has been shown to be suitable for fusion without compromising virus replication.¹³ Figure 3a shows the design and construction of the RC vector with an integrated thymidine kinase gene

isolated from herpes simplex virus (*hsvtk*) gene fusion at aa 389 of the nsp3 protein (RC-Sindbis/Nsp3-tk/Fluc). We first tested whether the vector proliferation can be controlled by systemic administration of the prodrug ganciclovir (GCV). As SCID mice, without tumors, cannot sustain active RC vector proliferation and, according to our previous finding, interferon-I/stat1 signaling plays an important role in early vector infection control,¹⁴ we used *stat1* knockout animals to check whether the prodrug GCV can suppress or delay RC vector propagation *in vivo*. Intraperitoneal injection of the RD Sindbis vector into *stat1* knockout mice causes strong infection in the liver and peritoneal fat in 24 h. However, a second challenge of the RD vector does not result in persistent infection and all infection signals dissipate in ~48 h, suggesting an interferon-independent mechanism that controls vector infection. Without GCV, a single intraperitoneal injection of RC-Sindbis/Nsp3-tk/Fluc into *stat1* knockout mice (no tumor) caused a strong intraperitoneal infection signal similar to the RD vector, which is mostly present in the peritoneal fat and gradually faded off by day 4. The fact that infection can be resolved by day 4 in *stat1* knockout animals suggests that this vector system could be very safe for clinical use. Interestingly, to further increase its safety, GCV treatments significantly suppressed vector propagation as shown in reduced bioluminescent signals in the whole body (Figures 3b and c). These results support the use of prodrugs to control the propagation of the RC vector after administration.

We later tested whether RC-Sindbis/Nsp3-tk/Fluc shows specific targeting and propagation in tumors. SCID mice carrying subcutaneous BHK tumors received a single intravenous injection of conventional RD-Sindbis/Fluc or RC-Sindbis/Nsp3-tk/Fluc vector. Bioluminescent imaging showed that the insertion of the

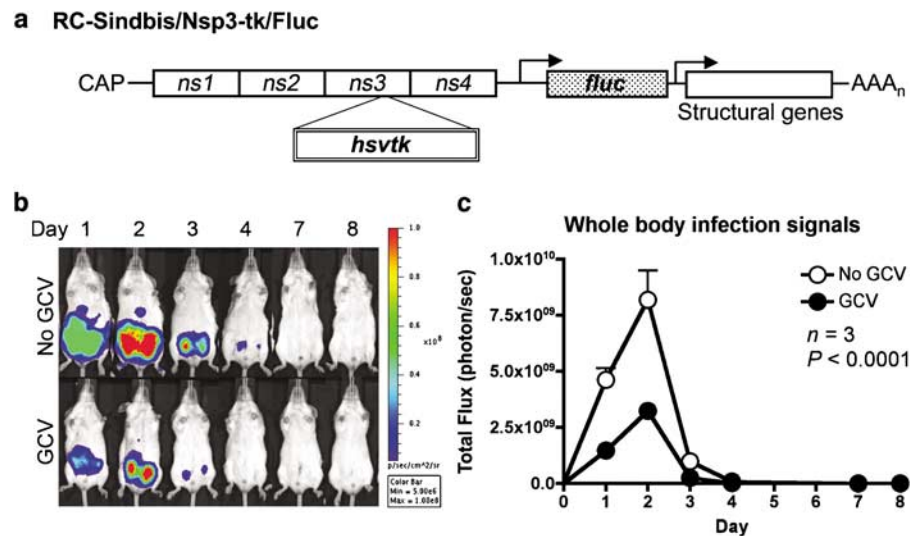


Figure 3 In-frame fusion of *hsvtk* suicide gene into *ns3* gene as a safety mechanism to control propagation of RC vectors in *stat1* knockout mice. (a) Scheme of RC-Sindbis/Nsp3-tk/Fluc vector design. This RC vector has a herpes simplex viral thymidine kinase (*hsvtk*) gene fusion in-frame with the viral *ns3* gene at the *SpeI* site on pSinRep5/Fluc-tBB. (b) On day 0, Balb/c *stat1* KO mice were intraperitoneally (i.p.) injected with a single dose of RC-Sindbis/Nsp3-tk/Fluc (10⁶ PFU) followed by daily i.p. injections of 25 mg/kg⁻¹ ganciclovir (GCV, CYTOVENE-IV; Roche Laboratories Inc., last dose injected on day 4). Serial bioluminescent imaging shows higher vector replication signal in animals without GCV treatment. (c) Quantitative analysis of RC-Sindbis/Nsp3-tk/Fluc propagation in Balb/c *stat1* KO mice after i.p. injection on day 0. Animals received GCV treatments showing significant reduction in vector proliferation as analyzed by two-way ANOVA. ANOVA, analysis of variance.

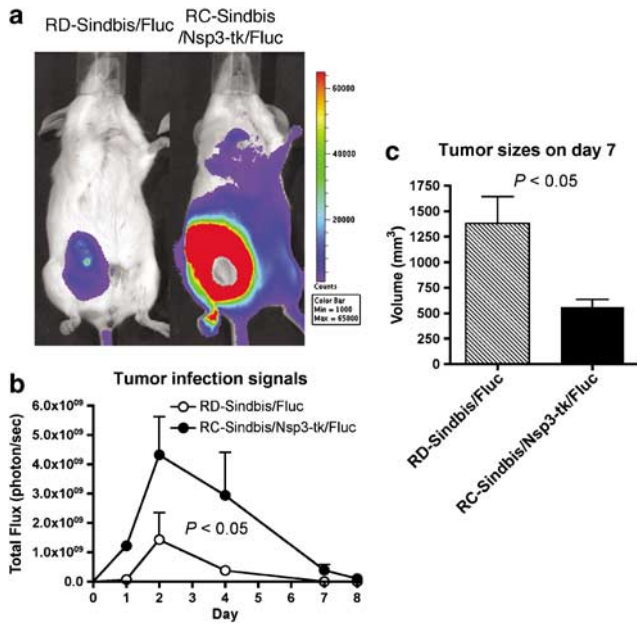


Figure 4 RC-Sindbis/Nsp3-tk/Fluc vector maintains tumortargeting activity and performs better than conventional RD-Sindbis/Fluc vector. (a) Bioluminescent imaging of subcutaneous (s.c.) BHK tumor-bearing mice after a single treatment of RD-Sindbis/Fluc or RC-Sindbis/Nsp3-tk/Fluc vector. As in Figure 2, BHK tumors were induced in female SCID mice by subcutaneous injection of 2 million BHK cells on day -10 . Started on day 0, tumor-bearing mice received a single intravenous vector treatment ($\sim 10^6$ vector particles), and bioluminescent images were acquired the day after (day 1). The hollow signals in the RC tumor resulted from saturation signals. (b) Quantitative representation of tumor luminescent signals after vector treatments. Significant difference was observed according to two-way ANOVA ($n=3$, $P<0.05$). (c) Tumor volume measured on day 7 using the formula: $\pi/6 \times \text{length} \times \text{width} \times \text{height}$. P -values are calculated using t -test ($n=3$, $P<0.05$). ANOVA, analysis of variance; SCID, severe combined immunodeficient.

hsvtk gene at the *ns3* region did not abolish the propagation of the RC vector in tumors. Higher luciferase activity (Figure 4a) allowing prolonged tumor-specific signals (Figure 4b) suggested that the vector actively propagates inside the tumor, resulting in significantly enhanced tumor growth suppression (Figure 4c).

In addition to serving as a safety feature, the fused suicide gene provides another advantage. The tumor cells that are selectively infected by the vector would be more susceptible and sensitive to prodrug treatment, as they would not only face being killed by the Sindbis infection, but also the toxic metabolites as a result of prodrug activation. In some cases, activated toxic metabolites can passively diffuse to neighboring uninfected tumor cells to further enhance tumor killing. Several suicide genes and their appropriate prodrugs have been designed and are suitable for insertion into the Sindbis vector as safety features. A specific prodrug, GCV, has been developed to be activated by *hsvtk* protein. GCV has been clinically approved for treatment of viral infection of cytomegalovirus and herpes simplex virus in humans. We have earlier shown that a conventional RD Sindbis vector carrying an *hsvtk* gene significantly enhances tumor killing and tumor *hsvtk*

activity can be monitor using positron emission tomography (PET).¹⁵ The capability to monitor *hsvtk* activity *in vivo* provides a mean to track RC vector propagation as a precautionary measure.

In summary, the single-component RC Sindbis vector system can dramatically enhance *in vivo* tumor targeting and killing capability, whereas the incorporation of the suicide gene provides an additional layer of protection to achieve 'controlled' propagation in tumors. This novel design of the Sindbis vector system should provide a promising reagent for successful cancer gene therapy.

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