Myxoma Virus Virotherapy for Glioma in Immunocompetent Animal Models: Optimizing Administration Routes and Synergy with Rapamycin

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Abstract
Oncolytic myxoma virus (MYXV) is being developed as a novel virotherapeutic against human brain cancer and has promising activity against human brain tumor models in immunocompromised hosts. Because an intact immune system could reduce its efficacy, the purpose of this study was to evaluate the oncolytic potential of MYXV in immunocompetent melanoma models. Here, we report that MYXV infects and kills all mouse cell lines and that its effects are enhanced by rapamycin. Intratumoral administration of MYXV with rapamycin improved viral replication in the tumor and significantly prolonged host survival. Similarly, coadministration via a method of convection-enhanced delivery (CED) enhanced viral replication and efficacy in vivo. Mechanisms by which rapamycin improved MYXV oncolysis included an inhibition of type I IFN production in vitro and a reduction of intratumoral infiltration of CD68+ macrophages. Our findings define a method to improve MYXV efficacy against gliomas by rapamycin coadministration, which acts to promote immune responses engaged by viral delivery. Cancer Res; 70(2); 598–608. ©2010 AACR.

Introduction
The current management of patients with malignant gliomas uses a combination of surgery, radiation, and chemotherapy (1) but is unsatisfactory because patients survive for only a year on average. Thus, new treatment approaches are desperately needed. Recently, oncolytic viruses have been evaluated against malignant gliomas in preclinical models (2–7) and in clinical trials (8–12).

We discovered that myxoma virus (MYXV) was oncolytic for several human cancers (5, 13–16). It is promising for human therapy because its genome has been sequenced, it is simple to engineer, its natural tropism is highly restricted to European rabbits (Oryctolagus cuniculus), and there is no acquired viral immunity in humans (17). Despite its extremely narrow host tropism in nature, MYXV infects many human cancer cells and certain nonrabbit cells in vitro (18), which have defects in their IFN responses (19, 20). Cancer cells, such as gliomas, which have activated Akt, are particularly susceptible to MYXV (21, 22).

The preclinical models traditionally used to evaluate oncolytic viruses in malignant gliomas have been in immunodeficient mice bearing human tumors (2–6). Few have used immunocompetent models (23–26). We have reported promising efficacy of MYXV in immunocompromised brain tumor models (5, 14, 15); there is one report of MYXV in an immunocompetent model of melanoma (16). The evaluation of an oncolytic virus in immunocompetent setting is important because both the innate and the acquired immune system may dramatically modulate oncolytic efficacy. This host modulation could either reduce [e.g., impairing viral infection or intratumoral (i.t.) distribution] or enhance (e.g., stimulate antitumor immune responses) oncolysis. Thus, a systematic evaluation of MYXV in immunocompetent glioma models is needed to address these issues. We chose a racine, rather than a spontaneously occurring murine genetic model, because this syngeneic line establishes reproducible and consistent tumor burden in its immunocompetent host and has a long track record (25). The more readily available transgenic murine models use (retro-) viral vectors in their derivation, which could confound the evaluation of immune responses in these gliomas. Hence, we chose an immunocompetent racine model of glioma in this study.

The best examples of modulating immune responses to improve oncolytic virus efficacy have been with herpes simplex virus (HSV). HSV is neutralized by complement when administered i.v. or by microglia (CD68 cells) and macrophages (CD163 cells) when administered i.t. Inhibiting both
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Materials and Methods

Cell lines, viruses, and reagents. Established rat glioma cell lines RG2, 9L, F98, and C6 and murine NIH3T3 fibroblasts were obtained from the American Type Culture Collection. All cells were grown in DMEM/F12 with 10% fetal bovine serum. The firefly luciferase gene plasmid (pGL3 enhancer vector; Promega) was cotransfected into RG2 cells as reported before (37). All the viruses used in this study were constructed in the laboratory of G. McFadden, and virus was propagated and titrated by focus formation on BGMK cells as described previously (5, 16, 21, 22, 38, 39). UV-inactivated MYXV [dead virus (DV)] was prepared by irradiating virus with UV light for 2 h. Rapamycin was obtained from LC laboratories, and RAD001 dry powder and cyclophosphamide (can prepare) or saline (5 μL; 5 × 106, 1 × 107, 5 × 107, and 1 × 108 plaque-forming units (pfu)/rat (10 μL; the highest feasible dose we can prepare)] or saline (5 μL), as we described previously (25). Animals were followed for up to 30 d. Animals losing ≥20% body weight or having other unacceptable symptoms were sacrificed as Animal Care Guidelines. After sacrificed, brains and major organs were processed for histologic examination.

In vivo studies in rodent orthotopic glioma model in immunocompetent hosts. RG2 cells (1 × 104 per rat) were implanted into Fischer 344 rats under anesthesia as described before (25). For experiments assessing survival, i.t. administration of 1 × 105 pfu (in 5 μL PBS) of either DV or MYXV at 5 d after implantation, animals were followed until they developed symptoms. To test the effect of combination therapy using MYXV and rapamycin or cyclophosphamide, RG2 tumor-bearing animals were divided into the following groups (five to six animals per group): (a) DV control, (b) cyclophosphamide or rapamycin alone (5 mg/kg rapamycin, i.p., five times per week for a total of 2 wk; or 60 mg/kg cyclophosphamide, one time only), (c) MYXV (1 × 107 pfu/rat, every other day for a total of three injections), and (d) MYXV plus rapamycin or cyclophosphamide. Animals were monitored daily and sacrificed when symptoms developed. We repeat the above experiment with F98 rat brain tumor model.

For experiments assessing the effects of rapamycin and cyclophosphamide on the immune response to viral replication, RG2-bearing animals were treated, as described above for the survival study, 8 d after implantation. Animals were sacrificed at 24 and 72 h and 7 d after treatment (three animals per treatment). When sacrificed, the brains were imaged and then embedded with OCT for immunohistochemistry, or the green fluorescent protein (GFP)—expressing tumor was saved in liquid nitrogen to quantify the virus titers as described previously (5, 14).

CED of MYXV plus rapamycin in RG2 glioma model of immunocompetent hosts. To determine if the delivery of MYXV via CED in combination with rapamycin would further increase the therapeutic benefit, we used the same infusion system and infusion rates as the Sugiyama group (36). RG2-bearing rats were divided into the following groups (five or six animals per group) 5 d after implantation: (a) DV (CED) control, (b) MYXV i.t. only (1 × 107 pfu, in 5 μL PBS), (c) MYXV CED only (1 × 107 pfu, in 20 μL PBS), (d) MYXV i.t. plus rapamycin (MYXV: 1 × 107 pfu; 5 mg/kg rapamycin, i.p., five times per week for a total of 2 wk), and (e) MYXV CED plus rapamycin. Animals were monitored daily for survival studies. To check the virus distribution, animals were sacrificed at 72 h and 7 and 12 d after virus treatment (three animals per treatment). After sacrifice, the same procedure with above was used to get the brain image and tumor tissue viral titers.

Immunohistochemistry. Frozen sections were exposed to primary antibody [a rabbit polyclonal myxoma antibody M-T7 (1:2,000) or mouse anti-rat CD68 (1:500; Serotec) and CD163 (1:300; Serotec)] in 10% normal goat serum for 1 h at pathways using cyclophosphamide increases viral replication and oncolysis (27–29). In our study, we chose rapamycin as a cotherapeutic with MYXV for several reasons. First, rapamycin enhances MYXV tumor cell infection in vitro [possibly by further activating Akt (30) or inhibiting a type I IFN response (31)] and in animal tumor models (14, 16). Second, rapamycin is a potent immunosuppressant that inhibits the development and/or activation of numerous arms of the innate or adaptive immune response, most well studied being the blocking of T-cell activation/proliferation during allograft rejection (32). Third, it has direct activity against several cancers/gliomas (33, 34) and is safer than cyclophosphamide (34). We therefore considered it an ideal candidate for combination therapy with MYXV in malignant gliomas.

We also evaluated a second strategy, convection-enhanced delivery (CED), to enhance the therapeutic potential of MYXV. CED can help optimize delivery and biodistribution (35, 36). Here, we assessed CED of MYXV, either singly or in combination with rapamycin, to further augment the efficacy of MYXV in an immunocompetent model.

The objectives of this study were to determine in immunocompetent models of gliomas (a) the safety/toxicity of the intracranial (i.c.) inoculation of MYXV, (b) the efficacy of MYXV alone or in combination with rapamycin against malignant gliomas, and (c) the efficacy of CED of MYXV combined with rapamycin in vivo.

Safety/toxicity of i.c. administration of MYXV in nontumor-bearing rats. Fischer 344 rats under anesthesia (ketamine and xylazine) received i.c. injection of the different doses of MYXV [in 5 μL; 5 × 106, 1 × 107, 5 × 107, and 1 × 108 plaque-forming units (pfu)/rat (10 μL; the highest feasible dose we can prepare)] or saline (5 μL), as we described previously (25). Animals were followed for up to 30 d. Animals losing ≥20% body weight or having other unacceptable symptoms were sacrificed as Animal Care Guidelines. After sacrificed, brains and major organs were processed for histologic examination.

For cell viability experiments, cell viability was measured at 72 h and 7 and 12 d after virus treatment (three animals per treatment). When sacrificed, the brains were imaged and then embedded with OCT for immunohistochemistry, or the green fluorescent protein (GFP)—expressing tumor was saved in liquid nitrogen to quantify the virus titers as described previously (5, 14).

Western blot analysis. Western blot was performed using polyclonal MYXV antibodies (M-T7 and Serp-1, recognizing early and late MYXV gene products, respectively), and horse-radish peroxidase (HRP)–conjugated secondary antibody was used to detect MYXV proteins, as described previously (5, 14).
room temperature after fixation and blocking. Biotinylated anti-rabbit IgG or anti-mouse IgG (Vector Laboratories) were used as a secondary antibody. Sections were then incubated with avidin conjugated to HRP, and staining was visualized by the addition of 3,3′-diaminobenzidine.

**In vivo bioluminescence imaging.** On days 7, 10, 13, 17, 20, 27, 33, and 41 after RG2-Fluc (1 × 10⁴ cells per rat) implantation, rats from different treatment groups—DV, rapamycin, MYXV, and MYXV + rapamycin (four or five rats per group)—were imaged with the Xenogen IVIS 200 system to record bioluminescent signal emitted from tumors. Data were analyzed based on total photon flux emission (photons/s) in the region of interest over the intracranial space as reported previously (40).

**Statistical analysis.** Statistical Analysis Software (SAS Institute, Inc.) and GraphPad Prism (version 4; GraphPad Software, Inc.) were used for statistical analyses. Survival curves were generated by the Kaplan-Meier method. All reported P values were two-sided and were considered to be statistically significant at P < 0.05.

**Results**

**MYXV infects and kills raceine glioma cell lines in vitro.** All four glioma cell lines (RG2, F98, C6, and 9L) were found to be permissive to MYXV (5 multiplicities of infection (MOI), 48 hours) as illustrated by the expression of GFP (early gene) and β-galactosidase (late gene). In contrast, control line NIH3T3 was poorly permissive to MYXV (Fig. 1A). Next, to assess whether the appearance of viral proteins correlated with cellular susceptibility in the glioma cells, MTT assays were performed. All four glioma cell lines were killed by MYXV (10 MOI, 48 hours; Fig. 1B). In contrast, >70% of the NIH3T3 cells were viable after exposure to MYXV.

**Pretreatment with rapamycin enhances viral replication and promotes MYXV oncolysis in raceine glioma cells in vitro.** Treatment of RG2 and F98 cells with MYXV plus rapamycin resulted in significantly greater cell killing in vitro than either treatment alone (5% versus 80% for RG2; 8% versus 75% for F98; 1 nmol/L rapamycin; **, P < 0.001, t test; Fig. 2A; doses of 0.1 nmol/L rapamycin showed no effect (Supplementary Fig. S1A and B)); similar results were seen with a second mammalian target of rapamycin (mTOR) inhibitor RAD001 (Supplementary Fig. S1C). There was no significant effect on NIH3T3 cells (3% versus 5%; Fig. 2A). To determine if the increase in cell susceptibility was the result of increased viral replication, we next assessed virus gene expression. F98 and RG2 treated with MYXV (1 MOI) following pretreatment with rapamycin (20 nmol/L) showed substantially more viral GFP and β-galactosidase (Fig. 2B, inset) expression than cells treated with virus alone 48 hours after infection (Fig. 2B). The increase was specific for the glioma cells, as no increase was detected in the NIH3T3 cells (Fig. 2B). Consistent with this, treatment of the raceine glioma lines with both MYXV (0.1 MOI) and rapamycin (20 nmol/L) resulted in a significant increase in viral titers 72 hours after infection as compared with virus alone (**, P < 0.05, t test; Fig. 2C).

**Safety/toxicity of i.c. administration of MYXV in nontumor-bearing animals.** We evaluated the toxicity of MYXV in nontumor-bearing immunocompetent rats with i.c. administration. MYXV was safe in nontumor-bearing rats with only a slight weight loss seen at the highest dose (1 × 10⁸ pfu), which slowly recovered within 2 weeks (Fig. 3A). There were no deaths (Fig. 3B), no observed pathologic changes at 30 days (Supplementary Fig. S2B), and no neurologic symptoms noted.
throughout the experiment. However, we found slight inflammation and virus staining in the brain at early time points (3 days) after i.c. administration (Supplementary Fig. S2A). For subsequent in vivo experiments, we therefore selected a MYXV dose of 1 × 10⁷ pfu.

**Combination therapy with rapamycin enhanced MYXV replication in two immunocompetent racine glioma models.** To assess viral distribution in the RG2 glioma model, animals were treated with i.t. MYXV ± rapamycin as described. Animals treated with MYXV alone showed limited viral GFP expression (approximately 3–5% of the tumor cells; Fig. 4A, top middle), and there was no detectable virus by day 3 (Supplementary Fig. S3A). In contrast, animals treated with MYXV plus rapamycin showed substantially more viral GFP expression (Fig. 4A, top right) and viral protein detected by immunohistochemistry (approximately 35–40% of tumor cells; Fig. 4A, bottom) 24 hours after infection. In addition, significantly higher viral titers were detected when using rapamycin, which persisted for at least 7 days (Supplementary Fig. S3A). Similar results were observed using the F98 glioma model, where...
MYXV–red fluorescent protein virus (5 × 10^6 pfu) was used in combination with rapamycin (Supplementary Fig. S3B). Significantly, no virus replication was observed in any normal brain tissues, either with or without rapamycin.

**Combination therapy of MYXV with rapamycin prolongs survival in two immunocompetent racine glioma models.** RG2 and F98 cells were implanted into rats to establish immunocompetent glioma models as described previously (25). Four days later, rapamycin was injected i.p., and the following day, DV or MYXV was injected i.t. Treatment with MYXV alone did not prolong survival of animals harboring RG2 tumors (P = 0.078, log-rank test; Fig. 4B) and only slightly prolonged the survival in animals with F98 tumors (P = 0.0439; Fig. 4D). In contrast, treatment with MYXV plus rapamycin significantly prolonged survival as compared with treatment with MYXV alone (P = 0.0098, Fig. 4B; P = 0.0411, Fig. 4D) or as compared with treatment with the DV (P = 0.0011, Fig. 4B; P = 0.0025, Fig. 4D) in both animal models. Rapamycin alone did prolong survival of animals bearing RG2 gliomas (P = 0.026; Fig. 4B) but had no effect on survival in animals bearing F98 gliomas (P = 0.1167; Fig. 4D). We confirmed these results using bioluminescence imaging (BLI). RG2-Fluc–bearing animals were imaged at varying time points in the presence or absence of MYXV and/or rapamycin using the Xenogen 200 system. Control (PBS) and MYXV-treated animals had a BLI that rapidly increased starting on day 7 after tumor implantation (6.34E+05, 5.73E+04), reaching a peak by day 13 (1.98E+07), slowly increasing between days 13 and 27 to reach a peak level by day 33 (2.04E+07); in contrast, a very low BLI signal from rats treated with MYXV + rapamycin first appeared on day 17 (7.71E+04), and very slowly increasing until day 27, but still did not reach peak levels by day 41 (1.04E+07, termination of the experiment) after tumor implantation (Fig. 4C; Supplementary Fig. S3C). These results were consistent with the histologic examination at day 13 (Fig. 4C, bottom).

**CED of MYXV further improves efficacy in vivo.** RG2-bearing animals were treated with rapamycin 4 days after tumor implantation. The following day, DV or MYXV was administered via CED as described above. We found that compared with DV-treated animals, MYXV delivered via CED significantly improved survival (P = 0.0045, log-rank test; Fig. 5A) and this was further increased when MYXV was delivered by CED in combination with rapamycin (P < 0.0007; Fig. 5A), with 50% of the animals subsisting as "long-term" survivors (>45 days). In addition, combined CED MYXV and rapamycin significantly prolonged survival as compared with CED MYXV alone (P = 0.0009; Fig. 5A) or i.t. administered MYXV in combination with rapamycin (P = 0.0398; Fig. 5A). To determine if the prolonged survival was the result of increased viral replication, we measured in vivo virus distribution and viral titers. Animals treated with MYXV delivered via CED together with rapamycin had significantly higher levels of viral GFP expression (Fig. 5B), with virus persisting longer than either CED MYXV alone or MYXV administered i.t. in the presence of rapamycin (***, P < 0.001, t test; Fig. 5B and C). Importantly, we found that CED MYXV alone increased virus replication and spread, with the presence of virus being detected up to 12 days after virus administration (Fig. 5B and C).

**Rapamycin blocks IFN inhibition of MYXV infection and inhibits macrophage/microglial infiltration in glioblastoma multiforme.** To begin to understand the mechanism(s) by which rapamycin enhances MYXV replication and oncolysis, we first confirmed that rapamycin inhibited the mTOR pathway and activated Akt as previously described (14, 30). Rapamycin alone, MYXV alone, or both increased the phosphorylation of both Ser1177 and Thr389 in both RG2 and F98 cells (Supplementary Fig. S4). In both cell lines, the levels of phosphorylated p70S6K were unchanged by infection with MYXV alone but were significantly inhibited by rapamycin, effectively inhibiting mTOR under the conditions used with as little as 1 nmol/L rapamycin (Supplementary Fig. S4). We next investigated the effect of IFN on the replication of MYXV in glioma cells in vitro. The decision to test IFN was based on the observation that the IFN response of the host cell triggered by MYXV accounts for resistance to poxvirus infection (18). In addition, many glioma cells are not believed to express IFNα or IFNβ (19, 41–44), but they respond to exogenous IFN (19, 45). RG2 and F98 were pretreated with racine-IFNα or racine-IFNβ for 6 hours before infection with
MYXV in the presence or absence of rapamycin. We found that the presence of either IFNα (50 units/mL) or IFNβ (100 units/mL) was able to limit MYXV (0.1 MOI) infection as assessed by GFP expression 48 hours (or 72 hours) after infection (Fig. 6A; Supplementary Fig. S5C), a limitation that was reversed by the presence of rapamycin (20 nmol/L; Fig. 6A; Supplementary Fig. S5C). As little as 1.0 nmol/L (but not 0.1 nmol/L) of rapamycin blocked the antiviral response found with IFN (Supplementary Fig. S6A–C). To confirm that the expression of GFP correlated with viral replication, following treatment, RG2 and F98 cells were lysed and Western blots were performed for M-T7. We found

Figure 4. Viral distribution and survival in RG2 and F98 glioma model following i.t. administration of MYXV ± rapamycin. A, representative photomicrographs of GFP-labeled virus in the brain tumor (n = 3 rats per group) and immunohistochemistry for the viral gene M-T7 (brown). Magnifications, ×20 (top) and ×25 (bottom). Arrow, area of GFP-positive tumor cells. B, Kaplan-Meier plot showing the survival of RG2-bearing rats following different treatments. C, top, representative BLIs were obtained 13 d after implantation of RG2-Fluc in control (PBS)-treated, rapamycin-treated, vMyxgfp-treated, and vMyxgfp + rapamycin–treated animals; bottom, representative histologic brain sections from rats on day 13. D, Kaplan-Meier plot showing the survival of F98-bearing rats following different treatments. Arrows, virus injections.
that cells treated with racine-IFNα or racine-IFNβ showed very little M-T7 viral protein expression, whereas pretreatment of cells with rapamycin 1 hour before virus treatment restored M-T7 viral protein expression (Supplementary Fig. S5A and B). Consistent with these results, as determined by foci formation assays on BGMK cells 72 hours following treatment, both IFNα and IFNβ significantly inhibited viral replication, and the presence of rapamycin negated this inhibitory effect (Fig. 6B). These functional data suggest that rapamycin may help MYXV replication and spread, at least in part, by inhibiting the ability of glioma cells to respond to type I IFN. Next, we wanted to determine if treatment with rapamycin blocked type I IFN responses to MYXV infection biochemically in the glioma cell line (Fig. 6C, lane 3). Exogenous IFNα induced IFN-related factor-7 (IRF-7) and ISG15, showing biochemically that these cells maintain their ability to respond to exogenous IFN, an effect that seemed to be unaffected by rapamycin. Further, MYXV induced IFNβ, IRF-7, and ISG15 in these cells, suggesting that these rat glioma cell lines can detect and mount a cellular innate viral response to the virus (Fig. 6C, lane 6), a response that was totally or partially blocked with rapamycin (Fig. 6C,
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A

B

C

D

MYXV  MYXV+Rap  MYXV+IFNα  MYXV+IFNβ  MYXV+IFNα+Rap  MYXV+IFNβ+Rap

Input virus

Viral titers (FFU/10^6 cells, Log10)

MYXV  MYXV+Rap  MYXV+IFNα  MYXV+IFNβ  MYXV+IFNα+Rap  MYXV+IFNβ+Rap

IFNα (50 units/mL)
Rap (20 nmol/L)
MYXV (1 MOI)

IFNβ
IRF-7
ISG15
β-Actin

DV  MYXV  Rap  MYXV+Rap

CD68

CD163
Finally, rapamycin partially inhibited the response of these genes to exogenous IFNβ with MYXV, most notably with level of IFNβ and IFN-β (Fig. 6C, lane 8). Together, these data suggest that rapamycin may help MYXV replication and spread, at least in part, by inhibiting the initial type I IFN response of glioma cells. However, as shown in Fig. 6A and B and Fig. 6C (lane 8), rapamycin can mitigate the protection from exogenous type I IFN in the presence of MYXV, suggesting that rapamycin may be playing a role in dulling the response of the tumor to type I IFN. This is a novel finding and one that is currently being investigated by our laboratory.

To further assess whether the increase in MYXV replication observed in vivo was also the result of a decreased host cellular immune response to the virus infection, we performed immunohistochemical staining for markers of microglia/macrophages (CD68/CD163). We found that the presence of MYXV in the tumor induced an increase in infiltrating CD68+ microglia/macrophages and CD163+ peripheral macrophages that was abolished when the animals were given rapamycin or cyclophosphamide (Fig. 6D; Supplementary Fig. S7D). This suggests that part of the enhanced viral replication observed in vivo is the result of the immunosuppressant functions of these drugs on the host innate cellular immune response to the virus infection.

Discussion

We show here for the first time that oncolytic MYXV has significant therapeutic activity and minimal toxicity in rat immunocompetent models of malignant glioma. We made several novel observations. First, there were no significant toxicities when MYXV was administered directly into the brains of immunocompetent rats. Second, unlike xenografted gliomas in immunodeficient mice, MYXV had no significant activity as monotherapy in vivo. However, survival was markedly prolonged when it was administered with rapamycin. The enhanced oncolytic effect of rapamycin is due to the reduction of type I IFN responses and the reduced infiltration of inflammatory [natural killer (NK; CD68+) and microglia/macrophage (CD163+)] cells into gliomas treated with MYXV. Finally, the efficacy of combination therapy with MYXV and rapamycin is improved by delivering the virus through CED.

This report supports the concept that a major barrier to effective oncolytic virus therapy for glioma patients will be innate antiviral immune responses. In immunocompromised mice, MYXV administered i.t. cured most animals (5) but was ineffective as a monotherapy in immunocompetent rats. Although it is possible that this reflects species differences in the tumor (i.e., human versus racine gliomas) or the host (i.e., CD-1 nude mice versus Fischer 344 rats), it is more likely that this is due to an intact antiviral response in the immunocompetent hosts. This is the first report to show that immunosuppression with rapamycin enhances oncolysis in gliomas in immunocompetent hosts in vivo. The mechanisms by which rapamycin affects innate immune responses within the gliomas and the surrounding normal host tissues are not completely understood; however, we do know that type I IFN is an important component of the innate response to MYXV. MYXV replication in mammalian cells is naturally sensitive to inhibition by IFN (38), and the host antiviral responses mediated by type I IFN are a major determinant of its restrictive host range outside the natural rabbit host (18). Moreover, RIG-I mediates the coinduction of tumor necrosis factor and type I IFN elicited by MYXV in primary human macrophages, and together, these two induced cytokines shut down virus replication in all primary untransformed human cells tested to date (41). In normal noncancerous cells, one model of mTOR signaling and viral infection proposes that rapamycin suppresses innate immune responses by limiting the translation of IRF-7 (31, 46), transcription factor needed for a robust IFN response. In plasmacytoid dendritic cells (47), which constitutively express IFN-β, phosphorylation of S6K1 and S6K2 mediates downstream signaling cascades following the recognition of viral nucleic acids by Toll-like receptors 7 and 9, S6K1 and S6K2, which are downstream of mTOR, facilitate the formation of the Toll-like receptor 9/MyD88 complex, which in turn activates IRF-7 and the transcription of type I IFN. Therefore, blocking mTOR with rapamycin leads to reduced phosphorylation of S6K1 and S6K2, the inactivation of IRF-7, and a reduced type I IFN response. Finally, rapamycin may also increase the levels of activated Akt in cancer cells (14, 16, 30). Although it is unknown if these mechanisms enhance MYXV viral infection in gliomas, we report here for the first time that rapamycin effectively blocks type I IFN responses, which enhance MYXV replication and cell killing in vitro. In vivo, the ability of rapamycin to increase MYXV replication and spread within glioma tissue is multifactorial and reflects the contributions from both tumor cells and the accompanying normal innate immune cells in the brain (especially the resident microglia, macrophages). Because rapamycin is an immunosuppressant in its own right, it can prevent T-cell proliferation/activation (48) and reduces the total population of CD4+ T cells (32), as well as the less studied effects of blocking B-cell and NK cell proliferation, among other immunomodulatory effects (49). In addition, the ability of rapamycin to restore the oncolytic effect to MYXV in the face of exogenous IFN may be particularly important because...
IFNs are likely released from the surrounding tumor stroma and immune cells of the brain in response to the local infection. Gliomas retain their ability to respond to exogenous IFN (19, 45) but many have lost the capacity to produce type I IFNs (42–45). So, presumably, IFNs released locally from the surrounding normal tissue in response to MYXV infection could block the viral replication and spread of MYXV. It is likely that these, and several other mechanisms, are relevant in vivo. A limitation of our study is that we may not have optimized the use of rapamycin in this setting. Rather, we used rapamycin on a standard “chemotherapy” regimen of a single course (for 2 weeks). Using it as a “switch” to control oncolytic viral replication in glioma patients might require repeated administration “tailored” to the response of the tumor.

Our data do not allow us to definitively determine if rapamycin is superior to cyclophosphamide in improving the oncolytic effect of MYXV in vivo. Using rapamycin, we found that the titers of live virus were higher, the survivors were longer, and the reduction of recruitment of CD68+ and CD163+ cells seemed more than when using the combination therapy with cyclophosphamide (Supplementary Fig.). Our study was not designed to test which of these drugs was superior because of several limitations. The dose of cyclophosphamide we used (60 mg/kg) was lower than other studies ([>100 mg/kg for rats (50) or even 300 mg/kg for mice (29)] because these higher doses were not tolerated in the animals used in this study. Hence, the degree of immunosuppression with cyclophosphamide in our hands may have been less than in other studies. We were also unable to definitively compare the suppression of recruitment of CD68+ and CD163+ cells because our assessment was based on immunohistochemistry and was qualitative and we did not perform a functional assessment of NK or microglial/macrophage functions. Lastly, given the limitations in generalizing results from rodents into humans, we consider combination therapy with either cyclophosphamide or rapamycin to be reasonable combination therapies that need a clinical trial to determine which is clinically superior.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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