Research Article

Synergistic anti-tumour effects of rapamycin and oncolytic reovirus

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Abstract

There are currently numerous oncolytic viruses undergoing clinical trial evaluation in cancer patients and one agent,Talimogene laherparepvec, has been approved for the treatment of malignant melanoma. This progress highlights the huge clinical potential of this treatment modality, and the focus is now combining these agents with conventional anti-cancer treatments or agents that enhance viral replication, and thereby oncolysis, in the tumour microenvironment. We evaluated the combination of reovirus with rapamycin in B16F10 cell, a murine model of malignant melanoma, based on potential mechanisms by which mTOR inhibitors might enhance viral oncolysis. Rapamycin was not immunomodulatory in that it had no effect on the generation of an anti-reovirus neutralising antibody response in C57/black 6 mice. The cell cycle effects of reovirus (increase G0/G1 fraction) were unaffected by concomitant or sequential exposure of rapamycin, However, rapamycin attenuated viral replication if given prior or concomitantly with reovirus and similarly reduced reovirus-induced apoptotic cell death annexin V/PI and caspase 3/7 activation studies. We found clear evidence of synergistic antitumour effects of the combination both in vitro and *in vivo*, which was sequence dependent only in the *in vitro* setting. In conclusion, we have demonstrated synergistic anti-tumour efficacy of reovirus and rapamycin combination.

Introduction

There has been considerable interest in the development of oncolytic viruses as anticancer agents[1]. A number of viruses have now been studied in the pre-clinical and clinical setting[2], with an oncolytic herpesvirus Talimogene laherparepvec now approved for the treatment of malignant melanoma[3]. However, the full potential of these agents will be in combination therapies, with indications of this from studies combining OV with cytotoxic agents, targeted therapy, radiotherapy and immune checkpoint inhibitors (reviewed recently in Choi et al. 2016). Reovirus is a double-stranded RNA virus that is ubiquitious in the environment and is not associated with any known disease in humans[5]. It has been shown to be oncolytic in a broad range of cancer cell lines[6-9], though the exact mechanism of this selective activity has yet to be fully determined. Activation of the Ras pathway in transformed cells, or their upstream or downstream elements, is an important factor in a cell's permissiveness to reovirus oncolysis[10]. This is in part due to the inability of Ras-activated cells to phosphorylate cellular PKR, but also due to enhancement of virus uncoating, particle infectivity and apoptosis-dependent release[11].

Tumour regression after intratumoral and systemic delivery of reovirus has been reported in both immunodeficient and immunocompetent murine models[10, 12]. Several clinical studies have now been completed using both intratumoral and systemic delivery of reovirus[13, 14]. These have confirmed that reovirus is safe, with only mild flu-like symptoms, and that reoviral infection of metastatic tumour deposits occurs when delivered systemically. However, while there was evidence of antitumor activity in patients with advanced cancers (recently reviewed in Gong et al., 2016) further strategies are clearly necessary for reovirus to become a useful anticancer agent

Rapamycin is an inhibitor of the Serine/Threonine protein kinase mTOR (mammalian target of rapamycin), which regulates cell growth and metabolism in response to environmental

cues. Rapamycin and its analogues bind FK506 binding-protein, which in turn binds to mTOR leading to the inhibition of downstream signalling. Rapamycin induces autophagy and causes cell cycle arrest in tumour cells, but also inhibits endothelial cell proliferation, hypoxia induced factor-1 (HIF-1), expression of vascular epithelial growth factor (VEGF), tumour-associated angiogenesis, and vascular permeability[16, 17]. These vascular effects of mTOR inhibitors, together with their direct anti-tumour and immunosuppressive affects, demonstrate the potential of these drugs as anticancer agents. A number of mTOR inhibitors have shown benefit in the treatment of cancers and are now in clinical use[18-20]. mTOR inhibitors are cytostatic and consequently there has been interest in combining them with other treatment modalities. Rapamycin has been shown to act synergistically with a number of chemotherapeutic agents[21-22], radiotherapy[23] and endocrine treatment[24]. Synergistic antitumour effects of rapamycin and oncolytic adenoviruses have been reported[25, 26] In this study, we have assessed the combination of rapamycin with reovirus in a murine melanoma model for therapeutically useful synergistic tumour kill. The potential mechanisms behind these interactions were also investigated.

Materials and Methods

Cell lines

The mouse melanoma cell line B16.F10 was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) at 37^{o}C and 10% CO_2. L929, a murine fibroblast-like line, was cultured in DMEM at 37^{o}C and 5% CO_2. Both cell lines were bought from ATCC. All media were supplemented with 2mM GlutaMAX-1 supplement (Invitrogen), 100 units/ml penicillin/streptomycin (Sigma) and either 10% (v/v) foetal calf serum (FCS) for routine passage or 2% (v/v) FCS for reovirus infection work. Mycoplasma testing was routinely carried out once a month using MycoAlert PLUS detection kit (Lonza, LT07-710)

Reovirus stock and chemotherapeutic agents

Reovirus type 3 Dearing strain Reolysin® was obtained from Oncolytics Biotech. Inc. (Calgary, Canada). Virus stock titre and virus stability was measured by standard plaque assay of serially diluted samples on L929 cells. Six-well plates were seeded with 1 x 10^6 L929 cells per well and infected with dilutions of viral stocks. After 3h incubation at 37^{o}C, the virus inoculum was removed and the wells were overlaid with a 1:1 mixture of 2% SeaPlaque agarose (Cambrex Bio Science Rockland, Inc, ME) and 2 x MEM (Invitrogen) supplemented to a final concentration of 5% (v/v) FCS, 100 units/ml penicillin/streptomycin and 2mM GlutaMAX-1. Wells were stained with 500µL 0.03% neutral red (Sigma) in PBS 72h post-infection and plaques were counted 3 to 4 h later. Rapamycin was purchased from Sigma.

In vitro survival and synergy assays

B16.F10 cells were plated in 96-well plates at a density of 7.5 x 10^3 cells per well. After 24 hours, they were infected with known dilutions of reovirus or rapamycin, either alone,

concomitantly or in sequence, and after 48h incubation, cell viability was quantified using the CellTiter 96 AQueous One Solution Cell Proliferation Assay reagent 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS; Promega) according to manufacturer's instructions. Briefly, 20µL of MTS reagent was added to each well and following incubation at 37^{o}C for 1-4 hours, absorbance was measured at 495nm. Survival was calculated as a percentage compared to untreated cells and the median effective dose (ED50) was determined.

The effect of the combination of reovirus and rapamycin on B16.F10 cell viability was assessed. Experiments were performed as described above using 4, 2, 1, 0.5 and 0.25 times the calculated ED50 of each agent in a constant ratio checkerboard design. The agents were applied either concomitantly or sequentially with a 24h interval and survival was assessed 48h after the first agent was applied.

CalcuSyn software (Biosoft, Ferguson, MO) was used to calculate combination-index (CI) values. Derived from the median-effect principle of Chou and Talalay[27], the CI provides a quantitative measure of the degree of interaction between two or more agents. A CI of 1 denotes an additive interaction, >1 antagonism and <1 synergy.

Virus recovery from *in vitro* cultures

The effect of rapamycin on viral replication *in vitro* was assessed. Flasks seeded with B16.F10 cells were exposed to reovirus alone, concomitant reovirus and rapamycin, rapamycin followed 24 hours later by reovirus, and reovirus followed 24 hours later by rapamycin. After 48 or 72 hours the flasks were transferred to a -80°C freezer and the virus titre in the resulting lysate was determined by plaque assay as described above.

Determination of apoptosis induction by Annexin V / PI staining

Following overnight seeding, B16.F10 cells were exposed to reovirus and rapamycin as single agents or in combination and 72 hours later the percentage of early and late apoptotic cells was estimated by flow cytometry. Adherent and non-adherent cells were collected, washed in cold PBS, re-suspended at 1 x 10^6 in 500µL PBS and then incubated for 15 min at room temperature, in the dark, in cold 1 x binding buffer containing Annexin V-FITC antibody, according to manufacturer's instructions (Merck Biosciences Ltd). The cells were pelleted and re-suspended in cold 1 x binding buffer. Cells were stained with 10µL propidium iodide (PI) at 30µg/mL and analysed by flow cytometry using a MACSQuant Analyser (Miltenyi Biotech Ltd), and MACSQuantify software (Miltenyi Biotech Ltd).

Determination of apoptosis induction by Caspase 3/7 activation

B16.F10 cells were exposed to reovirus and rapamycin as single agents or in combination as decribed above and 72 hours later caspase 3/7 activation was measured using the CaspaseGlo assay (Promega). At the time point of interest, 100µl of CaspaseGlo was added to each well and the plate shaken to ensure adequate mixing. The plate was incubated at room temperature for 1 hour and then the luminescence measured at 565nM using a Beckman Coulter plate reader.

Cell cycle analysis of B16.F10 cells exposed to reovirus and/or rapamycin

B16.F10 cells were plated and incubated at 37^0C overnight. Cells were infected with Reovirus at MOI of 10 and 10nM Rapamycin (*Sigma*). The treated B16.f10 cells were trypsinised and washed in ice cold PBS. The cells were centrifuged at 1500rpm at 4^0C for 3min and resuspended in 300ul of ice cold 50% FCS/PBS. 900ul of ice cold 70% ethanol was added drop wise during vortexing. The resulting cell suspend was incubated overnight at 4^0C. The cells were washed twice with ice cold PBS cells. The pellet was resuspended in

200ul of 5µg/ml propidium iodide and 1mg/ml Ribonuclease and incubated at 37^0C for 30 min in the dark. Analysis was carried out by flow cytometry using a MACSQuant Analyser (Miltenyi Biotech, Bergisch Gladbach, Germany) and MACSQuantify software (Miltenyi Biotech).

Autophagy Analysis

B16.F10 cell were seeded at $5x10^5$ cells/well and incubated overnight at 37ºC. Cells were then treated with either medium with 10% FCS, 10nM rapamycin, reovirus MOI 10, or a combination of rapamycin 10nM and reovirus MOI 10 either concomitantly or in sequence with 24 intervals per treatment. After 48 hours, cells were trypsinised with StemPro® Accutase (Thermo Fisher) and then washed 3x with 1x assay buffer (Enzo). Each sample was then resuspended in 250µl of 1x assay buffer 5% FBS. Cyto-ID® stain (Enzo) was diluted 1:1000 in 1x assay buffer and 250µl was then added to each sample, and then incubated for 30 minutes at 37ºC**.** Cells were then washed 3x with 1x assay buffer and were resuspended in 500µl 1x assay buffer before being analysed on a MACSQuant® Analyzer 10 (Miltenyi Biotec).

PI3K-mTOR signalling cascade analysis

B16.F10 cells were prepared the same way as the autophagy analysis samples, in terms of reovirus MOI, rapamycin concentration and term points. After 48 hours, cells were trypsinised with StemPro® Accutase (Thermo Fisher) and then washed. The cells were then fixed and permeabilizated using the FlowCellect PI3K-mTOR signalling cascade mapping kit (Millipore FCCS025210 | FlowCellect™ PI3K-mTOR Signaling Cascade kit). Resulting cells were stained with Anti-phospho-Ribosomal Protein S6 (Ser235) PerCP conjugate Monoclonal Antibody and Antiphospho-Akt1/PKBα (Ser473) Alexa Fluor 488 conjugate Monoclonal Antibody (Millipore kit as above). Cells were then washed with 1x assay buffer

and were resuspended in assay buffer before being analysed on a MACSQuant® Analyzer 10 (Miltenyi Biotec).

In vivo studies

All procedures were approved by the United Kingdom Home Office and University of Surrey AWERB. The design of individual experiments will generally involve obtaining maximal information from the minimum resources. Sample sizes will be set using power analysis and calculated using Graph StateMate. Female C57Bl/6 mice (5-6 weeks old) (were purchased from B and K Universal Ltd and all animal experiments were repeated at least three times. Subcutaneous tumours were established on the flank of each mouse by injecting 5 x 10^5 cells in a volume of 100µL Hanks Balanced Salt Solution (HBSS; Sigma). Animals were examined thrice weekly for tumour development. Three orthogonal tumour diameters (d_1, d_2, and d_3) were measured using Vernier callipers and tumour volume was calculated from the formula V =(length x width)/2 . Animals were killed when tumour size exceeded 15mm in any one dimension. Reovirus was administered intratumorally as described previously[28].

Once tumours were established, (average tumour diameter 4.5-5.5mm, approximately 10-12 days), mice were randomly assigned to five treatment groups: (a) HBSS 100µL intraperitoneal (i.p.) control, (b) reovirus 3 x 10^8 pfu reovirus in 100µL volume intratumourally on day 1 and day 4, (c) 5mg/kg of rapamycin i.p. alone, or (d) concomitantly with rapamycin on day1 and 4, or (e) 24h after reovirus on day 2 and 5. All animals receiving rapmycin had further i.p. rapamycin on day 8 and 11 (N=8 per group) .

Viral recovery from tumours

Tumours were established in mice as described above. Following random assignment to treatment groups, mice received either 3 x 10^8 pfu reovirus in 100µL volume intratumourally

alone, concomitant reovirus and 5mg/kg of rapamycin, or reovirus followed 24h later by rapamycin.

Mice were sacrificed 4 days after commencement of treatment and tumours, livers, lungs and hearts excised. Tumour and organs were weighed and homogenized in a TissueLyser (Qiagen) at 30 Hz for 2 min. Following centrifugation to clarify the virus lysate, virus titre was determined by plaque assay on L929 cells as described above and expressed as pfu/g tissue.

Serum analysis for presence of neutralising antireoviral antibodies.

The methodology used for analysis of neutralising antireovirus antibody (NARA) has been reported recently[29]. Briefly, serum samples collected from individual mouse groups on day 4 after treatment were batched and analyzed simultaneously. To determine a suitable virus dilution for subsequent assay, L929 cells were plated in 96-well plates at 2.5×10^4 per well and incubated overnight at 37°C and 5% CO_2. Reovirus stock (3.5×10^{10}/mL) was added in two dilution series (2- and 10-fold) across the plate such that the final dilutions of the two series were 1:32,768 and 1:1012. After 2 h, the reovirus inoculum was removed and replaced with growth medium. After a further 48 h, cell survival was measured by MTT assay. To establish a suitable dilution series for the estimation of neutralising antibody levels in the serum, the above experiment was repeated with a constant titre of reovirus (known to cause 80% cell death) that was preincubated with a dilution series of goat polyclonal antireoviral antibody, and cell survival was measured at 48 h by MTT assay.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software Inc.). Comparisons between groups were done using the t test and of variance (ANOVA) models. Survival curves were generated using the Kaplan-Meier method. *In vivo* survival data was analysed using the logrank test.

Results

Concomitant Reovirus and Rapamycin is antagonistic in B16.F10 Cells

To determine the effect of reovirus and rapamycin on the mouse melanoma cell line, B16.F10, cell viability was first assessed after 48 hours of exposure to each agent, using the MTS assay. B16.F10 was found to be permissive to reovirus with a medium effective dose (ED50) between 1 and 5 MOI (figure 1). Heat inactivated reovirus had no effect on B16F10 cells (data not shown). Rapamycin also decreased cell viability with an ED50 between 1nM and 5nM. Previous reports have demonstrated synergistic anti-tumour effects of rapamycin and chemotherapy combinations[30]. All cells were assessed for viability by MTS assay, 48 hours after the first treatment was given. Having established the ED50s, rapamycin and reovirus were then combined at 0.25, 0.5, 1, 2, 4 times the ED50 of each agent using a constant ratio chequerboard design. The effect of rapamycin and reovirus on cell viability was reproducible with less than 0.01% chance of randomly observing an effect of this large with an experiment of this size (2-way ANOVA; $p<0.0001$). Bonferroni post-tests showed no significant difference between the means at each dose level for single agent and combination treatment. The effect of the combination of reovirus and rapamycin on cell viability was assessed by isobologram analysis and the calculation of combination indices (CI). With the concomitant administration of reovirus and rapamycin there was evidence of antagonism with a combination index at ED50, ED75, and ED90, of 1.6, 1.7 and 2.6 respectively (figure 2).

Cytotoxicity is affected by the sequence of combination treatment

Given that synergy with rapamycin is sequence dependent with certain chemotherapeutic agents[30], we wanted to evaluate the effect of the sequence of reovirus and rapamycin on cell viability. Cells were plated on 96-well plates and exposed to rapamycin, reovirus or a combination as described earlier. However, two further groups were added: reovirus followed 24 hours later by rapamycin, and rapamycin followed 24 hours later by reovirus.

The 24 hour time point was used following a report of optimal cell kill when an mTOR inhibitor was given 24 hours after paclitaxel[30] in a breast cancer model. All cells were assessed for viability by MTS assay, 48 hours after the first treatment was given. Once again the experiments were reproducible, with a significant difference between the treatments using 2-way ANOVA ($p<0.0001$). When rapamycin was given before reovirus there was no difference between the means at each dose level between that of reovirus or rapamycin alone. However, when rapamycin was given after reovirus there was a significant difference between the means at each dose level compared with either agent alone ($p<0.001$). Also, the combination of rapamycin and reovirus was found to be synergistic by isobologram analysis only when reovirus was followed by rapamycin, with a CI value of 0.5, 0.4, and 0.4 for the ED50, ED75 and ED90 respectively. When rapamycin was given before reovirus there was similar evidence of antagonism as seen with concomitant treatment with a CI value of 1.3, 1.9, and 2.7 for the ED50, ED75 and ED90 respectively (Figures 1 and 2).

The effect of reovirus, rapamycin or combination on cell cycle.

B16F10 cells were exposed to reovirus, rapamycin, both concomitantly or in sequence. For comparison, NIH3T3 cells were similarly exposed in the same permutations. In NIH3T3 cell, rapamycin exposure led to increased G0/G1 fraction and marked lowering of G2/M and S phase fractions which was unaffected by the later addition of reovirus. Reovirus exposure also resulted in reduction in G2/M and S phase, but to a lesser extent than with rapamycin, and this reduction was largely unaffected by the concomitant or sequential exposure to rapamycin. In B16 F10, the effects of all treatment permutations resulted in more marked increases in G0/G1 fraction, and more marked reduction in G2/M and S phase fractions. The highest reduction in G2/M and S phase fractions associated with rapamycin were similar to NIH3T3 cells where the effects of reovirus were not overcome by rapamycin in the combination groups (figure 3).

Concomitant reovirus and rapamycin shuts down viral production

The effect of rapamycin on viral replication was assessed by plaque assay. Flasks seeded with B16.F10 cells were exposed to reovirus alone, concomitant reovirus and rapamycin, rapamycin followed 24 hours later by reovirus, and reovirus followed 24 hours later by rapamycin. After 48 or 72 hours, the flasks were transferred to a -80°C freezer and the resulting supernatant serially diluted and allowed to transfect L929 cells before an overlay was added.

Both pre-treatment with rapamycin, or concomitant reovirus and rapamycin treatment, significantly reduced the number of plaques (Bonferroni post-test; $p<0.001$) (figure 4). However, when reovirus preceded rapamycin there was no significant difference in plaque number compared to reovirus alone (Bonferroni post-test; $p<0.001$). These results indicate that rapamycin inhibits viral replication when given together or prior to reovirus but that this effect is lost if rapamycin is given after the reovirus exposure. Significantly more plaques were seen at 72 hours than 48 hours, but the difference between the treatment groups remained the same indicating that the effect of rapamycin on reovirus persists over this additional time period (figure 4).

B16F10 cell apoptosis is reduced with concomitant administration of reovirus and rapamycin

To assess the contribution of apoptosis to the mechanism of cell death with the combination of reovirus and rapamycin, two apoptosis assays were used.

Using the Annexin V/PI assay, the difference in the percentage of early apoptotic cells between treatments was significant (1-way ANOVA; $p<0.001$). Bonferroni's multiple comparison test showed there was a significant reduction in the percentage of early apoptotic cells when rapamycin was given prior to, or concomitantly with, reovirus ($p<0.05$).

However, when rapamycin was sequenced after reovirus there was no significant difference compared with reovirus alone (figure 5).

Caspase-3/7 is a recognised marker of apoptotic induction inside cells. The presence of activated caspase-3/7 suggests a cell that is proceeding towards a terminal pathway of cell death. Caspase-3 is an 'executioner' caspase whose activation leads to the cleavage of a wide range of proteins including PARP and ICAD in the downstream events during apoptosis. Treatment with reovirus alone led to a significant increase in caspase 3/7 activation compared with untreated cells (1-way ANOVA). As seen with the Annexin V / PI assay, when rapamycin was used as a single agent, or before or concomitantly with reovirus there was a reduction in the activation of caspase 3/7 ($p<0.001$). However, when reovirus infection preceded rapamycin treatment there was no significant difference in caspase 3/7 activation compared to reovirus treatment alone (figure 5).

Assessment of the effect of combination treatment on cell signalling.

To determine the effect of reovirus on mTOR pathway signalling with concomitant or sequential treatment of B16F10 cells a flow cytometry based assay was performed (figure 6). Focusing on the PI3K-mTOR signalling cascade we looked for changes in the levels of phosphorylated-Akt (p-Akt) above mTOR and the phosphorylated-ribosomal S6 protein (p70S6K) downstream of mTOR. Reovirus infection alone resulted in a marked increase in p70S6K expression. In contrast B16.F10 cells treated with rapamycin alone, showed a large decrease in phosphorylation of ribosomal protein S6 with only a slight effecting on the phosphorylation of AKT. All combinations of rapamycin and reovirus showed similar results as rapamycin alone. Which suggest that rapamycin has the most dominant effect on the PI3K-mTOR signalling pathway.

Reovirus inhibits Rapamycin induced autophagy

Rapamycin is also an inducer of autophagy, as inhibition of mTOR mimics cellular starvation by blocking signals required for cell growth and proliferation. We investigated whether induction of autophagy by rapamycin was influenced by the pro-apoptotic effects of reovirus. Induction of autophagy was assessed through detection of autophagic vacuoles using a cytoplasmic dye in a flow cytometric assay. Cells treated with 10nM of rapamycin (Figure 7b) caused a rapid increase in autophagy when compared to control cells (Figure 7a), but cells treated with reovirus MOI 10, Figure 7c, exhibited a less dramatic increase in autophagy. Sequential treatment with reovirus then rapamycin, Figure 7d, and rapamycin then reovirus, Figure 7e, resulted in significantly different levels of autophagy. Cells treated with reovirus before rapamycin had autophagy levels similar to cells treated with reovirus alone, and when the treatment sequence was reversed, autophagy levels were similar to rapamycin alone. When cells were treated with both agents concomitantly, figure 7f, autophagy levels was similar to treatment with reovirus alone. These results indicate that the reovirus dictated the mode of cell death when combined concomitantly with rapamycin.

Combined reovirus and rapamycin treatment enhances tumour growth delay and prolongs survival in a mouse melanoma model

The *in vivo* effects of reovirus, rapamycin and their combination were assessed in a mouse melanoma model using B16.F10 cells in C57/BL6 mice. Cells were implanted subcutaneously in the flank of the mice and treated when they had reached an average diameter of 4.5-5.5mm, usually 10 days after implantation. Mice were then treated with intratumoural reovirus, intraperitoneal rapamycin, or both, as described earlier. There were no obvious toxic effects of either single agent or combination treatments and experiments were concluded if tumour growth exceeded 15mm in any one dimension or there was ulceration of the tumour.

Both rapamycin and reovirus alone resulted in a significant reduction in tumour growth compared to control (2-way ANOVA $p<0.0001$), however the combination of the two, whether concomitant or in sequence, resulted in the most effective growth delay (figure 8). The median survival for the control, rapamycin, reovirus, sequence, and concomitant groups was 8, 11, 14, 15, and 17 days respectively. There was no significant difference in median survival between concomitant and sequenced treatment, and the difference between sequenced treatment and reovirus alone did not reach significance either. However, the difference in survival between the concomitant group and reovirus alone did reach significance (Log-rank test; $p=0.02$).

Viral replication in tumour and organs of treated mice

To assess the effect of rapamycin on reoviral replication *in vivo*, we evaluated viral replication in tumours and organs 4 days after treatment with either reovirus alone, concomitant reovirus and rapamycin, or reovirus followed 24 hours later by rapamycin. Following sacrifice, viral titres were determined in tumour and organs using plaque assays as described early. As expected, the highest viral yield was in the resected tumour, the liver harboured the most reovirus of all the organs evaluated. There was no difference in virus titre in the lung, liver or heart between the treatment groups. There was, however, a difference in the viral titre in the resected tumours with a significant increase in virus seen in the sequenced group compared to the reovirus alone group (unpaired t-test; $p=0.03$). There was no significant difference between the sequence or concomitant groups and neither was there a difference between the concomitant and reovirus alone groups (figure 9).

Neutralising anti-reoviral antibody levels are not reduced by the rapamycin

Rapamycin is known to have immunosuppressive properties. The combination of other immunosuppressive agents with reovirus has been shown to reduce the humoral response and improve the antitumour effects of reovirus[31]. Similarly, other oncolytic viruses have

shown improved efficacy in combination with immunosuppressive agents[23, 33]. To determine whether the administration of rapamycin affected the anti-reovirus humoral response, we assessed the NARA response to reovirus and the reovirus/rapamycin combination, both concomitant and sequential, in C57BL/6 mice. Using a previously reported assay[29], we found that, at the dose of 5mg/kg, rapamycin had no significant effect on the NARA response whether given concomitantly or sequentially (Figure 10).

Discussion

Reovirus is a wild-type oncolytic virus that has activity in a broad range of cancers. It has been shown to be safe and tolerable when given both by intratumoural and intravenous injection[14, 34] While responses have been seen with reovirus alone in patients with advanced malignancies the current focus is on combining reovirus with other treatments to try and improve efficacy[35]

In this study we assessed the combination of reovirus with the mTOR inhibitor rapamycin in a conventional immunocompetent murine melanoma model. Malignant melanoma is increasing in incidence and despite key developments in targeted therapies and immune checkpoint inhibitor antibody therapy[36], the majority of patients with metastatic disease still progress and die from their disease in a short period of time. There is therefore still an urgent need for novel treatment strategies for this disease.

The mTOR inhibitors have been shown to have anticancer activity and are now in use in the clinical setting in patients with advanced renal cell carcinoma and in combination with aromatase inhibitors in breast cancer. The Akt-mTOR pathway is upregulated in up to 70% of malignant melanomas[37]. While there is some preclinical evidence of efficacy in melanoma models[38], a phase II trial of single agent rapamycin in metastatic malignant melanoma was disappointing with only one objective response in 34 patients[39]. However, the combination of mTOR inhibitors with both conventional and novel therapies has been shown to improve response rates in pre-clinical[21, 38-41] and clinical studies[42].

Three different oncolytic viruses have been combined with mTOR inhibitors [43,44] and all demonstrated synergistic anti-tumour effects in combination, with different hypotheses given for how the agents interact to increase cancer cell death. The potential mechanisms by which mTOR inhibitors might enhance viral oncolysis include targeting of alternative signalling pathways, suppression of the antiviral immune response, and alteration in tumour

vasculature through their antiangiogenic properties. Furthermore. mTOR inhibitor induced autophagy may generate decomposed cellular molecules as nutrients to support viral replication.

In this study, we assessed the novel combination of reovirus and rapamycin and also found evidence of synergistic anti-tumour cytotoxicity. However, at least *in vitro*, this synergy appeared to be sequence dependent with the combination being antagonistic unless reovirus is given prior to rapamycin. Rapamycin is known to act as a cytostatic agent, being a potent inducer of G1 cell cycle arrest. This may explain the antagonism seen in cells exposed to rapamycin before or concomitantly with reovirus, as we have previously reported that reovirus oncolysis is cell cycle dependent, occurring in S phase[46]. However, we have also shown that pre- or concomitant treatment with rapamycin shuts down reovirus replication, as determined by viral plaque assay, which could account for the antagonism seen with these combinations. It would also explain the reduction in apoptosis seen after pre- and concomitant treatment with rapamycin. Rapamycin is known to increase myxoma virus replication in certain cancer cell lines[47] *in vitro*. However, this is thought to be due to rapamycin-induced Akt activation, and the increased tropism of myxoma virus to cells with hyperactivation of Akt. Reovirus tropism is intimately related to the Ras pathway and there is now evidence that inhibition of mTORC1 leads to Ras/MAPK pathway activation[48]. Increasing activated S6 kinase levels lead to PI3K inhibition, via a negative feedback loop, leading to a reduction in Ras/MAPK signalling. A similar feedback loop can lead to Akt activation in certain cell lines5. In our study, we observed that reovirus exposure increases pS6 phosphorylation and slight increase p-AKT.

Though a cytostatic agent, rapamycin has also been shown to induce apoptosis in cells lacking functional p53[49-52]. In our model, rapamycin reduced induced autophagy and reduced apoptosis in B16.F10 cells compared to untreated controls, whereas reovirus induced apoptosis, as has been demonstrated previously[26]. The shutdown in reoviral replication and

subsequent reduction in apoptosis and autophagy may explain this antagonism. Reovirus also induced autophagy in B16.F10 cells to a small extent but the dominant effect on the tumour cells was through reovirus where pre- or concomitant treatment with reovirus markedly reduced the pro-autophagy effect of rapamycin. However, this may be a cell specific phenomenon as a previous study showed a reduction in lung metastases after rapamycin treatment in a B16 mouse melanoma model reported no evidence of autophagy[38]. In the *in vivo* experiments, both single agent alone, and combination of rapamycin and reovirus resulted in a significant reduction in tumour growth and median survival. Combination treatment was significantly more effective than either treatment alone, however, unlike in the *in vitro* setting, there was no significant difference between concomitant and sequenced treatment. This may simply reflect the difference in treatment delivery, with the reovirus given intratumourally and the rapamycin via the peritoneum. One possibility for the synergy seen in other oncolytic virus models may be due to a reduction in the NARA response caused by rapamycin. Rapamycin as an immunosuppressant could potentially have an effect of reducing host immune response to viral infection. However, we found no significant attenuation in the NARA response, which may reflect differences in the dose scheduling of rapamycin or the timing of NARA assessment after viral administration. It is also possible that attenuation of the immune response may reduce the efficacy of reovirus-induced immune priming[53]. Rapamycin reduces the interferon responses to viral infection[54], but also suppresses T-cell function[55]. This suppression not only effects T-regulatory cells, which maybe undesirable, but also T-cell mediated viral responses which may actually improve reovirus infection.

The mTOR pathway is considered a key regulator of cell metabolism, and rapamycin has been reported to elicit paradoxical effects on glucose metabolism and oxidative phosphorylation. The stimulatory or inhibitory effect on metabolism depends most likely on the nature of the dysregulation of cell signalling in tumour cells, so in turn, virus replication may be enhanced or inhibited in combination exposure in tumour cells[56,57]

Another possible mechanism of synergy is the effect of rapamycin on the tumour blood supply. Alteration of the tumour vasculature due to antiangiogenic therapy such as rapamycin can lead to a 'normalisation window' whereby disorganised blood vessels transiently reorganise[56], reducing hydrostatic barriers to virus delivery. The subsequent withdrawal of rapamycin could then trap the virus within the tumour, as has been hypothesised in a colorectal mouse model combining adenovirus with the mTOR inhibitor, everolimus. While this model confirmed ablation of the primary antibody response with everolimus in immunocompetent mice, synergy was also seen in an immunosuppressed xenograft model. The authors argued that the withdrawal of regular mTOR inhibitor causes vascular disruption trapping the virus and facilitating viral spread within the tumour.

In this study, we have demonstrated that the combination of reovirus with the mTOR inhibitor, rapamycin, is synergistic in a mouse melanoma model. While this appears to be sequence dependent *in vitro*, concomitant administration significantly improves survival *in vivo*. The mechanism for this synergy is uncertain though attenuation of the humoral response was not demonstrated. The combination of reovirus with other anticancer agents is currently being investigated in phase I and II trials.

In conclusion, combination of rapamycin may enhance the therapeutic potential of oncolytic reovirus through number of mechanisms. This combination warrants clinical evaluation.

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Conflict of Interest

All authors declare no conflicts of interest

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Figure Legends

Figure 1. Rapamycin enhances reovirus cytotoxicity in a sequence dependent manner

Cells were exposed to rapamycin and reovirus at 0.25, 0.5, 1, 2, 4 x calculated ED50 for given exposure time and combined as follows: **A,** rapamycin followed 24 hours later by reovirus. **B**, concomitant rapamycin and reovirus. **C**, reovirus followed 24 hours later by rapamycin. 48 hours after exposure to first drug, cell viability was assessed using the MTS assay.

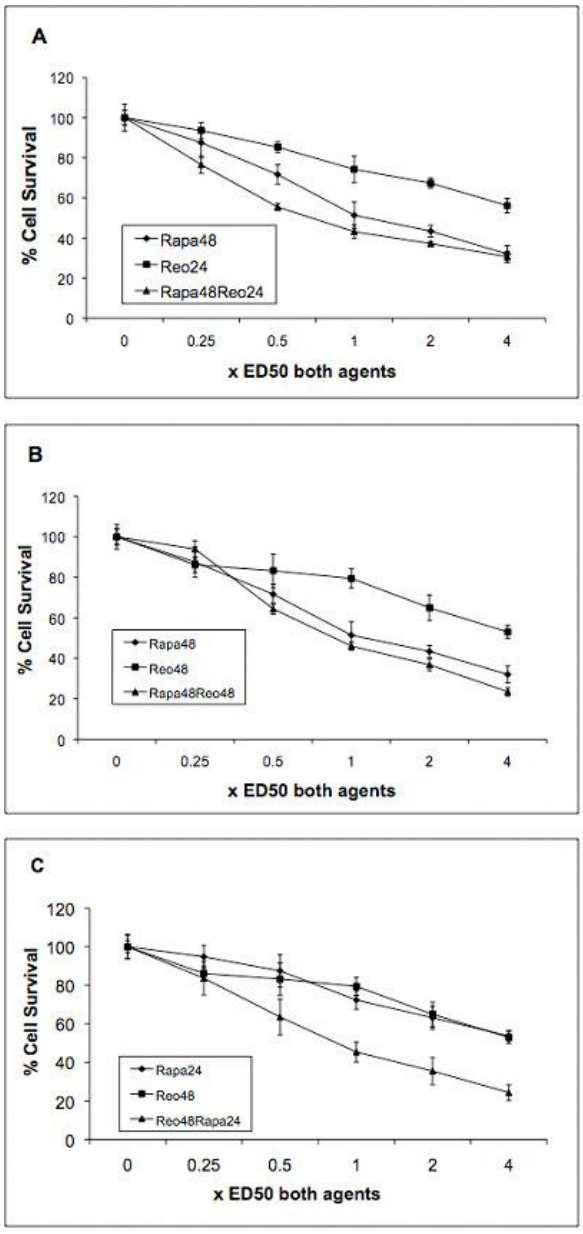


Figure 2. Sequence of reovirus then rapamycin is synergistic

The classic isobologram for the sequenced combination of reovirus followed by rapamycin shows synergy at all effective dose levels (observed ED50, ED75, ED90 lie to the left of their respective hypothenuse).

Combination Indices were generated using Calcusyn software. Data is presented as combination index value ± standard error of the mean at the effective dose indicated. Synergy was only seen when rapamycin was given 24 hours after reovirus.

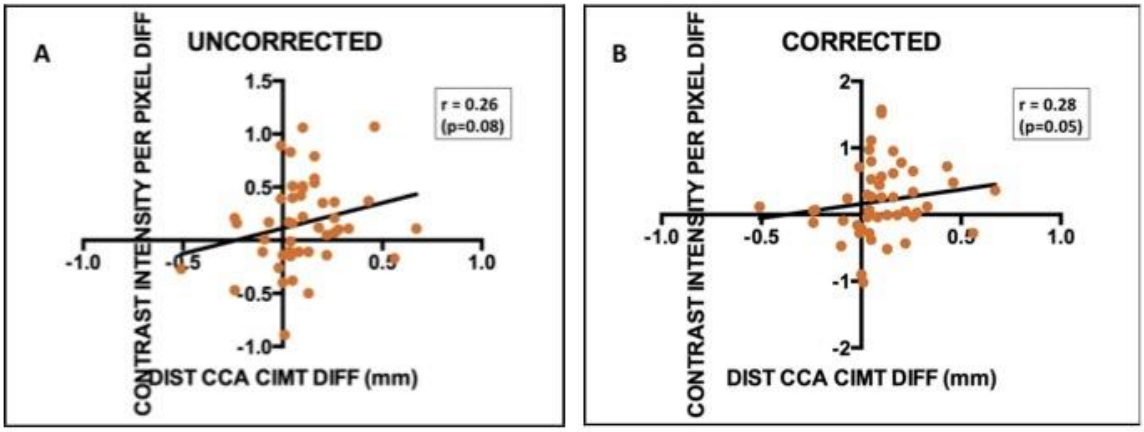


Figure 3. The effect of reovirus and/or rapamycin on cell cycle. Rapamycin exposure results in most marked reduction of G2/M and S phase fractions. In both NIH3T3 cells and B16F10 cells, prior or concomitant exposure to reovirus reduces this effect.

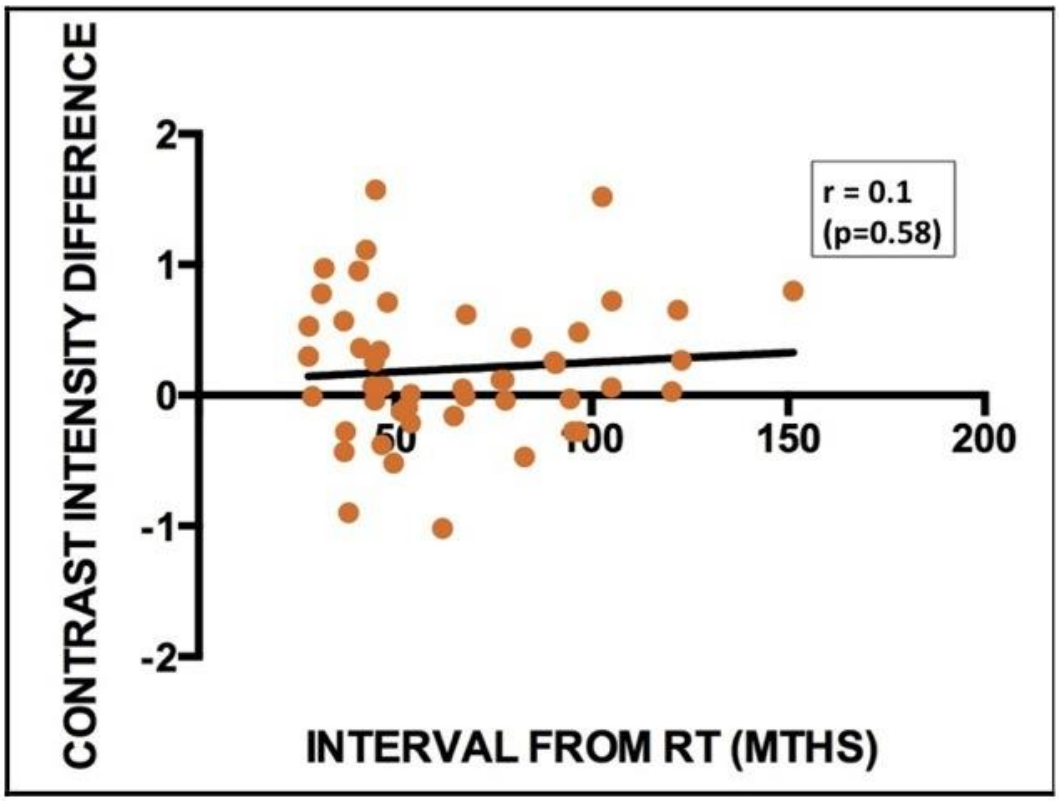


Figure 4. Rapamycin shuts down viral replication

Plaque assays were performed in 6 well plates using a L929 cell monolayer. B16.F10 cells were infected with reovirus with or without rapamycin, concomitantly or in sequence. Serial dilutions, 10^{-4} to 10^{-9}, of resulting supernatant at 48 or 72 hours were prepared. Rapamycin reduces reovirus replication when given before or concomitantly with reovirus. Rapamycin given after reovirus does not effect reovirus replication.

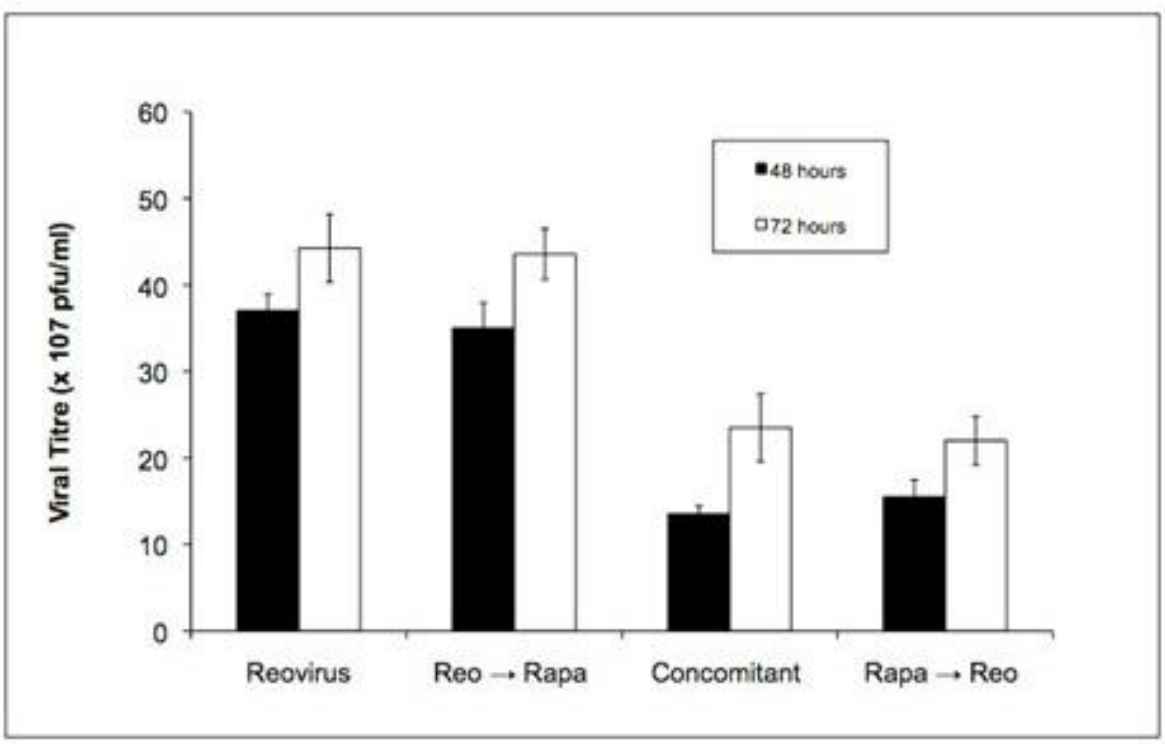
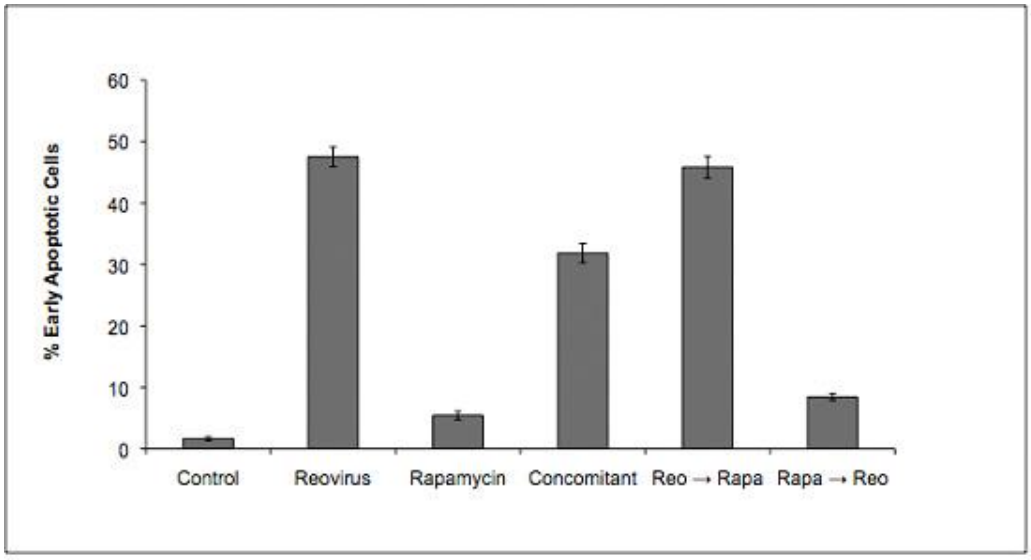


Figure 5. Reovirus-induced apoptosis is reduced by pre- or concomitant treatment with rapamycin, as determined by Annexin/PI staining by FACS analysis (top), and Caspase 3 activation using CaspaseGlo Assay (bottom)



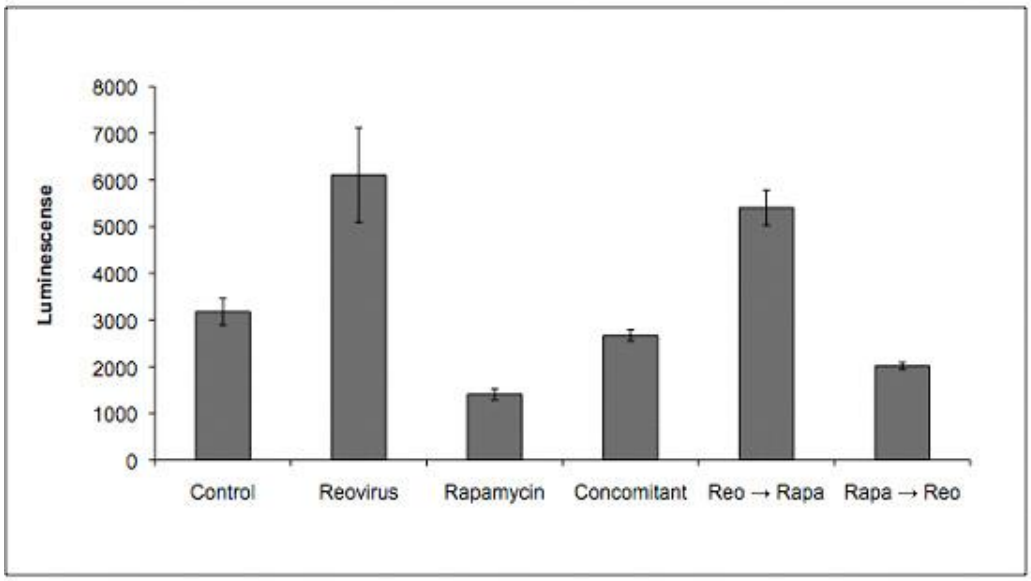


Figure 6. Assessment of the effect of combination treatment on cell signalling of the mTOR pathway by Flow based assay. A: Untreated B16.f10 B: Reovirus was added and incubated for 48 hrs. C: Rapamycin was added and incubated for 48 hrs.D: Reovirus was added for 24 hrs then removed and rapamycin added for 24 hrs. E: Rapamycin was added for 24 hrs then removed and reovirus added for another 24 hrs. F: Both reovirus and rapamycin were added and incubated for 48 hrs.

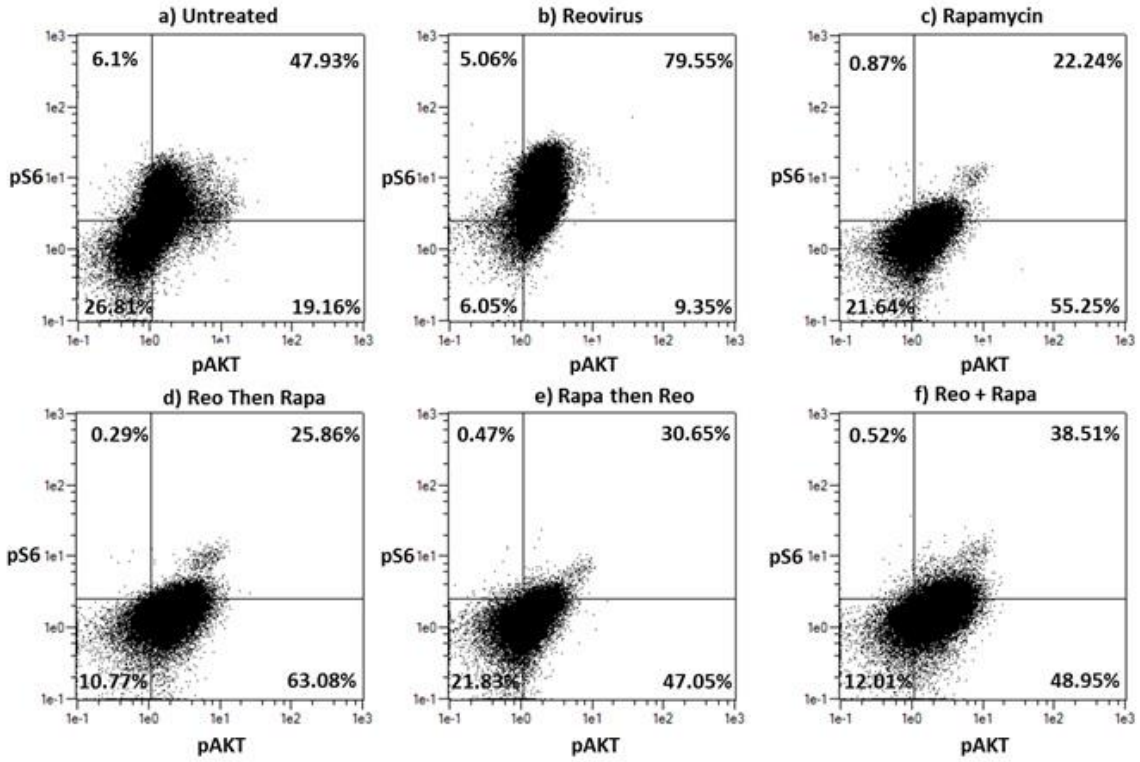


Figure 7: Autophagy levels were determined via Cyto-ID staining with FACs analysis. Cells were treated with either A) media 10% FBS, B) 10nM of rapamycin for 48 hours, C) Reo MOI 10 for 48 hours, D) Reo MOI 10 for 24 hours then rapamycin 10nM for 24 hours, E) rapamycin 10nM for 24 hours the Reo MOI 10 for 24 hours or F) Reo MOI 10 and rapamycin 10nM for 48 hours concomitantly. FACs plots gated on autophagy positive cells.

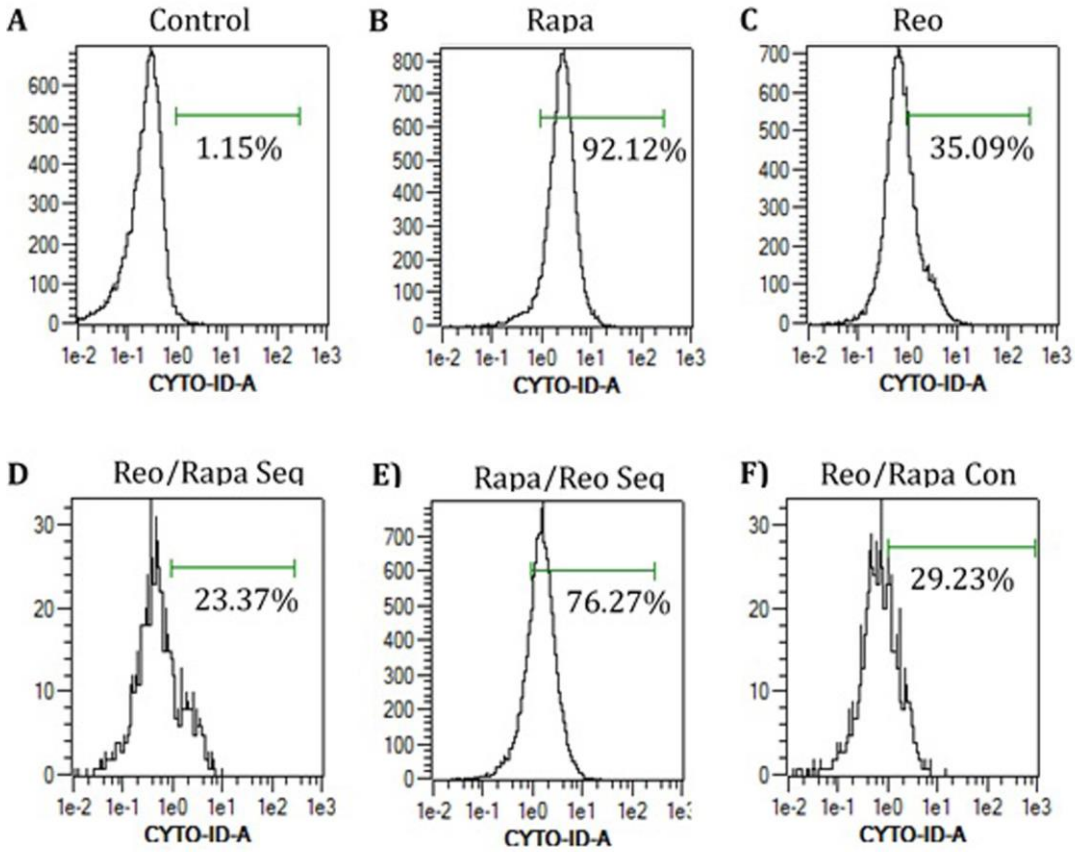
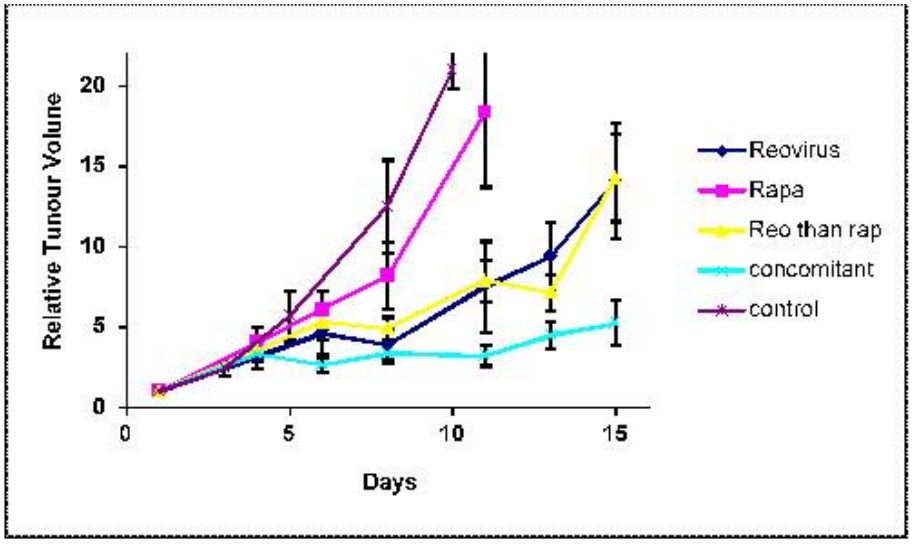


Figure 8. Combination of reovirus and rapamycin reduces tumour growth and improves survival in mouse melanoma model

C57Bl/6 mice (N=8) with a B16.F10 flank tumour were treated with i.t. reovirus (days 1 and 4), i.p. rapamycin (days 1, 4, 8,and 11), or a combination (concomitant or with rapamycin 24hrs after reovirus). Growth was expressed as tumour volume relative to volume at commencement of treatment. Concomitant treatment led to significantly slower tumour growth compared to either agent alone and a significant improvement in survival compared to reovirus or rapamycin alone (log-rank test; p0.02). There was no significant difference between combination treatment given in sequence or concomitantly.



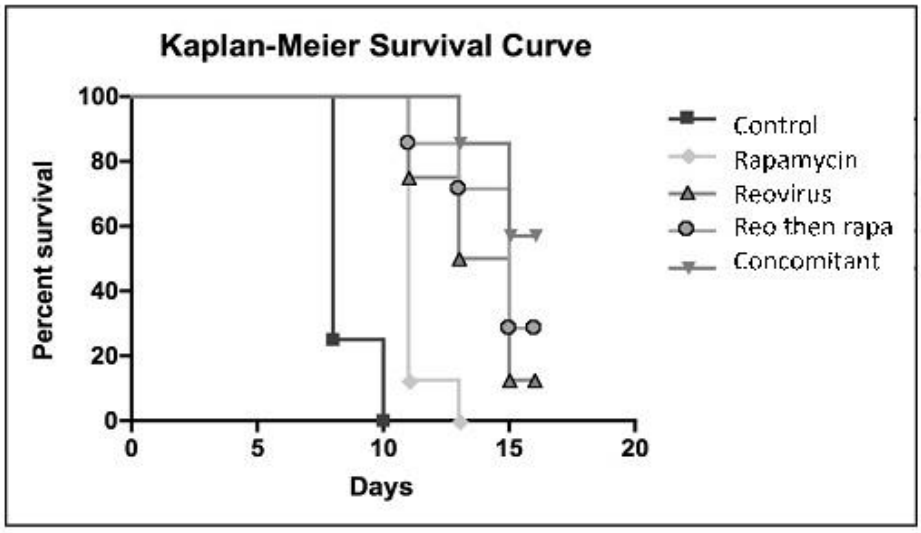


Figure 9. B16.F10 tumours were seeded on the flanks of C57Bl/6 mice. Mice were treated with reovirus i.t. either alone or in combination with i.p rapamycin on days 1 or 2, viral titre in tumour (a), liver (b), lungs (c) and heart (d) was determined using plaque assay. Data shown is representative of mean plaque forming units per gram of tissue. There was a significant difference (*) in pfu/g of tissue in the tumour group between mice treated with reovirus alone and the sequenced combination (unpaired t-test;p=0.03).

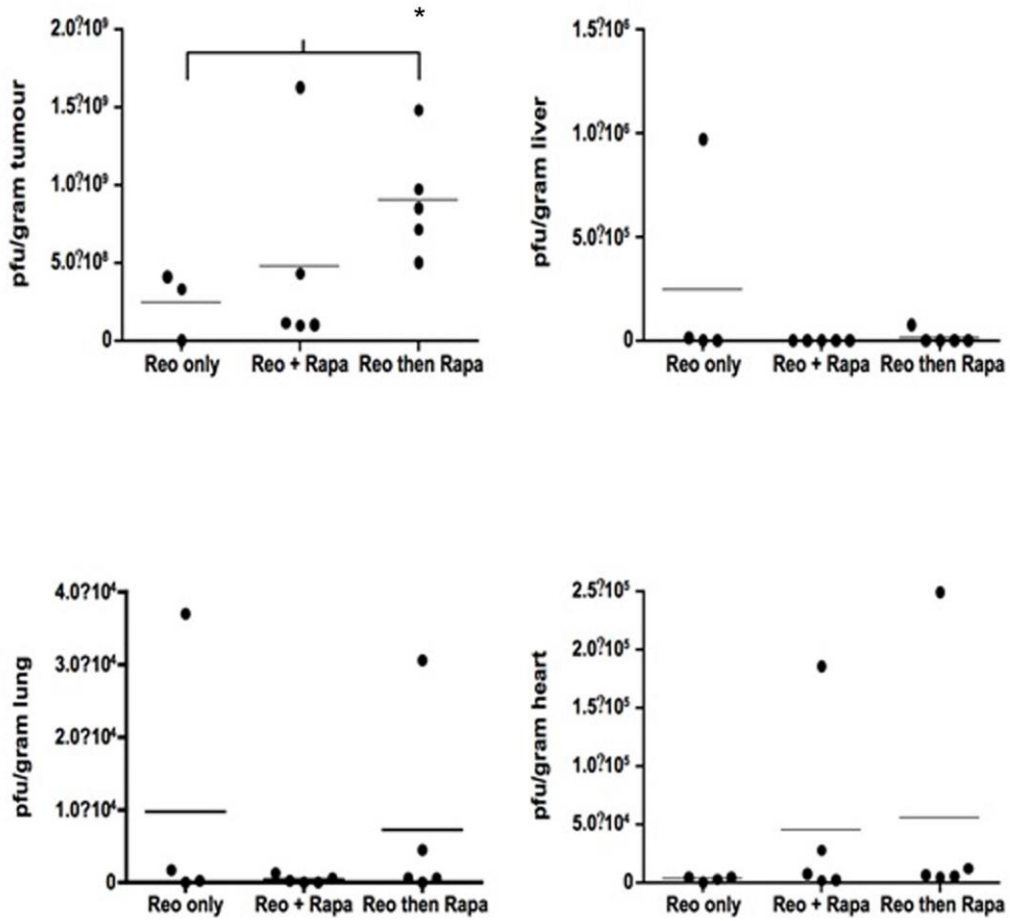


Figure 10 Rapamycin does not affect the humoral response to reovirus at the dose of 5mg/kg. The level of neutralising antibody in the serum of tumour bearing mice treated with either reovirus alone, or in concomitant or sequenced combination was determined. Unpaired student T –tests were carried out to compare reovirus NARA endpoint titre vs reovirus then rapamycin endpoint titre, also reovirus endpoint titre vs concomitant endpoint titre. These unpaired student T –tests showed no significant difference P= >0.1