

Synergistic Effects of Oncolytic Reovirus and Cisplatin Chemotherapy in Murine Malignant Melanoma

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Abstract Purpose: To test combination treatment schedules of reovirus and cisplatin chemotherapy in human and murine melanoma cell lines and murine models of melanoma and to investigate the possible mechanisms of synergistic antitumor effects.

Experimental Design: The effects of reovirus \pm chemotherapy on *in vitro* cytotoxicity and viral replication were assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay and plaque assay. Interactions between agents were assessed by combination index analysis. Mode of cell death was assessed by Annexin V/propidium iodide fluorescence-activated cell sorting–based assays; gene expression profiling of single versus combination treatments was completed using the Agilent microarray system. Single agent and combination therapy effects were tested *in vivo* in two immunocompetent models of murine melanoma.

Results: Variable degrees of synergistic cytotoxicity between live reovirus and several chemotherapy agents were observed in B16.F10 mouse melanoma cells, most significantly with cisplatin (combination index of 0.42 ± 0.03 at ED₅₀). Combination of cisplatin and reovirus exposure led to increased late apoptotic/necrotic cell populations. Cisplatin almost completely abrogated the inflammatory cytokine gene up-regulation induced by reovirus. Combination therapy led to significantly delayed tumor growth and improved survival *in vivo* ($P < 0.0001$ and $P = 0.0003$, respectively). Cisplatin had no effect on the humoral response to reovirus in mice. However, cisplatin treatment suppressed the cytokine and chemokine response to reovirus *in vitro* and *in vivo*.

Conclusion: The combination of reovirus and several chemotherapeutic agents synergistically enhanced cytotoxicity in human and murine melanoma cell lines *in vitro* and murine tumors *in vivo*. The data support the current reovirus/chemotherapy combination phase I clinical studies currently ongoing in the clinic. (Clin Cancer Res 2009; 15(19):6158–66)

There has been rapid preclinical and clinical development of viruses with oncolytic activity as anticancer therapeutics (1). As with other novel agents, the true potential of this approach to cancer treatment may only be realized in combination with

other treatment modalities. Reoviruses (respiratory enteric orphan viruses) are ubiquitous, nonpathogenic viruses that have been isolated from the human respiratory and gastrointestinal tract. They are nonenveloped icosahedral viruses with a segmented genome composed of double-stranded RNA. To date, reovirus infection in humans has not been associated with any known disease. Reovirus has innate oncolytic potential in an extensive range of murine and human tumor cells, and this is, at least in part, dependent on the transformed state of the cell (2, 3). The precise mechanism of reoviral tropism and selective oncolysis in malignant cells is yet to be fully determined. In normal cells, the presence of an intact double-stranded RNA-activated protein kinase system prevents the establishment of a productive infection. In malignant cells with activated Ras pathway (or up-regulation of upstream or downstream components of the cell signaling pathway) through either Ras mutation or up-regulated epidermal growth factor receptor (EGFR) signaling (4–6), this cellular antiviral response mechanism is perturbed, and viral replication and subsequent lysis of the host cell results. Reovirus has been shown to cause tumor regression after intralesional injections in immunodeficient mice and after systemic administration in immunocompetent mice (4, 5). In

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Translational Relevance

Oncolytic reovirus is a potentially attractive cancer therapeutic in view of its intrinsic targeting of cancer cells with dysregulated Ras pathway signaling (and therefore relevance to a wide range of malignancies), lack of host toxicity, and safety in phase I studies. The true potential of oncolytic viruses may only be realized in combination with other modalities, such as chemotherapy, targeted therapy, and radiotherapy. This study shows synergistic cell kill *in vitro* and a survival benefit *in vivo* in malignant melanoma in murine models and human cell lines when reovirus was combined with a range of commonly available chemotherapeutics. The approach has direct relevance to the clinic, and currently, several phase I studies are evaluating reovirus/chemotherapy combinations. The finding that cisplatin almost negates the inflammatory response to reovirus may influence study design and ultimately allow an increase in the dose levels of reovirus without increasing systemic toxicity.

In addition to the exploitation of oncogene signaling, reovirus activates the host immune response to potentially enhance antitumor responses through the efficient induction of type I IFNs (7) and local inflammatory responses generated by reovirus-infected tumor cells cause bystander toxicity against reovirus-resistant tumor cells and activation of human myeloid dendritic cells (8). Several phase I clinical studies of intratumoral (i.t.) or systemic reovirus as a single agent have been completed, with evidence of significant antitumor activity (9, 10).

We have recently shown synergistic *in vitro* antitumor activity when reovirus and radiotherapy were combined across a range of tumor cell lines. The combined effect was greatest in cell lines that were only moderately susceptible to reovirus alone. Interestingly, this effect did not depend on treatment sequence or schedule. Sensitization was due to an increase in apoptosis in cells treated with combined therapy. Furthermore, *in vivo* studies using xenograft (HCT116 and SW480) and syngeneic (B16.F10) tumors showed enhanced activity of combined treatment relative to reovirus or radiation alone (11). To date, oncolytic viruses have been safely combined with systemic chemotherapy in the clinic.

In this study, we have investigated the potential for increasing the antitumor effects of reovirus in murine and human melanoma cell lines in combination with chemotherapeutics and the possible mechanisms underlying these interactions.

Materials and Methods

Cell lines. The mouse melanoma cell line B16.F10 was cultured in DMEM at 37°C and 10% CO₂. K1735, another mouse melanoma, and L929, a murine fibroblast-like line, were cultured in DMEM at 37°C and 5% CO₂. The human melanoma cell line Mel 888 and two early-passage melanoma lines SM and OM (passage 22 and 24, respectively; both cell lines were derived from metastatic malignant melanoma deposits and were positive for S100 and HMB45 staining) were cultured in RPMI 1640 at 37°C and 5% CO₂. All media were supplemented with 2 mmol/L GlutaMAX-1 supplement (Invitrogen), 100 units/mL penicillin,

and 100 units/mL streptomycin (Sigma) and either 10% (v/v) FCS for routine passage or 2% (v/v) FCS for experimental work.

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

Cisplatin [*cis*-diamminedichloroplatinum(II); Mayne Pharma Plc], DTIC (dacarbazine; Ben Venue Laboratories, Inc.), gemcitabine (Ely Lilly and Co.), paclitaxel (Bristol-Myers Squibb Co.), and carboplatin (Bristol-Myers Squibb) were all obtained from Royal Surrey County Hospital pharmacy.

In vitro survival assay. Cells were plated in 96-well plates at a density of 7.5×10^3 per well. After 24 h, they were infected with known dilutions of reovirus and chemotherapeutic drug, either alone or in combination. After 48 h of 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 the manufacturer's instructions. Briefly, 20 μL of MTS reagent were added to each well, and following incubation at 37°C for 1 to 4 h, absorbance was measured at 495 nm. Survival was calculated as a percent compared with untreated cells. All experiments were repeated at least five times.

In vitro synergy assay. The effect of the combination of reovirus and chemotherapy on cell proliferation was assessed by calculating combination index (CI) values using CalcuSyn software (Biosoft). Derived from the median-effect principle of Chou and Talalay (12), 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. Experiments were done as described for the *in vitro* survival assay using 4, 2, 1, 0.5, and 0.25 times the calculated ED₅₀ of each agent in a constant ratio checkerboard design.

Reovirus one-step growth curves. Test cells were seeded overnight and then infected with multiplicity of infection (MOI) 10 reovirus in DMEM with 2% FCS. At various times after infection, samples were transferred to -80°C. Following three freeze-thaw cycles between -80°C and room temperature, lysates were collected and virus titer was determined by plaque assay as described above.

Inactivation of reovirus by UV irradiation and heat. Reovirus was UV inactivated by exposing 50 μL aliquots of viral stock at 1.2×10^{10} plaque-forming unit (pfu)/mL to 720 mJ irradiation using a Stratalinker UV Crosslinker 2400 (Stratagene) to cross-link viral RNA. Heat inactivation was done by heating 200 μL aliquots of viral stock at 1×10^9 pfu/mL for 20 min at 60°C.

Fluorescence-activated cell sorting analysis of cell survival and apoptosis. Following overnight seeding, B16.F10 cells were treated with 50 μmol/L cisplatin and/or MOI 1 reovirus for 48 h. Adherent and non-adherent cells were collected, washed in cold PBS, resuspended at 1×10^6 in 500 μL PBS, and then incubated for 15 min at room temperature in the dark in cold 1× binding buffer containing Annexin V-FITC antibody according to the manufacturer's instructions (Merck Biosciences Ltd.). The cells were pelleted and resuspended in cold 1× binding buffer. Cells were stained with 10 μL propidium iodide (PI) at 30 μg/mL and analyzed on a Coulter Epics XL flow cytometer (Beckman Coulter) using EXPO32 ADC software (Beckman Coulter).

Caspase inhibition assay. B16.F10 cells were seeded overnight and then treated for 45 min with 50 μmol/L Z-VAD-FMK (R&D Systems) before exposure to 50 μmol/L cisplatin and/or MOI 1 reovirus in DMEM with 2% FCS for 24, 48, or 72 h. Cell viability was quantified at these time points as described above for the *in vitro* survival and synergy assays.

Microarray analysis. Total RNA was extracted from test cells using the RNeasy Mini kit (Qiagen, Inc.) according to the manufacturer's recommendations. Fluorescent-labeled cRNA was produced using the One-Color Low RNA Input Linear Amp kit (Agilent Technologies UK Ltd.) and purified using the RNeasy Mini kit. The labeled cRNA was fragmented and hybridized overnight to Whole Mouse Genome (4x44K) Oligo Microarray slides (Agilent) using the Agilent Gene Expression Hybridization kit (Agilent). The slides were washed and scanned using an Agilent DNA Microarray Scanner (Agilent). Data were extracted using the Agilent Feature Extraction software and subsequently analyzed using GeneSpring (Agilent) using the fold change of each gene based on normalized data. Only changes calculated to be significant ($P < 0.05$) are included in the results.

In vitro studies. All procedures were approved by United Kingdom Home Office and institutional boards. All animal experiments were repeated at least thrice. Mice were purchased from B&K Universal Ltd. S.c. tumors were established in the flank of each mouse by injecting 5×10^5 cells in a volume of 100 μ L HBSS (Sigma). Animals were examined thrice weekly for tumor development. Three orthogonal tumor diameters (d_1 , d_2 , and d_3) were measured using Vernier calipers, and tumor volume was calculated by the formula $V = \pi/6 (d_1 \cdot d_2 \cdot d_3)$. Animals were killed when tumor size exceeded 15 mm in any one dimension.

Reovirus (1×10^8 pfu in 100 μ L volume) was administered using a single cutaneous puncture site. Once in a s.c. location, the 25-gauge needle was redirected along multiple tracks within the tumor to achieve maximal dispersal of the reovirus. It was possible to achieve direct i.t. injection without backflow of the injectate. Cisplatin (2.5 mg/kg) was administered i.p. in a total volume of 100 μ L. Control animals received an equivalent volume of HBSS alone.

Virus titration from tumor and organs. Tumor and organs (heart, lungs, and liver) were weighed and homogenized in a TissueLysar (Qiagen) at 30 Hz for 2 min. Following centrifugation to clarify the virus lysate, virus titer was determined by plaque assay on L929 cells as described above and expressed as pfu/g tissue.

Serum analysis for presence of neutralizing antireoviral antibodies. The methodology used for analysis of neutralizing antireovirus antibody (NARA) has been reported recently (13). Briefly, serum samples from individual mouse groups 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% CO₂. 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 neutralizing antibody levels in the serum, the above experiment was repeated with a constant titer 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.

Cytokine analysis. Culture supernatant was collected from B16.F10 cells treated with either reovirus (MOI 1) or cisplatin (50 μ mol/L) either alone or in combination at 0, 6, 12, 24, 48, and 72 h after treatment. Samples were stored at -80°C before analysis using a Mouse Bio-Plex Cytokine Assay (Bio-Rad) according to the manufacturer's instructions. Levels of interleukin (IL)-1 α , IL-6, IL-12 (p70), IL-17, MIP-1 α , and RANTES were measured in 50 μ L of cell supernatant, carried out in duplicate.

Serum was collected on day 4 from C57BL/6 mice bearing B16.F10 tumors and treated with reovirus and cisplatin alone or in combination as described above on days 0 and 3. Serum samples were stored at -80°C before analysis for cytokines as described above.

Statistical analysis. Comparisons between groups were done using the *t* test and two-way ANOVA. Survival curves were estimated using the Kaplan-Meier method, and significance was assessed using the χ^2 and log-rank test. Statistical analysis was done using GraphPad Prism 4 (GraphPad Software, Inc.).

Results

Reovirus cytotoxicity in NIH3T3, L929, and tumor cell lines. The effect of reovirus infection over 48 hours was assessed in murine and human malignant melanoma cell lines, L929 cells, and NIH3T3 fibroblasts. Cells were infected with reovirus at MOI from 0.001 to 100. Differential sensitivity to reovirus across the cell lines was observed. As expected, L929 cells were extremely sensitive to reoviral cytotoxicity with <1% survival at MOI 0.01 at 48 hours. Figure 1A shows the superiority of reovirus cytotoxicity in B16.F10 lines compared with NIH3T3 cells where toxicity was only observed at high (≥ 10) MOI. Consistent with this observation was the ability of B16.F10 cell to support viral replication to a higher degree than NIH3T3 cells (Fig. 1B).

Reovirus cytotoxicity is enhanced by combination with cisplatin. B16.F10 cells were infected with reovirus at a range of MOIs from 0.01 to 1 at cell concentration of 7.5×10^3 per well for 48 hours, either alone or with concomitant 10 or 100 μ mol/L of cisplatin (Fig. 1C). A MTS survival assay was done after 48 hours. The addition of cisplatin significantly enhanced tumor kill compared with reovirus alone across all MOIs in a cisplatin dose-dependent manner ($P < 0.0001$, two-way ANOVA).

Synergistic interaction between reovirus and chemotherapeutic agents in malignant melanoma cell lines. The effect of the combination of reovirus and chemotherapy on cell proliferation was assessed by isobologram analysis by calculating CIs. 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. Experiments were done using 4, 2, 1, 0.5, and 0.25 times the calculated ED₅₀ of each agent in a constant ratio checkerboard design. Chemotherapy and reovirus were administered to cells concomitantly.

The combination of reovirus and the chemotherapeutic agents cisplatin, dacarbazine, gemcitabine, paclitaxel, and carboplatin on B16.F10 cells was assessed. We observed synergistic cell kill with all agents. This was more pronounced with cisplatin and paclitaxel with CI values at the ED₅₀ of 0.42 ± 0.03 and 0.44 ± 0.09 , respectively (Table 1A). We further evaluated the combination of cisplatin (as it was associated with the lowest CI) with another murine malignant melanoma cell line, K1735, and human malignant melanoma lines Mel 888, SM, and OM (early-passage lines, described in Materials and Methods). Synergistic effects of combined agents were observed with the early-passage lines but with higher CI values at ED₅₀. However, the combination was antagonistic in the Mel 888 cell line with a CI of 1.37 ± 0.02 at ED₅₀ (Table 1B).

Live virus is required for efficient cell killing. We established that only live, and not inactivated, virus is capable of cytotoxicity, either alone or in combination with cisplatin.

B16.F10 cells were treated with live, heat-inactivated, or UV-inactivated virus for 48 hours at MOIs up to 100, and survival was assessed by MTS assay. Live virus resulted in the most efficient cell kill; this effect was markedly reduced at all but the highest MOI after UV inactivation and almost completely negated by heat-inactivated virus (Fig. 1D).

Live, heat-inactivated, and UV-inactivated reovirus was combined with cisplatin at a fixed ratio of cisplatin to reovirus (12 μ mol/L cisplatin to MOI 1 reovirus) over five 2-fold dilutions for 48 hours. The combination of live reovirus with cisplatin enhanced cell kill compared with cisplatin alone. This

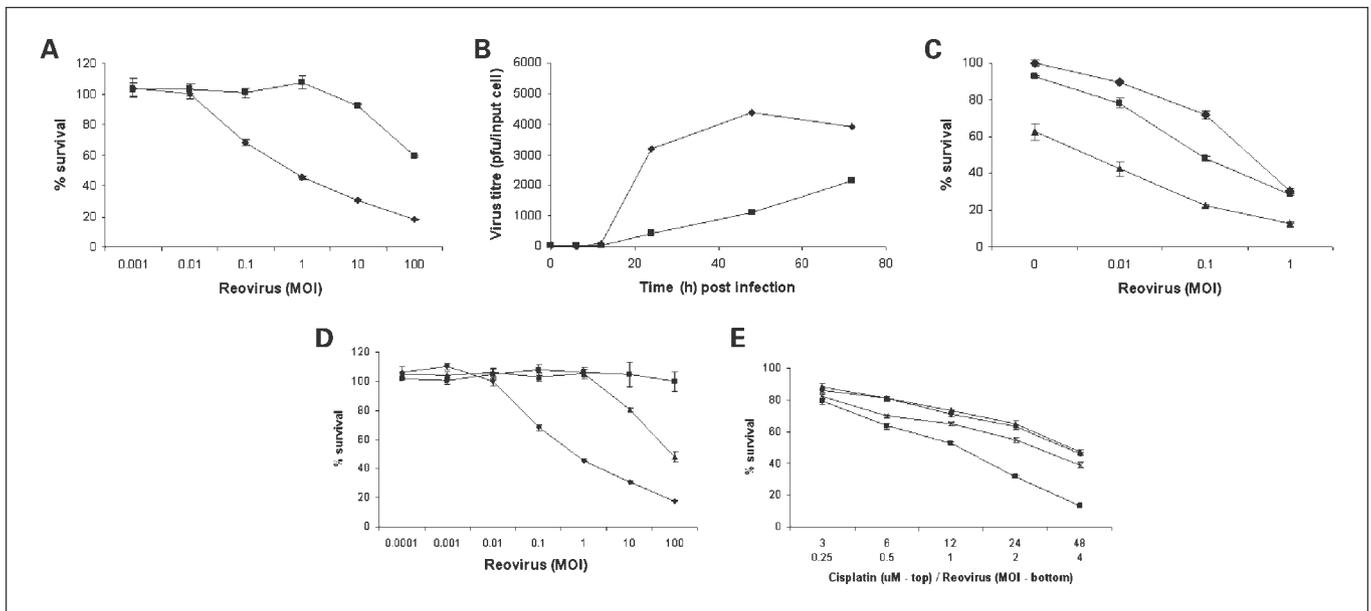


Fig. 1. Superior reovirus cell kill and viral replication in B16.F10 mouse melanoma cells compared with mouse fibroblast-like NIH3T3. **A**, B16.F10 (◆) and NIH3T3 (■) cells (7.5×10^3 per well) were treated with live virus for 48 h at the MOI indicated. Cell survival was measured at this time by MTS assay. **B**, B16.F10 (◆) and NIH3T3 (■) cells were treated with live virus at a MOI of 10. Samples were collected at the indicated time points, and viral titer was determined by plaque assay. **C**, reovirus-induced cell kill is enhanced by the addition of cisplatin in a dose-dependant manner. B16.F10 cells (7.5×10^3 per well) were treated with reovirus for 48 h at the MOI indicated either alone (◆) or with the addition of 10 $\mu\text{mol/L}$ (■) or 100 $\mu\text{mol/L}$ (▲) of cisplatin. Survival was measured at this time using MTS ($P < 0.000$, two-way ANOVA). **D**, live virus is required for efficient cell kill. B16.F10 cells (7.5×10^3 per well) were treated with live (◆), heat-inactivated (■), or UV-inactivated (▲) virus for 48 h at the MOI indicated. Survival was measured at this time by MTS assay. **E**, cisplatin-enhanced reovirus kill requires live virus. B16.F10 cells (7.5×10^3 per well) were treated with cisplatin alone (◆) or cisplatin with live reovirus (■), heat-inactivated reovirus (▲), or UV-inactivated virus (x) for 48 h at the concentration and MOI indicated. Survival was measured at this time by MTS assay ($P < 0.0001$, two-way ANOVA).

enhancement of cell kill was not apparent when heat-inactivated or UV-inactivated reovirus was used ($P < 0.0001$, two-way ANOVA; Fig. 1E).

Enhanced apoptotic cell death with cisplatin and reovirus combination. The nature of the synergy of cell kill with reovirus and cisplatin treatment observed was further investigated. We assessed the mode of cell death of B16.F10 cells treated with MOI 1 reovirus, 50 $\mu\text{mol/L}$ cisplatin, or both agents together at 24 and 48 hours by Annexin V/PI staining (Fig. 2A and B). At 24 hours, there was a small increase in late apoptotic/necrotic population (A^+/PI^+) in all groups but slightly more so in the combination. By 48 hours, this effect had increased considerably with the majority of cells in the combination group A^+/PI^+ and concomitant reduction of intact cells (A^-/PI^-). The effect of reovirus alone also caused a degree of apoptotic death in this cell line.

Caspase inhibition assay. B16.F10 cells were seeded overnight and then treated with the pan-caspase inhibitor Z-VAD-FMK before exposure to 50 $\mu\text{mol/L}$ cisplatin and/or MOI 1 reovirus (Fig. 2C-E). The viability of cells treated with reovirus alone was reduced to 25% at 72 hours; addition of Z-VAD-FMK to reovirus-infected cells significantly reversed the reovirus-induced cytotoxicity, suggesting that reovirus cytotoxicity was through a caspase-mediated apoptotic pathway. No similar reversal was seen with cisplatin alone, but in combination with reovirus, some reversal was observed.

Gene expression profile of B16.F10 treated with cisplatin, reovirus, or combination. We investigated the effect of individual agents and their combination on global gene expression. Total RNA was extracted from B16.F10 cells after exposure to cisplatin (100 $\mu\text{mol/L}$), reovirus (MOI 25), or both at two time

points. Significant changes in gene expression were observed only after 24 hours (12-hour data not shown). The effects of reovirus alone included the up-regulation of proinflammatory cytokines, apoptosis genes, MHC class I, oncogenes such as *Rous sarcoma*, and interestingly *EGFR1*. A 5-fold increase in expression of *EGFR1* was confirmed by quantitative PCR (data not shown). Cisplatin alone was associated with increases in apoptosis genes such as *Fos*. The expression profile with combined cisplatin and reovirus was almost identical to that of cisplatin alone with almost complete abrogation of inflammatory cytokine expression. Only *sonic hedgehog (Shh)* expression seemed to be uniquely up-regulated as a result of the combined treatments (Fig. 3).

Combined reovirus and cisplatin treatment enhances tumor growth delay in two immunocompetent murine models of malignant melanoma. The *in vivo* effects of combined reovirus and cisplatin were evaluated in two models in view of the observed *in vitro* synergy: B16.F10 cells in C57BL/6 mice and K1735 in C3H mice. For both models, 5×10^5 cells were implanted s.c. Treatment was initiated when tumors reached an average diameter of 4.5 to 5.5 mm at approximately 10 to 12 days for B16.F10 and 8 to 10 days for K1735. Mice were treated with i.t. reovirus, i.p. cisplatin, or both on days 0 and 3. Control mice received the same tumor inoculum and were treated with an equivalent volume of saline i.t. and i.p. administered in an identical manner. There were no obvious toxic effects of single agent or combination treatments in all mice treated, and experiments were concluded as a result of tumor growth reaching 15 mm in any one dimension.

In the B16.F10 C57BL/6 model, the combination of cisplatin and reovirus resulted in the most effective response in terms of

tumor growth retardation ($P < 0.0001$, two-way ANOVA), although both cisplatin and reovirus monotherapies resulted in delayed tumor growth (Fig. 4A). The median survival for the control, cisplatin, reovirus, and combination groups was 6, 8, 12, and 17 days, respectively ($P = 0.0003$, log-rank test; Fig. 4B). We found a similar advantage for combination therapy in K1735 tumor/C3H mouse model (data not shown).

Viral replication in tumors and organs of treated mice. We evaluated viral replication in tumors and organs at day 4 after therapy. B16.F10 tumors were seeded on the flanks of C57BL/6 mice. Mice were treated with i.t. reovirus either alone or in combination with i.p. cisplatin on days 0 and 3, and following sacrifice on day 4, viral titer in tumor, liver, lungs, and heart was determined using plaque assay (Fig. 5A). The highest viral yield was in resected tumor as expected, with over three times more viruses detected in mice receiving the combination treatment than those receiving reovirus alone. Of the organs tested, the liver supported most viral growth but much less (>5 logs) than tumor. No difference in virus titer in the liver, lungs, or heart was observed between treatment with reovirus alone or in combination with cisplatin.

Applying the same approach, we evaluated viral yield from organs from similarly treated cohorts of mice at point of sacrifice when tumor had reached maximum allowed size (15 mm

in any dimension) and/or ulcerated. After treatment with reovirus alone (median, 10.5 days), virus grew from all tumors and occasionally in the heart and liver. However, in combination with cisplatin (median, 10 days), virus was grown from three of six tumors and also from four of six livers (Table 1C).

Cisplatin does not affect the humoral response to reovirus. The antitumor effects of reovirus have been enhanced by the coadministration of agents that reduce the humoral response to reovirus (5). This response is vigorous and is generally initiated within 48 hours. Abrogation of the humoral response results in increased viral effects, both antitumor and organ toxicity (14). Using a recently reported assay (13), we assessed the NARA response to reovirus and reovirus/cisplatin combination in C57BL/6 mice. We found equivalent end-point titers in both groups, indicating that cisplatin did not affect the NARA response to reovirus when administered concomitantly (Fig. 5B).

Cisplatin reduces inflammatory cytokine response to reovirus. The microarray analysis of B16.F10 cells treated with reovirus and cisplatin in combination showed marked reductions in the expression of a range of proinflammatory cytokine genes. Culture supernatant was collected from B16.F10 cells treated with either reovirus (MOI 1) or cisplatin (50 $\mu\text{mol/L}$) alone or in combination at 0, 6, 12, 24, 48, and 72 hours after treatment. Samples were stored at -80°C before analysis using a Mouse Bio-Plex Cytokine Assay. At 48 hours, concomitant exposure of tumor cells to reovirus and cisplatin resulted in a marked reduction and, in some cases, almost complete abrogation in cytokine production in a range of Th1 and Th17 cytokines as well as MIP-1 α and RANTES (Fig. 5C).

This was further evaluated in the *in vivo* model. Blood was collected from B16.F10 tumor-bearing mice on day 4 following treatment as described above, and the serum was assessed for cytokines by cytometric bead array in the same way as the *in vitro* experiment. Once again, the concomitant administration of cisplatin markedly reduced the production of the same cytokines (Fig. 5D).

Table 1. Interactions of reovirus and chemotherapy

A. Synergistic interaction of reovirus and chemotherapy on B16.F10

Chemotherapeutic	ED ₅₀	ED ₇₀	ED ₉₅
Cisplatin	0.42 \pm 0.03	0.30 \pm 0.02	0.24 \pm 0.01
DTIC	0.80 \pm 0.10	0.66 \pm 0.08	0.56 \pm 0.10
Gemcitabine	0.47 \pm 0.09	0.47 \pm 0.07	0.48 \pm 0.04
Paclitaxel	0.44 \pm 0.09	0.38 \pm 0.11	0.35 \pm 0.14
Carboplatin	0.62 \pm 0.01	0.56 \pm 0.02	0.50 \pm 0.03

B. Interaction of reovirus and cisplatin on other melanoma cell lines

Cell line	ED ₅₀	ED ₇₀	ED ₉₅
K1735	0.92 \pm 0.02	0.78 \pm 0.01	0.57 \pm 0.01
Mel 888	1.37 \pm 0.02	1.02 \pm 0.03	0.77 \pm 0.04
SM	0.84 \pm 0.02	0.54 \pm 0.01	0.36 \pm 0.01
OM	0.88 \pm 0.04	0.67 \pm 0.04	0.52 \pm 0.03

C. Presence of live virus in tumor tissue and selected organs of reovirus-treated mice

	No. animals positive for virus by plaque assay			
	Tumor	Liver	Lungs	Heart
Reovirus	6/6	1/6	0/6	1/6
Combination	3/6	4/6	0/6	0/6
Cisplatin	0/3	0/3	0/3	0/3
Control	0/3	0/3	0/3	0/3

NOTE: Data are presented as CI values \pm SE at the effective dose indicated. Median survival (days): reovirus, 12; combination, 17; cisplatin, 8; control, 6.

Discussion

In this study, we have shown evidence of synergistic anticancer activity of oncolytic wild-type reovirus with chemotherapy in a range of murine and human melanoma cell lines *in vitro* plus two *in vivo* models of murine melanoma. Metastatic malignant melanoma is a particularly aggressive cancer that has a modest response to conventional chemotherapy and therefore justifiably a target for novel cancer therapies (15). Synergistic cell kill was observed across several chemotherapeutic agents with reovirus; cisplatin was selected for detailed evaluation following the demonstration of marked *in vitro* synergy, the documented clinical activity (albeit modest) of cisplatin in advanced melanoma (as both a single agent and in combination), and that, for future human studies, a reovirus/cisplatin combination would be feasible as patients' disease may have already progressed while on dacarbazine, the current chemotherapeutic standard of care.

Several chemotherapy/oncolytic virus combinations have been evaluated to date and have been shown to result in marked antitumor effects without compromising safety. The adenovirus Onyx-015 enhanced clinical efficacy by combining i.t. Onyx-015 with systemic cisplatin and 5-fluorouracil when

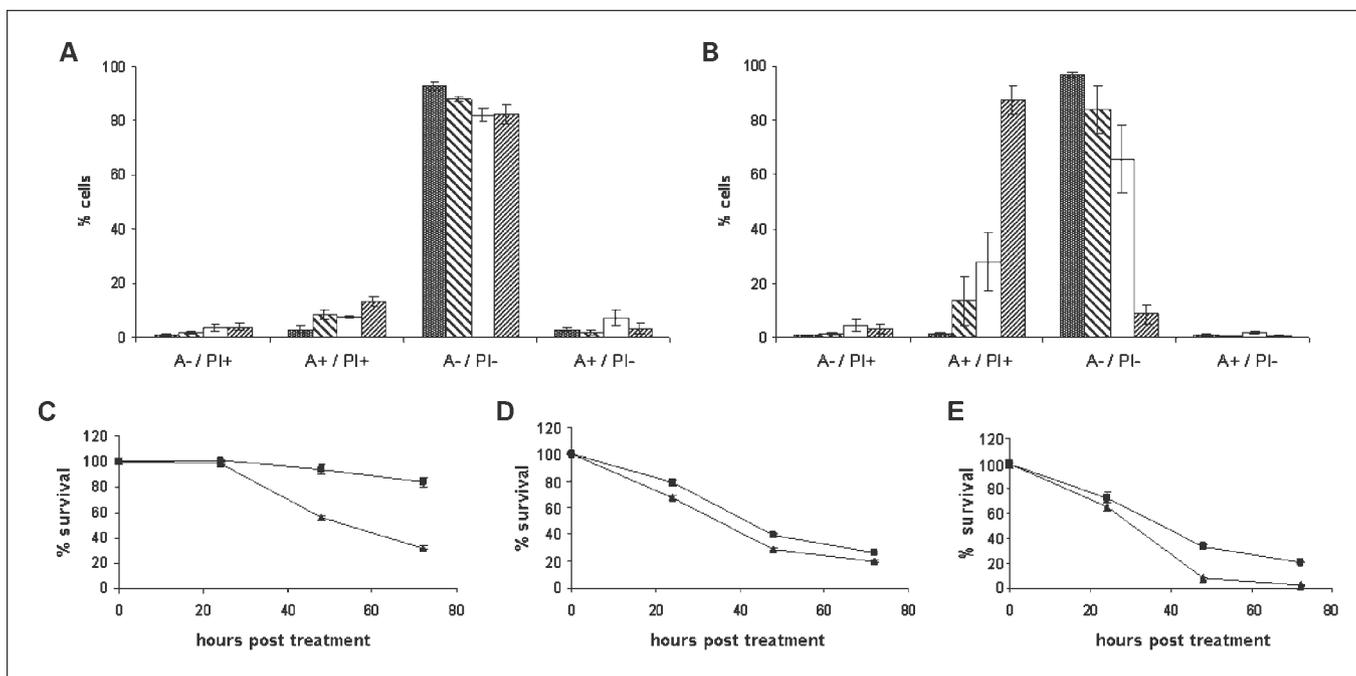


Fig. 2. A and B, enhanced apoptotic cell death of B16.F10 cells treated with reovirus and cisplatin in combination. B16.F10 cells were treated with MOI 1 reovirus (white columns), 50 $\mu\text{mol/L}$ cisplatin (wide diagonal striped columns), or both agents together (narrow diagonal striped columns) for 24 h (A) or 48 h (B) before staining with Annexin V and PI and analyzing using flow cytometry. Untreated cells (dark gray columns) were included as a negative control. Addition of each agent alone resulted in an increased late apoptotic/necrotic population (A⁺/PI⁺) and concomitant reduction of intact cells (A⁻/PI⁻) compared with untreated cells. There was a dramatic increase in this effect at 48 h when the two agents were combined. C to E, enhanced survival of cells treated with reovirus and/or cisplatin following pretreatment with the general caspase inhibitor Z-VAD-FMK. B16.F10 cells were pretreated for 45 min in the presence (■) or absence (▲) of 50 $\mu\text{mol/L}$ Z-VAD-FMK before culture with reovirus (C), cisplatin (D), or a combination of the two agents (E). Survival was determined at 24, 48, and 72 h by MTS assay.

compared with chemotherapy alone (16). E1A-expressing adenoviral E3B mutants combined with cisplatin and paclitaxel (17) showed synergistic activity *in vitro* and *in vivo*. A combination of oncolytic herpesviruses, such as G207, HSV-1716, or NV1066, with chemotherapeutic agents showed higher anti-tumor activity than treatment with the virus alone. G207 combined with cisplatin, and HSV-1716 with mitomycin C, resulted in synergistic activity *in vitro* and *in vivo* (18–21). The mechanism underlying the observed synergy is incompletely understood in these examples. Cell kill has in some cases been due to enhancement of viral replication leading to increased cytolysis (19, 22, 23). However, no increase in viral replication was observed in other studies, such as NV1020 in combination with 5-fluorouracil, SN38 (irinotecan), or oxaliplatin, where viral replication was clearly reduced if chemotherapeutics were added before, simultaneously, or after virus treatment (24). Other similar combination studies have shown either no effect of chemotherapeutics on viral replication (25–27) or actually reduced viral replication (22). Recently, synergistic cytotoxic effects of rat parvovirus H-1PV and gemcitabine in a pancreatic cancer model were observed in gemcitabine-resistant cell lines (28).

Recently completed phase I studies by our group and others using reovirus type 3 (Dearing) have confirmed its potential as an anticancer agent as well as its safety and tolerability in humans (9, 10). It is most likely that reovirus may achieve its potential by a combination approach with other modalities. The aim of combination treatment is to exploit additive or synergistic effects between agents, allow reovirus to target drug-resistant

subpopulations of tumor cells, and use chemotherapy to improve the biodistribution or penetrance of reovirus in the tumor and also for the attenuation of local and systemic immune responses to reovirus, allowing virus to persist in the tumor environment for longer periods.

We recently reported that the combination of reovirus and radiotherapy synergistically enhances cytotoxicity in a range of tumor cells through the enhancement of apoptosis *in vitro*, with confirmation of synergy *in vivo* (11). Consistent with our findings in human melanoma lines (8), where reovirus-induced cytotoxicity of human melanoma cell lines was reversed with the addition of a pan-caspase inhibitor ZVAD, when B16.F10 cells were treated in combination with cisplatin, partial reversal was observed, suggesting that at least some of the treated cells were dying through reovirus-associated apoptotic death mechanisms, as cisplatin-induced apoptosis is not thought to be caspase dependent. Annexin V/PI staining indicated significant proportions of late apoptotic/necrotic cells rather than purely apoptotic cell death, which may explain the partial reversal of cytotoxicity with ZVAD inhibition. Analysis of reovirus distribution in organs 4 days after injection indicated increased i.t. viral replication with combination therapy. This seems a little at odds with the observed *in vitro* synergy and increased apoptosis. Consistent with recent reports with an oncolytic herpes simplex virus, it may be that the apoptotic effects of cisplatin may have altered tumor architecture and interstitial pressure to facilitate increased viral uptake and diffusion, thereby allowing more viral replication (29). Furthermore, the observation of high levels of reovirus in tumor in the combination group, which then

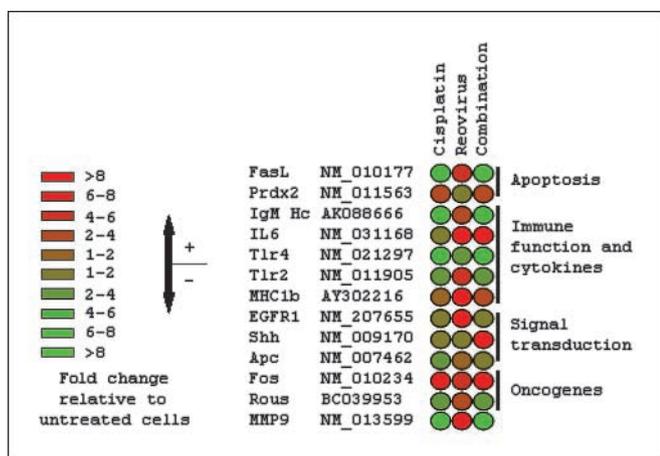


Fig. 3. Microarray analysis of transcriptional changes in B16.F10 cells treated with cisplatin, reovirus, or a combination of both. Changes in expression are shown relative to untreated cells.

were reduced to lower levels than reovirus alone, may be explained by the increased tumor destruction in the combination group and thereby less tumor mass later to support viral replication. The persistence of reovirus in liver 10 days after treatment may raise, theoretically, potential safety issues, but to date, the clinical studies of reoviral therapy as a single agent or in combination with chemotherapy have not identified significant hepatotoxicity.

Gene expression profiling of reovirus-treated tumor cells versus controls showed marked up-regulation of proinflammatory cytokines and activation of proapoptosis genes such as *NF- κ B*. Similar effects have been reported in previous microarray analyses of reovirus-infected HEK293 cells and murine neural cells (30, 31). Notably, we found that cotreatment with cisplatin *in vitro* and *in vivo* markedly reduced inflammatory cytokine response to reovirus. The clinical implications of this may be the possibility of escalating the reovirus dose delivered to enhance tumor kill. Reovirus treatment alone also increased the expression of *EGFR1* in B16.F10 cells as well as other murine and human tumor cell lines (data not shown), although this too was prevented by cotreatment with cisplatin. *EGFR* has been shown to enhance reoviral infection efficiency by what would seem to be the opportunistic use of an already activated signal transduction pathway (32). The transfection of relatively resistant cell lines with the *EGFR* gene confers significantly higher susceptibility to reovirus infection (32), and so an increase in endogenous *EGFR1* expression could be expected to potentiate reovirus infection and enhance cell killing.

Only one gene was found to be up-regulated uniquely by the combination of cisplatin and reovirus, the secreted signaling protein *Shh*, part of a key signaling pathway involved in both early development and cancer. In the latter, *Shh* secretion may modify the behavior of stromal cells to facilitate metastasis, although malignant cells that express high levels of *Shh* may also require *Shh* signaling to survive and are thus sensitive to *Shh* antagonists (33). Future work will determine whether cells treated with cisplatin and reovirus become more sensitive to *Shh* antagonism.

The host immune response to oncolytic viruses may ultimately influence their success in the clinical arena, both in

terms of humoral responses to neutralize virus and also the priming of antigen-specific T cells from *in situ* tumor destruction after capture by local antigen-presenting cells and migration to regional lymph nodes. Our recent clinical study showed the induction of a brisk NARA response to repeated systemic reovirus infusion (14). In this study, concomitant administration of cisplatin did not abrogate this response. However, in combination, cisplatin acted to drastically reduce the inflammatory cytokine response of B16.F10 cells to reovirus *in vitro* and *in vivo*. Cytokine and chemokine production by tumor cells has been extensively reported; the profiles observed have been specific to individual cell lines and culture/medium conditions (34). Cisplatin forms primarily intrastrand cross-link adducts, which activate several signal transduction pathways, including those involving ATR, p53, p73, and mitogen-activated protein kinase, and culminate in the activation of apoptosis. Cisplatin exposure induces IL-6, granulocyte colony-stimulating factor, and basic fibroblast growth factor secretion from specific cell lines (34).

We recently reported that reovirus-induced death of other human melanoma cell lines and human melanoma explants

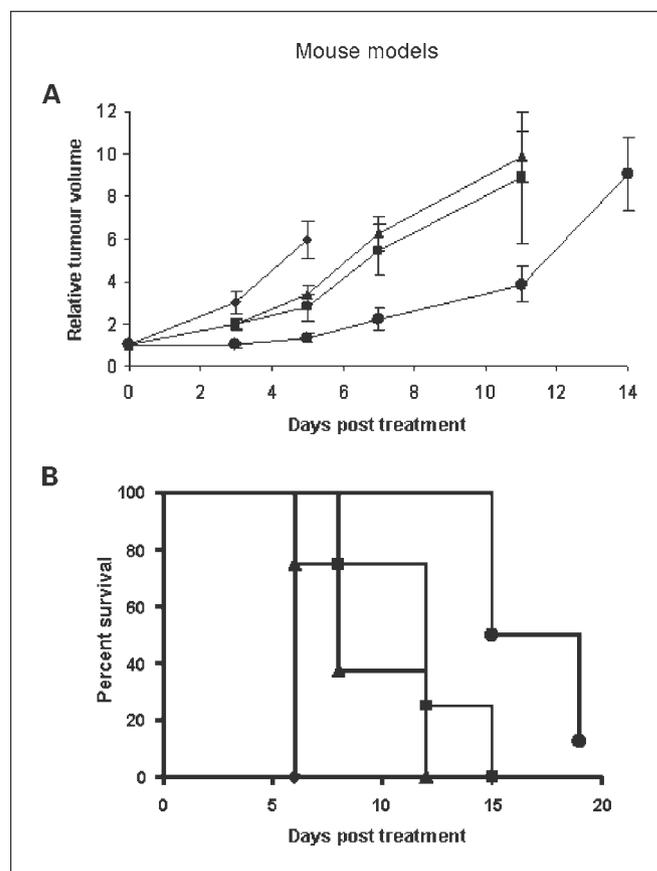


Fig. 4. Reduced tumor growth and increased survival following reovirus/cisplatin combination therapy. C57BL/6 mice bearing s.c. B16.F10 tumors were treated on days 0 and 3 with either reovirus alone i.t. (▲), cisplatin alone i.p. (■), or reovirus and cisplatin in combination (●). Control-treated mice (◆) received PBS. A, tumors were measured on the days indicated, and tumor volume was expressed as tumor volume relative to volume at commencement of treatment ($P < 0.0001$, two-way ANOVA). B, mice were euthanized when tumors exceeded 15 mm in any one dimension or tumor site ulcerated. Survival is expressed as Kaplan-Meier plots ($P = 0.0003$, log-rank test).

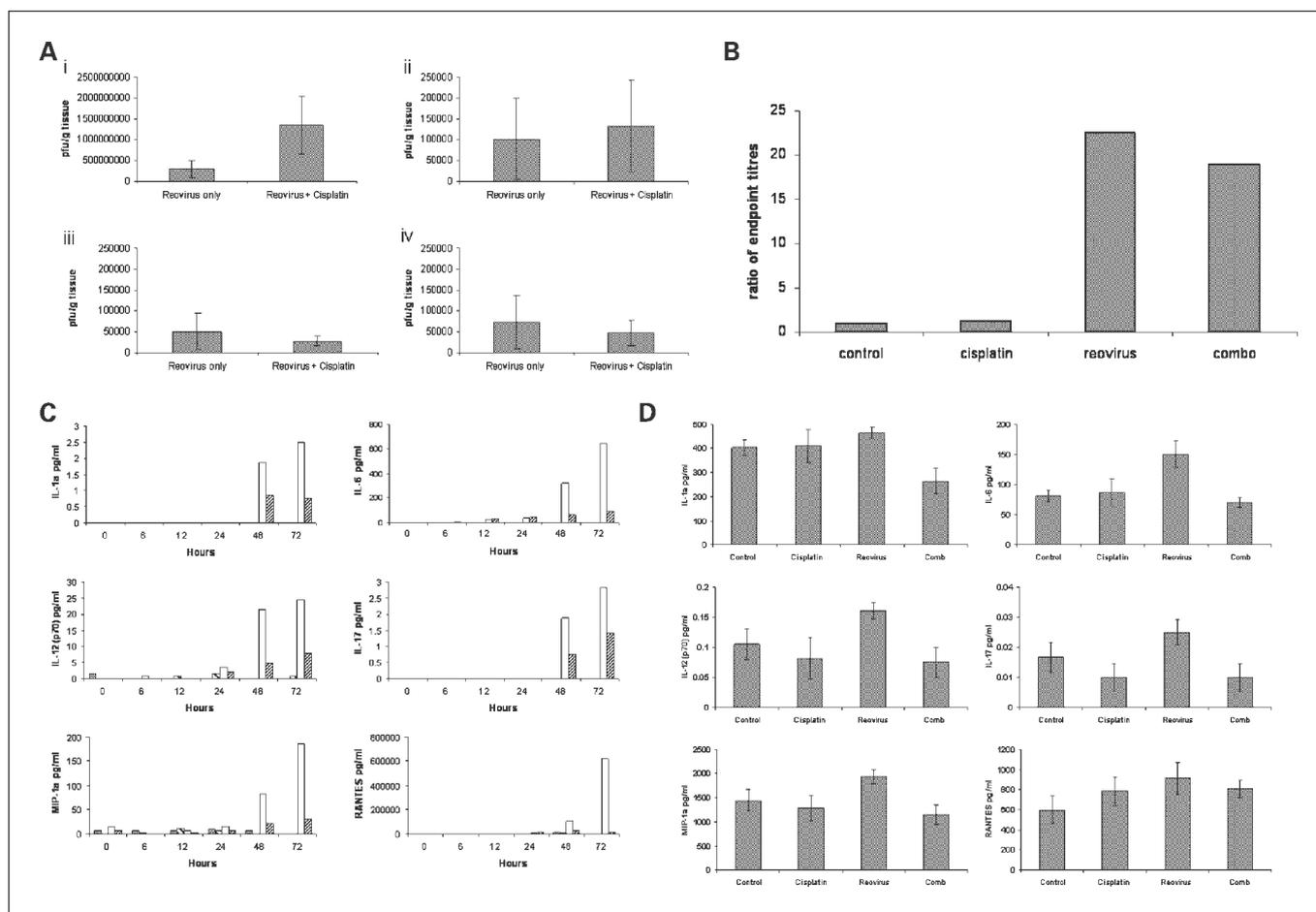


Fig. 5. A, analysis of virus distribution following i.t. injection. B16.F10 tumors were seeded on the flanks of C57BL/6 mice. Mice were treated with reovirus i.t. either alone or in combination with i.p. cisplatin on days 0 and 3, and following sacrifice on day 4, viral titer in tumor (i), liver (ii), lungs (iii), and heart (iv) was determined using plaque assay. Data are mean pfu/g of tissue. B, cisplatin does not affect the humoral response to reovirus. The level of neutralizing antibody in the serum of tumor-bearing mice treated with either reovirus or cisplatin alone or in combination was determined. C, cisplatin suppresses reovirus-induced cytokine responses *in vitro*. Culture supernatant was collected from B16.F10 cells at the time points indicated following treatment with reovirus (white columns) or cisplatin (wide diagonal striped columns) alone or in combination (narrow diagonal striped columns). Untreated cells (dark gray columns) were included as a negative control. Cytokine levels were determined using the Mouse Bio-Plex Cytokine Assay. Data are representative of two individual experiments tested in duplicate. D, cisplatin suppresses reovirus-induced cytokine responses *in vivo*. Serum was collected from B16.F10 tumor-bearing C57BL/6 mice on day 4 following treatment with reovirus or cisplatin given as monotherapies or in combination. Cytokine levels were determined using the Mouse Bio-Plex Cytokine Assay. Columns, mean of six individual mice; bars, SE.

released a range of inflammatory cytokines and chemokines, whereas IL-10 secretion was abrogated. Reduced cytokine and chemokine production by coadministration of cisplatin may allow increase in the dose of reovirus delivered to the tumor site. We and others have shown that the efficacy of reoviral cytotoxicity is increased in the setting of immune suppression mediated through reduction in humoral and cellular immunity with cyclosporine and cyclophosphamide (5, 13, 35).

However, the abrogation of a local inflammatory cell kill by reovirus in this way may also be undesirable ultimately as it may reduce the efficiency of immune priming at the site of reoviral cytolysis and reduce migration of tumor-loaded antigen-presenting cells to regional lymph nodes and the possibility of generating antigen-specific T-cell immunity against the tumor (36). In addition, blunting the inflammatory response may also have a detrimental effect by counteracting the increased virus-associated vascular permeability and vasodilation, thereby potentially restricting viral distribution within tumor (37).

The combination of systemic reovirus and several chemotherapeutic agents is currently being evaluated as phase I studies (REO-). These include attempts to enhance cytotoxicity with gemcitabine (REO09), docetaxel (REO10), and carboplatin/paclitaxel (REO11, REO15, and REO16) in several indications. We are also attempting to exploit the immune-modulating potential of chemotherapy using escalating doses of cyclophosphamide to attenuate the NARA response to reovirus (REO12).

Disclosure of Potential Conflicts of Interest

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