Synergistic effects of oncolytic reovirus and cisplatin chemotherapy in murine malignant melanoma

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Statement of translational relevance

Oncolytic reovirus is a potentially attractive cancer therapeutic in view of its intrinsic targeting of cancer cells with dysregulated ras pathway signalling (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 demonstrates 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.

Abstract

Purpose

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

Experimental design

Effects of reovirus +/- chemotherapy on *in vitro* cytotoxicity and viral replication were assessed using MTS assay and plaque assay. Interactions between agents were assessed by combination index (CI) analysis. Mode of cell death was assessed by annexin/PI FACS based assays; gene expression profiling of single versus combination treatments were completed using the Agilent microarray system. Single agent and combination therapy effects were tested *in vivo* in two immune competent models of murine melanoma.

Results

Variable degrees of synergistic cytotoxicity between live reovirus and a number of chemotherapy agents were observed in B16.F10 mouse melanoma cells, most significantly with cisplatin (CI 0.42+/-0.03 ED50). Combination cisplatin and reovirus exposure led to increased late apoptotic/necrotic cell populations. Cisplatin almost completely abrogated the inflammatory cytokine gene upregulation induced by reovirus. Combination therapy led to significantly delayed tumour 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 a number of chemotherapeutic agents synergistically enhanced cytotoxicity in human and murine melanoma cells lines *in vitro*, and murine tumours *in vivo*. The data supports the current reovirus/chemotherapy combination phase I clinical studies currently ongoing in the clinic.

Introduction

There has been rapid pre-clinical and clinical development of viruses with oncolytic activity as anti-cancer 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, non-pathogenic viruses that have been isolated from the human respiratory and gastrointestinal tract. They are non-enveloped 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 upregulation of upstream or downstream components of the cell signaling pathway) through either Ras mutation or up-regulated epidermal growth factor receptor signaling (4, 5, 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, 7). 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 interferons (8) and local inflammatory responses generated by reovirus-infected tumor cells causes bystander toxicity against reovirus-resistant tumor cells and activation of human myeloid dendritic cells (9). A number of phase I clinical studies of intratumoral or systemic reovirus as a single agent have been completed, with evidence of significant antitumor activity (10, 11)

We have recently demonstrated 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. Sensitisation was due to an increase in apoptosis in cells treated with combined therapy. Furthermore, *in vivo* studies using xenograft (HCT116, SW480) and syngeneic (B16.F10) tumors demonstrated enhanced activity of combined treatment relative to reovirus or radiation alone (12). 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 Dulbecco's Modified Eagle's Medium (DMEM) at 37^{o}C and 10% CO_2. K1735, another mouse melanoma, and L929, a murine fibroblast-like line, were cultured in DMEM at 37^{o}C and 5% CO_2. 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 Medium at 37^{o}C and 5% CO_2. All media were supplemented with 2mM GlutaMAX-1 supplement (Invitrogen), 100 units/ml penicillin, and 100 units/ml streptomycin (Sigma) and either 10% (v/v) fetal calf serum (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. (Calgary, Canada). Virus stock titer 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 solution 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.

Cisplatin (cis diamminedichloroplatinum; Mayne Pharma Plc, UK), DTIC (dacarbazine; Ben Venue Laboratories, Inc, Bedford, OH), gemcitabine (Ely Lilly and Co., Indianapolis, IN), paclitaxel (Bristol-Myers Squibb Company, New York, N.Y.) and carboplatin (Bristol-Myers Squibb Company, New York, N.Y.) 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 x 10^3 cells per well. After 24 hours, they were infected with known dilutions of reovirus and chemotherapeutic drug, either alone or in combination. 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 percent compared to 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, Ferguson, MO). Derived from the median-effect principle of Chou and Talalay (13), 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 performed as described for the *in vitro* survival assay using 4, 2, 1, 0.5 and 0.25 times the calculated median effective dose (ED50) of each agent in a constant ratio checkerboard design.

Reovirus one-step growth curves

Test cells were seeded overnight and then infected with reovirus MOI 10 in DMEM with 2% FCS. At various times after infection, samples were transferred to -80^{o}C. Following three freeze-thaw cycles between -80^{o}C and room temperature, lysates were collected and virus titre determined by plaque assay as described above.

Inactivation of reovirus by UV-irradiation and heat

Reovirus was UV inactivated by exposing 50µL aliquots of viral stock at 1.2 x 10^{10} pfu/mL to 720 millijoules irradiation using a Stratalinker UV Crosslinker 2400 (Stratagene, LA Jolla, CA) to cross link viral RNA. Heat inactivation was performed by heating 200µL aliquots of viral stock at 1 x 10^{9} pfu/mL for 20min at 60^{o}C.

FACS analysis of cell survival and apoptosis

Following overnight seeding, B16.F10 cells were treated with 50µM cisplatin and/or reovirus MOI 1 for 48h. 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 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 45min with 50μM Z-VAD-FMK (R&D Systems) prior to exposure to 50μM cisplatin and/or reovirus MOI 1 in DMEM with 2% FCS for 24, 48 or 72h. 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 Qiagen 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 RNeasy Mini Kit (Qiagen). 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 was extracted using the Agilent Feature Extraction software and was subsequently analysed using GeneSpring (Agilent), using the fold change of each gene based upon normalised data. Only changes calculated to be significant ($p<0.05$) are included in the results.

***In vivo* studies**

All procedures were approved by United Kingdom Home Office and institutional boards. All animal experiments were repeated at least three times. Mice were purchased from B and K Universal Ltd. Subcutaneous tumours were established in 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 $V = \pi/6\ d_1 \cdot d_2 \cdot d_3$. Animals were killed when tumour size exceeded 15mm in any one dimension.

Reovirus (1 x 10^8 pfu in 100μL 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 tumour to achieve maximal dispersal of the reovirus. It was possible to

achieve direct intratumoral injection without backflow of the injectate. Cisplatin (2.5 mg/kg) was administered intraperitoneally in a total volume of 100μL. 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 TissueLyser (Qiagen) at 30Hz for 2 minutes. Following centrifugation to clarify the virus lysate, virus titer was determined by plaque assay on L929 cells as described above and expressed as plaque forming units (pfu)/g tissue.

Serum analysis for presence of neutralizing anti-reoviral antibodies (NARA)

The methodology used for analysis of NARA has been reported recently (14). Briefly, serum samples from individual mouse groups day 4 post 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 x 10^4 cells/well and incubated overnight at 37^{o}C and 5% CO_2. Reovirus stock (3.5 x 10^{10}/ml) was added in two dilution series (2-fold and 10-fold) across the plate such that the final dilutions of the two series were 1:32,768 and 1:10^{12}. After 2 hours, the reovirus inoculum was removed and replaced with growth medium. After a further 48 hours, cell survival was measured by MTT assay. In order 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 pre-incubated with a dilution series of goat polyclonal anti-reoviral antibody and cell survival was measured at 48 hours by MTT assay.

Cytokine analysis

Culture supernatant was collected from B16.F10 cells treated with either reovirus (MOI 1) or cisplatin (50μM) either alone or in combination at 0, 6, 12, 24, 48 and 72h post treatment. Samples were stored at -80^{o}C prior to analysis using a Mouse Bio-Plex Cytokine Assay (Bio-Rad) according to the manufacturer's instructions. Levels of IL-1α, 6, 12 (p70), 17, MIP-1α, and RANTES were measured in 50μL 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^{o}C prior to analysis for cytokines as described above.

Statistical analysis

Comparisons between groups were done using the t test and 2-way ANOVA. Survival curves were estimated using the Kaplan-Meier method, and significance was assessed using the χ^2 and logrank test. Statistical analysis was performed 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 cytoxicity with less than 1% survival at MOI 0.01 at 48 hours. Figure 1a demonstrates the superiority of reovirus cytotoxity in B16.F10 lines compared to 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 x 10^3/well for 48 hours, either alone or with concomitant 10 or 100μM cisplatin (Fig 1c). An MTS survival assay was performed after 48 hours. The addition of cisplatin significantly enhanced tumor kill compared to reovirus alone across all MOIs in a cisplatin dose dependent manner (2-way ANOVA, $p<0.0001$).

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 combination indices (CI). 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 performed using 4, 2, 1, 0.5 and 0.25 times the calculated median effective dose (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 the materials section). 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 (12μM cisplatin to MOI 1 reovirus) over five two-fold dilutions for 48 hours. The combination of live reovirus with cisplatin enhanced cell kill compared to cisplatin alone. This enhancement of cell kill was not apparent when heat inactivated or UV-inactivated reovirus was used (Fig 1e) 2-way ANOVA $p<0.0001$.

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 reovirus MOI 1, cisplatin 50μM or both agents together, at 24 and 48 hours by annexin/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 prior to exposure to 50μM cisplatin and/or reovirus MOI 1 (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 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 (100μM), 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 upregulation of proinflammatory cytokines, apoptosis genes, MHC class I, oncogenes such as *Rous sarcoma*, and interestingly *epidermal growth factor receptor 1* (*EGFR1)*. A five-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 appeared to be uniquely upregulated as a result of the combined treatments (Figure 3).

Combined reovirus and cisplatin treatment enhances tumor growth delay in two immune competent 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 C57/BL6 mice and K1735 in C3H mice. For both models, 5×10^5 cells were implanted subcutaneously. Treatment was initiated when tumors reached an average diameter of 4.5-5.5mm at approximately 10-12 days for B16.F10 and 8-10 days for K1735. Mice were treated with intratumoral reovirus, intraperitoneal cisplatin or both on day 0 and day 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 15mm 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 (2-way ANOVA $p<0.0001$), although both cisplatin and reovirus monotherapies resulted in delayed tumor growth (Fig 4a). The median survival for the control, cisplatin, reovirus and combination groups were 6,

8, 12 and 17 days respectively (Fig 4b) logrank test p=0.0003. 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 tumours and organs at day 4 post therapy. 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. cisplatin on days 0 and 3, and following sacrifice on day 4 viral titre in tumour, liver, lungs and heart was determined using plaque assay (Fig 5a). The highest viral yield was in resected tumour as expected, with over three times more virus 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 (more than 5 logs) than tumour. No difference in virus titre 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 tumour had reached maximum allowed size (15mm 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 3/6 tumors and also 4/6 livers (Table 1c).

Cisplatin does not affect the humoral response to reovirus

The antitumor effects of reovirus have been enhanced by the co-administration of agents which 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 (15). Using a recently reported assay (14), we assessed the neutralizing anti-reovirus antibody (NARA) response to reovirus and reovirus/cisplatin combination in C57BL/6 mice. We found equivalent endpoint 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 demonstrated marked reductions in the expression of a range of pro-inflammatory cytokine genes. Culture supernatant was collected from B16.F10 cells treated with either reovirus

(MOI 1) or cisplatin (50μM) alone or in combination at 0, 6, 12, 24, 48 and 72h post treatment. Samples were stored at -80^{o}C prior to 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-1α 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).

Discussion

In this study, we have demonstrated evidence of synergistic anti-cancer 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 which has a modest response to conventional chemotherapy and therefore justifiably a target for novel cancer therapies (16). Synergistic cell kill was observed across a number of 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.

A number of 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 intratumoral Onyx-015 with systemic cisplatin and 5-fluorouracil (5FU) when compared to chemotherapy alone (17). E1A-expressing adenoviral E3B mutants combined with cisplatin and paclitaxel (18) showed synergistic activity *in vitro* and *in vivo*. A combination of oncolytic herpesviruses such as G207, HSV-1716, or NV1066, with chemotherapeutic agents shown higher antitumor 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* (19, 20, 21, 22). 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 (23, 20, 24). However, no increase in viral replication was observed in other studies such as the NV1020 in combination with 5-FU, SN38 (irinotecan), or with oxaliplatin where viral replication was clearly reduced if chemotherapeutics were added before, simultaneously, or after virus treatment (25). Other similar combination studies have shown either no effect of chemotherapeutics on viral replication (26,27, 28), or actually reduced viral replication (29). Recently, synergistic cytotoxic effects of rat parvovirus H-1PV and gemcitabine in a pancreatic cancer model were observed in gemcitabine-resistant cells lines (30).

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 (10,11). 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, utilize 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.

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* (12). Consistent with our findings in human melanoma lines (9), 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 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/propidium iodide 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 post injection indicated increased intratumoral viral replication with combination therapy. This appears 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 (31). Furthermore, the observation of high levels of reovirus in tumour in the combination group, which then were reduced to lower levels than reovirus alone may be explained by the increased tumour destruction in the combination group, and thereby less tumour mass later to support viral replication. The persistence of reovirus in liver 10 days post 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 upregulation of proinflammatory cytokines and activation of pro-apoptosis genes such as

NF-κB. Similar effects have been reported in previous microarray analyses of reovirus infected HEK293 cells and murine neural cells (32, 33). Notably, we found co-treatment 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 co-treatment with cisplatin. EGFR has been shown to enhance reoviral infection efficiency by what would appear to be the opportunistic use of an already activated signal transduction pathway (34). The transfection of relatively resistant cell lines with the EGFR gene confers significantly higher susceptibility to reovirus infection (34) 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 both in 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 (35). 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 (15). 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/media conditions (36). Cisplatin (cis diamminedichloroplatinum(II)) forms primarily intrastrand crosslink adducts, which activate several signal transduction pathways, including those involving ATR, p53, p73, and MAPK, and culminate in the activation of apoptosis. Cisplatin exposure induces IL-6, G-CSF, βFGF secretion from specific cell lines (36).

We recently reported that reovirus-induced death of other human melanoma cell lines and human melanoma explants released a range of inflammatory cytokines and chemokines, while IL-10 secretion was abrogated. Reduced cytokine and chemokine production by co-administration 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 (7, 14, 37).

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, 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 (38). 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 tumour (39).

The combination of systemic reovirus and a number of chemotherapeutic agents is currently being evaluated as phase I studies (REO-). These include attempts to enhance cytotoxicity with gemcitabine (REO 09), docetaxel (REO10) and carboplatin/paclitaxel (REO11, REO15 and REO16) in a number of 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).

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