Epithelial ovarian cancer (EOC) is the most lethal of the gynaecologic malignancies as 75% of cases are diagnosed after extensive metastasis. EOC metastasis involves shedding of cancer cells from the primary tumor directly into the peritoneal cavity where EOC cells form multicellular aggregates or spheroids that implant in and invade the peritoneal mesothelium to initiate secondary tumors. EOC spheroids undergo dormancy in suspension to promote survival and in turn demonstrate resistance to standard chemotherapeutics such as carboplatin. In an in vitro model of EOC metastasis, we have recently demonstrated that Myxoma virus (MYXV) effectively kills adherent EOC cells and reduces spheroid reattachment. In order for MYXV to advance toward clinical use as a first-line therapy for ovarian cancer, its compatibility with current chemotherapeutics must be assessed. We propose that combination treatment using MYXV and carboplatin will decrease the viability of EOC spheroids. Herein, we show that carboplatin does not impede MYXV replication or oncolytic potential in EOC cells in monolayer or spheroid culture. Administration of different sequential regimens of carboplatin and virus does not enhance sensitivity to oncolysis of EOC cell lines in monolayer culture. Furthermore, co-treatment of MYXV and carboplatin did not consistently induce oncolysis in spheroids generated from EOC cell lines or patient ascites. However, upon reattachment, spheroids treated with various combination regimens revealed additive cytopathic effects. Remarkably, we also encountered a patient sample which responded to MYXV only in the presence of carboplatin suggesting that chemotherapy-sensitive primary EOC spheroid cells are possibly eliminated by MYXV-mediated oncolysis during co-treatment. We hope to include more patient sample analyses that allow for strong pre-clinical evaluation of this combination treatment.

Keywords: Myxoma virus/MYXV, Ovarian cancer, Spheroid, Carboplatin, Oncolytic virotherapy
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INTRODUCTION

Ovarian cancer is the most lethal of the gynecologic malignancies[1] as 75% of cases are diagnosed at late stages when prognosis is poor. Epithelial ovarian cancer (EOC) accounts for 90% of all human ovarian tumors which show remarkable diversity in clinical, morphological and molecular presentation[2]. Ovarian cancer metastasis does not follow the traditional model of blood-borne disease; rather, it involves shedding of cancer cells from the primary tumor directly into the peritoneal cavity[3]. Malignant EOC cells promote the build-up of a voluminous exudative fluid in the abdominal space termed ascites, a distinct feature of advanced-stage ovarian cancer[4]. Furthermore, EOC cells often form multicellular aggregates or spheroids that implant in and invade the peritoneal mesothelium thereby seeding metastatic disease[5].

Treatment for ovarian cancer begins with cytoreductive surgery to remove secondary tumors found throughout the abdominal cavity. It is then followed by adjuvant chemotherapy often involving the administration of a platinum-based agent (cisplatin or carboplatin) coupled with a taxane (paclitaxel)[6]. These drugs function by interfering with mechanisms that control cell proliferation; the platinum-based therapies prevent DNA replication and taxanes affect microtubule formation[6]. The platinum-taxane regimen elicits a positive initial response but most EOC patients face recurrent chemo-resistant disease. Heterogeneous EOC spheroids display anchorage-independent growth, dormancy and anti-apoptotic properties that allow them to escape the selective pressures of these traditional treatments[5, 7, 8]. Clinical trials have been exploring the use of other cytotoxic drugs and molecular-targeted agents against EOC but neither has shown significant responses in women with platinum-resistant disease[9]. It is clear that novel therapeutic strategies are necessary to better eradicate metastatic EOC cells and improve overall patient survival.
Over the last two decades, viral oncolytics has emerged as a strong area of research for cancer treatment. Oncolytic virotherapy relies on naturally-occurring or genetically-modified viral strains to preferentially infect and lyse cancer cells, while leaving normal cells intact[10]. A wide variety of oncolytic viruses have been applied to EOC models; some examples include conditionally-replicating adenovirus, reovirus, and vesicular stomatitis virus[11]. Myxoma virus (MYXV), a rabbit poxvirus, is a promising candidate with antitumoral effects reported across many cancer types[12]. The efficacy of MYXV infection depends on the interaction between viral protein M-T5 and activated PI3K-AKT cellular signaling[13, 14], a pathway commonly hyper-activated in human cancers including ovarian cancer. MYXV has been shown to infect and replicate within immortalized human EOC cell lines[14, 15, 16]. Correa et al. were the first to demonstrate susceptibility to MYXV infection using primary human EOC cells directly isolated from patient ascites[16]. Adherent cultures of EOC cells were effectively killed by MYXV while EOC spheroids permitted the virus’ entry into cells but were resistant to MYXV-mediated cell-killing. However, when MYXV-infected EOC spheroids were permitted to reattach by reintroduction into adherent culture (to model secondary metastasis), the spheroids gained sensitivity to viral oncolysis[16]. AKT activity was described as the molecular basis responsible for the differential success of MYXV-mediated oncolysis throughout the metastatic process[16]. These findings suggest that advanced-stage EOC could potentially be targeted and eliminated by MYXV oncolytic virotherapy.

In order for an oncolytic virus such as MYXV to advance toward clinical use as a first-line therapy for ovarian cancer, its compatibility with the current standard of care for EOC patients must be assessed. Chemotherapy is often criticized for its toxicity and lack of specificity as it targets all proliferating cells instead of just tumor cells[9] while oncolytic viruses, natural or genetically engineered, are known for their selective tropism for cancer cells[10]. These
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disparate models of antitumoral activity may result in synergistic therapeutic benefits[17]. Moreover, EOC spheroids have shown a reduced chemotherapeutic response *in vitro*[7] as well as resistance to MYXV-mediated cell killing[16]. It is possible that dual administration of both treatments could positively alter EOC spheroid responses. It was hypothesized that combination treatments using MYXV and carboplatin would have cytopathic effects on EOC metastasis, particularly in spheroids derived from ovarian cancer cell lines and patient ascites.

**MATERIALS & METHODS**

**Cell culture.** The ovarian cancer cell line HEY was purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in RPMI medium. The ovarian cancer cell line HEY A8 (obtained from Dr. Gordon Mills, MD Anderson Cancer Center, Houston, TX) and baby green monkey kidney (BGMK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wisent, St. Bruno, Canada). OVCA429 cells (gift of Dr. Barbara Vanderhyden, OHRI) were cultured in alpha MEM. All media were supplemented with 5% fetal bovine serum (FBS; Wisent) and 50 μg/ml penicillin–streptomycin (Wisent). Cells were maintained in a 37°C humidified atmosphere of 95% air and 5% CO₂.

Ascitic fluid collected from chemotherapy-naïve patients at time of paracentesis or debulking surgery was used to generate primary human EOC cell cultures as described previously[18]. Primary cultures of ascites cells were maintained in a 1:1 mixture of MCDB 105 medium: medium 199 medium (Sigma, St. Lewis, MO) supplemented with 10% FBS (Wisent) and 50 μg/ml penicillin–streptomycin (Wisent). All work with patient materials has been reviewed and approved by the university Human Subjects Research Ethics Board.

Adherent cells were maintained on tissue culture-treated polystyrene (Sarstedt, Newton, NC). Spheroids were maintained on Ultra-low Attachment (ULA®) cultureware (Corning,
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Corning, NY), which is coated with a hydrophilic, neutrally-charged hydrogel to prevent cell attachment. We have determined empirically that seeding single cell suspensions of 50,000 cells/ml using ULA® tissue culture plastic and allowing three days for aggregation results in multi-cellular spheroids that closely resemble those observed in patient-derived ascites. Media changes in spheroid culture consisted of the careful removal of 800 µL of media from individual wells of 24-well ULA® cluster dishes while leaving spheroids untouched and the addition of equivalent volume of fresh complete growth medium. Spheroids were reattached to standard tissue culture plastic by transferring spheroids in a volume of <200 µL from individual wells of 24-well ULA® cluster dishes to individual wells of 24-well tissue culture-treated plates containing 800 µL of complete growth medium. These approaches are applicable to working with both cell lines and primary cells (Fig. 1).

**Virus and drug treatments.** Preparation and titering of MYXV (*i.e.* vMyxGFP[19]) using BGMK cells was performed as described previously[20]. For preparation of UV-inactivated MYXV, virus stock was UV-irradiated for 5 minutes using a Stratalinker UV crosslinker (Stratagene); this incubation time was determined empirically to inactivate vMyxGFP to undetectable fluorescence in BGMK cells at MOI 10. Primary EOC cells and cell lines were infected with MYXV by adding serially-diluted virus (diluted in growth media) to cells to achieve various MOIs, as indicated in each experiment. Virus infection was monitored over 24-96 hours by phase contrast and fluorescence microscopy using an Olympus IX70 inverted microscope and photos were acquired using ImagePro image capture software.

Carboplatin (Bristol Myers Squibb) was purchased from the London Health Sciences Centre pharmacy (London, ON, Canada) as a powder and stored at 4°C. Stock solutions were prepared in MilliQ water at 10 mM and further diluted in complete growth media for treatments. Stock solutions were stored at -20°C. DMSO diluted in complete growth medium was
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Figure 1. Modelling intra-peritoneal EOC metastasis in an *in vitro* tissue culture system. EOC metastasis occurs when cells are shed from the primary tumour and aggregate into multicellular spheroids that eventually implant in the peritoneal mesothelium to induce secondary tumour formation. To model this process *in vitro*, isolation and primary culture of patient ascites-derived EOC cells is performed; a similar approach can be applied to work with ovarian cancer cell lines. EOC cells can be grown in monolayer or seeded to ULA® cultureware to generate spheroids in suspension. Spheroids transferred to normal tissue culture plastic will typically adhere and grow out. Phase contrast images depicting the various culture conditions reveal that cultured EOC spheroids closely resemble those observed directly in patient ascites.
temporarily used as a vehicle control. Carboplatin dose response curves were constructed for specific ovarian cancer cell lines in adherent and spheroid cultures to determine the optimal doses for combination treatment experiments.

MYXV (M) and carboplatin (C) treatments were initiated simultaneously, M/C, or sequentially, M+C and C+M. In all three models, EOC cells were seeded 24h prior to treatment to form adherent monolayers or EOC cells were seeded to wells of ULA® plates and allowed to form spheroids for 3 days prior to treatment. In this report, we share many timelines of treatment that were explored in preliminary studies. The most efficient treatment timeline for EOC spheroids derived from ovarian cancer cell lines demands a 12 day *in vitro* experiment for M/C, C+M and M+C as follows: day 0-3 for spheroid formation as described above, day 3 marks treatment 1 (M/C, C or M), day 6 marks treatment 2 (media change alone, media change and M, or media change and C), day 9 marks spheroid reattachment as described above, day 12 marks spheroid dispersion assay as described below (Fig. 2).

**Cell number and viability assays.** Viability of spheroids post-treatment was assessed using alamarBlue® (Invitrogen, Burlington, ON), as per manufacturer’s instructions with some modifications. In pilot experiments to determine optimal incubation times, day 6 spheroids post-co-treatment were incubated from 4 h to overnight with readings taken at several timepoints; spheroids formed from cell lines gave the most reliable readings after 6 h and spheroids generated from patient samples gave comparable results for 6 h to overnight incubation, hence these were adopted for future studies. In one method, 50% of the culture medium of spheroid cultures (500µL per well of 24-well plate) was removed carefully and an equal volume alamarBlue® reagent diluted 1:10 in complete medium was added. Cultures were incubated for 6 h at 37°C in a humidified atmosphere of 95% air and 5% CO2, and fluorescence was then quantified with a Wallac plate reader using 560/590 nm excitation/emission filter settings. In the
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**MYXV + Carboplatin (M/C)**

- **Day 0**: Seed to form spheroids
- **Day 3**: Carboplatin + MYXV
- **Day 6**: Change media (remove treatment)
- **Day 9**: Reattach spheroids
- **Day 12**: Fix, stain and image

**Carboplatin → MYXV (C+M)**

- **Day 0**: Seed to form spheroids
- **Day 3**: Carboplatin
- **Day 6**: Change media + MYXV
- **Day 9**: Reattach spheroids
- **Day 12**: Fix, stain and image

**MYXV → Carboplatin (M+C)**

- **Day 0**: Seed to form spheroids
- **Day 3**: MYXV + carboplatin
- **Day 6**: Change media
- **Day 9**: Reattach spheroids
- **Day 12**: Fix, stain and image

**Figure 2.** Combination treatment regimens for EOC spheroids derived from ovarian cancer cell lines demand 12-day *in vitro* experiments. Treatments included: MYXV (MOI 1, 5) or UV-inactivated MYXV control and carboplatin (0, 25, 50 µM). Treatment timelines for M/C, C+M and M+C are as follows: day 0-3 for spheroid formation, day 3 marks treatment 1 (M/C, C or M), day 6 marks treatment 2 (media change alone, media change and M or media change and C), day 9 marks spheroid reattachment, day 12 marks spheroid dispersion assay. Quantification of spheroid dispersion areas serves as the means of characterizing the cytotoxicity of combination treatments.
other method, treated EOC spheroids were transferred to standard tissue culture plastic to facilitate reattachment as described above. Three days post-reattachment, cell viability was assessed using alamarBlue® reagent as described above but with overnight incubation. Attached spheroids were then fixed, stained and visualized for qualitative assessment of cell viability.

The effects on cell proliferation in adherent culture by the drug or virus alone or in combination treatments were considered. At the assay time point, adherent cells were washed with PBS, trypsinized and counted on a Beckman Coulter Z1 Particle Counter (Beckman, Mississauga, ON, Canada), as per manufacturer’s instructions.

**Spheroid dispersion assay.** Spheroids were transferred in a volume of <200 µL from individual wells of 24-well ULA® cluster dishes to individual wells of 24-well tissue culture-treated plates containing 800 µL of complete growth medium to facilitate reattachment. After three days, dispersing spheroids were fixed and stained using Hema-3 Stain kit (Fisher, Kalamazoo, MI). Reattached spheroids were visualized using an Olympus stereomicroscope and ImagePro image capture software in earlier studies and a Zeiss stereomicroscope and EyeImage image capture software in later studies. The dispersion area of individual reattached spheroids was quantified using NIH ImageJ (NIH, Bethesda, MD) area measurement tool.

**RESULTS**

We performed MYXV and carboplatin co-treatment experiments in human ovarian cancer cell lines and primary EOC cells and initiated sequential treatment studies in cell lines. An *in vitro* tissue culture system effectively modelling stages of intra-peritoneal EOC metastasis allows for the testing of MYXV and carboplatin as a combination treatment for ovarian cancer (Fig. 1). Studies were primarily performed in spheroid culture with a few parallel studies in adherent culture. Spheroid culture reflects the dormant, often drug-resistant cellular aggregates
that function as the metastatic vehicles \textit{in vivo} and thus demands our focus whereas monolayer is thought to represent the primary tumor\cite{5, 7}. Reattachment of spheroids post-treatment assesses their capability for secondary tumor formation and therefore acts as a measure of EOC spheroid viability (Fig. 1).

\textbf{Preliminary co-treatment studies in EOC spheroids}

A study by Correa \textit{et al.} states that EOC spheroids are effectively targeted by MYXV with slower infection kinetics but this initial delay is overcome with prolonged infection \cite{16}. We show that this ability of MYXV to enter and replicate in ovarian cancer spheroids is not affected by co-treatment with carboplatin, as evidenced by visualization of GFP expression from the vMyxGFP recombinant virus that reveals almost entirely GFP positive spheroids (Fig. 3).

Although EOC spheroids are susceptible to MYXV infection, it has been demonstrated that the virus is unsuccessful at reducing the overall bulk viability (\textit{i.e.} induce oncolysis) of cells in spheroids\cite{16}. To determine if MYXV and carboplatin co-treatment could have stronger cytopathic effects, we performed alamarBlue\textsuperscript{®} fluorometric viability assays on EOC spheroids after three days of treatment. Prior to their use in this study, monolayer cultures of OVCA429 and HEY ovarian cancer cell lines and ascites-derived primary EOC cells (EOC33 and EOC57) were all shown to be sensitive to MYXV-mediated oncolysis as seen by effective cell death at the three MYXV doses, MOI 0.1, 1, and 10 (data not shown). All spheroids formed from the tested cell lines and patient samples display a 50\% reduction in cell viability after exposure to carboplatin alone, as expected from the known IC\textsubscript{50} (Fig. 4). However, the spheroids formed from patient samples yield overall lower values for cell viability than the spheroids formed from cell lines across all treatments. This is likely attributed to the fact that EOC patient samples are generally less metabolically active than cell lines (data not shown) in culture so their ability to respond to the alamarBlue\textsuperscript{®} reagent is already hindered prior to treatment. Spheroids demonstrate
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Figure 3. Susceptibility to MYXV infection in EOC spheroids is not affected by co-treatment with carboplatin. After three days of spheroid formation, OVCA429 spheroids were infected with MYXV (MOI 10) or UV-inactivated MYXV control and simultaneously dosed with carboplatin (50 µM) or DMSO vehicle control. Phase contrast and fluorescence images were captured 24 h post-treatment to assess MYXV infection.

Figure 4. MYXV and carboplatin co-treatment is not effective in reducing viability of EOC spheroids. OVCA429 and HEY cells and two independent ascites-derived primary EOC samples (EOC33 and EOC57) were grown in spheroid culture. After three days of spheroid formation, spheroids were co-treated with MYXV (MOI 0.1, 1, 10) or UV-inactivated MYXV control and carboplatin (50 µM) or DMSO vehicle control. At 72 h post-treatment, cell viability was assessed using alamarBlue® reagent with 6h incubation. The cytotoxicity of the co-treatment is not notably different from single agent carboplatin-induced cytotoxicity across the subset of analyzed cell lines and patient samples.
no benefit in response upon co-treatment with carboplatin compared to MYXV alone. EOC spheroids subjected to this co-treatment also displayed minimal difference in viability in comparison to those dosed with single agent carboplatin (Fig. 4). Co-treatment is unable to induce additive oncolysis in EOC spheroids under these particular conditions.

Correa et al. assessed the efficacy of MYXV infection across a number of independent patient ascites samples to reveal a differential sensitivity to MYXV infection that highlights the heterogeneity and complex genetic make-up of ovarian cancer [16]. In continuing to evaluate the co-treatment strategy in spheroids formed from primary EOC samples (EOC138, EOC154 and EOC149), we performed alamarBlue® fluorometric viability assays on EOC spheroids post-reattachment. EOC138 and EOC154 responded similarly to previously discussed patient samples. On the other hand, EOC149 cell viability is reduced during co-treatment to a greater extent than carboplatin with UV-inactivated MYXV. This suggests EOC138 and EOC154 to be carboplatin-resistant while EOC149 may be carboplatin-sensitive (Fig. 5A). When the attached spheroids are fixed and stained, EOC154 spheroids disperse to a greater extent than EOC149 spheroids where carboplatin-induced cytotoxicity interferes with proliferation (Fig. 5B).

**Combination treatment studies**

Wennier et al. investigated sequential use of MYXV and gemcitabine treatment against pancreatic cancer cells to show that the combination regimen successfully increases oncolysis but found the optimal order of treatment administration to be cell type dependent[21]. Given that simultaneous administration of MYXV and carboplatin did not result in significant synergy, sequential combination treatments involving these anticancer agents demanded evaluation. We thus explored three regimens, M/C co-treatment, M+C and C+M sequential treatments with continually evolving timelines. Experiments were conducted in OVCA429 and HEY A8 cell
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Figure 5. Chemotherapy-sensitive primary EOC spheroid cells are effectively eliminated by MYXV-mediated oncolysis during co-treatment. Three independent ascites-derived primary EOC samples (EOC138, EOC154 and EOC149) were grown in spheroid culture. Three-day spheroids were co-treated with MYXV (MOI 0.1, 1, 10; MOI 1 visualized) or UV-inactivated MYXV control and carboplatin (50 µM) or DMSO vehicle control. At 72 h post-treatment, spheroids were reattached to standard tissue culture plastic. (A) At 72 h post-reattachment, cell viability was assessed using alamarBlue® reagent with overnight incubation. Co-treatment in E0138 and EOC154 spheroids shows no benefits to MYXV alone. In contrast, EOC149 exhibits a response to MYXV that is only observed by co-treatment with carboplatin. These differences between patient samples could be attributed to chemosensitivity thus deeming EOC154 carboplatin-resistant and EOC149 carboplatin-sensitive. (B) Attached spheroids were fixed, stained and visualized by phase contrast microscopy to qualitatively confirm the chemosensitivity index of EOC patient samples.
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lines in both adherent monolayers and spheroids to consider any differences in treatment response both across different cell lines and between culture conditions.

**Adherent culture.** Combination treatment studies in OVCA429 monolayer reveal that carboplatin does not impede MYXV replication or oncolytic efficacy in adherent culture as the anticancer agents are able to successfully stimulate oncolysis in this cell line regardless of the order of treatment. A basic dose-dependent increase in cytotoxicity is visible and only differs between the regimens at 25 µM likely because M+C and C+M are exposed to carboplatin earlier than M+C. There appears to be no differential sensitivity to cell death based on type of combination treatment in OVCA429 adherent cells (Fig. 6).

For HEY A8 adherent cells, carboplatin dose response experiments were conducted in systems that mimicked C+M and M+C. Cells seeded at 10,000 cells/ml became too confluent during the treatment regimen and cell growth plateaued. Carboplatin targets proliferating cells and thus sensitivity to carboplatin was impaired in experiments using 10,000 cells/ml making 5,000 cells/ml the optimal seeding density (Fig. 7A, B). We were able to select a single dose of carboplatin (10 µM) which yielded 25% cell killing so that we could clearly observe any potential additive or synergistic effects upon combination with MYXV in monolayer (Fig. 7A, B). One follow-up M+C co-treatment experiment was completed in HEY A8 adherent cells using these determined conditions and yielded a clear dose-dependent decrease in cell proliferation but the other two regimens need to be explored in HEY A8 monolayer for comparison (Fig. 7C).

**Spheroid culture.** M/C, C+M and M+C combination regimens were performed in spheroid culture in 12-day *in vitro* experiments (Fig. 2). Preliminary cytotoxicity profiling experiments revealed the need for 3 days between all manipulations (data not shown). Dispersion area was
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Figure 6. MYXV and carboplatin display no antagonism and are able to induce oncolysis of OVA429 cells in monolayer irrespective of order of administration. OVCA429 cells were seeded at 10,000 cells/well in monolayer culture at day 0. Treatments included: MYXV (MOI 0.1, 1, 10) or UV-inactivated MYXV control and carboplatin (0, 10, 25 μM). Treatment timelines for M/C, M+C and C+M are as follows: day 1 marks treatment 1 (M/C, C or M, respectively), day 3 marks treatment 2 (media change alone, media change and M or media change and C, respectively), day 5 marks cell counting. Considering the nearly identical trend visible at 10 μM across all three regimens, order of administration appears to have no effect on combination treatment efficacy in OVCA429 monolayers.
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Figure 7. Carboplatin dose response curves for HEY A8 cells in adherent culture reveals optimal seeding density and carboplatin dose for future combination treatments. HEY A8 cells were seeded at (A) 5000 cells/ml and (B) 10,000 cells/ml on day 0 and counted using the Coulter Counter at day 5. Carboplatin Day 1, thought to mimic C+M, represents cells dosed with carboplatin (0, 10, 25, 50 µM) on day 1 with a media change on day 3. Carboplatin Day 3, thought to mimic M+C, represents both a media change and carboplatin treatment on day 3. The optimal seeding density was determined to be 5000 cells/ml and the optimal carboplatin dose to achieve 25% cell killing was determined to be 10 µM. (C) The M/C co-treatment was then evaluated in adherent HEY A8 cells using these conditions. Cells seeded at 5000 cells/ml were co-treated with MYXV (MOI 0.1, 1, 10) or UV-inactivated MYXV control and carboplatin (0, 10 µM) on day 1; media was changed on day 3; cells were counted using the Coulter Counter on day 5. All treatments were performed in duplicate and two counts were performed per replicate. A basic dose-dependent increase in cytotoxicity is visible.
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quantified from images of fixed and stained re-attached spheroids to evaluate the efficacy of combination treatments.

For combination treatment studies conducted in HEYA8 and OVCA429 cell lines, spheroids of comparable sizes were visible to the naked eye in all wells prior to fixation but in reality, the extent of reattachment and therefore viability of spheroids varied quite greatly between treatments, as evidenced by images of fixed and stained spheroids (Fig. 8). In the MOI 1 treatment group in M/C in HEYA8 spheroids, cell viability is reduced to a greater extent upon combination with carboplatin than the virus-infected spheroid (Fig. 8A). In OVCA429 spheroids, C+M is least effective in inducing additive oncolysis across all of the tested MOIs. In the MOI 1 treatment group in M/C and M+C in OVCA429 spheroids, combination treatment decreases the number of cells emanating away from the spheroids compared to virus or drug controls (Fig. 8B). Single agent carboplatin has cytopathic effects during C+M but not M/C in HEYA8 spheroids (Fig. 8A) and has the opposite effect in OVCA429 spheroids (Fig. 8B). This is an unusual finding given that carboplatin is administered and removed at the exact same timepoints in both M/C and C+M in both cell lines. Equally peculiar is the absence of spheroids under control conditions at MOIs 5 and 10 in OVCA429 (Fig. 8B). These observations are likely attributed to variability in experimental conditions.

DISCUSSION

Given the established role of carboplatin in the management of ovarian cancer, the prospect of combining this chemotherapy with MYXV is of high clinical relevance; our results provide the first consideration of this innovative anticancer strategy tested not only in human ovarian cancer cell lines, but also in primary human EOC cells. By focussing studies on our in vitro spheroid culture model system that mimics tumor pathobiology in a three-dimensional environment, we
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Figure 8. EOC spheroids subjected to different carboplatin and MYXV combination regimens reveal varying responses to treatment between regimens and across cell lines. (i) (A) HEYA8 and (B) OVCA429 spheroids were subjected to M/C, C+M and M+C combination regimens. Representative phase contrast images of fixed and stained spheroids at the completion of the regimens are shown. (ii) ImageJ software was used to quantify dispersion area from images of 3-10 attached spheroids per cell line and treatment. Single agent carboplatin affects HEYA8 spheroid viability in C+M but not M/C and vice versa in OVCA429 spheroids. (A) HEYA8 spheroids exhibit a response to MYXV at MOI 1 during M/C that is only observed by combination with carboplatin treatment. (B) In C+M, combination does not enhance sensitivity to oncolysis. In the MOI 1 treatment group in M/C and M+C, combination with carboplatin substantially reduces the number of cells migrating away from the attached spheroids compared to spheroids treated with virus or drug alone.
have initiated an evaluation of this combination treatment at a therapeutically resistant and critical stage of the EOC metastatic process [7, 16].

Carboplatin binds and cross-links cellular DNA and the subsequent recognition of DNA damage triggers apoptotic pathways [22]. Given that the drug is a DNA alkylating agent, it could be imagined to have a negative influence on viral replication and decrease the pathogenicity of MYXV as an oncolytic virotherapeutic [17, 23]. Indeed, many chemotherapy and virus combinations are not successful due to such adverse interactions [21, 24]. We herein show that there is no inhibitory effect on MYXV replication or MYXV oncolytic potential in EOC cells in monolayer or EOC spheroids infected in the presence of carboplatin suggesting that the two treatments are appropriate for simultaneous administration. Moreover, the reduced ability of MYXV-infected spheroids to reattach and disperse is not negatively affected by treatment with the drug [16]. Therefore, carboplatin does not represent an impediment to the effectiveness of MYXV-mediated oncolysis in controlling EOC metastasis.

When evaluating co-treatment efficacy in spheroids generated from EOC cell lines and patient samples, neither the 6-day nor 9-day treatment timeline reliably induces oncolysis. Consistent with the findings of Correa et al., spheroids maintained a resistance to MYXV-mediated cell killing and this resistance is not notably attenuated by co-treatment with carboplatin at any of the tested MOIs [16]. It is important to consider that the poor compatibility of our EOC spheroid culture system and the alamarBlue® fluorometric assay may be compromising our results. The assay relies on the reducing power of healthy living cells to convert the reagent into a detectable fluorescent product which is used as an indicator of cell viability. It has been shown that EOC cells present in multi-cellular spheroids enter a state of cellular quiescence with significant reduction in cell proliferation [8]. Studies also propose a role of autophagy in regulating dormancy in human ovarian cancer cells [25]. As seen in untreated
control samples with poor reduction of alamarBlue® reagent, the dormant phenotype of EOC spheroids may be masking the effects of the co-treatment. Perhaps the strongest modified version of the alamarBlue® assay was when it was conducted on reattached spheroids due to their more metabolically active phenotype but this method still presents problems for alamarBlue reagent penetration when working with more dense spheroids.

The identification of an accurate direct viability assay well suited to spheroid culture has obviously been a concern for our experimental design as discussed in difficulties using metabolic readouts in the last paragraph. Given that EOC spheroids possess chemoresistance [7] and slower MYXV infection kinetics[16], any effect of the combination regimens on spheroids could possibly only come into play meaningfully upon reintroduction into adherent culture when the cells of the spheroid are metabolically reactivated from dormancy. The spheroid dispersion assay was thus adopted as the most appropriate means of evaluating the effects of MYXV and carboplatin combination treatments on EOC spheroids in vitro. Interestingly, in extending the co-treatment regimen to 12 days, allowing whatever virus or drug that was in the spheroids to remain there for an extra 6 days, and in adopting the spheroid dispersion assay, responses to co-treatment were observed. In fact, out of all the combination regimens tested, HEYA8 spheroids responded best to M/C.

Wennier et al. conducted in vitro and in vivo studies that showed MYXV and gemcitabine could be combined sequentially to treat pancreatic tumors in mice but the critical determinant was a cell type-dependent timing of virus administration[21]. Similarly, our findings differ both between HEYA8 and OVCA429 cell lines and in between adherent and spheroid cultures. MYXV was shown to sensitize pancreatic cancer cells to the chemotherapy in immunocompetent mice[21]. In our studies in OVCA429 spheroids, the M/C and M+C regimens at level of the MOI 1 treatment group, the success of the combination treatment suggests that
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MYXV may be sensitizing the spheroids to carboplatin. In these early sequential treatment studies, the findings could be attributed to variations in experimental conditions. For example different passage numbers of cell lines were used for each regimen but ideally, all regimens within one cell line should be conducted with the same culture of cells. Moreover, MYXV MOI 5 was too high for HEYA8 spheroids that the cytopathic effect was too strong without carboplatin and 50 μM carboplatin is too strong for OVCA429 spheroids such that it masks the effects of the combination regimen. Therefore, all experiments must be repeated to make more definitive conclusions.

The phenomenon of virotherapy sensitizing cancer cells to chemotherapy and vice versa are both being heavily explored in the field of oncolytics across many different cancer types[17]. In ovarian cancer, mortality is often associated with treatment failure after the development of drug resistance[22]. It therefore remains highly significant to test the possibility of MYXV as an adjuvant treatment for platinum-resistant tumors. This can certainly be explored in vitro using cell lines and primary EOC samples that demonstrate low sensitivity to carboplatin, such as those we have encountered in our preliminary experiments. We hope to include more analyses in patient samples that allow for strong pre-clinical evaluation of this combination treatment and concurrently elucidate the specific mechanisms involved in oncolysis driven by carboplatin and MYXV that are likely entrenched within the underlying biology of EOC metastasis.

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