

Synergistic anti-tumor efficacy of immunogenic adenovirus ONCOS-102 (Ad5/3-D24-GM-CSF) and standard of care chemotherapy in preclinical mesothelioma model

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Malignant mesothelioma (MM) is a rare cancer type caused mainly by asbestos exposure. The median overall survival time of a mesothelioma cancer patient is less than 1-year from diagnosis. Currently there are no curative treatment modalities for malignant mesothelioma, however treatments such as surgery, chemotherapy and radiotherapy can help to improve patient prognosis and increase life expectancy. Pemetrexed-Cisplatin is the only standard of care (SoC) chemotherapy for malignant mesothelioma, but the median PFS/OS (progression-free survival/overall survival) from the initiation of treatment is only up to 12 months. Therefore, new treatment strategies against malignant mesothelioma are in high demand. ONCOS-102 is a dual targeting, chimeric oncolytic adenovirus, coding for human GM-CSF. The safety and immune activating properties of ONCOS-102 have already been assessed in phase 1 study (NCT01598129). In this preclinical study, we evaluated the antineoplastic activity of combination treatment with SoC chemotherapy (Pemetrexed, Cisplatin, Carboplatin) and ONCOS-102 in xenograft BALB/c model of human malignant mesothelioma. We demonstrated that ONCOS-102 is able to induce immunogenic cell death of human mesothelioma cell lines *in vitro* and showed anti-tumor activity in the treatment of refractory H226 malignant pleural mesothelioma (MPM) xenograft model. While chemotherapy alone showed no anti-tumor activity in the mesothelioma mouse model, ONCOS-102 was able to slow down tumor growth. Interestingly, a synergistic anti-tumor effect was seen when ONCOS-102 was combined with chemotherapy regimens. These findings give a rationale for the clinical testing of ONCOS-102 in combination with first-line chemotherapy in patients suffering from malignant mesothelioma.

Malignant mesothelioma is an aggressive and a rare form of cancer that develops from mesothelium. MM is primary caused by exposure to asbestos and exhibits a long latency period,¹ usually >30 years. The median survival time for

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mesothelioma patients after diagnosis is typically only 9–12 months.² MM affects the pleura (85.5%), peritoneum (13.2%), pericardium (0.5%) and tunica vaginalis (0.8%).³ MM tumors are often poorly responsive to standard therapies and incidence is constantly increasing worldwide.^{2,4–7} The low incidence of MM has for a long time limited the discovery of new drugs,³ therefore, new treatment modalities are highly needed.⁸

Oncolytic adenoviruses are promising and potentially powerful immunotherapy tools for treatment of cancer. This approach exploits the high immunogenicity of adenovirus.^{9,10} In addition, oncolysis releases tumor epitopes for processing of antigen presenting cells (APC) and may ultimately lead to the development of adaptive cellular immune responses specific for tumor epitopes.^{11,12} Immunogenicity of adenovirus can be further enhanced by arming the virus with an immune-stimulatory transgene.¹³ The overall antitumor efficacy can be potentially enhanced by combining viral immunotherapy with chemotherapy or radiotherapy.^{14–16}

ONCOS-102 (Ad5/3-D24-GM-CSF) is a serotype 5 adenovirus, comprising a chimeric capsid for enhanced gene delivery to cancer cells and a 24 bp deletion in Rb binding site of

What's new?

Oncolytic adenoviruses are promising and potentially powerful immunotherapy agents for advanced cancer patients. Their efficacy may be enhanced by combined therapy with chemotherapy or radiotherapy. In this study, authors have shown a synergistic antineoplastic activity of combinatorial use of ONCOS-102 with standard of care chemotherapy (Pemetrexed, Cisplatin, Carboplatin) in human mesothelioma xenograft model in BALB/c nude mice. Presented findings support clinical application of ONCOS-102 in combination with first-line chemotherapy for treatment of malignant mesothelioma.

E1A region for cancer cell restricted replication. ONCOS-102 is armed with granulocyte-macrophage colony-stimulating factor (GM-CSF) for an enhanced immunostimulatory effect.^{11,17} Safety and immunological activity of ONCOS-102 has already been demonstrated in a phase I clinical study.^{11,12} In this phase I study, local treatment of pleural mesothelioma with ONCOS-102 induced a systemic anti-tumor CD8⁺ T cell response and infiltration of CD8⁺ T cells into tumors in the last line refractory malignant pleural mesothelioma patient.

In practice, the treatment of malignant pleural mesothelioma has remained unchanged since 2003.¹⁸ The most commonly used chemotherapeutic agents in MPM treatment are Pemetrexed (Pemetrexed Disodium, Alimata), Cisplatin (Platinol), Carboplatin (Paraplatin), (SoC chemotherapy) and their combinations.¹⁹

Moreover recent elucidations on the type of induced cell death²⁰ has highlighted the importance of combining standard chemotherapy with oncolytic viruses. In fact, it has been shown that cancer cell death can be immunogenic or non-immunogenic.²⁰ Immunogenic cell death (ICD) comprises changes in the structure of the cell surface and leads to the release of pro-immunogenic factors.²¹ Subsequently it attracts APCs to take up tumor antigens, process them and finally elicit anti-tumor immune response (specific anti-tumor T cells).²⁰

The success of the cancer treatment, whether using chemotherapy, oncolytic viruses or a combination of the two, relies on the induction of immunogenic tumor cell death and induction of anti-tumor immune responses.²² It is known that some chemotherapeutics^{23,24} and oncolytic adenoviruses²⁵ act as potent inducers of ICD, and thus have a beneficial impact on anti-cancer immune responses, contributing to anti-tumor activity. ICD can be evaluated by the presence of ICD biomarkers such as calreticulin (CRT) in the outer plasma membrane, followed by extracellular release of high-mobility group box 1 protein (HMGB1) and adenosine triphosphate (ATP).^{21,22,26}

In this study, we assessed an antitumor activity of ONCOS-102 alone and in combination with first-line chemotherapeutics *in vitro* and in a xenograft model of human malignant mesothelioma.

Material and Methods**Cell lines, virus and chemotherapeutic agents**

Human epithelioid mesothelioma cell line JL-1 (ACC 596, purchased from DSMZ, Germany) was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 20%

heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin and streptomycin. Human malignant biphasic mesothelioma MSTO-211H (ACC 390, purchased from DSMZ, Germany) and human epithelial mesothelioma NCI-H226 (H226, CRL-5826, purchased from ATCC, Manassas, VA) were cultured in RPMI 1640 supplemented with 10% h.i. FBS, 2 mM L-glutamine, 1% penicillin and streptomycin. All cell lines were incubated at 37°C with 5% CO₂. The construction and characterization of chimeric oncolytic adenovirus coding for human GM-CSF (ONCOS-102) has been described previously.¹⁷ ONCOS-102 was produced by Biovian (Turku, Finland) according to good laboratory practice (GLP) and stored at -80°C until use. Pemetrexed Disodium, Cisplatin and Carboplatin were all purchased from Santa Cruz Biotechnology (Dallas, TX) and reconstituted in sterile water before use.

Cell viability—*In vitro* tumor cell killing assay

Mesothelioma cells were seeded at 1×10^4 cells per well on 96-well plates. After overnight incubation the cells were infected with ONCOS-102 with a viral particles/cell ratio of 10 (VP/cell). The virus and chemotherapeutic agents were diluted in media containing 5% FBS. Pemetrexed, Cisplatin and Carboplatin were tested at the following sub-optimal, previously selected concentrations of 0.625 mg/ml, 0.0026 mg/ml, 0.0625 mg/ml (H226 cells); 0.625 mg/ml, 0.0006 mg/ml, 0.0019 mg/ml (JL-1 cells); 0.083 mg/ml, 0.0026 mg/ml, 0.0625 mg/ml (MSTO-211H cells), respectively. Eight different treatment combinations were evaluated: ONCOS-102 alone, Pemetrexed + Cisplatin, Pemetrexed + Carboplatin, ONCOS-102 + Pemetrexed + Cisplatin (administered simultaneously), ONCOS-102 + Pemetrexed + Carboplatin (administered simultaneously), ONCOS-102 + Pemetrexed + Cisplatin (priming: virus first, chemotherapy added 24 hrs after infection), ONCOS-102 + Pemetrexed + Carboplatin (priming: virus first, chemotherapy added 24 hrs after infection) and mock (growth media only). Cell viability was determined 3 days later by CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay (MTS) according to manufacturer's instruction (Promega, Madison, WI).

Analysis of apoptotic and necrotic cells *in vitro*

Mesothelioma cells were seeded onto six well plates at 5×10^5 cells/well. Cells were infected with 10 VP/cell of ONCOS-102 and supplemented with chemotherapeutics according to

the treatment scheme mentioned above. The amount of apoptotic and necrotic cells was measured 48 hrs later with a TACS Annexin V-FITC kit by flow cytometer (Trevigen Inc., Gaithersburg, MD) according to manufacturer's instructions.

Immunogenicity of tumor cell death *in vitro*

CRT exposure. Cell lines were seeded in duplicate onto 6 well plates at 5×10^5 cells/well. Cells were infected with 10 VP/cell of ONCOS-102 and/or with chemotherapeutic agents according to the treatment combinations presented above. 24 (H226, JI-1) and 48 (MSTO-211H) hours later cells were harvested and stained with 1:1000 diluted rabbit polyclonal anti-Calreticulin antibody (Abcam, Cambridge, UK) for 40 min at 4°C and subsequently with 1:100 diluted Alexa-Fluor 488 secondary antibody (Invitrogen, Carlsbad, CA) and analyzed by flow cytometry (LSR II, BD, Franklin Lakes, NJ).

HMGB-1 release. Cell lines were seeded in triplicate onto 96 well plates at 1×10^4 cells/well and infected with 10 VP/cell of ONCOS-102 and/or with chemotherapeutic agents according to the treatment combinations presented above. 72 hrs later, supernatants were collected and HMGB-1 was measured with an Elisa kit according to manufacturer's instruction (MBL International, Woburn, MA).

ATP release. Cell lines were seeded in triplicates onto 96 well plates at 1×10^4 cells/well and treated as mentioned above. Supernatants were collected after 48 (JI-1, MSTO-211H) and 72 (H226) hours and analyzed with ATP Determination Kit according to manufacturer's protocol (Promega, Madison, WI) for luminometric analysis (Varioscan Flash, ThermoFisher Scientific, Waltham, MA).

Virus infectivity *in vitro*—Immunocytochemistry assay (ICC)

Mesothelioma cell lines were seeded in five replicates onto 24 well plates at 3×10^5 cells/well and treated with eight different treatment combinations mentioned above. 24 hrs later, supernatant was aspirated and cells were fixed by incubation with ice-cold methanol for 15 min. The determination of the ONCOS-102 infectivity was based on visual quantification of viral hexon protein in infected cells. Briefly, cells were stained with 1:2,000 diluted mouse anti-hexon antibody (Novus Biologicals, Littleton, CO) for 1 hrs at RT in the dark and subsequently with 1:500 diluted Biotin-SP-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA) for 1 hrs at RT in the dark. Subsequently the Extravidin-peroxidase was added at 1:200 and incubated for 30 min at RT in the dark (Sigma-Aldrich, Germany). Finally, the infected cells were visualized by adding the stain: DAB up to 5 min (Sigma-Aldrich, Germany). For each five replicates (wells) five images of non-overlapping fields was acquired using an AMG EVO XL microscope (ThermoFisher Scientific, Waltham, MA). Infectivity data is presented as the average number of spots in five wells.

CAR, CD46 and DSG2 expression in mesothelioma cell lines

CAR, CD46 and DSG2 expression level in H226, JI-1 and MSTO-211H cells was assessed by staining with mouse monoclonal anti-CAR antibody (Santa Cruz Biotech, Dallas, TX); mouse monoclonal anti-CD46 antibody (Abcam, Cambridge, UK) and subsequently with 1:2,000 diluted Alexa-Fluor 488 secondary antibody (Abcam, Cambridge, UK); mouse monoclonal anti-DSG2 antibody (Abcam, Cambridge, UK) and subsequently with 1:2,000 diluted Alexa-Fluor 488 secondary antibody (Abcam, Cambridge, UK) respectively for flow cytometry analysis (LSR II, BD, Franklin Lakes, NJ).

Human mesothelioma xenograft model

Animal experiments were approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Mice were obtained from Scanbur (Karlslunde, DK) at the age of 6–8 weeks and quarantined for 1 week before the experiment started. Mice were anesthetized with isoflurane (Baxter, Deerfield, IL) and NCI-H226 cells in 50 μ l were injected into both flanks (6E + 06/flank). Tumors were let to grow 8 days prior to the treatments. Two pilot studies have been performed prior the main experiment (Supporting Information Figs. S1 and S2). In the main study (Fig. 4) viruses were administered every 6 days. One group received ONCOS-102 only, two groups received ONCOS-102 and chemotherapy (Pemetrexed + Cisplatin or Pemetrexed + Carboplatin) simultaneously every six days, while two other groups received ONCOS-102 priming followed by combinatorial treatment of chemotherapy (Pemetrexed + Cisplatin or Pemetrexed + Carboplatin) and ONCOS-102 in 3-day cycles by turns. Mock animals were treated with 0.9% saline. ONCOS-102 was diluted into 0.9% saline and injected intratumorally at a dose of 5×10^7 VP per tumor (two tumors per animal). Injections were given in a fan-like pattern to ensure even distribution throughout the tumor. Pemetrexed, Cisplatin and Carboplatin were diluted in 0.9% NaCl and administered intraperitoneally at doses of 10 mg/kg, 1.5 mg/kg and 8 mg/kg, respectively. The injection volume was 100 μ l per chemotherapeutic agent.

Tumor size was measured with caliper on two dimensions every 3 days, starting on the first treatment day. The longest and shortest diameter were recorded and the tumor volume was calculated using a formula of $0.52 \times \text{length} \times (\text{width})^2$. The tumor size progression was indicated as percentage of the first measurement point on Day 0 which was arbitrarily set as 100%. All animals were observed for clinical signs, morbidity or mortality daily during acclimation and administration period and additionally 30 minutes after each treatment. In the course of the experiment, the mice were killed due to tumor reaching the maximum allowed (1.5 cm) diameter.

Human GM-CSF Elisa

Total proteins from harvested BALB/c nude tissue samples (tumor and liver) were extracted using Tissue Extraction Reagent I (Invitrogen, Carlsbad, CA) supplemented with protease inhibitor cocktail (Sigma-Aldrich) according to manufacturer's instructions. Protein extracts and previously collected serum were analyzed for human GM-CSF concentration using Elisa (Abcam, Cambridge, UK) according manufacturer's instructions.

Quantitative real-time PCR

qPCR for adenovirus E4 copy number was carried out according to the protocol previously described ((primer FW: 5'-GGA GTG CGC CGA GAC AAC-3', primer RV: 5'-ACT ACG TCC GGC GTT CCA T-3', probe E4: 5'-(6FAM)-TGG CAT GAC ACT ACG ACC AAC ACG ATC T-(TAMRA)-3').¹⁷ Total DNA was extracted from BALB/c nude murine samples (tumors, livers, blood) using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Subsequently isolated DNA was analyzed for adenoviral E4 copy number normalized to murine beta-actin (liver, blood) and human beta-actin (tumor), respectively ((primer FW: 5'-CGA GCG GTT CCG ATG C-3', primer RV: 5'-TGG ATG CCA CAG GAT TCC AT-3', probe murine beta-actin: 5'-(6FAM)-AGG CTC TTT TCC AGC CTT CCT TCT TGG-(TAMRA)-3'; (primer FW: 5'-CAG CAG ATG TGG ATC AGC AAG-3', primer RV: 5'-CTA GAA GCA TTT GCG GTG GAC-3', probe human beta-actin: 5'-(6FAM)-AGG AGT ATG ACG CCG GCC CCT C-(TAMRA)-3'). Samples were analyzed using LighCycler qPCR machine (LighCycler 480, Roche, Basel, Switzerland).

Statistical analysis

Statistical significance was analyzed by using one-way ANOVA with Tukey's Multiple Comparison test and non-parametric Mann-Whitney test. Survival curves and their statistical analysis were performed using Kaplan-Meier test. All statistical analysis, calculations and tests were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Results are presented as mean \pm SEM. All *p* values were two-sided, considered statistically significant when ≤ 0.05 .

Results

Combination with first-line chemotherapy improves the *in vitro* efficacy of ONCOS-102 in mesothelioma

Oncolytic potency of ONCOS-102 was tested in three mesothelioma cell lines *in vitro* (Fig. 1a). MSTO-211H (malignant biphasic), H226 (epithelial morphology) and JL-1 (epithelioid mesothelioma) cells appeared to be relatively resistant to oncolysis as 10 VP/cell (sub-optimal dose) killed 24%, 11% and 18% of cells respectively in 3 days. H226 and JL-1 cell lines were more resistant to chemotherapy-mediated cytotoxicity compared to MSTO-211H cells (Pemetrexed + Cisplatin or Pemetrexed + Carboplatin). Incubation with chemothera-

peutics only killed 11–12% of H226 and 10% of JL-1 cells in 3 days. In contrast, 63 and 73% of MSTO-211H cells were killed by Day 3 in culture with Pemetrexed + Cisplatin and Pemetrexed + Carboplatin, respectively. Compared to the results observed in the single-treatment (virus alone and chemotherapy alone treatment group), the combination of ONCOS-102 with chemotherapeutics significantly increased cytotoxicity in H226 and JL-1 cells. Slightly increased cytotoxicity was seen in MSTO-211H cells when chemotherapy was combined with ONCOS-102 compared to virus alone and chemo alone treatment group. In general, H226 and JL-1 cells were more resistant to both oncolysis (ONCOS-102 alone), and cytotoxic effect of chemotherapeutics (alone) than MSTO-211H cells which were particularly more sensitive to chemotherapy. Importantly, ONCOS-102 combined with chemotherapeutics showed a synergistic anti-tumor effect ($R > 1$) in mesothelioma cell lines (Table 2).

In line with the cell viability results, the number of apoptotic JL-1 and cells H226 was generally low in all treatment groups, but a combination treatment slightly increased the number of apoptotic cells in comparison to monotherapies. The highest amount of the necrotic cells (within all tested groups) has been observed in MSTO-211H cell line compared to H226 and JL-1 cells (Fig. 1b). Similar trend in the proportion of apoptotic cells was observed within three cell lines at tested treatment regimes (Fig. 1c).

Immunogenic cell death and viral replication

Markers for immunogenic cell death, such as the exposure of calreticulin to cell surface and the extracellular release of ATP and HMGB1 were measured from mesothelioma cell cultures after exposure to ONCOS-102, chemotherapeutic agents, or combination of both. The most immunogenic tumor cell death was induced by treatment with ONCOS-102 + chemotherapy in all cell lines (Fig. 2).

ONCOS-102 has shown its oncolytic property at different concentration of viral particles in three tested mesothelioma cell lines *in vitro* (Fig. 3a).

All mesothelioma cells lines (MSTO-211H, H226 and JL-1) expressed high level of CD46 (98, 96 and 98%, respectively) and DSG2 (95%, 7% and 64% respectively) on their surfaces. Finally, H226 (88%) and JL-1 (15%) expressed CAR, while MSTO-211H was negative for CAR (Fig. 3b).

We also assessed the impact of chemotherapy on ONCOS-102 replication *in vitro* (Fig. 3c). The combination of ONCOS-102 and chemotherapeutics significantly decreased the number of infected cells as compared to the control cells treated with each agent alone ($p < 0.001$ vs. ONCOS-102).

ONCOS-102 in combination with pemetrexed and cisplatin or carboplatin showed improved anti-tumor efficacy in human xenograft mesothelioma model

In order to study the synergy of oncolytic virus and chemotherapeutics on anti-tumor treatment we have performed animal study. Subcutaneous human mesothelioma H226 tumors were treated

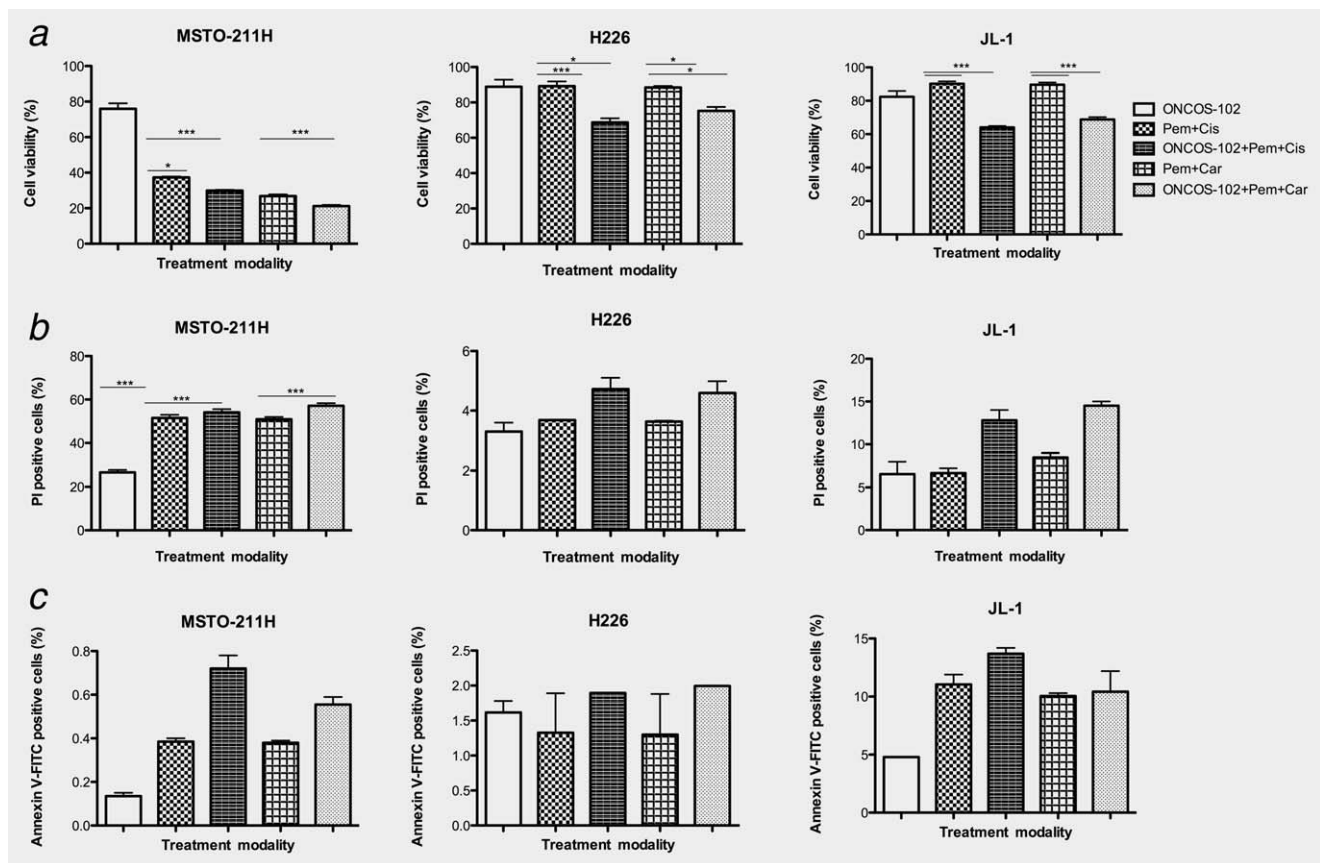


Figure 1. Treatment of human mesothelioma cells: JL-1, MSTO-211H and H226 with ONCOS-102 (10VP/cell), Pemetrexed, Cisplatin or Carboplatin—*in vitro* efficacy study. (a) Antineoplastic efficacy was measured by MTS cell-viability assay in three human mesothelioma cell lines. Cell viability was determined against untreated cells (mock) by MTS assay 72 hrs post the treatment. The amount of (b) necrotic cells (PI), (c) early apoptotic (FITC-labeled Annexin-V) 48 hrs post the treatment were analyzed by flow cytometry. Error bars, mean \pm SEM: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

according to the treatment regime presented in Table 1. Tumors appeared to be refractory against standard chemotherapeutics (Pemetrexed + Cisplatin, Pemetrexed + Carboplatin), as none of the treatments significantly reduced tumor growth (Fig. 4a, Supporting Information Figs. S1 and S2). Chemotherapy alone was the most inefficient treatment modality against mesothelioma, probably in part due to the suboptimal dose regimen used. One animal treated with ONCOS-102 + Pemetrexed + Cisplatin showed a complete tumor regression (both tumors) by Day 21. In addition, one animal treated with ONCOS-102 priming + Pemetrexed + Cisplatin showed a complete regression of both tumors by Day 45. Indeed, this regimen was the most effective with 97% of initial tumor size at Day 60 vs. 473% (mock), 563% (Pemetrexed + Cisplatin) and 672% (Pemetrexed and Carboplatin). Additionally, in all combination regimes (ONCOS-102 + chemotherapy) we observed the most significant anti-tumor activity compared to other groups (e.g. initial tumor size: 97% (virus priming + Pemetrexed + Cisplatin), 138% (virus + Pemetrexed + Cisplatin) vs. 206% (virus alone), 473% (mock), 563% (Pemetrexed + Cisplatin) at Day 60), (Fig. 4a). Importantly, ONCOS-102 combined with chemotherapeutics showed a strong synergistic anti-tumor effect ($R > 1$) on Day 21, 48 and 60 (Table 2).

ONCOS-102 replicates locally in tumor and produces human GM-CSF

Quantification for adenovirus E4 copy number and the level of human GM-CSF in murine organs (tumor, liver) and serum was done by qPCR and ELISA. ONCOS-102 was present only in tumors (locally). Adenoviral particles were not detected in serum or liver in any of the groups (Fig. 4d). Additionally, we detected human GM-CSF produced by ONCOS-102, and the highest concentration was detected in tumors (Fig. 4c).

Discussion

Mesothelioma is a treatment-resistant cancer to standard therapies with no effective curative options for advanced cancer patients.^{2,4} The most effective standard treatment against MM to date is a combination of Pemetrexed and Cisplatin, with a 41% response rate (RR).^{1,27}

In preclinical studies, the combination of Pemetrexed with Cisplatin presented activity against human non-small cell lung cancer cell lines, suggesting potential benefit in MPM.^{27,28} This combination caused a greater than additive growth delay in H460 human non-small cell lung carcinoma

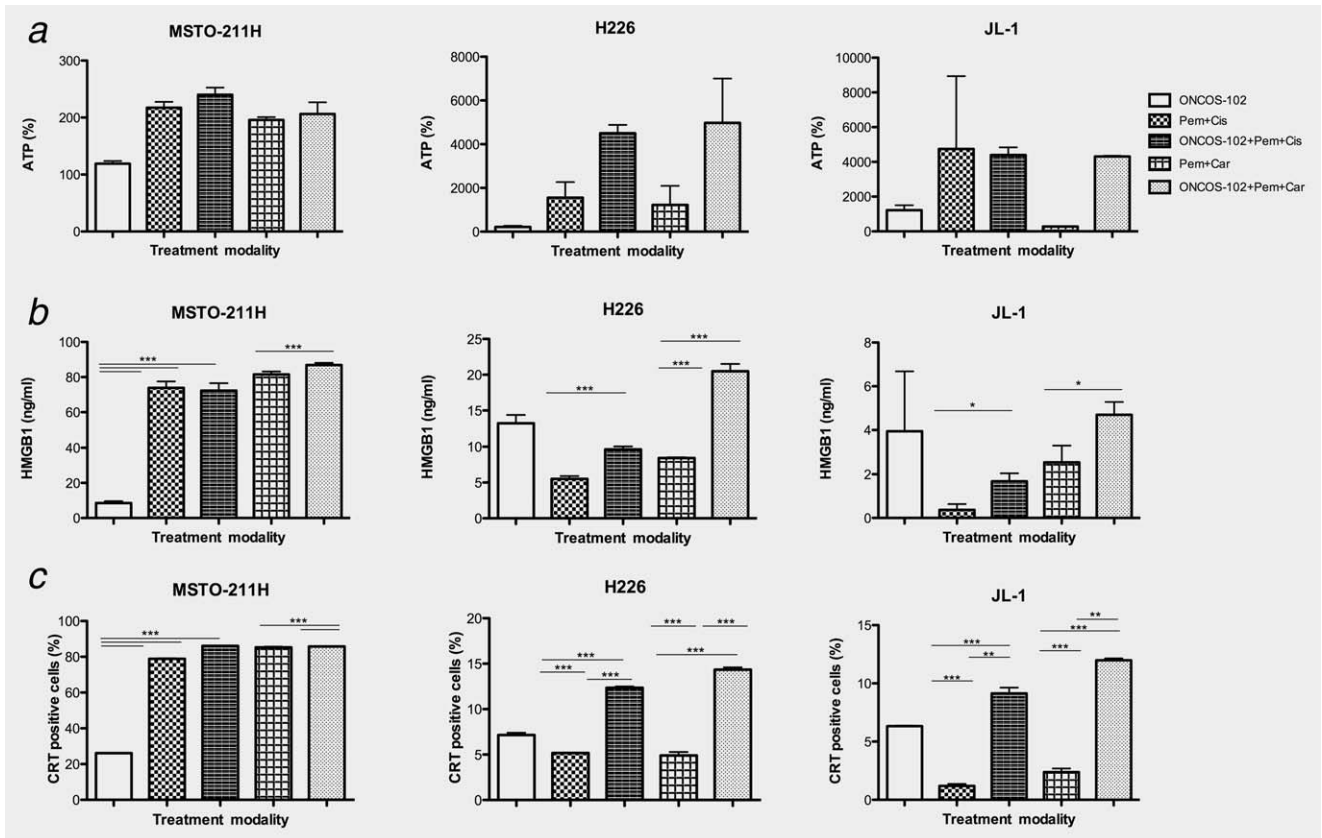


Figure 2. Combined treatment of human mesothelioma cells: JL-1, MSTO-211H and H226 with ONCOS-102 (10VP/cell), Pemetrexed, Cisplatin or Carboplatin—*in vitro* immunogenic tumor cell death. (a) Extracellular ATP was measured from supernatant 48 (JL-1, MSTO-211H) and 72 (H226) hours post the treatment using ATP determination kit. (b) Extracellular HMGB1 secretion into the supernatant was measured 3 days post the treatment utilizing ELISA assay. (c) Calreticulin exposure on outer cell surface of tested human mesothelioma cells was measured 24 (H226, JL-1) and 48 (MSTO-211H) hours post the treatment by flow cytometer. Data was normalized based on the mock group (untreated cells). Error bars, mean \pm SEM: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(NSCLC) and Calu-6 NSCLC xenografts in nude mice.²⁹ In the phase III EMPHACIS trial, an improved overall survival was seen, when patients were treated with the combination versus Cisplatin alone. The median survival of patients in the combination group was 12 months compared to a median survival of 9 months in patients treated with only Cisplatin ($p = 0.02$).²⁹ Subsequently, FDA approved this combination for the treatment of unresectable MPM (500 mg/m² and 75 mg/m² respectively).³⁰ Due to high toxicity of Cisplatin in many mesothelioma patients, Carboplatin has been tested as an alternative.³¹ Although this combination improves the survival of mesothelioma patients compared to single-agent chemotherapy,^{1,32} MM is still a lethal disease with a median PFS/OS of 12 months from the initiation of treatment, and thus, new treatment modalities are needed.

Importantly, it has been shown that oncolytic adenovirus and doxorubicin-based chemotherapy resulted in synergistic antitumor activity against soft-tissue sarcoma.²³ To this end, we decided to combine chemotherapy with ONCOS-102 in order to enhance anti-tumor effect. In addition to that, our findings will be used in clinical study design toward mesothe-

lioma treatment, since ONCOS-102 replicates specifically in tumors and shows high tropism toward mesothelioma cells.

Tumors are highly heterogeneous complex of cells, containing stromal cells, cancer cells, tumor infiltrating lymphocytes (TILs) like regulatory T cells (Tregs) which stimulate tumor progression and maintain an immunosuppressive environment.³² Tumors develop many mechanisms for evading the innate immune responses.³³ Many cell types such as Tregs, myeloid suppressor cells (MDSC) and type 2 macrophages (M2) negatively regulate anti-tumor immune responses. Additionally, tumors themselves can promote suppression of antitumor immunity by expression of inhibitory ligands such as programmed death-ligand 1 (PDL1),³⁴ programmed death-ligand 2 (PDL2),³⁵ NKG2D and MICA/B that inhibit the functionality of natural killer (NK) and T cells³⁶ and accelerate the production of immunosuppressive CD4+ T cells.³⁷ Further, soluble mediators (IL-10, histamine, hydrogen peroxidase, adenosine) produced by tumor cells can block cytotoxic T lymphocytes (CTLs).³⁸ Therefore, efficacy and antitumor responses induced by monotherapy may not be sufficient to eradicate cancer cells.

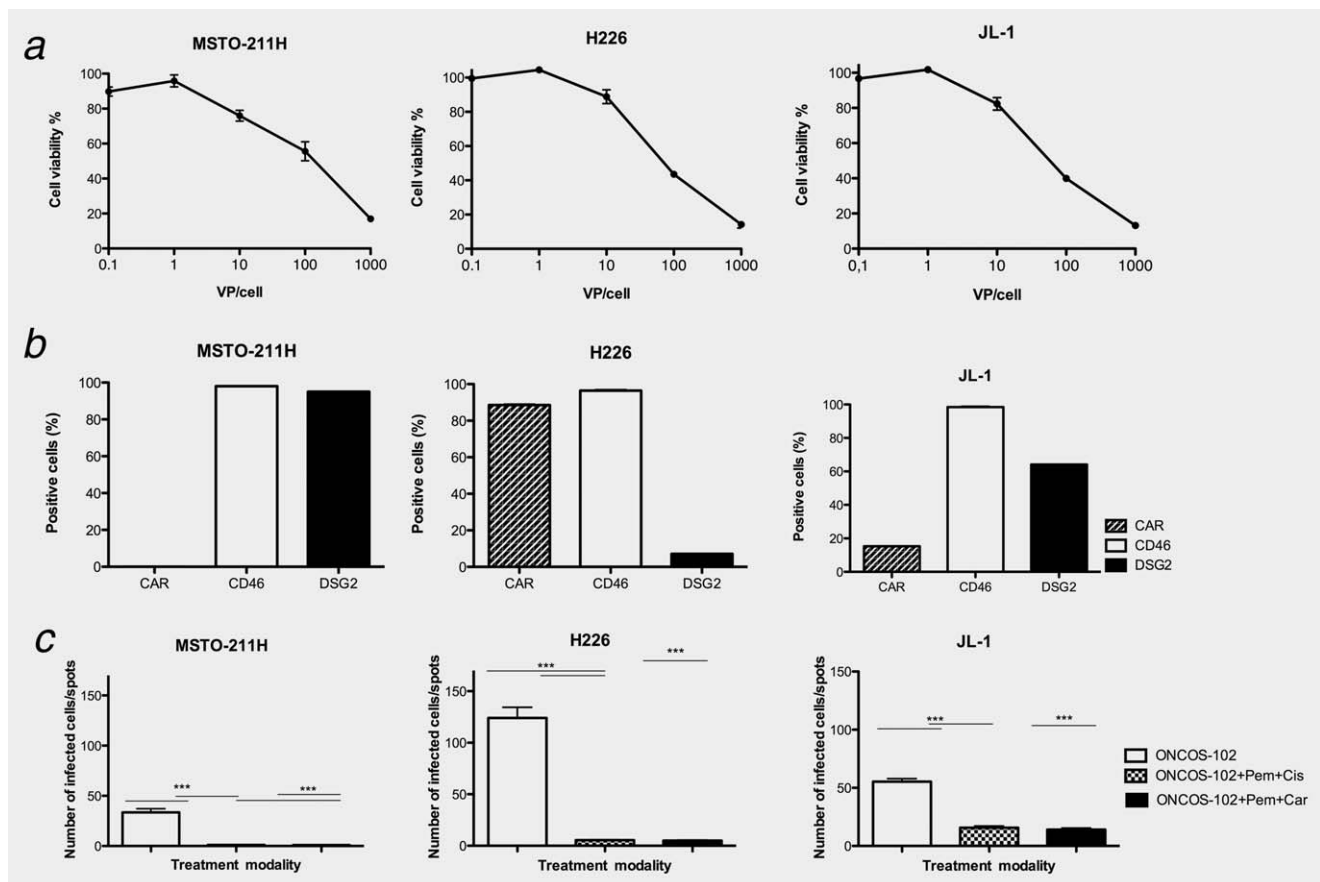


Figure 3. Oncolytic efficacy of ONCOS-102 measured by MTS cell-viability assay in three mesothelioma cell lines (JL-1, MSTO-211H, H226); receptor expression profile for mesothelioma cells and virus infectivity assay in combined treatment groups toward mesothelioma treatment. (a) Oncolytic efficacy of ONCOS-102 (0.1; 1; 10; 100 and 1,000 VP/cell) was measured by MTS cell-viability assay 3 days post the treatment initialization. Cell viability profile was determined against untreated cells (mock). (b) Mesothelioma positive cells for CAR, DSG2 and CD46 were measured by flow cytometry, followed by specific antibody staining. (c) ONCOS-102 infectivity assay was performed for three treatment groups. The determination of the ONCOS-102 infectivity was based on visual quantification of infected cells after the staining of virus hexon protein and finally the calculation for detected spots. For each five replicates five images of non-overlapping fields have been acquired using an AMG EVO XL microscope. For infectivity comparison, the data have been presented as the average number of spots in five wells. Error bars, mean \pm SEM: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Oncolytic viruses exhibit different mechanism of action from conventional anticancer approaches (chemotherapy, radiotherapy), giving a possibility for additive or synergistic interactions in cancer therapy. Further, combined therapies may lead to reduced toxicity and increased efficacy, without additional side effects.

This study was designed to evaluate the oncolytic potency of ONCOS-102 in combination with SoC chemotherapy in human mesothelioma xenograft model.

The immunomodulatory functions of the transgene GM-CSF are a central mechanism of action of ONCOS-102.^{13,17} Further, adenovirus itself is a strong activator of the immune system and this significantly contributes to the overall anti-tumor efficacy of the virus.¹³ Previously it has also been shown that ONCOS-102 produces functional human GM-CSF, inducing tumor-specific immunity^{23,39} indicating that GM-CSF might be involved in the recruitment of dendritic cells (DC) and activation at the tumor site, leading to subse-

quent stimulation of T-cells and finally, induction of anti-tumor immunity. Unfortunately, in our mesothelioma cancer model, immune modulatory functions of GM-CSF have been lost due to the immunodeficient nature of the chosen murine xenograft model. However, GM-CSF utility in immune system modulation against mesothelioma has been already shown in a phase I clinical study, where clinical data demonstrated that ONCOS-102 was able to prime a tumor specific immune response (induction of cytotoxic tumor-specific CD8+ T cells) in chemotherapy refractory malignant pleural mesothelioma patient.^{11,12}

Most of chemotherapeutics induce cancer cell death by triggering an immune responses against cancer.²² ICD involves specific changes in the composition of the cell surface (Calreticulin) as well as the release of soluble biomarkers (ATP and HMGB1). Such mediators interact with the receptors on the surface of APCs and lead to the presentation of tumor antigens to T cells, determining a long-term success of

Table 1. Antineoplastic activity of ONCOS-102 in combination with chemotherapeutic agents (Pemetrexed, Cisplatin and Carboplatin) toward mesothelioma treatment was evaluated in eight tested groups

Group	Day 1	Day 4	Day 7	Day 10, and every 3 days thereafter
A: Mock	NaCl 50 µl/tumor i.t.	NaCl 100 + 100 µl i.p. (as the chemos)	NaCl 50 µl/tumor i.t.	NaCl 100 + 100 µl i.p. (as the chemos)
B: ONCOS-102	1E8 VP/mouse i.t. (5E7 VP in 50 µl NaCl/tumor)	NaCl 100 + 100 µl i.p. (as the chemos)	1E8 VP/mouse i.t. (5E7 VP in 50 µl NaCl/tumor)	NaCl 100 + 100 µl i.p. (as the chemos)
C: Pem + Cis	Pemetrexed 10 mg/kg in 100 µl i.p. + after 30 min Cisplatin 1.5 mg/kg in 100 µl i.p.	NaCl 50 µl/tumor i.t.	Pemetrexed 10 mg/kg in 100 µl i.p. + after 30 min Cisplatin 1.5 mg/kg in 100 µl i.p.	NaCl 50 µl/tumor i.t.
D: ONCOS-102 + Pem + Cis	1E8 VP/mouse i.t. (5E7 VP in 50 µl NaCl/tumor)- + Pemetrexed 10 mg/kg in 100 µl i.p. + after 30 min Cisplatin 1.5 mg/kg in 100 µl i.p.	No injection	1E8 VP/mouse i.t. (5E7 VP in 50 µl NaCl/tumor)- + Pemetrexed 10 mg/kg in 100 µl i.p. + after 30 min Cisplatin 1.5 mg/kg in 100 µl i.p.	No injection
E: ONCOS-102 priming + Pem + Cis	1E8 VP/mouse i.t. (5E7 VP in 50 µl NaCl/tumor)	Pemetrexed 10 mg/kg in 100 µl i.p. + after 30 min Cisplatin 1.5 mg/kg in 100 µl i.p.	1E8 VP/mouse i.t. (5E7 VP in 50 µl NaCl/tumor)	Pemetrexed 10 mg/kg in 100 µl i.p. + after 30 min Cisplatin 1.5 mg/kg in 100 µl i.p.
F: Pem + Car	Pemetrexed 10 mg/kg in 100 µl i.p. + after 30 min Carboplatin 8 mg/kg in 100 µl i.p.	NaCl 50 µl/tumor i.t.	Pemetrexed 10 mg/kg in 100 µl i.p. + after 30 min Carboplatin 8 mg/kg in 100 µl i.p.	NaCl 50 µl/tumor i.t.
G: ONCOS-102 + Pem + Car	1E8 VP/mouse i.t. (5E7 VP in 50 µl NaCl/tumor)- + Pemetrexed 10 mg/kg in 100 µl i.p. + after 30 min Carboplatin 8 mg/kg in 100 µl i.p.	No injection	1E8 VP/mouse i.t. (5E7 VP in 50 µl NaCl/tumor)- + Pemetrexed 10 mg/kg in 100 µl i.p. + after 30 min Carboplatin 8 mg/kg in 100 µl i.p.	No injection
H: ONCOS-102 priming + Pem + Car	1E8 VP/mouse i.t. (5E7 VP in 50 µl NaCl/tumor)	Pemetrexed 10 mg/kg in 100 µl i.p. + after 30 min Carboplatin 8 mg/kg in 100 µl i.p.	1E8 VP/mouse i.t. (5E7 VP in 50 µl NaCl/tumor)	Pemetrexed 10 mg/kg in 100 µl i.p. + after 30 min Carboplatin 8 mg/kg in 100 µl i.p.

Different treatment regimes, chemotherapys and virus concentrations were tested in human mesothelioma xenograft model in BALB/c nude mice during 2 months.

anticancer therapy.²¹ It is well studied that the efficacy of anticancer chemotherapies is related to cytotoxic effects of the antineoplastic agent on tumor cells. Chemotherapeutics can stimulate immune effector cells either directly or by restoring the immunosuppressive environment inhibiting antitumor immune responses.⁴⁰ Additionally, some chemotherapeutics induce tumor cell death in such a way that specific immune responses are evoked.⁴¹

Ads are excellent immunotherapeutic vectors with properties to prime and boost immune responses. Oncolytic adenoviruses lead to immunogenic cancer cell death and subsequent release of tumor specific antigens for APC cells, resulting in the priming of potent tumor-specific immunity.¹²

The combination of oncolytic viruses with chemotherapy has a potential for improved anticancer efficacy and induction of antineoplastic immunity. Further, armed ONCOS-102 with chemotherapy can be a powerful tool in order to overcome the major obstacle of immune suppressive microenvironment in tumor⁴² due to the immunogenic tumor cell death²¹⁻²⁴ it causes and the subsequent induction of anti-cancer immune responses.^{22,43,44}

We have shown that both ONCOS-102 and chemotherapeutics induce ICD in a preclinical setting. Our findings suggest that their combination elevates immunogenic cell killing *in vitro*, further suggesting that the virus-induced ICD plays a part in the antitumor T-cell activation observed in humans

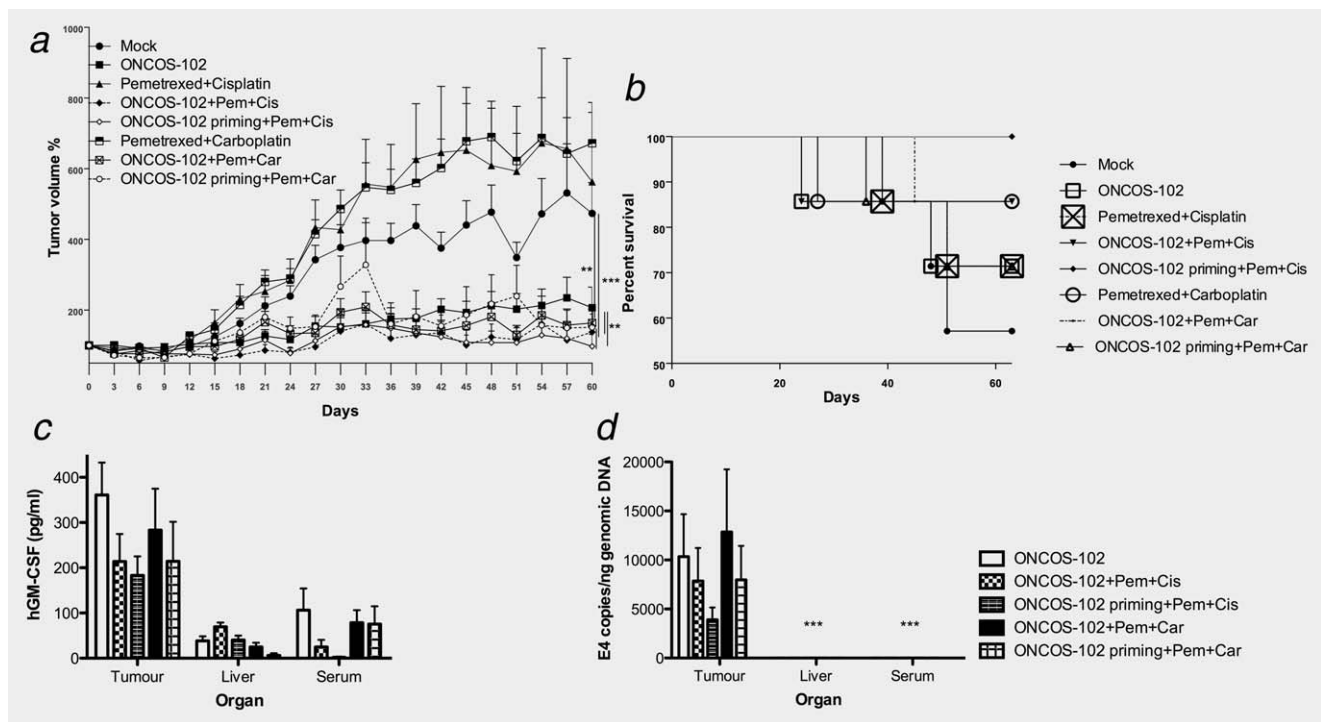


Figure 4. Main study *in vivo*: antitumor efficacy of ONCOS-102 (1E + 8 VP/mouse i.t.) in combination with Pemetrexed (10 mg/kg in 100 μ l IP), Cisplatin (1.5 mg/kg in 100 μ l IP) or Carboplatin (8 mg/kg in 100 μ l IP) in human mesothelioma xenograft model in BALB/c nude mice (seven mice per group, 14 tumors per group). (a) BALB/c nude mice bearing s.c. H226 tumors (6×10^6 cells/tumor) were treated according the treatment scheme (Table 1) with ONCOS-102 (i.t.) and chemotherapies (IP) in eight studied groups. Animals were treated every 3 days throughout 2 months. (b) Survival profile was calculated by Kaplan–Meier test. (c) Measurement for human cytokine: GM-CSF produced by ONCOS-102 was performed from harvested organs (tumor, liver, serum) after animal euthanasia utilizing Elisa technique. (d) Adenoviral copies toward E4 gene were measured by qPCR from euthanized mice’s organs (tumor, liver and serum) at the end of the treatment. Error bars, mean \pm SEM: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 2. Combined treatment of mesothelioma with ONCOS-102 and Pemetrexed-Cisplatin/Pemetrexed-Carboplatin

In vitro									
Cell line	Pem+Cis	Pem+Car	ONCOS-102	ONCOS-102+Pem+Cis			ONCOS-102+Pem+Car		
				Exp.*	Obs.**	Ratio	Exp.*	Obs.**	Ratio
MSTO-211H	0.39	0.27	0.77	0.3	0.299	<u>1</u>	0.2	0.21	0.95
H226	0.89	0.89	0.89	0.8	0.69	<u>1.16</u>	0.8	0.75	<u>1</u>
JL-1	0.9	0.89	0.82	0.74	0.64	<u>1.15</u>	0.73	0.69	<u>1</u>
In vivo									
Day	Pem+Cis	ONCOS-102	ONCOS-102+Pem+Cis			ONCOS-102 prim+Pem+Cis			
			Exp.*	Obs.**	Ratio	Exp.*	Obs.**	Ratio	
21	1.12	0.60	0.72	0.40	<u>1.80</u>	0.72	0.54	<u>1.32</u>	
48	1.28	0.45	0.57	0.26	<u>2.19</u>	0.57	0.23	<u>2.5</u>	
60	1.19	0.44	0.52	0.29	<u>1.78</u>	0.52	0.20	<u>2.5</u>	
Day	Pem+Car	ONCOS-102	ONCOS-102+Pem+Car			ONCOS-102 prim+Pem+Car			
			Exp.*	Obs.**	Ratio	Exp.*	Obs.**	Ratio	
21	1.32	0.60	0.79	0.79	1.0	0.79	0.85	0.93	
48	1.44	0.45	0.64	0.38	<u>1.70</u>	0.64	0.46	<u>1.41</u>	
60	1.42	0.44	0.62	0.35	<u>1.8</u>	0.62	0.32	<u>1.94</u>	

Assessment of therapeutic synergy with FTV calculation method. FTV (mean value of cell viability experimental/mean value of cell viability mock) or (mean tumor volume experimental)/(mean tumor volume control). *(Mean FTV of Chemotherapy) \times (mean FTV of Virus). **(expected FTV by the observed FTV). A ratio of >1 indicates a synergistic effect, and a ratio of <1 indicates a less than additive effect.

treated with ONCOS-102.^{11,12} The molecular mechanisms underlying this phenomenon is still unknown, however several hypothesis can be put forth. Further, the production of adenovirus E1A proteins has been shown to sensitize cancer cells to chemotherapy-induced cell killing and chemotherapeutics have also been shown to change the viral tropism toward the cancer cells.⁴⁵ Importantly, ICD seems to be beneficial or even necessary for the induction of anticancer immunity and efficacy, and is in conjunction with exposure and release of ATP and HMGB1.⁴⁴

In our studies combined treatment showed synergistic effects and was the most effective therapy against mesothelioma (over SoC therapy) thus we suggest that ONCOS-102 combined with SoC can be used as a new treatment modality against mesothelioma. Our observations are in line with our previously reported findings where ONCOS-102 combined with Doxorubicin resulted in synergistic antitumor effect against soft tissue sarcoma (STS) in Syrian hamsters.²³ Similar finding has been reported when combining Ad5/3-delta24 and gemcitabine, resulting in synergistic effect to ovarian cancer *in vitro* and *in vivo*.⁴⁶ Similar findings have been reported as well by many other research groups testing various combinations of oncolytic viruses with chemotherapeutic agents. The combinatory therapy of reovirus and Cisplatin synergistically improved anticancer properties in human and murine mel-

noma cell lines *in vitro* and murine tumors *in vivo*.⁴⁷ Triple combination therapy with reovirus, Cisplatin and Paclitaxel demonstrated synergistic cytotoxic effect *in vitro* in head and neck cancer, giving a rationale for clinical study.⁴⁸ Also the combination of Newcastle disease virus (NDV) with standard of care chemotherapeutics agent 5-Fluorouracil (5-FU) has shown synergistic effect on different tumor cell lines *in vitro*. The combination of virus and chemotherapy had the strongest cytotoxic efficacy than virus and 5-FU alone, suggesting this treatment regime for new adjuvant therapy.⁴⁹ Next the application of herpes simplex virus (G207) in combination with Paclitaxel and Doxorubicin for the therapy of ATC (anaplastic thyroid cancer) *in vitro* and *in vivo* has shown anticancer synergy as well. Combinatory therapy (G207 plus Paclitaxel) in athymic nude mice bearing KAT4 flank tumors significantly reduced tumor volume compared with virus, chemotherapy and control alone. Importantly no morbidity *in vivo* has been reported in combinatory treatment group.⁵⁰

In summary our findings support combining ONCOS-102 with standard of care chemotherapy for the treatment of malignant pleural mesothelioma.

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