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PRINCIPAL INVESTIGATOR: Dr John Hiscott

CONTRACTING ORGANIZATION: Vaccine & Gene Therapy Institute of Florida
Port Saint Lucie, FL 34987

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Dr John Hiscott

E-Mail:jhiscott@vgtifl.org

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14. ABSTRACT

Replication competent vesicular stomatitis virus (VSV) can exert a dual antitumor effect by triggering direct tumor lysis and eliciting tumor specific immunity. VSV can also deliver tumor associated epitopes or other immunomodulatory molecules to enhance the antitumor immune responses. This is particularly desirable for lung tumors, which are usually poorly immunogenic, and quickly develop drug resistance. In order to examine the oncolytic effects of VSV in drug-resistant lung cancer cells, we utilized mouse lung cancer cells KLN205 (K-CP0) to generate cisplatin-resistant cells, K-CP3 and K-CP6. Two cell lines were generated by prolonged exposure to increasing concentrations of cisplatin, and both K-CP3 and K-CP6 were sensitive to the cytopathic effect of VSV. Cisplatin-resistant cells had decreased level of p-Akt and upregulated several markers of autophagy, including the scaffold protein Beclin 1 and microtubule-associtaed light chain protein 1. When grown subcutaneously in immunocompetent DBA/2 mice, both K-CP0 and K-CP6 formed tumors. Intratumoral injection of VSV into either K-CP0 or K-CP6 tumors led to a delay in tumor growth. Histological examination of K-CP0 and K-CP6 tumors revealed a decrease in necrotic areas in the VSV-treated tumors, and the levels of infiltrating leukocytes were similar across the VSV-treated tumors. Altogether the data indicate that VSV-based therapy is effective against a cisplatin-resistant lung tumor model.

15. SUBJECT TERMS
Drug resistance, oncolytic virotherapy, vesicular stomatitis virus, lung cancer immunotherapy

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1. INTRODUCTION

Lung cancer is the leading cause of cancer-related death in the US with an overall 5-year survival of less than 15 % (1). In addition to surgery and radiotherapy, chemotherapy remains the major treatment intervention option. The most widely used drug for lung cancer is cisplatin, often administered in combination regiments with other small molecule therapies such as paclitaxel, captothecin or gemcitabine (2). Despite initial positive responses to therapy, the majority of patients develop resistance to chemotherapy, ultimately leading to relapse of the disease. The heterogenic nature of drug-resistant cancers requires multimodal therapies for successful elimination of resistant cells. Small molecule-based therapies frequently share common resistance mechanisms, and second-line therapies that kill cells through novel mechanisms have a potential to overcome such resistance mechanisms (3). Self-replicating biotherapeutics such as oncolytic viruses (OVs) can eliminate tumors via both oncolysis and induction of specific tumor-targeted immune responses in the host (4). Because viral oncolysis has a potential to induce tumor antigen release and induce inflammatory cytokine production, it can adapt the tumor microenvironment to one that facilitates tumor antigen uptake and thus the maturation of antigen presenting cells such as dendritic cells (5). Direct cytopathic effect of an oncolytic virus can be enhanced via the tumor-specific delivery of therapeutic genes or cancer-associated epitopes which, upon infection of tumor, cells, may act as adjuvants and prime the host's immune system. Vesicular Stomatitis Virus (VSV), an enveloped, negative-sense RNA virus of the family Rhaboviridae, has served as a prototype oncolytic virus – a potent, non-human, non-pathogenic, replication competent oncolytic virus (6). In normal cells and tissues, VSV multiplication is sensitive to the antiviral effects of type 1 interferons (IFN), and other innate immune effectors. Malignant cells on the other hand acquire during their tumorigenic evolution diminished responsiveness to IFN action and are specifically infected and killed by VSV (7). When VSV was used as a highly immunogenic platform for gene delivery, it cured established prostate tumors of the same histological type (8). Suboptimal vaccination, on the other hand, resulted in therapy escape variants that were readily treated with a second vector delivery of a cDNA library created from tumor tissues that escaped previous therapeutic intervention. Therefore, it is possible to target a population of cancer cells that escaped previous therapeutic intervention using viral delivery of cDNA from the same cell population. We hypothesize that the delivery of a tumor antigen library

derived from a drug resistant population will target that specific tumor cell population for elimination by the immune system.

2. KEY WORDS

Lung cancer, vesicular stomatitis virus, oncolytic virotherapy, apoptosis, autophagy, mouse tumor models, drug resistance

3. ACCOMPLISHMENTS

Aim1. Generate and characterize cisplatin-resistant KLN205 and LLC1 cells.

Our first goal was to generate and characterize LLC1 and LKN205 cisplatin-resistant cells. Drug-resistant cells are typically generated by continuous exposure of tumor cells to sub-lethal doses and such dose is increased until cells acquire a resistant phenotype. Because cisplatin is highly mutagenic, it has an ability to quickly induce genetic changes in cancer cells usually resulting in Darwinian selection and generation of cells that acquire a permanent resistant phenotype (9).

We initially exposed both KLN205 and LLC1 to increasing concentrations of cisplatin in order to generate cisplatin-resistant cells. However, upon subcutaneous injection (above right flank) of LLC1 cells in mice, a fast growth of lesions and quick appearance of ulceration (within 7 days of tumor injection) were observed, and animals had to be humanely euthanized. Therefore, we performed all subsequent experiments using KLN205 cells that, when injected subcutaneously in mice, did not form ulcers *in vivo* in a short time period (2-3 weeks). Four month exposure of KLN205 cells to the vehicle (K-CP0) or to increasing concentrations of cisplatin, 0.5-3 μM for one set of cells

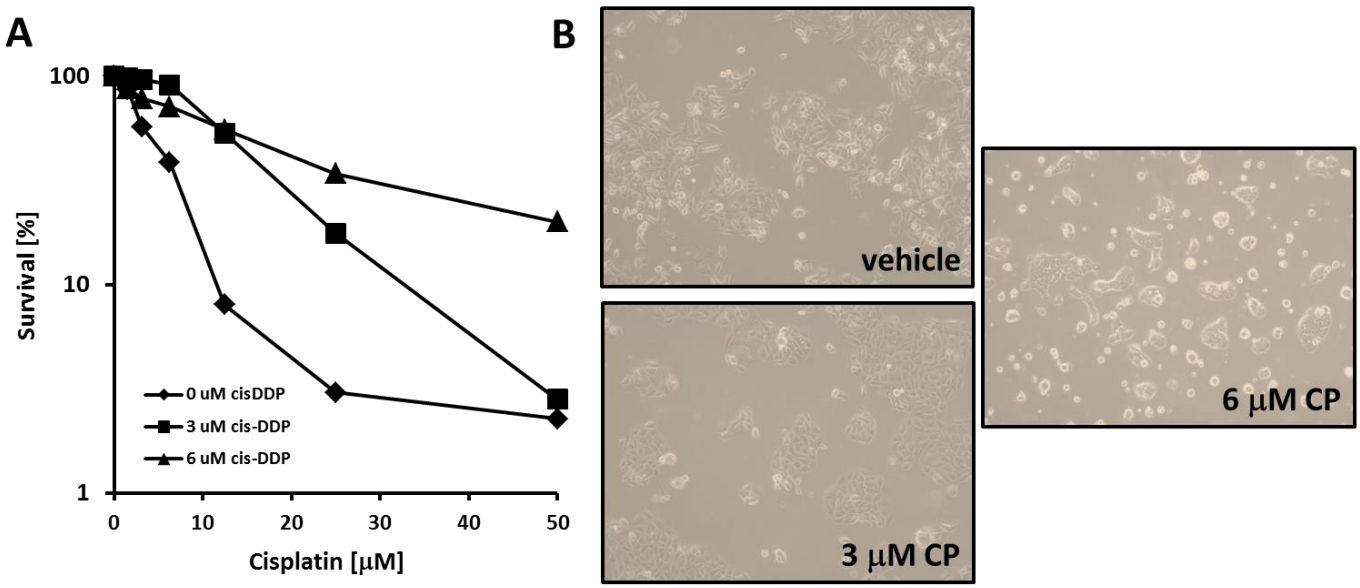

Figure 1: Characterization of cisplatin-resistant cells. A) KLN205 cells were exposed to cisplatin for 4 months and assessed for sensitivity to increasing concentrations of the drug. B) KLN205 cells exposed to the indicated concentrations of cisplatin were visualized using bright field microscopy and 400X objective.

(K-CP3), and 1-6 μM for the other set (K-CP6), resulted in a maximum of 1.5 log difference in sensitivity (at 25 μM cisplatin) between K-CP0 and K-CP6 cells (Figure 1A). Further increase in cisplatin concentration did not result in increased resistance. Cells were then continuously passed without the drug for 4 months and subsequent cytotoxicity tests confirmed generation of cisplatin-resistant cell lines that did not revert to their original sensitivity. The two cell lines acquired different phenotypes (Figure 1B) and growth rates; K-CP6 cells grow in clumps and have a lower growth rate at 72 h – about 40% slower compared to K-CP0 or K-CP3. We first tested oncolytic activity of VSV against K-CP3 and K-CP6 cells *in vitro* by measuring VSV replication and induction of apoptosis. Cells were exposed to low multiplicities of infection (MOIs) of VSV expressing green fluorescent protein (GFP) and analyzed by flow cytometry and plaque assay for VSV replication and induction of

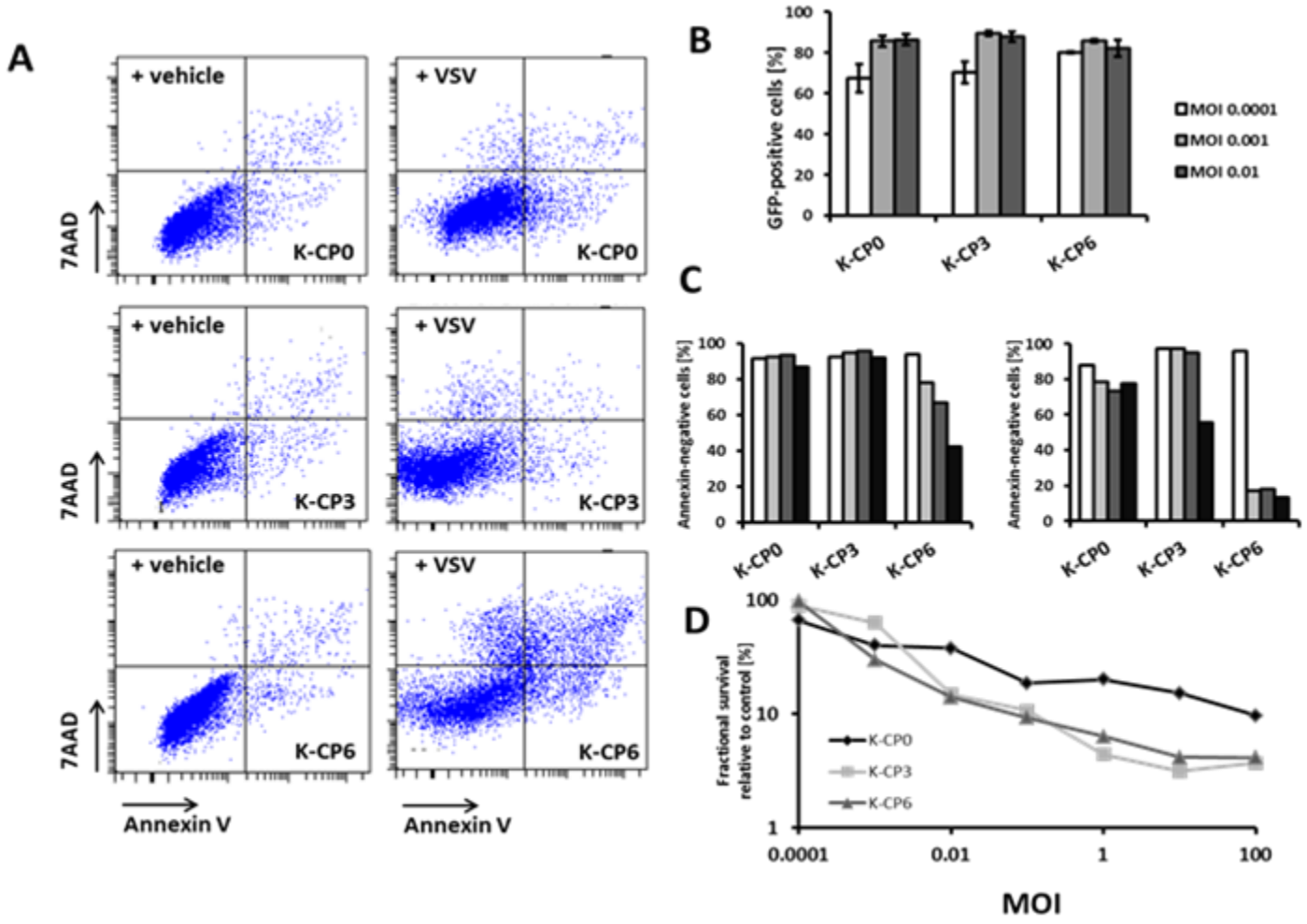

Figure 2: VSV replication and induction of apoptosis in cisplatin-resistant cells. A) Annexin V staining of K-CP0, K-CP3 or K-CP6 cells exposed to VSV; B) Quantification of VSV-GFP replication by flow cytometry in the three cell lines indicated at the bottom; C) Cell survival upon exposure to VSV measured as the number of annexin-negative cells at 24 h (left) or 48 h (right) post-infection; White bars indicate untreated cells, light shaded MOI=0.0001, dark shaded MOI=0.001 and black bars indicate MOI=0.01; D) Three cell lines were exposed to VSV for 72 h and cytotoxicity was measured with the SRB assay.

apoptosis (annexin V and 7AAD staining) at 24 and 48 h post-infection (Figure 2). VSV infection induced an increase in annexin V positive cells as early as 24 h post-infection (Figure 2A) and VSV-GFP replication was similar in all three cell lines (Figure 2B). The highest increase in annexin-positive cells was observed in K-CP6 cells at 48 h (Figure 2C). Based on the results of SRB assay which measures total cell survival, K-CP3 and K-CP6 cells are more sensitive to the cytopathic effect of VSV. Taken together, our data indicate that VSV replicates and induces apoptosis in all three cell lines, while K-CP3, KLN-CP6 appear to be more sensitive to oncolytic effect of VSV.

Our recent work indicated a role of autophagy in potentiating VSV oncolysis (10), so next we examined several markers of autophagy, including p-Akt, Beclin- 1 (BECN), and microtubule-associated light chain protein 1, LC3-I (Figure 3A). Immunoblotting analysis revealed increased levels of LC3-I and BECN, and decreased levels of p-Akt in K-CP6 cells (lanes 5-8) compared to K-CP0 cells (lanes 1-4), and such changes were observed both in cells infected (lanes 3, 4, 7, and 8) or (lanes 1, 2, 5, and 6) not with the virus. Addition of the autophagy inhibitor 3-methyladenine (3MA) did not impact increases in levels of LC3-I or BECN in K-CP6 cells; however, because 3MA inhibits PI3K, levels of p-Akt were decreased in both cell lines upon addition of 3MA.

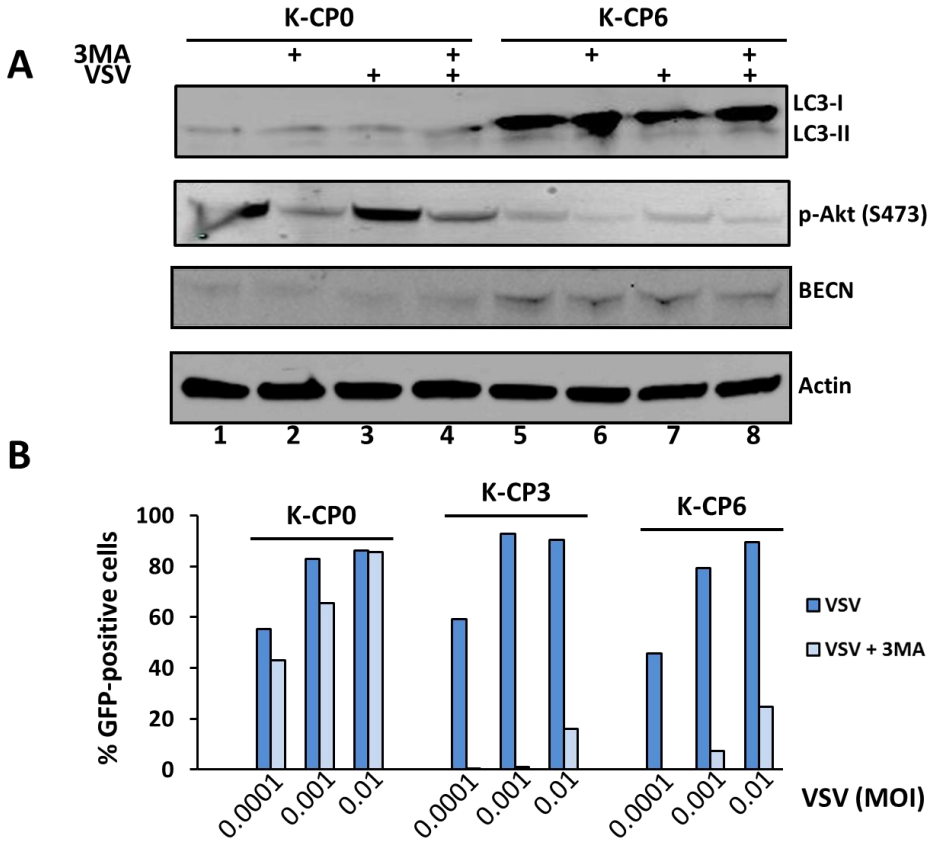

Figure 3: Autophagy-mediated VSV replication in cisplatin-resistant cells. A) K-CP0 and K-CP6 cells were exposed or not to VSV alone or in combination with 3MA as indicated on the upper panel. Levels of proteins indicated on the right were assessed by immunoblotting; B) VSV replication in K-CP0, K-CP3 and K-CP6 cells was examined by quantifying of GFP-positive cells (flow cytometry) that were pre-treated (1 h) with 3MA.

We next blocked the autophagy pathway in cells by pre-treating them with 3MA and examined replication expressing GFP transgene after exposing them to various MOIs of VSV by measuring the number of GFP-positive cells. While only a slight decrease in the number of GFP-positive cells was observed upon 3MA treatment in K-CP0 cells, 3MA treatment drastically decreased the number of GFP-

positive K-CP3 and K-CP6, indicating that pharmacological inhibition of autophagy in these cell lines has an inhibitory effect on VSV replication.

Aim 2. Test antitumor activity of VSV against cisplatin-resistant KLN205 cells *in vivo*.

Our next goal was to test VSV as a monotherapy against cisplatin resistant cells using syngeneic mouse model that possesses complete immune system and allows for evaluation of immune responses. Because of the lack of overlap between resistance mechanisms, we hypothesize that VSV will be efficient in inducing apoptosis in cisplatin-resistant lung tumor cells by inducing apoptotic cell death, vascular shutdown and inflammation.

To evaluate the antitumor effect of VSV against cisplatin-resistant cells *in vivo*, we utilized a syngeneic subcutaneous (sc) lung tumor model. DBA/2 mice were injected with either KLN-CP0 or KLN-CP6 cells (0.5×10^6 cells) and randomized into four treatment groups: 1) K-CP0 + vehicle, 2) K-CP0 + VSV, 3) K-CP6 + vehicle, and 4) K-CP6 + VSV. Three weeks after tumor innoculation, VSV (1×10^8 pfu) was administered three times intratumorally (treatments and procedures are described in Figure 4A). We also injected VSV-naïve K-CP0 or K-CP6 tumors (n=3 for each) with VSV 48 h prior to animal euthanasia for comparison. Tumor growth was measured three times per week with a caliper and is represented as a volume increase relative to day 1 (Figure 4B). The growth rate of K-CP0 and K-CP6 cells was comparable and a statistically significant difference in tumor growth ($p<0.05$) was observed at day 15 between vehicle- and VSV-injected tumors. Assessment of survival based on Kaplan-Meier analysis also revealed increased survival in the VSV-treated groups. No VSV-associated toxicity was observed and animal weight remained constant throughout the entire experiment (data not shown). Altogether, these data indicate that intratumoral injection of VSV leads to delayed growth of K-CP0 and K-CP6 cells in vivo.

We next examined cross sections of subcutaneously growing K-CP0 and K-CP6 tumors in order to assess histopatological changes induced by VSV treatment. Upon termination of experiment, tumors were excised and fixed in 4 % paraformaldehyde followed by paraffin embedding and H&E staining. Tumors were encapsulated and gross examination of the stomach did not reveal any obvious lesions; color and position of major organs were also within normal limits, indicating the absence of adverse reactions to VSV injection. Cross sections were further examined for cell shape, nuclear shape, mitotic figures, apoptosis, necrosis, and inflammation. In all four groups tumor masses were densely cellular; cell borders were indistinct and up to 20 mitotic figures were observed per 400x field, about 30-40% of which were irregular. The number of mitotic figures was decreased in the VSV-injected tumors, and both VSV-injected and vehicle-injected K-CP6 tumors possessed a slightly lower number of mitotic figures compared to K-CP0 tumors (Figure 5A). Apoptosis in tumors was assessed

by TUNEL staining; K-CP6 tumors appeared to have a slightly higher number of TUNEL-positive nuclei and no statistically significant differences were observed between vehicle- and VSV-injected tumors (Figure 5B) in tumors that were harvested 2 weeks post-treatment. However, TUNEL staining of tumors that were harvested 48 h post-VSV injection revealed ~3-4 fold increase in TUNEL-positive nuclei compared to control, indicating induction of apoptosis in both K-CP0 and K-CP6 tumors by VSV injection *in vivo*. Taken together, these data indicate that VSV treatment leads to a reduction of mitotic bodies and induction of apoptosis that delays tumor growth in both K-CP0 and K-CP6 tumors.

Further histological examination revealed almost complete absence of necrotic areas in the VSV-injected tumors (Figure 5C). When necrotic regions were present in tumors, they usually comprised 30-40% of the total tumor mass and displayed high levels of inflammation (tumor-infiltrating lymphocytes). (Figure 5C and 5D). Tumors with high necrotic areas were also found to contain higher number of blood vessels, based on the number of smaller non-stained areas that were

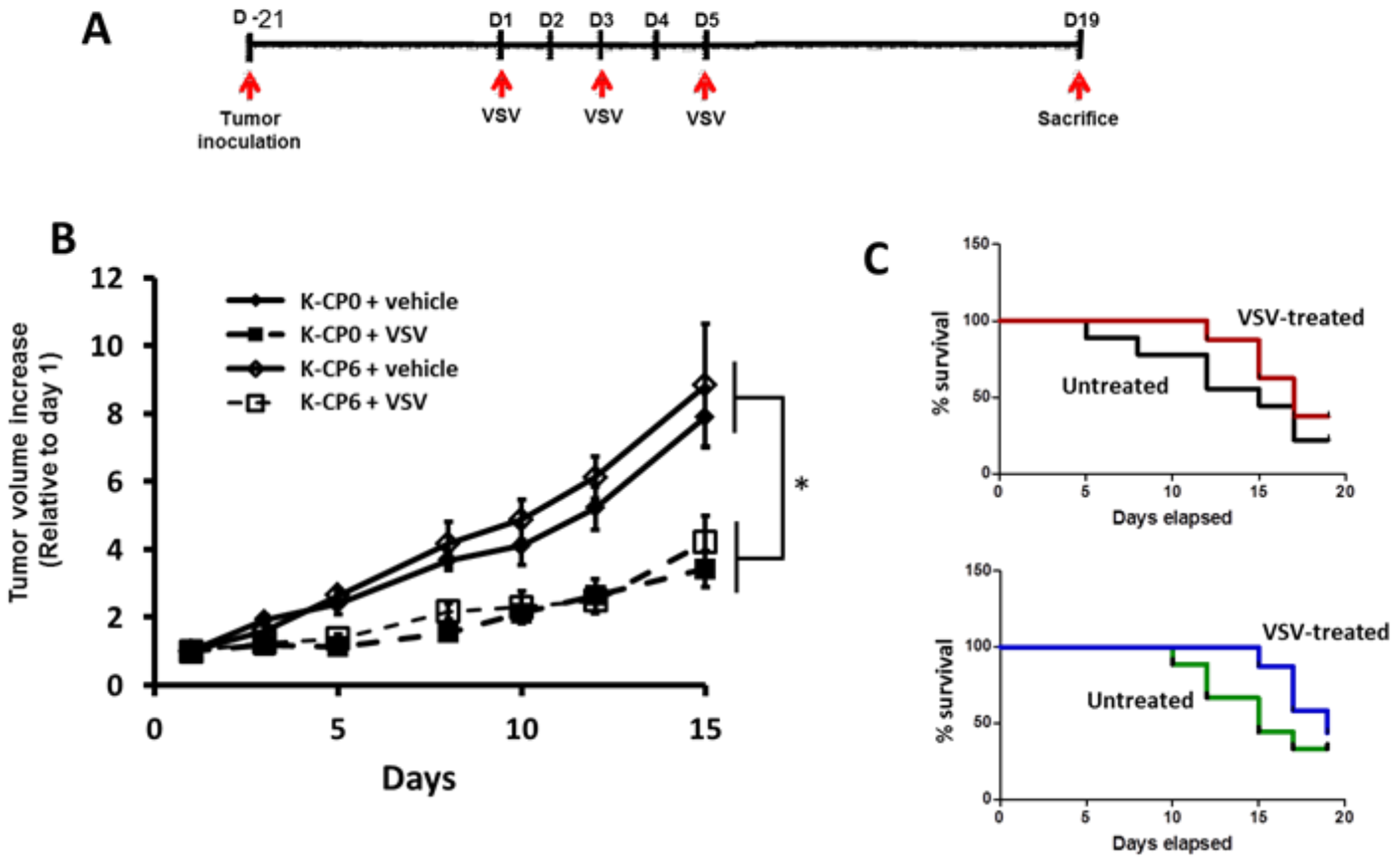

Figure 4: Antitumor activity of VSV against cisplatin resistant tumors *in vivo*. A) Flowchart describing the schedule and time points for the animal procedures. B) Oncolytic activity of VSV against K-CP0 or K-CP6 cells in DBA/2 mice (n=8) injected with respective tumor cells and treated as described in A). C) Estimation of survival based on the Kaplan-Meier analysis. Top diagram represents survival of mice injected with K-CP0 cells and bottom diagram represents mice injected with K-CP6 cells. Respective treatments are indicated next to the curves. * $p<0.05$

aligned with the endothelium which we identified as blood vessels. This indicates that VSV treatment leads to a decrease in tumor necrosis and vascular shutdown.

The overall conclusion is that VSV delays tumor growth in cisplatin resistant cells to the same extent as in cisplatin sensitive cells. The examined parameters were similar between K-CP0 and K-CP6 tumors but the difference in parameters examined were observed between VSV- and vehicle-treated tumors.

Conclusions: VSV possesses oncolytic activity against cisplatin-resistant cancer cells which is facilitated by upregulation of the autophagy pathway in such cells. The *in vivo* mechanism of VSV antitumor activity is likely multimodal and includes direct tumor cell oncolysis, vascular shutdown and

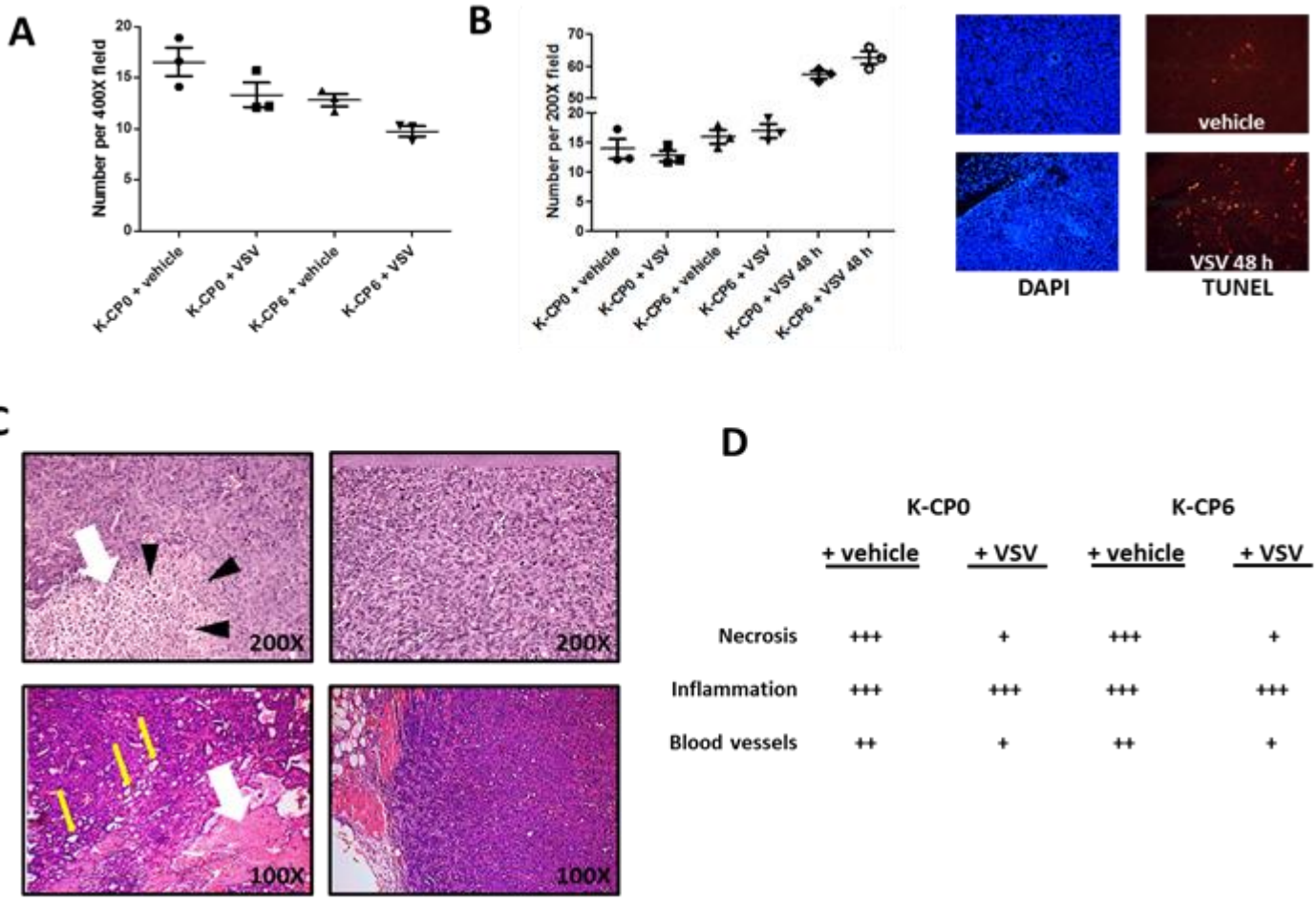

Figure 5: Histopatological characterization of K-CP0 and K-CP6 tumors injected with VSV. A) Quantification of mitotic figures using bright-field microscopy. Respective treatments are indicated at the bottom. B) Quantification of the number of TUNEL-positive cells. Two examples of images used for TUNEL displayed on the right. Blue color spots are total nuclei while red spots indicate TUNEL-positive nuclei. C) H&E staining of K-CP6 tumors treated with the vehicle (left) or VSV (right). White arrows indicate necrotic area, black arrows indicate infiltrating lymphocytes and yellow arrows indicate blood vessels. The absence of necrotic areas and decrease in blood vessels is visible in the VSV treated tumors (right) D) Quantification of necrosis, inflammation and blood vessels in tumors exposed to indicated treatments (top). +++ = high, + = low.

a decrease in necrotic areas in tumors. Therefore, VSV-based therapeutic approaches could be efficient in lung tumor patients that develop resistance to standard-of-care therapies.

4. IMPACT

- Two new cell lines resistant to cisplatin and sensitive to oncolysis were generated; such cell lines can be used in projects that will examine novel therapies against cisplatin resistant lung tumor cells.
- Intratumorally delivered VSV is efficient in inducing apoptosis in such resistant cells *in vitro* and it delays tumor growth *in vivo*. Oncolytic viruses are inadequately exploited therapeutics and our data indicate that VSV can be used in treatments of drug-resistant lung tumors.
- Autophagy pathway facilitates VSV oncolysis of cisplatin-resistant cells. While it has been known that drug-resistant cells occasionally up-regulate autophagy pathway for survival, this is a novel role for autophagy pathway in facilitation of VSV replication in drug-resistant cells.

5. CHANGES/PROBLEMS

We were able to synthesize cDNA library from resistant cells. Unfortunately, due to the low yield during the VSV rescue phase, we were unable to obtain sufficient number of VSV clones to generate a library of drug-resistant clones. We tried both Maraba virus rescue protocol and Vaccinia virus rescue protocol, but in each case we were unable to rescue substantial number of clones. We have instead decided to examine whether autophagy contributes to VSV oncolysis in cisplatin-resistant cells.

6. PRODUCTS

- Review paper: “The use of oncolytic viruses to overcome lung cancer drug resistance” By Beljanski V, Hiscott J. *Curr Opin Virol.* 2012 Oct;2(5):629-35
- The concept that autophagy contributes to VSV oncolysis was also a theme of our recent publication [Shulak L, Beljanski V, et al. J. Virol. 88(5):2927-40 (2014)] and was used as part of preliminary data for NIH R21 grant that was scored top 6% after first submission.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

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Current Opinion in Virology

The use of oncolytic viruses to overcome lung cancer drug resistance

Vladimir Beljanski and John Hiscott

Intrinsic and acquired drug resistance remains a fundamental obstacle to successful applications of anticancer therapies for lung cancer. Combining conventional therapies with immunotherapeutic approaches is a promising strategy to circumvent lung cancer drug resistance. Genetically modified oncolytic viruses (OVs) kill tumor cells via completely unique mechanisms compared to small molecule chemotherapeutics typically used in lung cancer treatment and can also be used to deliver specific toxic, therapeutic or immunomodulatory genes to tumor cells. Recent pre-clinical and clinical studies with oncolytic vaccine approaches have revealed promising combination strategies that enhance oncolysis of tumor cells and circumvent tumor resistance mechanisms. As clinical trials with oncolytic vaccines progress, and as the knowledge acquired from these studies builds a foundation demonstrating OVs safety and efficacy, novel combination approaches could soon have a major impact on the clinical management of patients diagnosed with lung cancer.

Address
Vaccine and Gene Therapy Institute of Florida, 9801 Discovery Way, Port Saint Lucie, FL 34987, United States

Corresponding author: Hiscott, John (jhiscott@vgtifl.org)

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This review comes from a themed issue on **Antivirals and resistance**

Edited by **Daniel Lamarre** and **Mark A Wainberg**

For a complete overview see the Issue and the Editorial

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Multidrug resistance in lung cancer chemotherapy

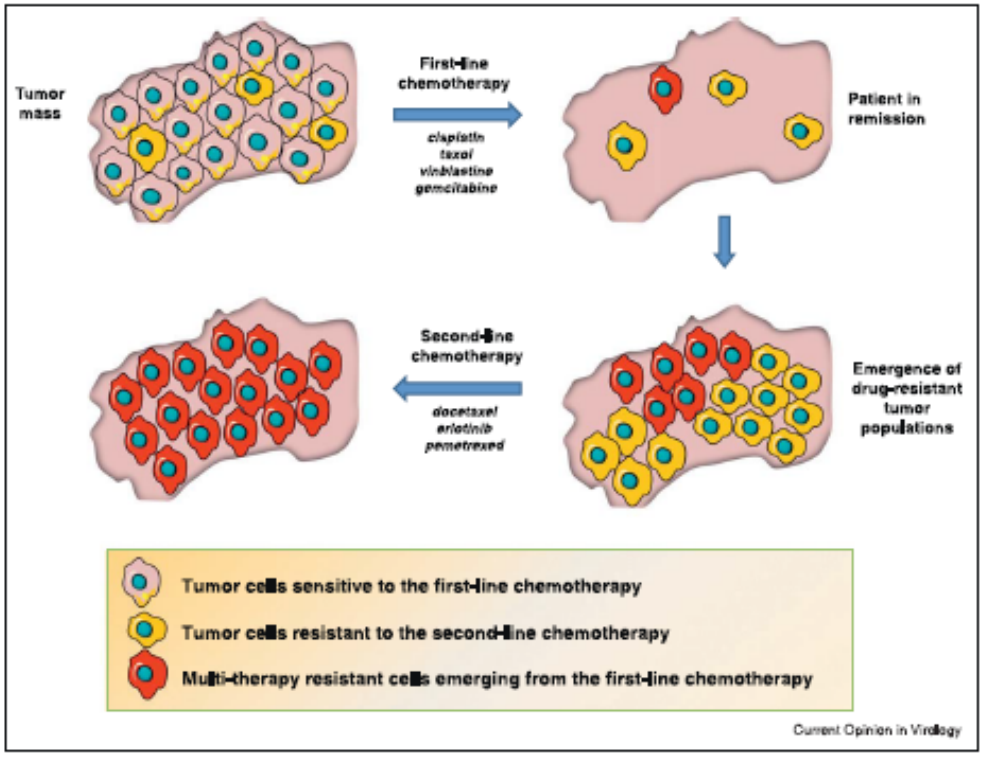
Multidrug resistance is the principal mechanism by which cancers develop resistance to chemotherapeutic drugs, and thus represents a major cause of chemotherapy failure in the clinic [1]. Tumors usually consist of mixed, genetically distinct populations of malignant cells, some of which can be eradicated with chemotherapy, while drug resistant populations remain therapy-resistant [2]. The therapy-resistant cell population continues to grow, is typically resistant to previously employed therapeutics and contributes further to the heterogeneity of the tumor population (Figure 1). Drug resistance has been observed in both solid and hematological malignancies and a number of molecular mechanisms such as overexpression of efflux transporters or antiapoptotic genes, changes in signaling pathways, and loss of or mutations in apoptotic genes, have all been described as contributing factors (reviewed in [3]).

The poor overall survival rate in lung cancer patients remains a major challenge in the clinical management of lung cancer and underscores the urgent need to develop novel therapeutic approaches that overcome intrinsic drug resistance. While non-small cell lung cancer (NSCLC) cells are often resistant to drugs at the beginning of the treatment, small-cell lung cancer (SCLC) cells usually acquire resistance during treatment [4]. The majority of patients at the time of diagnosis already present a drug-resistant phenotype, resulting in a poor 5-year prognosis that remains less than 15% for NSCLC and 5% for SCLC [4]. Numerous studies shed light on resistance mechanisms, and it is now recognized that therapy-resistant lung cancer cells: (1) overexpress membrane transporters such as ABC transporters that function as drug efflux pumps; (2) overexpress sulfur containing proteins and peptides that bind to and inactivate small molecules such as cisplatin; (3) upregulate DNA repair enzymes that reverse therapy-induced DNA lesions; and (4) lose intracellular apoptosis-mechanisms leading to prolonged survival even in the presence of cytotoxic therapies (reviewed in [4]). In addition, drug-specific mechanisms involving mutation of small molecule binding sites on target proteins have also been described [5]. A number of oncogenes that give rise to lung cancer development have been identified: EGFR (mutations), EML4-ALK (fusion), K-RAS (mutations), PIK3CA (mutations), and MET (mutations) (reviewed in [6]). This diversity of oncogenes complicates design of therapies for lung cancer, and a successful treatment will probably require a several chemotherapeutic agents to be administered simultaneously. This diversity of resistance mechanisms highlights the need for therapeutic approaches that will complement or even bypass 'classical' small-molecule based therapies.

OVs as chemotherapeutics

Development of immunotherapies is a rapidly maturing field of experimental cancer research that has the potential to yield major breakthroughs in cancer treatment. Oncolytic viruses (OVs) are inadequately exploited immunotherapeutics that can be either selected or genetically engineered to specifically replicate in cancer cells [7–9]. Tumor cells often display deregulated or defective host antiviral response mechanisms – a 'so-called' Achilles heel of cancer cells – that permits selective

Figure 1



Formation of multidrug-resistant lung cancer cells. First-line chemotherapy with cisplatin, taxol, vinblastine and gemcitabine eliminates drug-sensitive cancer cells but fails to eradicate drug resistant variants because of the genetic and epigenetic changes in drug-exposed tumor cells. These resistant cell populations will give rise to new, multidrug-resistant cells (colored in red). Second line chemotherapy is applied concomitantly (docetaxel, erlotinib and pemetrexed) and the majority of tumor cells resistant to first-line chemotherapy are now eliminated, but multidrug-resistant tumor cells still possess clonal potential and after a short period of remission expand further and acquire metastatic potential.

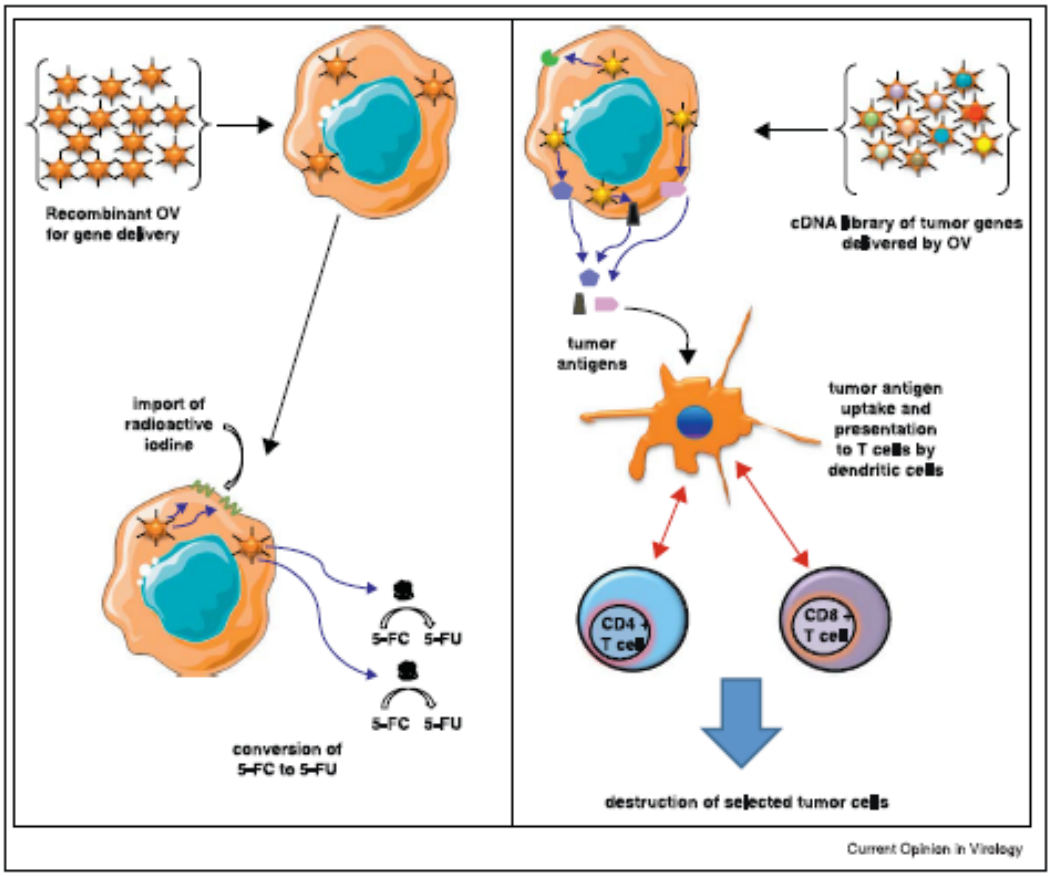
tumor-specific viral replication, ultimately resulting in cancer cell apoptosis. OVs can also stimulate the adaptive immune response against tumor antigens, resulting in the immune elimination of tumor cells and/or antitumor vaccination [10**,11–13]. Finally, OVs lack genotoxicity [14] and offer potential for delivery of immunomodulating genes, pro-drug metabolizing genes, pro-apoptotic genes and other therapeutic genes, that open numerous possibilities for therapeutic interventions [15,16] (Figure 2). Several OV variations are currently being evaluated in phase 2 and 3 clinical trials (reviewed in [17]), with highly encouraging response rates of 30–70% being reported [18**].

On the contrary, therapeutic management of complex cancers with immunotherapies – including OVs – is a formidable task, although research during the past decade has identified relevant tumor-specific antigens, delivery vectors and adjuvants/combination approaches that bypass the immunosuppressive environment of NSCLC [19]. Lung cancer may also be an attractive target for OV therapy, partly because of the possibility of intranasal delivery of therapeutic viral particles [20]. The majority of OVs evaluated in preclinical models of NSCLC were adenoviruses [21–23], but therapeutic efficacy with other viruses such as herpes simplex virus [24], coxsackievirus [25], Newcastle disease virus [26], Seneca valley virus [27], reovirus [28,29] has also been reported. TG4010 is an OV-based anticancer vaccine, based on highly immunogenic, modified vaccinia virus vector expressing MUC1 antigen, together with IL-2 as an immunoadjuvant to reverse suppression of T-cell response [30]. This genetically modified virus was evaluated in open-label phase IIb clinical trials in MUC1-positive NSCLC patients [31*]. When combined with first-line chemotherapy, TG4010 delayed advanced NSCLC progression [19].

Combination of OVs and other anticancer therapies

The real potential of oncolytic viruses may be fully appreciated only when used in combination with other therapeutic approaches such as chemotherapy, targeted

Figure 2



Oncolytic viruses as a tool to eliminate multidrug-resistant lung cancer cells. Multidrug-resistant lung cancer cells are exposed to oncolytic viruses that replicate preferentially in cancer cells. The left panel represents a recombinant OV that will deliver a symporter gene or a drug-converting enzyme to tumor cells. This strategy yields higher drug concentrations in tumor cells (as in the case of radioactive iodine) or leads to local high concentrations of cytotoxic drugs (5-FU, 5-fluorouracil), administered as non-toxic pro-drugs (5-FC, 5-fluorocytosine). The latter approach will also lead to 'bystander killing' of tumor cells that are not infected with viruses. The right panel represents viral delivery of cDNA libraries generated from drug-resistant tumor cells. This library will express many known and unknown resistance-associated tumor epitopes that can be taken up by dendritic cells and presented to CD4+ and CD8+ T cells leading to priming of adaptive immunity and antitumor vaccination.

therapy and/or radiation therapy. The combination of oncolytic vaccines together with small molecule inhibitors or immune modulators has been studied largely as means to facilitate virus replication and cell killing in tumors that are resistant to viral oncolysis [32,33], and for transient immunosuppression that facilitates viral delivery by reducing the protective effect of neutralizing antibodies [34,35]. In addition, because they kill cancer cells through virus-mediated oncolysis, OVs also have the potential to eradicate drug-resistant populations, during or after treatment with conventional therapeutics [36].

Vesicular stomatitis virus (VSV) is an excellent prototypical OV for several reasons: because VSV is not a human pathogen, most individuals do not possess humoral antibodies against VSV [37]; VSV possesses a broad host range because of the envelope G glycoprotein and thus infects most cell types; VSV replicates exclusively in cytoplasm and thus does not have a mutational capacity or transforming ability, often associated with integrating viral vectors [38]. Furthermore, VSV is easily manipulated by molecular techniques that permit therapeutic gene insertion [39] and rescue of high titer infectious recombinant virus. VSV specifically replicates in tumor cells with acquired defects in antiviral interferon signaling pathways and about 75% of tumor cell lines examined are susceptible to VSV oncolysis [20]. Tumor cells defective in Ras, p53 and c-Myc signaling pathways are likewise susceptible to VSV infection and replication [40].

Despite the distinct advantages of VSV, a number of primary tumor specimens and some cancer cell lines remain resistant to oncolysis. Several groups, including ours, have investigated various combinations of OVs with small molecules to improve oncolysis [41•,42•,43••]. For example, OVs in combination with histone deacetylase inhibitors (HDIs) such as vorinostat (SAHA) and MS-275, or the mTOR inhibitor rapamycin – small molecules that modulate gene expression and immune responses – dramatically enhance OV replication and tumor cell lysis [33,43••]. An excellent example of such approach is the combination of VSV and vorinostat in a hormone-refractory prostate tumor model resistant to oncolysis; when VSV was combined with vorinostat, VSV replication and apoptotic cell death was increased in tumor cell lines and in murine models of prostate, colon and melanoma cancer [43••]. Similarly, in primary tumor specimens resistant to VSV infection, addition of vorinostat enhanced VSV replication, and remarkably, this effect is cancer tissue-specific [43••]. Combination strategies that increase viral replication also help to circumvent the low bioavailability of systemically administered OVs, and addition of such small molecules may improve pharmacoavailability of OVs [44].

Modulating immune responses with epigenetic modulators such as HDIs is not the only therapeutic combination that can be utilized to increase viral oncolysis. In patients with primary chronic lymphocytic leukemia (CLL), *ex vivo* primary CLL samples are resistant to various chemotherapies [45], including VSV oncolysis [46]. Resistance to oncolysis in CLL is partly owing to the overexpression of the anti-apoptotic Bcl-2 protein that binds to and inactivates pro-apoptotic proteins BAD and BAX, and thus blocks intrinsic mitochondrial dependent apoptosis [47]. We hypothesized that therapies targeting Bcl-2 could sensitize CLL cells to oncolytic VSV [48,49]. Indeed, Bcl-2 antagonists blocked heterodimerization of Bcl-2 and BAX, and the combination of VSV and Bcl-2 inhibitors resulted in mitochondrial dependent apoptosis in CLL cells, with a therapeutic index of 18 for this combination therapy [49].

Another strategy that combines OVs with small molecules, termed 'suicide gene therapy' [50], is the delivery of genes encoding prodrug-converting enzymes to cancer cells in order to locally modify a nontoxic prodrug into a pharmacologically active agent. This approach limits systemic toxicity, and leads to increased local bioavailability and increases local 'bystander killing' of non-infected tumor cells [51]. This strategy was studied using recombinant VSV to deliver toxic enzymatic activities such as the HSV thymidine kinase enzyme that phosphorylates the prodrug ganciclovir, or the human sodium iodine symporter to tumor cells, resulting in accumulation of radioactive iodine at the tumor site [38,52]. We investigated this synergistic approach by combining recombinant VSV expressing the cytosine deaminase/uracil phosphoribosyltransferase protein (VSV-C) and 5-fluorocytosine (5FC) pro-drug in a panel of cancer cell lines and found increased apopotosis in bystander non-infected cells *in vitro* [53]. These data were further corroborated in an animal model of syngeneic TSA mammary adenocarcinoma, where administration of VSV-C and 5FC led to increased animal survival compared to animals treated with single agents. The option of therapeutic gene insertion into the VSV vector thus permits the augmentation of viral biological activity with specific mechanisms to kill cells, maximize anticancer activity, and re-introduce apoptosis-inducing genes that are frequently incapacitated in cancer cells [54].

Combination of OV-based therapies in lung cancer treatment

Various combination strategies have been tested in lung cancer models to evaluate both wild-type and recombinant OVs in combination with small molecule therapies, and even radiation therapy [55,56]. The first genetically engineered OV that was evaluated in clinical trials was ONYX-015, a human adenovirus with specific cytolytic effect in tumor cells with nonfunctional p53 [57]. *In vitro*, ONYX-015 showed synergistic effect (5–10-fold) with standard NSCLC chemotherapy in two of the four primary tumor specimens, when combined with low doses of cisplatin or paclitaxel [58]. Antitumor activity in lung and several other solid tumors was observed when two genes that mediate cancer cell death (TNF-related apoptosis-inducing ligand [TRAIL] and IL-24) were simultaneously delivered to cancer cells via tumor-specific adenoviral vector ZD55 in an approach termed 'dual gene virotherapy' [59]. IL-24 is an attractive gene for cancer therapy as it negatively regulates several oncogenic pathways, suppresses angiogenesis, and stimulates antitumor immune responses, while TRAIL binds to TNF-related death receptors and induces caspase-8-dependent apoptosis. [60,61]. Adenoviral ZD55 vector expressing IL-24 was also evaluated in combination with standard chemotherapeutics, cisplatin or doxorubicin, in xenograft models of lung tumor and, compared to single treatment groups, the tumor growth of co-administration group was remarkably delayed [60].

The synergistic effect of Reovirus type 3 Dearing strain combined with standard cancer chemotherapies has been evaluated in a panel of NSCLC cell lines [29]. In these experiments, synergism was only observed in drug-sensitive cells when the virus was combined with cisplatin, gemcitabine and vinblastine; the combination of Reovirus with paclitaxel was synergistic in all cell lines, and correlated with increased PARP cleavage compared to other co-treatments. Interestingly, increased virion production was observed in cell lines treated with reovirus + paclitaxel, but the increase in virion production was also observed in reovirus + vinblastine without synergistic effect. Additional therapeutic benefit can be achieved

by 'arming' virus with a fusogenic glycoprotein from gibbon ape leukemia virus (GALV), which should facilitate the spread of virus by inducing cell-to-cell fusions [62]. Genetically engineered HSV, expressing yeast cytosine deaminase/uracil phospho-ribosyltransferase fusion protein to convert 5-fluorocytosine to 5-fluorouracil and GALV generated a highly potent oncolytic virus that was evaluated in combination with 5-fluorocytosine in a number of solid tumor models, including lung tumors [63].

Another highly promising approach in combating lung cancer drug resistance is to utilize highly immunogenic OVs to prime adaptive immunity upon viral delivery of drug-resistance associated antigens, leading to enhanced antitumor immunity [10**,13]. Recombinant VSV carrying a normal prostate tissue cDNA library was used to treat prostate tumors of the same histological type [10**]. A suboptimal therapeutic dose resulted in accumulation of therapy-resistant population that was eliminated with a second VSV vector delivery of a cDNA library created from tumors that escaped the first tumor vaccination. Therefore, it is possible to target a population of cancer cells that escaped previous therapeutic interventions using viral delivery of cDNA isolated from the therapy-escape cell population. Because the mechanisms of resistance in lung cancer frequently include overexpression and mutation of oncogenes [4], a highly immunogenic viral platform that delivers drug-resistance associated epitopes has the potential to prime antitumor immunity and activate CD8+ T cells for lung tumor elimination. Interestingly, no pre-clinical data are available for these highly immunogenic virotherapies in combination with HDIs. OVs and HDI combinations could induce complex immune responses to virotherapies in cancer patients and further examination of adaptive and innate immune responses to such therapies are required.

Future prospects of OVs in lung cancer treatment

Although the majority of pharmaceuticals currently used in cancer treatment are small molecule drugs, the emergence of immunotherapies including oncolytic vaccines is having an increasingly important impact on the development of cancer therapies. Several obstacles remain in the development of oncolytic virotherapies, the most important of which include the optimization of systemic OV delivery and the stimulation of adaptive immunity against tumor rather than viral antigens. Promising pre-clinical and clinical studies with the TG4010 cancer vaccine in combination with standard chemotherapy illustrate the potential of oncolytic vaccines in combination with first or second line chemotherapies (Figure 1). Finally, oncolytic specific targeting of drug-resistant cancers with cDNA libraries, coupled with controlled optimization of viral replication with HDIs, opens the possibility to fight drug resistance and modulate the immune response in OV-based therapies.

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- •• of outstanding interest

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