

Improving immunotherapy using vanadium-based compounds

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Abstract

Heterogeneity of response and tumor resistance are preventing oncolytic viruses from reaching their full therapeutic potential in the clinic. We recently reported that vanadium-based compounds improve oncolysis and induce long-term antitumor immunity when used in conjunction with oncolytic virus VSV Δ 51 in murine models of carcinoma. Given the known capacity of vanadium compounds to inhibit multiple phosphatases (PPases), we hypothesized that vanadium compounds work by altering PPase/kinase homeostasis and set out to explore this idea using a targeted screening approach. Based on previous observations, we further hypothesized that a vanadium/OV regimen could be effective in sarcoma and synergize with immune checkpoint blockade.

A high-throughput screen using a library of small interfering RNAs targeting human PPases revealed multiple PPases that significantly increase VSV Δ 51 infection and cytotoxicity. A complementary kinase inhibitor screen and pathway analysis further unveiled kinase/PPase substrates that may contribute to vanadate's mechanism of action, as well as novel viral sensitizers, which were validated in *in vitro* and *ex vivo* models. The vanadium/VSV Δ 51 combination therapy was tested in mice bearing rhabdomyosarcoma tumors. While tumor progression was delayed, no significant improvement in survival was observed in the dual vanadium/VSV Δ 51 test group compared to VSV Δ 51 monotherapy. An aggressive triple-combination regimen of vanadium-VSV Δ 51 with immune checkpoint blockade led to high tumor cure rates but was not well tolerated and led to serious adverse events including treatment-related deaths.

Overall, this project provides insights into vanadium's mechanism of action and identifies promising novel small molecule enhancers of VSV Δ 51 virotherapy. Our studies also

warrant that further treatment regimen optimization and exploration be carried for the safe and effective use of vanadium-OV containing immunotherapy regimens in the context of sarcoma.

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List of abbreviations

CAF	Cancer associated fibroblast	NAE	NEDD8-activating enzyme
CDK	Cyclin dependent kinase	NFkB	Nuclear factor k-B
CPI	Checkpoint inhibitor	NSCL	Non-small cell lung cancer
CTLA4	T-lymphocyte-associated protein 4	OV	Oncolytic virus
DAMP	Damage-associated molecular pattern	PAMP	Pathogen-associated molecular pattern
EGF	Epidermal growth factor	PCR	Polymerase chain reaction
HAD	haloacid dehalogenase	PD-L1	PD-1 ligand
HDAC	Histone deacetylase	PPase	Phosphatase
hpi	Hours post infection	ppm	Parts per million
HSV-1	Herpes simplex virus-1	PPM	Metal-dependent protein phosphatase
HTS	High-throughput screen	PPP	Phosphoprotein phosphatase
i.p.	Intraperitoneal	PRR	Pattern recognition receptors
i.t.	Intratumoral	PTP	Protein tyrosine phosphatase
IFN	Interferon	RIG-I	Retinoic acid-inducible gene I
IL	Interleukin	RISC	RNA-induced silencing complex
IRF	Interferon regulatory factor	RMS	Rhabdomyosarcoma
ISG	Interferon stimulated gene	STAT	Signal transducer and activator of transcription
ISRE	Interferon-stimulated response element	TAA	Tumor associated antigens
JAK	Janus kinase	TGF-β	Transforming growth factor beta
LDLR	Low density lipoprotein receptor	TLR	Toll-like receptor
MDA5	Melanoma differentiation-associated gene 5	TME	Tumor microenvironment
MDSC	Myeloid-derived suppressor cells	TYK	Tyrosine kinase
MHC	Major histocompatibility complex	Van	Vanadate
MOA	Mechanism of action	VS	Vanadyl sulfate
MOI	Multiplicity of infection	VSV	Vesicular Stomatitis virus

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1. Introduction

1.1 Cancer

1.1.1 Statistics: global and Canadian trends

Research and development in technology and medicine have culminated in revolutionary advancements. The 30-year increase in human life expectancy over the last century in some parts of the world is a clear tribute to the scientific progress¹. Yet, cancer remains a serious global concern. Even if mortality rates have been steadily decreasing over the past decades in Canada, the fact remains that an estimated one out of four Canadians will succumb from a form of cancer². Latest studies indicate that cancer incidence, in Canada and globally, are on the rise partly because of prolonged lifespans and growing populations^{2,3}. Worldwide, the numbers are striking; over 18 million new cancer diagnoses and nearly 10 million cancer-related deaths were estimated for the year 2018 alone³. The urgency is further captured by the initiative implemented by the United Nations to decrease by a third the number of premature deaths attributable to noncommunicable diseases, including cancer, by year 2030³. In addition to its profound repercussions on families and quality of life, a cancer diagnosis is a burden to the healthcare system and the Canadian economy⁴. To this end, increased efforts in prevention, education and research are being deployed.

1.1.2 Cancer biology and the tumor microenvironment

Malignancies are heterogeneous on many levels; their diverse cell and genetic composition^{5,6} as well as their wide spectrum of phenotypic and clinical presentations⁷ are challenges for modern medicine and contribute to treatment resistance, morbidity and

mortality. Over the years, a set of distinctive cancer characteristics has been outlined as research improved our understanding of tumorigenesis: rewired cell metabolism, immunoevasion, continuous proliferation, defective growth inhibition and cell death circuitries, angiogenesis and metastasis⁵. The body's natural innate and adaptive surveillance mechanisms allow for the detection and immune-mediated elimination of malignancies, but this selective pressure causes cancers to evolve ways to minimize their immunogenicity through immunoediting⁸. For example, malignant cells can downregulate MHC class I⁹ or directly block CD8 T cell effector functions by expressing programmed cell death protein ligand 1 (PD-L1) thereby conferring cancer tolerance¹⁰. Tumors can also capitalize on the expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA4) on regulatory or activated T cells, which competes with the CD28 costimulatory molecule for B7 on an antigen presenting cell¹¹. CTLA4 is an inhibitory molecule with a higher affinity for B7 than CD28 and thereby constitutes an important regulator of immunological homeostasis and tolerance¹². When CTLA4 engages with a B7 molecule in lymphoid organs, it results in T cell anergy and this systemic halt in T cell response can promote tumor progression because it suppresses T cells exposed to tumor associated antigens (TAAs)¹¹. In addition, cancer cells secrete immunosuppressive cytokines, like transforming growth factor beta (TGF- β) and anti-inflammatory interleukin 10 (IL-10), or rely on cell populations like immune cell infiltrates (regulatory T cells, myeloid-derived suppressor cells (MDSCs), type II macrophages), cancer associated fibroblasts (CAFs) and vascular cells to resist eradication^{6,13,14}. How these cellular subpopulations and their relationship with various hallmarks of cancers can be leveraged in the clinic to benefit patients is a field in rapid development.

1.1.3 Modern therapeutic strategies

Chemotherapy, radiotherapy and surgery are well established in clinics. Chronologically, surgery is the most ancient practice with reports of tumor resections as early as 3000 B.C, followed by radiotherapy which was formally implemented in the early 1900's¹⁵. After the First World War, nitrogen mustard unintentionally set the foundations of chemotherapy¹⁶. Considered a pivotal point in the history of medical oncology, the chemotherapy arsenal now includes a variety of compounds: DNA alkylating agents (e.g. cyclophosphamide), antimetabolites (e.g. methotrexate), antimitotics (e.g. vincristine) and antineoplastic antibiotics (e.g. doxorubicin)¹⁵. Much like vaccination, chemotherapy is considered by many as one of medicine's most successful discoveries of the 20th century¹⁵. However, these treatment modalities have extensive chronic and latent side effects including infertility, pain, organ toxicity, cardiovascular and neurological complications¹⁷. Furthermore, recent work has shown that the immune system is down-modulated after cancer surgery which therefore increases the risks of postoperative relapse¹⁸.

In light of these serious limitations and with the advent of molecular breakthroughs, targeted therapies were developed in an attempt to reduce the off-target and debilitating effects witnessed in previous interventions. These biologics (e.g. HER2 monoclonal antibody Trastuzumab) or pharmaceutical products (e.g. EGFR kinase inhibitor Gefitinib) act on select molecular drivers of oncogenesis; however, this makes them highly susceptible to drug resistance mechanisms¹⁹.

Important events such as (but not limited to) the recent Nobel prize awarded to James P. Allison and Tsuku Honjo for their discoveries on immune checkpoint blockade²⁰ and the approval of modified oncolytic herpes simplex virus 1 (HSV-1) Imlygic® by

European, Australian and American health agencies²¹ have recently drawn the public's attention towards immunotherapies. This class of therapy holds significant promise because it reshapes a patient's own immune system to not only directly recognize and eliminate a tumor but also helps build a durable line of defence against cancer recurrence.

1.1.4 Current sarcoma research landscape

Sarcomas are malignancies that stem from mesenchymal tissues in contrast with carcinomas, which arise from epithelial cells²². In Canada, soft tissue sarcomas and malignant bone tumors have the second and third poorest 5-year observed survival among all childhood diagnostic groups and constitute about 11% of all cancer diagnoses in that age category²³. Treatment modalities involve surgery, radiotherapy and chemotherapy²⁴. Although relatively rare, sarcomas are aggressive with about a third of patients presenting with metastasis at time of diagnosis⁷, in which case the five-year conditional survival is only 33-38% at time of diagnosis²⁵. Despite developments in molecular biology, the survival rates have not significantly improved in the last few decades²⁶. Targeted therapies result in modest therapeutic benefits. For example, VEGF1-3 inhibitor Pazopanib, resulted in only a 2 month increase in overall survival relative to placebo in a phase 3 clinical trial involving patients with advanced soft-tissue sarcoma which subsequently led to its approval²⁷. Despite breakthroughs achieved with immunotherapies in the treatment of other malignancies, like melanoma (e.g. checkpoint inhibitors²⁸, OV Imlygic®²¹), immune-based therapies like checkpoint blockade^{29,30}, vaccines³¹ and cytokine-based³² therapies have not been successful in sarcomas³³. Few OV's have been explored in clinical studies such as JX-594 (vaccinia encoding GM-CSF)³⁴. Although generally well tolerated, JX-594 exhibited

limited efficacy in sarcoma³⁴. Given the heterogeneity of the disease, sarcoma patients might benefit from combination therapies.

1.2 Oncolytic viruses

1.2.1 The multiplexed mechanism of action of OVs

Oncolytic viruses (OV) such as Imlygic® are a class of immunotherapy that consists of the infection of malignant cells by viruses with engineered or innate oncospecificity³⁵. Unlike normal cells, tumors frequently have compromised antiviral defences because tumorigenic and antiviral networks serve incompatible functions³⁶. Upon infection, OVs replicate and directly lyse cancer cells thereby releasing newly synthesized infectious particles into the tumor microenvironment (TME) along with damage- and pathogen-associated molecular patterns (DAMPs and PAMPs) and TAAs which can be captured by antigen presenting cells³⁷. Upon lytic infection, the OV spreads within the TME where it can also infect CAFs³⁸ and endothelial cells to disrupt tumor blood supply³⁹. In summary, the actions of OVs culminate in immunogenic cancer cell death, *in situ* vaccination, vascular collapse, disruption of the stroma and stimulation of an innate and adaptive immune response³⁷⁻³⁹.

OVs with DNA backbones (e.g. vaccinia⁴⁰) and RNA genomes (e.g. vesicular stomatitis virus⁴¹) are actively being pursued. An important advantage of OVs is that they do not depend on the discovery of neoantigens, which often constitutes a challenging rate limiting step in the development of other immunotherapies like cancer vaccines⁴². Also, as OVs follow the course of their viral life cycles, the number of infectious viral particles is gradually increased therefore multiplying the therapeutic dose readily available in the TME. Most importantly their genome can be modified to encode therapeutic genes (e.g.

GM-CSF cytokine in vaccinia virus JX-594⁴³), or to improve their safety profiles (e.g. deletion of methionine 51 in the matrix protein of vesicular stomatitis virus⁴⁴).

1.2.2 Vesicular stomatitis virus (VSV)

VSV, belonging to the *Rhabdoviridae* family, is an enveloped virus composed of a negative single-stranded RNA genome⁴⁵. Its RNA contains sequences encoding five key proteins: nucleoprotein (N), phosphoprotein (P), RNA-dependent-RNA-polymerase (L), the matrix protein (M) and the glycoprotein (G)^{45,46}. Adsorption relies on the engagement of the viral G protein with host cell low density lipoprotein receptor (LDLR)⁴⁷ which subsequently triggers clathrin-dependent endocytosis of the viral particle⁴⁸. Next, endosomal acidification culminates in G protein conformational changes and the release of the encapsidated genome into the cytosol through fusion of viral and endosomal membranes⁴⁹. The L and P proteins catalyze the transcription of mRNAs using the viral N protein-coated RNA as template for the translation of viral proteins^{46,50}. Viral genome replication operates first by generating a positive RNA strand from which a complementary negative strand, identical to the parental genome, can be produced⁴⁶. In parallel, G proteins are processed by the secretory pathway and accumulate at the cell surface⁵¹. Finally, the newly synthesized viral proteins and the ribonucleocapsid are shuttled to the cell membrane for assembly and budding of virions.

The M protein is a known neurovirulence factor in the context of wildtype VSV and is involved in shutting down nucleocytoplasmic movement through the nuclear pore complex thereby obviating host antiviral gene expression⁵². To increase its safety profile, a VSV variant carrying a deletion of the 51st residue (methionine) has been generated⁴⁴. Building on the observation that the majority of neoplastic cells have short-circuited antiviral

networks⁴⁴, the resulting mutant thus has improved oncotropism since viral infection is possible in cancer cells but dampened in normal cells with active innate defense mechanisms.

VSV is a practical candidate for the development, testing and clinical translation of virotherapies. Viral production yields high titers in a short time span, its cytoplasmic replication avoids the risks of genotoxicity or host gene mutations and humans are generally naïve hosts with no preestablished immunity against the virus since horses, cattle and pigs are typical VSV hosts in nature⁵³. Furthermore, this virus is amenable to intravenous administration.

1.3 The innate immune response and viral sensitizers

A variety of OV-based immunotherapies have undergone clinical testing, but they have generally fallen short of their anticipated clinical efficacies as monotherapies⁵⁴. Despite promising pre-clinical evidence, the major issues in the clinic include subpar oncolytic activity and antitumor immunity⁴¹. Many factors can justify this poor response, one of which is functional antiviral cascades in transformed cells^{44,55,56}.

Pattern recognition receptors (PRRs) are sensors of microbial elements (PAMPs). In the context of RNA virus infection, there are three major PRRs present in endosomes, at the cell membrane or in the cytoplasm: toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5)⁵⁷. TLRs engage with exogenous proteins, lipids, peptides and nucleic acids⁵⁸. RIG-I's and MDA5's PAMP is RNA⁵⁹ although RIG-I has a marked affinity for sequences harbouring a triphosphorylated 5' end (5'ppp)⁶⁰. Upon PRR-PAMP engagement, a signalling cascade is triggered leading to the activation of transcription factor nuclear factor kB (NFkB) and IFN

response factors (IRFs) for expression of pro-inflammatory cytokines and type I IFNs respectively⁵⁸.

IFNs are cytokines secreted in response to microbial invasion to fight infection, prevent spread and activate cells of the immune system⁶¹. There are presently three families of IFNs: type I (e.g. IFN α and IFN β), type II (IFN γ) and the newly discovered type III (IFN λ s)⁶¹. Once synthesized, type I IFNs are secreted and interact with type I IFN receptors (IFNAR1/2)⁶². This activates a signal transduction cascade that first involves receptor dimerization, then the activation of tyrosine kinase 2 (TYK2) and Janus activated kinase 1 (JAK1) found on the cytoplasmic receptor tails, followed by the subsequent phosphorylation and dimerization of STAT1 and STAT2⁶³. Together with IRF9, they migrate to the nucleus, where they recognize the IFN stimulated response element (ISRE) within the promoter region of IFN-stimulated genes (ISG)⁶³. ISGs fight infection by interfering with nearly every step of the viral life cycle like viral entry (e.g. IFITM3)⁶⁴, transcription and replication (e.g. MxA)⁶⁵ or budding (e.g. tetherin)⁶⁴.

Unlike type I IFNs, type II IFN are usually secreted from natural killer cells or T cells and act through IFN γ receptor (IFNGR), JAK1 and JAK2⁶¹. In a similar fashion, STAT1 is phosphorylated but this time homodimerizes and translocates to the nucleus to bind DNA at the IFN γ -activated sequence (GAS)⁶³. The resulting gene signature is proinflammatory (e.g CXCL9, IRF1)⁶⁶.

The activity level of the type I IFN axis is variable in transformed cells and remains a problem for cancer virotherapy^{44,55,56}. In an effort to improve clinical outcomes, our lab and others have explored and identified a series of small molecules that potentiate OV activity and induction of antitumor immunity. The term “viral sensitizer” or “VSe” was

first coined by Diallo et al. to describe compounds that synergistically increased viral infection in tumor cells⁶⁷. A >12,000 compound high-throughput screen, has led to the discovery of VSe1 which yielded a 1,000-fold enhancement in viral titers in refractory renal cell carcinomas⁶⁷. Some of the first drugs tested in combination with OV_s include chemotherapeutics⁶⁸ and histone deacetylase inhibitors^{69,70}. Since then, many new viral sensitizer drugs have been identified. For example, dimethyl fumarate (DMF), a drug indicated for the treatment of multiple sclerosis, enhances VSVΔ51 infection by shutting down the type I IFN cascade through NFκB inhibition⁷¹. Most recently, vanadium-based compounds were identified as novel VSe_s exhibiting the capacity to enhance VSVΔ51 bystander killing and spread⁷². In aggressive murine models of brain, breast and colon cancers, the combination potentiated tumor-targeted infection, prolonged survival and led to some durable cancer-specific immunity⁷². This proposed a new clinical application for vanadium compounds, which have been studied in the past mostly for their insulin mimetic and more recently antineoplastic actions.

1.3.1 Vanadium and human health

Vanadium is a transition metal naturally found in mammalian tissues; in fact, it is estimated that 1 mg of vanadium can be found in the human body⁷³. At physiological pH, vanadium forms vanadyl (VO^{2+}) or vanadate (VO_4^{3-}) with oxidation states V(IV) or V(V) respectively^{74,75}. It predominantly accumulates in bones⁷⁶ and has a wide spectrum of reported functions in health and disease. For example, vanadium is important for bone development, a feature likely attributed to its ability to activate osteoblasts⁷⁷. Vanadium-based compound vanadyl sulfate is commonly used as a bodybuilding supplement although its efficacy remains a topic of controversy⁷⁸. Vanadium derivatives like

bis(ethylmaltolato)oxovanadium(IV) have been studied clinically for the treatment of type 2 diabetes due to their insulin-like effects and ability to regulate glycemia⁷⁹. Mechanistically, this involves restoring signal transduction through the insulin receptor and glucose intake via GLUT4 by inhibiting PTP1b⁸⁰. However because of the heterogeneity in responses, the small sample size, the side effects (e.g. gastrointestinal distress) and the lack of evidence on long-term efficacy and safety, vanadium clinical trials have not progressed to market approval and the patent has since expired⁸¹.

Vanadium-based compounds have been explored for their anti-cancer properties due to their potential to impinge on specific hallmarks of cancer⁸². For example, vanadate can limit growth of hepatocellular carcinoma cells by inducing a G2/M cell cycle arrest and ultimately apoptosis⁸³. Therapeutic efficacy has been reported in an animal model of hepatic cancer where delayed tumor progression was observed with sodium orthovanadate⁸³. Interestingly, the invasion and migratory potential is diminished in *in vitro* models of osteosarcoma upon treatment with V(IV) complexes⁸⁴.

The exact impact of vanadium on the immune system is poorly understood. Sodium metavanadate confers radioprotection by promoting proliferation and activation of B cells in mice originally immunocompromised by γ -ray irradiation⁸⁵. In human renal cell adenocarcinoma cells, the treatment of vanadate stimulated the production of pro-inflammatory cytokines and the effect was further improved when the vanadate treatment was combined with oncolytic VSV Δ 51⁷². However, in a study conducted on chickens, diets supplemented with > 30 ppm of ammonium metavanadate were associated with lowered blood IL-2 and IL-6 levels and led to inferior percentages of splenic CD4 and CD8 T cells relative to the control diet test group⁸⁶. Inversely, when fed diets containing 5 ppm of

ammonium metavanadate, modest but statistically significant increases in the percentage of splenic CD4 T cells and IL-6 serum levels were observed⁸⁶. Furthermore, a sodium metavanadate dose of 100 μ M suppresses human peripheral blood mononuclear cell proliferation as well as IFN- γ and IL-5 production whereas a concentration of 0.1 μ M increased TNF- α and IL-5 secretion⁸⁷. Evidently, vanadium derivatives modulate the activity of various immune subsets⁸⁸. Compound concentration and chemistry (e.g. oxidation state, stability) may be contributing factors.

A major limitation in vanadium-based medicine is that knowledge on the mechanism of action is highly fragmented. Due to its structural analogy with phosphate, vanadate is believed to enter cells through phosphate channels or endocytosis⁸⁰. Although vanadate has been shown to inhibit various classes of PPases including serine/threonine PPases⁸⁹, its affinity is highest for protein tyrosine PPases⁹⁰. The mechanisms by which vanadium compounds suppress PPase activity, and likely exert their antineoplastic and immunoregulatory actions, are through reactive oxygen species (ROS)-mediated oxidation of the active site's cysteine residue⁸⁰, and by competitively blocking the active site⁹¹.

1.3.2 Vanadium-based viral sensitizers

Despite mounting evidence supporting their therapeutic potential and the vast repertoire of vanadium derivatives designed for medicinal use, none have been approved for use in the clinic thus far^{75,78}. We have proposed a novel application for vanadium compounds as small molecule enhancers of virotherapy^{72,92}. This year, we documented improvements in VSV Δ 51 virotherapy by vanadium (V) dipicolinate derivatives, a phenomenon consistent with complex degradation and accumulation of vanadate under physiological conditions⁹². We also discovered that pan-PPase inhibitor vanadate can in

fact improve VSV Δ 51 infection, tumor control, survival and adaptive immune responses in aggressive models of breast, colon and brain cancers relative to monotherapies⁷². This can be attributed to the subversion of an antiviral type I IFN cascade into a pro-inflammatory type II IFN response in VSV Δ 51-infected cancer cells, with sharp upregulation of chemokine expression (e.g. CXCL9/10)⁷². The combination therapy increased T cell infiltrates and activation of CD8 T cells⁷². The addition of vanadate, as a form of adjuvant, to oncolytic virotherapy is a promising pursuit that manifestly improved immune recruitment to the TME through enhanced immunostimulation.

1.4 Post-translational modifications of proteins: phosphatases and kinases

Protein phosphorylation plays an essential role in protein function and signal transduction. The covalent addition of the negatively charged phosphate group results in conformational changes that can modify a protein's activity, for example by blocking or making a binding site available⁹³. This post-translational modification is orchestrated by phosphatases (PPases) and kinases. Kinases transfer a phosphate group from a molecule of ATP onto a serine, threonine or tyrosine residue of the target protein⁹⁴. In turn, PPases remove the phosphate group and the protein regains its original conformational state.

Representing about 2% of the genome, nearly 520 genes encode for kinases in mammals⁹⁴. In contrast, there are only 189 phosphatases in the human genome⁹⁵. The ratio between the two opposite enzymes is seemingly disproportional, but to compensate, PPases associate with numerous regulatory subunits which enables precise and efficient dephosphorylation. For example, there are 45 regulatory subunits for PP1c (protein phosphatase 1 catalytic subunit) and association with these subunits dictates substrate selectivity and determines the location of the PPase complex within intracellular

compartments⁹⁶. There are four distinct PPase superfamilies; protein tyrosine phosphatases (PTP; Tyr), haloacid dehalogenase (HAD; Ser/Thr/Tyr), phosphoprotein phosphatase (PPP; Ser/Thr) and the protein phosphatase metal (magnesium or manganese) dependent (PPM; Ser/Thr)⁹⁷. PTPs dephosphorylate protein tyrosine residues but also phospholipids and glycogen⁹⁸. With a total of 107 members, this superfamily is the largest and also include dual-specificity PPases (Ser/Thr/Tyr residues)⁹⁸. On the other hand, human kinases are classified as typical (similar catalytic domain sequences) or atypical, the former being subdivided based on Ser/Thr or Tyr phosphorylation activity⁹⁴.

1.4.1 The kinome and the phosphatome in sickness and health

Phosphorylation is an important post-translational modification involved in cellular homeostasis and its manipulation holds tremendous therapeutic potential. However, PPases have had a long-standing reputation as being poor drug targets due to their structural complexity, the diversity of the cell processes they modulate and the misconception that they are less selective than kinases^{97,99}. Instead, kinases have dominated the scientific efforts in the last decades. In fact, the number of FDA-approved protein kinase inhibitors has skyrocketed from 17 small molecule inhibitors in 2012⁹⁷ to a total of 48 in 2019¹⁰⁰. Today, there are 212 protein kinase inhibitors in clinical trials¹⁰¹ and it is estimated that this enzyme family is the focus of 20-33% of global drug development initiatives¹⁰⁰. However, drug resistance is omnipresent in the clinic and tactics to overcome this problem include pharmacological combination strategies such as some involving PPase effectors^{97,99}. The major hurdle in developing selective PPase inhibitors is the strong physical similarity and conserved sequence of the active sites between PPases¹⁰². However, recent development in drug discovery methodologies have since challenged the doubts surrounding the potential

of PPases as drug targets and today six PPases inhibitors have entered clinical trials⁹⁹. Although, the study of PPase-directed medicine is in its infancy, it is nevertheless on the rise and is greeted with great optimism by the research community.

1.5 Rationale and hypothesis

We previously reported an improvement VSV Δ 51 oncolytic activity when combined with vanadium-based viral sensitizers⁷². Mechanistic explorations at the time led us to conclude that vanadium compounds simultaneously dampen the type I IFN response while boosting the type II IFN response, an effect associated with differential phosphorylation of STAT1 and STAT2⁷². However, a clear and comprehensive delineation of its mechanism of action, in terms of the specific phosphatases and kinases involved is still lacking. Given the promising effects of vanadium compounds, which are pan-PPase inhibitors, we consider that identifying specific PPases and kinases modulating resistance to oncolytic virotherapy will provide promising leads for development of improved immuno-virotherapy regimens. Also, while it enhances RNA viruses such as VSV Δ 51, vanadate does not enhance DNA OV_s that carry viral PPases or other proteins that co-opt cellular PPases as virulence factors⁷². Therefore, finding host PPases conferring OV resistance and selectively inhibiting them will broaden the applications of this viral sensitizing strategy for other OV platforms, including HSV and vaccinia which are in more advanced stages of clinical development. Since vanadate is a pan-inhibitor of PPases⁹⁰, we hypothesize that the suppression of PPase(s) activity and resulting alteration in PPase/kinase and phosphoproteome homeostasis is driving its viral sensitizing effect.

We have previously observed improved VSV Δ 51 spreading and cytotoxicity in a variety of murine, canine and human sarcoma cell lines upon vanadate pre-treatment¹⁰³ (manuscript also in preparation). We thus hypothesize that vanadium-based VSes can improve therapeutic outcomes of VSV Δ 51 oncolytic virotherapy in murine models of sarcoma. Within this framework, the vision of the current study is to improve the efficacy of OV-based immunotherapy through the manipulation of the cellular phosphoproteome, with an emphasis on improving sarcoma therapy that is in dire need of innovation.

1.6 Specific research objectives

1. Identification of PPases and kinases critical for OV therapy enhancement. This will be accomplished through two complementary screens: (i) a human PPase siRNA screen and (ii) a *reverse screen strategy* using vanadate and a kinase inhibitor library followed by pathway analysis of hits.
2. Identification of novel small molecule enhancers of virotherapy. This will be accomplished in parallel with objective 1, through the use of the kinase inhibitor library without vanadate.
3. Evaluate the impact of a vanadate/OV immunotherapy regimen in a syngeneic rhabdomyosarcoma murine model.

2. Materials and methods

2.1 Drugs

The vanadium-based compounds explored in this project were vanadium (IV) oxide sulfate hydrate ($\text{VOSO}_4 \times \text{H}_2\text{O}$, Sigma-Aldrich, cat. 233706) and sodium orthovanadate (Na_3VO_4 , Sigma-Aldrich, cat. 450243). Both compounds were dissolved in water. Sodium orthovanadate was pH-adjusted to 10 at which stage a color change from clear to yellow was observed. It was then boiled until translucent then cooled to room temperature. pH adjustment, heating and cooling steps were repeated until the solution remained translucent at pH 10 and were necessary to ensure the formation of stable monomeric vanadate¹⁰⁴. Stock solutions were stored in aliquots at -20°C and thawed on ice for *in vitro* testing. For *in vivo* experiments, vanadium (IV) oxide sulfate hydrate was prepared fresh by reconstituting in sterile PBS.

2.2 Cell culture

All cell lines were kept at 37°C in 5% CO_2 humidified incubators. 786-O (human renal cell adenocarcinoma, cat. CRL-1932), B16F10 (murine melanoma, cat. CRL-6475), Vero (African green monkey kidney cells, cat. CCL-81) and CT26WT cells (murine colon carcinoma, cat. CRL-2638) were acquired from the American Type Culture Collection (ATCC). 76-9 (murine rhabdomyosarcoma) were generously provided by Drs. Korneluk and Holcik from the Children's Hospital of Eastern Ontario. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone cat.10-013-) supplemented with 1% (v/v) penicillin-streptomycin (Gibco), 30mM HEPES buffer and 10% (v/v) serum. The

serum was prepared by mixing 3 parts HyClone newborn calf serum (Fisher cat. SH3011803) to 1 part fetal bovine serum (Gibco cat. 12483020).

2.3 Viruses

The oncolytic virus tested in this project was derived from the vesicular stomatitis virus (VSV), the Indiana subtype. It was propagated, purified and titered by standard plaque assay as previously published¹⁰⁵. A variant carrying a deletion of methionine 51 (VSV Δ 51) and expressing the green fluorescent protein (VSV Δ 51-GFP)⁴⁴ or the firefly luciferase (VSV Δ 51-fluc)^{106,107} were used for cell-based assays and animal experiments.

2.4 Human PTP siRNA screen

The MISSION® siRNA Human Phosphatase Library (cat. SI03200) targeting 303 phosphatase genes (1131 siRNA total) was acquired from Millipore Sigma and reconstituted by diluting in molecular grade RNase, DNase, protease-free water (Sigma, cat. W4502) to a final stock concentration of 2.5 μ M and stored at -20°C until use. Prior to transfection, library plates were further diluted to a final working concentration of 0.2 μ M with molecular grade water and used immediately. 786-O cells were transfected using Invitrogen's Lipofectamine® RNAiMAX reagent (cat.13778150) in a 96-well microplate format. As per supplier's instructions, RNAiMAX lipofectamine was mixed with serum-free Opti-MEM I Medium (Thermo Fisher Scientific, cat. 31985070) then incubated for 5 minutes at room temperature. Then, the RNAiMAX solution was mixed with the diluted siRNA and incubated for 20 minutes at room temperature to allow siRNA-lipid complexes formation. Lipocomplexes were subsequently mixed with a 786-O suspension leading to a final cell density of 16,000 cells per well. The final siRNA concentration per well was

20nM. Dilutions were performed using the BioTek™ Precision™ Microplate Pipetting System (Thermo Fisher Scientific) and solutions were dispensed using the MicroFlo Select™ Microplate Dispenser (Biotek™) and the manual Liquidator 96 Pipetting System (Mettler Toledo). Cells were incubated for 48 hours at 37°C in a 5% CO₂ humidified incubator. Prior optimization experiments showed stable knockdown at 48 hpi (data not shown). Subsequently, samples were infected with VSVΔ51-fluc at a multiplicity of infection (MOI) of 0.05 (using serum-free DMEM). 48 hours post infection (hpi) infectious viral particles were quantified by high-throughput titration and the relative metabolic activity was assessed using resazurin sodium salt (Millipore Sigma, cat. SI03200), as previously described¹⁰⁶, by measuring fluorescence (530nm excitation, 590nm emission) with the BioTek Microplate Reader and Gen5 2.07 software. Each microplate consisted of 80 library siRNAs, two scramble siRNA controls (Mission® siRNA Universal Negative Control #1, Sigma, cat. SIC001), two vanadate-treated wells (125μM) and two RIG-I (Millipore Sigma, cat. NM_014314, siRNA ID SASI_Hs01_00047980) knockdowns acting as transfection control (**Supplemental figure 1**). Experiment was conducted in triplicate. See **Supplemental figure 2** for a visual depiction of the high-throughput screen (HTS) strategy. The *viral sensitizer factor*⁶⁷ was calculated (**Equation 1**) to rank the primary hits based on viability data and was defined as the logarithm of the viability of transfected cells divided by the viability of transfected infected cells at 48 hpi. Viability and titer thresholds for identification of top hits were determined relative to the plate controls.

Equation 1

$$\text{Log}_{10}\left(\frac{\text{Viability PPase siRNA alone}}{\text{Viability PPase siRNA} + \text{VSV}\Delta 51}\right)$$

2.5 Kinase inhibitor screen

The Ontario Institute for Cancer Research (OICR) small molecule screening library consisting of 480 kinase inhibitors and 320 tool compounds was generously provided by the W. Stanford lab from the Ottawa Hospital Research Institute. 786-O cells were seeded at a density of 30,000 cells per well of a 96-well microplate. Cells were pre-treated with kinase inhibitor (final concentration of 1 μ M) and/or vanadate (125 μ M) for four hours prior to infection with VSV Δ 51-fluc at a MOI of 0.1 (using serum-free DMEM). Viral output, as quantified by high-throughput titration, and the relative metabolic activity using resazurin were measured at 48 hpi¹⁰⁶. Each microplate consisted of 80 library compounds, four vanadate-treated and four untreated wells acting as positive and negative controls respectively. Experiment was conducted in triplicates. Refer to **Supplemental figure 3** for a visual depiction of the HTS workflow and see **Supplemental figure 2** for an understanding of the HTS strategies. A *viral sensitizer factor*⁶⁷ was calculated to rank and further refine primary hits. It was defined as the logarithm of the viability of 786-O cells treated with library compound alone, divided by the viability of the treated infected cells (**Equation 2**). Viability and titer thresholds for identification of top hits were determined relative to the plate controls.

Equation 2

$$\text{Log}_{10}\left(\frac{\text{Viability drug alone}}{\text{Viability drug} + \text{VSV}\Delta 51}\right)$$

2.6 Pathway analysis

To identify the substrate(s) driving vanadate's viral sensitization, the top hits obtained from the human PPase siRNA screen, specifically the PPases that increase viral titers once silenced, were cross-referenced with the kinase inhibitors from the OICR

reverse screen that were found to prevent vanadate's enhancement of viral titers and oncolysis. To do so, PPase substrates were obtained from the human DPhOsphorylation Database (version 1.1)¹⁰⁸ and kinase inhibitor targets were sourced from the OICR product information files and confirmed using Drugbank (5.1.4)¹⁰⁹ or Pubchem (National Center for Biotechnology Information)¹¹⁰. The open source software Cytoscape¹¹¹ was used to intercept PPase and kinase substrates. The transparency of the colors for kinases and the PPase hits were set to reflect the resulting cell viability at 48 hpi: an opaque kinase represents high viability, and inversely, an opaque PPase represents a low viability. Therefore, network interceptions involving opaque colors are of interest as they will include the kinase that prevents vanadate's oncolysis (high viability, opaque), the PPase that enhances oncolysis upon knockdown (low viability, opaque) and their common substrate. Lastly, substrates of PPases that had no influence on VSVΔ51 titers when silenced are excluded from the network. Pathway analysis was conducted by Adrian Pelin.

2.7 High-throughput titration

Assay was conducted as previously described with minor modifications¹⁰⁶. Vero cells were seeded at a cell density of 2.5E4 cells/well (final volume of 100μL) on an opaque white bottom 96-well microplate (Thermo Fisher Scientific, cat. 07-200-628). 48 hpi, 20μL of supernatants were transferred from treated 786-O cells onto Vero cells. Controls with known PFU/mL (standard curve) were also loaded onto each Vero plate. Plates were then centrifuged for 5 min at 1,400 RPM and incubated for 5 hours at 37°C, 5% CO₂. Subsequently, 25μL of a luciferin solution (2mg/mL prepared with sterile PBS, Cedarlane Lab cat. 122799(PE)) was dispensed in each well of the Vero microplate using the Biotek Microplate Reader, and mean luminescence was read 30 second after a brief mixing step

(Gen5 2.07 software). With the standard curve on each individual plate, a linear regression equation was generated on Microsoft Excel from which Log₁₀(PFU/mL) were calculated for each plate control and drug tested. Values were normalized against infected untreated 786-O control (mock) to obtain fold change.

2.8 Standard plaque assay

Infectious viral particles were quantified by standard plaque assay as described previously with slight modifications¹⁰⁵. Vero cells were seeded onto 12-well plates at a final density of 3E5 cells per well. Supernatants were collected and stored at -80°C until use. Samples were serially diluted using serum-free DMEM, transferred (500μL) onto Vero cells and incubated at 37°C, 5% CO₂ for 45 minutes. Virus was subsequently removed from each well and replaced with 1mL of an agarose overlay (1-part 1% agarose mixed with 1-part 2XDMEM supplemented with 20% fetal bovine serum). After a 24-hour incubation, plates were fixed with a fixative solution (3-part methanol mixed with 1-part glacial acetic acid) for a minimum of 1 hour then stained for 30 minutes with a Coomassie Blue solution prepared by mixing 4g Coomassie Brilliant Blue R (Sigma, cat. B0149) with 800mL methanol, 400mL acetic acid and 2800mL distilled water. Plates were washed, dried then titers (expressed as PFU/mL) were calculated using **Equation 3** where “D” is defined as the corresponding dilution. The assay was conducted with a minimum of three biological replicates.

Equation 3

$$Titer (PFU/mL) = \frac{(number\ of\ plaques) \times 10^D}{volume\ in\ mL\ transferred\ onto\ veros}$$

2.9 Animal models

Subcutaneous injection of the right hind limb with syngeneic 76-9 (1E6) or B16F10 (5E5) cells suspended in serum-free DMEM was performed in six-week-old female C57BL/6 mice (Charles River Laboratories). Tumor volume was defined as $\frac{width^2 \times length}{2}$ and measurements were performed weekly with an electronic caliper. Once tumors grew to approximately 5 mm x 5 mm (9-14 days post implantation), treatment regimen was initiated and consisted of an intra-tumor (i.t.) injection of vanadyl sulfate (50-100mg/kg or PBS vehicle control, final volume of 50 μ L) followed by an i.t. injection of VSV Δ 51-fluc (1E8 plaque forming unit or vehicle, final volume of 25 μ L) and intraperitoneal injection of immune checkpoint antibody (100-250 μ g anti-CTLA4 or vehicle) 4 hours post-treatment. The anti-CTLA4 antibody is the clone 9H10 (Leinco Technologies Inc., cat. C1614). Vanadyl sulfate doses are calculated for each mouse according to their weight. Animals were euthanized once tumors measured >1,500mm³. Quantification of viral replication was performed using *in vivo* imaging (IVIS, Perkin Elmer) to quantify bioluminescent signals as previously described¹⁰⁷. Briefly, mice received 200 μ L intraperitoneal injections of a 10mg/mL solution of XenoLight D-Luciferin prepared with PBS (Cedarlane Labs, cat. 122799PE). They were anaesthetized with 3% isoflurane five minutes after injection. Signals were measured using the software Living Image v2.50.1. To control for the effects of anaesthesia, all mice were exposed to 3% isoflurane. The experiment was conducted in conformity with the University of Ottawa Animal Care and Veterinary Services guidelines for animal care (protocol OHRI-2265). Andrew Chen and Mohammed Selman assisted with the animal experiments.

2.10 Flow cytometry

The van/OV treatment regimen was tested in a murine rhabdomyosarcoma model. Cured mice were re-implanted on the opposite limb with 1E6 76-9 cells on day 82. Two of the three mice from the van/OV therapy test group re-developed tumors. On day 131, these mice were euthanized, tumors were excised and dissociated using the mouse tumor dissociation kit (Miltenyi Biotec, cat. 130-096-730) following manufacturer's protocol. Briefly, for each tumor sample, an enzyme preparation was prepared by combining 3mL RPMI, 50 μ L enzyme R, 12.5 μ L enzyme A and 0.6mg collagenase (amsbio, cat. 17454.01). Tumors were first mechanically dissociated, then mixed with the enzyme preparation and transferred into a gentleMACS C tube then inserted into the gentleMACS dissociator under the program m_impTumor_02. Samples were then filtered with a 70 μ m cell strainer, washed with media (RPMI supplemented with 10% fetal bovine serum) then centrifuged at 500g for 5 minutes. The cell pellets were then resuspended in 300 μ L ACK buffer (Thermo Fisher Scientific, cat. A1049201) for 5 minutes to lyse red blood cells. Samples were centrifuged once more, supernatants discarded, and pellets resuspended in media. Cells were counted manually using a hemacytometer. A total of 1 E6 cells were stained with viability dye-APC-Cy7 (1:1000, Thermofisher Scientific, cat. 65-0865-14) diluted in PBS for 15 minutes at room temperature. Then, the cells were washed in PBS and stained with Fc block (CD16/CD23, BD Bioscience, cat 553141) diluted 1:100 diluted in FACS buffer (PBS supplemented with 0.5% BSA) for 5 minutes at 4°C. The cells were then stained with CD45-BV786 (BD bioscience, cat. 564225) and H2kb-PE (eBioscience, cat. 12-5958-82) for a 30 minute incubation at 4° C. As controls, a single stain condition for compensation was prepared using beads (BD bioscience, cat. 340487) as well as a

fluorescence minus one (FMO) condition were included. As a reference, *in vitro* 76-9 cells were also analyzed. After staining, cells were washed with FAC buffer and fixed in 1% paraformaldehyde. Cells were acquired on BD flow cytometry (Fortessa) and analyses were performed using FlowJo software v9. Cells were first gated based on size (FSC-A) and granularity (SSC-A), then gated on singlet cells (FSC-AxFSC-H). Then the singlet cells were gated on live only cells (APC-Cy7+). To distinguish hematopoietic cells, cells were gated into CD45+ and CD45- populations. And finally, non-hematopoietic cells were assessed for MHC expression. Flow cytometry and data analysis were performed by Samriti Birdi.

2.11 Ex vivo tumor models

76-9 and CT26wt cells were implanted in syngeneic mice. Once tumors reached a volume $> 1,500 \text{ mm}^3$, animals were sacrificed, tumor and normal tissues (lung, brain, muscle, spleen) were isolated. Coring procedure was adapted from previously published protocols^{112,113} where tissues were sliced and resected by punch biopsy into cores measuring 2mm x 2mm. Samples were maintained in DMEM supplemented with 10% serum, 30 mM HEPES, 1% (v/v) penicillin-streptomycin and 0.25 mg/L amphotericin B. Cores were kept in humidified incubators at 37°C, 5% CO₂. Samples were assessed for viability after processing using the metabolic dye resazurin and cores featuring low viability were excluded from the study. Cores were pre-treated with drugs or vehicle at indicated concentrations for 4 hours prior to infection with 3E4 plaque forming units (PFU) of VSVΔ51-GFP. Supernatants were collected and fluorescent microscopy images were captured at 48 hpi using the EVOS microscope (Thermo Fisher Scientific). Infectious viral particles were quantified by standard plaque assay from supernatants collected at 48 hpi¹⁰⁵.

2.12 Murine hepatocyte model

Validation of novel viral sensitizers was also performed using primary murine hepatocytes generously supplied by Dr. Morgan Fullerton and Conor O'Dwyer (University of Ottawa). Hepatocyte isolation has been previously described¹¹⁴. Briefly, livers of anesthetized mice were perfused first with an EGTA solution (140mM NaCl, 6.7mM KCl, 10mM HEPES, 500 μ M EGTA, pH adjusted to 7.4) then with a collagenase solution (0.5% collagenase, 67mM NaCl, 6.7mM KCl, 4.7mM CaCl₂·2H₂O, 100mM HEPES, pH adjusted to 7.6). Cells were filtered (100 μ m), centrifuged (1000 rpm, 5 min) and seeded in Williams Medium E supplemented with 10% serum (3 parts HyClone newborn calf serum to 1 part fetal bovine serum), 1% (v/v) penicillin-streptomycin and 1% (v/v) L-glutamine (Gibco). Final cell density was 75,000 cells/well of a 96-well microplate.

2.13 Viral sensitizer validation screens

To validate the novel viral sensitizers, 786-O cells or primary hepatocytes were seeded onto microplates (3E4 and 7.5E4 cells/well respectively) and treated with serially diluted compounds or vehicle control (5% DMSO). AT7519 (Cedarlane, cat. HY-50943), Flavopiridol (Sigma, cat. F3055), CDK1/2 inhibitor III (Cedarlane Labs, 18859-5) and Pevonedesitat (Sigma, cat. 5054770001) were reconstituted in DMSO. Treatments were done using the BioTek Precision™ Microplate Pipetting System (Thermo Fisher Scientific) and 4 hours post-treatment, plates were infected with virus at indicated MOI or mock infected with media. 24 hpi, GFP images were captured by fluorescence imaging using the Cellomics ArrayScan High Content Screening reader (ThermoFisher Scientific) and GFP foci were counted using the HCS Studio (Version 6.6.0).

2.14 Statistical analysis

Statistical significance was determined using GraphPad Prism 6.0 and Microsoft Excel. Statistical tests are described in figure captions: log-rank (Mantel-Cox) test for Kaplan-Meier survival curves, unpaired T-Test assuming unequal variances for volcano plots and ANOVAs. Error bars indicate the standard error of the mean (SEM). Statistical significance was defined as $p \leq 0.05$.

3. Results

3.1 Identification of PPases involved in resistance to VSV Δ 51 virotherapy

The role of host PPases in mediating oncolytic virotherapy and the mechanism of action behind vanadate's viral sensitization are poorly understood. To this end, a HTS composed of small interfering ribonucleic acids (siRNA) targeting human PPases was performed to identify PPases that promote oncolysis and increase viral titers in the context of virotherapy (**Supplemental figure 1**).

siRNAs are valuable biotechnological tools as they provide means to selectively suppress the expression of genes and study their ensuing impact on cell functions. Double stranded siRNAs can be delivered through viral vectors, exosomes or nanoparticles (e.g. liposomes)¹¹⁵. Once released into the cytosol, siRNA duplexes associate with Argonaute and other cofactors to form the RNA-induced silencing complex (RISC)¹¹⁶. The guide RNA strand binds to cellular messenger RNA (mRNA) due to the complementarity of their respective nucleotide sequences thereby bringing translation of targeted transcripts to a halt and ultimately their degradation by processing bodies¹¹⁷.

786-O cells were transfected with the arrayed human PPase siRNAs library and 48 hours later infected with VSV Δ 51-fluc. 48 hpi, readouts for the screen were viability, quantified with the metabolic dye resazurin, and viral titers calculated using bioluminescence readings of luciferase-expressing VSV Δ 51 following re-infection in sensitive Vero cells. To assess transfection efficacy, a human retinoic acid-inducible gene I (RIG-I) siRNA control was used. As discussed in **section 1.3**, RIG-I is an intracellular PRR that detects and confers immunological protection against a variety of RNA viruses including VSV¹¹⁸. RIG-I's PAMP is RNA⁶⁰, therefore upon infection with VSV Δ 51, 786-

O cells with successfully silenced RIG-I should therefore have compromised antiviral immune defenses and feature decreased viability and increased viral replication. As shown in **Figure 1** (in green) all RIG-I knockdowns lead to statistically significant declines in viability which therefore validates the efficacy of the high-throughput transfection protocol. Overall median viability values 48 hpi for vanadate, RIG-I and scramble plate controls were 21%, 1% and 64% respectively. Viability was normalized to infected untreated 786-O plate controls. A total of 236 of 1131 siRNAs significantly increased cytotoxicity (<40% viability) when combined with VSV Δ 51 at 48hpi. To rank these primary hits, the *viral sensitizer factor* was calculated and is defined as the logarithm of the viability of transfected cells divided by the viability of transfected infected cells (see **Equation 1**). Thus, this formula accounts for the cytotoxicity generated upon gene silencing alone and assigns a positive score to PPase siRNAs that robustly decreased viability upon infection with VSV Δ 51. The average viral sensitizer factor for vanadate corresponds to 0.43 and a total of 54 PPase targets scored higher than the vanadate controls (**Figure 2**). As summarized in **Table 2**, slingshot homolog 3 (SSH3) scored the greatest viral sensitizer factor followed by Mg²⁺/Mn²⁺ dependent protein phosphatases 1M and 1B (PPM1M and PPM1B), protein phosphatase 1 regulatory subunit 16A (PPP1R16a) and phosphoserine phosphatase (PSPH).

It is important to note that four PPase targets scored negative viral sensitizer factors meaning that these PPases, once silenced, may confer resistance to oncolysis upon infection (**Figure 2**). However, only three are statistically significant: ENPP3 (ectonucleotide pyrophosphatase/phosphodiesterase family member 3), ENTPD8 (ectonucleoside triphosphate diphosphohydrolase 8) and NT5C2 (cytosolic purine 5'-nucleotidase) with

viral sensitizer factors of -0.46, -0.26 and -0.27 respectively. These targets likely do not hold biological significance, since the transfection of the three siRNA alone (in absence of virus) resulted in a cell viability inferior to 20% suggesting that these phosphatases play a role in cell survival. No statistically significant fluctuation in viral titers, when compared to mock, was observed for these three targets, which is likely due to the inability of the transfected cells to sustain viral replication because of toxicity associated with knockdown of these targets.

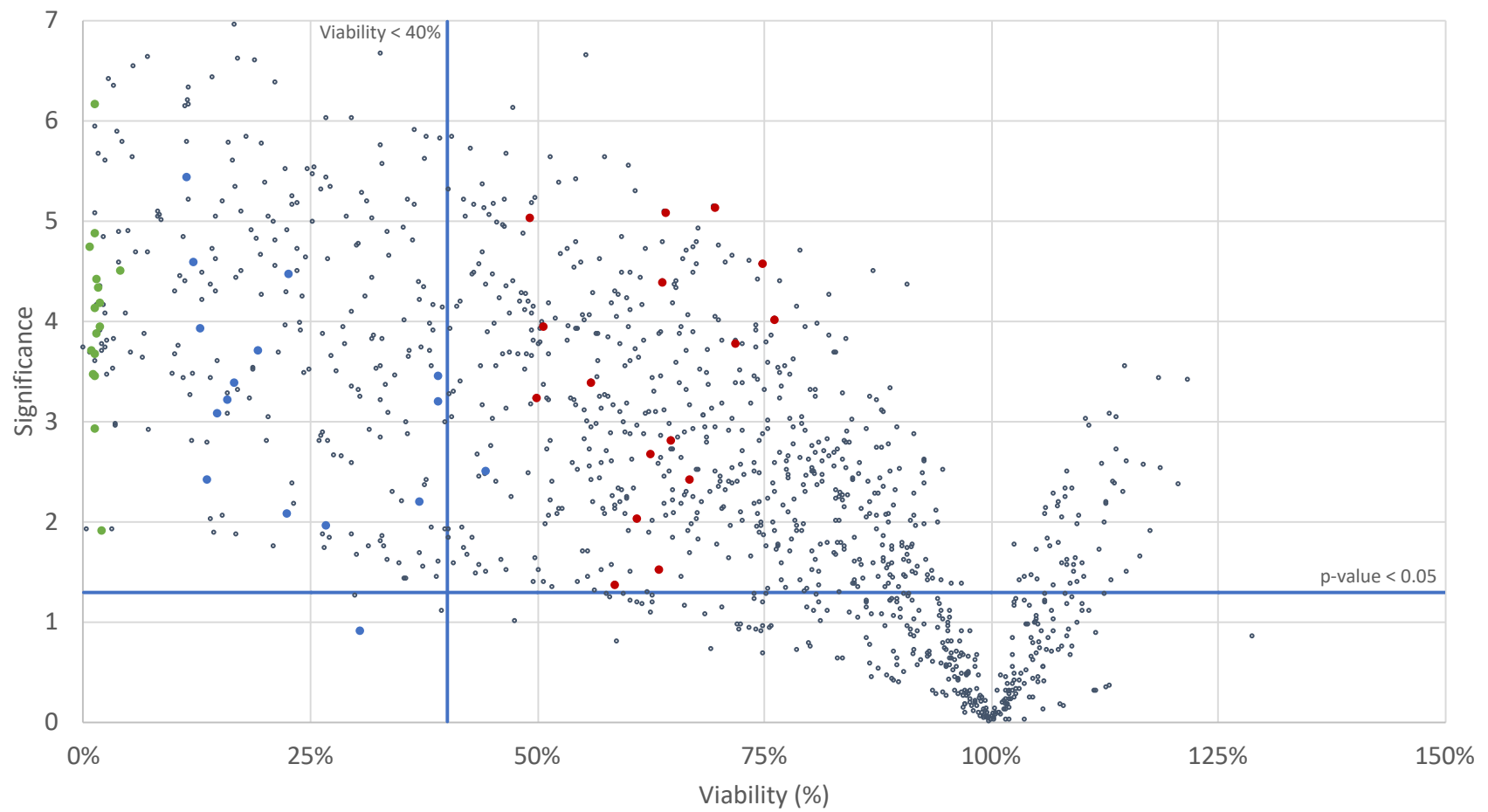


Figure 1. Volcano plot of viability 48 hpi

Reverse transfection was performed using human PPase siRNAs and the RNAiMAX reagents with a final siRNA concentration of 20nM. 48 hours post transfection, cells were infected (or mock infected) with VSV Δ 51 MOI 0.05. Viability values were blank-controlled then normalized against infected untreated 786-O control (mock) as measured by resazurin at 48 hpi. An unpaired T-test assuming unequal variances was performed to assess significance between untransfected infected cells and PPase siRNA-transfected infected cells ($-\log_{10}(\text{p-value})$). As a reference, 786-O cells were pre-treated with vanadate ($125\mu\text{M}$) for 4 hours prior to infection (depicted in blue). As a transfection control, 786-O cells were transfected with 20nM RIG-I siRNA (green) prior to infection and a scramble siRNA (20nM) control is presented in red. N = 3

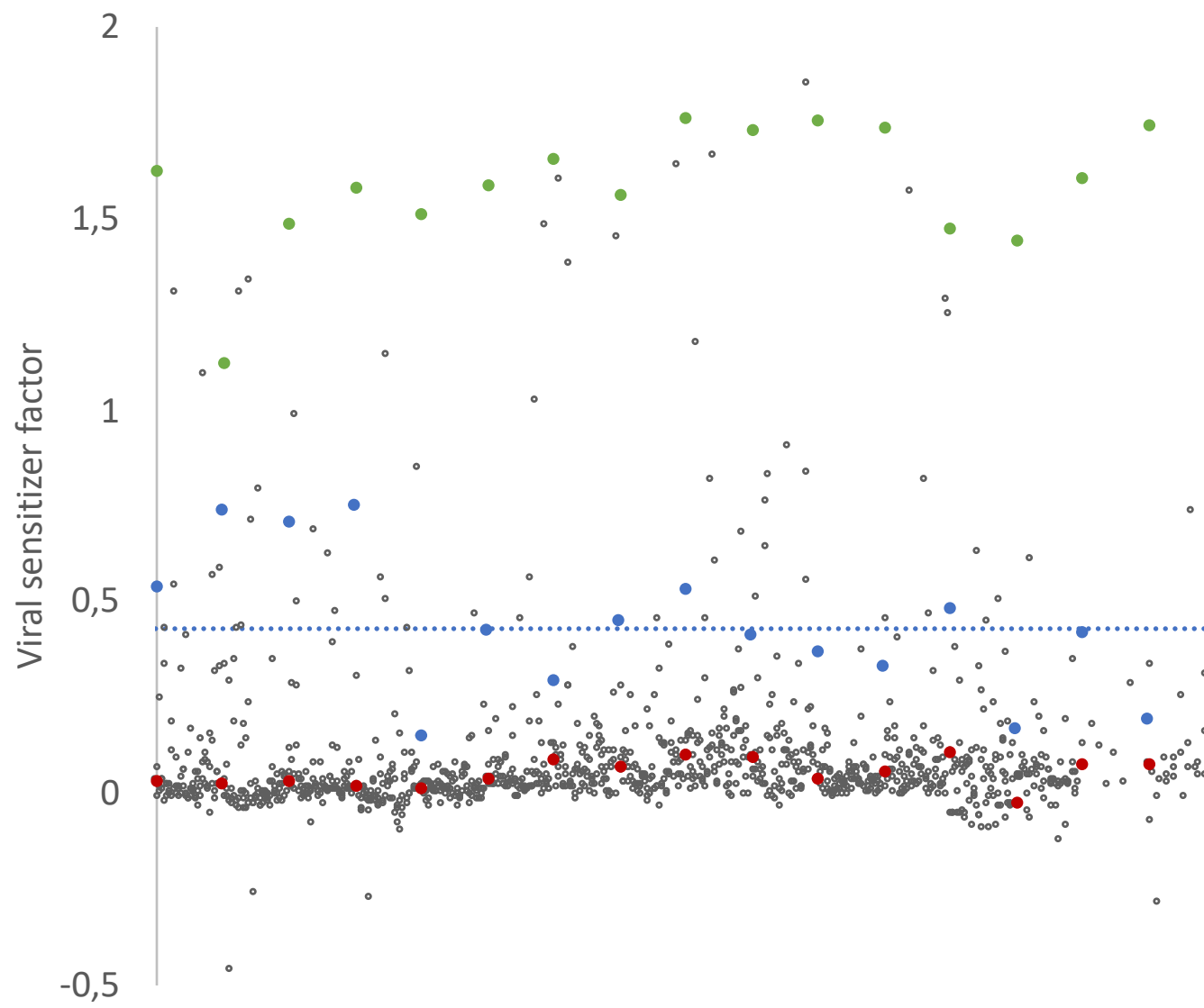


Figure 2. Viral sensitizer factors associated with PPase silencing

786-O cells were transfected using human PPase siRNAs and the RNAiMAX system. 48 hours post transfection, cells were infected with VSV Δ 51-fluc at a MOI of 0.05. 48hpi, viability was measured using resazurin. As a means to rank the PPase targets found in **Figure 1** based on their ability to enhance cytotoxicity upon infection, the *viral sensitizer factor* was calculated (see **Equation 1**). As a reference, 786-O cells were pre-treated with vanadate (125 μ M) for 4 hours prior to infection (depicted in blue). The average viral sensitizer factor for vanadate was 0.43 and is represented by the blue line. As a transfection control, a RIG-I siRNA is presented in green, and a scramble siRNA is shown in red.

High-throughput titration of VSV Δ 51-fluc at 48 hpi was conducted by measuring bioluminescence signals. By preparing a standard curve from a previously titered VSV Δ 51-fluc stock (by plaque assay in PFU/mL), a linear equation was generated from which viral expression units (VEUs) were calculated based on luminescence. Titers, expressed as Log(VEU/mL), were obtained for each control and siRNA tested. Fold change in viral titers was calculated by dividing the titers (Log(VEU/mL)) of each sample, by the titers (Log(VEU/mL)) of the untransfected infected 786-O plate control. A total of 76 siRNAs lead to a statistically significant increase in viral replication by a fold change value of 1.5 or more in Log(VEU/mL) (**Figure 3**). Average fold change in Log(VEU/mL) in viral output for the vanadate, RIG-I and scramble siRNA plate controls were 1.86, 2.37 and 1.04 respectively. siRNAs targeting PTPRN, PPP1R16A and PSPH lead to the strongest potentiation of viral titers with fold change values of 2.95, 3.00 and 2.92 in Log(VEU/mL) respectively (**Table 2**). **Figure 4** compiles and further refines the siRNAs that increased viral titers by a fold change value 1.6 or more in Log(VEU/mL) and simultaneously decreased viability to 40% or below. A total of 52 PPase siRNA meet the statistical significance threshold ($p < 0.05$) and are listed in **Table 2**. Several distinct siRNAs targeting various subunits of phosphatases were uncovered: protein phosphatase 1 (subunits PPP1R12b, PPP1R16a, PPP1R14a, PPP1R2 and PPP1R10) protein phosphatase 2A (PPP2R5E, PPP2R2C) and protein phosphatase 4 (PPP4R1, PPP4C, PPP4R1L). EYA2, ACP2 and ENTPD4 were identified as top hits twice by different siRNAs which further affirms their role in innate immunity. Based on their ability to enhance cytotoxicity and viral titers when silenced, these findings suggest that EYA2, PP1, PP2A, PP4, ACP2,

ENTPD4, PPM1M, PPM1B, SSH3 and PSPH are host PPases involved in resistance to VSV Δ 51 virotherapy.

3.2 PPase siRNAs that enhance titers without increasing oncolysis

Several PPase siRNAs were shown to enhance viral titers without robustly decreasing viability (**Figure 4, Table 1**). Five of these hits, have viabilities exceeding 70% upon VSV Δ 51 infection and among them is the PP1 regulatory subunit PPP1R14B which has inhibitory actions against PP1 polymeric enzymes¹¹⁹. The highest enhancement in viral titers was achieved with the PTPRV siRNA yielding a fold change increase in Log(VEU/mL) of 2.7. Protein tyrosine phosphatase receptor type V (PTPRV), is a pseudogene in humans^{95,120–122}.

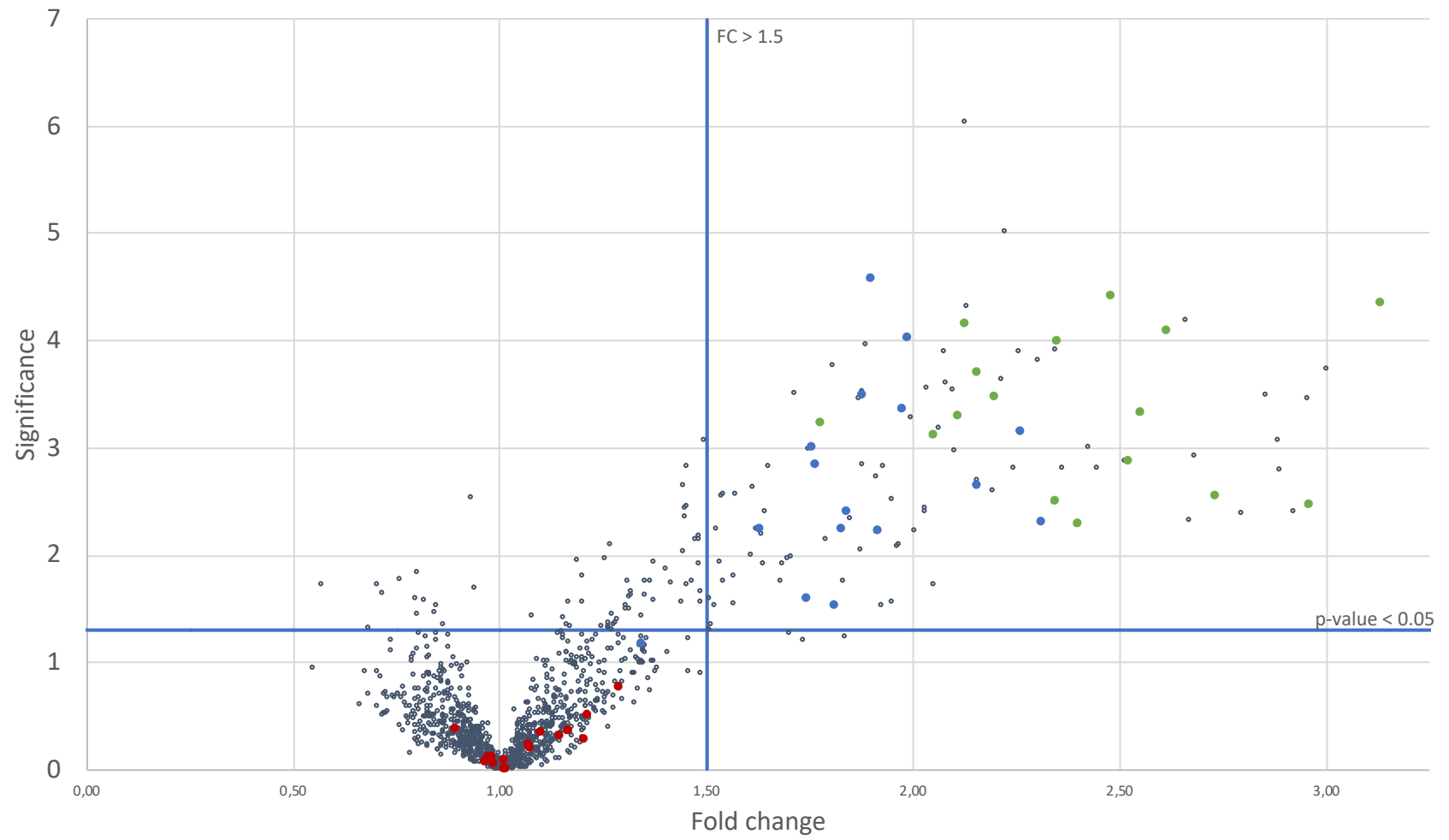


Figure 3. Volcano plot of viral titers 48 hpi

786-O cells were treated as described in **Figure 1**. At 48 hpi, a high-throughput titration was performed. Fold change against statistical significance ($-\text{Log}_{10}(\text{p-value})$) is presented. Fold change in $\text{Log}(\text{VEU/mL})$ is in reference to untransfected infected 786-O controls. As a positive control, 786-O cells pre-treated 4 hours with vanadate ($125\mu\text{M}$) prior to infection are shown in blue. As a transfection control, cells transfected with RIG-I siRNA or scramble siRNA are shown in green and red respectively. Statistical analysis was determined via an unpaired T-Test assuming unequal variances to establish significance between untransfected infected controls and PPase siRNA transfected infected conditions. $N = 3$.

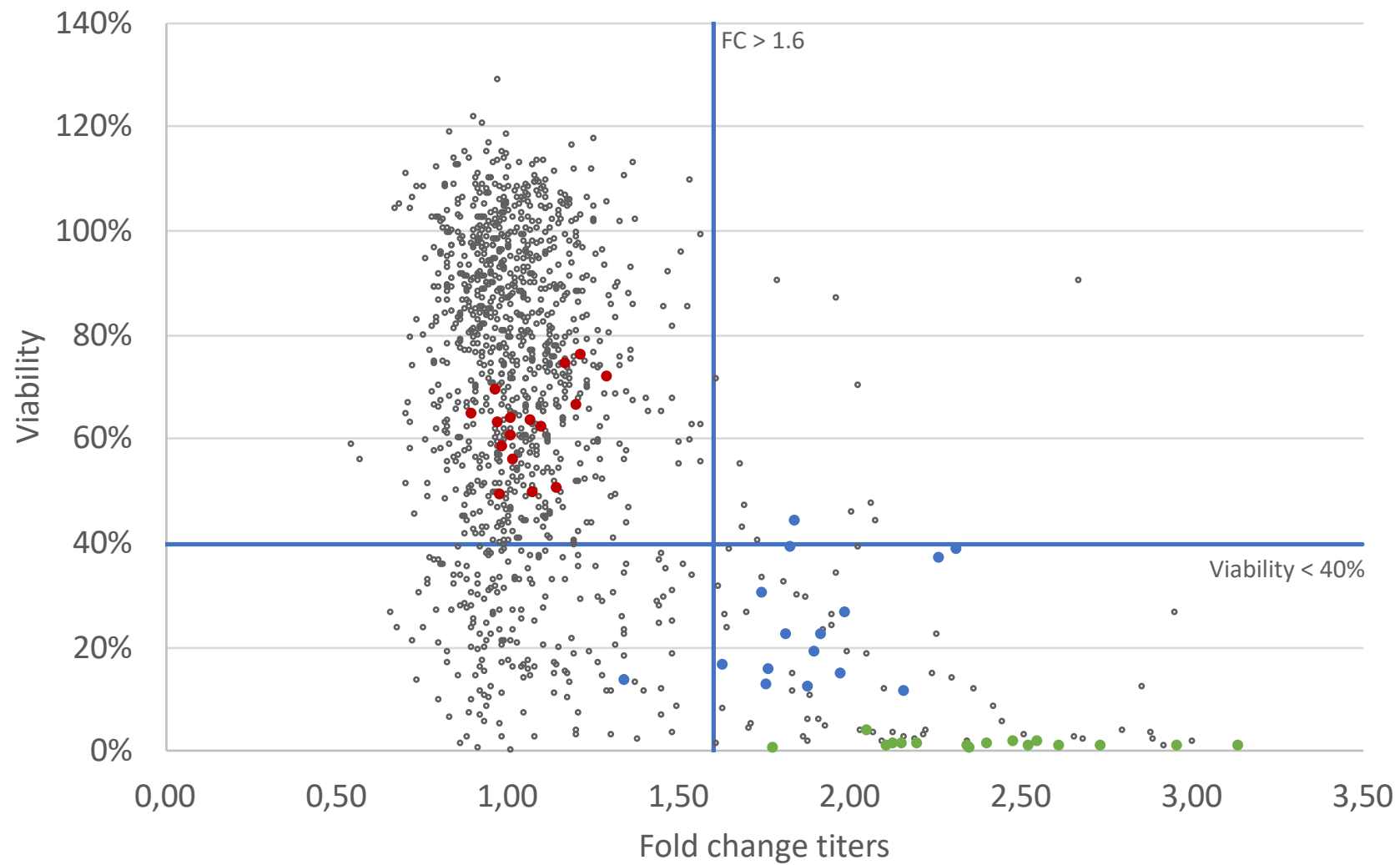


Figure 4. Impact of PPase siRNAs on viability and viral titers

Cells were treated as previously described. Viability for the transfected infected conditions as well as the fold change in Log(VEU/mL) for each transfected condition are graphed. As a reference, vanadate-treated controls are presented in blue. As a transfection control, RIG-I siRNA transfected cells are shown in green and scramble siRNA controls are shown in red. N = 3.

ID	Viability siRNA + VSV	Viability siRNA alone	FC in Log(VEU/mL)	Target	Gene ID	Gene Description
PTP7D6	91%	100%	1.8	PPP1R14B	NM_138689	Protein phosphatase 1 regulatory inhibitor subunit 14B
PTP12D9	90%	106%	2.7	PTPRV	NR_015444	Protein tyrosine phosphatase pseudogene
PTP13F9	87%	108%	2.0	CTDP1	NM_048368	CTD phosphatase subunit 1
PTP6F10	72%	87%	1.6	PNKP	NM_007254	Polynucleotide kinase 3'- phosphatase
PTP4F9	70%	100%	2.0	PFKFB4	NM_004567	6-phosphofructo-2- kinase/fructose-2,6- biphosphatase 4
PTP12E11	55%	69%	1.7	TENC1	NM_015319	Tensin 2
PTP9F2	47%	69%	2.1	PPFIA4	NM_015053	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 4
PTP1H11	47%	100%	1.7	EYA4	NM_004100	EYA transcriptional coactivator and phosphatase 4
PTP7C6	46%	109%	2.0	PPP1R14A	NM_033256	Protein phosphatase 1 regulatory inhibitor subunit 14A
PTP6A11	44%	69%	2.1	PPAP2B	NM_003713	Phospholipid phosphatase 3
PTP16E2	43%	77%	1.7	PPP2R2B	NM_004576	Protein phosphatase 2 regulatory subunit beta
PTP13D7	40%	86%	1.7	PTPN13	NM_006264	Protein tyrosine phosphatase non-receptor type 13

Table 1. PPase siRNAs that increase viral titers without robustly compromising cell viability

Twelve siRNA sequences were associated with fold changes (FC) in viral titers exceeding 1.6 (Log(VEU/mL)) and cell viabilities exceeding 40% (**Figure 4**). The cell viability upon transfection of the siRNA alone as well as the viability upon combined transfection and infection are presented as a reference along with respective PPase description.

ID	Accession Number	Gene	Gene description	Viability siRNA	Viability siRNA + VSV	Viral sensitizer factor	FC titers
PTP1C2	NM_001610	ACP2	Acid phosphatase 2, lysosomal	109%	31%	0.5	1.6
PTP3A9	NM_001946	DUSP6	Dual specificity phosphatase 6	104%	11%	1.0	1.9
PTP10G10	NM_018276	SSH3	Slingshot homolog 3 (Drosophila)	102%	1%	1.8	2.1
PTP9D4	NM_144641	PPM1M	Protein phosphatase, Mg2+/Mn2+ dependent, 1M	99%	2%	1.7	2.7
PTP6E4	NM_019892	INPP5E	Inositol polyphosphate-5-phosphatase, 72 kda	95%	33%	0.5	1.7
PTP11F6	NM_002832	PTPN7	Protein tyrosine phosphatase, non-receptor type 7	91%	39%	0.4	2.0
PTP9D9	NM_020416	PPP2R2C	Protein phosphatase 2, regulatory subunit B, gamma	90%	22%	0.6	2.3
PTP3F11	NM_005244	EYA2	Eyes absent homolog 2 (Drosophila)	88%	30%	0.5	1.9
PTP8H2	NM_001033556	PPM1B	Protein phosphatase, Mg2+/Mn2+ dependent, 1B	86%	2%	1.6	2.3
PTP7B11	NM_005134	PPP4R1	Protein phosphatase 4, regulatory subunit 1	80%	3%	1.4	2.9
PTP14B8	XM_931711	PTPN20A	Protein tyrosine phosphatase, non-receptor type 20A	78%	19%	0.6	2.0
PTP12F8	NM_002846	PTPRN	Protein tyrosine phosphatase, receptor type, N	78%	27%	0.5	3.0
PTP2G4	NM_033331	CDC14B	CDC14 cell division cycle 14 homolog B (S. Cerevisiae)	76%	34%	0.3	2.0
PTP1C3	NM_203488	ACYP1	Acylphosphatase 1, erythrocyte (common) type	76%	4%	1.3	2.2
PTP2D5	NM_004715	CTDP1	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase, subunit 1	75%	3%	1.3	2.1
PTP12H10	NM_014188	SSU72	SSU72 RNA polymerase II CTD phosphatase homolog (S. Cerevisiae)	74%	4%	1.3	2.8
PTP7H10	NM_002720	PPP4C	Protein phosphatase 4, catalytic subunit	70%	2%	1.5	2.7
PTP7A7	NM_032902	PPP1R16A	Protein phosphatase 1, regulatory subunit 16A	69%	2%	1.6	3.0
PTP9C6	NM_033256	PPP1R14A	Protein phosphatase 1, regulatory (inhibitor) subunit 14A	68%	24%	0.5	1.6
PTP16F4	NM_030668	PTPRO	Protein tyrosine phosphatase, receptor type, O	66%	12%	0.7	2.9
PTP2C2	NM_001610	ACP2	Acid phosphatase 2, lysosomal	65%	3%	1.3	2.2
PTP3A11	NM_004901	ENTPD4	Ectonucleoside triphosphate diphosphohydrolase 4	61%	32%	0.3	1.8
PTP6F11	NM_032728	PPAPDC3	Phosphatidic acid phosphatase type 2 domain containing 3	59%	6%	1.0	2.4
PTP4H9	XM_930828	PGAM4	Phosphoglycerate mutase family member 4	59%	8%	0.8	1.6
PTP12F2	NM_001001484	PTER	Phosphotriesterase related	56%	9%	0.8	2.4
PTP13H3	NM_001114089	ENTPD6	Ectonucleoside triphosphate diphosphohydrolase 6 (putative)	54%	23%	0.4	1.9
PTP4D3	NM_176799	ILKAP	Integrin-linked kinase-associated serine/threonine phosphatase	53%	15%	0.6	2.2
PTP1A11	NM_004901	ENTPD4	Ectonucleoside triphosphate diphosphohydrolase 4	52%	24%	0.3	1.9
PTP9E7	NM_006241	PPP1R2	Protein phosphatase 1, regulatory (inhibitor) subunit 2	52%	39%	0.1	1.7
PTP1F11	NM_005244	EYA2	Eyes absent homolog 2 (Drosophila)	45%	4%	1.1	2.1
PTP13G3	NM_203468	ENTPD2	Ectonucleoside triphosphate diphosphohydrolase 2	45%	14%	0.5	2.3
PTP14C5	NM_021090	MTMR3	Myotubularin related protein 3	44%	26%	0.2	1.9
PTP6F5	NM_022126	LHPP	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase	43%	12%	0.6	2.4
PTP12H11	NR_023314	THTPA	Thiamine triphosphatase	41%	2%	1.3	2.9
PTP6H2	NM_138387	G6PC3	Glucose 6 phosphatase, catalytic, 3	41%	1%	1.5	1.6
PTP4D9	NM_001018053	PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	40%	3%	1.1	2.5
PTP11A9	NM_002850	PTPRS	Protein tyrosine phosphatase, receptor type, S	39%	26%	0.2	1.6
PTP10E6	NM_002831	PTPN6	Protein tyrosine phosphatase, non-receptor type 6	36%	4%	0.9	1.7
PTP7B10	NM_006246	PPP2R5E	Protein phosphatase 2, regulatory subunit B', epsilon isoform	35%	19%	0.3	2.0
PTP12D2	NM_004577	PSPH	Phosphoserine phosphatase	35%	1%	1.6	2.9
PTP9B5	NM_002714	PPP1R10	Protein phosphatase 1, regulatory subunit 10	32%	2%	1.2	2.2
PTP9C11	NM_018498	PPP4R1L	Protein phosphatase 4, regulatory subunit 1-like	27%	4%	0.8	2.0
PTP2D8	NM_024025	DUSP26	Dual specificity phosphatase 26 (putative)	26%	5%	0.7	1.7
PTP4G9	NM_000290	PGAM2	Phosphoglycerate mutase 2 (muscle)	24%	12%	0.3	2.1
PTP5E3	NM_005536	IMPA1	Inositol(myo)-1(or 4)-monophosphatase 1	18%	15%	0.1	1.8
PTP2E5	NM_021198	CTDSP1	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 1	12%	2%	0.8	2.1
PTP3E10	NM_178543	ENPP7	Ectonucleotide pyrophosphatase/phosphodiesterase 7	11%	3%	0.6	1.9
PTP9G5	NM_032103	PPP1R12B	Protein phosphatase 1, regulatory subunit 12B	9%	6%	0.2	1.9
PTP7D2	NM_003625	PPFIA2	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 2	9%	6%	0.2	1.9
PTP3D2	NM_001111034	ACP5	Acid phosphatase 5, tartrate resistant	8%	2%	0.7	1.9
PTP11E6	NM_002831	PTPN6	Protein tyrosine phosphatase, non-receptor type 6	6%	5%	0.1	1.9
PTP14H2	NM_001611	ACP5	Acid phosphatase 5, tartrate resistant	6%	3%	0.3	2.2

Table 2. PPase siRNAs simultaneously increase viral titers and cytotoxicity

The PPase siRNAs associated with higher viral replication and cytotoxicity from **Figure 4** are listed and ranked according to cell viability following siRNA transfection (no infection). Respective viral sensitizer factors (as shown in **Figure 2**) are listed and calculated following **Equation 1**. These siRNAs all meet statistical significance for viability and titers.

3.3 Kinase inhibitors that prevent viral sensitization

Vanadate exerts its viral sensitization by inhibiting the action of PPases and thereby causing the accumulation of phosphorylated substrate. Because of the interplay between kinases and phosphatases, the next objective consisted of identifying kinases that counteract the action of vanadium's viral sensitizing PPase targets. 800 compounds (kinase inhibitors and tool compounds) from various developmental stages (e.g. approved, undergoing pre-clinical testing or in clinical trials) were tested in a *reverse screen* strategy (**Supplemental figure 3, Supplemental figure 2**). 786-O cells were pre-treated for 4 hours with an optimal dose of vanadate (125 μ M) and library compound (1 μ M), then subsequently infected with VSV Δ 51. Primary hits were defined as kinase inhibitors that limit viral replication (as quantified by high-throughput titering) and oncolysis (measured using the resazurin metabolic dye) when combined with vanadate. On average, vanadate plate controls increased viral replication by a fold change value of 1.8 in Log(VEU/mL) and decreased viability to 4%. A total of 23 compounds reversed vanadate's effect (**Figure 5**). Nearly half of these compounds target the epidermal growth factor receptor (EGFR); the majority of which are already clinically approved or are currently undergoing clinical testing (**Table 3**). Three drugs target the MAPK/ERK kinase 1 and 2 (MEK1 and MEK2) and two target the NF κ B inhibitor kinase (IKKb).

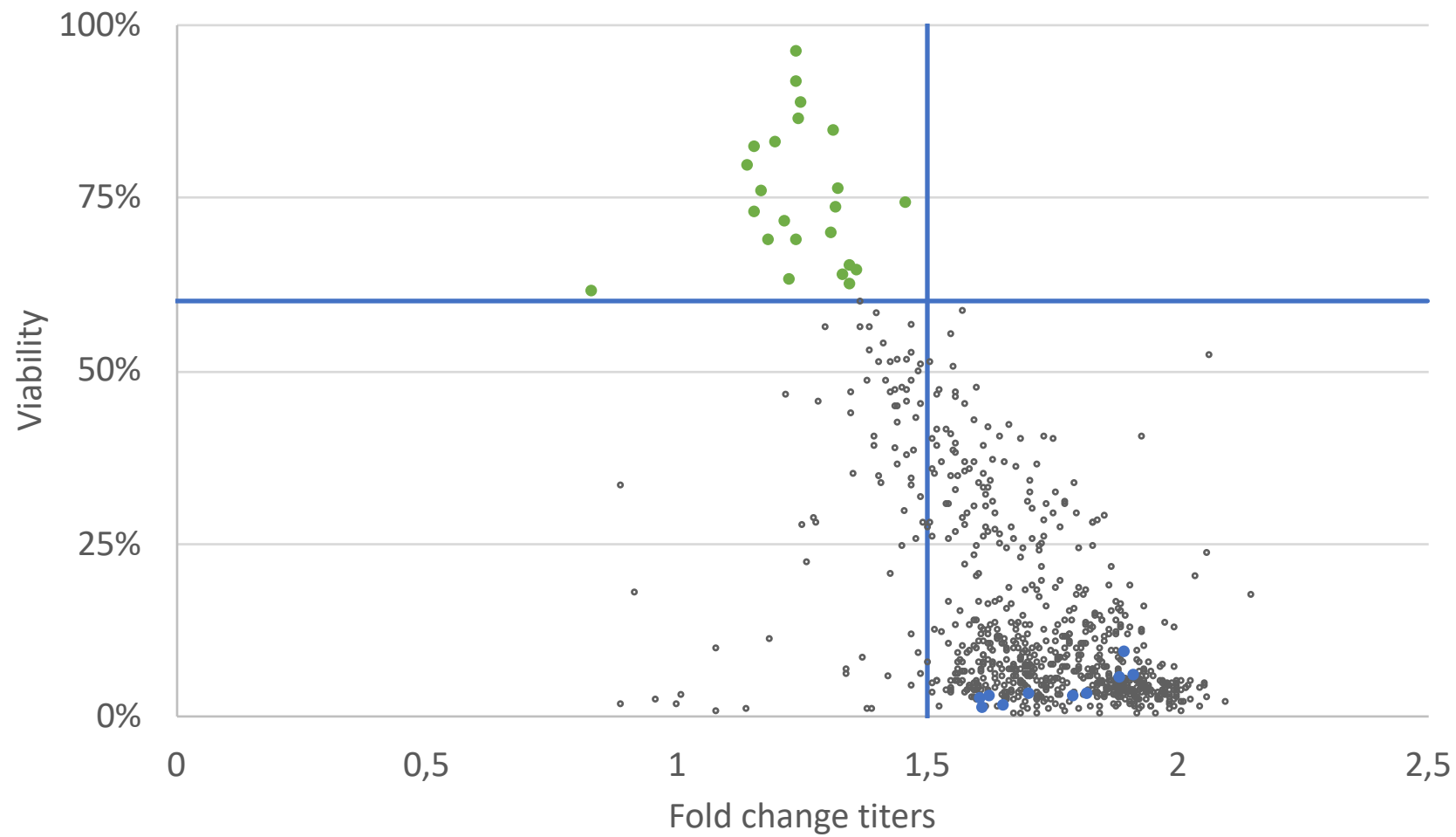


Figure 5. Drugs that prevent vanadate's viral sensitization

786-O cells were pre-treated with the OICR kinase inhibitor drug library ($1\mu\text{M}$) and with vanadate ($125\mu\text{M}$). Cells were subsequently infected 4 hours later with VSV $\Delta 51$ -fluc at a MOI of 0.1. 48 hpi, the relative metabolic activity was measured using resazurin. Viability is blank-controlled and normalized against mock (no vanadate, no drug, only VSV). Titers were normalized against infected untreated 786-O control to obtain the fold change (expressed in $\text{Log}(\text{VEU/mL})$). As a reference, 786-O pretreated with vanadate alone prior to infection are shown in blue. Drug combinations yielding viabilities superior to 60% and fold changes in viral titers of less than 1.5 are presented in green. N= 2-3.

ID	Drug	Target(s)	Developmental stage
KI1C5	Gefitinib, Iressa, ZD1839	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)	Approved: metastatic non-small cell lung cancer
KI1C6	Lapatinib ditosylate, Tykerb, GW572016	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)	Approved: advanced or metastatic breast cancer
KI1C10	Vandetanib, Zactima, ZD6474, AZD-6474	EGFR (ERBB1, HER1), KDR (VEGFR2, VEGFR, FLK1), RET, ABL1 (ABL), KIT (c-KIT), FLT1 (VEGFR1), FLT4 (VEGFR3), TRKA (TRK)	Approved: local or metastatic medullary thyroid cancer
KI1E2	Erlotinib HCl, CP-358774, OSI-774, Tarceva, NSC-718781, RG-1415, Ro-50-8231	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)	Approved: metastatic non-small cell lung cancer, metastatic pancreatic cancer.
KI1E10	GW2974	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)	Pre-clinical
KI1F2	GW583340 dihydrochloride	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU)	Pre-clinical
KI1G10	BIBX 1382 dihydrochloride, Falnidadamol	EGFR (ERBB1, HER1)	Clinical trial: adult solid tumors.
KI2B6	PD-153035, AG-1517, Compound 32, SU-5271, ZM-252868, WHI-P79	EGFR (ERBB1, HER1)	Pre-clinical
KI2C2	BIBW-2992, Tovok, Afatinib	EGFR (ERBB1, HER1)	Approved: NSCLC
KI2C5	CI-1033, Canertinib, PD-183805, SN-26606	EGFR (ERBB1, HER1), ErbB2 (TKR1, HER2, NEU) - Irreversible	Clinical trials: breast cancer
KI1B6	PD-184352, CI-1040	MAP2K1 (MEK1), MAP2K2 (MEK2), Erk2 (ERK, p38), RAF1 (c-Raf)	Clinical trials: lung, breast, pancreatic and colorectal cancers
KI2B11	AZD6244, ARRY-142886, AZD-6244, Selumetinib, ARRY-886	MAP2K1 (MEK1), MAP2K2 (MEK2), Erk2 (ERK, p38)	Clinical trials: carcinoma, NSCLC, melanoma
KI2E2	SL327	MAP2K1 (MEK1), MAP2K2 (MEK2)	Pre-clinical
KI3G11	IMD 0354, IMD-0354	IKKb (IKK2)	Pre-clinical
KI2C6	SC-514	IKKb (IKK2)	Pre-clinical
KI1F4	IRAK-1/4 Inhibitor I	IRAK1 (IRAK)	Pre-clinical
TC2B6	NVP-AUY922, AUY922, VER-52296	Hsp90 inhibitor	Clinical trials: lymphoma, breast cancer, hematologic neoplasms
KI2B5	NU-7026, LY-293646	DNAPK (DNA-PKcs)	Pre-clinical
KI2B8	Sal003	eIF2a	Pre-clinical
KI2C8	Bosutinib, SKI-606, Bosulif	BCR, ABL1 (ABL), SRC (c-SRC), FGR (SRC2), LYN	Approved: leukemia
KI2D2	Ro-31-8220 mesylate, Bisindolylmaleimide IX	PKC	Pre-clinical
KI2E5	SU 4312, DMBI	KDR (VEGFR2, VEGFR, FLK1)	Pre-clinical
KI2F10	API-2, Triciribine, NSC154020, TCN, Tricibine, VQD-002	AKT1 (PKBa)	Clinical trials: leukemia, ovarian and breast cancer

Table 3. Drugs that abrogate vanadate's viral sensitizing effects

Name, targets and developmental stages are provided for each of the lead compounds identified in **Figure 5**. Lead compounds are grouped according to their cellular targets. Developmental stages were manually searched using PubChem (National Center for Biotechnology Information)¹¹⁰ and DrugBank (5.1.4)¹⁰⁹.

3.4 Pathway analysis of the phosphatome and kinome

Thus far, the PPase siRNA screen identified 76 PPases that once silenced significantly enhanced VSVΔ51 growth (**Figure 3**). In parallel, the *reverse screen* (**Supplemental figure 3, Supplemental figure 2**) conducted using the OICR library revealed kinase inhibitors that reverse vanadate's viral sensitizing effect and prevent enhancement of viral titers and oncolysis (**Figure 5, Table 3**). Mechanistically, we hypothesize that vanadate potentiates OV's by inhibiting PPases thereby leading to the accumulation of phosphorylated substrates. The objective was to identify the substrates that, once phosphorylated, exert viral sensitizing effects. The data generated from both screens were integrated by cross-referencing the substrates of lead PPases with that of the antagonistic kinases. Using DEPOD (version 1.1)¹⁰⁸, Drugbank (5.1.4)¹⁰⁹ and Pubchem (National Center for Biotechnology Information)¹¹⁰ databases, the drug targets and substrates of both kinases and PPases were tabulated and ran into Cytoscape¹¹¹ to intercept the common substrates shared between PPases and kinases (**Figure 6**). The viability data generated is included into the network and is reflected by the transparency of the PPase and kinase nodes. As described in the previous section, numerous EGFR inhibitors interfere with vanadate's mechanism of action. The kinase-phosphatase relationships identified evolve around 8 main substrates: EGFR, KIT, NTRK1, KDR, SRC, MAPK1, LYN and IKBKB. This suggests that increased phosphorylation of these common substrates (presumably), either by decreased PPase activity or increased kinase activity towards them, functionally correlates with viral sensitization.

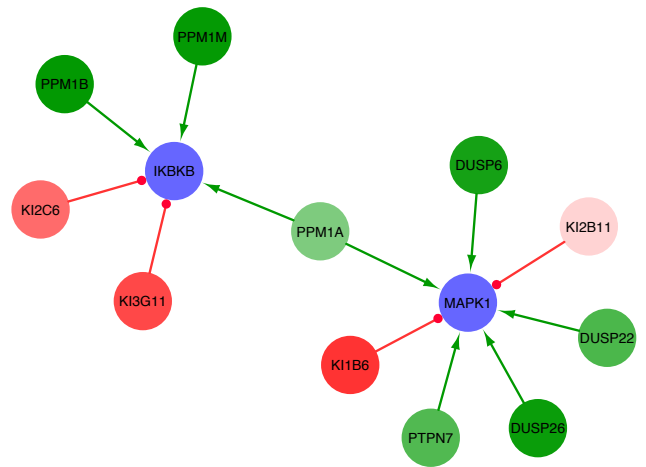
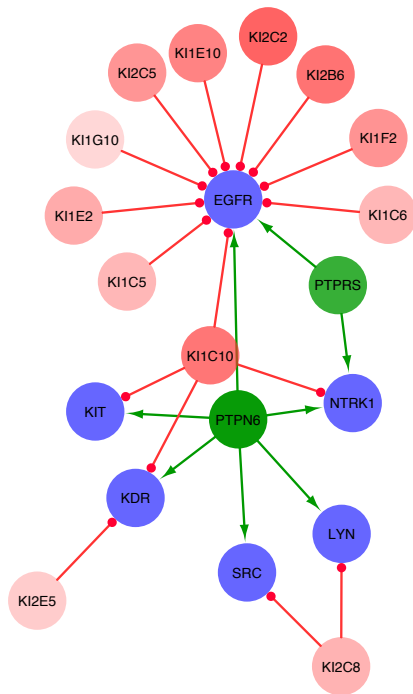


Figure 6. Pathway analysis of *reverse screen* and PPase siRNA screen

The primary hits uncovered from the human PPase siRNA screen, specifically the PPases that increase viral titers once silenced (**Figure 3**), were cross-referenced with the kinase inhibitors from the *reverse screen* that were found to prevent vanadate's enhancement of viral titers and oncolysis (**Figure 5, Table 3**). Substrates (blue) were obtained from the human DEDPhosphorylation Database (version 1.1)¹⁰⁸, Drugbank (5.1.4)¹⁰⁹ and Pubchem (National Center for Biotechnology Information)¹¹⁰. Cytoscape¹¹¹ was used to generate the PPase-kinase-substrate networks. The transparency of the colors for kinases (red) and the PPase (green) hits were set to reflect the resulting cell viability at 48 hpi: an opaque kinase represents high viability, and inversely, an opaque PPase represents a low viability. PPase substrates that did not increase viral titers when silenced are excluded from the network. Dots indicate inhibition of the substrate (blue) by the kinase inhibitor (red). Arrows indicate dephosphorylation of the blue substrate by the green Ppase. This pathway analysis was performed by Adrian Pelin.

3.5 Identification of novel small molecule enhancers of oncolytic virotherapy

In parallel with the *reverse screen*, 786-O cells were also treated with the OICR library compounds alone and infected with VSV Δ 51. Interestingly, several compounds were found to significantly increase cytotoxicity and viral titers on their own (without vanadate). A total of 69 library compounds significantly decreased cell viability to less than 50% when combined with VSV Δ 51 (**Figure 7**). As a means to further refine these small molecules and to account for drug-associated toxicity, a *viral sensitizer factor* was calculated according to **Equation 2**. Calculated scores are graphed in **Figure 8** and positive values suggest enhancement in virus-mediated oncolysis. A total of 4 compounds generated scores that exceed the average viral sensitizer factor for vanadate plate controls. These compounds are TC2D6 (histone deacetylase (HDAC) inhibitor Dacinostat), TC2B8 (HDAC inhibitor Panobinostat), TC2E6 (HDAC1 inhibitor quisinostat) and KI1E3 (pan kinase inhibitor staurosporine). Panobinostat and staurosporine did not meet the statistical or fold change thresholds.

Fold changes in viral titers 48 hpi for each library drug are plotted in **Figure 9**. 23 compounds significantly increased viral output and they can be classified into five broad categories (**Table 4**): antimitotic drugs (fold changes ranging 1.6-1.9 in Log(VEU/mL)), cyclin-dependent kinase (CDK) inhibitors (1.5-1.9), HDAC inhibitors (1.5-1.7) Janus kinase (JAK) inhibitors (1.7-1.9) and others (1.5-1.9). 22 of the 23 compounds significantly decreased viability below the 50% threshold (**Figure 7**). Pevonedistat is a promising candidate in advanced stages of clinical development, with a potent fold change value of 1.9 in Log(VEU/mL) and constitutes the only lead compound directly targeting the protein degradation pathway (NEDD8). While antimitotic drugs¹²³, JAK¹²⁴ and HDAC^{69,70}

inhibitors have already been explored by our group and others, the OICR kinase inhibitor screen revealed an unprecedented potential role for cell cycle proteins and the NEDD8 network in improving oncolytic virotherapy (**Supplemental figure 4**).

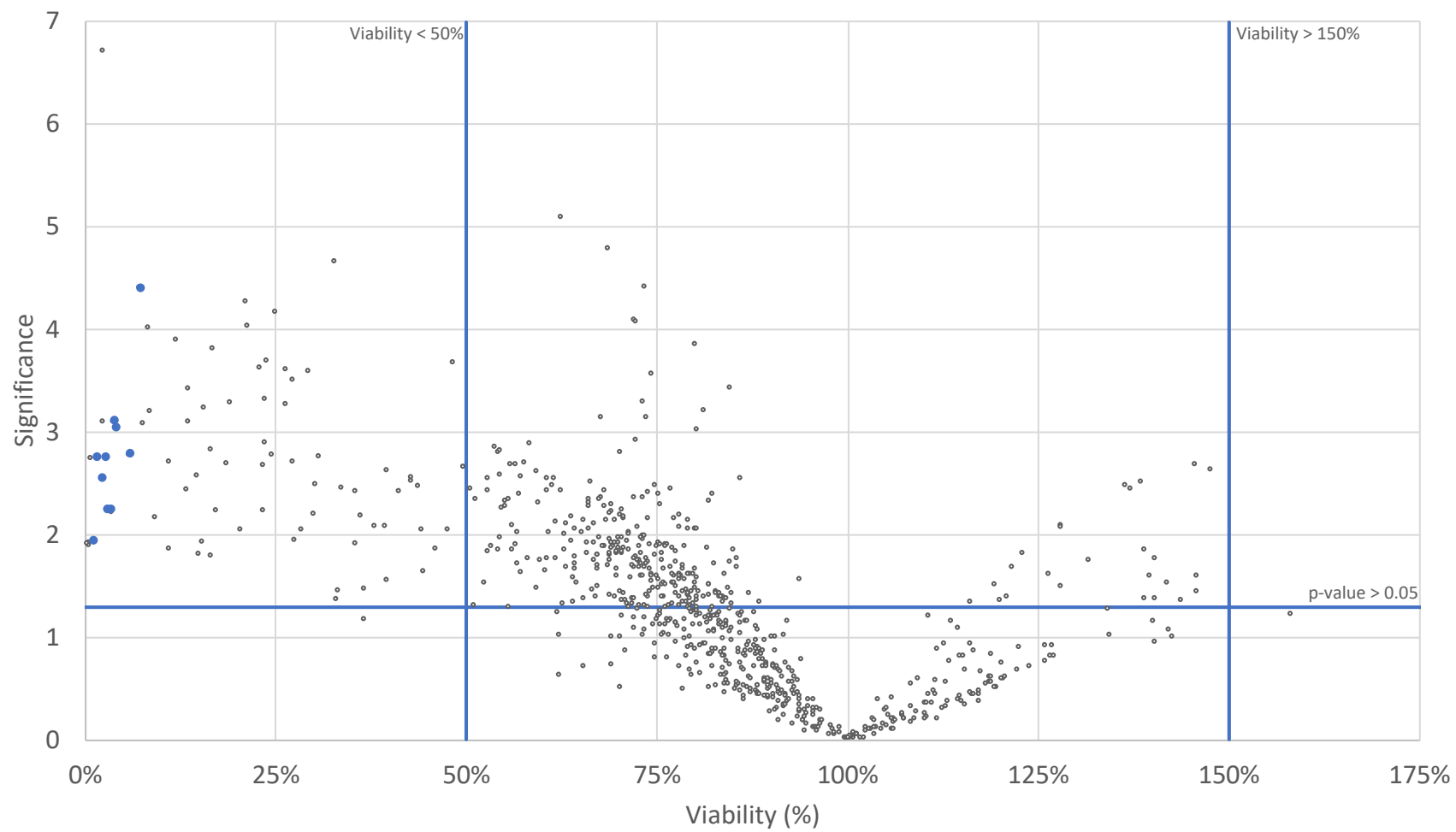


Figure 7. Volcano plot of viability 48hpi

786-O cells were pre-treated with the OICR kinase inhibitor drug library ($1\mu\text{M}$) for four hours and subsequently infected with VSV $\Delta 51$ -fluc at a MOI of 0.1. As a positive control, 786-O cells pre-treated with vanadate ($125\mu\text{M}$) prior to infection are shown in blue. 48 hpi, the metabolic activity was measured using resazurin. Values were blank-controlled then normalized against infected untreated 786-O control (mock). To establish statistical significance, an unpaired t-test assuming unequal variances was performed. The y-axis represents the calculated $-\text{Log}(\text{p-value})$. A total of 69 drugs have statistically significant viability values of less than 50% when combined with VSV $\Delta 51$. $N = 2-3$.

Figure 8. Viral sensitizer factor

786-O cells were treated as described in **Figure 7**. The viral sensitizer factor for each drug compound was calculated following **Equation 2**. As a reference, vanadate plate controls are shown in blue. Four compounds (TC2D6, TC2B8, TC2E6 and KI1E3) scored higher than the average of vanadate plate controls (depicted by the blue line). N = 2-3.

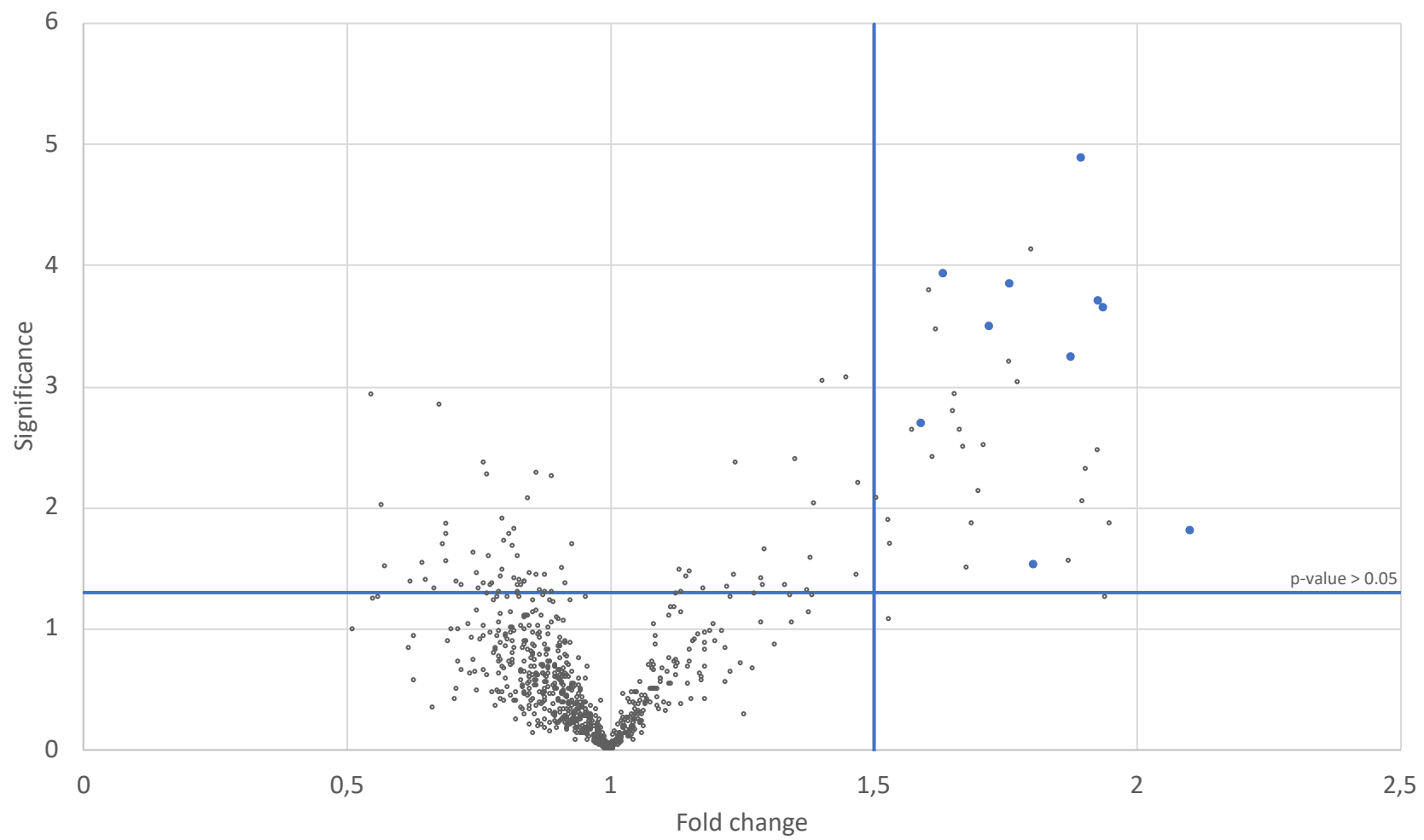


Figure 9. Volcano plot of viral titers 48hpi

786-O cells were treated with the OICR kinase inhibitor drug library at a final concentration of $1\mu\text{M}$. As a reference, vanadate pre-treated cells ($125\mu\text{M}$) are shown in blue. 4 hours post-treatment, cells were infected with VSV Δ 51-fluc at a MOI of 0.1. 48hpi, high-throughput titration was conducted. $\text{Log}_{10}(\text{VEU/mL})$ were calculated for each drug tested and values were normalized against infected untreated 7876-O control (mock) to obtain fold change. An unpaired t-test assuming unequal variances was done. A total of 23 drugs yield a statistically significant fold change superior to 1.5 in $\text{Log}(\text{pfu/mL})$. N = 2-3.

ID	Drug	Target(s)	Developmental Stage	FC Titer	VSe Factor
TC3A7	Vincristine sulfate	Tubulin polymerization inhibitor, antimitotic drug	Approved: acute leukemia, lymphoma	1.9	0.69
TC3G11	Vinblastine sulfate	Tubulin polymerization inhibitor, antimitotic drug	Approved: breast and testicular cancer, lymphomas, neuroblastoma	1.9	0.63
TC2F8	Vinorelbine	Tubulin polymerization inhibitor, antimitotic drug	Approved: breast cancer, non-small cell lung cancer (NSCLC)	1.7	0.69
TC1C11	Docetaxel	Microtubule stabilizing agent, antimitotic drug	Approved: breast, ovarian, NSCLC	1.6	0.56
TC1D11	Ispinesib	Kinesin-like spindle protein inhibitor, antimitotic drug	Clinical trials: breast, liver cancers	1.6	0.53
KI4C11	GSK461364	PLK1, antimitotic drug	Clinical trials: lymphoma	1.7	0.49
KI5G6	Rigosertib	PLK1, antimitotic drug	Clinical trials: myelofibrosis, myelodysplastic syndrome	1.6	0.78
KI5E3	Cdk1/2 Inhibitor III	CDK1/2	Pre-clinical	1.7	0.91
KI6E6	AT-7519 hydrochloride	CDK1/Cyclin B, CDK2/Cyclin A, CDK3/Cyclin E, CDK4/Cyclin D1, CDK5/p35, CDK6/Cyclin D3, CDK9	Clinical trial: metastatic solid tumors	1.9	0.74
KI4C9	Alvocidib (Flavopiridol)	CDC2, CDK2, CDK4, CDK6, CDK7, CDK9, Bcl-2, Mcl-1, Survivin, XIAP	Clinical trials: pancreatic cancer, lymphoma, prostate cancer. Orphan drug status for acute myeloid leukemia (FDA).	1.5	0.61
TC4F11	CUDC-907	HDAC and PI3K inhibitor	Clinical trials: lymphoma, thyroid and breast cancers, multiple myeloma	1.5	0.94
TC2E6	Quisinostat	HDAC1 inhibitor	Clinical trial: lymphoma, myelodysplastic syndrome, leukemia	1.7	1.82
TC2D6	LAQ824	Broad HDAC inhibitor	Clinical trial: phase I pharmacokinetics	1.7	2.10
KI4E3	AZ-960	JAK2	Pre-clinical	1.9	0.75
KI5D6	Ruxolitinib	JAK1/2/3, TYK2	Approved: bone marrow cancer, myelofibrosis	1.8	0.57
KI5H6	AZD1480	JAK1/2/3	Clinical trials: myelofibrosis, solid malignancies	1.8	0.79
KI1D5	Lestaurtinib	FLT3, TRKA, Jak2	Clinical trials: neuroblastoma, leukemia, prostate cancer	1.7	0.03
KI4D9	AT9283	AuroraA, AuroraB, JAK2, JAK3, ABL1 mutant, FLT3	Clinical trials: lymphoma, leukemia, multiple myeloma	1.7	0.40
KI6C5	Hesperadin	AuroraB, tyrosine kinase inhibitor	Pre-clinical	1.5	0.55
TC2G4	JK184	SHH antagonist	Pre-clinical	1.6	0.70
KI5C10	APY 82	IRE1	Pre-clinical	1.8	0.80
KI6A3*	GLPG-0259	MAPKAPK5	Clinical trials: rheumatoid arthritis	1.7	0.40
TC4G5	Pevonedistat (MLN4924)	NAE Inhibitor	Clinical trials: lymphoma, multiple myeloma, leukemia. Orphan drug status for the treatment of myelodysplastic syndrome and acute myeloid leukemia.	1.9	0.66

Table 4. Kinase inhibitor library uncovers novel viral sensitizers

786-O cells were treated as described in **Figure 9**. Drugs that significantly increased viral titers by a fold change (FC) of 1.5 or in Log(VEU/mL) more are described. Their respective viral sensitizer factor (VSe factor) are listed along with their developmental stage according to PubChem (National Center for Biotechnology Information)¹¹⁰ and DrugBank (5.1.4)¹⁰⁹. Drugs are classified into five broad categories based on their targets: antimitotic drugs, CDK, HDAC and JAK inhibitors, or others. KI6A3* did not meet statistical significance for viability (**Figure 7**).

3.5.1 Characterization of novel VSes: dose-response analysis

To obtain comprehensive viral output and viability data, a secondary validation screen was conducted in 786-O using the novel viral sensitizers: CDK inhibitors (AT7519, Flavopiridol and CDK1/2 inhibitor III) and Pevonedistat. 786-O cells were pre-treated 4 hours with serially diluted compounds at a final concentration of 0.0002-40 μ M (0.008-800 μ M for Pevonedistat). Samples were then infected with VSV Δ 51-Fluc or VSV Δ 51-GFP at a MOI of 0.05. 24hpi fluorescent images were captured and analyzed for GFP foci. 40 hpi, high-throughput titration, measured as Log(VEU/mL), was conducted using Vero cells as done previously¹⁰⁶. 48 hpi, cell viability was evaluated using resazurin, a metabolic dye. As indicated in **Figure 10**, the four VSes robustly increased viral output. AT7519 (2.56 μ M), Flavopiridol (0.41 μ M) and CDK1/2 inhibitor III (1.02 μ M) enhanced viral titers by a Log of 1.6, 2.1 and 2.5 respectively relative to untreated control. The concentrations at peak viral titers is similar to that used in the context of the HTS (1 μ M). Pevonedistat on the other hand, presents a broader effective concentration range, with peak viral output attained at 0.32 μ M which represents nearly a 3-Log increase in viral titers when compared to mock. Concentration at peak GFP count overlaps with the concentration range associated with a viability decline in cells co-treated with VSe and VSV Δ 51 (**Figure 11**). As reflected by the calculated LD₅₀, the combination of these novel VSes with VSV Δ 51 leads to a robust drop in cell viability (**Table 5**). The Pevonedistat and VSV Δ 51 combination led to a 99.5% reduction in LD₅₀ which constitutes the sharpest augmentation in cytotoxicity among all four novel VSes tested.

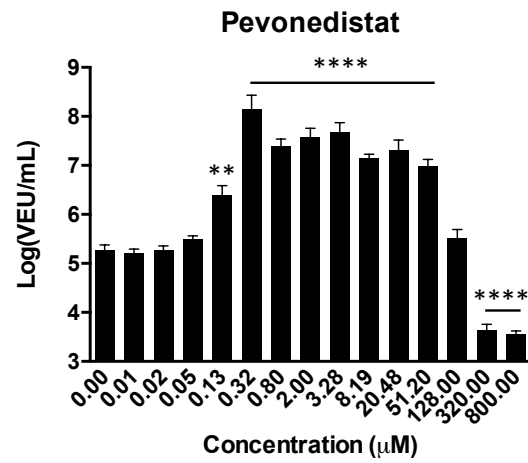
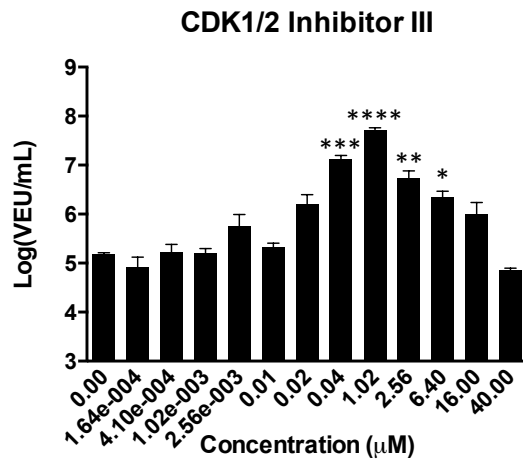
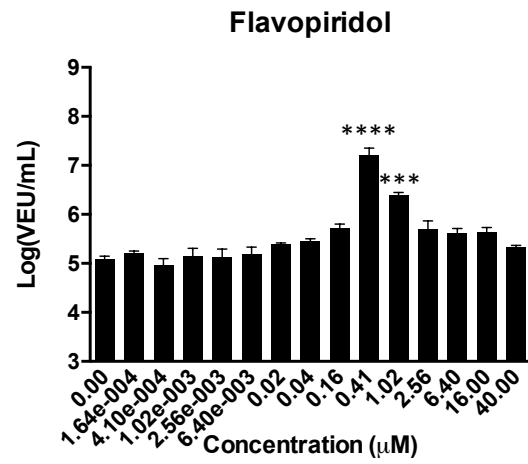
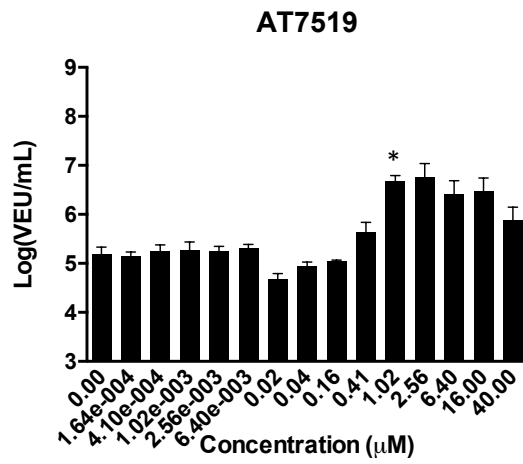
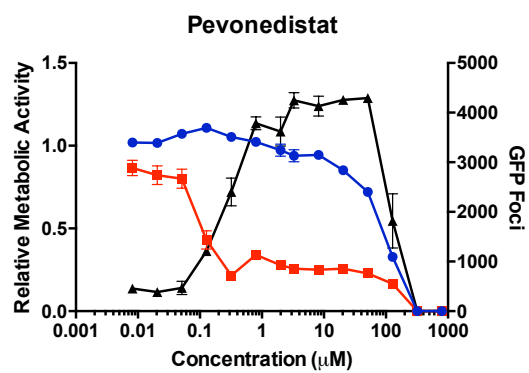
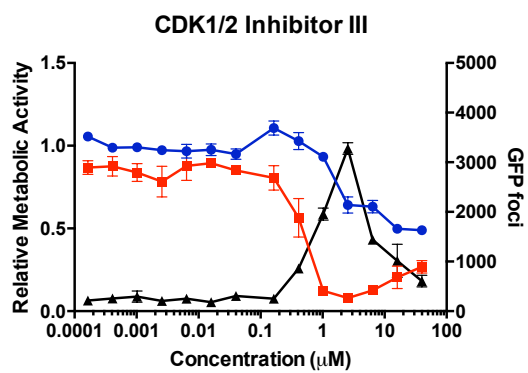
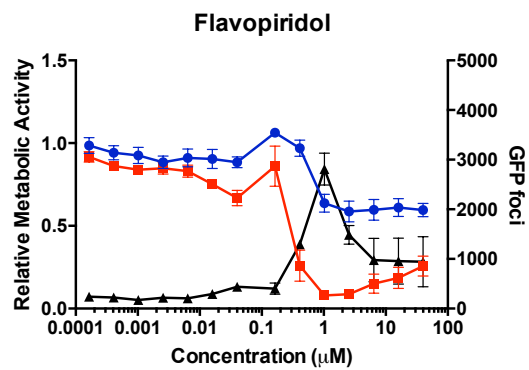
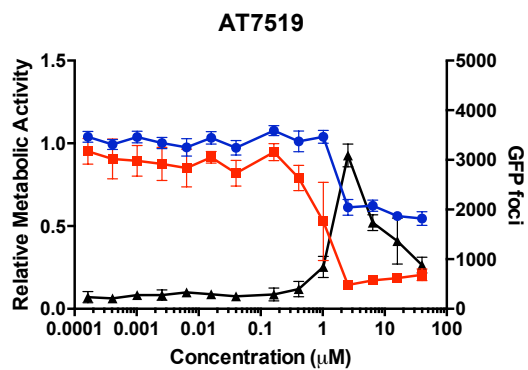


Figure 10. Dose-dependent enhancement of viral output by novel VSes

786-O cells were pre-treated for 4 hours with serially diluted compound at indicated concentration (μM) then infected with VSV Δ51 -Fluc at a MOI of 0.05. 40 hpi, viral titers, expressed as Log(VEU/mL) was quantified. N = 2-4 biological replicates. Error bars indicate standard error of the mean (SEM). One-way ANOVA *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 relative to untreated control as calculated on GraphPad Prism6.



—●— Drug
 —■— Drug+Virus
 —▲— GFP foci

Figure 11. Dose response of novel VSes on GFP count and viability

Cells were treated as described in **Figure 10**. 24 hpi fluorescent images were captured and total GFP foci was quantified. 48 hpi the relative metabolic activity was assessed using resazurin. Fluorescence is blank-subtracted and normalized to untreated uninfected cells. N = 2-4 biological replicates. Error bars indicate standard error of the mean (SEM).

Drug	LD₅₀ drug alone	LD₅₀ drug + VSVΔ51
AT7519	20.88	1.59
Flavopiridol (Alvocidib)	20.58	0.52
CDK1/2 Inhibitor III	16.80	0.85
Pevonedistat	74.81	0.37

Table 5. Novel VSe and VSVΔ51 synergistically decrease LD₅₀

786-O cells were treated as in **Figure 10**. The drug concentration (μ M) resulting in death of half the cell population (lethal dose, LD₅₀) is calculated using GraphPad Prism6 based on the viability data presented in **Figure 11**.

3.5.2 Validation of novel VSes in ex vivo tumor cores

To expand on the tumor selectivity and broaden the oncolytic potential of these compounds, tissues of mice bearing 76-9 (murine rhabdomyosarcoma) or CT26wt tumors were processed and viable specimens (tumor, lung, brain, muscle, spleen) were treated with VSes AT7519, Flavopiridol, CDK1/2 inhibitor III and Pevonedistat, then infected with VSV Δ 51-GFP four hours later. 48 hpi, fluorescence images were taken, and supernatants were collected for subsequent quantification of viral titers. CDK1/2 inhibitor III is the most potent enhancer of viral replication in both *ex vivo* models. 76-9 cores pre-treated with this compound generated the strongest GFP signal intensity and nearly a full Log increase in viral titers (**Figure 12**). In the CT26wt model, which is refractory to VSV Δ 51 infection⁶⁷, a 2-Log increase in viral output was attained in presence of CDK1/2 inhibitor III (**Figure 13**). Most importantly, pre-treatment of normal tissues does not increase viral replication past viral input values (horizontal lines **Figure 12, Figure 13**) thereby suggesting that VSV Δ 51 maintains its tumor tropism when combined with VSes.

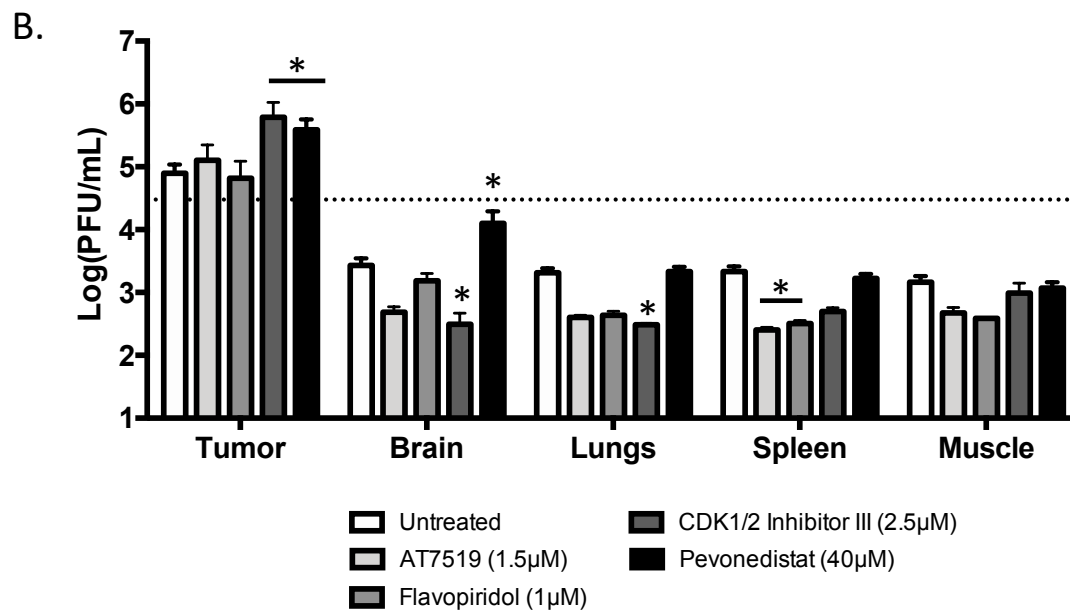
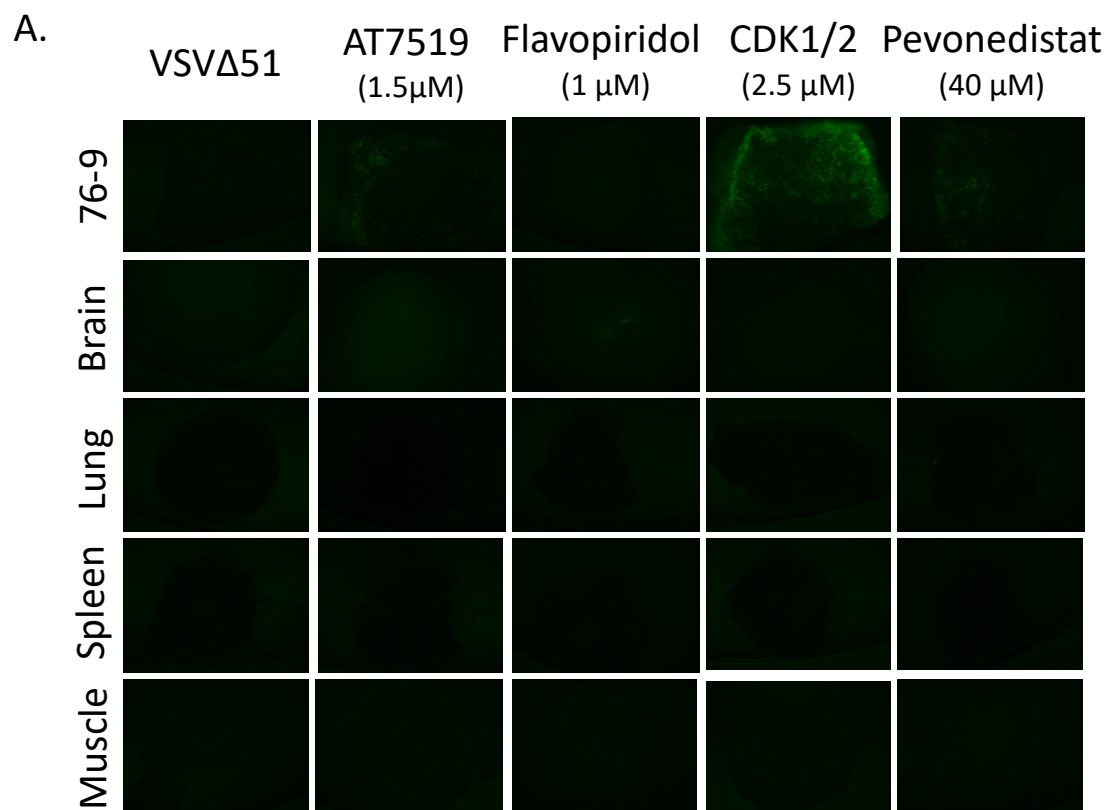


Figure 12. Impact of VSes on viral replication in ex vivo 76-9 model

76-9 (murine rhabdomyosarcoma) tumors were implanted subcutaneously in C57BL/6 mice. Once tumors reached 1500mm³, tissue samples were isolated, cored and treated for four hours with compounds at indicated concentrations prior to infection with 3E4 plaque-forming units of VSVΔ51-GFP (indicated by the horizontal line in **B.**). To monitor viral infection, GFP images were captured (**A.**) and supernatants were collected at 48 hpi. Representative GFP images are shown for tumor and normal tissues (brain, lung, spleen, muscle). (**B.**) Infectious viral particles were quantified by standard plaque assay. N = 3-12 cores per tissue, error bars indicate SEM. Two-way ANOVA *p<0.05 relative to untreated control of each tissue as calculated on GraphPad Prism6.

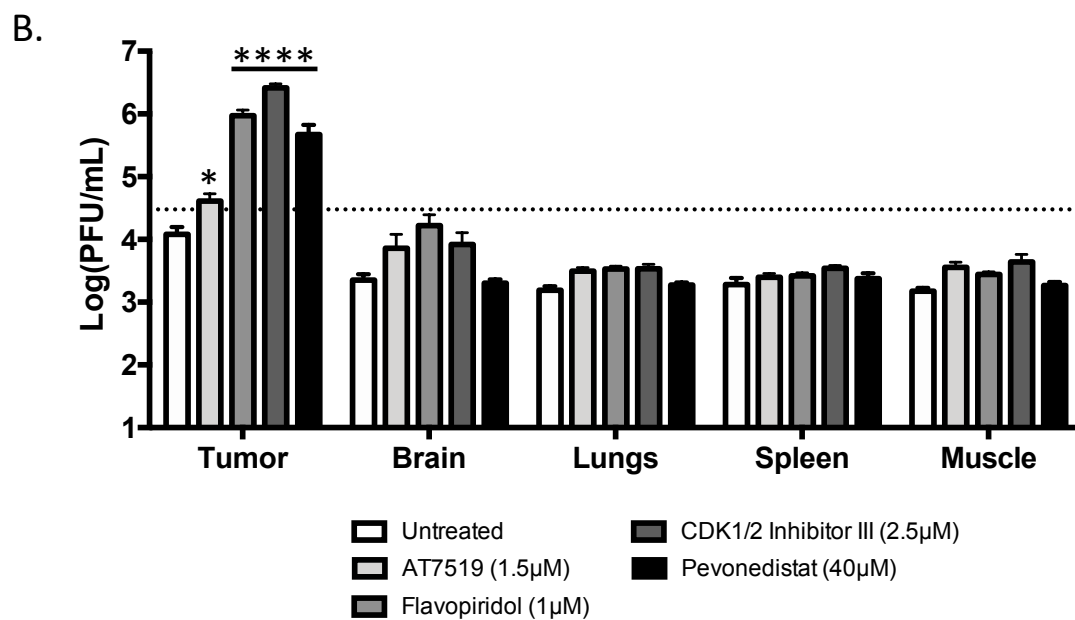
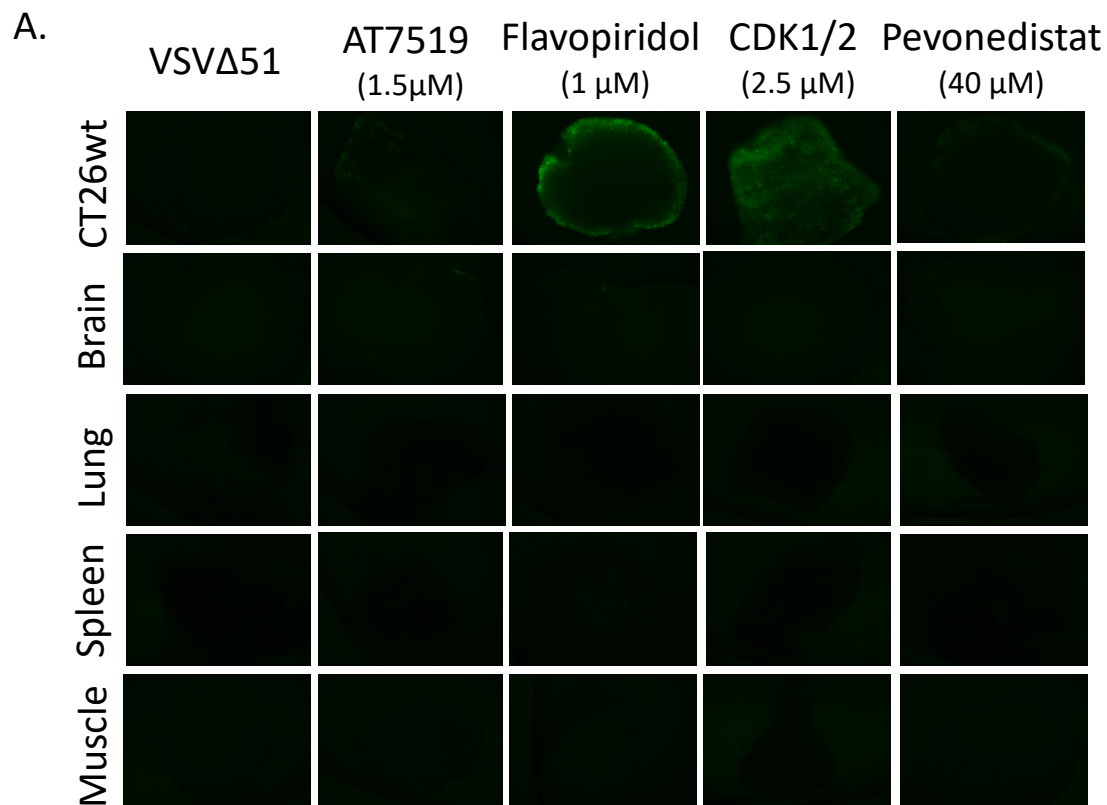
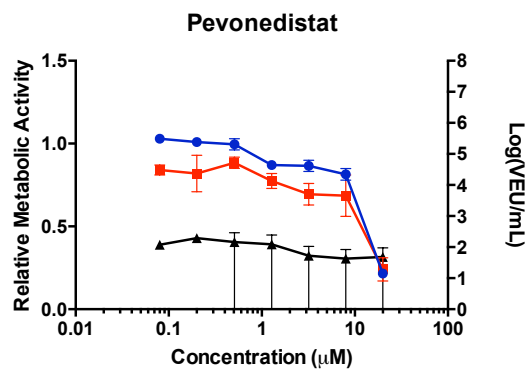
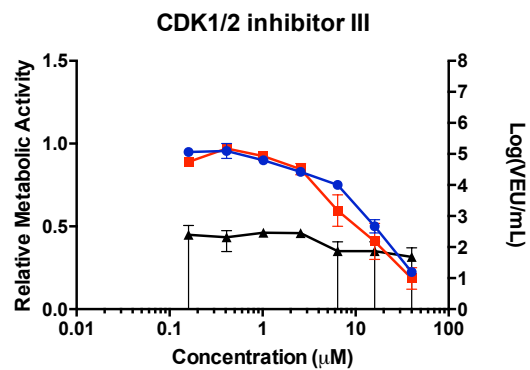
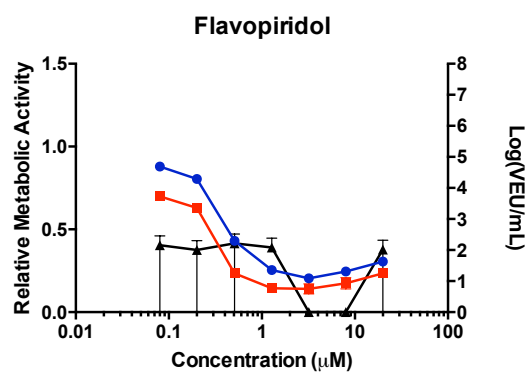
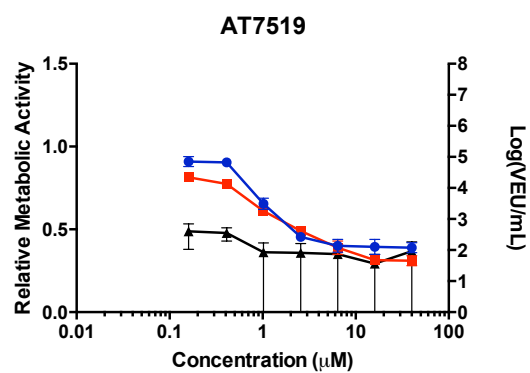


Figure 13. Impact of VSes on viral replication in resistant ex vivo CT26wt model

CT26wt (murine colon carcinoma) tumors were grown in BALB/c mice until they reached a volume of 1500mm³. Tissues were collected, cored and treated for four hours with compounds at indicated concentrations prior to infection with 3E4 plaque-forming units of VSVΔ51-GFP (indicated by the horizontal line in **B.**). To monitor viral infection, GFP images were captured (**A.**) and supernatants were collected at 48 hpi. Representative GFP images are shown for tumor and normal tissues (brain, lung, spleen, muscle). **B.** Infectious viral particles were quantified by standard plaque assay. N = 4-16 cores per tissue, error bars indicate SEM. Two-way ANOVA *p<0.05 and ****p<0.0001 relative to untreated control of each tissue as calculated on GraphPad Prism6.

3.5.3 Validation of novel VSeS in vitro using normal murine hepatocytes

The four VSeS were tested on primary murine hepatocytes to further characterize their impact on normal cells and on VSV Δ 51's selectivity. As shown in **Figure 14**, viral replication is not enhanced in VSe-pretreated normal murine hepatocytes. When treated with drug alone, cell viability declines in a dose-dependent fashion but the addition of VSV Δ 51 does not augment cytotoxicity. Therefore, VSV Δ 51 specificity for cancer cells is maintained upon VSe treatment.



● Drug ■ Drug+Virus ▲ Log(VEU/mL)

Figure 14. Novel VSes do not increase VSV Δ 51 infection of murine hepatocytes

Primary murine hepatocytes were freshly isolated and treated with compounds for four hours prior to infection with VSV Δ 51 (MOI 0.05). 40 hpi viral output was quantified by high-throughput titration and viability was assessed 48 hpi with resazurin. Viability is blank-controlled and normalized to uninfected untreated controls. N = 2, error bars represent SEM. Cells were isolated and provided by Dr. Morgan Fullerton and Conor O'Dwyer.

3.6 Van/OV combination therapy in murine sarcoma model

We have previously showed that rhabdovirus VSV Δ 51 is a promising virotherapeutic for sarcoma; in a direct comparison of four OV platforms, VSV Δ 51 and MG1 treatment of a panel of murine and human sarcoma cell lines resulted in robust infection and cell death¹²⁵. Furthermore, in a variety of sarcoma cell lines, vanadate increased cytotoxicity and viral spreading when used in conjunction with VSV Δ 51¹⁰³ (manuscript also in preparation). Building on these findings, we next sought to test the combination of vanadate and VSV Δ 51 in a 76-9 (murine rhabdomyosarcoma) tumor model.

Mice bearing 76-9 tumors (10-13 mice per group) were administered 100 mg/kg of vanadyl sulfate (VS) and 1E8 PFU of VSV Δ 51-fluc four hours later, repeated three times over seven days (**Figure 15**). The combination therapy resulted in enhanced viral replication (**Figure 15C**) and tumor control (**Figure 15D**) compared to the monotherapies; however, the dual therapy and the VSV Δ 51 test groups ultimately yielded statistically indistinguishable survival rates. Survivors from the study were re-implanted with 76-9 cells on the opposite limb to assess whether a tumor-specific adaptive immune response was developed. Two of the three mice from the dual therapy and none of the two mice from the VSV Δ 51 group developed tumors. We postulated that these tumors may escape immunological cell death by downregulating MHC class I expression. We thus measured MHC class I expression on the tumors of the 2 mice from the dual combination test group. However, MHC class I expression was observed in the CD45- population (**Supplemental figure 5**). This led us to hypothesize that the two mice that relapsed evaded CD8 T cell-mediated killing through other immunosuppressive mechanisms, such as the expression of checkpoint proteins that block T cell activation for example.

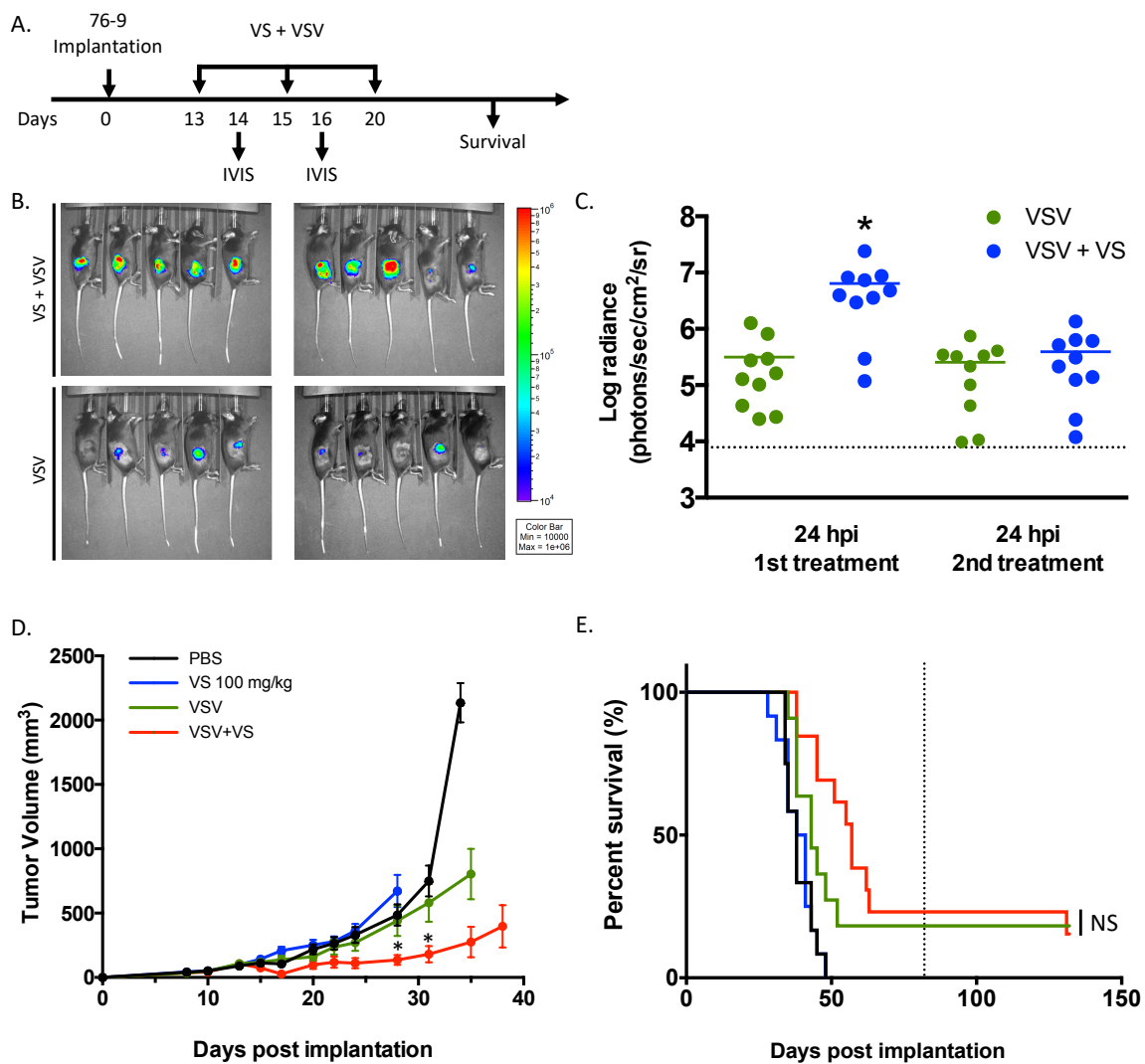


Figure 15. Vanadyl sulfate improves infection and tumor control

A. 76-9 cells were engrafted subcutaneously in C57BL/6 mice. Treatment was initiated 13 days post implantation and consisted of an intratumoral (i.t.) vanadyl sulfate (100 mg/kg) or vehicle injection followed by an i.t. injection of VSV Δ 51-fluc (1E8 PFU). Treatment was repeated three times over 7 days. Viral replication was evaluated by IVIS 24 hours after the first and second treatment. IVIS images 24 hpi after the first treatment are shown (**B**) and bioluminescence signal quantification for individual mice is presented (**C**). * $p < 0.05$ by a 2-tailed T-test comparing the VSV and dual therapy test groups. Horizontal bars represent mean. **D.** Average tumor volume over time, error bars represent standard error of the mean (SEM). Curve ends when the first mouse of a cohort reaches end-point. Two-way ANOVA * $p < 0.05$ relative to PBS control as calculated on GraphPad Prism6. **E.** Kaplan-Meier curve. Log-rank test (Mantel-Cox) reveals that VSV ($p < 0.05$) and the dual therapy ($p < 0.0001$) significantly improve survival when compared to PBS or VS controls. The dual therapy and VSV curves are not statistically different ($p = 0.25$, NS). Tumor re-challenge was done on day 82 (dotted vertical line). IVIS data (**B-C**) was generated by Mohammed Selman and Andrew Chen. $N = 10-13$ mice per group. Error bars represent standard error of the mean (SEM).

3.7 Immune checkpoint inhibitors and van/OV combination therapy

Many groups are exploring the combination of OV_s with immune checkpoint blockade antibodies (e.g. ipilimumab¹²⁶ or pembrolizumab¹²⁷ and T-Vec in melanoma patients) and results thus far indicate robust enhancement in overall responses in the combination therapy arms¹²⁸. A small pilot experiment was conducted using the B16F10 model. Mice were implanted with 5E5 cells subcutaneously, and treatment regimen was initiated on day 11 and consisted of three sets of injections over five days: an i.t. VS injection (100mg/kg), followed by an i.t. VSVΔ51-fluc injection (1E8 PFU) and a checkpoint inhibitor (CPI) anti-CTLA4 or anti-PDL1 (250μg) intraperitoneal (i.p.) injection four hours later (**Supplemental figure 6**). The triple combination anti-CTLA4 + VS + VSV was the most efficient at delaying tumor progression and led to one cure (N = 3). In contrast, the CPI + VSV dual therapies, VSV + VS and the anti-PDL-1 + VS + VSV combinations all generated modest tumor control compared to monotherapies and failed to robustly improve survival rates. Two mice succumbed post-treatment in this study (1 mouse anti-PDL1 + VS + VSV cohort, 1 mouse from the VS + VSV group), indicating toxicities for the combination regimen in this model.

Based on these results, we proceeded to test the triple combination of van/OV paired with immune checkpoint inhibitor anti-CTLA4 in the 76-9 model to see (i) if CPI can improve the anti-tumor immune response of the van/OV regimen and (ii) how the CPI-VSV treatment schedule compares to the triple combination and monotherapy regimens. The treatments consisted of three VS (100mg/kg, i.t.), VSVΔ51-fluc (1E8 PFU i.t.) and anti-CTLA4 (200μg, i.p.) injections over five days (**Figure 16**). VS caused a significant drop in weight which was most prominent in the triple combination condition (14% relative

to weight on day 7). Two mice from the triple combination therapy cohort and one mouse from the VS + CPI test group succumbed from toxicity and weight loss. Scabs at the site of VS injection were also observed. Quantification of viral infection by IVIS was not performed to minimize toxicity. Measurements of tumors bearing skin lesions were excluded until fully healed if they obstructed accurate caliper measurements. Recorded onset of skin lesions on tumors was at day 15 and they fully resolved by day 35. Despite adverse effects, the triple combination therapy led to cures. In comparison to results obtained in **Figure 15**, the dual VSV+ VS therapy delayed tumor progression but while overall survival was improved (40%) compared to VSV alone (20%) survival curves were not statistically different ($p = 0.27$). Notably, the combination regimen ultimately gave worse outcomes than VS alone (60% survival) in sharp contrast with results obtained in **Figure 15**. Strikingly, CPI alone was efficient at curing 80% of mice. While cure rates were not improved by combination with either VS (~56%) or VSV (~56%), the addition of CPI to the VS+VSV dual therapy led to 100% cures ($p < 0.001$ when compared to PBS, VSV or VS+VSV). However, there were significant adverse events in this group and two mice succumbed to this treatment combination. Overall, whether there is a true therapeutic benefit in combining CPI with VS and VSV Δ 51 is unclear, but the treatment-related adverse events evidently warrant further treatment regimen optimizations.

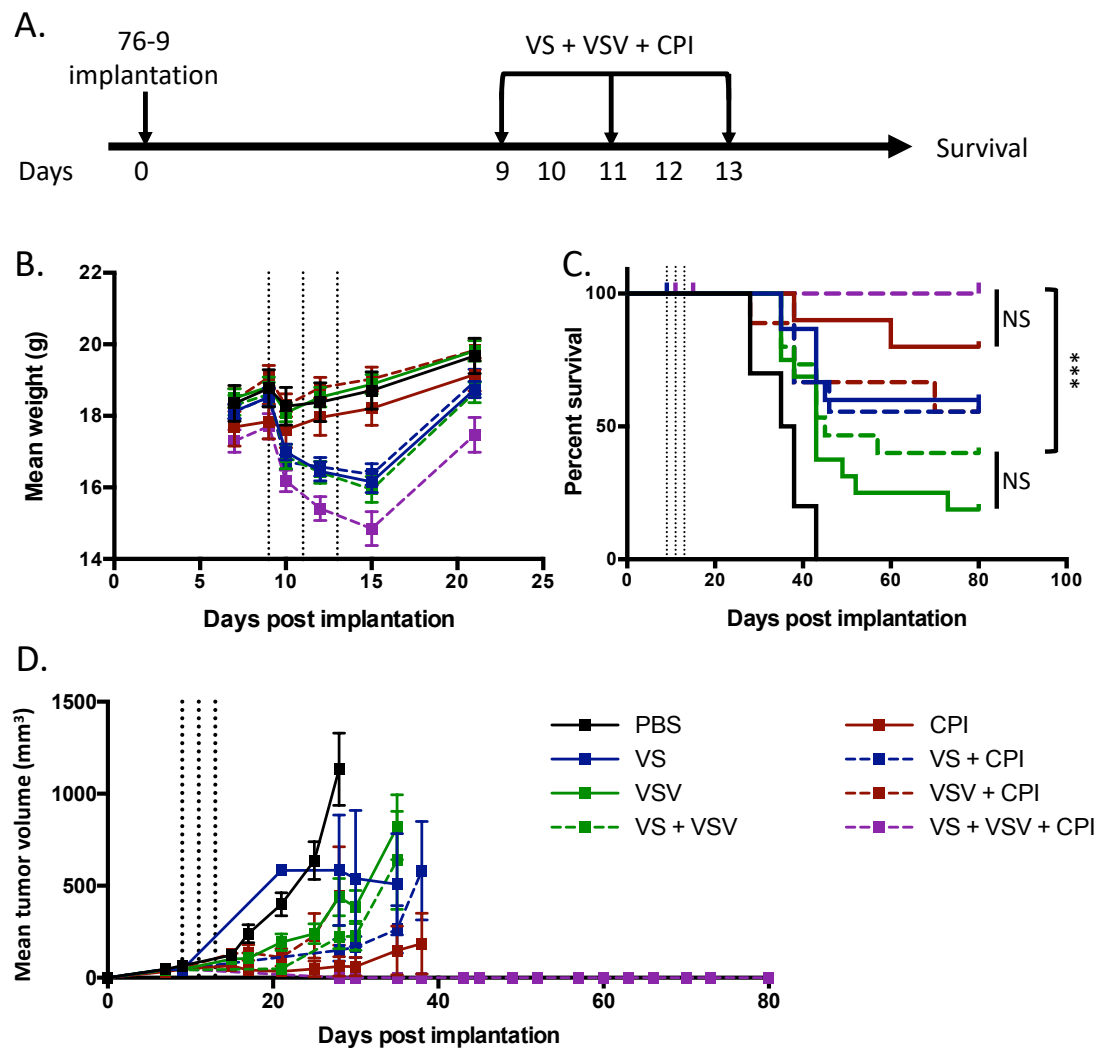


Figure 16. Effect of the triple combination therapy on survival and tumor progression

A. Treatment regimen. Mice were implanted subcutaneously with 1E6 76-9 cells. 9 days post-engraftment, mice were treated with VS (100mg/kg) i.t. and four hours later with 1E8 PFU of VSV Δ 51-fluc (i.t.) and checkpoint inhibitor (CPI) i.p. (anti-CTLA4, 200 μ g). **B.** Average weight over time. **C.** Kaplan-Meier survival curve. Censored due to toxicity: 2 mice from the triple combination therapy test group and 1 mouse from the VS+CPI cohort. A log-rank (Mantel-Cox) test was performed to assess statistical significance. **D.** Tumor progression over time. Curves were ended once the first mouse of a test group is euthanized. Doted vertical lines indicate days of treatments. Error bars represent standard error of the mean (SEM). N = 9-15. ***p<0.001, NS = not significant.

3.8 Treatment regimen optimization

A dose escalation study was performed to assess drug tolerability and select a safe vanadyl sulfate (VS) dose that simultaneously leads to optimal viral infection in the 76-9 model. Treatment consisted of an i.t. VS injection (dose of 50, 75 or 100mg/kg) followed by an i.t. VSV Δ 51-fluc injection (1E8 PFU) 4 hours post treatment. Treatment was repeated three times over 5 days. Weight loss was observed in all VS-treated mice but the 50 and 75 mg/kg cohorts showed signs of recovery (**Figure 17**). Viral replication was monitored by IVIS and revealed a statistically significant enhancement in mice treated with 50 and 75 mg/kg of VS compared to VSV Δ 51 counterparts. Importantly, one mouse from the test group receiving 100mg/kg succumbed on the second day of treatment. Skin lesions at the site of injection were observed (scabs) in mice treated with the highest doses (75 and 100 mg/kg). 7 days post treatment, mice were euthanized to analyze T cell infiltration and checkpoint protein expression, but scabs obstructed tumor excision and sample sizes were insufficient to draw robust conclusions (data not shown). Overall, the 50 mg/kg treatment was deemed the safest dose still enhancing viral replication *in vivo*. We thus proceeded to test the impact of the 50 mg/kg VS dose in combination with VSV Δ 51 on long-term tumor control and survival.

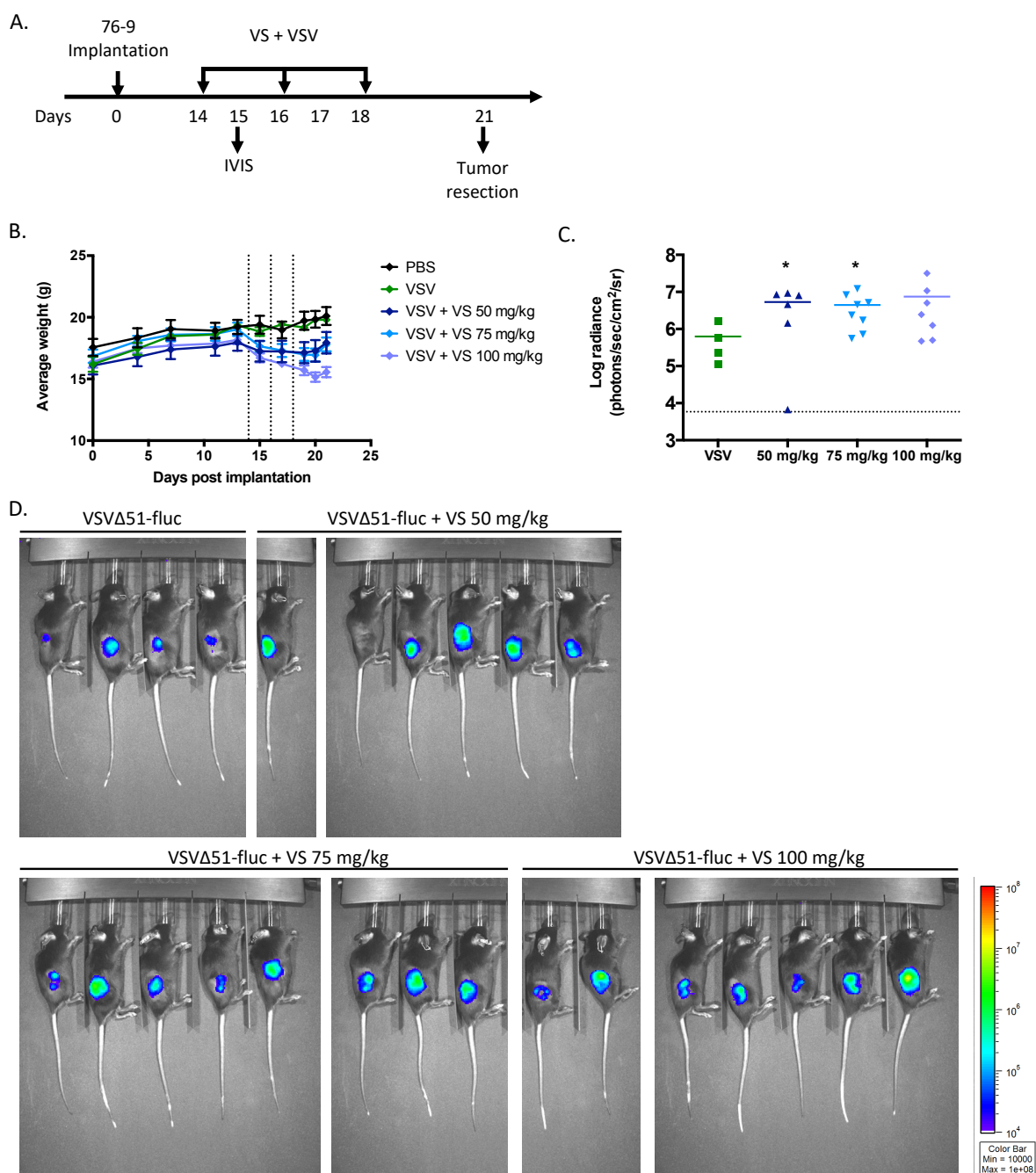


Figure 17. Combination therapy regimen optimization

76-9 cells were subcutaneously implanted in C57BL/6 mice. 14 days after engraftment, treatment was initiated and mice were injected intratumorally (i.t.) with vanadyl sulfate (50, 75 or 100 mg/kg dissolved in PBS) and with 1E8 PFU of VSV Δ 51-fluc four hours post treatment. Treatment was repeated for a total of three times over a five-day period (**A**). Weights were monitored over time (**B**). 24 hours after the first treatment, viral infection was assessed by IVIS and bioluminescence values are plotted for each individual mouse as well as the average per condition (horizontal bars). Dotted horizontal line indicates background signal (**C**). A two-tailed unpaired t-test was performed on GraphPad Prism6 to determine statistical significance between VS-treated test groups and the VSV Δ 51-fluc control. IVIS images are shown (**D**). * $p < 0.05$, $N = 4-8$. Vertical lines indicate treatment days. Error bars represent standard error of the mean (SEM).

3.9 Checkpoint inhibitor anti-CTLA4 prolongs survival in sarcoma

Since anti-CTLA4 alone previously resulted in 80% survival and improved tumor control, a lower dose of 100 μ g was tested in order to more clearly test for synergy upon combination with VS and/or VSV Δ 51. Treatment regimen was initiated 14 days after engraftment once tumors were 89 mm³ on average (**Figure 18**). One mouse succumbed 16 hours post infection (first treatment, 15 days post implantation) from the VSV+VS cohort. Scabs over the tumor were observed in mice receiving VS but were healed by day 27. Weight loss was overall minimal; a drop was observed in the VSV+VS cohort but mice recovered by day 23. Viral replication, as quantified by IVIS, was not significantly enhanced in neither the VSV+VS nor the triple combination test groups when compared to the VSV Δ 51 cohort. However, a trend towards increased luminescence is observed in presence of VS (nearly a 13-fold increase in signal intensity between VSV+VS and VSV). No relationships were found between the strength of the bioluminescence signal and therapeutic outcome. Mice receiving anti-CTLA4, most notably the triple combination and the VSV+CPI cohorts, achieved better tumor control. In order to assess whether a tumor-specific adaptive immune response was mounted, 76-9 cells were implanted on the opposite limb of cured mice on day 91. None of the challenged mice re-developed tumors thereby indicating the presence of an antitumor immunological memory. Overall VS, CPI, VSV+VS and the triple combination therapy generated similar survival rates although only CPI and the triple therapy meet statistical significance when compared to PBS control (p-value of 0.012 and 0.002 respectively). A head-to-head comparison of the triple and the CPI+VSV combination therapies indicate that the presence of VS at 50 mg/kg lowers the efficacy of the CPI+VSV dual therapy at prolonging survival. The CPI dose of 100 μ g is

much less effective than the previously tested 200 μ g dose. In fact, as a single-agent, the survival rate of mice treated with 200 μ g CPI was 80% (**Figure 16**), but this value drops to 20% when the dose is 100 μ g (**Figure 18**). Survival rates were 60% in the VSV+CPI test group which is similar with the previous experiment (**Figure 16**, 55% survival rate) despite having used half less CPI. Overall, the combination of VSV Δ 51 with CPI anti-CTLA4 results in a robust improvement in survival and leads to long-lasting cures.

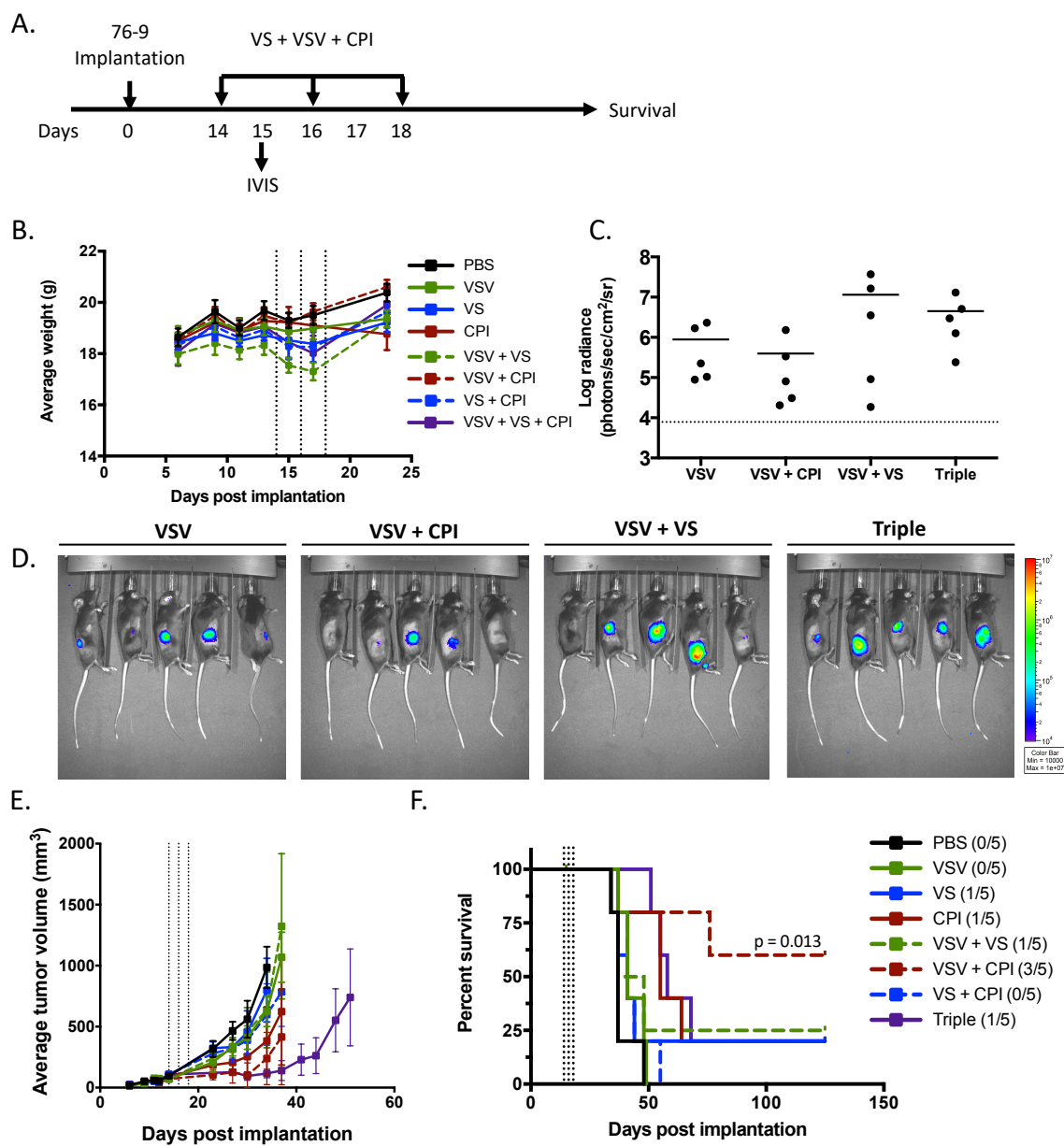


Figure 18. Impact of the triple combination therapy on viral infection, tumor control and survival

76-9 cells were subcutaneously injected in C57BL/6 mice. 14 days after engraftment, treatment was initiated, and mice were injected intratumorally (i.t.) with vanadyl sulfate (VS, 50 mg/kg dissolved in PBS) followed by a 1E8 PFU i.t. injection of VSV Δ 51-fluc and CPI anti-CTLA4 (100 μ g) i.p. four hours post treatment. Treatment was repeated for a total of three times over a five-day period (**A**). **B**. Weights were monitored over time. **C-D**. 24 hours after the first treatment, viral infection was quantified by IVIS. Luminescence is plotted and horizontal bars represents test group average. The dotted horizontal line indicates background signal. A two-tailed unpaired t-test was performed, none of the dual or triple combination therapies led to statistically significant enhancement in viral replication when compared to the VSV Δ 51 test group. **E**. Tumor volume over time. Curves end once the first mouse of a cohort reaches end-point. **F**. Kaplan-Meier survival curves. Censored due to adverse event: 1 mouse from the VSV+VS test group (16h post first treatment). A Log-rank (Mantel-Cox) test reveals that VSV+CPI significantly prolongs survival ($p=0.013$). Number of cures are listed in the legend in brackets. A tumor re-challenge was performed 91 days post implantation on the opposite limb and none of the mice redeveloped tumors. $N = 5$. Vertical lines represent treatment days. Error bars indicate standard error of the mean (SEM).

4. Discussion

4.1 PPases in viral infection

The innate antiviral response is subject to various regulatory mechanisms, including protein phosphorylation by kinases and PPases, which can be exploited by pathogens. In fact, some viruses upregulate host PPases expression to circumvent innate defenses. For example, the hepatitis C virus increases cellular PP2Ac levels to block transcription of ISGs¹²⁹. Other viruses have naturally evolved to encode PPases to overcome host viral defences. For instance, vaccinia virus carries the dual specificity phosphatase VH1, an important virulence factor involved in directly dephosphorylating STAT1 thereby shutting the IFN- γ cascade¹³⁰. The ICP34.5 protein of HSV-1 is a viral regulatory subunit of PP1 that contributes to immune-evasion by dephosphorylating eIF2 α (α subunit of translation initiation factor 2) and reinstating protein synthesis in the infected cell¹³¹. This antagonizes the action of the host protein kinase R (PKR) which, in response to stress (e.g. viral RNA), normally phosphorylates eIF2 α to shutdown translation and prevent viral spread¹³². With such understanding, researchers have notably designed oncolytic viroceuticals with enhanced safety profiles and cancer selectivity like the FDA-approved Imlygic®, a HSV-1 virus among others with an ICP34.5 deletion^{21,133}.

In response to OV infection, immunostimulatory cytokines are released and TAAs are presented to T cells thereby eliciting a cancer-specific immune protection. However, mounting clinical data suggests that OVs as stand-alone agents are not reaching their anticipated therapeutic efficacies⁵⁴. This is owing to multiple factors including sufficiently functional antiviral pathways in a significant proportion of cancer cells^{44,55,56}. To this end, combination therapies including pharmacoviral strategies or genetic engineering of OVs with therapeutic

transgenes have gained significant research momentum¹³⁴. The use of small molecules potentiators of OV, like vanadium-based compounds, holds many advantages; they pre-condition the tumor microenvironment to improve susceptibility to OV infection and can act as adjuvants. Previous studies have shown enhancement of virotherapy in aggressive glioma, colon and breast carcinoma models with vanadate⁷². In this study, we sought to provide a comprehensive approach to delineate its mechanism of action.

4.2 Identification of PPases involved in viral sensitization

In hopes of identifying the PPases likely targeted by pan-PPase inhibitor vanadate and driving its viral sensitizing effects, a human PPase siRNA library was screened to uncover targets that enhance VSV Δ 51 oncolysis and viral output. We identified several such host PPases whose knockdown led to simultaneously improved VSV Δ 51 growth and oncolysis (**Table 2**). These top genes are: EYA2, PP1, PP2A, PP4, ACP2, ENTPD4, PPM1M, PPM1B, SSH3 and PSPH. Of these, seven would not have been easily predicted from the literature (PP2A, ACP2, ENTPD4, PPM1M, PPM1B, SSH3 and PSPH) because they are poorly characterized and/or have no comprehensive relationships with innate antiviral immunity thus far.

4.2.1 Haloacid dehalogenase (HAD) PPases

Eye absent (EYA) proteins were initially described in *Drosophila* ocular development but in recent years, their threonine and tyrosine PPase activity along with their roles in human pathologies and immunity have been characterized^{135,136}. In vertebrates, the four EYA proteins (EYA1-4) are HAD PPases involved in organ formation¹³⁵. Intriguingly, mouse embryonic fibroblasts transduced with EYA1-4 have increased IFN β mRNA levels upon stimulation with

TLR3 agonist poly(I:C)¹³⁷. EYA4 physically interacts with signal transducers of innate antiviral response MAVS, STING and NLRX1 resulting in an improved IRF3-dependent ISG expression and inhibition of viral replication¹³⁷. While EYA4 silencing did not increase VSVΔ51 cytotoxicity passed the 40% viability threshold (**Table 1**), the combination of EYA4 knockdown with VSVΔ51 infection nevertheless led to a viral sensitizer factor of 0.33 and significantly increased viral titers. EYA2 is among the top PPases which, upon silencing, generated a strong viral sensitizer factor and an enhancement of VSVΔ51 titers (**Table 2**). It would be interesting to validate if this potentiation of VSVΔ51 infection in the context of EYA silencing is caused by an impaired PAMP-PRR cascade. This could be done by looking at protein-protein interactions (e.g. STING-TBK1 by immunoprecipitation), protein-DNA interactions (e.g. IRF3-DNA by chromatin immunoprecipitation) and/or ISG expression (e.g. IFNβ, MX2 by PCR). PSPH also belongs to the HAD PPase superfamily but has no reported role innate immunity¹³⁶.

4.2.2 Phosphoprotein PPases (PPP)

The PPP family of Ser/Thr PPases includes PP1, PP2A, PP3-6⁹⁷. Combined, PP1 and PP2A are responsible for 90% of a cell's Ser/Thr dephosphorylation¹³⁸ and both were shown to impede interferon responses via inactivation of IRFs^{139,140}. Specifically, PP1 acts as a negative regulator of antiviral defenses by dephosphorylating IRF7¹³⁹ and IRF3¹⁴⁰. As shown in **Table 2**, PPP1R16A, PPP1R14A, PPP1R2, PPP1R10 and PPP1R12B were top hits. The regulatory subunit PPP1R16A, also known as the myosin phosphatase-targeting subunit 3 (MYPT3), prevents dephosphorylation of PP1 substrates (e.g. phosphorylase a, myosin light chain and myosin substrate)¹⁴¹. PPP1R14A, also referred as CPI-17, negatively controls the catalytic subunit of PP1, with enhanced inhibition of PP1 activity when the PPP1R14A subunit

is phosphorylated¹⁴². Similarly, PPP1R2¹⁴³ (commonly named IPP2), PPP1R10¹⁴⁴ (alternatively named PNUTS) and phosphorylated PPP1R12b¹⁴⁵ (also known as MYPT2) are PP1 inhibitors. In absence of these PP1 regulatory subunits (such as in the present siRNA HTS), PP1 subcellular location and substrate specificity are thus likely modified to favor viral replication and oncolysis. This could be tested by measuring IRF3/7 phosphorylation (western blot) and nuclear translocation (fractionation and western blot or immunofluorescence), ISG expression and viral titers in VSV Δ 51-infected 786-O cells devoid of PP1R16A/14A/2/12B expression. PP1 cellular localization could be assessed by immunofluorescence and microscopy.

The trimeric PP2A holoenzyme is composed of a catalytic, a structural and a regulatory subunit¹³⁸. PP2A is involved in human diseases including cancer and most recently, its role in dephosphorylating IRF3 via the adaptor protein RACK1 has been described¹⁴⁶. The regulatory subunit PPP2R5E, also known as PR61 ϵ , and PPP2R2C, commonly known as PR55 γ , were primary hits (**Table 2**). However, knowledge on the activity of PP2A holoenzymes is fragmented given the broad variety of multi-subunit complexes that exists. There is evidence supporting the role of PPP2R2C in directly suppressing c-SRC signaling¹⁴⁷ and SRC stimulates the TBK1-IRF3 antiviral cascade¹⁴⁸. Therefore, the identification of PPP2R2C as an enhancer of viral titers and oncolysis when silenced is unexpected given the literature and reiterates the need to validate the primary PPase siRNA hits before drawing final conclusions. For example, the phosphorylation patterns of TBK1 and IRF3 along with type I IFN expression should be evaluated in the context of PPP2R2C knockdown and VSV Δ 51 infection.

The dimeric PP4 holoenzyme, consisting of a catalytic and a regulatory subunit, plays a role in a myriad of cellular functions including microtubule organization, chromatin

remodeling, immunity and cell proliferation¹⁴⁹. Histone deacetylase 3 (HDAC3) activity is downregulated by the PPP4R1-PPP4C PP4 complex¹⁵⁰. In macrophages devoid of PP4, IFN α /IFN β expression is upregulated and VSV replication is greatly reduced; a phenomenon reported to be driven by PP4 directly dephosphorylating and inactivating TBK1¹⁵¹. In parallel, overexpression of PPP4C was found to upregulate NF κ B-mediated gene expression¹⁵². Therefore, the observed enhancement in titers and cytotoxicity upon PPP4R1 and PPP4C silencing is thus likely occurring via a mechanism homologous to previously described viral sensitizer MS-275⁶⁹ (HDAC1/3 inhibitor) and dimethyl fumarate⁷¹ (NF κ B inhibitor). Lastly, the function of PPP4R1L remains poorly understood.

4.2.3 Metal-dependent protein PPases (PPM)

The PPM family of Ser/Thr PPases consists of metal-dependent enzymes and unlike PPPs, PPMs are monomeric and their activity is not modulated by regulatory subunits¹⁵³. PPM1B has been recently identified as a suppressor of TBK1-mediated antiviral signalling by directly dephosphorylating and deactivating TBK1¹⁵⁴. Furthermore, PPM1B is an IKK β phosphatase and prevents NF κ B nuclear translocation¹⁵⁵. Therefore the identification of PPM1B is surprising because in the context of this screen, PPM1B silencing would imply NF κ B activation which stimulates ISG expression¹⁵⁶. The phosphorylation status of NF κ B as well as TBK1 should be tested upon PPM1B knockdown and VSV Δ 51 infection to validate this primary hit. Little is known about the PPM1M enzyme although it was shown to have no effect on TBK1-induced IFN β expression¹⁵⁴ and thus likely serves distinct cellular functions and have substrate affinities that differ from PPM1B's.

4.3 The roles of PPases in human diseases

While some PPase confer protection against illnesses, aberrant PPase activity can also drive pathogenesis. This is reflected by the discovery of oncogenes (e.g. SHP2) and tumor-suppressing (e.g. PP2A) PPases⁹⁷. For decades, PPases were perceived as “undruggable”, but recent breakthroughs, namely in our understanding of PPases but also in drug development strategies, are challenging this misconception^{97,99,102}. Some PPase inhibitors have been discovered by chance while others were approved and used in clinics before they were shown to exert their therapeutic effects through the suppression of PPase activity. For example, Guanabenz is an α 2-adrenergic agonist indicated for the treatment of hypertension with newly discovered inhibitory actions against PP1’s regulatory subunit PPP1R15A¹⁵⁷. Other potent inhibitors of EYA2 (NCGC00241225)¹⁵⁸ and PP2A (LB100)^{159,160} are undergoing pre-clinical or clinical testing for a variety of indications including cancer. LB100 is a newly discovered small molecule inhibitor of PP2A currently investigated in phase 1 and 2 clinical trials for the treatment of myelodysplastic syndrome (NCT03886662) and glioblastoma (NCT03027388)¹⁶¹. Interestingly, LB100 in combination with chemo- or radiotherapy¹⁵⁹ as well as immune checkpoint blockade¹⁶⁰ are the focus of growing scientific efforts. Based on our findings, pairing these small molecule effectors with OV_s could constitute rational combination therapies worth investigating.

To validate that the provided shortlist of lead PPase genes are indeed involved in vanadate’s mechanism of action, biochemical analysis of PPase activity in presence of vanadate should be conducted. Since vanadate-mediated potentiation of oncolytic virotherapy is associated not only with enhanced oncolysis and viral growth but also with promotion of a type II inflammatory response, resulting in sharp induction of cytokines like CXCL9 and

CXCL10⁷², an ELISA should be performed to measure cytokine secretion upon PPase silencing and VSV Δ 51 infection. Nevertheless, it is possible that the full effect of vanadate is the result of targeting multiple PPases with viral sensitizing effects simultaneously.

4.4 New horizons for immunotherapies: targeting PPases to boost efficacy

The PPases identified in this screen have the potential to guide the development of novel pharmacoviral regimens or the genetic engineering of OV's capable of tailoring the immune response and improve therapeutic gains. It would be pertinent to test these gene targets in other clinically relevant OV platforms including HSV-1, vaccinia and adenovirus. This would overcome one of the major limitations of pan-PPase inhibitor vanadium-based VSEs being that it prevents growth of DNA viruses including HSV-1 and vaccinia⁷² likely through the inhibition of their viral PPases, H1¹⁶² and ICP34.5¹³¹ respectively.

The selective regulation of PPases to potentiate immunovirotherapy could be achieved by genetically modifying OV's to express short hairpin RNA (shRNA) transgenes against PPases. Zhang et al. have developed a one-two punch approach that pairs the oncolytic activity of conditionally replicative adenovirus with oncogene silencing: shRNA are transcribed during viral replication and are processed by Dicer into siRNAs that silence gene expression through RISC¹⁶³. However, this approach relies on successful viral entry and replication; which may pose a problem since some cancers have responsive innate antiviral defenses^{44,55,56}. Because some of the shortlisted PPase genes are upregulated in cancers (e.g. EYA2 in prostate cancer¹⁶⁴), successful PPase silencing, and consequently potentiation of virotherapy, would thus require significant production of viral transcripts.

Alternatively, PPase activity can be modulated using small molecule inhibitors or RNA interference (RNAi) therapy. As discussed in the previous section, pharmacological

manipulation of PPase activity to benefit virotherapy and other immunotherapies holds promise and the selection of small molecules will likely broaden as the PPase-directed drug hunt evolves. In the same line of thinking, RNAi is also a promising strategy. RNAi is routinely used in laboratory settings to study gene function, but they have recently made an appearance in the clinic. Last year, the FDA approved the first siRNA therapy, Onpattro, for the treatment of polyneuropathy after a successful phase 3 clinical trial¹⁶⁵. Compared to viral engineering, drug treatments have the advantage of pre-conditioning the TME and priming tumor cells for successful OV infection. As Onpattro trailblazes in clinics, important lessons can be learned to design improved second-generation RNA-based therapies. Importantly, the current drawbacks of such interventions include: low success rate of transfection, short life span of RNA duplexes, delivery-associated toxicity and off-target effects¹⁶⁶

Overall, the combination of siRNA and OVs could be an interesting pursuit; whether by co-treatment of siRNAs and OVs or by transgenic expression of shRNAs in OVs. Regardless, the impact of PPase knockdown in healthy tissues and in the various cell populations that form the TME should be verified. Furthermore, in the event that off-target gene silencing occurs in these cells, we must ensure that OVs retain their (i) cancer tropism (ii) safety profiles (iii) ability to induce a durable antitumor defense.

Lastly, we have also identified a series of PPase siRNA that improved viral output without robustly compromising cell viability (**Table 1**). Lead hits based on cytotoxicity and titers include PP1's regulatory subunit PPP1R14B and protein tyrosine phosphatase pseudogene (PTPRV)^{95,120–122}. Designing stable cell lines with specific PPase deletion for cell-based vaccine production could help produce high titers of viruses, improve cost-efficiency of the manufacturing process and prevent shortages of vaccines.

4.5 EGFR inhibitors antagonize vanadate's viral sensitizing effects

In the context of the *reverse screen* strategy with the kinase inhibitor library, our results indicate that, from the 800 compounds tested, 23 abrogated vanadate's viral sensitizing effects (**Figure 5, Table 3**). Nearly half directly inhibit the EGF pathway indicating that vanadate may enhance virotherapy by activating this pathway.

Under normal physiological conditions, signal transduction downstream of epidermal growth factor receptors (ErbBs) is highly pleiotropic and ultimately modulates proliferation, survival and differentiation of epithelial cells¹⁶⁷. The ErbBs family consists of four receptor tyrosine kinases: EGFR (HER1), HER2, HER3 and HER4¹⁶⁷. The EGF axis is often mutated and hyperactive in many forms of cancers^{167,168} and there is a growing body of evidence linking the EGF pathway with IFN and antiviral cascades. For example, during airway epithelial cell infection, EGFR downregulates IRF1 and IFN- λ expression thereby promoting influenza A virus replication¹⁶⁹. In human glioblastoma and carcinoma cell lines, signal flux through the EGFR induces STAT1 expression^{170–172}. Others have found that overexpression of RAS and Raf1 improves VSV-mediated lytic infection because the RAS/MEK/ERK pathway limits expression of IFN α -stimulated genes like MxA¹⁷³. Conversely, the use of small molecule MEK1/2 inhibitor restored type I IFN antiviral response¹⁷³. Also there is evidence suggesting that the EGF axis increases STAT3 phosphorylation upon hepatitis C virus infection in IFN α -treated cells¹⁷⁴ and that phosphorylated STAT3 impairs antiviral defenses^{174,175}. Interestingly, Wu et al., recently discovered that HER2 blocks the STING cascade through AKT1-mediated phosphorylation of TKB1 (S510) thereby shutting down the innate antiviral response¹⁷⁶. This is particularly important because many of the EGFR inhibitors identified as vanadate's antagonists are also HER2 inhibitors (**Table 3**). Therefore, vanadate's ability to enhance

virotherapy might rely on an active HER2-STING pathway to subdue cancer antiviral responses. While unrestrained EGF signalling might confer a survival advantage to cancer cells, these lines of evidence prove that it can be exploited in the context of OV therapy. Furthermore, these findings can be valuable in the context of clinical trials as they could foreseeably be employed as antidotes and help control the activity of vanadium-based viral sensitizers in the event that vanadate-related toxicities are observed.

The combination of vanadate with oncolytic virus VSV Δ 51 promotes a type II IFN gene signature in cancer cells; an observation that may be explained by the increase in STAT-1 phosphorylation, homodimerization and nuclear translocation⁷². These lines of evidence suggest that the EGF cascades exacerbate the antiviral response which, mechanistically, could be complementary to vanadate's mechanism of action. Another avenue by which the EGFR axis potentially contributes to vanadate's enhancement in viral replication is by increasing viral entry. It has been shown that signal transduction through the EGFR pathway leads to LDLR upregulation in murine models of glioblastoma¹⁷⁷. Whether this holds true in other cell models remains to be tested. The phosphorylation pattern and nuclear translocation of STATs should be explored in cells treated with EGFR inhibitors, vanadate and VSV Δ 51 to characterize the intracellular events and the relationship between the EGFR and JAK-STAT axes. Overall, this screen identified kinases that antagonize vanadate's phenotype, and suggests that vanadate-mediated viral sensitization is dependent on the action of upstream kinases of the EGFR pathway.

4.6 Insights into vanadate's mechanism of action

In the present study, we have cross-referenced by means of pathway analysis the top hits of the PPase screen with that of the OICR kinase inhibitor's *reverse screen* to find common

substrates between viral PPases and antagonistic kinases. To summarize, the human PPase siRNA screen uncovered 76 siRNAs that significantly enhanced viral output of VSV Δ 51-fluc by a fold change of 1.5 or more at 48 hpi (**Figure 3**). On the other hand, the kinase inhibitor screen identified 23 compounds that abrogated vanadate's OV potentiation (**Figure 5, Table 3**). By integrating the PPase, kinases and their substrates in Cytoscape¹¹¹, a network is generated highlighting the common substrates shared by PPases and kinases. Overall the data from the *reverse screen*, the PPase siRNA screen and the pathway analysis indicate that eight substrates are likely involved in viral sensitization of 786-O to VSV Δ 51: EGFR, KIT, NTRK1, KDR, SRC, MAPK1, LYN and IKBKB. Four of which are growth factor receptors (EGFR, KIT, NTRK1 and KDR).

Due to its role in converting type I to type II IFN responses^{178,179}, which is analogous to vanadate's mechanism of action, the identification of PTPN6 (SHP1) in the pathway analysis comes as no surprise. Mechanistically, this involves PTPN6 dephosphorylating JAKs, thereby preventing downstream phosphorylation of STAT1¹⁷⁹. The phosphorylation pattern and dimerization of STATs directs gene expression; activated STAT1 homodimers will translocate to the nucleus and bind to the gamma-activating sequences (GAS) whereas activated STAT1-STAT2 heterodimers will preferentially bind to IFN-stimulated response element (ISRE)⁶³. PTPN6 is a PPase that simultaneously increased viral titers and cytotoxicity (**Figure 4, Table 2**). PTPN6 is involved in dephosphorylating the tyrosine residues on EGFR which in return inactivates the receptor¹⁸⁰. As discussed in the previous section, the EGFR axis is involved in diverting the type I IFN response through a process that likely involves STAT1¹⁷⁰⁻¹⁷². Although PTPN6 silencing alone resulted in a sharp decrease in viability, this is likely because of its role

in a plethora of cellular pathways as reflected by its broad phosphatase activity (**Figure 6**). PTPN6 is thus likely one of the PPases targeted by vanadate.

As revealed by the pathway analysis (**Figure 6**), PTPN6 is a central PPase directly involved in modulating the activity of growth receptors EGFR, KIT, NTRK1 and KDR as well as downstream proteins SRC and LYN. EGFR (presented in **section 4.5**), the stem cell growth factor receptor KIT, the nerve growth factor receptor NTRK1 and the vascular endothelial growth factor receptor VEGFR2 (encoded by the KDR gene) are tyrosine kinase receptors that can drive tumorigenesis^{181,182}. These growth factor receptors have overlapping downstream signalling networks. In fact, instigation of signal transduction through these receptors culminates in JAK-STAT, RAS-ERK1/2(MAPK1) circuits, among others, for cell proliferation and survival^{39,181,182}. Whether KIT, NTRK1 and KDR functionally exert viral sensitizing effects through a mechanism analogous to EGF's (e.g. through STING, STAT3 or STAT1 see **section 4.5**) should be investigated. Previous work conducted by our group supports the identification of phosphorylated VEGFR2 as a viral sensitizing candidate in the present HTS. We reported a potentiation in viral replication consistent with a suppression of type I IFN response upon VEGFR2 stimulation³⁹. This was found to be modulated through STAT3 and ERK1/2 phosphorylation resulting in PRD1-BF1 expression, a down-modulator of innate immunity³⁹. Also, the SRC family kinases SRC and LYN were listed as substrates involved in viral sensitization and both regulate STAT3 phosphorylation and activation^{183,184}. For example, STAT3 phosphorylation is dependent on LYN in stem cell growth factor (SCF)-treated bone marrow mast cells¹⁸³. This is particularly important because STAT3 is a down-modulator of antiviral defenses¹⁷⁵. Whether these phosphorylated substrates are exerting their viral sensitizing effects through STAT3 should be tested for example by looking at activation

of STAT3 (western) and gene signature of growth factor-treated or substrate-silenced cells infected with VSV Δ 51 (e.g. assess ISG expression by PCR).

Lastly the phosphorylated IKK2 (encoded by the IKBKB gene) reportedly plays an important role in modulating vanadate's viral sensitization. The inhibitor of NFkB (IkB) kinase subunit B (or simply IKK2) phosphorylates two of IkB's serine residues which in turn liberates and activates NFkB for nuclear translocation and expression of antiviral and proinflammatory genes¹⁸⁵. This piece of evidence is intriguing as it defies the mechanism of action of another VSe, namely the NFkB inhibitor DMF⁷¹.

As vanadate is a broad PPase inhibitor, we cannot exclude the possibility that its viral sensitization is the result of pleiotropic dephosphorylation and consequently the accumulation of several phosphorylated substrates. Future steps should include validating these substrates one by one (and perhaps in combinations) and look at the impact of their gene silencing, their overexpression and the effect of inducing their phosphorylation on OV efficacy. Also, the phosphorylated residues driving the viral sensitizing activity of these substrates should be identified.

4.7 CDK and NAE inhibitors are novel potent VSes

In addition to the *reverse screen*, 786-O cells were pre-treated with the kinase inhibitor drug library alone and infected with VSV Δ 51-fluc. High-throughput titration and measurement of viability were performed at 48 hpi. 23 compounds significantly increased viral titers and simultaneously scored positive *viral sensitizer factors* (**Table 4**). These top hits can be classified into five broad categories: antimetabolic drugs, CDK, JAK or HDAC inhibitors and others. Several have already been explored in the context of virotherapy, but CDK1/2 inhibitor

III, AT7519, Flavopiridol and Pevonedistat are novel VSEs in advanced stages of clinical development and merit consideration (**Supplemental figure 4**).

Antimitotic drugs are a class of chemotherapeutic agents that impairs microtubule kinetics and thus impedes cell division¹⁵. They have already been explored as small molecules enhancers of oncolytic virotherapy. For example, microtubule-destabilizing agents such as colchicine and Vinorelbine (also identified in the HTS) potentiate VSV Δ 51 spread and bystander killing¹²³. On the other hand, Paclitaxel, a microtubule-stabilizing drug, was also found to improve virotherapy, this time in a rodent model of breast cancer with oncolytic Maraba MG-1¹⁸⁶. Because antimitotic drugs like Vinorelbine and Paclitaxel are approved and available on the market, they make promising candidates for pharmacoviral therapeutic strategies. Several clinical trials are exploring such interventions. For example, a study exploring the combination of Paclitaxel with Imlygic® (T-Vec) is currently recruiting breast cancer patients (NCT02779855)¹⁶¹.

Epigenetic modifications reversibly modulate protein expression without altering gene sequences and the process relies on the action of two antagonistic enzymes; histone deacetylases (HDACs) and histone acetyltransferases (HATs)¹⁸⁷. HDAC inhibitors have been shown to enhance viral oncolysis by our group and others^{69,70}. For example, HDAC inhibitors SAHA (Vorinostat) and MS-275 sensitize resistant cancer cells to VSV Δ 51 *in vitro*, *in vivo* and in primary human tumor samples⁶⁹. SAHA, a broad inhibitor of class I and II HDACs (HDAC1, HDAC2, HDAC3, HDAC6) currently approved for the treatment of cutaneous T-cell lymphoma¹⁰⁹ and MS-275, a HDAC1/3 inhibitor indicated for Hodgkin's lymphoma, were not lead viral sensitizers in this screen. This may be because of the concentration (1 μ M) is inferior to that used in these previously published studies⁶⁹. This HTS uncovered broad HDAC

inhibitors CUDC-907, Quisinostat and LAQ824 (**Table 4**). LAQ824 is the most effective of its class, with a robust 2.1 viral sensitizer factor and a fold change in viral titers of 1.7 in Log(VEU/mL). LAQ824 is a more potent HDAC inhibitor than FDA-approved Vorinostat, however, LAQ824 was associated with severe dose limiting toxicities (e.g. atrial fibrillation and death) and no objective responses were reported in a phase I clinical trial involving patients diagnosed with solid tumors¹⁸⁸.

Upon infection, type I IFN are secreted and, in a paracrine or autocrine fashion, bind to IFN receptors at the cell membrane. This results in the phosphorylation of STATs by receptor-bound Janus kinases (JAKs)⁶³. JAK inhibitors, like Ruxolitinib, sensitize head and neck cancer cell lines to VSV Δ 51 infection by downregulating expression of genes involved in virus detection and antiviral response (including *IRF7*, *IRF9* and *OAS1*)¹²⁴. The JAK inhibitors identified in the screen include: AZ-960, Ruxolitinib, AZD1480, Lestaurtinib and AT9283. In preclinical murine models of NSCLC, Ruxolitinib combined with VSV encoding IFN β (VSV- IFN β) marginally prolonged survival relative to VSV-IFN β monotherapy¹⁸⁹. The combination of Ruxolitinib with VSV-IFN β -NIS is undergoing clinical evaluation for the treatment of endometrial cancer (NCT03120624) as well as myeloma, leukemia and lymphoma (NCT03017820)¹⁶¹. As reflected in its modest viral sensitizer factor, Lestaurtinib does not enhance cytotoxicity when combined with virus; the viability of 786-O cells treated with the drug alone or with VSV Δ 51 were 52% and 48% respectively. Today, only two JAK inhibitors are approved in the clinic: Ruxolitinib (JAK1/2/3, TYK2 inhibitor) and Tofacitinib (JAK3 inhibitor)¹⁹⁰. Tofacitinib was not a primary hit likely because JAK3 is a kinase bound to the gamma chain of type-I cytokine receptors unlike JAK1/2, which are associated with type-II IFN receptors¹⁹⁰. Broad inhibitors may cause off-target effects; in the case of AZD1480

(JAK1/2/3 inhibitor), a phase I clinical trial was terminated early because of neuropsychiatric dose limiting toxicities in patients with solid tumors¹⁹¹. Novel selective JAK2 inhibitor AZ-960 has not yet been tested in the clinic but has shown promising pre-clinical results in patient-derived acute myelogenous leukemia cells¹⁹².

Inhibitors of cell cycle proteins were identified as novel viral sensitizers. Cell division is a highly regulated process characterized by sequential activation of kinases and subsequent phosphorylation of substrates involved in DNA replication and mitosis. The mitotic kinome is involved in unlocking cell cycle checkpoints and includes, among others, the Aurora family kinases as well as CDKs¹⁹³. Two lead compounds, Hesperadin and AT9283, were found to broadly target Aurora A and or AuroraB (**Table 4**). AuroraA is involved in the G2/M checkpoint by activating CDK1 whereas AuroraB is a member of the chromosomal passenger complex and supports cytokinesis¹⁹³. Because of their roles in cell division, Aurora proteins like AuroraA are considered tumorigenic; in fact, Aurora kinases have been explored as therapeutic targets for several cancers including breast cancer and their expression are recognized as an indicator of prognosis¹⁹⁴. Another group of cell cycle regulators with prominent roles in cancer biology are CDKs. Flavopiridol is a first generation pan-CDK inhibitor and since its discovery, it has been studied in more than 60 clinical trials with some therapeutic benefit observed in haematological cancers¹⁹³. Mechanistically, Flavopiridol induces G1 and G2 cell cycle arrest¹⁹⁵. However, because of toxicities, derivatives were synthesized such as AT7519 which is currently undergoing phase II clinical testing¹⁹³. Unlike Flavopiridol and AT7519, CDK1/2 inhibitor III is a more selective inhibitor but has not been explored in clinical settings. These small molecule CDK inhibitors were identified in the HTS screen and generated some of the most potent viral sensitizer factors (second to the HDAC

inhibitors). There is a growing body of evidence that supports the implication of the cell cycle clock in antiviral immunity^{196–198}; several viruses including the human immunodeficiency virus (HIV)¹⁹⁶ and coronavirus¹⁹⁷, hijack the cell cycle to promote their viral replication and spread. And recently, a screen of small molecule cell cycle modulators revealed that a G2/M cell cycle arrest in human pancreatic ductal adenocarcinomas potentiates VSVΔ51 growth¹⁹⁸. Mechanistically, these compounds (paclitaxel and colchicine among others) exerted diminished type I IFN production and also impaired ISG expression, a consequence the authors attribute to a shutdown of host transcription during the G2/M phase¹⁹⁸. Whether the cell cycle modulators identified in the present screen exert their viral sensitizing actions by promoting a G2/M cell cycle arrest warrants investigation.

Protein turnover is essential to maintain homeostasis and impacts cellular functions. It is achieved by tightly regulating gene expression and through post-translational modifications. An example of the latter is the covalent addition of ubiquitin, which tags proteins for proteasomal digestion through the canonical ubiquitin-proteasome system¹⁹⁹. Mounting evidence support the existence of analogous mechanisms that rely on the action of ubiquitin-like proteins such as neural cell developmentally downregulated 8 (NEDD8). In a process often referred as “neddylation”, NEDD8 covalently binds to a cullin-RING ligase (CRL) to initiate its ubiquitin ligase function^{200,201}. CRL substrates include several proteins including some involved in the cell cycle and survival²⁰². Initiation of this signalling cascade relies on the activation of NEDD8 by the NEDD8-activating enzyme (NAE)^{202,203}. Since CRL substrates have implications in oncogenesis, it led to the development of novel cancer targeted strategies including Pevonedistat, an inhibitor of NAE²⁰². It was shown to cause apoptosis and prevent nuclear translocation of p65 and p52 thereby blocking NFκB signalling in a lymphocytic

leukemia B cell model²⁰⁴. This is particularly relevant because NFkB controls expression of type I IFN as well as other ISG¹⁵⁶ and suggests that this compound potentially exerts its viral sensitizing actions via NFkB. With 23 currently ongoing clinical trials, Pevonedistat is being tested as a single agent and in combination with chemotherapeutics for various indications including leukemia, NSCLC and myelofibrosis (ClinicalTrials.gov)¹⁶¹. Moreover, it holds an orphan drug designation from the Food and Drug Administration for the treatment of acute myeloid leukemia²⁰⁵. Thus far, completed clinical testing revealed overall drug tolerability and evidence of a modest therapeutic benefit as a single agent but also an improved objective response rate when combined with the chemotherapeutic drug azacitine²⁰⁶. Pevonedistat robustly enhanced virotherapy with a fold change of 1.9 in Log(VEU/mL). The impact of neddylation inhibition on NFkB signaling in the context of infection should be explored.

A secondary validation screen on AT7519, Flavopiridol, CDK1/2 inhibitor III and Pevonedistat in the same 786-O model was conducted using a wider concentration range. At a nanomolar concentration (320nM), Pevonedistat increased viral titers by nearly 3 Logs (**Figure 10, Figure 11**). Furthermore, a very broad effective concentration range was observed for this compound which is indicative of a wide therapeutic window. Most notably, Pevonedistat combined with VSVΔ51 results in a synergistic 99.5% reduction of LD₅₀ (**Table 5**). However, Pevonedistat did not robustly enhance viral infection in *ex vivo* 76-9 and CT26wt cores. Instead, CDK1/2 inhibitor III was the most potent in tumor cores with up to a 2-log increase in viral titers in the CT26wt model (**Figure 13**). Infection in VSe-treated cores is heterogeneous between models (76-9 and CT26wt) but also between drugs. Concentrations were picked based on the dose-response data (**Figure 11**) which was conducted in 786-O.

Perhaps more concentrations should be tested *ex vivo* and dose-responses should be performed in other cell lines.

Overall, the murine hepatocyte model and the *ex vivo* core experiments indicate that these novel sensitizers do not enhance viral infection in normal tissues and that VSV Δ 51 retains its cancer tropism (**Figure 12, Figure 13, Figure 14**). The low concentration (1 μ M) selected for the kinase inhibitor panel is an important consideration since it may increase the number of false negatives. However, higher concentrations entailed a risk of toxicity and higher rate of false positives. Since we have previously found that a minimum of a four hour drug pre-treatment with vanadate is most efficacious at enhancing viral titers using other viral sensitizers⁷², we have arbitrarily pre-treated cells with kinase inhibitors for four hours as well. However, it would be important to test different incubation times, and also determine the kinetics of VSV Δ 51 infection by conducting single (high MOI) and multistep (low MOI) growth curves. This would reveal if viral replication and/or spread is enhanced. To determine the impact of these findings on the treatment of human malignancies, these novel Vses should be tested in human patient biopsies and matched normal tissues, but also in animal models and in combination with other clinically relevant OV platforms including vaccinia and HSV-1.

4.8 Evaluation of van-OV immunotherapy regimens in rodent 76-9

rhabdomyosarcoma model

Previous work conducted in our lab has revealed improved survival rates in aggressive *in vivo* models of breast carcinoma (4T1), glioma (DBT) and colon carcinoma (CT26wt) when mice were treated with the vanadate and VSV Δ 51 dual therapy⁷². In hopes of developing novel immunotherapeutic strategies for sarcomas, we sought to explore the combination of VSV Δ 51 with vanadium-base viral sensitizers because (i) VSV Δ 51 infects and kills sarcoma cells¹²⁵

and (ii) vanadate has been shown to improve VSV Δ 51 spreading and cytotoxicity in *in vitro* models of sarcoma¹⁰³ (manuscript also in preparation).

The first pilot experiment demonstrated improved tumor control in the VS+VSV cohort but relative to the VSV monotherapy, VS+VSV ultimately failed to enhance survival rates (**Figure 15**). Furthermore, following a tumor re-challenge, two of the three mice from the dual therapy test group relapsed despite expressing MHC class I (**Supplemental figure 5**). We thus predicted that tumor re-development was caused by the absence of a tumor-specific adaptive immunity through the inhibition of CD8 T cell by checkpoint protein expression. This hypothesis was reinforced by results from the B16F10 model where in sharp contrast, the triple combination of anti-CTLA4 + VS + VSV successfully delayed tumor progression and improved survival rates compared to the monotherapy and VS+VSV cohorts (**Supplemental figure 6**).

In the 76-9 model, increased tumor control and cures (100%) in mice treated with the VS+VSV+anti-CTLA4 triple combination therapy were observed (**Figure 16**). However, VS resulted in side effects that include skin lesions (scab) on tumors, weight loss and death. We next sought to test the triple combination treatment with the revised 50mg/kg VS doses to see if similar therapeutic gains can be achieved without side effects. However, when the triple treatment schedule was repeated, side effects persisted: one mouse succumbed post treatment and scabs were observed on tumors of mice treated with VS. The triple combination therapy delayed tumor progression but in the long run, it resulted in survival rates comparable to that of the VS, CPI (100 μ g) and the VSV +VS cohorts (**Figure 18**). Relative to the VSV+ 50mg/kg VS dose (**Figure 18**), the 100mg/kg dose results in a steeper weight drop but more cures are achieved in the VSV+100mg/kg cohort (**Figure 16**). A comparison of **Figure 16** and **Figure**

18 reveals that an anti-CTLA4 dose of 200 μ g is more efficient at prolonging survival (80% cures) than 100 μ g (20% cures) but combining VSV+CPI (100 μ g) synergistically generate 60% cures. There may be benefits in using treatment schedules with lower doses of CPI – if equivalence in efficacy is achieved – because they are costly and immunological adverse events can ensue²⁰⁷. In fact, these antibodies are worth nearly 4000 times their weight in gold and depending on the treatment schedules, the cost to treat a single patient can be as high as a million USD which is economically unsustainable²⁰⁸. Nevertheless, the positive response of 76-9 to anti-CTLA4 is surprising given that clinical studies have not successfully demonstrated therapeutic benefits in using anti-CTLA4 for the treatment of sarcoma^{29,209}. This observation attests for the heterogeneous nature of the disease.

There are a few experimental distinctions between the *in vivo* 76-9 experiments of **Figure 15** and **Figure 16**: in the first, the three treatments were administered over the span of seven days whereas in the second, they occurred within five days. This may impact the efficacy of the treatment regimen because an antiviral immune response may be developed past day five²¹⁰. Also, it is possible that this compressed timeline causes more toxicities owing to vanadium pharmacokinetics. Furthermore, the initial tumor size at time of treatment is potentially influencing the therapeutic efficacy. In **Figure 15-16** and **Figure 18** mean tumor volumes at the start of treatment were 102, 48 and 89 mm³ respectively.

Whether scabs are conducive to tumor regression and cures remains to be determined. Scabs on tumors upon van/OV treatment were observed in previous models and led to positive outcomes⁷². Interestingly, in a vaccinia virus (vvDD) phase I clinical trial, intratumoral virus injection of cutaneous tumors resulted in necroses, an ulcerated, necrotic and inflamed lesion associated with vvDD replication²¹¹. Necroses have been observed in rhesus macaques

studies of oncolytic vaccinia virus²¹², and in immunocompromised subjects receiving smallpox vaccination²¹³. Since VS increases viral replication (**Figure 15**) and skin lesions were predominantly observed in the cohorts receiving VS and VSVΔ51, we can thus predict that the formation of scabs is consistent with VSVΔ51 replication within the TME and virus-induced tissue destruction.

While the triple combination with 100mg/kg VS was highly effective but led to toxicity, the triple combination therapy with 50mg/kg of VS did not enhance survival. Intriguingly, improvements in tumor control at early timepoints were observed in both cases. T cell exhaustion in the 50mg/kg triple combination regimen could explain this phenomenon but a more detailed analysis of PDL1 and other exhaustion markers on TILs within the recurrent tumors would be necessary to make this claim. A way of overcoming T cell dysfunction is with antibodies against PD1/PDL1, but conceptually the addition of a second CPI is not ideal because it likely involves more side-effects and higher risks of severe complications, not to mention costs to the healthcare system. Also, the use of such strategies is hard to justify considering that in our experiments, anti-CTLA4 (200μg) and VSV + anti-CTLA4 (100μg) generated cures in over 60% of mice (**Figure 16, Figure 18**). It is evident however that the present 76-9 rhabdomyosarcoma model behaves differently to the van/OV treatment regimen than the previously tested CT26wt model (colon carcinoma, Balb/c mice) where 20% cures without lethal toxicities were achieved, and an anticancer adaptive immune response was induced⁷². Study of the underlying reasons for these differences in responses and toxicities would be warranted and could lead to clinical biomarkers that would help identify patients more likely to safely benefit from vanadium-OV regimens. For example, the expression profile

of vanadate's viral sensitizing PPases (e.g. PTN6, EYA2, PP1 etc.) could be an indicator of vanadium-based pharamacoviral therapy efficacy.

5. Conclusion

The development of oncolytic virotherapies has made significant strides in the last decades and show promise in some pre-clinical and clinical studies. But the heterogeneous nature of tumors is a major hurdle that limits the efficacy of these biotherapeutics. Our lab has discovered small molecule enhancers of virotherapy such as the pan-phosphatase inhibitor vanadate. Using a kinase inhibitor library of 800 compound and a human phosphatase siRNA library targeting 303 phosphatase genes, we have uncovered potential PPases that could explain the mechanism of action of vanadate. We provide several lines of evidence supporting the dependence of vanadate on an active EGF pathway in order to exert its viral sensitization. Also, we have shortlisted ten phosphatase gene candidates that, once silenced, improve cytotoxicity and viral titers of VSV Δ 51. Pathway analysis identified eight potential kinase-phosphatase substrates that, if phosphorylated may drive vanadate's viral sensitization.

Despite having achieved therapeutic benefits in murine models of breast, colon and brain cancer with the combination of vanadate and VSV Δ 51⁷², we have not observed a significant improvement in overall survival rates when the dual therapy was tested in a murine model of rhabdomyosarcoma albeit some evidence of tumor control was found at earlier timepoints. Treatment regimens incorporating the immune checkpoint inhibitor anti-CTLA4 alone and in combination with VSV were notably also efficient in our 76-9 rodent model of rhabdomyosarcoma.

Lastly, we have discovered that CDK and NAE inhibitors improve OV efficacy in *in vitro* and *ex vivo* models of colon and renal cell carcinomas as well as rhabdomyosarcoma and may present new therapeutic strategies for this latter type of

malignancy. Importantly, these novel viral sensitizers do not increase VSV Δ 51 infection in normal tissues indicating that OV enhancement is selective to cancer cells.

How vanadate influences the various immune cell populations to potentiate OVs and mount an anticancer defense remains to be elucidated, but overall, this project answers several key signalling questions. These findings have significant implications for the development of novel oncolytic virus-based interventions.

6. References

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7. Collaborations and contributions

Dr. Mohammed Selman contributed to the acquisition of the IVIS data presented in **Figure 15**.

Samriti Birdi assisted in generating and analyzing the flow cytometry data shown in **Supplemental figure 5**.

Anna Jirovec, Michael Phan, Naveen Haribabu and Nouf Alluqmani helped run the HTS.

Dr. Jean-Simon Diallo and Dr. Rozanne Arulanandam helped with experimental design and thesis editing.

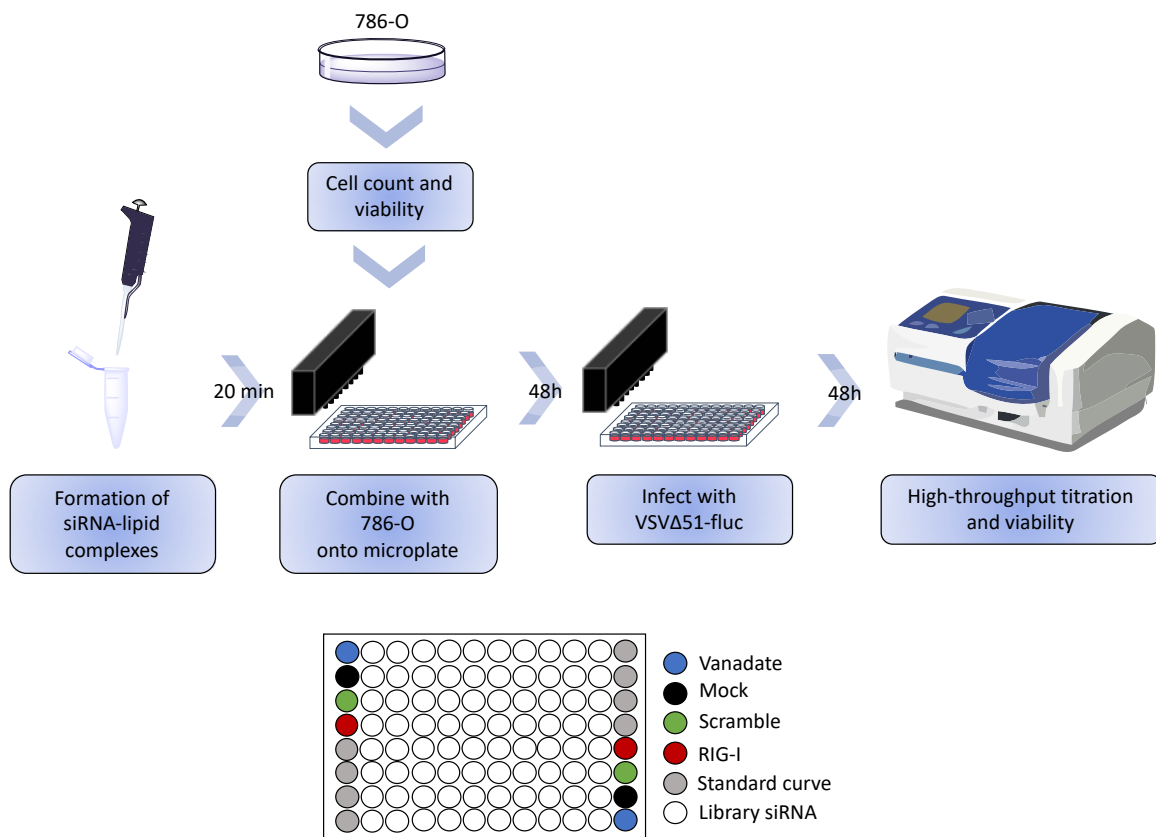
Andrew Chen helped perform the *in vivo* manipulations (injections, caliper measurements), virus propagation and purification.

Dr. Julien Yockell-Lelievre provided guidance for the OICR kinase inhibitor screen and the OICR drug library was generously provided by Dr. William Stanford's lab at the Ottawa Hospital Research Institute.

Adrian Pelin from Dr. John Bell's lab conducted the pathway analysis depicted in **Figure 6**.

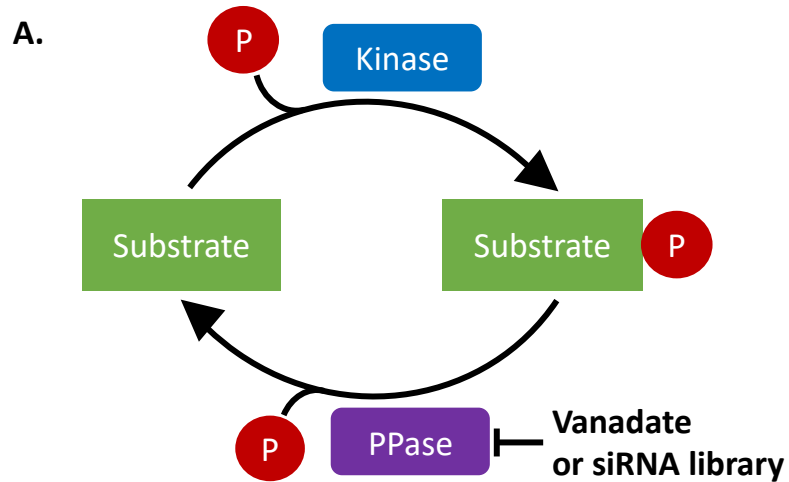
Dr. Morgan Fullerton and Conor O'Dwyer provided the primary hepatocytes for **Figure 14**.

8. Appendix: supplemental figures



Supplemental figure 1. Human PPase siRNA HTS workflow and plate layout

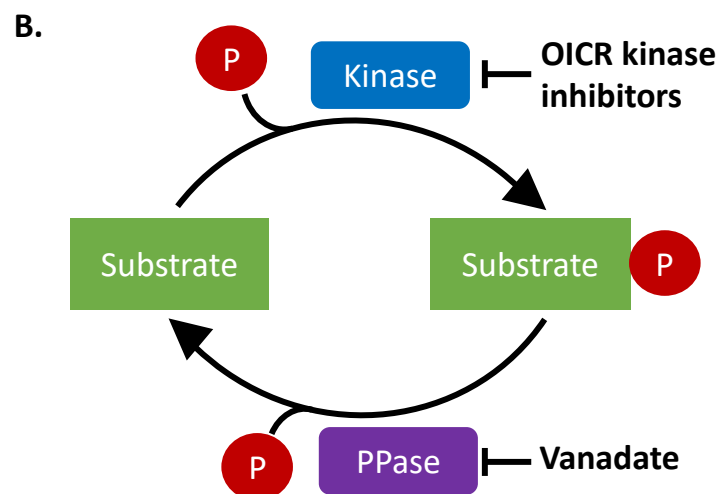
Visual depiction of the siRNA HTS. Graphics were generated on ChemDraw Prime 15.1. Plate map indicates location of controls and samples. The standard curve wells were designated space for the loading of known VSV Δ 51-fluc plaque forming units (PFUs) for high-throughput titration.



1. Human PPase siRNA HTS

PPase of interest:

Enhances viral replication and oncolysis when silenced



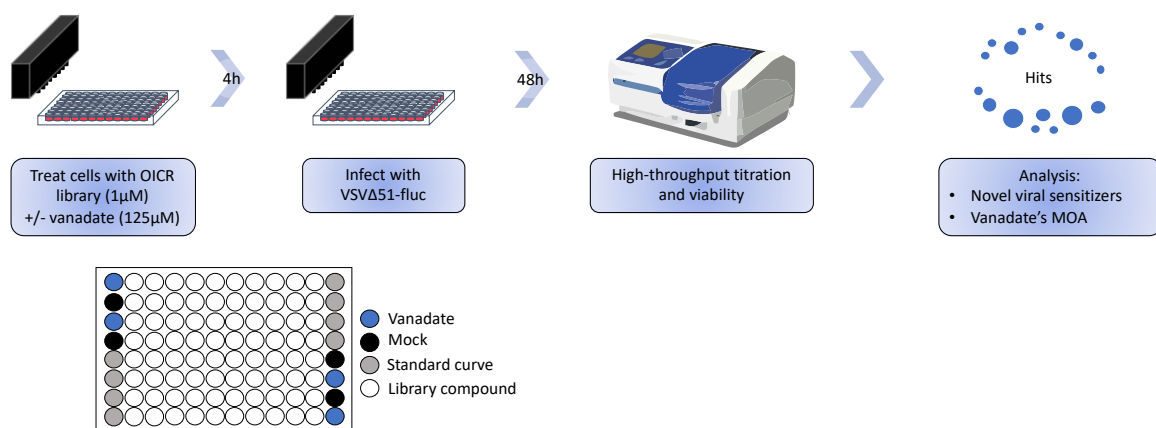
2. OICR *reverse screen* strategy

Kinase of interest:

Limits viral replication and oncolysis when inhibited

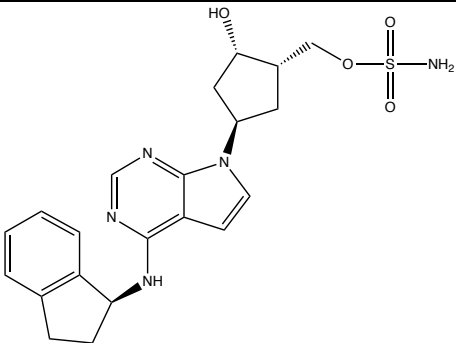
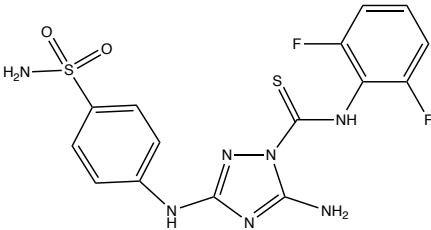
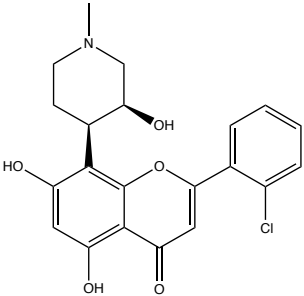
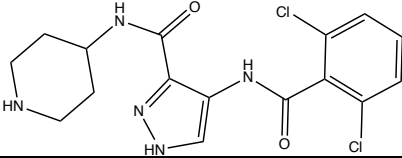
Supplemental figure 2. Overview of the HTS strategies.

A. The primary hits for the human PPase siRNA screen were PPases that once silenced, enhanced VSV Δ 51 infection and oncolysis. This screen uncovered possible PPases involved in vanadate's mechanism of action. **B.** The lead compounds for the reverse screen strategy were kinase inhibitors that prevented viral sensitization by vanadate. 786-O cells were co-treated with kinase inhibitors (1 μ M) and vanadate (125 μ M) prior to infection with VSV Δ 51.



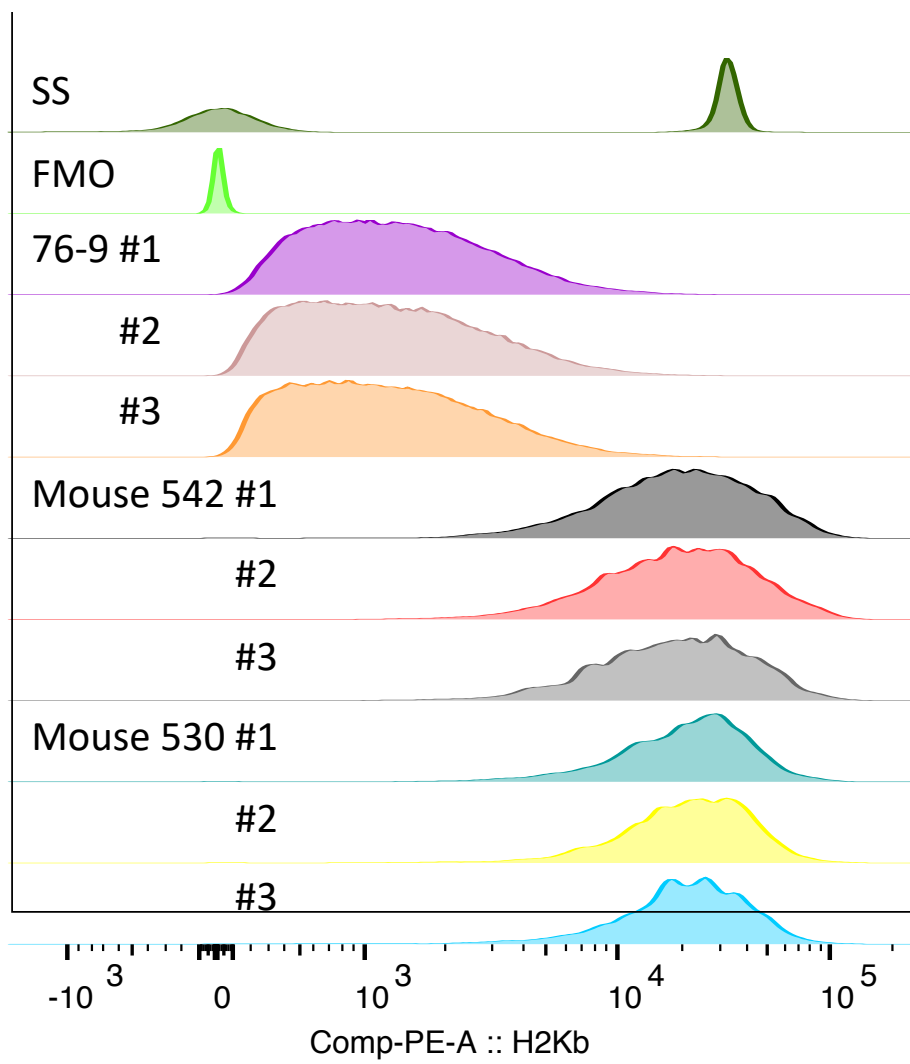
Supplemental figure 3. Kinase inhibitor HTS workflow and plate layout

Visual depiction of the OICR HTS. Graphics were generated on ChemDraw Prime 15.1. The plate map indicates the location of controls and tested library drugs. The standard curve wells were reserved for loading of known VSV Δ 51 PFUs for high-throughput titration.

Name	Structure	Molecular formula	Molecular weight (Da)
Pevonedistat (MLN4924)		C ₂₁ H ₂₅ N ₅ O ₄ S	443.519
CDK1/2 inhibitor III		C ₁₅ H ₁₃ F ₂ N ₇ O ₂ S ₂	425.436
Flavopiridol (Alvocidib)		C ₂₁ H ₂₀ ClNO ₅	401.840
AT7519		C ₁₆ H ₁₇ Cl ₂ N ₅ O ₂	382.245

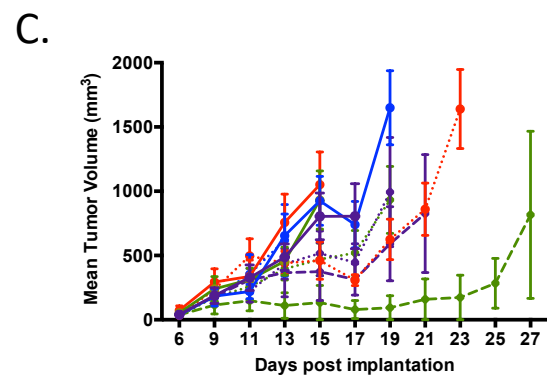
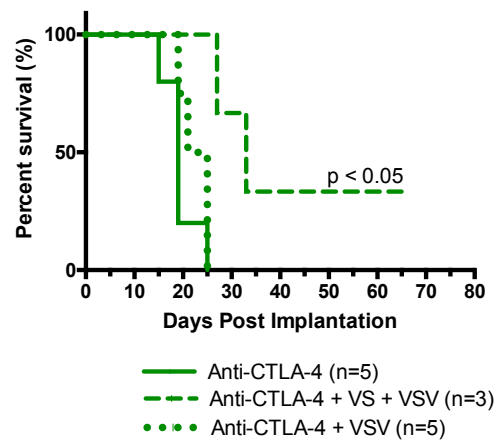
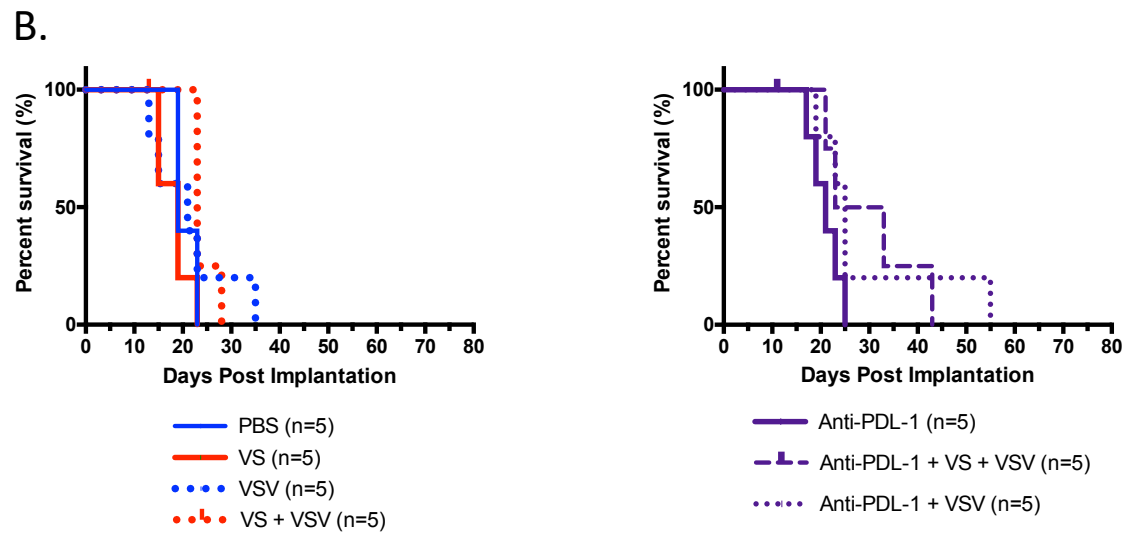
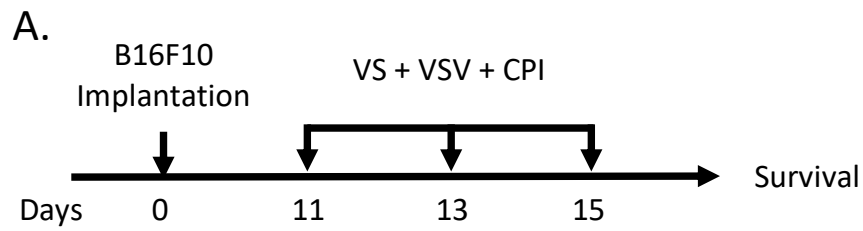
Supplemental figure 2. Structure and characteristics of novel viral sensitizers

Four novel viral sensitizers were identified using the OICR kinase inhibitor drug library. These compounds are cell cycle modulators or effectors of the NEDD8 degradation pathway. Compound structures were generated on ChemDraw Prime 15.1.



Supplemental figure 3. 76-9 tumor MHC class I expression from relapsed mice

Two of the three mice (#542 and 530) from the VS + VSV Δ 51 test group re-developed tumors post 76-9 re-challenge (**Figure 15**). On day 131, the relapsed mice were euthanized, and their tumors were analyzed for MHC class I expression by flow cytometry. 1E6 cells were stained for viability, CD45-BV786 and H2kb-PE. Controls include a single stain (SS) for compensation and a fluorescence minus one (FMO). As a reference, in vitro 76-9 cells were also assessed. Cells were gated on size (FSC-A), granularity (SSC-A), viability of singlets, and CD45-. MHC class I expression was measured on the non-hematopoietic cell population. N = 3. The analysis and data acquisition were performed by Samriti Birdi.



Supplemental figure 4. Effect of the van/OV regimen and immune checkpoint blockade combination in B16F10 melanoma model

A. 5E5 murine melanoma cells (B16F10) were implanted subcutaneously in C57BL/6 mice. 11, 13 and 15 days post implantation (DPI) vanadyl sulfate (VS; 100mg/kg) or vehicle (PBS) was administered intratumorally (i.t.). 4 hours post-treatment, VSV Δ 51-fluc (1e8 PFU) was injected IT as well as checkpoint inhibitor anti-CTLA4 and anti-PDL-1 (checkpoint inhibitor; CPI, 250 μ g) intraperitoneally. **B.** Kaplan-Meier survival curves for controls and combination therapy. Censored due to toxicity: one mouse from the anti-PDL1+VS+VSV cohort and one from the VS+VSV succumbed on day 11 and 13 post injection. Log-Rank test reveals that the van/OV regimen combined with anti-CTLA4 prolongs survival over PBS ($p = 0.0123$), anti-CTLA4 ($p = 0.0116$) and VS ($p = 0.0120$) alone. **C.** Tumor progression over time. Curves are terminated once the first mouse reaches end-point. $N = 3-5$. Error bars represent SEM.

9. Curriculum Vitae

Education

Bachelor of Science (BSc)

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Publications

Published in the International Journal of Cancer

Le Boeuf, F. *et al.* Oncolytic maraba virus MG1 as a treatment for sarcoma. *Int. J. Cancer* **141**, 1257–1264 (2017).

Published in Molecular Therapy

Selman, M. *et al.* Multi-modal potentiation of oncolytic virotherapy by vanadium compounds. *Mol. Ther.* **26**, 56–69 (2018).

Published in Science Translational Medicine

Selman, M. *et al.* Dimethyl fumarate potentiates oncolytic virotherapy through NF- κ B inhibition. *Sci. Transl. Med.* **10**, eaao1613 (2018).

Published in Biometals

Bergeron, A. *et al.* Enhancement of oncolytic virotherapy by vanadium(V) dipicolinates. *BioMetals* **32**, 545–561 (2019).

Poster presentations

Bergeron A, Selman M, Chen A, Tzelepis F, Arulanandam R, Abdelbary H, Werier J, Crans D and Diallo JS. Potentiating oncolytic virotherapy in sarcoma using vanadium-based compounds.

- BioCanRx Summit for Cancer Immunotherapy, June 25-28, 2017, Ottawa-Gatineau, Canada.
- Ottawa Hospital Research Institute (OHRI) Research Day, November 7, 2017, Ottawa, Canada.

- Keystone's Cancer Immunotherapy: Combinations, March 23-27, 2018, Montréal, Canada.
- 1st Annual Interdisciplinary Student Research Conference in Healthcare (ISRCH), April 7, 2018, Ottawa, Canada.
- 11th International Biometals Symposium – Biometals 2018, July 15-19, 2018, Ottawa, Canada.
- BioCanRx Summit for Cancer Immunotherapy, October 27-30, 2018, Banff, Alberta.
- Connective Tissue Oncology Society (CTOS) 2018, November 14-17, 2018, Rome, Italy.

Oral presentations

Bergeron A, Selman M, Chen A, Chen A, Tzelepis F, Arulanandam R, Abdelbary H, Werier J, Crans D and Diallo JS. Améliorer les virus oncolytiques avec le vanadate. University of Ottawa's Three Minute Thesis (3MT), March 13, 2018, Ottawa, Canada.

Bergeron A, Selman M, Chen A, Tzelepis F, Arulanandam R, Abdelbary H, Werier J, Crans D and Diallo JS. Improving oncolytic virotherapy using vanadium-based compounds in sarcoma. Hans K. Uthoff Research Day, April 26, 2019, Ottawa, Canada.

Research positions

Research Assistant **June 2013 – August 2015**

University of Ottawa, Department of Cellular and Molecular Medicine | Ottawa, ON
Principal Investigator: Dr. Qiao Li, PhD

Research Assistant **May 2016 – July 2017**

Ottawa Hospital Research Institute | Ottawa, ON
Principal Investigator: Dr. Jean-Simon Diallo, PhD

Honour's Student **September 2016 – April 2017**

University of Ottawa and Ottawa Hospital Research Institute | Ottawa, ON
Principal Investigator: Dr. Jean-Simon Diallo, PhD

Master's Student **September 2017 – September 2019**

University of Ottawa and Ottawa Hospital Research Institute | Ottawa, ON
Principal Investigator: Dr. Jean-Simon Diallo, PhD

Awards and accolades

- Undergraduate Research Scholarship, University of Ottawa, **2013-2014**
- National Essay Contest by *French for the Future*, University of Ottawa, **2013**

- Admission Scholarships – Undergraduate Studies, University of Ottawa, **2013-2017**
- Dean’s Honour List, University of Ottawa, **2013-2017**
- Undergraduate Student Research Award, NSERC, **2015**
- 2016 BioCanRx Summer Studentship Award, BioCanRx, **2016**
- Gold Award – Best Overall Seminar Presentation, Ottawa Hospital Research Institute’s summer student seminar series, **2016**
- Admission Scholarship – Graduate Studies, University of Ottawa, **2017-2018**
- Recipient of the Roger Guindon Scholarship Fund, University of Ottawa, **2017**
- Recipient of the Hans K. Uhthoff Graduate Fellowship, University of Ottawa, **2017-2018**
- 1st Place Graduate Poster Presentation, Interdisciplinary Student Research Conference on Healthcare, University of Ottawa, **2018**
- 3rd Place Graduate Poster Presentation, 11th International Biometals Symposium, **2018**
- Excellence Scholarship – Graduate Studies, University of Ottawa, **2018-2019**
- Queen Elizabeth II Graduate Scholarships in Science and Technology (QEII-GSST), University of Ottawa, **2018-2019**