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(57) Abstract: The present disclosure provides for a method for treating a cancer, comprising administering to a subject in need thereof an oncolytic vaccinia virus and a tyrosine kinase inhibitor. Further disclosed herein is a method for treating a cancer, comprising administering to a subject in need thereof an oncolytic vaccinia virus and an adenosine receptor blocker.

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COMBINATORIAL STRATEGIES TO ENHANCE VACCINIA VIRUS IN VITRO AND IN VIVO ONCOLYSIS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority to, and the benefit of, U.S. Provisional Application No. 63/459,363 filed on April 14, 2023, the disclosure of which is hereby expressly incorporated by reference herein in its entirety.

BACKGROUND

The global incidence of Renal Cell Carcinoma (RCC) is increasing, with an estimated 431,000 new cases per year and almost 180,000 deaths. North America carries the highest burden with 10.9 new cases per 100,000, followed by western Europe and Australia/New Zealand. In the United States, RCC is the ninth most common malignancy, responsible for 79,000 new cancer cases and 14,000 deaths. While localized RCC is amenable to curative resection, up to half of patients undergoing surgery have local or distant recurrences, causing significant suffering to innumerable patients and their families. Advances in the understanding of RCC pathogenesis, including the role of VHL-HIF axis, increased tumor angiogenesis, and the critical role of the tumor microenvironment (non-immune and immune), have led to the development of more effective (approved) treatment options. While these advances have made a positive impact in progression free and overall survival, most patients eventually progress and succumb to refractory disease.

While RCC can be cured in most cases by surgery when diagnosed and treated early, renal cell cancer recurs in about half of those patients. Due to its aggressive nature, spread to vital organs, and the resistant nature of the cancer, RCC is a fatal disease when advanced. Many patients with metastatic RCC patients eventually stop responding to treatment and succumb to their disease. Therefore, there is an urgent need to find new strategies to overcome resistance and cure renal cell cancer.

The methods disclosed herein address these and other needs.

SUMMARY

In accordance with the purposes of the disclosed materials and methods, as embodied and broadly described herein, the disclosed subject matter, in one aspect, relates to vaccinia virus and methods of using thereof.

Thus, in one example, a method of treating a cancer is provided, including administering to a subject in need thereof an oncolytic vaccinia virus and a tyrosine kinase inhibitor.

In a further example, a method of treating a cancer is provided, including administering to a subject in need thereof an oncolytic vaccinia virus and an adenosine receptor blocker.

Additional advantages will be set forth in part in the description that follows, and in part will be obvious from the description, or may be learned by practice of the aspects described below. The advantages described below will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying figures, which are incorporated in and constitute a part of this specification, illustrate several aspects described below.

FIGS. 1A-1B show effects of JX-594 and cemiplimab in in advanced clear cell RCC. (1A). Waterfall plot of best responses (by RECIST 1.1) in treated patients. (1B). Spider plot showing changes in the sum of target lesions over time.

FIGS. 2A-2F show in vitro effects of oncolytic vaccinia virus in RCC. JX-594 induced cytotoxicity in human 786-0 (2A), A498 (2B), Caki-1 (2C), ACHN (2D), and murine (RENCA, mJX-594 2E) cells. Cell growth was determined using (xCELLigence assay). Bar graphs represent average (5 replicates +/- SD) cell growth, relative to controls. * $p<0.001$ vs. controls. 2F. JX-594 (786-0, ACHN) and mJX-594 (RENCA) viral replication at 24, 48, 72, 96, and 120 hours.

FIGS. 3A-3F show RPPA (functional proteomics) analysis of 786-0 cells treated with JX-594. Cells were treated with JX-594 (MOI=0.1); cell lysates were obtained at 48 hours for RPPA analysis (MD Anderson RPPA core), and data were analyzed as previously reported. (3A). Heat map showing differences in protein expression between controls (upper rows) and JX-594 treated cells (lower rows). Significantly up and down regulated proteins were grouped by cell cycle (3B), apoptosis (3C), necrosis (3D), autophagy (3E) and stress responses (3F). Bars represent fold-change in selected proteins in JX-594-treated relative to untreated 786-0 cells. Bioinformatics analysis was performed as reported.

FIG. 4 shows that generation of adenosine in the tumor microenvironment leads to the suppression of multiple immune subsets. Arrows indicate increased expression or activation. T bars indicate inhibition or reduced activity. Figure is taken from: *Int. J. Mol. Sci.* 2018, *19*(12), 3837; https://doi.org/10.3390/ijms19123837.

FIGS. 5A-5G show in vitro effects of vaccinia virus on the adenosine pathway in human RCC. A. CD73 expression was assessed in JX-594 infected 786-0 cells (MOI=0.1 for 72 hours) using anti-CD73 antibodies (Abcam). I: Mock treatment; II: JX-594 infected cells. B. CD73 expression was quantitated by assessment of mean fluorescence intensity (MFI) of triplicate samples. AU=Arbitrary units. C, D: Methods were similar as in A and B, using anti CD39 antibodies (Abcam) E. Effects of JX-594 on ATP release. 786-0 cells were treated with JX-594 (MOI=0.1 and 1) for 72 hours. Extracellular ATP was measured from supernatants using an ATP detection Kit (Thermo Fisher Scientific). CD39 expression in JX-594 or mock infected cells.. F. Effects of A2AR inhibition (SCH58261, 100 nM) on viral replication. G. Effects of SCH58261 on JX-594 induced viral oncolysis. p NS between JX-594 vs A2Ari+JX594 at comparable time points. Bars represent average percent viability (triplicate experiments) compared to mock treated cells.

FIGS. 6A-6C show effects of MV-muPA on the tumor microenvironment. (5A). MV-muPA delays in vivo murine colon cancer (CT26) progression in immunocompetent mice. CT-26 bearing mice were treated with either IV (tail vein) PBS or MV-muPA ($1.5x10^6$ $TCID_{50}$, x 3), as reported . Statistical differences (p value) were analyzed at study day 23. (5B). Characterization of inflammatory infiltrates in tumors resected at day 5 by flow cytometry. (5C). IHC staining of CD8, CD39 and CD73 in virus treated and untreated (Ctrl) tumors. Scalebar:100 μm.

FIG. 7 shows mJX-594 engineered to express GM-CSF, as well as TK gene disruption and GFP.

FIGS. 8A-8G show in vivo effects of mJX-594 on murine renal cancer immune microenvironment. BALB/C mice were implanted with RENCA cells and received mock treatment. or mJX-594 ($1x10^7$ PFU x 3) via tail vein when tumors reached 5 mm. Tumor volumes were followed in control (6A) and virus (6B) treated mice. 6C, 6D. Additional RENCA bearing mice (n=3 per group) were treated with mock or mJX-594 $1x10^7$ PFU x 2) *when tumors reached 7-8 mm* (~ 200 mm3). Tumors were removed at day 5 of treatment for flow cytometry analysis of CD73, CD3, CD4, CD8, and PD-1. Analysis was performed using FlowJo (v10.8.1, BD Biosciences). 6C. Analysis of CD4, CD8, and PD-1 expression on

$CD3^+CD73^-$ T cells. 6D. Using the algorithm embedded in FlowJo, a t-SNE analysis of CD3, CD4, CD8, PD-1, and CD73 expression (gated on live cells) in control and treated groups in the concatenated file was generated using the following parameters – Iterations: 1000, Perplexity: 30, Learning configuration: Auto (opt-SNE), Learning rate: 7147, Algorithm: Barnes-Hut. 6E-6G: IHC analysis of CD8 (6E), CD39 (6F) and CD73 (6G) in control vs. virus treated tumors (day 5). Representative pictures of whole tumors (I), magnified pictures (II) and quantification of staining intensity (III). Quantitative analysis of IHC staining intensity was performed using image J software, as reported . Data are presented as relative (%) changes in marker expression (average +/- SD of triplicates) staining intensity in treated compared to controls (100%). Scale bars: I: 20 μm; II: 100 μm.

FIGS. 9A-9D show in vivo effects of mJX-594 and A2AR inhibition on RENCA progression in vivo: ctrl (7A), A2ARi (7B), mJX-594 (7C), AsARi+mJX-594 (7D). RENCA bearing BALB/C mice (n=6-7) per group were randomized to either mock treatment, SCH58261 (A2AR inhibitor), 1 mg/kg, intraperitoneally, daily, for 14 days, mJX594 ($1x10^7$ PFU in 100 μL PBS, vial tail vein, x 3 one day apart), of mJX-594 (x3 treatments) and SCH58261 (x 14 days) combination. Red arrows indicate day of treatment initiation. Statistical analysis (ANOVA, followed by post hoc pairwise comparisons) compare mean tumor volumes among groups at day 24 after tumor implantation.

FIG. 10 shows treatment scheme for efficacy studies of VV (mJX-594) alone and in combination with adenosine receptor (A2AR=Adenosine 2 A receptor, A2BR=Adenosine 2 B receptor) blockers, including the timeline of the virus and adenosine blocker treatments. Schema illustrates single agents, vs. doublets vs. triplet combinations.

FIG. 11 shows the results of the different treatments (single agents and combinations) on murine renal cell carcinoma progression. Each graph displays growth curves of individual tumors in each treatment group. VV=vaccinia virus (mJX-594); A2AR= Adenosine 2 Receptor blocker; A2BR: Adenosine 2 B receptor Blocker.

Figs. 12A-12D. In vivo effects of mJX-594, A2AR and A2BR inhibitors on RENCA progression in vivo. RENCA bearing BALB/C mice per group were randomized to either mock treatment or to VV, A2AR and A2BR inhibitors alone (A) Another treatment group was added to see the effect of A2AR inhibitor in combination with VV or A2BR inhibitor (B). And similarly the effect of A2BR inhibitor in combination with VV or A2AR inhibitor (C). The mice with mJX-594 were treated with 3 doses (1.5 × 107) by IV injection every other day. The mice with 1mg/mL of A2AR inhibitor (shc58261, Selleckchem) were treated with

28 doses of injection intraperitoneally every day. The mice with 1mg/mL of A2BR inhibitor (PSB1115, Tocris) were treated with 28 doses of injection intraperitoneally every day. Tumor volume after 21 days is lowest with administration of VV, VV and A2AR inhibitor, and VV and A2AR and A2BR inhibitors (D).

FIG. 13 shows RCC: histological subtypes.

FIG. 14 shows RCC staging.

FIG. 15 shows renal cell carcinoma management and treatment options.

FIG. 16 shows oncolytic viruses and the oncolytic vaccinia virus.

FIGS. 17A-17J shows in vitro effects of oncolytic vaccinia virus alone and with Cabozantinib on human and murine renal cancer. X-594 induced cytotoxicity in human 786-0 (A), A498 (B), Caki-1 (C), UOK262 (D), ACHN (E)., and murine (RENCA, F, treated with mJX-594) renal cancer G, H. * $p < 0.0001$, vs. control. Cell growth in 786-0 cells treated with JX-594 (MOI=0.1) (G) or RENCA cells (H) treated with MOI=0.1 mJX-594alone and in combination with cabozantinib. Bars represent the average cell growth relative to untreated controls, +/- SD. Viral replication of JX-594 (I) and mJX-594 (J) at 48 and 72 hours after VV infection alone (MOI=0.1) or in combination with cabozantinib (200 nM). * $p < 0.05$; ** <0.001. (vs controls).

FIGS. 18A-18D show mechanisms of VV oncolysis in vitro. 21A. Schema depicting cell preparation for functional proteomic analysis (RPPA) of 786-0 cells treated with JX-594. 21B-21C. Pathway analysis. Charts represent top significantly regulated Canonical pathways at 24 B. and 48 C. hours. Bar length indicates number of differentially expressed proteins; bar label indicates ratio of # of differential expression proteins to all proteins belonging to the pathway, and the significance of associated enrichment test. 21D. JX-594 induced changes in expression of cancer associated pathways in 786-0 cells at 24 and 48 hours after virus treatment.

FIGS. 19A-19D show in vivo effects of mJX-594 on murine renal cancer immune microenvironment. BALB/C mice were implanted with RENCA cells and received either PBS or mJX-594 (1x107 PFUx3) IV, when tumors reached 5 mm. Tumor volumes were followed in control (22A). and virus (22B). treated mice. 22C. Additional RENCA bearing mice (n=3/group) were treated with mock or mJX-594, and tumors were removed at day 5 of treatment for flow cytometry analysis of CD73, CD3, CD4, CD8, and PD-1. Analysis was performed using FlowJo (v10.8.1, BD Biosciences). Analysis of CD4, CD8, and PD-1 expression on CD3+CD73- T cells. 22D. Tumor sections from each group were stained (IF) for A27L (VV), ki67, CD31 and TUNEL. Scale bar =100 μm.

FIGS. 20A-20K show in vivo effects of mJX-594 and Cabozantinib on syngeneic murine renal cancer. 23A. RENCA bearing BALB/C mice were randomized into 8 treatment groups, as shown in the table (n= 10/group). Tumor volumes were calculated (W2 x L x 0.52. (23B-23I). Spider plots displaying individual tumor volume changes over time in the controls vs. single, double and triple therapy. 23J. Differences in average tumor volume. Graph represents average tumor volume (+/- SEM) of 10 mice per treatment group. Triplet (Day-31) vs: Cabo + VV, p=< 0.0001; vs. Cabo, p=0.04; vs. Cabo+PD-1:, p=0.024. 23K. Kaplan-Meier curve representing survival rate of mice in this experiment. Triplet vs: Cabo, p=0.04; vs. Cabo+PD1, p=0.0029; vs. all other groups, p< 0.001.

DETAILED DESCRIPTION

The following description of the disclosure is provided as an enabling teaching of the disclosure in its best, currently known embodiments. Many modifications and other embodiments disclosed herein will come to mind to one skilled in the art to which the disclosed compositions and methods pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the disclosures are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. The skilled artisan will recognize many variants and adaptations of the aspects described herein. These variants and adaptations are intended to be included in the teachings of this disclosure and to be encompassed by the claims herein.

Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

As can be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present disclosure.

Any recited method can be carried out in the order of events recited or in any other order that is logically possible. That is, unless otherwise expressly stated, it is in no way intended that any method or aspect set forth herein be construed as requiring that its steps be performed in a specific order. Accordingly, where a method claim does not specifically state in the claims or descriptions that the steps are to be limited to a specific order, it is no way intended that an order be inferred, in any respect. This holds for any possible non-express

basis for interpretation, including matters of logic with respect to arrangement of steps or operational flow, plain meaning derived from grammatical organization or punctuation, or the number or type of aspects described in the specification.

All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided herein can be different from the actual publication dates, which can require independent confirmation.

It is also to be understood that the terminology used herein is for the purpose of describing particular aspects only and is not intended to be limiting. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosed compositions and methods belong. It can be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the specification and relevant art and should not be interpreted in an idealized or overly formal sense unless expressly defined herein.

Prior to describing the various aspects of the present disclosure, the following definitions are provided and should be used unless otherwise indicated. Additional terms may be defined elsewhere in the present disclosure.

Definitions

As used herein, "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps, or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps, or components, or groups thereof. Moreover, each of the terms "by", "comprising," "comprises", "comprised of," "including," "includes," "included," "involving," "involves," "involved," and "such as" are used in their open, non-limiting sense and may be used interchangeably. Further, the term "comprising" is intended to include examples and aspects encompassed by the terms "consisting essentially of" and "consisting of." Similarly, the term "consisting essentially of" is intended to include examples encompassed by the term "consisting of."

As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example,

reference to "a compound", "a composition", or "a disorder", includes, but is not limited to, two or more such compounds, compositions, or disorders, and the like.

It should be noted that ratios, concentrations, amounts, and other numerical data can be expressed herein in a range format. It can be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it can be understood that the particular value forms a further aspect. For example, if the value "about 10" is disclosed, then "10" is also disclosed.

When a range is expressed, a further aspect includes from the one particular value and/or to the other particular value. For example, where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the disclosure, e.g., the phrase "x to y" includes the range from 'x' to 'y' as well as the range greater than 'x' and less than 'y'. The range can also be expressed as an upper limit, e.g., 'about x, y, z, or less' and should be interpreted to include the specific ranges of 'about x', 'about y', and 'about z' as well as the ranges of 'less than x', less than y', and 'less than z'. Likewise, the phrase 'about x, y, z, or greater' should be interpreted to include the specific ranges of 'about x', 'about y', and 'about z' as well as the ranges of 'greater than x', greater than y', and 'greater than z'. In addition, the phrase "about 'x' to 'y'", where 'x' and 'y' are numerical values, includes "about 'x' to about 'y'".

It is to be understood that such a range format is used for convenience and brevity, and thus, should be interpreted in a flexible manner to include not only the numerical values explicitly recited as the limits of the range, but also to include all the individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range is explicitly recited. To illustrate, a numerical range of "about 0.1% to 5%" should be interpreted to include not only the explicitly recited values of about 0.1% to about 5%, but also include individual values (e.g., about 1%, about 2%, about 3%, and about 4%) and the sub-ranges (e.g., about 0.5% to about 1.1%; about 5% to about 2.4%; about 0.5% to about 3.2%, and about 0.5% to about 4.4%, and other possible sub-ranges) within the indicated range.

As used herein, the terms "about," "approximate," "at or about," and "substantially" mean that the amount or value in question can be the exact value or a value that provides equivalent results or effects as recited in the claims or taught herein. That is, it is understood that amounts, sizes, formulations, parameters, and other quantities and characteristics are not and need not be exact but may be approximate and/or larger or smaller, as desired, reflecting tolerances, conversion factors, rounding off, measurement error and the like, and other factors known to those of skill in the art such that equivalent results or effects are obtained. In some circumstances, the value that provides equivalent results or effects cannot be reasonably determined. In such cases, it is generally understood, as used herein, that "about" and "at or about" mean the nominal value indicated ±10% variation unless otherwise indicated or inferred. In general, an amount, size, formulation, parameter or other quantity or characteristic is "about," "approximate," or "at or about" whether or not expressly stated to be such. It is understood that where "about," "approximate," or "at or about" is used before a quantitative value, the parameter also includes the specific quantitative value itself, unless specifically stated otherwise. As used herein, the term "substantially free," when used in the context of a composition or component of a composition that is substantially absent, is intended to refer to an amount that is then about 1 % by weight or less, e.g., less than about 0.5 % by weight, less than about 0.1 % by weight, less than about 0.05 % by weight, or less than about 0.01 % by weight of the stated material, based on the total weight of the composition.

The term "subject" preferably refers to a human in need of treatment with an anti-cancer agent or treatment for any purpose, and more preferably a human in need of such a treatment to treat cancer, or a precancerous condition or lesion. However, the term "subject" can also refer to non-human animals, preferably mammals such as dogs, cats, horses, cows, pigs, sheep and non-human primates, among others, which are in need of treatment with an anti-cancer agent or treatment.

The term "treating" refers to the medical management of a patient with the intent to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder. This term includes active treatment, that is, treatment directed specifically toward the improvement of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventative treatment, that is, treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological

condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological condition, or disorder.

Method

Method of Treating a Cancer – Tyrosine Kinase Inhibitor

The present disclosure, in one aspect, provides for a method of treating a cancer, comprising administering to a subject in need thereof an oncolytic vaccinia virus and a tyrosine kinase inhibitor. In some examples, the method of treating a cancer comprises administering to a subject in need thereof an oncolytic vaccinia virus, a tyrosine kinase inhibitor, and an immune checkpoint inhibitor.

Vaccinia virus is a large, complex, enveloped DNA virus that belongs to the poxvirus family. It has a linear, double-stranded DNA genome approximately 190 kb in length and encodes approximately 250 genes. Vaccinia virus includes, but is not limited to, strains Lister, Wyeth, and Western Reserve. In some examples, the vaccinia virus is genetically engineered to add or delete specific genes. In further examples, vaccinia virus is oncolytic in that it infect cancer cells, which in some examples is a result of its sensitivity to type I interferon.

In further examples, the oncolytic vaccinia virus is thymidine kinase (TK) defective, TK deficient, or lacks a functional TK gene.

In some examples, the oncolytic vaccinia virus encodes GM-CSF. GM-CSF refers to granulocyte-macrophage colony-stimulating factor (GM-CSF), which is a monomeric glycoprotein secreted by macrophages, T cells, mast cells, natural killer cells, endothelial cells and fibroblasts that functions as a cytokine. It is also a white blood cell growth factor. The human gene has been localized in close proximity to the interleukin 3 gene within a T helper type 2-associated cytokine gene cluster at chromosome region 5q31.

In further examples, the oncolytic vaccinia virus is JX-594. JX-594 refers to an oncolytic virus designed to target and destroy cancer cells (also referred to as Pexa-Vec or pexastimogene devacirepvec). It is a modified Copenhagen strain (or Wyeth strain) vaccinia poxvirus engineered by addition of the GM-CSF gene and the deletion of the thymidine kinase gene, thereby limiting viral replication to cells with high levels of thymidine kinase. The virus kills infected/cancer cells by oncolysis and can help initiate an anti-tumor immune response.

In some examples, the TK gene in the JX-594 strain is deactivated. In further examples, the TK gene is deactivated because of the insertion of the GM-CSF gene in the JX-594 strain.

In some examples, the oncolytic vaccinia virus is mJX-594, also referred to as the Western Reserve strain of oncolytic vaccinia virus.

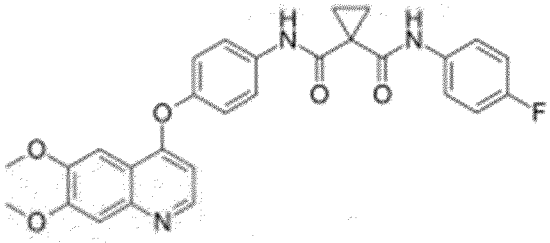
In further examples, the oncolytic vaccinia virus is a Lister strain. The Lister strain is a strain of oncolytic vaccinia virus.

In specific examples, the oncolytic vaccinia virus is a Copenhagen strain. The Lister strain is a strain of oncolytic vaccinia virus.

In some examples, the oncolytic vaccinia virus is a replicative oncolytic vaccinia virus.

In certain examples, the tyrosine kinase inhibitor comprises afatinib, alectinib, axitinib, bosutinib, cabozantinib, ceritinib, cobimetinib, crizotinib, dabrafenib dasatinib, erlotinib, gefitinib, ibrutinib, idelalisib, imatinib, lapatinib, lenvatinib, nilotinib, osimertinib, palbociclib, ponatinib, pazopanib, regorafenib, ruxolitinib, sorafenib, sunitinib, trametinib, vandetanib, vemurafenib, pharmaceutically acceptable salts thereof, and combinations thereof.

In specific examples, the tyrosine kinase inhibitor comprises cabozantinib. Cabozantinib is an anti-cancer medication small molecule inhibitor of tyrosine kinases with the following formula:



In further examples, the cancer is bladder cancer, breast cancer, cervical cancer, colorectal (colon) cancer, gynecologic cancers, head and neck cancers, kidney cancer, liver cancer, lung cancer, mesothelioma, myeloma, ovarian cancer, prostate cancer, skin cancer, uterine cancer, or vaginal and vulvar cancers.

In certain examples, the cancer is breast cancer. Breast cancer is a cancer that starts in breast tissue and occurs when cells in the breast change and grow out of control. Symptoms of breast cancer include a lump in the breast, bloody discharge from the nipple, and changes in the shape or texture of the nipple or breast.

In specific examples, the cancer is colon cancer. Colon, or colorectal, cancer is a growth of cells that form in the lower end of the digestive tract. Most of these cancers start as noncancerous growths called polyps. Symptoms may vary depending on the cancer's size and

location. Symptoms might include blood in the stool, abdominal discomfort, and a change in bowel habits, such as diarrhea or constipation.

In some examples, the cancer is renal cell carcinoma (RCC). Renal cell carcinoma is a disease in which cancer cells are found in the lining of tubules in the kidney. Signs of RCC include blood in the urine and a lump in the abdomen, as well as loss of appetite, weight loss, and anemia. Smoking and misuse of certain pain medicines can affect the risk of RCC.

In further examples, the renal cell carcinoma comprises Stage I, Stage II, Stage III or Stage IV renal cell carcinoma. In Stage I, the tumor is 7 centimeters or smaller and is found in the kidney only. In Stage II, the tumor is larger than 7 centimeters and is found in the kidney only. In Stage III, either the cancer in the kidney is any size and has spread to nearby lymph nodes; or cancer has spread to blood vessels in or near the kidney, to the far around the structures in the kidney that collect urine, or to the layer of fatty tissue around the kidney. The cancer also may have spread to nearby lymph nodes. In stage IV, one of the following occurs: cancer has spread beyond the layer of fatty tissue around the kidney and may have spread into the adrenal gland above the kidney with cancer or to nearby lymph nodes; or cancer has spread to other parts of the body, such as the bones, liver, lungs, brain, adrenal glands, or distant lymph nodes.

In certain examples, renal cell carcinoma comprises conventional (clear-cell) renal cell carcinoma, papillary renal cell-carcinoma, chromophobe renal carcinoma, onco-cytoma, or collecting-duct carcinoma.

In certain examples, the cancer is a solid tumor. Solid tumors that are cancerous are mainly of monoclonal origin and manifest six essential alterations: growth signals, intensification of anti-growth signals, cell and tissue apoptosis or necrosis, limitless replicative and proliferative potential, prolonged angiogenesis, and organ/tissue invasion followed by metastasis. Solid tumors can include adenocarcinoma, small cell carcinoma, neuroendocrine tumor, transitional cell carcinoma, or sarcoma, as described below.

In some examples, the cancer comprises Stage I, Stage II, Stage III, or Stage IV cancer. Stage I means the cancer is small and only in one area. This is also called early-stage cancer. Stage II and III mean the cancer is larger and has grown into nearby tissues or lymph nodes. Stage IV means the cancer has spread to other parts of the body and can also be called advanced or metastatic cancer.

In specific examples, the oncolytic vaccinia virus and the tyrosine kinase inhibitor are administered in combination with at least one additional cancer therapy.

In some examples, the additional cancer therapy comprises surgery, chemotherapy, immunotherapy, ionizing radiation, or a combination thereof.

In further examples, surgery comprises a radical nephrectomy, simple nephrectomy, partial nephrectomy, arterial or tumor embolization, or any combination thereof.

In some examples, surgery includes curative surgery, preventive surgery, diagnostic surgery, staging surgery, debulking surgery, palliative surgery, supportive surgery, and restorative surgery, as well as cryosurgery, laser surgery, electrosurgery, and microscopically controlled surgery.

In some examples, chemotherapy can include R-CHOP (Rituxan, cyclophosphamide, doxorubicin, vincristine, and prednisone), VcR-CAP (bortezomib, rituximab, cyclophosphamide, doxorubicin, and prednisone), R-hyperCVAD (rituximab, cyclophosphamide, vincristine, doxorubicin, and dexamethasone alternating with high-dose cytarabine and methotrexate), B+R (bendamustine and rituximab), R-FCM (rituximab, fludarabine, cyclophosphamide, and mitoxantrone), R-DHAP (rituximab, dexamethasone, cytarabine and cisplatin), R-CVP (rituximab, cyclophosphamide, vincristine, and prednisone), or R-CBP (rituximab, cyclophosphamide, bortezomib, and prednisone), or any combination thereof. These chemotherapies can be administered intravenously or via mouth.

In further examples, ionizing radiation includes radiation therapy using high-energy particles or waves, x-rays, gamma rays, electron beams, or protons.

In some examples, the surgery comprises stem cell transplantation (SCT). In specific examples, the surgery comprises stem cell transplantation (SCT). Stem cell transplantation (SCT), also referred to as a bone marrow transplant, is a procedure in which a subject receives healthy stem cells to replace damaged stem cells. This can include autologous transplantation in which the transplantation uses the subject's own stem cells. SCT can also include allogeneic transplantation, which uses stem cells from a donor.

In autologous transplantation, the subject stem cells are collected and stored. The cells are frozen and then returned to the subject after receiving intensive high-dose chemotherapy either with or without radiation therapy. This procedure can be used in clinically symptomatic subjects that are fit, young, and have few or no coexisting illnesses.

In further examples, cancer therapy can include R-CHOP followed by an autologous SCT, R-CHOP followed by higher doses of cytarabine and further followed by an autologous stem cell transplant, or R-hyperCVAD with autologous SCT, or any combination thereof.

In some examples, immunotherapy includes monoclonal antibodies, checkpoint inhibitors, CAR T-cell therapy, and cytokines.

Immunotherapy refers to the treatment of disease by activating or suppressing the immune system. Immunotherapies designed to elicit or amplify an immune response are classified as activation immunotherapies, while immunotherapies that reduce or suppress are classified as suppression immunotherapies.

In certain examples, the immunotherapy comprises administering an immune checkpoint inhibitor. Immune checkpoint inhibitors are drugs that work by block checkpoint proteins from binding with their partner proteins. This allows for T cells to kill cancer cells.

In specific examples, the immune checkpoint inhibitor comprises an anti-PD1 antibody. Anti-PD1 antibody is an antibody that binds to and inhibits PD-1 and its downstream signaling pathways, thereby restoring immune function through the activation of T-cells and cell-mediated immune responses against tumor cells.

In some examples, the anti-PD1 antibody comprises pembrolizumab, nivolumab, cemiplimab, avelumab, or any combination thereof.

In further examples, the method further comprises administering an additional therapeutic agent. In specific examples, the additional therapeutic agent comprises abiraterone acetate, apalutamide, bicalutamide, cabazitaxel, darolutamide, degarelix, docetaxel, enzalutamide, flutamide, goserelin acetate, leuprolide acetate, lutetium Lu 177 vipivotide tetraxetan, mitoxantrone hydrochloride, nilutamide, olaparib, radium 223 dichloride, relugolix, rucaparib camsylate, sipuleucel-t, or any combination thereof.

In some examples, administration occurs intratumorally, intravenously, intraarterially, intraperitoneally, or any combination thereof. In some examples, the components administered to the subject via the disclosed method are administered individually via the administration routes. In other examples, the components administered to the subject via the disclosed method are administered in any combination via the administration routes.

Method of Treating a Cancer – Adenosine Receptor Blocker

Further provided herein is a method for treating a cancer, comprising administering to a subject in need thereof the oncolytic vaccinia virus and an adenosine receptor blocker.

Vaccinia virus is a large, complex, enveloped DNA virus that belongs to the poxvirus family. It has a linear, double-stranded DNA genome approximately 190 kb in length and encodes approximately 250 genes. Vaccinia virus includes, but is not limited to, strains Lister, Wyeth, Western Reserve, and Copenhagen. In some examples, the vaccinia virus is genetically engineered to add or delete specific genes. In further examples, vaccinia virus is

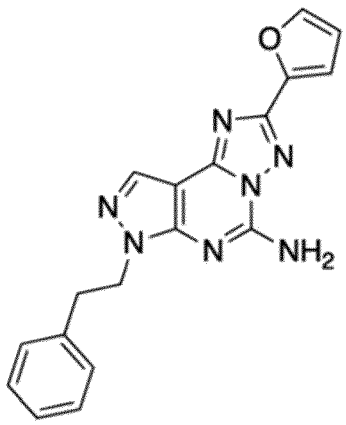
oncolytic in that it infect cancer cells, which in some examples is a result of its sensitivity to type I interferon.

In some examples, the oncolytic vaccinia virus encodes GM-CSF. GM-CSF refers to granulocyte-macrophage colony-stimulating factor (GM-CSF), which is a monomeric glycoprotein secreted by macrophages, T cells, mast cells, natural killer cells, endothelial cells and fibroblasts that functions as a cytokine. It is also a white blood cell growth factor. The human gene has been localized in close proximity to the interleukin 3 gene within a T helper type 2-associated cytokine gene cluster at chromosome region 5q31.

In further examples, the oncolytic vaccinia virus is JX-594. JX-594 refers to an oncolytic virus designed to target and destroy cancer cells (also referred to as Pexa-Vec or pexastimogene devacirepvec). It is a modified Copenhagen strain (or Wyeth strain) vaccinia poxvirus engineered by addition of the GM-CSF gene and the deletion of the thymidine kinase gene, thereby limiting viral replication to cells with high levels of thymidine kinase. The virus kills infected/cancer cells by oncolysis and can help initiate an anti-tumor immune response.

In certain examples, the adenosine receptor blocker comprises an A2AR inhibitor. A2AR refers to the adenosine A_{2A} receptor, which is an adenosine receptor that's a member of the G protein-coupled receptor family, which possess seven transmembrane alpha helices, as well as an extracellular N-terminus and an intracellular C-terminus. The gene encodes a protein which is one of several receptor subtypes for adenosine. Inhibitors of A2AR include, but are not limited to, ATL-444, KW-6002, MSX-3, SCH-420,814, SCH-58261, SCH-412,348, SCH-442,416, ST-1535, Ver-6623, Ver-6947, Ver-7835, BIIB-014, and ZM-241,385.

In specific examples, the A2AR inhibitor comprises SCH58261. SCH58261 refers to a drug that acts as a potent and selective antagonist for the adenosine receptor A_{2A}, which has the following formula:



In further examples, A2AR inhibitors include, but are not limited to, ciforadenant, imaradenant, etrumadenant, NIR178, EOS100850, Inupadenant, CS3005, PBF-999, INCB106385, or any combination thereof.

In some examples, the adenosine receptor blocker comprises an A2BR inhibitor. A2BR refers to the adenosine A_{2B} receptor, which is a G-protein coupled adenosine receptor that is encoded by the A_{2b} receptor gene. In some examples, inhibitors of A2BR include, but are not limited to, compound 38, ISAM-R56A, ISAM-140, ISAM-R324A, ATL-801, CVT-6883, MRS-1706, MRS-1754, OSIP-339, OSIP-391, PSB-603, PSB-0788, PSB-1115, and PSB-1901, etrumadenant, or any combination thereof.

In further examples, the A2BR inhibitor comprises PSB1115, or any combination thereof. PSB1115 refers to a selective A2B adenosine receptor antagonist that inhibits the 2,4,6-trinitrobenezenesulfonic acid (TNBS)-induced contraction inhibition of acetylcholine (ACh).

In certain examples, the A2AR inhibitors and/or the A2BR inhibitors are combined with atezolizumab, daratumumab, pembrolizumab, durvalumab, oleclumab, abiraterone, enzalutamide, mFOLFOX-6, zimberelimab, bevacizumab, domvanalimab, PDR001, spartalizumab, or any combination thereof.

In certain examples, the adenosine receptor blocker comprises the A2AR inhibitor and the A2BR inhibitor. In some examples, the adenosine receptor blocker comprises an inhibitor of both A2AR and A2BR. In further examples, the inhibitor of both A2AR and A2BR includes, but is not limited to, etrumadenant.

Etrumadenant is an orally bioavailable antagonist of both A2AR and A2BR. Upon administration, etrumadenant competes with adenosine for binding to A2AR and A2BR expressed on intra-tumoral immune cells, such as dendritic cells, natural killer cells, macrophages, and T lymphocytes.

In specific examples, the cancer is renal cell carcinoma. Renal cell carcinoma is a disease in which cancer cells are found in the lining of tubules in the kidney. Signs of RCC include blood in the urine and a lump in the abdomen, as well as loss of appetite, weight loss, and anemia. Smoking and misuse of certain pain medicines can affect the risk of RCC.

In some examples, the renal cell carcinoma comprises Stage I, Stage II, Stage III or Stage IV renal cell carcinoma. In Stage I, the tumor is 7 centimeters or smaller and is found in the kidney only. In Stage II, the tumor is larger than 7 centimeters and is found in the kidney only. In Stage III, either the cancer in the kidney is any size and has spread to nearby lymph nodes; or cancer has spread to blood vessels in or near the kidney, to the far around

the structures in the kidney that collect urine, or to the layer of fatty tissue around the kidney. The cancer also may have spread to nearby lymph nodes. In stage IV, one of the following occurs: cancer has spread beyond the layer of fatty tissue around the kidney and may have spread into the adrenal gland above the kidney with cancer or to nearby lymph nodes; or cancer has spread to other parts of the body, such as the bones, liver, lungs, brain, adrenal glands, or distant lymph nodes.

In further examples, the renal cell carcinoma comprises conventional (clear-cell) renal cell carcinoma, papillary renal cell-carcinoma, chromophobe renal carcinoma, onco-cytoma, or collecting-duct carcinoma.

In certain examples, the oncolytic vaccinia virus and the adenosine receptor blocker are administered in combination with at least one additional cancer therapy.

In specific examples, the additional cancer therapy comprises surgery, chemotherapy, immunotherapy, ionizing radiation, or a combination thereof.

In some examples, surgery comprises a radical nephrectomy, simple nephrectomy, partial nephrectomy, arterial or tumor embolization, or any combination thereof.

Immunotherapy refers to the treatment of disease by activating or suppressing the immune system. Immunotherapies designed to elicit or amplify an immune response are classified as activation immunotherapies, while immunotherapies that reduce or suppress are classified as suppression immunotherapies.

In further examples, the immunotherapy comprises administering an immune checkpoint inhibitor. Immune checkpoint inhibitors are drugs that work by block checkpoint proteins from binding with their partner proteins. This allows for T cells to kill cancer cells.

In certain examples, the immune checkpoint inhibitor comprises anti-PD1 antibody. Anti-PD1 antibody is an antibody that binds to and inhibits PD-1 and its downstream signaling pathways, thereby restoring immune function through the activation of T-cells and cell-mediated immune responses against tumor cells.

In specific examples, the anti-PD1 antibody comprises pembrolizumab, nivolumab, cemiplimab, avelumab, or any combination thereof.

In some examples, administration occurs intratumorally, intravenously, intraarterially, intraperitoneally, or any combination thereof. In some examples, the components administered to the subject via the disclosed method are administered individually via the administration routes. In other examples, the components administered to the subject via the disclosed method are administered in any combination via the administration routes.

A number of embodiments of the disclosure have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

By way of non-limiting illustration, examples of certain embodiments of the present disclosure are given below.

EXAMPLES

The following examples are set forth below to illustrate the methods and results according to the disclosed subject matter. These examples are not intended to be inclusive of all aspects of the subject matter disclosed herein, but rather to illustrate representative methods and results. These examples are not intended to exclude equivalents and variations of the present invention, which are apparent to one skilled in the art.

Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric. There are numerous variations and combinations of reaction conditions, e.g., component concentrations, temperatures, pressures, and other reaction ranges and conditions that can be used to optimize the product purity and yield obtained from the described process. Only reasonable and routine experimentation will be required to optimize such process conditions.

Example 1: Combinatorial Strategies to Enhance Vaccinia Virus In Vitro And In Vivo Oncolysis

Oncolytic viruses (OVs) are novel biological anticancer agents, which have demonstrated antitumor efficacy in preclinical models, and safety in humans [1]. Vaccinia virus has several advantages over other OV platforms, making it an attractive agent for preclinical and clinical development [6, 7]. These include its efficient life cycle, multiple mechanisms of viral spread, large genome, allowing acceptance of foreign therapeutic DNA inserts, proven safety in humans, due to its use as smallpox vaccine. In addition, the ability of the virus for immune evasion, and to target tumor vasculature makes it attractive for systemic (IV administration) in advanced malignancies.

While oncolytic viral vectors as single agents have shown safety and early evidence of efficacy, they alone may not be curative in the majority of patients. Among the potential

mechanisms leading to resistance to viral and immune therapies is the tumor microenvironment (TME), which mediates both decreased viral spread within the tumor, and immune suppression [8, 9]. Previous research has focused on identifying tumor stromal mechanisms that mediated OV resistance and immunosuppression, and OV based combinations with currently approved checkpoint inhibitors [10]. However, many other tumor environmental factors mediate OV resistance and immunosuppression. Angiogenic pathways including VEGF, HGF/c-met, and AXL not only promote tumor angiogenesis, but also are associated with immunosuppression in the TME by promoting accumulation of suppressive cytokines and immunosuppressive cell phenotypes [11-13]. An immunosuppressive and proangiogenic stromal resistance mechanism is the adenosinergic pathway, which encompasses the ectonucleotidases CD39 and CD73 (which catalyze cleavage of ATP →AMP and AMP →adenosine, respectively), and adenosine receptors (AR, A2AR, A2BR and A3R) [14-17]. Increased adenosine production enhances polarization of myeloid and T-cell subsets to proangiogenic and immunosuppressive phenotypes, enhancing tumor growth and survival. Moreover, adenosine negatively affects effector immune cells, NK cells, and $CD8^+$ T cells, among other effects. Tumor CD73 expression (which leads to adenosine production), is induced by inflammation, necrosis and hypoxia, and has been detected in a multiple malignancies, including colorectal, prostate, renal cell carcinomas and sarcomas, among others [16]. As many oncolytic viruses induce inflammation and necrosis, a potential mechanism explaining decreased efficacy of OVs (as single agents) is increased adenosine production caused by virus induced inflammatory/necrotic changes, eventually resulting in adenosine mediated immunosuppression. As the adenosine pathway is thought to be involved in resistance to checkpoint inhibitors, it is suggested that blocking the adenosine pathway can enhance the antitumor effects of Vaccinia virus/anti-PD-1 combinations.

Herein, the in vivo oncolytic and immunomodulatory effects of vaccinia virus-GM-CSF were enhanced in syngeneic, immunocompetent models of renal cell, colon and mammary cancer. The vaccinia virus antitumor efficacy (in separate experiments) was enhanced by targeting two tumor stromal pathways, namely tumor angiogenesis and the adenosine pathway in combination with vaccinia virus. Characterized herein are the safety, efficacy and antitumor effects of novel combinations of vaccinia virus and targeted agents (TKIs), or novel immunomodulatory agents (e.g. agents that block adenosine pathway alone or in combination with anti PD-1 agents). TKIs (e.g. cabozantinib) enhanced viral antitumor effects by direct anti-stromal effects, including "normalizing" disrupted tumor vasculature, as well as modulation of the immune microenvironment, a well-recognized effect of anti-

VEGF agents. Moreover, targeting the adenosine pathway, by either blocking adenosine production (with anti-CD73antibodies), or action (A2AR blockade) enhanced antitumor immune responses induced by vaccinia virus in vivo alone and in combination with anti-PD-1 agents, leading to significant improvement in outcomes in vivo.

The example herein describes as follows:

1) Characterization of in vitro effects of JX-594/PEXA-VEC (human) and MJX-594 (Murine) vaccinia viral vectors in human and murine cancer cell lines, alone and in combination with TKIs and adenosine receptor blockers.

a) Characterization of in vitro cytotoxicity of mpJX-594 and mJX-594 in Human and murine renal cell and breast/mammary cancer alone and in combination with cabozantinib or adenosine blockade (A2AR blockers). Representative murine and human cell lines include 786-0, A498, ACHN, Caki-1 (human renal cancer), RENCA (murine renal cancer), MDA-MB231 (triple negative human breast cancer), 4T1 (murine mammary cancer). Cytotoxic effects of virus and combinations were measured Trypan blue exclusion (using Vi-cell viability analyzer), and/or xCELLigence real time cell analysis.

b) Characterization of the molecular mechanisms of vaccinia virus' cytotoxic effects by functional proteomic analysis (RPPA). Cell lysates from untreated and virus treated cells were prepared at different time points (8, 24, 48, hours) for functional proteomic analysis. RPPA represents an antibody-based functional proteomic analysis for both tumor tissue and cultured cells. RPPA characterizes the basal protein expression and modification levels, growth factor- or ligand-induced effects, and time-resolved responses appropriate for systems biology analysis. It provides information to integrate the consequence of genetic aberrations in cancer, to validate therapeutic targets, to demonstrate on- and off-target activity of drugs, and to evaluate drug pharmacodynamics. RPPA is performed at the RPPA core facility at MD Anderson Cancer Center (fee for service). Up to 400 analytes were assessed for analysis pathways altered by virus-tumor cell interactions. Results of the analyses identified potential mechanisms of OV resistance, which provided important information on novel combinations to enhance the VV's oncolytic effects. Analysis was conducted in human (786-0) and murine (RENCA) renal cancer cells.

2) Determining the in vivo effects of adenosine pathway blockade on the antitumor efficacy of MJX-594 alone and MJX-594 plus PD-1 blockade in murine syngeneic models of cancer.

a) Efficacy studies: Murine renal cell (RENCA), and triple negative mammary (4T1) cancer were established. Once tumors reached 3-4 mm, mice were

randomized in 6 treatment groups: 1) Vehicle control; 2) MJX-594, intravenous, X 3, every other day; 3) anti PD-1200 ug IP x 4, every 3 days; 4) adenosine 2 receptor (A2AR) blocker (SCH58261) daily, IP, x 21 days; 5) MJX-594+anti PD-1, as above. Anti PD1 was started after last dose of the virus was given ; 6) MJX-594+A2AR blocker; A2AR inhibitor was started after last dose of virus is given; 7) MJX-594+A2AR blockade+anti-PD1, as above. Endpoints of the study were tumor progression (tumor volume) and survival (time to sacrifice). Number of mice per group: 10. All murine cell lines grew in BALB/C mice. For RENCA tumors, groups had an equal number of males and females. For 4T1, groups were only female mice.

b) Tumor correlative studies: Additional 10 tumor bearing mice per group were treated as above. At 5 and 10 days, mice were sacrificed, and tumors were resected, processed and tumor cell suspensions were prepared for flow cytometry analysis of the immune and non-immune stroma, using antibody panels available at the laboratory, for immunophenotypic characterization of tumor leukocytes (CD45+), to determine changes in macrophages, MDSCs, NK cells, B and T cells, as well as tumor infiltrating lymphocytes (T cell subsets). Moreover, immunophenotypic characterization of tumor endothelial cells (CD31 and CD105) fibroblasts (FAP) was performed. Tumor experiments were performed in selected treatment groups and controls, for comparative analysis of changes in the immune microenvironment induced by MJX-594 alone, in combination with PD-1, and in combination with anti PD-1 and A2AR blockade.

3) To determine the in vivo antitumor activity and mechanisms of JX-594/PEXA-VEC (human) or MJX-594 (murine) alone and in combination with RTKIs in models of renal and mammary cancer.

a) Characterization of in vivo antitumor and anti-stromal effects of MJX-594 alone and in combination with cabozantinib in syngeneic, immunocompetent murine renal cell and mammary Cancer . In this set of experiments, RTKI-virus combination was assessed in immune competent models. Murine renal cell (RENCA) was established as previously reported by the group [18, 20]. Once tumors reached 3-4 mm, mice were randomized into 4 groups: 1) Control; 2) MJX-594; 3) Cabozantinib; 4) mJX-594 and cabozantinib. mJX-594 was given intravenously (tail vein) x 3 doses, every other day; Cabozantinib was given via oral gavage 5 out of 7 days, for 21 days. Mice were followed for tumor progression (increase in tumor volume), and survival (time to sacrifice). Endpoints of the study were tumor progression (tumor volume) and survival (time to sacrifice. Number of

mice per group: 10. All murine cell lines grew in BALB/C mice. For RENCA, groups had an equal number of males and females.

i) Tumor correlative studies: Additional 5 tumor bearing mice per group were treated as above. At 5 days, mice were sacrificed and tumors were resected, processed and tumor cell suspensions were prepared for flow cytometry analysis of the immune and non-immune stroma, using antibody panels available at the laboratory, for immunophenotypic characterization of tumor leukocytes (CD45+), to determine changes in macrophages, MDSCs, NK cells, B and T cells, as well as tumor infiltrating lymphocytes (T cell subsets). Moreover, immunophenotypic characterization of tumor endothelial cells (CD31 and CD105) and fibroblasts (FAP) was performed.

a) Characterization of in vivo effects of JX-594/Pexa-Vec alone and in combination with cabozantinib in human renal cell (786-0) carcinoma. The rationale for doing such experiments in immunodeficient models was to determine the effects of virus-RTKI combinations in the tumor cells and non-immune stroma. **Methods:** Human xenograft experiments were performed in nude mice (males and females) to characterize the virus-host (non-immune stroma)-tumor interactions and its effects on tumor progression and survival. Mice were implanted with human cancer cell lines and when they reached 4-5 mm in diameter, mice were randomized into 4 groups: 1) Control; 2) mJX-594; 3) Cabozantinib; 4) mJX-594 and cabozantinib. mJX-594 were given intravenously (tail vein) x 3 doses, every other day; Cabozantinib was given via oral gavage 5 out of 7 days, for 21 days. Mice were followed for tumor progression (increase in tumor volume), and survival (time to sacrifice).

i) **Tumor correlative studies**. Additional 5 tumor bearing mice per group were treated as above, and sacrificed at day 5 after last dose of virus. Tumors were resected, fixed and processed for tumor studies. IHC and IF were performed to determine markers of angiogenesis (CD31 and CD105), proliferation (Ki67), apoptosis (TUNEL), and viral targeting (b-gal staining). Changes in the above markers were compared among treatment and control groups, and correlations were made between degree of antitumor activity by the virus or combinations and changes in the tumor/tumor stroma.

The above experiments provided valuable new knowledge that elucidated stromal mediated mechanisms of resistance or response, and the novel concept of adenosine pathway blockade as a strategy to enhance the immunostimulatory effects of OVs.

References

1. Fukuhara, H., Y. Ino, and T. Todo, *Oncolytic virus therapy: A new era of cancer treatment at dawn.* Cancer Sci, 2016. **107**(10): p. 1373-1379.
2. Martinez-Quintanilla, J., et al., *Oncolytic viruses: overcoming translational challenges.* J Clin Invest, 2019. **130**: p. 1407-1418.
3. Park, S.H., et al., *Phase 1b Trial of Biweekly Intravenous Pexa-Vec (JX-594), an Oncolytic and Immunotherapeutic Vaccinia Virus in Colorectal Cancer.* Mol Ther, 2015. **23**(9): p. 1532-40.
4. Breitbach, C.J., et al., *A Phase 2, Open-Label, Randomized Study of Pexa-Vec (JX-594) Administered by Intratumoral Injection in Patients with Unresectable Primary Hepatocellular Carcinoma.* Methods Mol Biol, 2015. **1317**: p. 343-57.
5. Breitbach, C.J., et al., *The emerging therapeutic potential of the oncolytic immunotherapeutic Pexa-Vec (JX-594).* Oncolytic Virother, 2015. **4**: p. 25-31.
6. Guo, Z.S., et al., *Vaccinia virus-mediated cancer immunotherapy: cancer vaccines and oncolytics.* J Immunother Cancer, 2019. **7**(1): p. 6.
7. Deng, L., et al., *Oncolytic cancer therapy with a vaccinia virus strain.* Oncol Rep, 2019. **41**(1): p. 686-692.
8. Wojton, J. and B. Kaur, *Impact of tumor microenvironment on oncolytic viral therapy.* Cytokine Growth Factor Rev, 2010. **21**(2-3): p. 127-34.
9. Kurozumi, K., et al., *Effect of tumor microenvironment modulation on the efficacy of oncolytic virus therapy.* J Natl Cancer Inst, 2007. **99**(23): p. 1768-81.
10. LaRocca, C.J. and S.G. Warner, *Oncolytic viruses and checkpoint inhibitors: combination therapy in clinical trials.* Clin Transl Med, 2018. **7**(1): p. 35.
11. Tazzyman, S., H. Niaz, and C. Murdoch, *Neutrophil-mediated tumthe angiogenesis: subversion of immune responses to promote tumthe growth.* Semin Cancer Biol, 2013. **23**(3): p. 149-58.
12. Motz, G.T. and G. Coukos, *The parallel lives of angiogenesis and immunosuppression: cancer and other tales.* Nat Rev Immunol, 2011. **11**(10): p. 702-11.
13. Coffelt, S.B., R. Hughes, and C.E. Lewis, *Tumor-associated macrophages: effectors of angiogenesis and tumor progression.* Biochim Biophys Acta, 2009. **1796**(1): p. 11-8.
14. Vijayan, D., et al., *Targeting immunosuppressive adenosine in cancer.* Nat Rev Cancer, 2017. **17**(12): p. 709-724.
15. Vigano, S., et al., *Targeting Adenosine in Cancer Immunotherapy to Enhance T-Cell Function.* Front Immunol, 2019. **10**: p. 925.
16. Leone, R.D. and L.A. Emens, *Targeting adenosine for cancer immunotherapy.* J Immunother Cancer, 2018. **6**(1): p. 57.
17. Vijayan, D., et al., *Targeting immunosuppressive adenosine in cancer.* Nat Rev Cancer, 2017. **17**(12): p. 765.
18. Jing, Y., et al., *In vivo safety, biodistribution and antitumor effects of uPAR retargeted oncolytic measles virus in syngeneic cancer models.* Gene Ther, 2014. **21**(3): p. 289-97.
19. Jing, Y., et al., *Tumor and vascular targeting of a novel oncolytic measles virus retargeted against the urokinase receptor.* Cancer Res, 2009. **69**(4): p. 1459-68.
20. Jing, Y., et al., *Role of plasminogen activator inhibitor-1 in urokinase's paradoxical in vivo tumor suppressing or promoting effects.* Mol Cancer Res, 2012. **10**(10): p. 1271-81.
21. Jing, Y., et al., *Molecular Effects of Stromal-Selective Targeting by uPAR-Retargeted Oncolytic Virus in Breast Cancer.* Mol Cancer Res, 2017. **15**(10): p. 1410-1420.

22. Jing, Y., et al., *In vivo anti-metastatic effects of uPAR retargeted measles virus in syngeneic and xenograft models of mammary cancer.* Breast Cancer Res Treat, 2015. **149**(1): p. 99-108.

Example 2: Oncolytic Vaccinia Virus in Advanced Renal Cell Carcinoma

Introduction

Oncolytic viruses (OVs) are promising biotherapies, which offer an advantage over standard treatments in that they selectively target, replicate, and kill cancer over normal cells. Genetic engineering has led to generation of safer (attenuated), targeted, and "armed" viruses. OVs have not been studied in RCC as extensively as in other human malignancies.

Among OV platforms, the oncolytic vaccinia virus (VV) offers several advantages, including an efficient life cycle, multiple mechanisms of viral spread, a large genome, allowing acceptance of foreign therapeutic DNA inserts, and proven safety in humans, due to its use as smallpox vaccine. In addition, the virus' immune evasion, and vascular targeting abilities makes VV attractive for systemic (IV) administration. Intravenous administration of oncolytic viruses is translationally relevant, as targeting multiple sites of metastasis (via the IV route) would be more effective and beneficial than intratumoral injection of few lesions. JX-594 (Pexavec) is an attenuated (TK deleted) oncolytic vaccinia VV, engineered to express GM-CSF and to selectively target tumor over non-tumor tissues. Intravenous administration of JX-594 has shown safety, tumor targeting, immune modulation, and preliminary evidence of efficacy. JX-594 is a potent oncolytic agent.

Tumor escape from oncolytic virotherapies and the role of the adenosine pathway

Experience from clinical trials suggests that similar to immunotherapy agents, patients can develop resistance to OVs. Current strategies to tackle virotherapy resistance include combinations of OVs with anti-PD1 or anti CTLA-4 agents. This strategy is associated with improved antitumor effects compared to OVs alone; however, the added benefit is not frequent. Mixed results from OV clinical trials have taught that, to uncover OV's true therapeutic potential, a better understand of the mechanisms of OV tumor escape was required, and to develop rational virus-drug combinations that go beyond anti PD-1 blockade.

The adenosine pathway encompasses the ectonucleotidases CD39 and CD73 (which catalyze cleavage of ATP released to the tumor microenvironment, to AMP and AMP to adenosine, respectively), and adenosine receptors (A2AR, A2BR and A3R). Factors known to increase expression of tumor CD39, CD73 and adenosine include cell death, cellular stress, hypoxia, necrosis, and inflammation, which commonly occur in aggressive cancers, or as a

result of treatment. Adenosine induces polarization of macrophage, myeloid and T-cell subsets to proangiogenic and immunosuppressive phenotypes. In addition, adenosine negatively affects immune effector cells, NK cells, and $CD8^+$ T cells, among other effects. Blocking adenosine, either by targeting CD39, CD73, or blocking A2A and/or A2B receptors showed improvement of effector cell function, decreased expression of co-inhibitory receptors (PD-1 and Lag-3) in effector T cells, and enhanced checkpoint inhibitor therapy in multiple preclinical models.

The adenosine pathway is a biologically and clinically relevant target in RCC, as overexpression of CD39, CD73 and A2AR are associated with poor prognosis . Moreover, early results from a recent clinical trial with A2AR blockers have shown promising clinical activity in human RCC . The above data demonstrate that immunosuppressive adenosine plays a critical role in tumor resistance mechanisms and constitute a novel, clinically relevant target for RCC combination biotherapies.

The adenosine pathway mediated tumor resistance to OV therapies. OVs induced potent oncolysis, necrosis and inflammation. While these are desirable effects, leading to potent, initial antitumor and immunostimulatory effects, they also increased adenosine production, with subsequent immunosuppression, and tumor progression. Discussed herein is the role of the adenosinergic axis in tumor resistance/escape to vaccinia virus RCC oncolysis, and to develop rational, novel oncolytic virus-immunotherapy combinations targeting adenosine.

Data

Oncolytic Vaccinia Virus in Advanced Renal Cell Carcinoma

A phase 1 multicenter trial of the novel oncolytic vaccinia vector JX-594 (Pexavec) in combination with cemiplimab was conducted in patients with advanced renal cell carcinoma (NCT03294083). Results from the cohort of patients treated with **intravenous** Pexavec and cemiplimab are discussed herein. Systemic (IV) administration of the combination was safe and associated with antitumor activity in subjects with advanced clear cell RCC. The majority of patients had a reduction in the tumor burden (Fig 1A), including one complete response. This cohort included patients who failed RTKI based therapies were CPI naïve (Fig. 1A-1B).

Examined herein were the oncolytic potency and mechanisms of these viruses in clear cell RCCs of different genetic backgrounds (VHL mutant 786-0, A498, VHL wild type Caki-1), non-clear cell RCC (ACHN, papillary RCC), and murine renal cancer cells (RENCA). As

shown in Figures 2A-2F, potent and persistent cytotoxic effects were induced by JX-594 in human, and by mJX-594 in murine renal cancer cells, at a magnitude (even at low doses - MOI-0.1, Figs 2.A-E), rarely observed with other agents tested in the lab, including other oncolytic viruses. Moreover, observed herein were efficient viral replication in human (786-0, ACHN) and murine (RENCA) cells (Fig. 2F), confirming RCC permissiveness to viral infection.

Mechanisms of JX-594 mediated oncolysis

Functional proteomics analysis of JX-594 treated 786-0 cells was performed by reverse phase protein array (RPPA, performed by MD Anderson RPPA core facility) to gain knowledge on potential mechanisms of viral oncolysis. As shown in Figure 3A-3F, JX-594 had significant effects inducing cell cycle disruption, apoptosis, necrosis autophagy and cellular stress responses (Fig. 3B-3F). These virus mediated-cellular and molecular effects increased tumor immunogenicity. On the other hand, cell death (necrosis, apoptosis) increased adenosine production, which may have led to subsequent immunosuppression.

Effects of vaccinia virus on the adenosine axis in vitro

Viral oncolysis leads to release of damage associated molecular patterns (DAMPs); among those is ATP. While a marker of immunogenic cell death, ATP is, on the other hand, the precursor of immunosuppressive adenosine. In vitro, it was observed that treatment with JX-594 induced marked ATP release at MOIs or 0.1 and 1 (Fig. 4A). Oncolytic vaccinia virus also induced increased expression of the ectoenzyme CD73 (Figs. 4B, 4C) and CD39 (Figs. 4D, 4E) in human 786-0 RCC cell lines (fluorescence intensity decreased in cells treated with MOI=1 because of cell loss (detachment) caused by potent viral oncolysis at 72 hours). Next, investigated herein was the in vitro effects of a A2AR blockade in combination with vaccinia virus on viral replication and viral induced oncolysis. No statistically significant changes (p= NS, Student T test) in virus replication (Fig. 4F) or viral induced cytotoxicity (Fig. 4G) were observed in cells treated with the drug combination vs. single agents, suggesting adenosine blockade did not interfere with vaccinia viral dynamics. Similar results were observed in the RENCA model.

Effects of oncolytic viruses on the adenosine axis in vivo

To extend these findings to syngeneic renal cancer models, pilot experiments were conducted to determine the effects of mJX-594 in RENCA tumor bearing mice. The RENCA model was established as reported. In efficacy experiments, tumor bearing mice were treated

with either vehicle or intravenous mJX-594 (via tail vein) when tumors reached 5 mm in diameter. As shown in Fig. 6B, mJX594 treatment was associated with a significant delay in tumor progression compared to mock virus treated mice (Fig. 6A). However, no complete or prolonged responses were observed after 3 treatments of mJX-594 as a single agent. Correlative studies were performed from tumor bearing mice treated with 2 IV treatments with mJX-594. To ensure enough tumor tissue was available for tumor studies, virus or mock treatments started with larger tumors, at approximately 200 mm^3 (7 to 8 mm). Tumors were resected 5 days after last treatment for flow cytometry and IHC studies. Limited, preliminary flow cytometry studies and analyses were performed. Flow cytometry studies showed a marked increase in the CD3 (from 6.41% to 24.8%) and CD8 (from 19.2% to 57.8%) lymphocyte population, compared to mock treated mice (Fig. 6C for quantitative analysis and 6D, for t-SNE analysis). On the other hand, a marked increase was observed in PD-1 expression in lymphocytes, as well as increase in CD73 density (Fig. 6D, t-SNE plot). As shown in Figure 6C and 6D, CD73 expression was observed at high levels even in untreated tumors, and the expression was visibly increased after virus treatment (Fig. 6D, t-SNE plot). Preliminary correlative IHC studies from treated vs control tumors confirmed an increase in CD8 as well as a clear increase in the expression in CD39 and CD73 expression. These preliminary observations showing OV modulation of the adenosine axis supported that viral oncolysis upregulated the adenosine axis.

In vivo effects of vaccinia virus and adenosine receptor blockade

By enhancing tumor CD39 and CD73 expression, vaccinia virus treatment promotes tumor adenosine production, leading to an immunosuppressive microenvironment and reduced long term in vivo efficacy. Adenosine targeting strategies can enhance and prolong vaccinia virus (mJX-594) antitumor effects in immunocompetent renal cancer models.

Characterization of the role of the adenosinergic axis on tumor escape mechanisms to vaccinia virus in renal cancer.

While VV had shown early evidence of antitumor efficacy, durable complete responses were not common in the clinical setting. Findings herein indicated that tumor escape from OV therapies was mediated by upregulation of immunosuppressive adenosine. Herein, the spatial and temporal dynamics of VV mediated modulation of the adenosine axis were characterized, and immunophenotypic changes with functional immune gene signatures, cytokine expression and tumor progression were correlated. Transcriptomic analysis of immune signatures in RCC emerged as a clinically valid biomarker, providing both predictive

and mechanistic information that is clinically relevant to immunotherapy strategies in RCC, including A2AR inhibitors. Discussed herein are mechanisms of tumor escape to OV therapies, necessary to develop novel and better OV based combinatorial strategies.

Results

Among the mechanisms responsible for limited treatment efficacy, the adenosine pathway plays a role. Adenosine, a by-product of ATP, is produced in tumors during hypoxia (low oxygen), tissue necrosis, cell death and inflammation, and often, because of anticancer treatments. Tumor adenosine causes treatment resistance by suppressing antitumor immune responses, limiting the action of the immune cells activated by viruses and immunotherapies. Therefore, targeting the adenosine pathway was promising to overcome resistance to immune and virus therapies.

Although current tyrosine kinase (TKI) and checkpoint inhibitor (CPI) combination strategies have improved clinical outcomes in advanced RCC, approximately 14,000 patients are expected to die of this disease in 2022. Developing novel, safe, and effective biotherapies to overcome resistance remains an unmet need in RCC. Oncolytic viruses (OVs) are promising biotherapies that offer an advantage over conventional oncology drugs, or other forms of gene therapy, because they can be genetically engineered to selectively target, replicate in, and ultimately kill tumor cells. In addition, they also act as immunomodulatory agents. While much translational work with OVs has been done in multiple solid tumors, few studies have been conducted in the development of OVs as potential systemic biotherapies in RCC. Among oncolytic viral platforms, the oncolytic vaccinia virus (VV) holds great potential in RCC, as it induces potent oncolysis, targets tumor vasculature and modulates the immune system, leading to important antitumor effects.

Oncolytic viruses' long-term benefit in human cancer trials, alone or in combination has been limited, after transient beneficial effects. This represents a significant barrier that limits progress in the field. A possible reason for the limited long-term success of OV based viroimmunotherapies, includes the development of resistant mechanisms, caused by the treatment, leading to an immunosuppressive tumor microenvironment. Among those, the adenosine pathway is a pertinent one, as it promotes proliferation of immunosuppressive cells and inhibition of immune effector cells. The adenosinergic pathway components (CD39, CD73, A2AR) are overexpressed and associated with poor prognosis in this disease.

Further discussed herein are the in vivo mechanisms of long-term resistance to oncolytic vaccinia virus and relevant strategies to enhance VV's efficacy by blocking

immunosuppressive adenosine. The data herein shows potent oncolytic activity of JX-594, an attenuated, "armed" oncolytic VV currently on clinical trials, and its "murine" counterpart, mJX594, in human and murine renal cancer models. Demonstrated herein is that while initial in vivo tumor control is achieved, VV oncolysis induced expression of CD39 and CD73, to cleave ATP to AMP and adenosine, respectively. Moreover, combined adenosine receptor blockade and VV led to improved antitumor efficacy compared to single agents. The results highlight the role of virus induced adenosine production as a potential mechanism of resistance and supports targeting adenosine to augment the therapeutic efficacy of oncolytic viruses.

This study and these results confirmed the concept of targeting the adenosinergic pathway as a strategy to improve OV efficacy.

While resistance to targeted and immune-based therapies is a common phenomenon affecting thousands of RCC patients, current second line and beyond strategies continue to focus on targeting the VHL-HIF-VEGF axis or immune checkpoints. In fact, FDA approved therapies for advanced RCC, resistant/refractory to first line TKIs or TKI-CPI combinations consist of either alternative TKIs and/or single agent checkpoint inhibitors. Targeting the same (angiogenic and immune) pathways in treatment refractory RCC lacks innovation and has been of limited benefit, as evidenced by poor progression free and overall survival rates in recent clinical trials. The oncolytic vaccinia virus (VV) as a viral platform uses non-cross resistant mechanisms of cytotoxicity, vascular targeting (different from RTKIs), and immune modulation (different from CPIs).

Discussed herein is targeting immunosuppressive adenosine to overcome secondary resistance mechanisms induced by viral oncolysis. Adenosine production was enhanced by in vivo viral oncolysis and targeting the adenosinergic pathway overcame resistance to oncolytic virotherapy. The dynamics and consequences of OV's mediated adenosine pathway activation were characterized in renal cancer models, as a mechanism of tumor OV escape. Strategies herein include combining VV vectors with antibody or pharmacological adenosine blockade, with or without checkpoint inhibitor therapy (doublet or triplet combinations). This represents a departure from efforts limited at targeting PD-1 or CTLA4 to enhance OV activity. Moreover, mJX-594 was used as a systemic therapy agent.

Example 3: Targeting the Adenosine Pathway to Enhance Vaccinia Renal Cancer Oncolysis In Vivo

The oncolytic vaccinia virus (VV) is a biotherapy that is undergoing active clinical development in human cancer. JX-594 is an oncolytic VV expressing human GM-CSF that

was studied in human renal cell carcinoma (RCC). While VV induced potent oncolytic and antitumor immune responses, experience from clinical trials shows that single agent OVs do not induce prolonged benefits or frequent complete responses. This suggests that tumors develop adaptive resistance to the therapy following the initial antitumor effects, leading to tumor progression. The adenosine pathway is a biologically and clinically relevant target in RCC, as overexpression of CD39, CD73 and A2AR are associated with poor prognosis. It has also been associated with adaptive resistance to immunotherapeutic agents.

The objectives herein were to characterize the in vitro and in vivo effects of targeting the adenosine pathway on vaccinia virus RCC oncolysis.

Methods and Results

Cell culture: VHL mutant 786-0, A498, VHL wild type Caki-1, non-clear cell RCC (ACHN, papillary RCC) and murine renal cancer cells (RENCA) were maintained in DMEM containing 10% FBS at 37 °C and 5% CO2.

Viral vectors: mJX-594 (JX), provided by SillaJen, Inc., is a Western Reserve strain of vaccinia virus, engineered to express GM-CSF in the vaccinia thymidine kinase gene locus (TK deleted), under the control of the p7.5 promoter. (FIG. 10)

ATP Release: The cells were treated with JX-594 MOI 0.1 and 1 for 72 hours. Extracellular ATP was measured from supernatants using an ATP detection kit (Thermo Fisher Scientific).

Tumor models: Tumors were implanted by subcutaneous injection of 2 × 105 RENCA cells into the right flank of wild-type BALB/c mice. When tumors reached >50 mm3, mice were treated with three doses of either PBS or 1 × 107 of mJX-594 by IV injection every other day.

For combination studies, when tumors reached >50 mm3 mice were randomized to the following tx groups: Group 1: PBS. Group 2: 3 doses of 1.5 × 107 mJX-594 by IV injection every other day. Group 3: 28 doses A2AR inhibitor (shc58261, Selleckchem) 1mg/kg by IP injection every day. Group 4: 28 doses A2BR inhibitor (PSB1115, Tocris) 1mg/kg by IP injection every day. Group 5: mJX-594+ A2AR inhibitor. Group 6: mJX-594+ ABAR inhibitor. Group 7: mJX-594+A2AR+A2BR inhibitor. Adenosine receptor inhibitors were given 5 days after last VV treatment.

Tumor studies: RENCA bearing mice were given mJX-594 (1 × 107 PFU, IV x2) or mock treatments. Tumors were resected 5 days after last treatment for tumor studies,

including Flow cytometry and IHC studies, to characterize the effects of VV on the immune microenvironment.

Figures 11A-11F shows the resulting in vitro effects of oncolytic vaccinia virus in RCC. Figure 12 shows the generation of adenosine in the tumor microenvironment leads to the suppression of multiple immune subsets. Figures 13A-13G show in vitro effects of vaccinia virus on the adenosine pathway in human RCC. Figures 14A-1C show in vivo effects of mJX-594 on murine renal cancer immune microenvironment. Figures 15A-15D show in vivo effects of mJX-594, A2AR, and A2BR inhibitors on RENCA progression in vivo.

Conclusions

Potent and persistent cytotoxic effects were induced by JX-594 in human, and mJX-594 in murine renal cancer cells. Mechanisms of VV oncolysis were presented in poster UO83.

While Vaccinia virus induced potent in vitro and in vivo renal cancer oncolysis, VV induced ATP release in vitro, as well as upregulation of CD73 and CD39 in renal cancer models vitro and in vivo. In vivo CD39 and CD73 upregulation were demonstrated by IHC as well as flow cytometry analysis of tumors after treatment with mJX-594. In addition, VV induced PD-1 expression in treated tumors.

mJX-594 given in combination with adenosine receptor blockers (A2AR and A2BR) overcame tumor escape to VV. Dual Adenosine (A2AR and A2BR) blockade led to enhanced in vivo antitumor effects compared to single agents or doublets.

Results suggest that, while VV induces potent in vitro oncolysis and cell death, a potential consequence of viral oncolysis is increased immunosuppressive adenosine production, by upregulation of the adenosine rate limiting enzymes CD73 and CD39.

As VV vectors and adenosine receptor blockers are undergoing clinical development, the results have the potential to be rapidly translated to novel clinical trials in RCC and other cancers.

Example 4: Systemically Administered Oncolytic Vaccinia Virus Enhances the In Vivo Antitumor Effects of RTKI and Anti-PD-1 Based Therapies in an Immunocompetent Renal Cancer Model

- Worldwide incidence/deaths: ~271,000/100,000
- United States incidence/deaths: ~82,000/14,500
- Males: 6th most common cancer

- Females: 10th most common cancer
- Average Age at Diagnosis: 64
- Risk Factors: Smoking, Obesity, Occupational
- Exposures, Family History

Stage at Presentation:

- Localized: 60%-70%
- Loco-Regional: 10%-15%
- Metastatic: 20%-25%
- Clear cell RCC is an immunogenic cancer with a complex tumor microenvironment.
- Tumor angiogenesis is a predominant feature, due to VHL silencing, leading to HIF-2α accumulation.

While significant advances have been made in the treatment of metastatic renal cell carcinoma (RCC), most patients develop treatment resistance and succumb to progressive disease. Limited options exist for patients checkpoint inhibitor (CPI) and/or receptor tyrosine kinase inhibitor (RTKI) based therapies, underscoring the urgent need to develop novel strategies for RCC. The oncolytic vaccinia virus (VV) is a promising antitumor agent undergoing clinical development in RCC and other cancers. Characterized herein are in vivo effects and mechanisms of oncolytic VV in combination with RTKIs and checkpoint inhibitors in RCC. As a single agent, VV showed limited benefit, underscoring the need to find novel virus-drug combinations for therapeutic gain.

Methods

Viral Vectors, Cell Lines, and Reagents. Human (786-0, A498, Caki-1, and ACHN) and murine (Renca), RCC cell lines were obtained from ATCC. Cell culture media was obtained from Corning. JX-594 and mJX-594 VV vectors were provided by SillaJen Inc.). Cabozantinib was obtained from Selleckem (Cat number: BMS-907351/XL-184).

Cytotoxicity Assays (xCELLigence). Cells were plated into 6 well plates (100,000 cells per well) and after 24 hours, they were infected with vaccinia virus (JX-594 or mJX-594) for 2 hours in Opti-MEM. After incubation, cells were washed, trypsinized and plated in xCELLigence plate (4,000 cells/well in quintuplicate) for cell growth real time monitoring. Data are displayed as average cell growth relative to controls (+/- SD).

Viral replication. Cell lines were infected with VV at an MOI of 0,1 for 24 to 120 hours. At each time point, cell lysates were prepared and underwent three cycles of

freeze/thaw. Serial dilutions of supernatants/lysates were prepared (in serum-free Opti-medium) and 1 ml of each dilution was plated onto a monolayer of U2OS cells (106 cells/well of a 6-well plate) the day before initiating the plaque assay. After 2 hours of incubation at 37°C, a semi-solid overlay of carboxyl methylcellulose in α-MEM supplemented with 10% FBS was applied. Plates were incubated for an additional 48 hours, at which time plaques were fixed and stained with 0.1% crystal violet in 80% methanol.

Reverse Phase Protein Array (RPPA). Cells were treated with JX-594 (MOI=0.1); cell lysates were obtained at 48 hours for RPPA analysis (MD Anderson RPPA core).

In Vivo Studies. Murine renal cancer (RENCA) cell lines (4.5 × 105) were implanted via subcutaneous injection into the right flank of immunocompetent BALB/c mice. Once tumors reached 4-5 mm in diameter, mice were randomized into 4 groups n=8-9 mice per group (See figure 4.A). Tumors were measured with calipers three times a week and tumor volume was calculated with the following formula: Width2 x Length x 0.52. The study endpoints were tumor progression. For tumor studies, RENCA bearing mice were treated as above. Tumors were resected 5 days after last virus treatment, and tumor sections underwent IF staining, KI67, CD31, A27L (VV protein), and TUNEL assay using commercially available antibodies/kits. Microphotographs were taken using Leica microscope. Additional mice were treated for tumor studies, including flow cytometry and immunofluorescence studies using standard protocols.

Statistical analysis. In vitro data are presented as means ± SD. Results from in vivo studies are shown as means ± SEM. Statistical analysis among groups was performed by ANOVA, and sub-group comparisons were made with the Tukey–Kramer test, as appropriate. p value < 0.05 were considered statistically significant. All statistical tests were two-sided.

Results

Figures 20A-20J show in vitro effects of oncolytic vaccinia virus alone and with Cabozantinib on human and murine renal cancer. Figures 21A-21D show mechanisms of VV oncolysis in vitro. Figures 22A-22D show in vivo effects of mJX-594 on murine renal cancer immune microenvironment. Figures 23A-23K show in vivo effects of mJX-594 and Cabozantinib on syngeneic murine renal cancer.

Conclusions

JX-594 and mJX-594 exerted potent and significant in vitro oncolytic effects on human and murine renal cancer. VV induced RCC oncolysis by downregulating survival, cell cycle, hypoxia and angiogenesis pathways, while upregulating pro-apoptotic pathways. In

vitro, Cabozantinib did not interfere with VV's viral replication or oncolytic effects in human and murine renal cancer.

In vivo studies demonstrated that mJX-594 in combination with Cabozantinib and PD-1 blockers was associated with significantly improved antitumor effects compared to single agents, as well as improved survival.

The Vaccinia Virus significantly enhanced the antitumor efficacy of checkpoint inhibitors and TKIs, in an immunotherapy resistant syngeneic renal cancer model. The triplet combination had the potential to enhance cure rates and improve outcomes in advanced RCC.

Example 5: Characterization of Adenosine Pathway

Spatial and temporal characterization of mJX-594 mediated changes on the renal cancer adenosine axis and immune cell infiltrates.

RENCA is an accepted preclinical model to investigate novel immune based strategies in renal cancer, including OV therapies, as its immune microenvironment has been well characterized. Therefore, the RENCA model is the most appropriate available model (acknowledging its limitations) to achieve results herein.

Mouse studies.

Procedures with animals: $4x10^5$ RENCA cells will be implanted into the right flank of BALB/C mice. When tumors reached 200 mm^3 (larger tumors to ensure enough tissue is available), mice will be mock treated (PBS 100 µl via tail vein) or treated with mJX-594: $1x10^7$ PFU x 2, one day apart. Tumor volumes will be calculated 3 x week to follow progression. Six mice per group will be sacrificed at days 5 (early), 12 (intermediate) and 19 (late) after treatment. Tumors and spleens will be weighed and extracted for correlative studies, as described below. Total number of mice: 36 (6 mice per group, 2 groups, 3 timepoints).

Flow cytometry.

Tumors and spleens were processed for flow cytometry as reported. Spleen studies will be performed to determine the systemic (in contrast to local, intra-tumor) immunomodulatory effects of VV treatment. Tissues will be dissected and sectioned into small fragments, digested (1 mg/mL of collagenase in complete RPMI media) prior to using Gentle MACS Dissociator. Cell suspensions will then be passed through a 70 µm nylon strainer to obtain a single cell population. For spleen studies, cell suspension will be centrifuged, and the pellet re-suspended in 1mL of RBC lysis buffer and incubated for 3 min

at RT. Single cell suspensions will be incubated with LIVE/DEAD™ Fixable Blue Dead Cell Stain (ThermoFisher) for 30 min at 4°C and washed x 2 in FACS buffer prior to staining in order to distinguish live from dead cells. Cells will then be incubated with Fc block for 20 min at 4°C and incubated for 30 min at 4°C with antibodies listed in Tables 1 and 2. Stained cells will be washed by FACS buffer to remove unbound antibodies and plated for flow cytometric analysis using the Aurora spectral flow cytometer (Cytek™ Biosciences). 100µL of sample will be counted and events analyzed as below.

Table 1. Antibodies for flow cytometry.

Marker	**Company**
CD45 (30-F11)	BD Biosciences
CD3 (145-2C11)	BD Biosciences
CD4 (GK1.5)	BioLegend
CD8a (53-6.7)	BD Biosciences
CD19 (6D5)	BioLegend
Ly-6C (HK1.4)	BioLegend
Ly-6G (1A8)	BD Biosciences
CD11b (m1/70)	BD Biosciences
CD11c (N418)	BioLegend
MHC II (2G9)	BD Biosciences
CD49b (HMα2)	BD Biosciences
CD117 (2B8)	Thermo Fisher Scientific
C103 (M290)	BD Biosciences
Mannose Receptor (857615)	R&D Systems
F4/80 (BM8)	BioLegend

Table 2. Antibodies for flow cytometry.

Marker	**Company**
CD366 (RMT3-23)	BioLegend
CD25 (PC61)	BD Biosciences
FoxP3 (FJK-16s)	Thermo Fisher Scientific
NKp46 (29A1.4)	BioLegend
CD163 (TNKUP)	Thermo Fisher Scientific

CD68 (FA-11)	BioLegend
CLEC9A (10B4)	BD Biosciences
CD44 (IM7)	Thermo Fisher Scientific
CD73 (eBioTY/11.8(TY/11.8))	Thermo Fisher Scientific
CD39 (Duha59)	BioLegend
Adenosine A2a Receptor (7F6-G5-A2)	Novus Biologicals
Adenosine A2b Receptor	Novus Biologicals
PD-1 (J43)	BD Biosciences
CD274 (10F.9G2)	BioLegend
Live/Dead Fix Blue	Thermo Fisher Scientific

Data analysis.

Flow cytometry data will be analyzed using FlowJo (v10.8.1, BD Biosciences). To normalize the number of events, each .fcs file will be down sampled to the smallest number of events collected among the six files in the experiment using FlowJo's Export/Concatenate function. The down sampled files will then be concatenated using the same function. All further analyses will be performed using the concatenated file. After initial gating using forward scatter (FSC) and side scatter (SSC), live cells will be identified as Live/Dead Fixable Bluelo/-. Further downstream gating will then be done to identify immune cell phenotypes. A tSNE analysis will run on the live cell gate using the embedded tSNE function, with the algorithm parameters described in Figures 6A-6G.

The frequency of adenosine pathway markers (CD39, CD73, A2AR and A2BR) in immune (CD45+) and non-immune (CD45-) cells in control and treated tumors at early, intermediate and late time points will then be determined. The antibody panels described above will then be characterized major components of the innate (NK cells, APC, M1 and M2 macrophages, MDSCs) and a tumor infiltrating lymphocyte (TIL) panel (table 2), which differentiated the major CD4 and CD8 lymphocyte subsets, including cytotoxic (CD8), activated (CD8+/GranzymeB) and exhausted (TIM3+, TIM3+/PD1+) CD8 cells. Comparisons will be made between treated and untreated groups in each timepoint, as well as intragroup comparisons using time as a variable. Comparisons of quantitative data (means) between groups will be performed by two-way ANOVA with treatment and time as factors. Significant overall effects will be further examined with post-hoc testing, with multiple comparison corrections to control the overall Type I error rate. Changes in the expression of

adenosine pathway markers will be correlated with changes in the immune cell populations described above, and with tumor volume changes over time. The results herein will confirm that mJX-594 oncolysis induced the adenosine pathway, shifting immune microenvironment from immunostimulatory to immunosuppressive.

Tumor IHC staining.

Further discussed herein is the characterization of changes in the distribution and location (within tumor tissues) of adenosine pathway markers and changes in immune cell infiltrates as a result of systemic therapy with mJX-594.

Procedures with mice.

Mice will be treated, and tumors will be extracted at similar time points. Resected tumors will be weighed and sectioned in two halves (one for PFA fixing and paraffin embedding, and the other will be flash frozen in OCT, and stored at -80C). Total numbers of mice: 36 Balb/C mice (18 male/18 female).

IHC studies.

Paraffin embedded tumor sections (5 micron) will be prepared for hematoxylin and eosin (H&E) staining, as well as IHC analysis of adenosine markers (CD39, CD73, A2AR, A2BR), as well as CD3, CD4, CD8, Granzyme B, FoxP3, NK cells, macrophages (total, and M1/M2), MDSCs, as well as PDL-1 and vaccinia virus using commercially available antibodies (see Table 3). Also performed herein will be IHC staining of tumor capillaries (CD 31) for determination of micro vessel density.

Table 3. Antibodies for IHC.

Marker	**Antibody name/Cat. Number**	**Company**
CD73	CD73/13160	Cell Signaling
CD39	CD39/223842	Abcam
A2AR	A2AR/3461	Abcam
A2BR	A2BR/ab229671	Abcam
CD39	CD3/16669	Abcam
CD4 T cells	CD4/183685	Abcam
CD8 T cells	CD8/217344	Abcam
Activated T cells/NK	Granzyme B/441535	Cell Signaling
Tregs	FoxP3/12653	Cell Signaling
NK	NK1.1 PK136/MA1-70100	Thermo Fisher
MDSC	MDSC/CD11b/133357	Abcam
Endothelial cells	CD31/MA5-16337	Thermo Fisher
Vaccinia Virus	Ab35219	Abcam

Image capture and data analysis.

IHC images were acquired using the SLIDEVIEW VS120 slide scanner, (Olympus Corporation) designed for high throughput image capture research and pathology. Images were processed using the Olympus OlyVIA 2.9 software. IHC images were analyzed in a blinded manner, for determination of viable vs. necrotic areas (H&E), virus presence, adenosine pathway markers and immune infiltrates as described. Blinded analysis was focused on determination of the frequency and spatial (central vs peripheral) marker distribution within the tumor (peripheral vs central regions), and staining intensity (Fig. 6). Intergroup (treated vs control) and intragroup (different time points within one group) comparisons were performed to determine treatment effect and time dependent effects among groups using statistical methods described above.

Determination of immune gene signatures and cytokine expression.

Discussed herein are the molecular mechanisms by which virus mediated modulation of the adenosine pathway induced the tumor immunophenotypes observed. Determined herein are the effects of VV on tumor immune gene signatures (targeted transcriptomic analysis) and tumor cytokine expression, to establish correlations between gene and cytokine patterns with immunophenotypic changes observed by flow cytometry and IHC.

Immune gene profiling.

Characterization of tumor immune pathways after IV administration of mJX-594 will be performed using the 770-gene panel mouse nCounter PanCancer Immune Profiling (Nanostring Technologies).

Procedures with animals.

Experiments will be performed from RENCA controls or mJX-594 treated tumors. Four mice per group (2 male/2 female) will be sacrificed and tumors extracted at early (5 days) and late (19 days) time points.

Methods.

Assays will be performed using PanCancer Immune profiling, following the manufacturer's instructions, with standard input of 100 ng and the codeset with the current XT chemistry using a NanoString nCounter Gen2 scanner and Prep-Station (NanoString Technologies). Samples will be verified by Agilent Bioanalyzer 2100 using Nano 6000 chips to determine RNA integrity number. Data was exported from NanoString's nCounter Raw Code Count Collector tool and loaded into R environment (v3.2.4) for analysis. Data

normalization will be performed using R package NanoStringNorm (v1.1.21), with a combination of multiple normalization steps.

Data analysis:

For differential expression analysis, R package limma (v3.26.9) will be used to perform two group comparison test, and raw *P* values based on empirical Bayes moderated *t* statistics will be adjusted for multiple testing with Benjamini–Hochberg FDR correction. Pathway enrichment and functional analyses will be generated using QIAGEN's Ingenuity Pathway Analysis (Qiagen). Immune gene signatures will be generated with the nSolver 2.6 and the mouse Pan Cancer Immune analysis software (NanoString Technologies). Gene expression data will be analyzed by the Sylvester Cancer Center Biostatistics/Bioinformatics core (fee for service).

Analysis of tumor cytokine profiles.

To further characterize tumor responses vs. tumor escape over time, changes in tumor cytokines will be assessed and immunophenotypes (aim 1. A and b), and immune gene signatures will be correlated. Proteome Profiler Mouse Cytokine Array Kit (R&D Systems), will be used to determine levels of 40 cytokines/chemokines, including Th1 (IFN gamma, IL-12, IL-2, TNF-alpha) and Th2 (IL-4, IL-6, IL-10).

Procedures.

RENCA tumor implantation, treatments, and tumor extraction (Day 5 and 19 after treatment) will be performed. Tissues were homogenized and prepared as per manufacturer's recommendations.

Data analysis.

Cytokine expression will be semi quantitatively analyzed by pixel density of spots (in duplicate) using image analysis software. Comparisons will be made between individual cytokine levels between treated and untreated tumors, and over time, using ANOVA, with post hoc analysis.

Results.

Based on the data, mJX-594 will induce a significant increase in tumor CD39 and CD73, leading increased adenosine, and in the tumor microenvironment form immunostimulatory (at early timepoints, day 5) to immunosuppressive (days 12 or 19), characterized by increased MDSC, M2 macrophages and Tregs, as well as increase in

exhausted CD8 cells. These findings will be associated with a "myeloid" gene signature, wherein the myeloid signature genes will be associated with immunosuppression. Finally, a predominant immunosuppressive cytokine pattern will be observed at later time points, with predominance of immunosuppressive Th2 cytokines, such as IL-4, IL-6, IL-10, which will be known to be expressed in the RENCA model. Increases in CD39 and CD73, and an immunosuppressive microenvironment will be more marked in "late" tumors, as they became more necrotic, amplifying, and perpetuating an immunosuppressive microenvironment and tumor escape.

Determining the effects and mechanisms of adenosine blockade in combination to vaccinia virus and anti PD-1 therapies in syngeneic RCC models

Targeting adenosine will overcome tumor escape from OV therapies alone or in combination with checkpoint inhibitors. Determined first will be the degree to which antibody or pharmacological inhibition of immunosuppressive adenosine enhanced mJX-594 efficacy ("pick the winner" trial). Once the optimal "doublet" is determined, it will be tested whether that adenosine blockade further enhanced the effects of vaccinia/anti-PD-1 combinations, by comparing a triple therapy (mJX-594, PD-1 and adenosine blockade), against doublets (mJX-594+anti PD-1, mJX-594+ adenosine blockade or anti-PD-1+ adenosine blockade). Correlative tumor studies will be performed to determine the effects of the combination therapies on immune gene signatures. Finally, immune depletion experiments will be performed to elucidate the role of effector cell type on the benefits observed by the triple vs double combination therapies.

Efficacy studies of mJX-594 in combination with adenosine blockade (pick "the winner") - In vivo combination studies.

mJX-594 will be combined with either antibody or pharmacological adenosine blockade and compared with appropriate controls as shown in Figures 8A-8B. Treatments will be administered to 6 groups of mice as in Fig 8. mJX-594 (groups B, E, F) given intravenously at a dose of $1x10^7$ PFU diluted in 100 µL PBS x 3 doses, one day apart, or PBS (groups A, C). Anti CD73 monoclonal antibodies (groups C, E), (TY/23, which inhibit its enzymatic activity, catalog # BE0209, BioXCell, Lebanon, NH), or Rat IgG2a Isotype Control (clone 2A3, BioXCell, groups B, D) will be administered intraperitoneally (IP) at a dose of 200 µg, starting at day 7 of treatment (2 days after virus or PBS injection) for 3 doses, 3 days apart. The A2AR inhibitor, SCH58261 (Selleckchem, Houston, TX, groups D, F) or PBS (for controls) will be administered IP at a dose of 1 mg/kg, daily, for 21 days. Endpoints:

Tumor progression (tumor volume), survival (time to sacrifice), safety (weight changes, signs of distress, overall well-being. Total Mice #: 10 mice per group (5 males/5 females) x 6 groups= 60 mice. These experiments will determine the best virus-adenosine blockade strategy to be used. The "winner" will be picked from the combination associated with significantly improved antitumor effects, survival, and safety.

Effects of adenosine blockade in combination to vaccinia virus and anti- PD-1 therapies in syngeneic RCC models.

The objectives of this in vivo experiment are to demonstrate the contribution of adenosine blocking strategies on the efficacy of mJX-594+anti-PD-1 combinations (JX-594 -Pexavec- and cemiplimab are currently being pursued in the clinic by the PI). The adenosine blocking strategy (antibody or pharmacological) that best synergizes with vaccinia virotherapy will be used for experiments using triplets. To ensure inclusion of all appropriate controls, a total of 7 groups will be tested, as shown in figure 9A.

Treatment regimens.

Treatments will be administered to 8 groups of mice as in fig 9. mJX-594 (groups B, F, G and H) will be given intravenously at a dose of $1x10^7$ PFU diluted in 100 µL PBS x 3 doses, one day apart,; Anti-mouse PD-1 Monoclonal Ab (clone RMP1-14, BioXCell, groups C, E, F, and H), given intraperitoneally at a dose of 200 µg, for 3 doses, starting at day 7 of treatment, 3 days apart; Adenosine blockade (groups D, E, G, H, either Anti CD73 antibodies (TY/23, catalog # BE0209, BioXCell, Lebanon, NH), administered IP at a dose of 200 µg,, for 3 doses, 3 days apart, starting at day 7 of treatment, *or* SCH58261, 1 mg/kg IP daily for 21 days, starting at day 6 of treatment. . Endpoints: Tumor progression (tumor volume), and survival (time to sacrifice). Total number of mice: 10 mice per group (5 males and 5 females) x 6 groups= 80 mice.

Tumor and functional immune studies.

Determined herein will be changes in the immune gene signatures associated with response in the treatment combinations, and the main mediator of enhanced antitumor immune responses as a result of the combinations, with immune depletion experiments. i. Immune gene signatures. Examining gene signatures in the triplet combination and comparisons with control and doublets will be focused on. Gene signatures procedures and analysis will be done as described herein. If necessary, changes in specific gene signatures (pathways) will be validated with IHC/IF or flow cytometry, as appropriate.

Immune depletion experiments.

To determine the role of immune effector cells on the antitumor responses from the triple therapy (mJX-594, anti-PD1, adenosine blockade), depletion experiments will be performed, using commercially available antibodies against mouse CD4 (clone GK 1.5, BioXCell), CD8 (clone 53-6.7, BioXCell), and natural killer (NK) cells (NK1.1, clone PK 136, BioXCell). Mouse studies. RENCA bearing mice established as above. When tumors reach 4-5 mm, mice will be randomized into 4 groups (8 mice per group) and treated as per Figure 9B. As the objective of the immune-depletion experiments is to "lose" the therapeutic effect of the combination, the "control" group will be group of mice (A) treated with the three-agent combination, and the experimental arms will be the treatment +targeted depletion of effector cells (groups B, C, D). Treatments with mJX-594, anti-PD1 antibody and adenosine blockade were administered. Anti CD4, CD8 and anti NK antibodies will be administered IP (200 μg of each antibody, IP) x 3 treatments, every three days, starting on day 1 of treatment. The endpoints will be tumor progression, safety, survival (time to sacrifice).

Data Analysis.

Tumor growth data (tumor volumes) will be analyzed by growth curve models to determine whether significant differences existed in rates of tumor progression among treatments. Differences in tumor volumes will be analyzed by one-way ANOVA, followed by post-hoc analyses with correction for multiple comparisons. Survival will be analyzed using Kaplan-Meier curves and log-rank tests. Analysis of tumor stromal, apoptosis, and proliferation markers will be done by one-way ANOVA with treatment as a factor and post-hoc testing as appropriate. Analysis of gene expression signatures will be performed with guidance of the Sylvester Cancer Center bioinformatics core facility.

Other advantages which are obvious, and which are inherent to the invention, will be evident to one skilled in the art. It will be understood that certain features and sub-combinations are of utility and may be employed without reference to other features and sub-combinations. This is contemplated by and is within the scope of the claims. Since many possible embodiments may be made of the invention without departing from the scope thereof, it is to be understood that all matter herein set forth or shown in the accompanying drawings is to be interpreted as illustrative and not in a limiting sense.

CLAIMS

What is claimed is:

1. A method for treating a cancer, comprising administering to a subject in need thereof an oncolytic vaccinia virus and a tyrosine kinase inhibitor.

2. The method of claim 1, wherein the oncolytic vaccinia virus encodes GM-CSF.

3. The method of claim 1, wherein the oncolytic vaccinia virus is JX-594.

4. The method of claim 1, wherein the oncolytic vaccinia virus is mJX-594.

5. The method of claim 1, wherein the oncolytic vaccinia virus is a Lister strain.

6. The method of claim 1, wherein the oncolytic vaccinia virus is a Copenhagen strain.

7. The method of any one of claims 1-6, wherein the oncolytic vaccinia virus is a replicative oncolytic vaccinia virus.

8. The method of any one of claims 1-7, wherein the tyrosine kinase inhibitor comprises afatinib, alectinib, axitinib, bosutinib, cabozantinib, ceritinib, cobimetinib, crizotinib, dabrafenib dasatinib, erlotinib, gefitinib, ibrutinib, idelalisib, imatinib, lapatinib, lenvatinib, nilotinib, osimertinib, palbociclib, ponatinib, pazopanib, regorafenib, ruxolitinib, sorafenib, sunitinib, trametinib, vandetanib, vemurafenib, pharmaceutically acceptable salts thereof, and combinations thereof.

9. The method of any one of claims 1-8, wherein the tyrosine kinase inhibitor comprises cabozantinib.

10. The method of any one of claims 1-9, wherein the cancer is renal cell carcinoma.

11. The method of claim 10, wherein the renal cell carcinoma comprises Stage I, Stage II, Stage III or Stage IV renal cell carcinoma.

12. The method of any one of claims 10-11, wherein the renal cell carcinoma comprises conventional (clear-cell) renal cell carcinoma, papillary renal cell-carcinoma, chromophobe renal carcinoma, onco-cytoma, or collecting-duct carcinoma.

13. The method of any one of claims 1-12, wherein the oncolytic vaccinia virus and the tyrosine kinase inhibitor are administered in combination with at least one additional cancer therapy.

14. The method of claim 13, wherein the additional cancer therapy comprises surgery, chemotherapy, immunotherapy, ionizing radiation, or a combination thereof.

15. The method of claim 14, wherein surgery comprises a radical nephrectomy, simple nephrectomy, partial nephrectomy, arterial or tumor embolization, or any combination thereof.

16. The method of any one of claims 14-15, wherein the immunotherapy comprises administering an immune checkpoint inhibitor.

17. The method of claim 16, wherein the immune checkpoint inhibitor comprises an anti-PD1 antibody.

18. The method of claim 17, wherein the anti-PD1 antibody comprises pembrolizumab, nivolumab, cemiplimab, avelumab, or any combination thereof.

19. The method of claim 17, comprising administering to a subject in need thereof the oncolytic vaccinia virus, cabozantinib, and the anti-PD1 antibody.

20. The method of any one of claims 1-19, wherein administration occurs intratumorally, intravenously, intraarterially, intraperitoneally, or any combination thereof.

21. A method for treating a cancer, comprising administering to a subject in need thereof an oncolytic vaccinia virus and an adenosine receptor blocker.

22. The method of claim 21, wherein the oncolytic vaccinia virus encodes GM-CSF.

23. The method of claim 21, wherein the oncolytic vaccinia virus is JX-594.

24. The method of any one of claims 21-23, wherein the adenosine receptor blocker comprises an A2AR inhibitor.

25. The method of claim 24, wherein the A2AR inhibitor comprises SCH58261.

26. The method of any one of claims 21-25, wherein the adenosine receptor blocker comprises an A2BR inhibitor.

27. The method of claim 26, wherein the A2BR inhibitor comprises PSB1115.

28. The method of any one of claims 26-27, wherein the adenosine receptor blocker comprises the A2AR inhibitor and the A2BR inhibitor.

29. The method of any one of claims 21-28, wherein the cancer is renal cell carcinoma.

30. The method of claim 29, wherein the renal cell carcinoma comprises Stage I, Stage II, Stage III or Stage IV renal cell carcinoma.

31. The method of any one of claims 29-30, wherein the renal cell carcinoma comprises conventional (clear-cell) renal cell carcinoma, papillary renal cell-carcinoma, chromophobe renal carcinoma, onco-cytoma, or collecting-duct carcinoma.

32. The method of any one of claims 21-31, wherein the oncolytic vaccinia virus and the adenosine receptor blocker are administered in combination with at least one additional cancer therapy.

33. The method of claim 32, wherein the additional cancer therapy comprises surgery, chemotherapy, immunotherapy, ionizing radiation, or a combination thereof.

34. The method of claim 33, wherein surgery comprises a radical nephrectomy, simple nephrectomy, partial nephrectomy, arterial or tumor embolization, or any combination thereof.

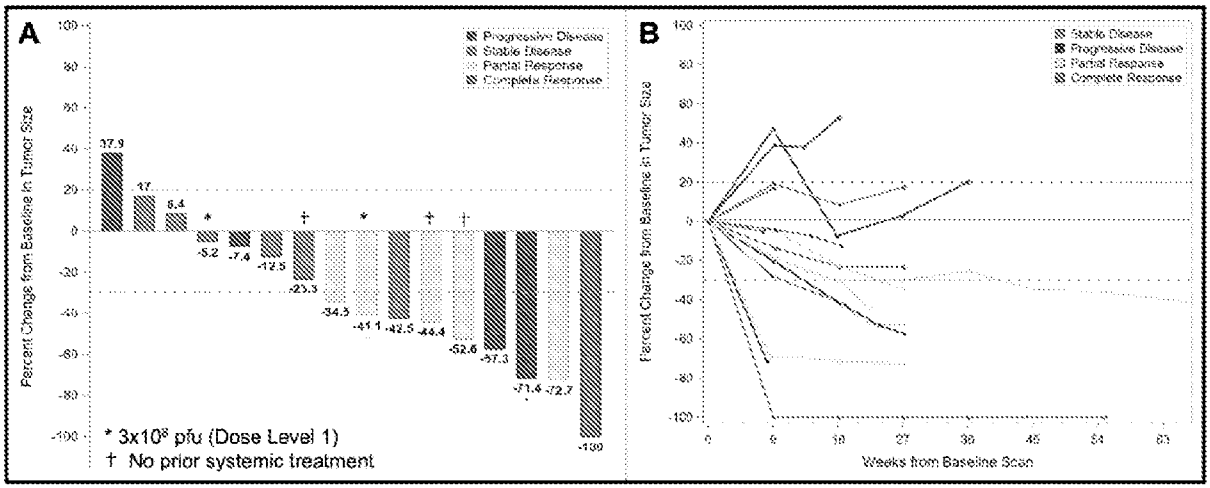
35. The method of any one of claims 33-34, wherein the immunotherapy comprises administering an immune checkpoint inhibitor.

36. The method of claim 35, wherein the immune checkpoint inhibitor comprises an anti-PD1 antibody.

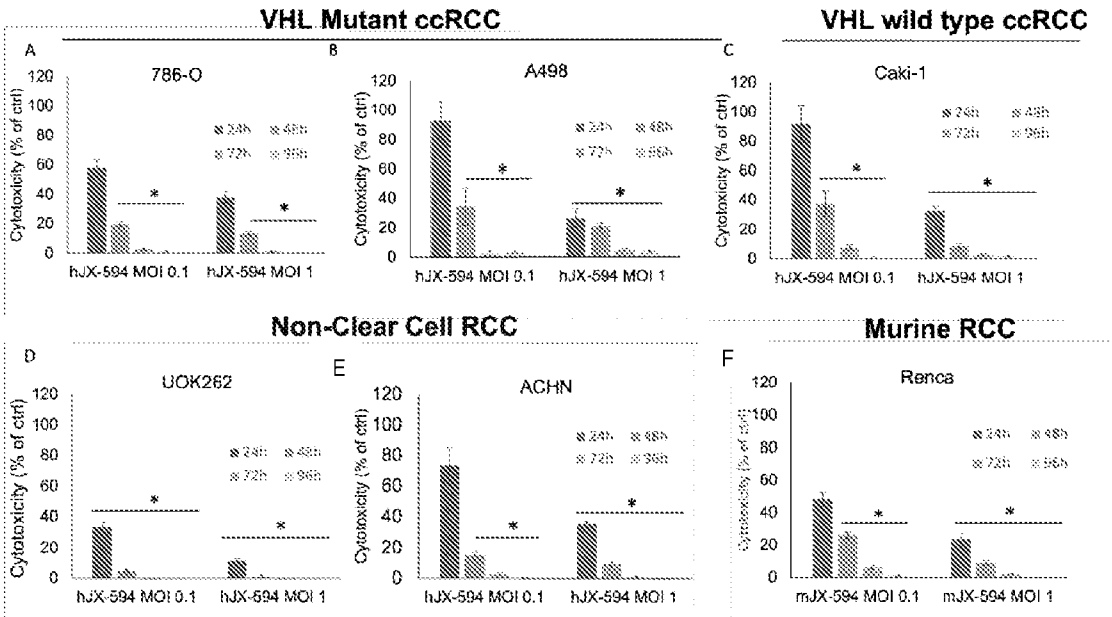
37. The method of claim 36, wherein the anti-PD1 antibody comprises pembrolizumab, nivolumab, cemiplimab, avelumab, or any combination thereof.

38. The method of any one of claims 21-37, wherein administration occurs intratumorally, intravenously, intraarterially, intraperitoneally, or any combination thereof.

FIGS. 1A and 1B



FIGS. 2A-2F



FIGS. 3A-3F

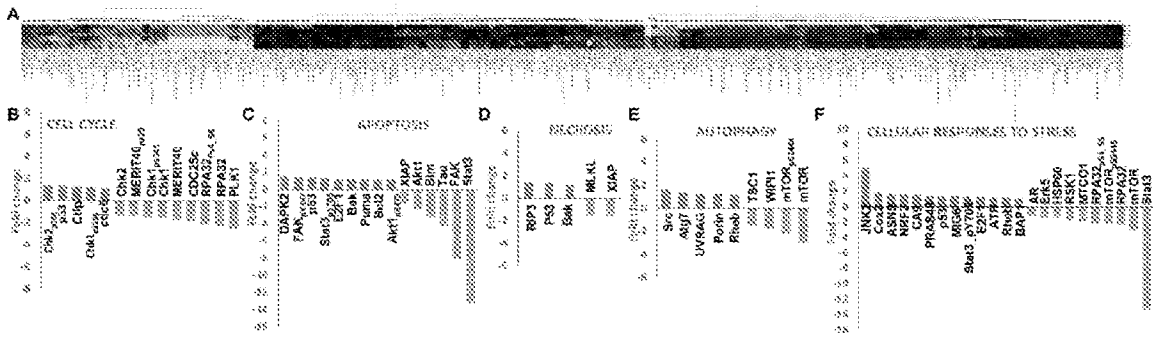
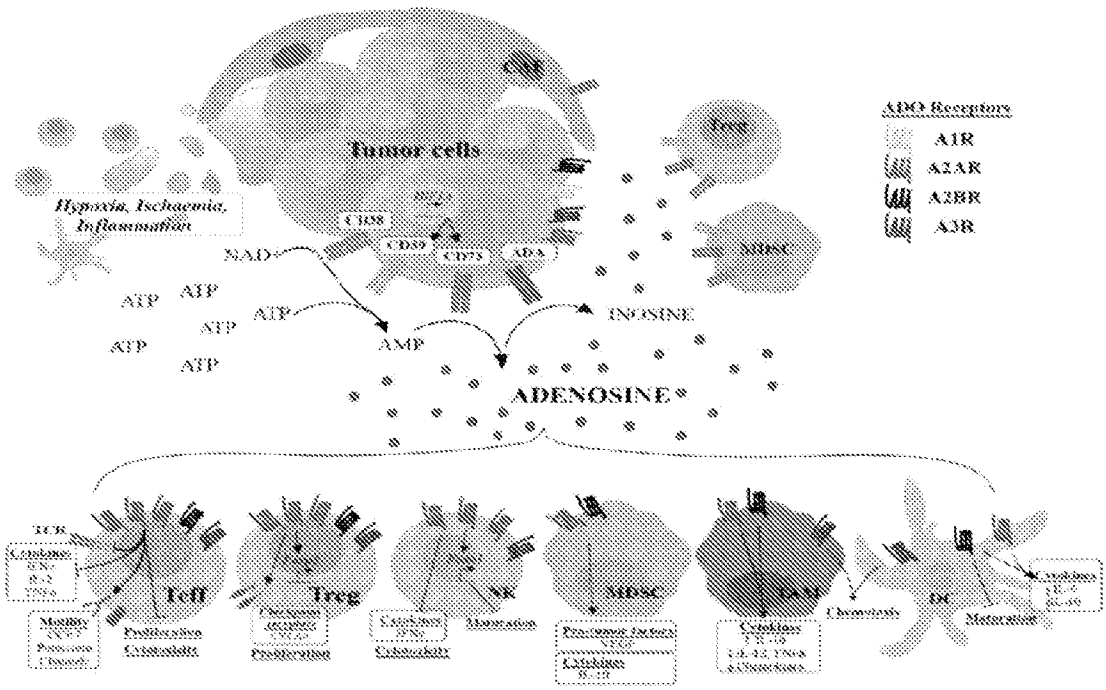
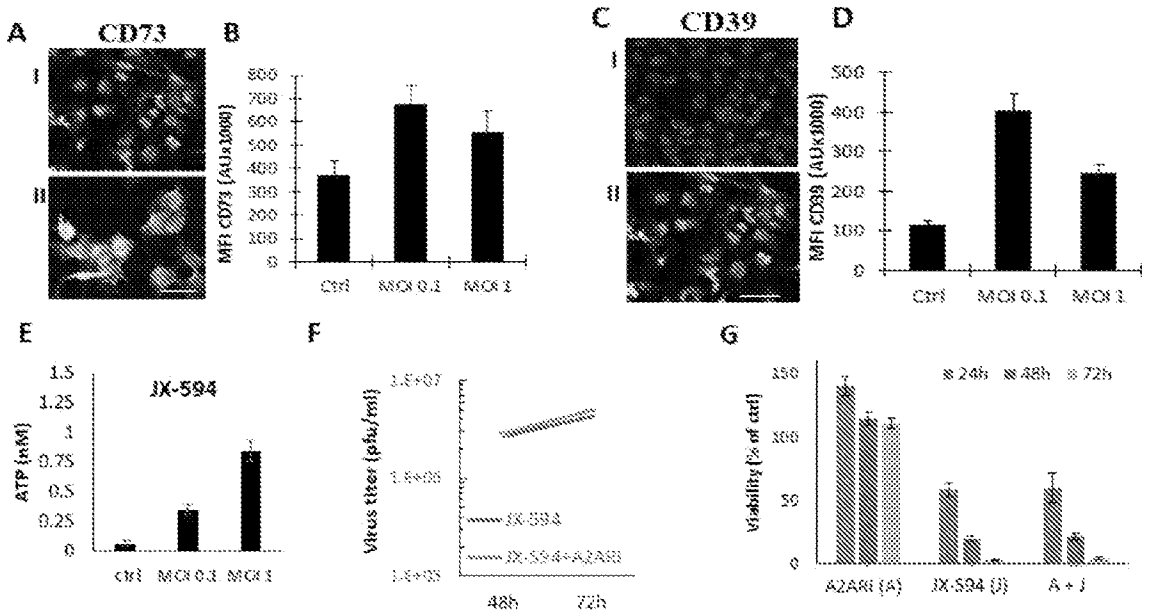


FIG. 4



(**Reference:** *Int. J. Mol. Sci.* **2018**, *19*(12), 3837; https://doi.org/10.3390/ijms19123837)

FIGS. 5A-5G



FIGS. 6A-6C

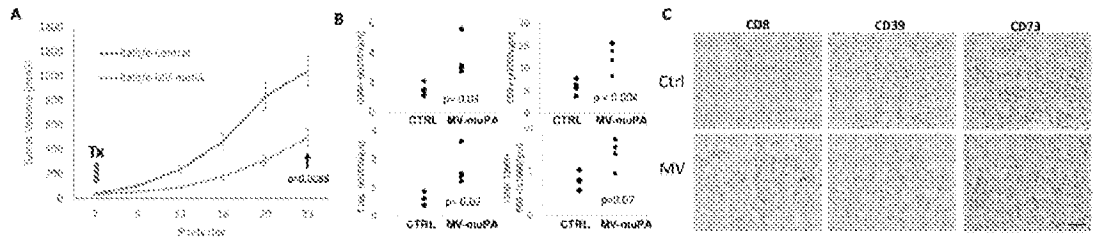
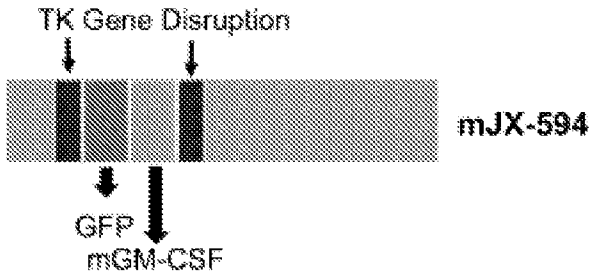
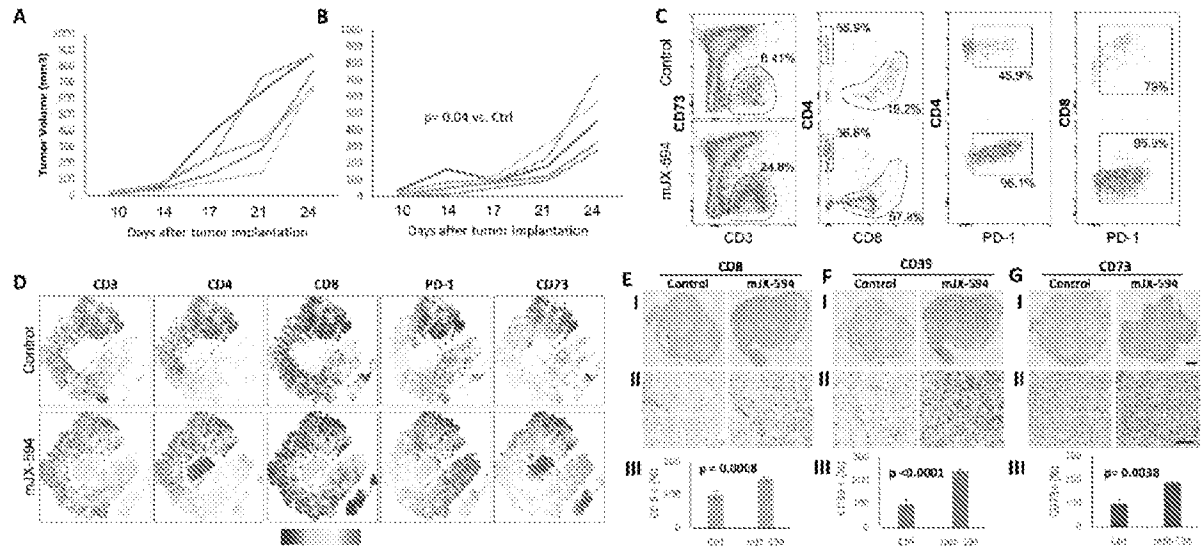


FIG. 7



FIGS. 8A-8G



FIGS. 9A-9D

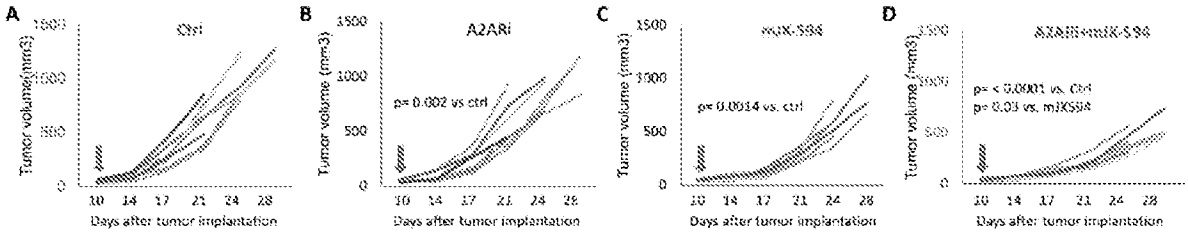


FIG. 10

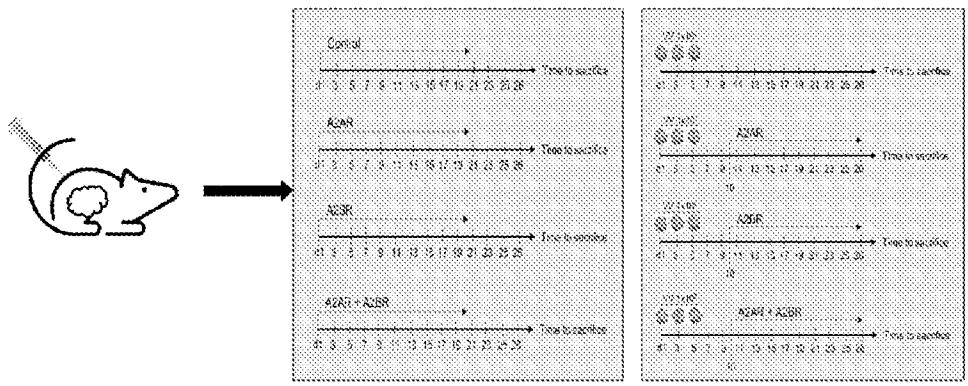
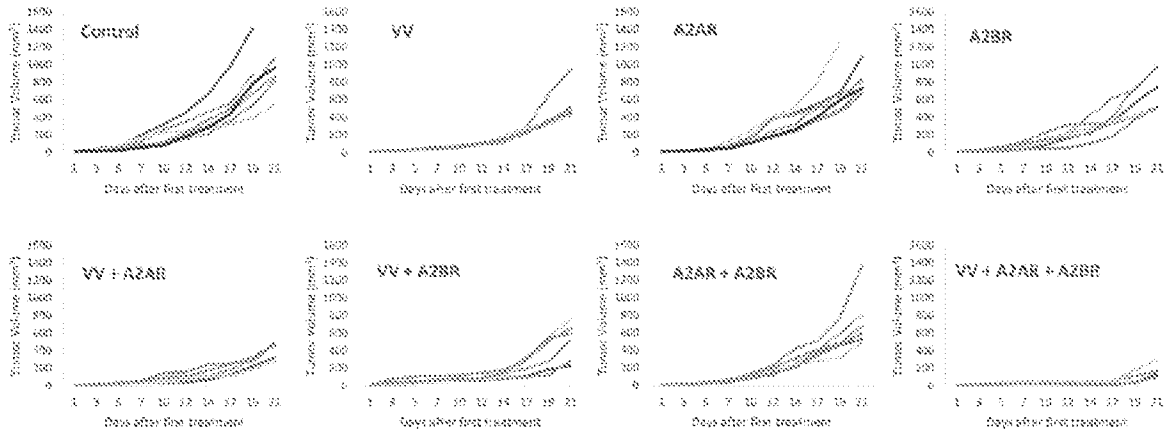


FIG. 11



FIGS. 12A-12D

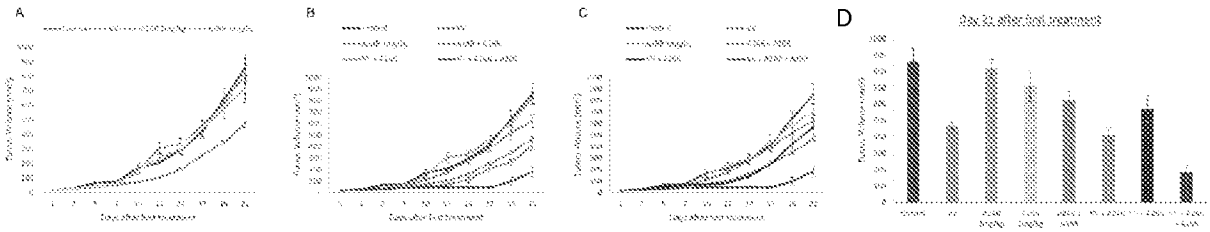


FIG. 13

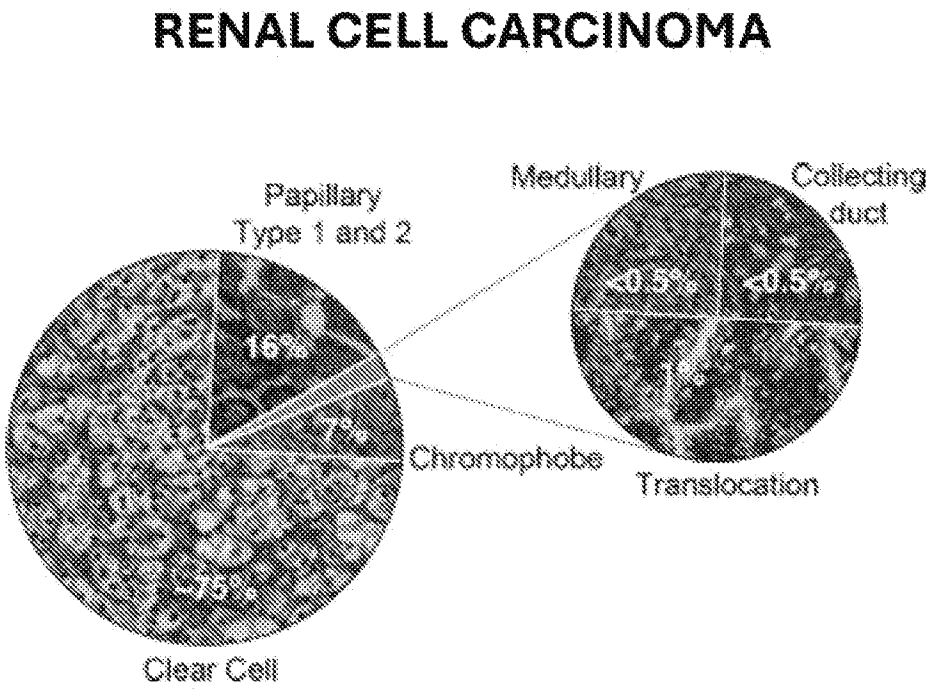

RENAL CELL CARCINOMA
Papillary
Type 1 and 2
16%
7%
75%
Clear Cell
Chromophobe
Medullary
Collecting
duct
<0.5%
<0.5%
Translocation

FIG. 14

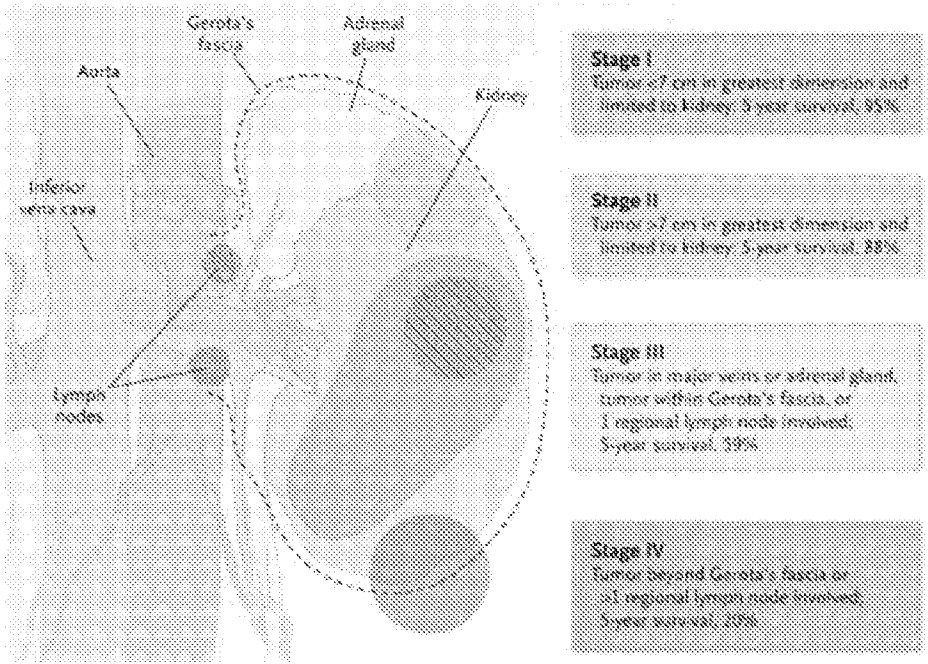

Gerota's fascia
Adrenal gland
Aorta
Kidney
Inferior vena cava
Lymph nodes
Stage I
Tumor ≤7 cm in greatest dimension and limited to kidney. 5-year survival, 95%.
Stage II
Tumor >7 cm in greatest dimension and limited to kidney. 5-year survival, 88%.
Stage III
Tumor in major veins or adrenal gland, tumor within Gerota's fascia, or 1 regional lymph node involved. 5-year survival, 39%.
Stage IV
Tumor beyond Gerota's fascia or ≥1 regional lymph node involved. 5-year survival, 8%.

FIG. 15

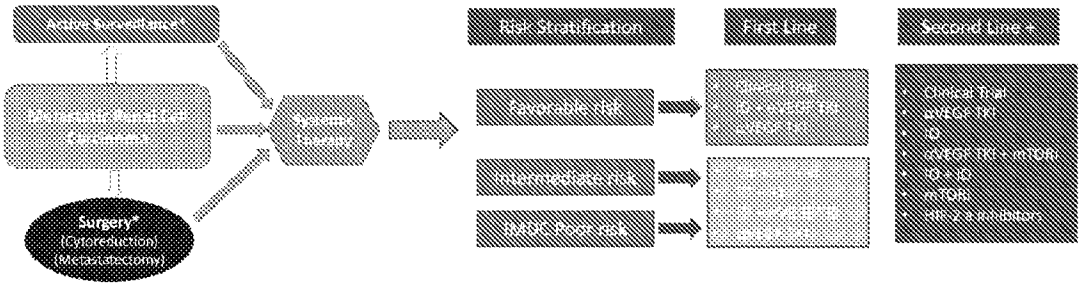
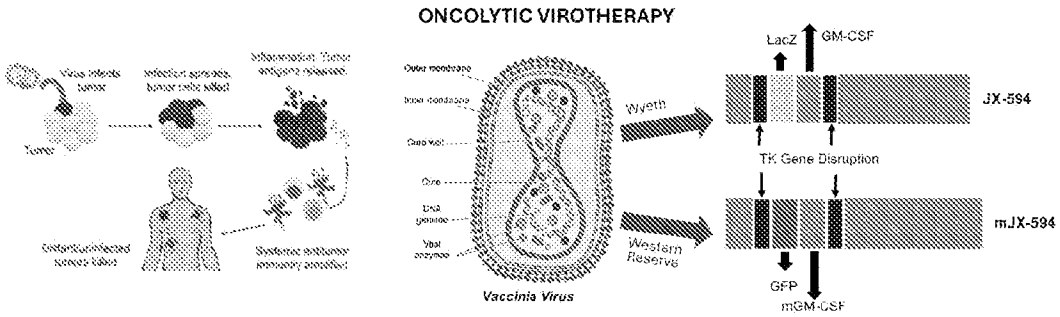
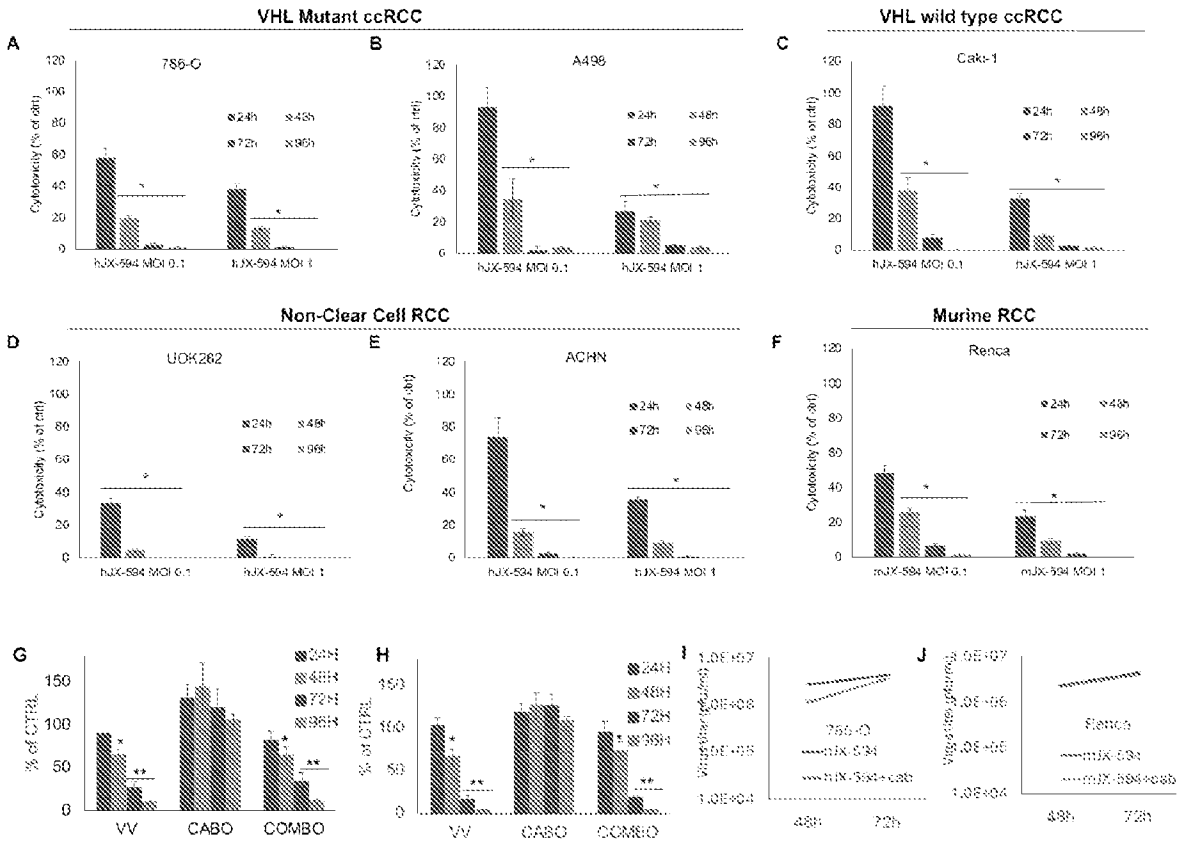

Risk Stratification
First Line
Second Line +
Surgery*
(Cytoreduction)
(Metastasectomy)

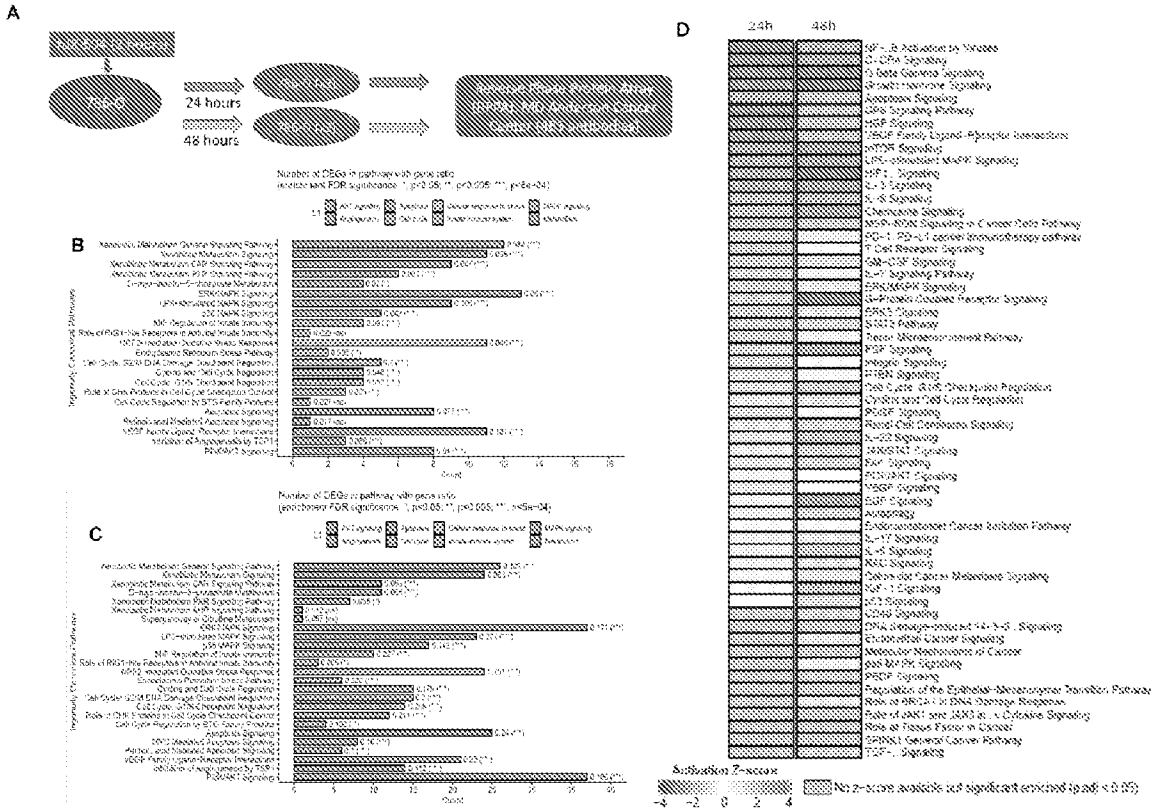
FIG. 16


ONCOLYTIC VIROTHERAPY
LacZ
GM-CSF
JX-594
TK Gene Disruption
mJX-594
GFP
mGM-CSF
Wyeth
Western Reserve
Vaccinia Virus

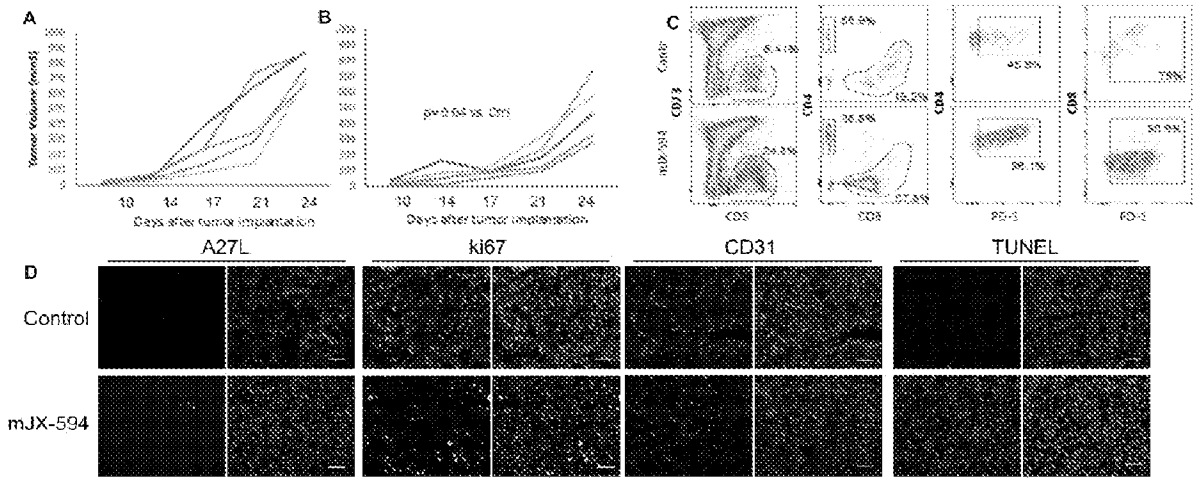
FIGS. 17A-17J


VHL Mutant ccRCC
VHL wild type ccRCC
Non-Clear Cell RCC
Murine RCC
A
786-O
B
A498
C
Caki-1
D
UOK262
E
ACHN
F
Renca
Cytotoxicity (% of ctrl)
24h
48h
72h
96h
hJX-594 MOI 0.1
hJX-594 MOI 1
mJX-594 MOI 0.1
mJX-594 MOI 1
G
H
% of CTRL
VV
CABO
COMBO
I
J
786-O
mJX-594
mJX-594+cabo
Renca
48h
72h

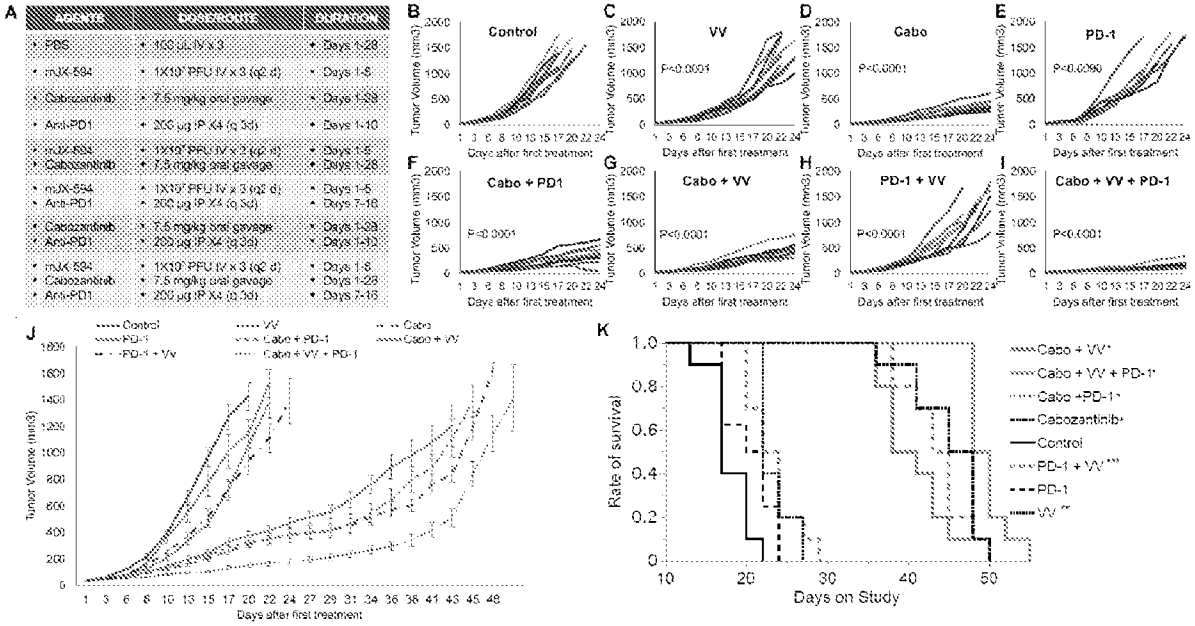
FIGS. 18A-18D



FIGS. 19A-19D



FIGS. 20A-20K


A
Control
Treatment
Schedule
PBS
150 µL IV x 3
Days 1-28
mJX-594
1X10⁷ PFU IV x 3 (q2 d)
Days 1-5
Cabozantinib
7.5 mg/kg oral gavage
Days 1-28
Anti-PD1
200 µg IP X4 (q 3d)
Days 1-10
mJX-594
Cabozantinib
Days 1-5
Days 1-28
mJX-594
Anti-PD1
Days 1-5
Days 7-16
Cabozantinib
Anti-PD1
Days 1-28
Days 7-16
mJX-594
Cabozantinib
Anti-PD1
Days 1-5
Days 1-28
Days 7-16
B
Control
C
VV
P<0.0001
D
Cabo
P<0.0001
E
PD-1
P<0.0090
F
Cabo + PD1
P=0.0001
G
Cabo + VV
P<0.0001
H
PD-1 + VV
P<0.0001
I
Cabo + VV + PD-1
P<0.0001
Tumor Volume (mm3)
Days after first treatment
J
Control
PD-1
PD-1 + Vv
VV
Cabo + PD-1
Cabo + VV + PD-1
Cabo
Cabo + VV
Tumor Volume (mm3)
Days after first treatment
K
Rate of survival
Days on Study
Cabo + VV*
Cabo + VV + PD-1*
Cabo +PD-1*
Cabozantinib+
Control
PD-1 + VV***
PD-1
VV **

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/024404

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***C12N 15/86*** (2024.01); ***A61K 31/519*** (2024.01); ***A61K 38/19*** (2024.01); ***A61P 35/00*** (2024.01); *C07K 14/535* (2024.01)

CPC: ***C12N 15/86; A61K 31/519; A61K 38/193; A61P 35/00;*** *C07K 14/535; C12N 2710/24142; C12N 2710/24143*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	US 2020/0140824 A1 (CALIDI BIOTHERAPEUTICS INC.) 07 May 2020 (07.05.2020)	
X	entire document	1-3, 5-7, 21-24
Y	entire document	4
	US 2022/0133889 A1 (NOVARTIS AG et al.) 05 May 2022 (05.05.2022)	
X	entire document	21, 24, 25
	US 2020/0085891 A1 (SILLAJEN INC) 19 March 2020 (19.03.2020)	
Y	entire document	4
	US 2014/0322173 A1 (JENNEREX INC.) 30 October 2014 (30.10.2014)	
A	entire document	1-7, 21-25
	WO 2018/017747 A2 (UNIVERSITY OF PITTSBURGH - OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION) 25 January 2018 (25.01.2018)	
A	entire document	1-7, 21-25

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 June 2024 (07.06.2024)	**13 June 2024 (13.06.2024)**

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US **Commissioner for Patents** **P.O. Box 1450, Alexandria, VA 22313-1450**	**MATOS** **TAINA**
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/024404

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☑ Claims Nos.: **8-20, 26-38**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).