Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans

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The efficacy and safety of cancer biologics (e.g. peptides, siRNA) could be dramatically increased if high concentrations could be achieved and amplified selectively in tumour tissues versus normal tissues following intravenous administration. This has not been achievable to date in humans. We hypothesised that a poxvirus, which evolved for blood-borne systemic spread in mammals, could be engineered for cancer-selective replication and used as a vehicle for the intravenous delivery and expression of transgenes in tumours. JX-594 is an oncolytic poxvirus engineered for replication plus transgene expression and amplification in cancer cells harboring activation of the Epidermal Growth Factor Receptor/ Ras pathway, followed by cell lysis and anti-cancer immunity[1]. In this clinical trial, we show that JX-594 selectively infects, replicates and expresses transgene products within cancer tissue after intravenous infusion in a dose-related fashion; normal tissues were not affected clinically. This platform technology opens up the possibility of multi-functional products that express high concentrations of multiple complementary therapeutic and imaging biologics in metastatic solid tumours selectively in humans.

Despite recent advances in cancer treatment, truly innovative approaches are required to move beyond the modest benefits achieved to date. One novel strategy is the use of replication-competent oncolytic viruses that selectively infect tumours[2,3]. Vaccinia and other poxviruses have a number of biological properties making them ideally suited for intravenous (IV) delivery and subsequent amplification of transgenes within tumours[1]. First, vaccinia evolved mechanisms for IV stability and spread to distant tissues, including resistance to antibody- and complement-mediated neutralisation in blood[4,5]. Vaccinia also evolved for rapid and motile spread within tissues[6-7]. Because of their relatively large size, vaccinia virions may preferentially deposit within tumours where neovasculature has increased permeability. Finally, vaccinia virus replication is dependent on Epidermal Growth Factor Receptor (EGFR)/Ras pathway signaling[8,9] which is commonly activated in epithelial cancers[10].

JX-594 is a Wyeth strain vaccinia vaccine-derived oncolytic virus engineered for viral *thymidine kinase* (TK) gene inactivation, and expression of the *human granulocyte-macrophage colony stimulating factor* (*hGM-CSF*) and *β-galactosidase* (*β-gal*) transgenes under control of the synthetic early-late and p7.5 promoters, respectively[11,12]. Selective replication in cancer cells is driven by EGFR/Ras pathway signaling, cellular TK and type-1 interferon resistance[1,9,13]. In a Phase 1 trial of intratumoural injection into liver tumours, JX-594 was well-tolerated and associated with replication, expression of biologically active hGM-CSF and tumour destruction[14].

The clinical trial described herein was designed to test whether JX-594 could infect metastatic tumours after IV infusion in patients. First, we assessed JX-594 selectivity for tumour tissue following *ex vivo* infection of paired viable tumour and adjacent normal tissue explants obtained from patients undergoing surgery. Within 24 hours following exposure, JX-594 was able to

selectively infect tumour tissue in 7 of 10 samples; most tumours had high intensity staining whereas normal tissues did not (Fig. 1). Peripheral blood mononuclear cells were also highly resistant to infection (data not shown).

We subsequently performed a Phase 1 dose-escalation trial of a single IV infusion of JX-594 in 23 patients with advanced, treatment-refractory solid tumours (Table 1). Patients were treated in one of six dose cohorts (1×10^5 - 3×10^7pfu/kg). JX-594 delivery, gene expression and replication within solid tumours were assessed. Safety (including determination of the maximum-tolerated dose (MTD)/ maximum feasible dose (MFD)), pharmacokinetics (PK) and anti-tumour activity were also evaluated.

For PK analyses, Quantitative Polymerase Chain Reaction (Q-PCR) was used to measure genome concentrations in blood during the one hour IV infusion and 4 hours afterwards. Maximum concentrations were detected during infusion. At doses of $\geq 10^7$ pfu/kg ($\geq 5 \times 10^8$ pfu/dose), genomes were still detectable in blood at 4 hours (Fig. 2a). Peak concentrations and the area-under-the-curve were both dose-related. JX-594 infectious units were also detected during IV infusion and at 4 hours in high dose patients (data not shown).

JX-594 was generally well-tolerated. Dose-escalation proceeded without dose-limiting toxicities. Therefore, $\geq 3 \times 10^7$ pfu/kg ($\geq$ approximately 2×10^9 pfu/dose) was the MFD; a fixed dose of 1×10^9 pfu was administered to patients in the expansion cohort (n=5 evaluable). The most common treatment-related adverse events consisted of Grade 1-2 flu-like symptoms lasting up to 24 hours: fever (78%), chills (56%), fatigue, headache, nausea, hypotension (22% each), vomiting (17%), tachycardia, hypertension, anorexia and myalgia (13% each). A single Grade 1 skin pustule was noted in two patients one week after infusion and resolved without sequelae.

Interferon-γ, tumour necrosis factor-α, interleukin (IL)-6 increased acutely in a dose-dependent manner (peak, eight hours; resolution, Day 4); IL-10 increased on Day 4-8. In contrast, IL-1 did not change significantly, and IL-4 decreased transiently (data not shown). Neutralising antibodies to vaccinia were detectable in six patients at baseline, and titers increased by Day 15 in all high dose patients. No correlation was demonstrated between antibody titers (baseline or induced) and JX-594 replication, safety or anti-tumour activity. Shedding to the environment was assessed (Supplemental Discussion).

Cancer-selective and dose-related JX-594 delivery and replication in tumours were demonstrated in biopsies obtained 8 - 10 days following infusion (Table 1; Fig. 2b). Of patients treated at approximately $\geq$ 1.5 x 10^7 pfu/kg ($\geq 10^9$ pfu/dose) and evaluable for biopsy analysis, 87% exhibited JX-594 positivity by Q-PCR and/or immunohistochemistry (IHC) analysis, whereas JX-594 was not detected in biopsies collected from subjects treated at lower doses. Of note, delivery and replication were demonstrated in a patient with baseline antibodies to JX-594/ vaccinia (Supplementary Fig. 1). Infection resulted in granular cytoplasmic IHC staining, indicative of virus replication (a.k.a. "virus factories") (Fig. 3 a, d; Supplementary Fig. 2). Diffuse infection and interspersed necrosis of malignant glandular structures were also evident. Adjacent and intermixed normal tissues were negative for replication by IHC (Fig 3i). In immediately adjacent normal squamous epithelium, low level diffuse staining without cytopathogenicity was suggestive of uptake without replication. Staining was absent in negative control biopsy samples (pretreatment tumour from same patient; Fig. 3c, f**;** no primary antibody, Fig. 3b, e). Three-dimensional visualisation of JX-594 infection within a tumour gland revealed diffuse infection and spread (Fig. 3g, h; Supplementary Movie 1).

IHC staining for the β-gal transgene product confirmed expression in JX-594 infected tumour cells (Fig. 3j, k, l). In addition, the induction of antibodies to β-gal protein was a surrogate marker for JX-594 replication and transgene expression (β-gal is not present in the product; significant expression requires replication). Anti-β-gal antibody induction was dose-related, occurring in 100% of high-dose patients (Spearman's Rank Correlation Coefficient $\rho = 0.975$; $p=0.005$) (Fig. 2c). Tumour biopsy positivity correlated strongly with β-gal antibody induction (Table 1). Finally, expression of the second JX-594 transgene (hGM-CSF) was assessed. In a previous clinical trial we demonstrated that high-level replication could result in detectable GM-CSF in blood when other inflammatory cytokines have returned to baseline (Days 4 to 15)[14], despite its short half-life (<2 hour)[15]. Therefore, GM-CSF detection in blood is indicative of high-level GM-CSF expression, and may be a specific but insensitive marker of transgene expression. Three patients had significant increases from baseline in plasma hGM-CSF concentrations (Days 4 – 15; all other inflammatory cytokines returned to baseline in < 24 hours). All had evidence of β-gal expression, tumour infection and/or anti-tumour activity (modified Choi and/ or fluoro-deoxy-d-glucose positron emission tomography (FDG-PET) criteria). GM-CSF protein-responsive white blood cell subsets (neutrophils, eosinophils and monocytes) peaked on Days 4 -15 (Supplementary Fig. 3).

Dose-related anti-tumour activity was demonstrated (modified Choi[16] or Response Evaluation Criteria in Solid Tumours (RECIST)[17] criteria) and correlated with delivery and replication of JX-594 (Table 1; Supplementary Fig. 4). Furthermore, new tumour outgrowth was less frequent at high doses than at low doses (Spearman's Rank Correlation Coefficient $\rho = -0.872$; $p=0.05$), suggesting suppression of microscopic tumour foci. Two of five high dose patients had anti-tumour activity by FDG-PET (>25% decrease in standardised uptake value).

Here we report in humans the first reproducible dose-related delivery, replication and transgene expression from a viral vector or oncolytic virus in metastatic solid tumours after IV administration. Engineered oncolytic poxviruses such as JX-594 can express multiple complementary therapeutic proteins[18] and/or siRNA[19] in metastatic tumours systemically in a cancer-selective fashion; therapeutic concentrations within tumour tissues should therefore be significantly higher than in normal tissues. Incorporation of transgenes for marker proteins can facilitate the monitoring of product replication and transgene expression through blood[20] or radiographic assessments[21,22]. Repeat IV dosing with JX-594 and related oncolytic poxviruses[23,24] should be assessed. While anti-viral immunity may in theory decrease delivery efficiency, all patients on this trial had a history of vaccination with live vaccinia virus as children, and delivery was demonstrated in a patient with neutralising antibodies present at baseline. In preclinical murine tumour models, IV vaccinia delivery and efficacy was feasible despite high titer antibodies[25]. Repeat IV delivery may be feasible because of the unique biology of vaccinia, including its ability to produce 'stealth' particles (extracellular enveloped virus, EEV) that can traffic in blood in the presence of neutralising antibody and complement[4,5]. In addition, IV pharmacologic dosing of JX-594 constitutes a route and dose that may transiently saturate native viral clearance mechanisms. In summary, JX-594 and related poxvirus constructs represent a novel systemic multi-functional cancer biotherapeutic platform.

METHODS SUMMARY

Patients

Twenty-three patients with treatment-refractory histologically-confirmed, advanced/metastatic solid tumours were enrolled and received a single IV infusion of JX-594 at one of six dose levels. This trial was registered with (clinical trials registration # NCT00625456).

All patients gave written informed consent according to Good Clinical Practice guidelines. Protocol and consent forms were approved by the United States Food and Drug Administration and Health Canada, as well as the Institutional Review and Infection Control Committees at each hospital. An independent data-safety monitoring board (DSMB) reviewed the clinical safety data from each patient cohort prior to each of the four dose escalations.

Tumour biopsy analysis

Biopsies (excisional, core needle, or fine needle aspirate [FNA]) were obtained from all subjects 8 to 10 days after treatment and formalin-fixed/paraffin-embedded. Sections were subjected to H&E staining, immunohistochemistry (IHC) staining for JX-594 proteins and for PCR (JX-594 genomes). IHC used anti-vaccinia polyclonal antibody (Quartett, Berlin, Germany) and secondary antibody kit (Vectastain, Vector Laboratories, Burlingame, CA). For IHC detection of β-gal, an anti-β-gal polyclonal antibody (Abcam, Cambridge, MA) was used. Negative controls were run without primary antibody and tumours from mice treated with JX-594 were included as positive controls. For PCR, DNA was extracted from 5 x 10 μm sections using FormaPure Kit (Agencourt, Agencourt Bioscience Corp, Beverly, MA) and amplified using primers corresponding to the vaccinia E3L gene TCCGTCGATGTCTACACAGG and ATGTATCCCGCGAAAAATCA, designed using Primer3 Software [26] using QuantiTect CYBR Green PCR Kit (Qiagen, Valencia, CA). Stained sections were digitised using the Aperio Scanscope (Aperio, Vista, CA) and analysed using ImageScope software. Adobe Photoshop CS software (Adobe, San Jose, CA) was used to apply linear adjustments to brightness and contrast across all compared stains where indicated.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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AUTHOR CONTRIBUTIONS

Study design: DHK, JCB. Data analysis, study write-up: CJB, DHK, THH, AM, RP, AP, JCB, AF. Enrollment & management of patients: JB, DJ, JS, AH, LC, JN. Laboratory work: FLB, JB, NDS, SC, JEJ, LE, YSL, KP, SHD, MD, JSD. CJB, AM, AP and DHK are employees of Jennerex Inc. CJB, JB, AM, AP, DHK, THH, KP, JCB hold stock options in Jennerex Inc. CJB and DHK had access to all the data in the trial. CJB and DHK had the final decision to submit for publication.

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Table 1. Overview of patient characteristics, JX-594 delivery to tumours and anti-tumour activity

Patient characteristics									JX-594 delivery, replication			Anti-tumour activity		
Pt #	Tumour type	Dose (pfu/kg)	Age (yrs)	Gen-der	# prior lines of therapy	# metastatic tumour sites	Sum LD (cm)	BL NAb	anti-βgal (day 29)	Biopsy (day 8-10) IHC	Biopsy (day 8-10) PCR	DC# (RECIST)	DC Duration	Mod. Choi
01	Lung (NSCLC)	1 x 10^5	64	F	2	6	16.2	NEG	NEG	NEG	NEG	No (PD)	NA	NR
02	Colorectal	1 x 10^5	72	M	5	3	14.7	POS	NEG	NEG	NEG	No (PD)	NA	NR
03	Lung (NSCLC)	1 x 10^5	59	F	5	4	3.1	NEG	NEG	NEG	NEG	Yes (SD)	10 wks+	NR
04	Colorectal	1 x 10^6	80	F	0	6	22.2	NEG	NEG	NEG	NEG	No (PD)	NA	NR
05	Melanoma	1 x 10^6	76	M	1	9	26.5	NEG	NEG	NEG	NEG	No (PD)	NA	n/d
06	Thyroid	1 x 10^6	66	M	2	1	3.2	POS	NEG	NEG	NEG	Yes (SD)	4 wks +	NR
07	Lung (NSCLC)	3 x 10^6	67	F	4	6	10	POS	NEG	NEG	NEG	Yes (SD)	4 wks +	NR
08	Pancreatic	3 x 10^6	75	F	3	4	7.1	NEG	POS	NEG	NEG	No (PD)	NA	R
09	Melanoma	3 x 10^6	52	M	3	5	13	POS	NEG	NEG	NEG	Yes (SD)	4 wks	R
10	Colorectal	3 x 10^6	64	M	2	1	5.4	NEG	NEG	NEG	NEG	Yes (SD)	10 wks+	NR
11	Ovarian	1 x 10^7	60	F	5	4	14.4	NEG	NEG	NEG	NEG	Yes (SD)	4 wks +	NR
12	Melanoma	1 x 10^7	60	M	4	3	37.1	NEG	n/d	NEG	NEG	n/d	n/d	n/d
13	Lung (NSCLC)	1 x 10^7	67	F	3	6	3.4	POS	NEG	NEG	NEG	Yes (SD)	10 wks	R
15	Gastric	1 x 10^7	61	F	5	5	11	NEG	POS	NEG	NEG	No (PD)	NA	R
14	Melanoma	3 x 10^7	79	F	3	6	15.1	NEG	POS	NEG	NEG	Yes (SD)	4 wks +	n/d
16	Leiomyosarcoma	3 x 10^7	55	F	3	6	17	NEG	POS	POS	NEG	Yes (SD)	16 wks+	NR
17	Lung (NSCLC)	3 x 10^7	43	M	3	4	13.5	NEG	POS	POS	POS	No (PD)	NA	n/d
18	Ovarian	1.5 x 10^7*	61	F	7	5	13	NEG	POS	POS	POS	Yes (SD)	16 wks+	n/d
20	Colorectal	1.5 x 10^7	67	F	3	8	22	NEG	POS	POS	POS	No (PD)	NA	R
21	Colorectal	1.5 x 10^7	57	F	2	7	17.8	NEG	POS	POS	POS	Yes (SD)	4 wks	n/d
22	Mesothelioma	1.5 x 10^7	68	F	2	4	12	NEG	POS	NEG	POS	Yes (PR§)	10 wks+	n/d
23	Colorectal	1.5 x 10^7	68	M	3	3	7.8	POS	POS	POS	NEG	Yes (SD)	4 wks	n/d

Note: Patient 19 not evaluable;*approximate dosage based on 66.7 kg weight; patients treated at 1 x 10^9 pfu (fixed dose); §Partial response (PR) determined according to modified RECIST for mesothelioma[17]; #SD or PR by RECIST criteria at Week 4 and/or 10; Abbreviations: Pt #: Patient number; pfu: plaque forming units, kg: kilograms, yrs: years; LD: longest diameter of tumour; cm: centimeter, BL: baseline; NAb: neutralising antibody to JX-594 (due to prior vaccinia vaccination); anti-βgal: antibody development to βgal marker transgene product between baseline and Day 29; IHC: immunohistochemistry; PCR: polymerase chain reaction; DC: disease control; RECIST: response evaluation criteria in solid tumours, Mod. Choi: Modified Choi response. NSCLC: non-small cell lung cancer, neg: negative, pos: positive, n/d: not determined, PD: progressive disease, SD: stable disease, PR: partial response, NR: no response, R: response, NA: Not Applicable

FIGURE LEGENDS

Figure 1. *Ex vivo infection of patient tumour and normal tissue explants reveals tumour-selective JX-594 gene expression***.** JX-594 expressing GFP (JX-594-GFP+/β-gal-) was used to infect primary live tissue specimens from cancer patients undergoing surgical resection. Matched tumour and adjacent normal tissues were infected overnight to assess the selectivity of transgene expression and replication. GFP expression from JX-594-β-gal+GFP infected cells was assessed using a fluorescence microscope. N= Normal tissue, C= Cancer tissue

Figure 2. *JX-594 is selectively delivered to, and amplified within, tumours following IV infusion.* (**a**) Acute pharmacokinetics of JX-594 genomes (Q-PCR) following a single IV infusion by dose cohort. Error bars are s.e.m. (**b**) Dose-dependent delivery of JX-594 as demonstrated by PCR and/or IHC analysis of tumour biopsies collected 8 to 10 days post-treatment. (**c**) Dose-dependent induction of antibodies to β-galactosidase in patients evaluable for this endpoint. ρ=Spearman's Rank Correlation Coefficient. n evaluable for each group indicated in graph.

Figure 3. *Immunohistochemical staining reveals JX-594 infection and β-galactosidase expression in tumours* (**a**) Vaccinia IHC (patient 20, 10 days post-treatment). Scale bar (SB) 200µm. (**b**) IHC, no primary antibody. SB 200µm. (**c**) Vaccinia IHC pre-treatment biopsy. SB 50µm. (**d-f**) as in (**a-c**) for patient 18 biopsy at 8 days. SB 50µm. (**g,h)** 3D reconstruction of vaccinia throughout tumour (patient 20). Green=vaccinia. (**i**) Low-magnification vaccinia IHC (patient 20). SB 500µm. Black arrows indicate tumour; red arrows indicate normal tissue. (**j**) β-gal IHC (patient 20). SB 50µm. (**k**)Vaccinia IHC. SB 50µm. (**l**) Negative control. SB 50µm. Linear adjustment to brightness and contrast applied to **j,k,l**.

METHODS

Ex vivo infection of tumour explants

Primary cancer and normal tissues specimens were obtained from consenting patients who underwent tumour resection. Institutional review board of Ottawa Hospital Research Institute has approved all human studies. A total of 10 tumour samples as well as accompanying adjacent normal tissue samples from the affected organ were assayed for JX-594-GFP+/β-gal- sensitivity. Samples were received in cell culture medium and processed within 2-16 hours. Samples were manually divided using a 15 mm scalpel blade into ~10-mm^3 pieces and placed on 12 wells plate with α-medium containing 10% fetal bovine serum under sterile techniques. Samples were inoculated with $1x10^7$ pfu JX-594 in 100 μL of α-MEM serum-free medium. JX-594 was allowed to adsorb for 2 hours at 37°C, at which time 1.9 mL of α-MEM supplemented with 10% FBS was added. Infected tissue specimens were incubated in a humidified incubator at 37°C for 24 hours prior to imaging JX-594-GFP+/β-gal- driven GFP expression.

Patients

Between July 21st, 2008 and February 9th, 2010, 23 patients were enrolled. Patients had treatment-refractory histologically-confirmed, advanced/metastatic solid tumours. At least one tumour mass had to be amenable to biopsy and/or fine-needle aspirate (FNA). Patients had adequate hematological function (leucocyte count > 3,500 cells/mm^3, CD4 count ≥ 200/mm^3, hemoglobin ≥ 10 g/dL, platelet count ≥ 100,000 PLT/ mm^3) and organ function (including aspartate aminotransferase [AST]/alanine aminotransferase [ALT] < 2.5 × upper normal limit, bilirubin ≤ 1.5 x upper normal limit and serum chemistries within normal limits (WNL) or Grade 1), coagulation status (INR ≤ [ULN + 10%]), and Karnofsky Performance Score (KPS) of ≥ 70.

Exclusion criteria included known central nervous system (CNS) malignancy, clinically significant and/or rapidly accumulating ascites, peri-cardial and/or pleural effusions, unstable cardiac disease, increased risk of vaccination complications (exfoliative skin conditions such as eczema, ectopic dermatitis), clinically-significant immunodeficiency, anti-cancer therapy within the preceding 4 weeks, pulse oximetry O_2 saturation <90% at rest.

All patients gave written informed consent according to Good Clinical Practice guidelines. Protocol and consent forms were approved by the United States Food and Drug Administration and Health Canada, as well as the Institutional Review and Infection Control Committees at each hospital. An independent data-safety monitoring board (DSMB) reviewed the clinical safety data from each patient cohort prior to each of the four dose escalations.

Manufacturing, product characterisation and release testing

Clinical trial material (CTM) lots which were used in this study were manufactured according to Good Manufacturing Practice guidelines (n=2). Virus was grown in adherent mammalian cells and purified through sucrose-gradient centrifugation or by tangential flow filtration. *In vitro* and *in vivo* comparability testing demonstrated equivalence of the two lots. Final product quality control tests included assays for sterility and endotoxin, DNA, protein, plaque-forming units (pfu) and genome concentration in the CTM; functional assays included potency and GM-CSF production. CTM was formulated in either phosphate-buffered saline with 10% v/v glycerol (pH 7.1) or 30 mM Tris with 10% (w/v) sucrose (pH 7.7). Immediately prior to the IV infusion, JX-594 was diluted in bicarbonate buffered saline in a total infusion volume of 250 mL.

Treatment

JX-594 was infused in 250 mL bicarbonate-buffered saline over 60 minutes. Patients in the dose-escalation portion of the trial received one of six dose levels (1×10^5 plaque-forming units per kilogram [pfu/kg] [cohort 1], 1×10^6 pfu/kg [cohort 2], 3×10^6 pfu/kg [cohort 3], 1×10^7 pfu/kg [cohort 4] and 3×10^7 pfu/kg [cohort 5]) in a group-sequential dose-escalation design (standard 3x3 design; two to six patients for each dose cohort) at one of four sites: the Billings Clinic, the Cancer Center of the Carolinas, the Ottawa Hospital Research Institute and the University of Pennsylvania. One additional patient could be treated in each dose cohort once that cohort was cleared for safety. An additional six patients were enrolled as part of an expansion cohort (at the approximate midpoint between cohort 4 and 5 doses, at a fixed dose of 1×10^9 pfu (approximately 1.5×10^7 pfu/kg, depending on patient weight). A starting dose of 1×10^5 pfu/kg was chosen based on GLP toxicology study safety findings, and demonstrated safe patient blood concentrations following intratumoural (IT) injection of JX-594 into liver-based tumours [14]. DLTs were defined as any of the following treatment-related adverse events (AEs) (through the 4-week evaluation period): 1) any Grade 4 toxicity (except isolated Grade 4 lymphopenia lasting $\leq$ 7 days), 2) Grade 3 or 4 hypotension, disseminated intravascular coagulation (DIC), or allergic reaction/hypersensitivity, 3) Grade 3 non-hematologic toxicity persisting > 7 days except if toxicity is transaminitis (which may last > 7 days if total bilirubin is normal or Grade 1) or flu-like symptoms that respond to standard treatments and 4) Grade 3 hematologic toxicity persisting for > 7 days (except isolated lymphopenia). The maximum tolerated dose (MTD) was defined as the dose immediately preceding that for which two or more dose-limiting toxicities (DLTs) were recorded. If no MTD was defined, the highest dose was defined as the maximum feasible dose (MFD).

Starting with a subset of patients in cohort 4, patients were pre-medicated with acetaminophen (plus every 6 hours as needed). Patients were advised to hydrate orally for 24 hours prior to treatment (e.g. $\geq$ 1 liter of solute-containing fluids), and they received hydration through 24 hours post-treatment (e.g. 1-2 liters of fluids, orally or intravenously). Patients were monitored after treatment in the hospital for 24 hours and for at least 29 days as outpatients. Physical exam and interval medical history were performed on each weekly study visit. Safety monitoring included adverse event-monitoring (National Cancer Institute Common Toxicity Criteria, version 3.0) and standard laboratory toxicity grading for hematology, liver and renal function, coagulation studies, serum chemistry and urinalysis.

Pharmacokinetic (PK) measurements in blood

JX-594 pharmacokinetics (PK) as well as gene expression and replication within solid tumours were assessed. Quantitative polymerase chain reaction (PCR) was used to measure the JX-594 genome concentration in blood as previously described [14,27]. Quantitative Polymerase Chain Reaction (Q-PCR) was used to determine the PK of JX-594 during and immediately after administration. Whole EDTA-blood samples were taken before the start of infusion; 15, 30, and 60 minutes after the start of infusion; and 30, 60, 120, and 240 minutes after the end of infusion. AUC, Cmax and $t_{1/2}$ were calculated using WinNonLin™, Version 5.2 (WinNonLin™ Copyright ©2010, Pharsight Corporation). The AUC was calculated by the linear trapezoidal method and Cmax was determined directly by inspection.

Antibody Titers to the β-gal Marker Transgene

Serum samples were obtained at baseline and on Days 15 and 29. Human IgG antibodies to β-gal were measured by ELISA. Briefly, plates (NUNC MaxiSorp, Thermo Fisher Scientific, Waltham, MA) with wells containing β-gal (Sigma, St. Louis, MO) or bicarbonate/carbonate

buffer-only were incubated overnight at 4°C and washed with PBS-Tween before incubation with blocking buffer (PBS with 1% bovine serum albumin (BSA, ELISA grade, Sigma, St. Louis, MO)). Diluted serum (1:50, 1:100, 1:200, in PBS + 0.05% Tween + 1% BSA) was added to β-gal-coated and control wells in duplicate and incubated at room temperature. Plates were washed and incubated with alkaline phosphatase-labeled goat anti-human IgG (AbCam, Cambridge MA) diluted 1:2000. After washing, colorimetric substrate pNPP (Sigma, St. Louis, MO) was added, and NaOH was added to stop color development after 10 minutes. Absorbance was read at 405nm, and absorbance of 630nm was subtracted. Control well values were subtracted to account for non-specific binding, and titers values were calculated by comparison to a standard curve of positive sera arbitrarily assigned a titer of 8,000.

Neutralising Antibody Titers to Vaccinia virus

This procedure is based on the ability of neutralising antibodies (NAb) in patient serum samples to reduce the cytopathic effect (CPE) caused by live vaccinia virus. Serum samples obtained at baseline and on Days 4, 8, 15, 22 and 29 were heat-inactivated, serially diluted in 96 well format (dilution factor 10 - 3,200,000) and incubated with vaccinia virus for 2 hours prior to transfer of mixture onto monolayers of A2780 cells. Cell viability was measured 3 days after inoculation by means of colorimetric assay based on live-cell mediated reduction of tetrazolium salt to formazan conversion (Cell Counting Kit-8 (CCK-8), Donjindo Laboratories, Kumamoto, Japan). NAb titer was defined as the reciprocal of the highest dilution of serum that results in ≥ 50% cell viability.

White blood cell induction

White blood cell count was performed by routine laboratory testing and was included in the safety assessment as defined in the protocol. WBC counts were assessed at baseline and on Days 4, 8, 15, 22 and 29.

Cytokine measurements

Endogenous cytokines IL-1β, IL-4, IL-6, IL-10, TNF-α, IFN-γ were measured in multiplex in plasma samples obtained at baseline, 3 hr and 8 hr after dosing, and on Days 4 and 8 using a Milliplex Kit as directed by the manufacturer (Millipore Corp, Billerica, MA). GM-CSF concentrations in plasma were determined at baseline, 3 hr and 8 hr after dosing and on Days 4, 8, 15, 22 and 29 using the Quantikine hGM-CSF sandwich ELISA kit as directed by the manufacturer (R&D Systems, Minneapolis, MN).

Tumour biopsy analysis

Biopsies (excisional, core needle, or fine needle aspirate [FNA]) were obtained from all subjects 8 to 10 days after treatment and formalin-fixed/paraffin-embedded. Sections were subjected to H&E staining, immunohistochemistry (IHC) staining for JX-594 proteins and for PCR (JX-594 genomes). IHC used anti-vaccinia polyclonal antibody (Quartett, Berlin, Germany) and secondary antibody kit (Vectastain, Vector Laboratories, Burlingame, CA). For IHC detection of β-gal, an anti-β-gal polyclonal antibody (Abcam, Cambridge, MA) was used. Negative controls were run without primary antibody and tumours from mice treated with JX-594 were included as positive controls. For PCR, DNA was extracted from 5 x 10 µm sections using FormaPure Kit (Agencourt, Agencourt Bioscience Corp, Beverly, MA) and amplified using primers corresponding to the vaccinia E3L gene TCCGTCGATGTCTACACAGG and ATGTATCCCGCGAAAAATCA, designed using Primer3 Software [26] using QuantiTect CYBR Green PCR Kit (Qiagen, Valencia, CA).

3D reconstruction

An excisional biopsy was obtained from Patient 20 on Day 10 and processed for immunohistochemical detection of vaccinia virus. 126 serial sections were cut and every other section was stained for virus. 2D pictures of the sections were then converted into a 3D volume using HTK Histology Toolkit software (Robarts Imaging Institute, University of Western Ontario). Volume reconstruction was completed using alignment and segmentation contouring algorithms which oriented each tissue section on top of one another. Each tissue section image, once oriented, was then converted from 2D pixels into 3D voxels. These 3D stacks were then rendered to generate the reconstructed tumour. Regions of infection were highlighted in green to aid in visualisation and in another representation in which the model can be viewed in orthogonal planes, image contrast was adjusted to 50 (Adobe Photoshop CS2; Adobe, San Jose, CA).

Tumour response analysis

Tumour response was assessed by contrast-enhanced CT imaging on Day 29 on all patients, and on Week 10 in patients who remained on study. Maximum tumour diameters and Hounsfield units (HU; density estimate) were obtained at all timepoints. Patients in the expansion cohort had PET-CT scans done at the same timepoints; standard uptake values (SUV) were determined from PET scans. Response Evaluation Criteria in Solid Tumours (RECIST)[28] and modified Choi criteria[16,29] for response were employed in image evaluations. Patient 22 (metastatic mesothelioma) was evaluated by modified RECIST for mesothelioma[17]. PET response was also determined for PET-evaluable patients.

Equipment and settings

GFP expression from JX-594+GFP/-β-gal in human tissue explants was visualized using the Leica M205FA microscope and Leica microsystem LAS AF6000 acquisition software(Leica Microsystem, Richmond Hill, Ontario, Canada) at 2-4X magnification. The same exposure time was used across all samples. Images of tumour and normal tissue samples for each patient were captured on the same day. Images were stored using Adobe Photoshop version 7.0 (Fig. 1).

Immunohistochemistry sections were digitised using the Aperio Scanscope (Aperio, Vista, CA) and analysed using ImageScope v10.2.2.2319 software (Aperio, Vista, CA). No image adjustments were applied to Fig. 3a-f, i. Adobe Photoshop CS software (Adobe, San Jose, CA) was used to apply linear adjustments to brightness and contrast across all compared stains where indicated (Fig. 3j, k, l).

Statistical analysis

The study sample size was set to assess safety issues. The primary objectives were to study the safety and to determine the MTD/MFD of JX-594 following IV infusion. Secondary objectives included pharmacokinetics and pharmacodynamics, immune responses (neutralising antibodies, anti-β-gal antibodies, cytokines) and delivery of JX-594 to solid tumours following IV infusion. The likelihood of dose escalation, given varying true DLTs in the treated population, was calculated as per routine in Phase 1 dose-escalation trials. Expected sample size was 18 to 24 patients.

The Spearman's correlation coefficient between ranks was used to calculate statistical dependence between antibody induction to β-galactosidase and dose cohort (percent of patients with antibody induction in Cohort 1-5) as well as appearance of new tumours and dose cohort (percent of patients with new tumours in Cohort 1-5)[30].

REFERENCE

30 Myers, J. L. W., Arnold D. *Research Design and Statistical Analysis*. second edition edn, 508 (Laurence Erlbaum, 2003).