Isolated limb perfusion with melphalan, tumour necrosis factor-alpha and oncolytic vaccinia virus delays tumour growth and prolongs survival in a rat model of locally advanced extremity sarcoma.

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Dear Sirs,

Please find herewith our manuscript, ‘Isolated limb perfusion with melphalan, tumour necrosis factor-alpha and oncolytic vaccinia virus delays tumour growth and prolongs survival in a rat model of locally advanced extremity sarcoma,’ for consideration for publication in the Annals of Surgery.

We can confirm that all authors made a significant contribution to the manuscript and qualify for authorship as determined by the guidance for authors. All authors have reviewed and approved the final manuscript. Should it be accepted, we agree to pay appropriate colour figure printing charges.

Many thanks for your consideration of our work. We look forward to hearing from you.

Yours sincerely,

Tim Pencavel

For and on behalf of the authors.
Mini Abstract:

The authors describe the use of oncolytic vaccinia virus in combination with existing isolated limb perfusion components in a model of soft-tissue sarcoma, and supporting in vitro data evaluating the relative contributions of each agent. Survival was prolonged and tumour growth reduced.
Structured Abstract

Objective:

To evaluate the addition of oncolytic vaccinia virus to existing isolated limb perfusion treatment regimens in soft-tissue sarcoma.

Summary Background Data:

Isolated Limb Perfusion (ILP) is a surgical technique for the treatment of locally advanced extremity sarcoma and in-transit melanoma. Extending this methodology to include administration of novel agents, such as cancer-selective oncolytic viruses, represents an exciting approach to improving the therapeutic efficacy of ILP. In addition, it raises the possibility of using a locoregional therapy to prime a systemic anti-tumour response.

Methods:

Standard in vitro assays were used to characterise single agent and combinatorial activities of melphalan, tumour necrosis factor-alpha (TNF-α) and Lister strain vaccinia virus (GLV-1h68) against BN175 rat sarcoma cells. A rodent model of advanced extremity sarcoma (BN175 in syngeneic Brown Norway rats) was used to evaluate tumour response and survival of animals after ILP with combinations of TNF-α, melphalan and GLV-1h68. The in vivo model was also used to study the locoregional and systemic biodistribution of virus after ILP.

Results:

The combination of melphalan and GLV-1h68 was synergistic in vitro when assessed using the median-dose effect principle of Chou and Talalay. ILP with virus was well tolerated. In a model of microscopic residual disease, the triple combination of TNF-α, melphalan and GLV-1h68 was no more effective than the TNF-α/melphalan combination. However, in the setting of macroscopic disease, the triple therapy resulted in increased tumour growth delay and enhanced survival when compared to other treatment regimens. Live virus was recoverable in large amounts from perfused regions, but in smaller amounts from systemic organs.
Conclusions:

The addition of oncolytic vaccinia virus to existing TNFα/melphalan-based ILP strategies results in survival advantage in an immunocompetent rat model of advanced extremity sarcoma. Virus administered in this way remains largely confined within the perfusion field, from which it can be recovered. Further evaluation and clinical translation of this approach is warranted.
Isolated limb perfusion with melphalan, tumour necrosis factor-alpha and oncolytic vaccinia virus delays tumour growth and prolongs survival in a rat model of locally advanced extremity sarcoma

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Conflict of Interest:
KJH received research funding in support of this work from Genelux GmbH/Genelux Corporation

Running Head:
Oncolytic viral therapy in ILP
Introduction

Isolated Limb Perfusion (ILP) is a regional chemotherapeutic technique used for the treatment of locally advanced extremity sarcoma and in-transit melanoma\textsuperscript{1-3}. It involves cannulation of the major blood vessels to the tumour-bearing region, isolation of the limb from the systemic circulation using a pneumatic or elastic tourniquet, and the administration of high-dose chemotherapy, typically melphalan, which is often used in conjunction with the cytokine tumour necrosis factor-alpha (TNF-\(\alpha\)). ILP with TNF\(\alpha\) is particularly useful as either a palliative or pre-resection procedure for patients with locally advanced sarcoma for whom the only other surgical option would be amputation, and for patients with bulky in-transit metastases from cutaneous melanoma\textsuperscript{4,5}.

Oncolytic virotherapy has been of interest to cancer researchers for several decades\textsuperscript{6}. Despite reassuring safety data and indications of efficacy in both pre-clinical and early-phase clinical studies, it has yet to realise its full potential\textsuperscript{7}. This is because achieving therapeutically-relevant titres at the target site following systemic delivery is problematic; the virus must overcome the combined barriers of the immune system of immunocompetent hosts and the relative impermeability of the tumour vasculature to large viral particles\textsuperscript{8,9}. Perhaps for this reason, current efforts are focussed on combining viral therapy with more conventional treatments such as chemotherapy and radiotherapy in an attempt to maximise efficacy\textsuperscript{10-12}.

The combination of ILP with viral agents is attractive for several reasons. First, it allows for the anatomically-targeted delivery of high viral titres directly to the tumour. Second, because the perfusion circuit is isolated from the systemic circulation the virus is protected from breakdown by the systemic immune system and reticulo-endothelial system during the initial stages of infection. Third, ILP allows for the co-administration of TNF\(\alpha\) which increases vascular permeability and, hence, viral extravasation into the tumour, further increasing the effective viral titre in the tumour microenvironment. The systemic toxicity of TNF\(\alpha\) is too great to allow administration other than by ILP. Finally, an existing network of established clinical ILP programmes offers the potential of rapid translation of this approach to early-phase clinical trials in this surgically challenging group of patients\textsuperscript{13}. Recent data on the combination of ILP with oncolytic viral therapy\textsuperscript{14}, and evidence of
efficacy against human sarcoma and melanoma cell lines\textsuperscript{15}, supports further investigation of such an approach.

In this study, we describe the use of a pre-clinical rat model of ILP for extremity sarcoma to evaluate tumour response and pharmacodynamic properties of oncolytic vaccinia virus (GLV-1h68) after locoregional administration.
Materials and Methods

In vitro studies

Cell lines:
The BN175 rat sarcoma cell line was kindly provided by Prof A Eggermont. This cell line is tumorigenic in Brown Norway rats\textsuperscript{16}. Cells were passaged in standard Roslyn Park Memorial Institute (RPMI) 1840 medium, supplemented with 5% heat inactivated foetal bovine serum, 2.5% L-glutamine and 1% penicillin/streptomycin. The CV-1 monkey kidney cell line was obtained from existing laboratory stocks and passaged in standard Dulbecco’s Modified Eagle’s Medium (DMEM), with 10% fetal bovine serum, 2.5% L-glutamine and 1% penicillin/streptomycin. Cells were cultured at 37 °C in an incubator maintaining a 10% carbon dioxide atmosphere.

Cytotoxic agents:
Melphalan (SigmaAldrich, St Louis, USA) was kept as a dry lyophilized powder as supplied until reconstitution, and used having been dissolved in 500 μL 100% ethanol with 3 drops of concentrated (1M) hydrochloric acid. This base solution was then diluted at least 1:100 in complete medium to give the first test solution. At this dilution, no effect of vehicle was seen on cell proliferation (data not shown). GLV-1h68 was produced and provided by Genelux Corporation (San Diego, USA). The structure has been described elsewhere\textsuperscript{17, 18} but in essence it is a Lister-strain Vaccinia virus which has been attenuated by insertion of LacZ (beta-galactosidase), gusA (beta-glucuronidase) and RUC-GFP (a fusion gene of Renilla luciferase and green fluorescent protein) into the J2R, A56R and F14.5L loci, respectively. Recombinant human TNFα was supplied as a lyophilized powder from First Link Ltd (Birmingham, UK) and was dissolved in PBS to a concentration of 100 units per mL. Aliquots of 1 mL solution were stored at -20 °C until use. Actinomycin D (SigmaAldrich, St Louis, USA) was obtained from existing laboratory stocks.

Cell survival assay:
Estimation of cell proliferation (as a surrogate for cytotoxicity) after exposure to cytotoxic agents alone or in combination was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide (MTT) assay\textsuperscript{19}. The experimental protocol was as follows: cells were plated in a clear-walled 96-well plate (Nunc, Denmark) at a density of 5000 cells per well. After 24 hours’ incubation, the cytotoxic agents were added at the required concentration. Forty-eight to 144 hours later, 20 \( \mu \text{L} \) MTT was added to each well and the plates were returned to the incubator for 5 hours. The medium was aspirated and the reduced MTT was dissolved in 200 \( \mu \text{L} \) dimethyl sulfoxide (DMSO) before being read on a plate reader (Victor 2, Perkin Elmer, Mass., USA) at an absorbance of 550 nm. Results were normalised to a 100% control population of untreated cells. Cell killing was calculated as 100 – surviving %. Where combinations of treatments were being investigated, analysis was performed using the median dose effect principle of Chou and Talalay\textsuperscript{20} on CalcuSyn software (Biosoft, Cambridge, UK).

Viral Plaque Assay:

CV-1 cells at a density of 2 \( \times \) 10\textsuperscript{5} cells per well were plated on a standard 24-well plate and incubated in standard medium for 48 hours until a confluent monolayer was seen. The test solution was added to each well and serially diluted. After 48 hours the cells were fixed with 2\% formaldehyde and 0.2\% glutaraldehyde in PBS and stained for 4 hours with 5 mM potassium hexacyanoferrat III, 5 mM potassium hexacyanoferrat II-trihydrate, 2 mM magnesium chloride hexahydrate and 0.6 mg/mL X-Gal (CalBioChem, Merck KGaA, Germany) in PBS and then washed with ultrafiltered (UF) water and dried. Macroscopic X-gal-stained viral plaques were counted manually.

\textit{In vivo} studies

Animals:

Inbred, specific pathogen-free adult male Brown Norway rats weighing between 225 and 275 g were obtained from Charles River (Margate, UK) or Harlan (Nottingham, UK). They were housed in compliance with all relevant regulatory requirements and fed standard chow and water \textit{ad libitum}. Experimental schedules were approved prior to commencement of the study by the UK Home Office and the Institute of Cancer Research’s Ethics board, and animals and operative techniques were
subject to regular oversight visits from a UK Home Office approved veterinary inspector to ensure compliance with high humane and ethical standards.

Tumour growth and humane endpoints:

Tumour growth was assessed every 48 hours by direct calliper measurement in two orthogonal dimensions. Tumour volume was calculated using the formula: Volume = \( \frac{1}{2} \times \text{width}^2 \times \text{breadth} \). Animals were culled at an agreed humane endpoint of 2 cm maximum tumour diameter. An additional humane endpoint of >10% weight loss was also defined but not reached in this study.

Tumour response experiments:

Two different tumour models were evaluated. In all aspects, these models were identical aside from the day on which ILP was carried out. BN175 cells were harvested from tissue culture flasks and washed in PBS three times prior to injection of \( 1 \times 10^7 \) cells in 500 μL PBS into the left hind limb of the rat. The needle was inserted at the ankle just proximal to the lateral malleolus and advanced in the subcutaneous plane for approximately 1 cm before the cell suspension was injected. This was to prevent immediate loss of cells through the injection track. For the “microscopic residual disease” model, ILP was performed 24 hours after injection of cells. For the “established tumour” model, ILP was performed between six and eight days after cell injection, when tumours had a maximum diameter between 0.8 and 1.2 cm. The ILP technique employed involved modification of a model that has previously been reported\(^2\). Briefly, in the anaesthetised and warmed animal the abdomen and proximal medial thigh were depilated and cleaned with ethanol. An incision was made over the landmark of the inguinal ligament and deepened to expose the femoral vessels. A tourniquet was placed under the inguinal ligament to prevent its distal migration. The vessels were cannulated and the circuit was run via a peristaltic pump at between 2–4 L min\(^{-1}\). Therapeutic agents were added to the perfusate reservoir at the following doses: melphalan - 40 μg, TNFα - 50 units, GLV-1h68 - \( 1 \times 10^7 \) p.f.u. The total perfusate volume was 14 mLs. At the end of the perfusion, the limb was washed out, the cannulae withdrawn and the vessels ligated to prevent haemorrhage. Therapeutic perfusion was performed for 13 minutes and washout for 2 minutes. To reduce hypoxia, the time between tightening
the tourniquet and commencing oxygenated perfusion was less than 15 seconds. The skin was closed and the animal recovered. The procedure took approximately 45 minutes in total, with a further 15 minutes for the animal to regain full consciousness. A degree of transient limb dysfunction (in the form of decreased limb movement) usually occurred, but full function in the perfused limb was seen within 2 hours of recovery. Tumour measurements began on the first post-operative day for both animal groups. All therapeutic groups consisted of eight animals.

Biodistribution experiments:

In order to obtain data on tumour uptake of virus, biodistribution experiments were performed at the same time point as the established tumour model, but with $1 \times 10^8$ p.f.u. of GLV-1h68. Rats were euthanized at 24, 48 and 72 hours post-perfusion and samples of tumour, skin, muscle and tibial bone marrow (by saline lavage) were taken from the perfused limb, as well as ipsilateral testis, liver, spleen, kidney, heart, lung and brain. Samples were weighed and 500 μL PBS was added before they were homogenised on a PreCellys 24 lysis machine at 2 cycles of 3000 rpm for 20 seconds with an intervening 90 second rest period. Aliquots of 100 μL were taken for DNA extraction and viral plaque assay (VPA). DNA extraction was performed using a QiaGEN DNEasy Tissue Kit (Qiagen Ltd, Crawley, UK) as per the manufacturer’s protocol. Primers to the vaccinia A21L gene were provided by Genelux GmbH (Bernreid, Germany); the Genelux GL-ONC1 VV-A21L kit provided all specific reagents for performing the PCR assay. A21L quantification was based on 50 cycles of real-time PCR (with hold times of 10s at 95 °C, 10s at 63 °C and 15s at 72 °C, and a temperature gradient of 20 °C/s). A LightCycler instrument (Roche Diagnostics, Burgess Hill, UK), along with the Faststart HybProbe kit (Roche Diagnostics) was used to detect the fluorescence produced (by the hybridisation of the test strand to the template DNA) at a wavelength of 640nm. Diluted A21L standards were used as positive controls, and a standard curve calculated from the standards for quantification of test samples using the software provided. Sterile filtered water was used as negative control. VPA was performed as described above, with the first two dilutions being 1:10 in UF water then 1:10 in PBS;
i.e., the first plated sample was 1:100 of the homogenized organ. Readings for both qPCR and VPA were quantified per gram of tissue. All biodistribution groups consisted of three animals.

Evans Blue perfusion:

Evans Blue can be used as a marker of vascular permeability. The Evans Blue perfusions were again performed in the established tumour model at three time points; pre-perfusion, 1 hr post-perfusion and 24 hours post-perfusion. ILP was performed in the standard manner. Evans Blue was administered through the tail vein, which was cannulated via an open cut-down technique for this purpose. The dye was allowed to circulate for 15 minutes prior to euthanasia. Tumour samples were taken and homogenised as described, this time using formamide as the diluent to increase Evans Blue extraction. Standard dilutions were also produced and these, with the samples, transferred to a black-walled 96-well plate. Quantification was performed using a plate reader for fluorescence, with the excitation frequency at 540 nm and emission frequency at 680 nm. Linear regression was performed using GraphPad software to interpolate the amount of Evans Blue per gram of tumour tissue. All data shown represent averages of separate experiments on three animals per time point.

Immunohistochemistry for viral capsid protein

Animals with established tumours were perfused with virus alone, virus and TNFα or TNFα/Melphalan/Virus. At 24, 48 or 72 hours after perfusion the animals were euthanised and the tumours excised ‘en bloc’ as a wide excision, taking overlying skin and underlying muscle. These specimens were bivalved and snap frozen in liquid nitrogen, before being stored at -80 °C. They were then mounted in paraffin blocks and representative sections mounted on glass slides. Some slides were stained according to routine laboratory protocol with H&E stain. The remainder, and the tissue blocks, were sent for immunohistochemistry, which was performed in collaboration with Dr Qian Zhang of Genelux Corporation (San Diego, USA). The primary antibody was a polyclonal antibody produced in rabbits to a synthetic peptide of the A27 IMV surface protein (custom made by GenScript.
USA Inc, NJ, USA). After 30 minutes’ incubation at a dilution of 1:1000 antibody in blocking serum, the slides were washed in PBS and the secondary anti-rabbit IgG labelled with horseradish peroxidase (Vector Laboratories Inc, CA, USA) antibody added at a dilution of 1 drop in 10mls blocking serum. The secondary antibody was then stained with ImmPACT DAB with brown cromogen (VECTASTAIN Elite ABC Kit, Vector Laboratories).

**Statistical Analysis**

Simple and descriptive statistical analysis was performed using Microsoft Excel (Microsoft Inc, Washington, USA). Kaplan-Meier survival analysis and group analysis, including ANOVA, was performed using GraphPad Prism software (GraphPad Software Inc, California, USA). Statistical significance was defined as a p-value of less than 0.05.
Results

Vaccinia virus is active against BN175 Rat sarcoma cells in vitro

Figure 1 shows the results of MTT-based analysis of cell survival following incubation of GLV-1h68 with BN175 cells. The action of vaccinia was both time- and dose-dependent. At high MOI (multiplicity of infection, the ratio of viral plaque-forming units to cells), cell killing was almost total. Maximum cell death was seen at 72 hours. At the later time point, some recovery of cell numbers was seen at the lower MOIs. The IC₅₀ values are presented in figure 1.

Vaccinia is synergistic with melphalan in vitro

Figure 2a shows the summary isobolographic analysis (using the median dose effect principle of Chou and Talalay²⁰) of combinations of GLV-1h68 with melphalan as determined by cell survival analysis using MTT assay. The combinations of doses were defined by the IC₅₀ doses of melphalan and vaccinia virus as 0.25, 0.5, 1, 2 and 4 times the IC₅₀ dose (sigmoidal dose-response curves for melphalan: data not shown). Given the time-dependent nature of the response to GLV-1h68 (Figure 1), the time point selected was 48 hours. This also represents a relevant time point to clinical perfusion systems, since the half-life of melphalan in vivo is approximately 60 minutes and, therefore, very little melphalan will persist after the first 24 hours. Figure 2b shows the combination indices for each dose combination, along with the interpretation. At higher doses, strong synergy was seen. Although the effect at lower doses was more variable, the overall trend was for increasing synergy at higher doses. Given the fact that ILP allows delivery of intratumoral melphalan concentrations 15–25 times greater than those achievable by systemic administration, this bodes well should a similar effect on delivery of high viral titres be achieved.

TNF-α exerts no effect on viral or melphalan activity in vitro

TNF-α has been shown to have differing effects on cell lines in vitro. For some lines it acts as a pro-survival mechanism, whilst for others it is cytotoxic²³–²⁵. Figure 3a shows the effect of TNF-α alone
and in combination with actinomycin D against the BN175 cell line. Actinomycin D acts as a cell cytoskeleton stabilizer, and is used in the standard cell-based assay of TNF-α activity\(^{25}\). With the addition of TNF-α to actinomycin D, some cytotoxicity was seen but there was minimal, if any, dose response. TNF-α alone was neutral or slightly cytostimulatory at high doses. Figures 3b and 3c show the effect of adding melphalan or GLV-1h68, respectively, to TNF-α. Here, normalisation is to the degree of cell death seen with the melphalan or GLV-1h68 IC\(_{50}\) dose, respectively. No additional cell death was seen with the addition of TNF-α, although there was some abrogation of the cytostimulatory effect seen with melphalan. Since the differences were small, it was not possible to calculate formal combination indices, but these results suggest that, whatever the explanation for the synergy seen in vivo by others between TNF-α and melphalan, or any increased effect from the addition of GLV-1h68, it is not mediated by TNF-α at the level of the cancer cells themselves.

**Adding GLV-1h68 to an ILP regimen for microscopic residual disease (MRD) is not superior to TNFα/Melphalan.**

Figure 4a shows the Kaplan-Meier survival analysis for the MRD model by treatment delivered. The median time to humane end point for untreated animals was 10 days. Median survival for animals treated by ILP using vehicle only was 11 days, which was not a statistically significant increase. The dose of melphalan used in the ILP groups with melphalan was 40 µg. This represents a low dose of melphalan when compared with previous studies, but was deliberately chosen such that the treatment effect due to melphalan alone would not obscure any differences between other therapeutic groups. All treatment groups showed prolongation of survival to humane end point, with the greatest prolongation seen in the TNF-α/melphalan/GLV-1h68 group (to 30 days) and the TNF-α/melphalan group (to 28 days). One animal, in the TNFα/melphalan/virus group, was cured of disease. This animal developed a tumour which subsequently regressed after treatment, with no recurrence 50 days after complete regression.

Fig 4b shows a box-and-whisker plot of survival to humane end point by treatment groups. Evaluating the data in this way indicates that the critical component in perfusate constituent that determines prolongation of survival is the melphalan. Log-Rank analysis of all survival data gave a P-value of
<0.0001 for the overall difference in survival between groups. To clarify the exact nature and significance of these differences, further data analysis was performed using an ANOVA with Tukey post-testing on the survival data, for which the results are shown in table 1. Significant differences were predominantly seen between groups containing melphalan and those not containing melphalan. Importantly, there was no significant difference with or without the addition of GLV-1h68 to TNF-α/melphalan perfusion. Using virus as the sole component of perfusion or in combination with TNF-α did not significantly increase survival.

Figure 4c shows a graph of tumour volume growth after injection of tumour cells at day 0. Although there was considerable heterogeneity of tumour growth rates, overall the TNFα/melphalan/virus, TNFα/melphalan and melphalan alone groups demonstrated slower growth to median survival than the other groups.

Overall, the procedure was well-tolerated, with only 2 deaths in the microscopic residual disease group, both of which had undergone TNFα/melphalan perfusion. Both deaths were in the early post-operative period (within 24 hours) and occurred after immediate recovery. Autopsy did not show an obvious structural cause or post-operative complication, such as major haemorrhage, to explain the deaths.

**Adding GLV-1h68 to ILP for established tumours results in delayed tumour growth and prolonged survival**

Figure 5a shows the Kaplan-Meier survival analysis for the established tumour model by treatment delivered. Sham perfusion in this model resulted in identical outcomes to sham perfusion in the MRD model. All other treatment groups showed prolongation of median survival, with the greatest improvement in the TNF-α/melphalan/GLV-1h68 group, in which median survival was prolonged to 24 days. Interestingly, survival after melphalan-alone perfusion was not significantly increased over sham perfusion, whereas the addition of virus to melphalan perfusion significantly improved survival over both sham and single agent melphalan regimens. This is in direct contrast to the findings in the MRD model.
Figure 5b shows a box-and-whisker plot of time to humane end point by treatment group for the established tumour model. This confirms that melphalan alone did not perform well as a single treatment. All groups containing TNF-α performed to a similar level. Interestingly, the melphalan/virus group also performed well. As for the microscopic model, Log-Rank analysis showed P<0.0001 for the differences across all groups. When analysed further by ANOVA with Tukey-Kramer post-testing (summarised in table 2), the greatest differences were seen between the TNF/melphalan/GLV-1h68 group and other groups.

Figure 5c shows a graph of tumour volumes against time in days. Day 0 represents the day on which cells were injected; as described above, ILP was performed at maximum tumour diameters of between 8 and 12 mm. Initial growth rates are similar in all groups with the exception of the TNF-α/melphalan/virus group and the melphalan/virus group, in which tumour growth was slower. TNF-α/melphalan perfusion showed a growth delay in animals which responded well to perfusion but the initial growth for the whole group was similar to the TNF-α/GLV-1h68 and GLV-1h68 alone groups.

Once again the procedure was well-tolerated. There were no deaths in the established tumour group. All animals in the TNFα/melphalan/virus group developed a mild, self-limiting rash which resolved by day 7 (Figure 5d). This had no functional consequences, and would be defined as a Wieberdink grade 1 ILP reaction.

**The administration of GLV-1h68 by ILP results in significant viral deposition in tumour tissue, with minimal systemic spread beyond the perfused limb.**

Figure 6A shows the results of qPCR analysis for viral genome copy numbers found in both perfused (skin, muscle, tumour and bone marrow) and non-perfused (testis, spleen, kidney, liver, heart, lung, brain) organs after perfusion with single agent GLV-1h68, i.e., no melphalan or TNF-α was administered. The perfusion dose was $1 \times 10^8$ p.f.u. of virus. At 24 hours post-perfusion, $1 \times 10^5$ copies of viral DNA were recovered per gram of tumour tissue. Each tumour weighed approximately 4 grams (data not shown). The number of copies of viral DNA increased significantly by 48 hours post-perfusion to $5 \times 10^7$ copies per gram of tissue, and remained stable at this level at 72 hours post-
perfusion. Significant amounts of virus were recovered from the samples obtained within the perfusion field, particularly skin, but virtually no virus was found outside the perfused limb except at 48 hours when small amounts were seen in the ipsilateral testis, spleen and kidney. To establish whether the number of viral copies indicated the presence of live virus, each sample was subjected to VPA analysis. Live virus was recovered from tumour and bone marrow samples but not from any other organ. The results of the VPA are depicted in table 3.

Co-administration of GLV-1h68 with TNF-α +/- melphalan results in higher initial intratumoural concentrations of virus but increases viral recovery from distant organs.

Figures 6b, 6c and 6d show viral DNA copy numbers recoverable from organs at 24, 48 and 72 hours for perfusions with three different treatment regimens. At 24 hours, the amount of virus recovered from tumour was significantly greater in the two regimens in which TNF-α was used. However, virus was recovered from a greater number of organs overall, and in the TNF-α/melphalan/GLV-1h68 arm vaccinia viral genomes were detected in all of the organs tested. At 48 hours, the amount of virus seen after virus alone and virus/TNF-α perfusions increased relative to the TNF-α/melphalan/GLV-1h68 perfusion. At 72 hours, the highest levels of genome copies in tumour were in the group that received TNF-α/melphalan/GLV-1h68 perfusions. At all time points, more virus was seen systemically in animals where TNF-α was used in the initial perfusion. This finding is reflected in table 3, which shows that live virus was recoverable from more organs. Interestingly, the only organ from which live virus could be recovered after TNF-α/melphalan/virus perfusion was spleen, despite the higher levels of qPCR-detected virus in the same samples.

Established tumours show immunohistochemical evidence of intratumoural vasculature, which is absent from the microscopic model

Fig 7 shows sections of the hindlimb of rats after the injection of tumour cells 1 week (7a,b,c) or 24 hours (7d,e) previously. Figure 7b, c and e have been stained for CD31, a marker of vascular endothelium. As would be expected, the tumour that has been present for longer is traversed by a
network of vessels, whereas the islets of cells present after 24 hours is in proximity to vessels already present within the stroma but not directly perfusing the tumour tissue (Fig 7e). Successful manipulation of the tumour vasculature might be expected to result in greater delivery of virus to tumour tissue, by allowing penetration of virus to deeper portions of the tumour through vasodilation and thus lower resistance to flow and extravasation of perfusate.

**Extravasation of Evans Blue dye is increased 1 hour after perfusion and has decreased to near pre-perfusion levels by 24 hours**

Evans Blue dye is a marker of vascular permeability, and is extravasated more easily when endothelial tissue has been injured or has undergone conformational change to increase the intercellular distance\(^\text{22}\). Figure 7f shows the results of Evans Blue administration to control animals before perfusion, 1 hour after perfusion with TNFα and at 24 hours after perfusion with TNFα. For the purposes of this experiment, and to prevent confounding factors, no additional therapy was used in the perfusate (e.g., melphalan is known to have a non-specific vasodilatory effect). The amounts of Evans Blue recovered per gram of tumour tissue are shown (values shown are averages of 3 repeats from 3 animals). The amount of Evans Blue recovered rose significantly in the 1 hour group before falling back to near baseline at 24 hours. Since all animals underwent ILP, the increased extravasation is most likely due to the action of TNFα in the perfusate.

**Immunohistochemistry shows an increase in viral capsid protein when perfusion is performed with TNFα/Virus and with TNFα/Melphalan/virus compared to virus alone perfusion**

Figure 8 shows the immunohistochemistry results from staining for the presence of vaccinia virus. The primary antibody was to viral capsid proteins, present either on mature virions or within the cytoplasm of cells in which virus was replicating. In the tumours perfused only with vaccinia virus, no appreciable staining could be seen at either 24 or 72 hours after perfusion. However, 24 hours after perfusion with TNFα and virus, areas of staining are seen within the tumour, which are wider and more diffuse after
triple therapy perfusion. The staining persists to 72 hours after TNFα/virus perfusion, but is more limited after triple therapy perfusion. Haematoxylin and eosin staining of adjacent section reveals a significantly greater degree of necrosis in triple therapy tumours at 72 hours, which may explain the weaker staining.
Discussion

Isolated limb perfusion has become a mainstay of treatment for locally advanced extremity sarcoma and melanoma since the introduction of TNF-α by LeJeune and colleagues in the 1980s\textsuperscript{27}. Whilst excision of tumours with a wide margin of normal tissue will always be the preferred approach, there remains a group of patients with locally advanced tumours in whom this is not possible, and these patients represent a significant surgical and oncological challenge. Often the only alternative surgical strategy would be an amputation, even though this would not necessarily result in cure\textsuperscript{2}.

In the current study, we have shown that in an experimental model of advanced extremity sarcoma the use of vaccinia virus as adjunct to the commonly-used melphalan and TNF-α regimens increases treatment efficacy in large established tumours, although it is not superior in a model of microscopic residual disease. These findings are similar to the finding in the clinical setting that there is no benefit to adjuvant ILP after primary resection of tumours (i.e. in the setting of no bulk disease)\textsuperscript{28}. Therefore, the addition of virus to existing ILP regimens would not be expected to lead to the reconsideration of ILP as an adjuvant therapy. In the context of large extremity sarcomas and in-transit melanoma, both of which represent situations more akin to the established tumour, gains in survival might be expected.

Although there have been many trials of viral therapy for cancer, including trials involving local perfusion, there has never been a treatment-related death directly attributable to the virus under study. In our study, no animal required euthanasia due to adverse effects of viral administration and only two animals, both in the microscopic residual disease model which had received ILP with TNF-α and melphalan, were culled as a consequence of the procedure. Given that the doses of virus administered were relatively high for a small-volume lower limb circuit, this represents excellent tolerability of the procedure with no increase in toxicity due to the virus.

Viral therapy has been shown to work consistently in immunodeficient models, particularly by intratumoural injection\textsuperscript{17, 28, 30}. Difficulties are seen in immunocompetent models due to the anti-viral action of host defence mechanisms. In addition, intratumoural injection is impractical in the setting of very large or multiple tumours. Here, we describe a mechanism of viral administration to a non-
immunogenic tumour type in an immunocompetent model that takes advantage of tumoural blood supply, allowing for the potential infection of multiple sites within large tumours, or for the simultaneous infection of multiple smaller tumours. The results of the biodistribution experiments indicate that virus is able to infect the tumours, as well as replicate within them, as indicated by the increase in viral titre between the 24, 48 and 72 hour time points. In addition, live virus was recovered from tumour, indicating that virus administered by ILP is able to infect and replicate within tumour tissue.

In ILP, one of the roles of TNF-α is to increase the vascular permeability within tumours and, thereby, the intratumoral concentrations of cytotoxic agents. The status of TNF–α as a cytotoxic agent independent of other effects is unclear. In both model systems, perfusion with TNF–α provided an advantage over single agent melphalan-based regimens, although this was not significant in the MRD model. No effect of TNF-α was seen in vitro, either alone or in combination with melphalan or vaccinia. These findings suggest a non-cytotoxic mechanism of action for TNF-α in this system and, given its known effect on nitric oxide synthesis in vessels, this activity in tumour-associated vasculature represents a more likely mechanism. The stromal component of a soft-tissue tumour is harder to model in vitro, explaining why there is a paucity of literature in non-clinical or animal series. Interestingly, in the biodistribution study, TNF-α did increase intratumoural concentrations of GLV-1h68 compared to non-TNF-α perfusions. A differential effect was also seen globally between the MRD and established/macroscopic tumour models, in that melphalan was the critical factor in microscopic disease whilst GLV-1h68 had more of an effect in established tumours. This is likely to reflect a difference in the vasculature in the two different models. In the MRD model, ILP did not lead to direct perfusion of tumour vasculature because it had not yet become established at the time of perfusion. This is demonstrated by the immunohistochemistry of tumour vessels within established tumours but the absence of vessels within the ‘nests’ of cells that were the target of perfusion in the microscopic model. Therefore, in the established/macroscopic tumour model, virus was administered directly in to the tumour vasculature. As a small molecule, melphalan could be expected to be extravasated relatively readily from the normal limb vasculature and thus ‘bathe’ tumour cells in a solution of melphalan, providing a cytotoxic effect. Further evidence of the importance of TNFα in
increasing the extravasation of virus comes from immunohistochemical staining for viral capsid protein at 48 hours after ILP. TNFα resulted in greater viral staining than the virus alone perfusion group. Interestingly, the triple therapy group showed the greatest staining. Whether this is due to a physical mechanism, such as melphalan’s action as a vasodilator, or a therapeutic mechanism similar to the in vitro synergy seen, has not been elucidated.

An aspect of viral therapy that has not been evaluated in this model is immune priming. Some authors have found immune-mediated cell killing in metastatic tumour deposits in immunocompetent mice\(^34\). Immunity represents an intriguing frontier for locoregional virotherapy\(^35\). Indeed, if locoregional virotherapy via ILP could be shown to provoke an anti-tumour immune response, this raises the possibility of a local treatment mediating a systemic effect. This is particularly clinically relevant in patients who undergo ILP for uncontrolled melanoma. The vast majority of these patients will die from the consequences of systemic spread rather than locoregional recurrence\(^2\). Such metastatic disease is often, although not invariably, occult at the time of perfusion and may be amenable to immune-mediated therapy (as demonstrated by the activity of anti-CTLA4 antibody therapy\(^36\)).
Conclusions

This paper provides evidence of the efficacy of oncolytic vaccinia virus as a therapeutic agent in ILP for locally advanced extremity sarcoma. The finding of in vitro synergy between melphalan and vaccinia virus, along with the ability of ILP to produce high viral titres at the tumour, represents a mechanism for increases in efficacy of both ILP and oncolytic viral therapy. In the light of the previous approval of such agents by regulatory authorities, their excellent safety profile and the availability of clinical ILP programmes, translation of this approach into clinical trials is suggested.
Figure Legends

Fig. 1: MTT cell survival assay of GLV-1h68 virus activity against BN175 cell line. Graph shows % cell survival against the log of MOI. Lines are best-fit sigmoidal dose-response curves; solid – 48 hours; dashed – 72 hours; dotted – 144 hours. Table shows IC_{50} dose by time point, illustrating the time-dependent nature of response.

Fig 2: (A) Fractional effect against combination index for the combination of melphalan and GLV-1h68 virus. Top and bottom curves represent 95% confidence intervals; points represent values calculated from experimental data. (B) Table showing the experimentally-derived values, with the interpretation suggested. Key: CI – Combination Index; Fa – Fractional Effect; s.d. – standard deviation

Fig 3: (A) TNFα activity against the BN175 cell line in vitro, with or without the addition of actinomycin D. (B) Activity of TNFα with or without melphalan against the BN175 cell line. (C) Activity of TNFα with or without Vaccinia virus against the BN175 cell line.

In all graphs, normalisation is to cell survival with no active agents. The doses of AcD, Melphalan and GLV-1h68 used were IC-50 doses. Curves shown are for 48 hours post addition of active agents.

Fig 4: Microscopic Residual Disease model. (A) Kaplan-Meier survival plot of survival vs time in days after injection of tumour cells. (B) Box-and-whisker plot for each group of time to humane end point. (C) Tumour growth to median survival.

Fig 5: Established Tumour Model. (A) Kaplan-Meier survival plot of survival vs time in days after injection of tumour cells. (B) Box-and-whisker plot for each group of time to humane end point. (C) Mean tumour volume vs time for all groups. Graph stopped at median survival for each group. (D) Vaccinia rash, which developed after ILP. From l-r: day 2, day 5, day 7
Fig 6: (A) qPCR data of number of copies of viral genome detected at 3 different timepoints after perfusion with Vaccinia virus alone. (B) qPCR data of viral copy numbers detected at 24 hours after perfusion with virus alone, virus and TNFα or virus, TNFα and melphalan. (C) qPCR at 48 hours. (D) qPCR at 72 hours.

Fig 7: Vascularity of tumours in rat hindlimb after cell injection, and the effect of TNFα on vascular permeability. Light micrographs: A, B and C – 1 week after tumour cell injection. D and E – 24 hours after injection. A: H&E stain, 10x magnification, showing densely-packed tumour cells and occasional chains of red blood cells. B. CD31 IHC, 10x. Arrow indicates linear staining consistent with an intratumoural vessel. C: CD31 IHC, 20x. Higher magnification of intratumoural vessel. D: H&E stain showing scattered cells within the target stroma. E: CD31 IHC showing a clump of tumour cells (circled, stained purple) in proximity to a stromal vessel but not clustered around it. F: Amount of Evans Blue recovered from tumour tissue at each time point after perfusion with TNFα alone.

Fig 8: IHC for viral capsid protein at 24 hours and 72 hours post -ILP. A – 24 hours, VV alone; B – 72 hours, VV alone; D – 24 hours, TNFα/Virus; E – 72 hours, TNFα/Virus; G – 24 hours, TNFα/Melphalan/Virus; H – 72 hours, TNFα/Melphalan/Virus. Also shown is H&E staining at 72 hours after VV alone (C); TNFα/Virus (F); TNFα/Melphalan/Virus (I). Representative sections from central portion of established tumours.
References

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Table 1: Summary of ANOVA with Tukey-Kramer post-testing for differences between mean survival by perfusion groups in the microscopic disease model. *** - p<0.001; ** - p between 0.001 and 0.01; * - p between 0.01 and 0.05; ns – p>0.05.
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Table 2: Summary of ANOVA with Tukey-Kramer post-testing for differences between mean survival by perfusion groups in the established tumour model. *** - p<0.001; ** - p between 0.001 and 0.01; * - p between 0.01 and 0.05; ns – p>0.05.
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Table 3: Summary of viral plaque assay for recovery of live virus at 3 different timepoints post-perfusion. Analysis was non-quantitative due to the effect of tissue samples on the CV1 monolayer; however, 4 serial dilutions of samples were performed. ++++ - live virus in all dilutions; +++ - live virus in 3 dilutions; ++ - live virus in 2 dilutions; + - live virus in most concentrated sample only.
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(A) qPCR data of number of copies of viral genome detected at 3 different timepoints after perfusion with Vaccinia virus alone. (B) qPCR data of viral copy numbers detected at 24 hours after perfusion with virus alone, virus and TNFa or virus, TNFa and melphalan. (C) qPCR at 48 hours. (D) qPCR at 72 hours.
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Statement of author involvement

All authors were involved in drafting/revising the article, and approved the final manuscript prior to submission. In addition, and with regards to section 1 of the guideline, the authors made significant contributions in the following areas:

Tim D Pencavel MRCS a,b – Study conception and design, data acquisition and interpretation/analysis.
Rohit Seth MRCS a – Study design, data acquisition, data analysis and interpretation.
Aadil A Khan MRCS a – Study design, data acquisition, data analysis.
Michelle J Wilkinson MRCS a,b – Study design, data acquisition.
David C Mansfield BSc a – Study design, data acquisition and analysis
Eleni M Karapanagiotou PhD a, - Data acquisition and analysis.
Victoria Roulstone BSc a – Study design, data acquisition
Richard J Aguilar PhD c – Study design, data interpretation.
Nanhai G Chen PhD c,e – Study design, data interpretation.
Aladar A Szalay PhD c,d,e – Study design, data interpretation.
Andrew J Hayes FRCS PhD b – Study conception and design, data interpretation and analysis.
Kevin J Harrington PhD a – Study conception and design, data acquisition, data interpretation.
This piece of the submission is being sent via mail.