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## Supplemental Information

# Oncolytic H-1 Parvovirus Shows Safety and Signs of Immunogenic Activity in a First Phase I/IIa Glioblastoma Trial

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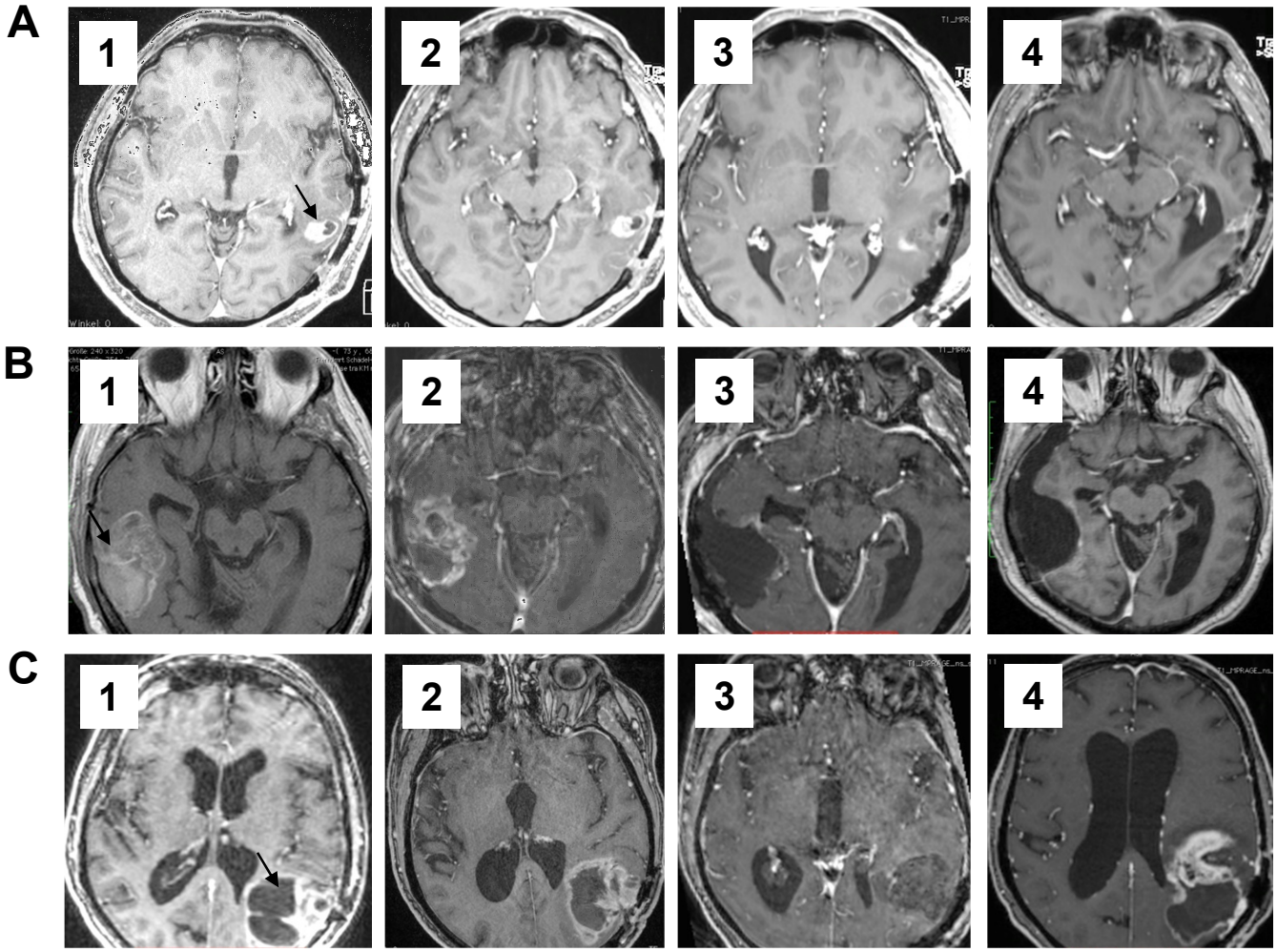


**Figure S1. Examples of regular study MRIs of patients from different treatment groups**

Upper panel (A1 to A4): patient 1-03, left temporal tumor (arrow). MRIs before treatment (A1), on day 9 after intratumoral virus injection prior to surgery (A2), on day 11 one day after surgery (A3) and after 6 months (A4) demonstrating stable disease. Middle panel (B1 to B4): patient 4-10, right temporal tumor (arrow). MRI before treatment (B1), on day 9 after the first intravenous virus injection prior to surgery (B2), on day 11 one day after surgery (B3) and after 2 months (B4) demonstrating that the patient died of unrelated causes (pneumonia). Lower panel (C1 to C4): patient 6-17, left temporo-occipital tumor (arrow). MRI before treatment (C1), on day 9 after intratumoral virus injection prior to surgery (C2), on day 11 one day after surgery (C3) and after 2 months (C4) demonstrating massive tumor progression.

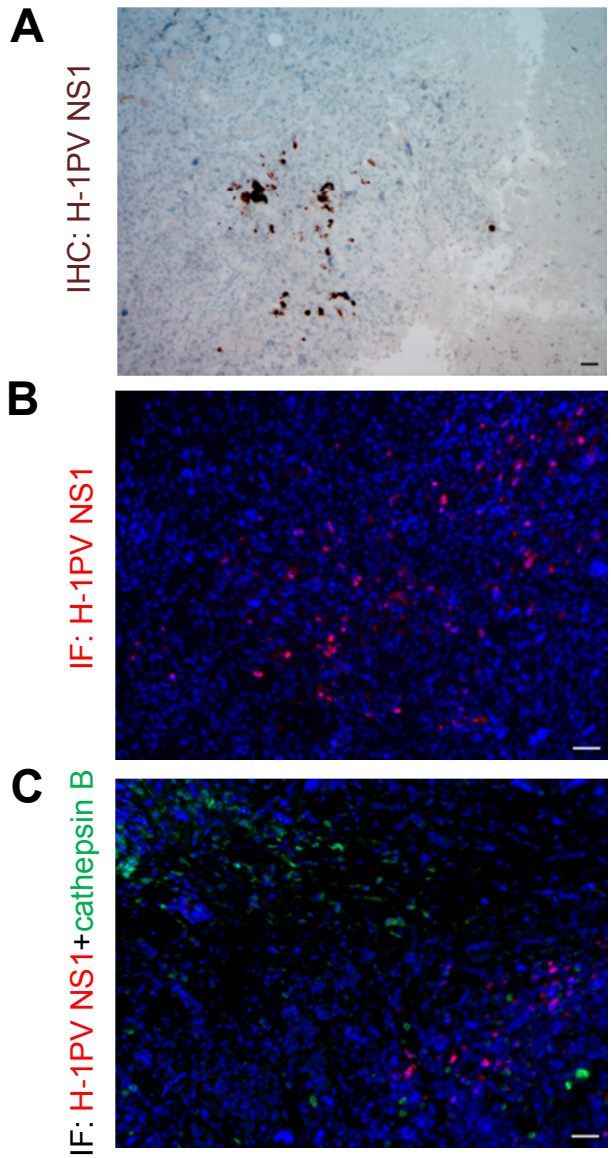


**Figure S2. Clustering of NS1-positive cells within solid tumor areas after local ParvOryx administration**
Tumor (patient 6-17) was resected 9 days after intratumoral ParvOryx (2.5E9 PFU) administration, and paraffin-embedded tumor tissue was subjected to analysis. (A) Immunohistochemical and (B) immunofluorescent NS1 protein detection using the 3D9 NS1-specific antibody revealed clusters of cells with high NS1 reactivity. (C) Strong cathepsin B expression (green) was seen in NS1-positive (red) tumor regions. Scale bars, 50 µm.

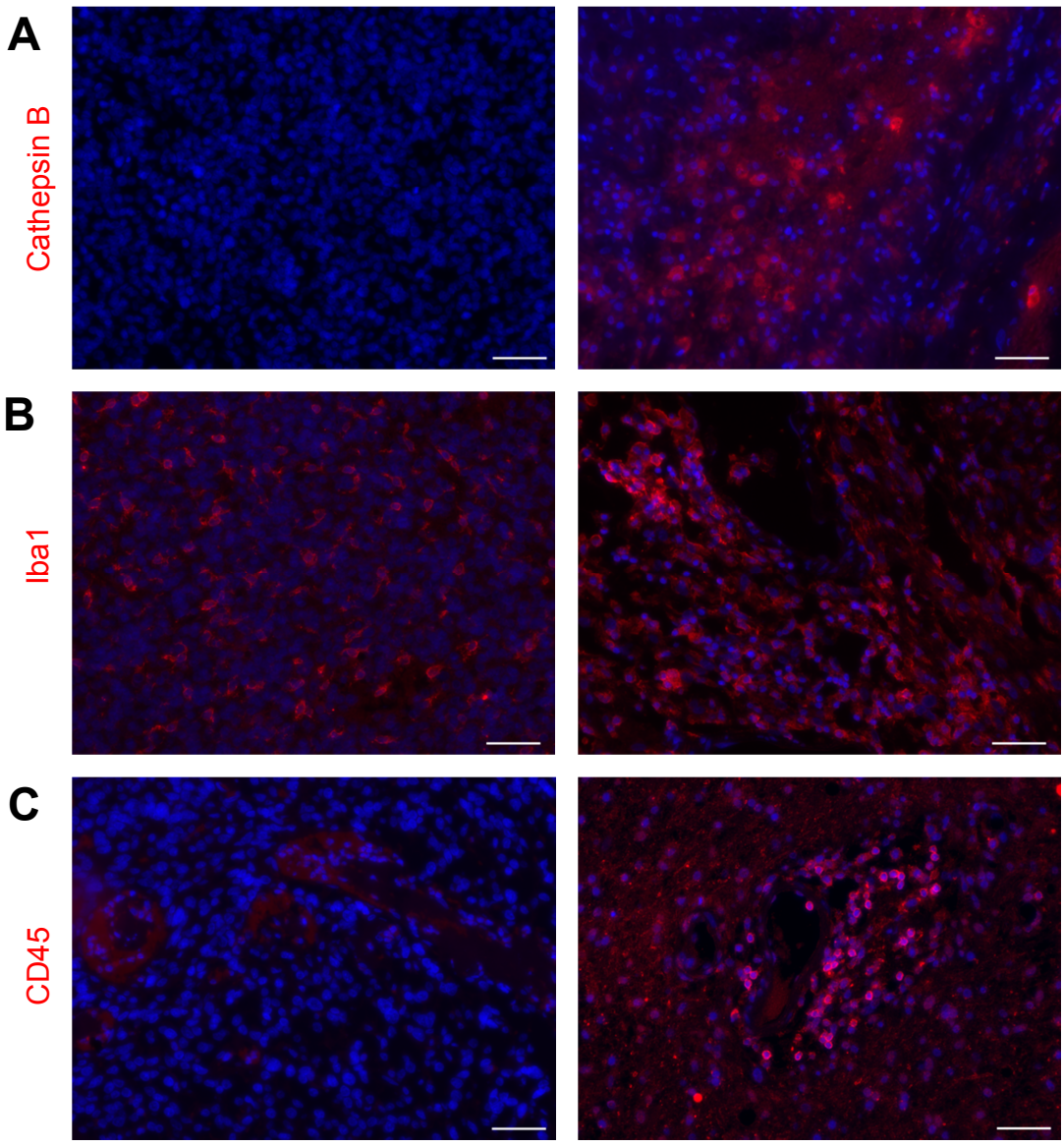


**Figure S3. Microglia/macrophage activation and leukocytic infiltration in a ParvOryx-treated recurrent glioblastoma compared with the corresponding primary tumor**

Recurrent glioblastoma (patient 1-02) was resected 9 days after intratumoral ParvOryx (5E5 PFU) administration, and paraffin-embedded tumor tissue was subjected to immunostaining (right panels). For comparison, the same analysis was performed with control samples from the resected primary tumor of the same patient (left panels). This primary tumor – which was not exposed to ParvOryx – gave rise, after resection to the recurrent tumor which was treated with ParvOryx in the framework of the present trial. (A) In contrast to the primary tumor, the ParvOryx-treated recurrent glioblastoma showed significant cathepsin B induction. (B) In comparison to pretreatment primary material, an increased microglia/macrophages phagocytic competence was noted after virus application. (C) Similarly, perivascular leukocytic infiltrates were detected only in the post-ParvOryx-treatment tumor tissue. Scale bars, 50 µm.

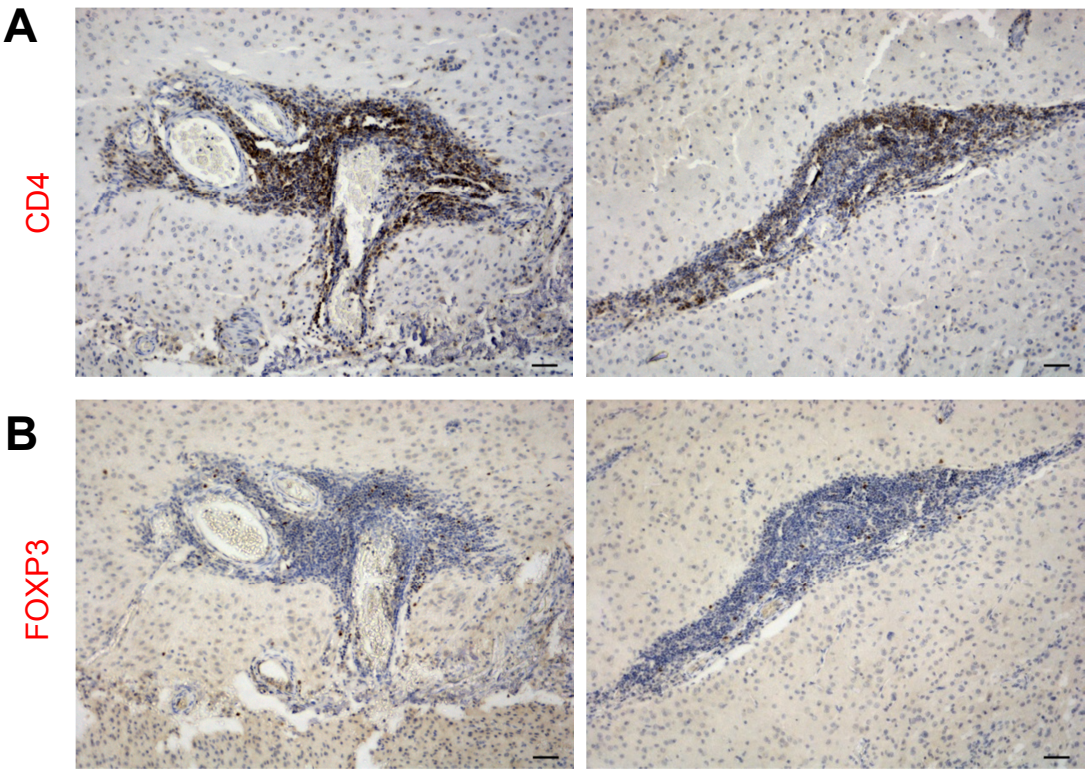


**Figure S4. Scarce Treg cell detection within tumor immune cell infiltrates**

Recurrent glioblastoma (patient 3-08) was resected 9 days after intratumoral ParvOryx (5E8 PFU) administration, and tumor-infiltrating immune cell populations were analyzed in situ through immunohistochemical staining. Prominent $CD3^+$ infiltrates were detected within the tumor and found to consist of both $CD8^+$ (not shown) and (A) $CD4^+$ T cells. (B) $FOXP3^+$ Treg cells represented only a very minor subpopulation within the $CD4^+$ tumor immune cell infiltrate. Scale bars, 50 µm.

**Table S1. Main characteristics of serious adverse events (SAEs)**

| Cohort | Subject ID | Preferred term | Start day | End day | Severity | Outcome | Relationship to ParvOryx |
|---|---|---|---|---|---|---|---|
| G1-L1 | 1-02 | Cerebrospinal fistula | 101 | 117 | Severe | Resolved | Unlikely |
| | | Post-operative wound infection | 101 | 117 | Severe | Resolved | Unlikely |
| G1-L2 | 2-04 | Deep vein thrombosis | 95 | 189 | Severe | Resolved | Unlikely |
| | | Pneumonia | 95 | 189 | Severe | Resolved | Unlikely |
| | | Pulmonary embolism | 95 | 189 | Severe | Resolved | Unlikely |
| | 2-06 | Cystitis | 80 | 87 | Severe | Resolved | Unlikely |
| | | Urosepsis | 116 | Unknown | Severe | Unknown | Unlikely |
| G1-L3 | 3-07 | Wound dehiscence | 31 | 129 | Severe | Resolved | Unlikely |
| | | Upper-limb fracture | 160 | 198 | Severe | Resolved | Unlikely |
| G2-L2 | 4-10 | Fall | 28 | 28 | Severe | Resolved | Not related |
| | | Head injury | 28 | 28 | Severe | Resolved | Not related |
| | | Subdural hygroma | 56 | Unknown | Severe | Unknown | Unlikely |
| | | Reduced level of consciousness | 71 | 98 | Severe | Not resolved | Unlikely |
| | | Pneumonia | Unknown | 98 | Fatal | Fatal | Unlikely |
| G3-L4 | 6-16 | Reduced level of consciousness | 12 | 185 | Severe | Not resolved | Possible |
| | | Secondary complications initiated by hydrocephalus | 15 | 185 | Fatal | Fatal | Possible |
| | | Occlusion of ventricular catheters due to high protein levels in CSF | 16 | Unknown | Life-threatening | Unknown | Possible |
| | 6-18 | Reduced level of consciousness | 11 | 102 | Severe | Not resolved | Unlikely |
| | | Convulsion | 16 | Unknown | Severe | Unknown | Unlikely |

**Table S2. Frequency of adverse events sorted by treatment cohort**

| AE subset | G1-L1 Frequency Count | | G1-L2 Frequency Count | | G1-L3 Frequency Count | | G2-L2 Frequency Count | | G2-L3 Frequency Count | | G3-L4 Frequency Count | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | P | E | P | E | P | E | P | E | P | E | P | E |
| Fatal events | . | . | 1 | 1 | . | . | 1 | 1 | . | . | 2 | 2 |
| Serious events | 1 | 2 | 2 | 5 | 1 | 2 | 1 | 5 | . | . | 2 | 5 |
| Related events | . | . | . | . | . | . | 1 | 1 | . | . | 1 | 4 |
| All events | 3 | 19 | 3 | 32 | 3 | 25 | 3 | 28 | 3 | 23 | 3 | 58 |

P, no. of patients; E, no. of events.

**Table S3. HLA class I-presented viral and glioma peptides on H-1PV-infected human glioma cells[a]**

| Viral protein | Peptide sequence/position (aa)[b] |
|---|---|
| NS1; NS2 | **NENVQLNGKDIGW/32-44** |
| NS1 | **NENITVVRI/486-494** |
| VP1 | AHIFVNQA/118-125 |
| VP1; VP2 | **SDGTETNQPDTGIANARVERSAD/145-166; 2-24** |
| | **TEQQGAGQDAIKVY/301-314; 159-172** |
| **Glioma protein** | |
| MAGEA1 | **KEADPTGHSY/160-169** |
| CSPG4 | **TMLARLASA/21-29** |
| | DTPLGTVALLQ/763-773 |
| SART1 | KLGLKPLEV/129-137 |
| | KTSSGDASSLSIE/109-121 |
| SART3 | TVKDLRLVTNR/790-800 |
| EPHA2 | NIMNDMPIY/57-65 |
| PTPRZ1 | VTGKVFAGIPTV/1344-1355 |
| MICA/B | **HLDGQPFLRY/50-59** |
| NRCAM | KVQALNDM/801-808 |

[a] Cells from a panel of 5 glioma lines showing broad HLA-class I allele expression were infected with H-1PV and processed for the identification of HLA I-presented peptides by immunoaffinity separation and sequencing mass spectrometry (see also Methods).

[b] Presented peptides identified as homologous (Expect Value <0.3) to fragment sequences (amino acid [aa] position) from H-1PV proteins[1] or known glioma antigens.[2] Peptides with an Expect Value <0.05, which is indicative of identity, are marked in **bold**. Peptide pools and individual peptides were used as stimulants in IFN-γ ELISpot assays. PBMCs were seeded on multi-well PVDF membrane plates pre-coated with anti-IFN-γ capture antibodies and incubated with (or without) a test or control stimulant for 18 h. Phytohemagglutinin (PHA, 25 µg/ml) and the CEF pool of common $CD8^+$ T cell epitopes (2.5 µM) were used as positive control stimulants.

1. Rhode, S.L. III, Paradiso, P.R. (1983). Parvovirus genome: Nucleotide sequence of H-1 and mapping of its genes by hybrid-arrested translation. J Virol. *45*, 173-184.
2. Dutoit, V., Herold-Mende, C., Hilf, N., Schoor, O., Beckhove, P., Bucher, J., et al. (2012). Exploiting the glioblastoma peptidome to discover novel tumour-associated antigens for immunotherapy. Brain. *135*, 1042-1054.

**Table S4. Main characteristics of dosing schedule**

**GROUP 1**

| Escalation level | Study day | Dose and route of administration | | Duration |
|---|---|---|---|---|
| **Level 1**<br>Total dose: 1E6 PFU | Day 1 | 5E5 PFU | intratumoral (via catheter) | 15 min |
| | Day 10 | 5E5 PFU | intracerebral (direct injection at several locations in resection wall) | 15–30 min |
| **Level 2**<br>Total dose: 5E7 PFU | Day 1 | 2.5E7 PFU | intratumoral (via catheter) | 15 min |
| | Day 10 | 2.5E7 PFU | intracerebral (direct injection at several locations in resection wall) | 15–30 min |
| **Level 3**<br>Total dose: 1E9 PFU | Day 1 | 5E8 PFU | intratumoral (via catheter) | 15 min |
| | Day 10 | 5E8 PFU | intracerebral (direct injection at several locations in resection wall) | 15–30 min |

**GROUP 2**

| Escalation level | Study day | Dose and route of administration | | Duration |
|---|---|---|---|---|
| **Level 1**[a]<br>Total dose: 1E6 PFU | Days 1–5 | 1E5 PFU | intravenous infusion | 2 h |
| | Day 10 | 5E5 PFU | intracerebral (direct injection at several locations in resection wall) | 15–30 min |
| **Level 2**<br>Total dose: 5E7 PFU | Days 1–5 | 0.5E7 PFU | intravenous infusion | 2 h |
| | Day 10 | 2.5E7 PFU | intracerebral (direct injection at several locations in resection wall) | 15–30 min |
| **Level 3**<br>Total dose: 1E9 PFU | Days 1–5 | 1E8 PFU | intravenous infusion | 2 h |
| | Day 10 | 5E8 PFU | intracerebral (direct injection at several locations in resection wall) | 15–30 min |

**GROUP 3**

| Escalation level | Study day | Dose and route of administration | | Duration |
|---|---|---|---|---|
| **Level 4 (single level)**<br>Total dose: 5E9 PFU | Day 1 | 2.5E9 PFU | intratumoral (via catheter) | 15 min |
| | Day 10 | 2.5E9 PFU | intracerebral (direct injection at several locations in resection wall) | 30–60 min |

[a] Planned as a possibility, but not implemented.

## Table S5. Schedule of trial procedures in patients of groups 1 and 3

| Study procedure | Study visit (day) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SC | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28[A] | FU[B] |
| Written informed consent | • | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Demography | • | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| HIV, HBV and HCV serology | • | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Pregnancy test | • | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Biopsy and histology [C] | | • | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Administration of ParvOryx [D] | | • | | | | | | | | | • | | | | | | | | | | | | | | | | | | | |
| Physical & neurological examination | • | | • | • | • | • | • | • | • | • | | • | • | • | • | • | • | • | • | | | | | | | | | | • | • |
| Vital signs [E] | • | | • | • | • | • | • | • | • | • | | • | • | • | • | • | • | • | • | | | | | | | | | | • | |
| 12-lead ECG | • | | • | | • | | | | | • | | • | | | | | | | • | | | | | | | | | | • | |
| Cl. chemistry, haematology, clotting [F] | • | | • | | • | | • | | | • | | | | | | | | | • | | | | | | | | | | • | |
| Serum protein electrophoresis | • | | | | | | | | | • | | | | | | | | | • | | | | | | | | | | • | |
| H-1 PV antibodies [G] | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | |
| MRI | • | | • | | | • | | | | • | | • [H] | | | | | | | | | | | | | | | | | • | • |
| Recording of adverse events | | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • |
| Shedding of H-1 PV [G] | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | |
| Tumour resection | | | | | | | | | | | • | | | | | | | | | | | | | | | | | | | |
| Survival status and PFS | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | • |

SC: screening; FU: follow-up; Cl.: clinical.

*A* Flexible: day 26–30 or earlier, if viral shedding had ceased or there was seroconversion (see text).

*B* 1 month (± 7 days) after day 28, then every 2 months (± 14 days) up to 6 months after day 1.

*C* At discretion of responsible investigator, i.e. only if imperative for unequivocal confirmation of diagnosis.

*D* Intratumoral administration on day 1, intracerebral administration on day 10.

*E* Pulse rate, blood pressure, body temperature.

*F* Selected parameters.

*G* Discontinuation of monitoring depending on previously obtained results.

*H* Either on day 11 or on day 12.

## Table S6. Schedule of trial procedures in patients of group 2

| Study procedure | Study visit (day) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SC | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28[A] | FU[B] |
| Written informed consent | • | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Demography | • | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| HIV, HBV and HCV serology | • | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Pregnancy test | • | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Biopsy and histology[C] | | • | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Administration of ParvOryx[D] | | • | • | • | • | • | | | | | • | | | | | | | | | | | | | | | | | | | |
| Physical & neurological examination | • | | • | • | • | • | • | • | • | • | | • | • | • | • | • | • | • | • | | | | | | | | | | • | • |
| Vital signs[E] | • | | • | • | • | • | • | • | • | • | | • | • | • | • | • | • | • | • | | | | | | | | | | • | |
| 12-lead ECG | • | | • | | • | | | | | • | | • | | | | | | | • | | | | | | | | | | • | |
| Cl. chemistry, haematology, clotting[F] | • | | • | | • | | • | | | • | | | | | | | | | • | | | | | | | | | | • | |
| Serum protein electrophoresis | • | | | | | | | | | • | | | | | | | | | • | | | | | | | | | | • | |
| H-1 PV antibodies[G] | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | |
| MRI | • | | • | | | • | | | | • | | • | [I] | | | | | | | | | | | | | | | | • | • |
| Adverse events | | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • |
| Shedding of H-1 PV[G,H] | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | |
| Tumour resection | | | | | | | | | | | • | | | | | | | | | | | | | | | | | | | |
| Survival status and PFS | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | • |

SC: screening; FU: follow-up; Cl.: clinical.

*A* Flexible: day 26–30 or earlier, if viral shedding had ceased or there was seroconversion (see text).

*B* 1 month (±7 days) after day 28, then every 2 months (±14 days) up to 6 months after day 1.

*C* At discretion of responsible investigator, i.e. only if imperative for unequivocal confirmation of diagnosis.

*D* Intravenous administration on days 1–5, intracerebral/intratumoral administration on day 10

*E* Pulse rate, blood pressure, body temperature.

*F* Selected parameters.

*G* Discontinuation of monitoring depending on previously obtained results.

*H* On days 1–5, viremia was monitored by taking three blood samples per day from each subject at the following times: pre-dose, interval from 1 h after start of infusion to 1 h after stop of infusion, and interval from 2 to 6 h after end of infusion.

*I* Either on day 11 or on day 12.

**Table S7. Sequence of locked nucleic acid (LNA) oligonucleotides used for FISH detection of H-1PV nucleic acids**

| | |
|---|---|
| **Sense probe** | |
| NS1 | 5DIGN/AATTCGCTAGGTTCAATGCGCT/3DIGN |
| VP | 5DIGN/TGACCTACCAACATCAGATACA/3DIGN |
| **Antisense probe** | |
| NS1 | 5DIGN/TCAGCACACAACAGATGGCAT/3DIGN |
| VP | 5DIGN/TACTATCCAGAGCAACCATCAT/3DIGN |

The sense and antisense probes were synthesized so as to recognize the negative or positive strands of H-1PV nucleic acids, respectively, and double digoxin (DIGN) labeled at their 3′ and 5′ ends. NS1- and VP-specific probes were used as a mix of equal amounts, to increase the hybridization signal. Signals were visualized by incubation with anti-DIGN antibody conjugated to horseradish peroxidase (Roche, Sigma-Aldrich, Munich, Germany) followed by incubation with the Tyramide Signal Amplification/cyanine 3 reagent (Perkin Elmer, Rodgau, Germany). Images were acquired with a motorized widefield Cell Observer® microscope and the ZEN blue image-processing software (Carl Zeiss, Göttingen, Germany).

**Table S8. Primary antibodies used for IF detection of tumor and tumor microenvironment markers**

| Marker | Primary antibody/Source | Provider |
|---|---|---|
| GFAP | ab7260/rabbit polyclonal | Abcam |
| EGFR | ab2430/rabbit polyclonal | Abcam |
| CD68 | MO814/mouse monoclonal | DAKO |
| CTSB | ab125067/rabbit monoclonal | Abcam |
| CD45 | ab8216/mouse monoclonal | Abcam |
| CD3 | ab5690/rabbit polyclonal | Abcam |
| CD4 | MA 1-80223/mouse monoclonal | Thermo Fisher Scientific |
| CD8 | ab4055/rabbit polyclonal | Abcam |
| GZMB | ab139354/mouse monoclonal | Abcam |
| Perforin | ab75573/mouse monoclonal | Abcam |
| IFN-γ | ab9657/rabbit polyclonal | Abcam |
| IL-2 | ab180780/rabbit polyclonal | Abcam |
| CD25 | ab154393/rabbit polyclonal | Abcam |
| CD154 (CD40L) | sc-978/rabbit polyclonal | Santa Cruz |
| Iba1 | ab15690/mouse monoclonal | Abcam |
| FOXP3 | ab20034/mouse monoclonal | Abcam |

Signals were visualized using cyanine 3/Oregon Green® 488-conjugated secondary antibodies. Images were acquired with a Cell Observer® microscope and the ZEN blue image-processing software (Carl Zeiss, Göttingen, Germany). GFAP, glial fibrillary acidic protein; EGFR, epidermal growth factor receptor; CTSB, cathepsin B; GZMB, granzyme B; IFN, interferon; IL, interleukin; Iba1, ionized calcium binding adaptor molecule 1; FOXP3, forkhead-box-protein P3.