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Antitumor Activity of Oncolytic Herpes Symplex Virus Type 1 and Photodynamic Therapy in *In Vitro* Preclinical Models of Glioblastoma

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Abstract

Background

Glioblastoma (GBM) remains the most aggressive primary brain tumor in adults, with limited therapeutic options and inevitable recurrence despite maximal standard-of-care treatment.

Photodynamic therapy (PDT) and oncolytic virotherapy have independently shown promise as alternative approaches, yet their combined potential in GBM remains incompletely explored.

Methods

We engineered a highly neuroattenuated herpes simplex virus type 1–based oncolytic virus (oHSV1), genetically related to talimogene laherparepvec (T-VEC), to express the photosensitizer KillerRed (KR) targeted to the cell plasma membrane (memKR). The cytolytic activity of this recombinant virus was evaluated in multiple human and murine GBM cell lines, patient-derived GBM cells, and three-dimensional (3D) spheroid models. We further assessed whether photoactivation of virus-encoded memKR or of the chemical photosensitizer phthalocyanine could enhance oHSV1-mediated cytotoxicity, including in spheroids infected via monocyte-based viral delivery.

Results

The recombinant memKR-encoding virus (oHSV1-KR) efficiently infected and killed GBM cells across 2D and 3D culture systems. Photoactivation of memKR significantly enhanced virus-mediated cytotoxicity in patient-derived GBM spheroids, particularly at lower viral doses. Similarly, phthalocyanine photoactivation augmented oHSV1-induced cell death, accelerating loss of viability in both monolayer cultures and spheroids. Monocyte-mediated delivery of oHSV1-KR resulted in effective viral transfer to GBM spheroids and retained responsiveness to photodynamic activation.

Conclusions

These findings demonstrate that combiningoHSV1–based virotherapy with photodynamic activation enhances cytotoxic efficacy in advanced preclinical models of GBM. While limited to *in vitro* and

ex vivo systems, this work establishes a modular and controllable therapeutic platform and provides a foundation for future *in vivo* and immunological studies aimed at translational development.

Keywords: Oncolytic Herpes Symplex Virus Type 1 (oHSV1); KillerRed (KR); Photodynamic therapy (PDT); Glioblastoma (GBM); Phthalocyanine.

Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor and is characterized by a dismal prognosis despite available treatments [1]. The standard therapeutic approach—surgical resection followed by chemotherapy—often fails due to recurrence, underscoring the urgent need for novel therapeutic strategies [2, 3]. Among emerging modalities, photodynamic therapy (PDT) and oncolytic virotherapy have gained increasing attention for the treatment of cancers, including GBM [4].

PDT employs a photosensitizing agent (PS), light, and oxygen to selectively induce cancer cell death [5]. Interstitial PDT (iPDT), which delivers light through stereotactically placed fiber optics, has been explored in brain tumors for more than three decades [6]. However, limitations in light penetration and photosensitizer design have restricted its broader clinical translation [5]. The INDYGO clinical trial (NCT03048240) evaluated intraoperative PDT following GBM resection via protoporphyrin IX (PpIX) induced by 5-aminolevulinic acid (5-ALA) [7]. The study has now been completed, with patient follow-up finalized in 2021. INDYGO demonstrated the feasibility and safety of intraoperative PDT in combination with fluorescence-guided surgery, providing encouraging signals of local tumor control. Based on these results, a follow-up DOSINDYGO trial was launched to explore higher light doses and enhanced tumor ablation depth, while additional clinical programs—including those at the U.S. National Cancer Institute (NCI) and UPMC—are

currently investigating dose-escalation intraoperative PDT protocols for newly diagnosed GBM. Together, these ongoing studies highlight the growing translational interest in light-based adjuvant therapies for GBM [5-7].

In previous work, we investigated the photosensitizer phthalocyanine in murine cancer models and demonstrated that its activation triggers apoptosis in both irradiated and neighboring bystander cells [8, 9]. Phthalocyanines, recognized as second-generation photosensitizers, have shown promising anticancer activity in preclinical studies [10, 11]. Their strong absorption in the near-infrared (NIR) region enables deeper tissue penetration than shorter wavelengths [12–14]. In addition, phthalocyanines exhibit high photostability and possess a modular chemical structure that allows optimization of their physicochemical and pharmacokinetic properties, although clinical translation remains influenced by formulation and delivery considerations [10]. To enhance tumor targeting, stability, and photodynamic efficacy, several groups have developed nanoparticle-based delivery systems for phthalocyanines [15].

In parallel, oncolytic viruses (OVs) have emerged as versatile therapeutic agents for cancers with poor prognoses [16]. OVs exploit defects in tumor antiviral signaling while directly lysing malignant cells and eliciting antitumor immune responses in immune-competent settings [16]. Most OVs are either attenuated human viruses or nonpathogenic wild-type viruses from other species. For example, oncolytic herpes simplex virus type 1 (oHSV1) is typically engineered with deletions in the γ34.5 gene to decrease neurovirulence [17–19]. Talimogene laherparepvec (T-VEC) was the first OV approved by the FDA and EMA for intratumoral treatment of unresectable melanoma. T-VEC is an oHSV1 variant deleted at γ34.5 and Us12. The latter deletion results in an increase of MHC-I mediated antigen presentation, thus improving the immune response against infected cells [20, 21]. While T-VEC additionally expresses granulocyte–macrophage colony–stimulating factor (GM-CSF) to enhance immune activation [20, 21], oHSV1 derivatives lacking immunostimulatory transgenes remain valuable for dissecting virolytic and combinatorial mechanisms in preclinical models [22]. Several oHSV1 variants are currently under clinical investigation for GBM [23–26].

Although early trials have demonstrated safety and biological activity following intracranial injection, direct delivery remains a major obstacle to broad clinical adoption, as stereotactic or intrathecal administration limits dosing frequency and scalability [23–27]. These limitations have motivated the exploration of alternative delivery strategies capable of improving tumor coverage while maintaining biosafety.

To address these challenges, carrier cell-based delivery of OVs has been proposed. *Ex vivo* loading of OVs into migratory cells enables systemic reinfusion and tumor-specific delivery [28]. In the case of GBM, such carriers must also traverse the blood–brain barrier (BBB). Monocytes, which constitute ~10% of circulating leukocytes, are promising candidates because of their ability to infiltrate the central nervous system and differentiate into tumor-associated macrophages (TAMs) [29, 30]. These cells can be isolated from peripheral blood, infected *ex vivo* with oHSV1, and reintroduced as Trojan horse carriers. Despite their potential, monocytes have rarely been explored as OV carriers and never with oHSV1 [31, 32]. Notably, even though wild-type HSV1 can infect monocytes, its replication is inefficient [33], a desirable property since carrier cells must remain viable and motile until tumor homing. We were the first to report that oHSV1-infected human monocytes migrate toward breast cancer cells *in vitro* and deliver their viral payload to tumor cells [34]. Using the *in ovo* chorioallantoic membrane (CAM) model [35, 36], we further showed that human monocytes can transport viral cargo to head and neck squamous cell carcinoma *in vivo* [34]. Moreover, employing a BBB-on-a-chip platform, we demonstrated that oHSV1-loaded monocytes efficiently traverse the blood–brain barrier, target human GBM spheroids, and release infectious virus shielded from neutralization by circulating immunoglobulins [37].

Recent studies have explored combining virotherapy with PDT [4, 38, 39]. This strategy offers the potential for spatiotemporal control of cytotoxicity through externally applied light, complementing virus-mediated tumor selectivity. This combinatorial strategy was shown to be effective in murine xenograft GBM models infected with oHSV1 expressing KillerRed (KR) [4], a genetically encoded photosensitizer whose photochemical properties and ROS-generating capacity have been

extensively characterized, enabling its use as a defined tool for photodynamic augmentation [40]. KR produces reactive oxygen species (ROS) upon illumination with 540–590 nm light [40], offering opportunities for targeted photodynamic virotherapy and combination immunotherapy [38-42].

Other genetically encoded photosensitizers have also emerged as attractive tools for photodynamic applications, and several variants have been developed, including KillerOrange [43], SuperNova [44], and miniSOG [45], which differ in their photophysical properties, activation wavelengths, and mechanisms of reactive oxygen species generation.

In the present study, KR was selected as a well-characterized and robust photosensitizer with established ROS-generating capacity and demonstrated compatibility with HSV1–based expression systems [4], serving as a proof-of-principle tool for integrating genetically encoded photodynamic activity into an oncolytic virotherapy platform. We therefore engineered a highly neuroattenuated oHSV1 variant expressing the KR protein targeted to the cell plasma membrane (memKR) to evaluate the potential of combining oncolytic virotherapy with PDT for GBM. We assessed the cytolytic effects of memKR-encoding oHSV1 (oHSV1-KR) in human and murine GBM cell lines and patient-derived cells in both 2D and 3D culture systems, including monocyte-mediated delivery. We then analyzed how memKR photoactivation enhances viral oncolysis and examined the combinatorial effects of oHSV1 and phthalocyanine. Collectively, our findings support the development of a multimodal therapeutic platform that integrates photodynamic and oncolytic strategies for the treatment of GBM. Alternative genetically encoded photosensitizers with longer activation wavelengths or improved optical properties may be explored in future studies to further enhance translational potential.

Materials and methods

Cells

Patient-derived GBM cells and ethics

Our research complied with all relevant ethical regulations and guidelines. This study was conducted under protocols approved by the IRBs and IACUCs of Padua University Hospital (2462P), Italy. All patients provided written informed consent for all clinical information, treatments, and prospective biopsy acquisition. All tissues were acquired following the tenets of the Declaration of Helsinki. Patient-derived GBM cultures were obtained from distinct surgical specimens and therefore reflect inter-patient biological heterogeneity; molecular subtype and treatment history were not used as stratification criteria in the present study.

Cells were isolated from GBM tumors following surgery and cultured as previously described [46]. Briefly, GBM samples were enzymatically and mechanically dissociated into single-cell suspensions. The cells were then placed on fibronectin-coated plates and grown as monolayers in DMEM/F12 (Biowest, Nuaillé, France, #11320033) supplemented with 10% BIT9500 (Stem Cell Technologies, Vancouver, Canada, #09500), 20 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml epidermal growth factor (EGF; both from Cell Guidance Systems Ltd., Cambridge, UK). GBM cells were maintained in an atmosphere of 2% oxygen, 5% carbon dioxide and balanced nitrogen in an H35 hypoxic cabinet (Don Whitley Scientific Ltd., Shipley, UK) to better recapitulate the hypoxic conditions of the GBM microenvironment [46, 47].

Cell lines

Human glioblastoma cell lines (LN-229, U87-MG), murine glioblastoma cell lines (GL-261, CT-2A), and non-tumor cell lines used for viral propagation and vector production are specified below. LN-229 (ATCC, Manassas, United States), U87-MG (ATCC), Vero CCL81 (ATCC), HEK-293T (ATCC), GL-261 (DMSZ, ACC 802) and CT-2A (SCC194, Sigma Aldrich, St. Louis, USA) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Monza, Italy, #11965092) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Thermo

Fisher Scientific, #A5256701) and 1% (v/v) penicillin–streptomycin (Pen-Strep; Gibco, Thermo Fischer Scientific, #15140122).

All cell lines were subjected to periodical screening for mycoplasma contamination via a Mycostrip Mycoplasma detection kit (InvivoGen, Toulouse, France, #rep-mys-10).

3D tumor spheroid cultures

3D tumor spheroid cultures were used to better approximate tumor architecture, cell–cell interactions, and diffusion constraints relevant to GBM biology compared with monolayer cultures.

U87-MG spheroids were generated as previously described [37]. Briefly, U87-MG cells (2×10^3 cells per well) were seeded in 100 µL of culture medium (DMEM supplemented with 10% v/v FBS and 1% v/v P/S) in ULA 96-well round-bottom plates (Corning®, New York, USA, #3474) and centrifuged at 1000 rpm for 10 minutes to facilitate spheroid formation.

For CT-2A spheroids, $5x10^3$ cells per well were seeded in 100 µL of culture medium (DMEM F12 supplemented with 10% v/v BIT9500, 20 ng/ml bFGF, 20 ng/ml EGF and 1% v/v P/S) inside ULA plates and centrifuged for 10 minutes at 1000 rpm.

For hGBM-82 and hGBM-15, $5x10^3$ cells per well were seeded in 100 µL of culture medium (DMEM F12 supplemented with 10% v/v BIT9500, 20 ng/ml bFGF, 20 ng/ml EGF and 1% v/v P/S) inside ULA plates and cultured for 5 days to allow spheroid formation.

oHSV1- KR construction, reconstitution and titration

Recombinant oHSV1 variants were generated via bacterial artificial chromosome (BAC) mutagenesis, as previously described [49, 50].

A BAC containing the full genome of the HSV1 strain 17+, with γ34.5 deletions and an FLuc expression cassette inserted into the UL55-UL56 intergenic region, was kindly provided by Beate Sodeik (Hannover Medical School, Germany). This BAC was further modified as follows:

1. The introduction of a Us12 deletion mirrored the deletion present in T-VEC.
2. Multiple mir124 target sequences were incorporated to enhance neuroattenuation [51].

   These first two steps led to the generation of the oHSV1 adopted in the phthalocyanine experiments (oHSV1). These modifications were designed to enhance neuroattenuation beyond γ34.5 deletion while preserving replication competence in tumor cells. Unlike T-VEC, this vector does not encode immunostimulatory transgenes, allowing focused analysis of virolytic and photodynamic interactions.
3. The FLuc cassette was replaced with a memKR expression cassette under the control of the human CMV (hCMV) promoter. The memKR expression sequence was obtained from https://evrogen.com/products/vectors/pKillerRed-membrane/pKillerRed-membrane.shtml.

All intermediate plasmids used for BAC mutagenesis and the final bacmids were verified via restriction analysis and/or sequencing.

Recombinant oHSV1s were reconstituted by transfecting HEK-293T cells with the corresponding bacmid DNA, followed by viral amplification and titration in highly permissive Vero cells, as previously described [50]. The same titration assay was adopted for the quantification of infectious viral particles released in the cell culture supernatants when requested.

Monocyte isolation, infection, and functional assays

Human monocyte isolation

Primary human monocytes were isolated from buffy coats obtained from healthy donors and provided by the Transfusion Center of the University Hospital of Padua, Italy. Peripheral blood mononuclear cells (PBMCs) were first separated via density gradient centrifugation via Ficoll-

Paque® Plus (Sigma–Aldrich, #GE17-1440-02) following the manufacturer's instructions. CD14+ monocytes were then purified from the PBMC fraction via the EasySep™ Human CD14 Positive Selection Kit II (StemCell Technologies, Vancouver, Canada, #100-0694) according to the provided protocol.

When needed, monocytes were cultured in cancer cell-conditioned medium derived from subconfluent U87-MG and LN-229 cells, which were subsequently grown in RPMI 1640 medium (Thermo Fisher Scientific, St. Louis, USA, #11875093) supplemented with 10% FBS and 1% P/S for 24 hours before the supernatant was harvested.

Monocyte infection and coculture assays

Primary human monocytes were infected with oHSV1 at an MOI of 3/5 PFU/cell, depending on the experimental conditions.

For coculture assays, infected monocytes were resuspended in complete culture medium and cocultured with confluent cancer cells at a 1:1 ratio. The supernatants were collected at fixed time points for viral titration.

Assessment of monocyte immune polarization, cytokine secretion, or long-term viability *in vivo* following viral loading was beyond the scope of the present study.

Migration assays

Migration assays were independently repeated using monocytes derived from multiple donors to ensure reproducibility. Monocyte migration was assessed via the use of 6.5 mm Transwell plates with 5.0 μm pore polycarbonate membrane inserts (Corning, Torino, Italy, #CLS3422). Uninfected and oHSV1-infected human monocytes (10^5 cells) were suspended in 100 μL of serum-free OptiMEM (Gibco, #11058021) and seeded into the upper chambers of Transwell inserts. The lower chamber contained 600 μL of either serum-free medium alone or cancer cell-conditioned

serum-free medium. Transwell plates were incubated for 3 hours at 37°C in a 5% CO_2 atmosphere with 98% humidity. Following incubation, the inserts were removed, and the migrated cells were stained with CellTracker™ Green Dye (Invitrogen, Thermo Fisher Scientific, Carlsbad, United States, #C7025) following the manufacturer's protocol. Migrated cells were counted across at least n = 3 different 10× microscopic fields.

GBM cell infection with oHSV1

GBM cells were seeded in appropriate multiwell plates, depending on the cell type and experimental requirements: 96-well plates: 1×10^4–2×10^4 cells/well; 24-well plates: 1×10^5–1.8×10^5 cells/well; and 6-well plates: 2.5×10^5–8.7×10^5 cells/well.

The following day, the cells were infected with recombinant oHSV1 variants at the appropriate MOIs in serum-free DMEM. Viral doses were selected based on preliminary titration experiments and prior studies to balance efficient infection with preservation of cell viability at early time points. After a 1-hour incubation at 37°C, the cells were washed with PBS and then maintained in DMEM supplemented with 2% FBS.

Infected cells were observed at various time points via fluorescence or confocal microscopy.

When needed, cell viability was analyzed via an MTT cell growth assay kit (Millipore Sigma, Burlington, USA, #11465007001) or via a Trypan blue exclusion assay (Thermo Fisher Scientific, #15250061), following the manufacturer's instructions.

Spheroid infection assays

GBM spheroids were infected with predetermined PFU of each oHSV1 variant. The number of PFU per spheroid was scaled to approximate the estimated number of cells per spheroid and to enable consistent infection across experiments. The desired viral dose was prepared in serum-free DMEM, and after washing with PBS, the virus was added to ULA 96-well round-bottom plates (Corning®) containing preformed spheroids.

After a 1-hour incubation at 37°C allowing for viral adsorption, the medium was discarded, a wash with PBS was performed, and the wells were filled with complete medium. Fluorescence microscopy was used in the following days to monitor viral infection and spread within the spheroids. When requested, cell viability was assessed by either an MTT cell growth kit (Millipore Sigma) or a Cell-Titer Glo® 3D cell viability assay (Promega, Madison, United States, # G9681) following the manufacturer's instructions.

GBM spheroid infection with oHSV1-loaded monocytes

Freshly isolated monocytes were labeled with CellTracker™ Green (Thermo Fisher) by incubating them in serum-free RPMI medium containing 0.5 µL of dye per 1 mL of solution for 20 minutes at 37°C. The labeled monocytes were pelleted via centrifugation (1000 rpm, 5 minutes), washed once with 1 mL of PBS, and resuspended in serum-free RPMI. Monocytes were then infected with oHSV1-KR at an MOI of 3 PFU/cell via incubation for 60 minutes at 37°C. Following infection, the cells were pelleted, washed twice with PBS, and resuspended in complete medium or PBS, depending on the experiment. oHSV1-loaded monocytes were added to preformed tumor spheroids at a final concentration of 5×10^3 monocytes per spheroid. Infection and viral spread were monitored over time via fluorescence microscopy. Cell viability was assessed with an MTT cell growth kit (Millipore Sigma) following the manufacturer's instructions.

Phthalocyanine treatment of 2D- and 3D-cultured hGBM-82 cells

Aluminum phthalocyanine chloride (Sigma–Aldrich, # 362530) was added to the cells at a final concentration of 10 µM together with pluronic F-127 (Thermo Fisher Scientific, # P6867) at a ratio of 1:100 v/v for 1 h at 37°C. Phthalocyanine concentration and incubation time were optimized to minimize dark toxicity while preserving robust photodynamic responsiveness. Next, the cells were washed 3 times with PBS and maintained in the appropriate complete medium during the following experiments. To assess cell viability upon phthalocyanine incubation and photoactivation, the cells

were stained with YO-PRO™ (Thermo Fisher Scientific, # Y3603) at a final concentration of 500 nM plus 2 drops/ml of NucBlue™ (Thermo Fisher Scientific, # R37605) in appropriate medium without FBS for 15 minutes at 37°C. Next, the cells were washed in PBS and incubated in complete medium for observation with a fluorescence microscope (Leica DFC7000T).

Cell photoactivation

For memKR photoactivation, we used a fiber-coupled 588 nm laser (MGL-U-588-100mW, Changchun New Industries Optoelectronics Tech.Co.Ltd). Laser output was recollimated with 20 mm aspheric lens (Thorlabs # **ACL2520U**) and coupled to the optical path of an upright spinning disk microscope (Olympus DSU) with a 50:50 broadband nonpolarizing beamsplitter cube (Thorlabs, # BS013) inserted above the objective. The latter was assembled by the juxtaposition of two achromatic doublets with focal lengths of 50 mm (Thorlabs #AC254-050-AB) and 75 mm (Thorlabs #AC254-075-AB), respectively. With an effective focal length of 30 mm, this objective permitted low-magnification imaging (approximately 3.5×,) and concurrent photoactivation of cells in 96 well plates **(Fig.S1A, B)**.

The laser was operated in pulsed mode, delivering 500 ms pulses at 1-s intervals (50% duty cycle) with an irradiance of I = 120 mW/cm^2, as measured at the beginning of each session using a USB Power Meter, Silicon Sensor 500 mW (PM16-121, Thorlabs). 2D cultures were irradiated for 1 hour (i.e. for an effective time interval Δt = 30 min, hence fluence $H = I \times \Delta t$ = 216 J / cm^2).

3D tumor spheroids were subjected to two rounds of photoactivation of 40 min each (fluence H = 144 J / cm^2) at 24-hour intervals. Thereafter, cell viability was evaluated via either the MTT Cell Growth Kit (Millipore Sigma) or the CellTiter-Glo® 3D Cell Viability Assay (Promega), following the manufacturer's protocols.

For Aluminum phthalocyanine chloride-loaded cells, the 588 nm laser was replaced by a 660 nm LED (M660L4, Thorlabs). Light pulses (500 ms, 50% duty cycle, I = 100 mW/cm^2) were delivered

for 10 minutes in 2D cultures (fluence H = 30 J/cm^2) and 20 minutes in tumor spheroids (fluence H = 60 J/cm^2).

Following photoactivation, cell viability was determined with the CellMeter™ Colorimetric Cytotoxicity Assay Kit (AAT Bioquest®, #22780) or the CellTiter-Glo® 3D Cell Viability Assay (Promega) according to the manufacturer's instructions.

All photoactivation experiments were performed at room temperature.

ROS detection

Cultures of hGBM-82 cells were grown on 12 mm round glass coverslips, infected or not with oHSV1-KR and loaded for 30 min at 37 °C in serum-free medium with 2',7'-Dichlorofluorescein Diacetate (DCF-DA, 10 μM, Merck # 287810), which enters cells and is cleaved by intracellular esterases to yield DCFH. This nonfluorescent intermediate is then oxidized by hydroxyl, alkoxyl, and peroxyl radicals, generating the fluorescent product DCF. In contrast, hydrogen peroxide and lipid hydroperoxides do not directly oxidize DCFH. Because DCFH distributes to both the cytosol and cellular membranes, it can interact with reactive species in aqueous as well as lipid environments. Accordingly, this probe is commonly used to assess overall free-radical production across cellular compartments. Similar experiments were also performed by loading cells with Liperfluo (1 μM, Bio-Techne # 8868/50U), a fluorescent probe for the detection and imaging of lipid hydroperoxides in living cells.

Cells were then transferred to the stage of Olympus DSU microscope equipped with a sCMOS camera (PCO.EDGE) and a 20× water immersion objective (XLUMPLFLN20XW, Olympus # N2699600, numerical aperture 1.0). Alternating excitation was provided by a custom-made LED illuminator at 470 nm (Thorlabs # M470L5) and 565 nm (Thorlabs # M565L3) to monitor DCF and KR fluorescence, respectively, using a multiband BrightLine® Pinkel filter set (IDEX/Semrock #S-001070/LED-DA/FI/TX-3X-B-000). Baseline images were acquired for 10 min, after which cells

were exposed to pulsed laser irradiation (588 nm, 500 ms pulses, 50% duty cycle, irradiance I = 120 mW/cm^2) for 35 min (fluence H = 126 J/cm^2).

For ROS-measurement experiments in cells loaded with aluminum phthalocyanine chloride and related non-loaded controls, irradiation was carried out with a 660 nm LED (M660L4, Thorlabs). Light pulses (500 ms, 50% duty cycle, irradiance I = 18 W/cm^2) were delivered for 5 minutes (fluence H = 2722 J/cm^2).

DCF fluorescence was quantified offline with Fiji (ImageJ) by averaging pixel signals within regions of interest (ROIs) encompassing each cell in the field of view. Data are shown as time-dependent departure from baseline, $\Delta F(t) = F(t) - F_0$, in arbitrary units (A.U., the same for all experiments) where F_0 denotes temporal mean of baseline (pre-photoactivation) fluorescence.

For measurements with a Fluorometric Intracellular ROS Kit (Merck # MAK143), hGBM-82 cells were grown in 96 well flat-bottom plates, infected or not with oHSV1-KR and irradiated continuously for 7 min with light from a 565 nm LED (Thorlabs # M565L3; irradiance I = 182 mW/cm^2, fluence H = 76 J/cm^2). Cells were then treated for 1 h with the Kit components as prescribed by the manufacturer. Immediately after, they were transferred to the stage of an inverted fluorescence microscope (DM IL LED Fluo, Leica Microsystems) and imaged with filter set I3 (Leica, # S 11513828, BP 450-490 excitation, RKP 510 dichroic, LP 515 emission) for ROS detection and N2.1 (Leica, # S 11513832, BP 515-560 excitation, RKP 580 dichroic, LP 590 emission) for memKR visualization. The microscope was equipped with a fluorescence illuminator (Leica LED5000 SLI, # 10450548), HI Plan L objectives (Leica, 20× # 11506272, 40× # 11506369) and a cooled CCD camera (Leica, DFC7000 T # 11547106). Acquired images were analyzed offline with Fiji (ImageJ). Fluorescence intensity was quantified by generating regions of interest (ROIs) on images of nuclei stained with NucBlue Live ReadyProbes (ThermoFisher Scientific # R37605) and imaged with filter set A (Leica, # S 11513824, BP 340-380 excitation, RKP 400 dichroic, LP

425 emission). These ROIs were then applied to the background-subtracted ROS images to determine the mean fluorescence value for each cell (A.U., the same for all experiments).

All ROS-detection experiments were performed at room temperature.

Statistical analysis

Statistical analyses were performed with GraphPad Prism or Matlab (R2025b). Unless otherwise noted, graphical data represent the mean ± standard deviation (SD) from at least n=3 independent samples. Comparisons between two groups were performed via Student's *t* test or Kruskal-Wallis test, depending on normality of distributions. Differences were considered statistically significant for P-values (p) < 0.05. No statistical methods were used to predetermine sample size.

Results and Discussion

oHSV1-KR efficiently replicates and kills human and murine GBM cells

To integrate oncolytic virotherapy with PDT, we engineered an oHSV1 expressing the photosensitizing protein memKR [40] (oHSV1-KR). The parental vector is an oHSV1 backbone closely related to the clinically approved T-VEC, which contains deletions in the γ34.5 and Us12 genes [20, 21]. Unlike T-VEC, our construct does not encode human granulocyte–macrophage colony–stimulating factor (GM-CSF). Instead, it carries a firefly luciferase (FLuc) reporter cassette inserted into the UL55-UL56 intergenic region under the control of the cytomegalovirus (CMV) immediate-early promoter. Importantly, to achieve enhanced neuroattenuation, beyond γ34.5 deletion, we incorporated the microRNA-124 (miR-124) target sequence—abundantly expressed in mature neurons—downstream of the UL29 gene, which encodes a DNA-binding protein that is essential for viral replication [37, 51–53]. This design yielded oHSV1-miR124, which was used here as the parental strain. From this backbone, we first generated oHSV1-miR124-mCherry, in

which FLuc was replaced by the mCherry fluorescent reporter. We recently demonstrated that oHSV1-miR124-mCherry effectively replicates in and kills spheroids derived from the human GBM U87-MG cell line [37].

In the new oHSV1-KR variant, the mCherry-encoding gene was substituted with memKR sequence. We evaluated oHSV1-KR infectivity and cytolytic activity in the human U87-MG and LN-229 GBM cell lines. Both cell lines were infected with a multiplicity of infection (MOI) of 1 plaque-forming unit (PFU) per cell and resulted highly susceptible to infection. Similarly, a marked decrease in cell viability was observed at 48 hours post-infection via the MTT assay (**Fig. 1A**). oHSV1-KR also efficiently infected and spread in murine GBM cells (**Fig. 1B**). Consistent with previous observations [54–56], GL-261 cells were significantly less permissive than CT-2A cells to oHSV1 infection, a feature that was reflected in viability measurements at 48 hours after infection.

Furthermore, when viable cells were quantified via a trypan blue exclusion assay, we observed a clear reduction in both viable U87-MG and CT-2A-infected cells, indicating that oHSV1-KR kills human and murine GBM cells (**Fig. 2**).

Crucially, oHSV1-KR was also capable of infecting and killing patient-derived GBM cells, namely, hGBM-82 cells, in an MOI-dependent manner (**Fig. 3**).

We next evaluated the ability of oHSV1-KR to infect, propagate, and kill murine and human GBM spheroids. CT-2A-derived spheroids were infected with 20,000 PFU and monitored via fluorescence microscopy. The dose of viral particles was selected based on the number of cells seeded to obtain spheroids. Cell viability, as measured by the MTT assay on days 3 and 7 post infection, revealed that oHSV1-KR efficiently infected murine GBM spheroids, significantly reducing viability compared with that of the uninfected controls (**Fig. 4A**).

Similar results were obtained with spheroids derived from hGBM-82 cells infected with 20,000 PFU of oHSV1-KR. These spheroids exhibited clear viral spread and a marked decrease in viability on both day 3 and day 7 post infection (**Fig. 4B**).

Interestingly, infection of spheroids derived from another patient (hGBM-15) with the same viral dose also resulted in robust viral replication and dissemination (**Fig. 4C**). However, the cytolytic effect in hGBM-15 spheroids was more pronounced than that in hGBM-82 spheroids at both time points, which is consistent with the well-known heterogeneity of GBM, which can influence susceptibility to oncolytic infection and killing. Overall, these results confirm and extend our previous findings in U87-MG spheroids [37], demonstrating that oHSV1-KR efficiently infects and kills both murine and human GBM spheroids under 3D growth conditions. At this stage, these experiments were designed to establish infectivity and cytolytic competence of the engineered oHSV1-KR platform across GBM models rather than to isolate the contribution of KR expression per se, which was addressed in subsequent combinatorial analyses.

Effect of memKR photoactivation on primary human GBM cells and spheroids

To evaluate the combined effects of viral infection and photoactivation in clinically relevant setting, hGBM-82 cells were infected with oHSV1-KR at two MOIs (0.1 and 1). Fluorescence microscopy confirmed the expected MOI-dependent spread of the virus (**Fig. 5**, left panels). At 48 hours post-infection (MOI=0.1) or 24 hours post-infection (MOI=1), the cells were photoactivated (irradiance I = 120 mW/cm^2, fluence H = 216 J/cm^2, **Fig. S1**), and the next day, viability was measured via the MTT assay (**Fig. 5**, right panels). Photoactivation significantly accelerated and enhanced virus-mediated cytotoxicity at lower MOIs (**Fig. 5**, right upper panel), indicating an additive effect between viral oncolysis and KR activation. At higher MOIs, the direct oncolytic effect of oHSV1-KR was predominant, masking the contribution of KR photoactivation.

To confirm ROS generation downstream of memKR photoactivation, we performed live-cell imaging in oHSV1-KR–infected hGBM-82 cells 24 hours post-infection (MOI=1) and non-infected controls.

Cells were loaded with DCFH-DA, which yields the green-fluorescent, ROS-sensitive product DCF, and imaged by alternating excitation at 470 nm and 565 nm to monitor ROS and KR

fluorescence, respectively (**Fig. 6A**). In oHSV1-KR–infected hGBM-82 cells, pulsed laser illumination (588 nm, irradiance I = 120 mW/cm^2, fluence H = 126 J/cm^2) induced progressive KR photobleaching, a pre-requisite for ROS generation. After 35 min of irradiation, the median reduction of KR emission amounted to ~40% (**Fig. 6B** and **Fig. S1B, C**). In parallel, DCF fluorescence emission grew steadily and, at the end of irradiation, the median variation ΔF in oHSV1-KR-infected hGBM-82 cells (**Fig. 6B**) exceeded that of non-infected controls (**Fig. 6C)** by 17.5-fold (**Fig. 6D**). Similar results were obtained with Liperfluo (1 μM), a fluorescent probe of lipid peroxidation (**Fig. S2),** as well as with a Fluorometric Intracellular ROS Kit (MAK143, **Fig. S3**),

We next extended our analysis to 3D culture conditions by using hGBM-82-derived spheroids, which were infected with oHSV1-KR (20,000 PFU/spheroid) and monitored by fluorescence microscopy. When most cells exhibited KR fluorescence (typically on day 2 or 3 post infection), morphological signs of viral replication became evident—such as the presence of rounded cells at the spheroid periphery—in a subset of spheroids, photoactivation was initiated via a 588 nm laser (120 mW/cm^2), 500 ms pulsed light, and a 50% duty cycle. The irradiation was applied for 40 minutes (thus, fluence H = 144 J/cm^2), a duration selected to approximate conditions that could be feasibly reproduced *in vivo* in murine models. Twenty-four hours later, a second photoactivation was performed under identical conditions, and cell viability was assessed the following day. A group of infected spheroids was not irradiated. As additional controls, uninfected spheroids were either subjected to the same photoactivations or left untreated. As shown in **Fig. 7**, after the first round of irradiation (middle left panels), a reduction in red fluorescence and the appearance of disaggregated cells around the spheroids were observed in the infected samples. Following the second light treatment (**Fig. 7**, lower left panels), spheroid disaggregation was further enhanced, and red fluorescence completely disappeared; however, this phenomenon was still observed in the infected, nonirradiated controls (**Fig. S4,** oHSV1-KR panels). Quantitative analysis confirmed a

marked reduction in cell viability in spheroids infected with oHSV1-KR compared with that in uninfected spheroids ($p < 0.0001$), whereas irradiation alone had no detectable effect on control spheroids ($p > 0.05$). Moreover, a statistically significant difference was observed between spheroids treated with the virus alone and those receiving combined oncolytic virotherapy and photoactivation, demonstrating a statistically significant enhancement of cytotoxic efficacy relative to either treatment alone (**Fig. 7,** right graph). These experiments were replicated in U87-MG-derived spheroids under the same conditions adopted for hGBM-82-derived spheroids, and in this case, the oHSV1-KR killing effect was significantly augmented by photoactivation **(Fig. S5**).

These experiments in advanced 3D preclinical models do not account for immune-mediated or vascular effects that may influence treatment responses *in vivo*.

Effect of carrier-mediated delivery of oHSV1-KR

To develop a feasible strategy for the systemic delivery of oHSV1 *in vivo*, we previously proposed the use of autologous human monocytes as cellular carriers [34]. In our earlier work, oHSV1-loaded primary monocytes were shown to migrate *in vitro* toward epithelial cancer cells of different origins. Moreover, human monocytic leukemia cells successfully delivered oHSV1 to human head and neck xenograft tumors grown on the chorioallantoic membrane (CAM) of fertilized chicken eggs following intravascular injection [34].

To test these findings, primary human monocytes were isolated from buffy coats of healthy donors and infected with oHSV1-mCherry at an MOI of 5, as previously described [34]. Migration assays demonstrated that oHSV1-infected monocytes efficiently migrated toward conditioned media derived from human GBM cells (**Fig. 8A**). Furthermore, when infected monocytes were cocultured with human GBM cells, the amount of infectious viral particles released into the supernatant progressively increased over time, as quantified by plaque assays (**Fig. 8B**).

We next evaluated whether primary human monocytes could similarly deliver oHSV1-KR to GBM spheroids, in light of our earlier observation that monocytes loaded with oHSV1-mCherry can

traverse an on-chip BBB model, shield the virus from neutralizing antibody and deliver viral payloads to U87-MG spheroids [37]. Monocytes were loaded with oHSV1-KR at a MOI of 3, as previously reported [37], and cocultured with hGBM-82-derived spheroids. Additional groups of spheroids were either directly infected with 15,000 PFU of free virus (**Fig. S6**) or left uninfected. Viral infection and propagation were monitored for 7 days post-treatment (**Fig. 9 and Fig. S6**). On day 1 (**Fig. 9**, upper panels), monocytes labeled with a green fluorescent vital dye were observed both around and within the tumor spheroids, which was consistent with their known tumor-homing behavior. No evident viral replication was detected at this early stage. By day 4 (96 hours, middle panels) and day 7 (lower panels), spheroids displayed widespread red fluorescence, indicating that monocytes had successfully transmitted oHSV1-KR infection to GBM cells. To assess the effects of viral spread, an MTT assay was performed on day 7 (**Fig. 9**, right panel). Compared with the untreated controls, both the monocyte-mediated and free-virus treatment groups presented a significant reduction in spheroid viability ($p < 0.05$) within this preclinical spheroid system. Importantly, no significant difference in cytotoxicity was observed between the two treatment modalities, demonstrating that monocytes effectively function as carrier cells for oHSV1-KR delivery. These experiments indicate that oHSV1-KR can be efficiently transmitted by human monocytes to hGBM-82 spheroids, resulting in a substantial loss of tumor cell viability comparable to that induced by direct viral infection (**Fig. 9**).

Next, primary human monocytes were loaded with oHSV1-KR (MOI=3) and cocultured with hGBM-82-derived GBM spheroids. Fluorescence microscopy was used to monitor infection dynamics. When viral transfer to GBM cells became evident (day 3 post coculture), a first round of photoactivation was performed via pulsed 588 nm laser light for 40 minutes, as described above (fluence H = 144 J/cm^2). A second photoactivation was conducted 24 hours later under identical conditions.

Compared with the control, the combination of monocyte-mediated viral infection and KR photoactivation resulted in a significant ($p < 0.05$) reduction in spheroid viability (**Fig. 10**). These

results indicate that KR photoactivation contributes to the antitumor effect even when oHSV1-KR is delivered via monocyte carriers. While monocyte-mediated delivery was effective in this model and in previously investigated *in vitro* and *in vivo* systems [34, 37], the impact of viral loading on monocyte immune function and behavior within an intact tumor microenvironment remains to be determined.

Phthalocyanines can be combined with oHSV1 to achieve stronger and faster cell death

In addition to genetically encoded PS, several chemical PS molecules have been developed as potent inducers of photodynamic cytotoxicity and have demonstrated efficacy against GBM in preclinical and clinical settings. Among these, our attention focused on aluminum phthalocyanine chloride, which we previously validated for its ability to generate ROS and trigger apoptosis upon photoactivation [8, 9].

Here, we investigated whether phthalocyanine loading could potentiate the oncolytic activity of oHSV1 against GBM spheroids. We first optimized the conditions for the phthalocyanine loading of hGBM-82 cells to ensure minimal baseline cytotoxicity. The cells were incubated with 10 µM phthalocyanine for 1 hour at 37°C, followed by photoactivation with 660 nm LED light (irradiance I = 100 mW/cm^2, 500 ms pulses, 50% duty cycle) for 10 minutes (fluence H = 30 J/cm^2) at room temperature. Twenty-four hours later, the cells were stained with YO-PRO (to identify dead cells) and NucBlue (to label all nuclei) and analyzed via fluorescence microscopy. Phthalocyanine was well tolerated by the cells in the absence of photoactivation, whereas a substantial increase in YO-PRO–positive cells was detected after photoactivation **(Fig. S7)**, confirming light-dependent cytotoxicity. At saturation, the associated median ROS production in phthalocyanine-loaded hGBM-82 cells exceed equally irradiated non-loaded controls by 24-fold (**Fig. S8**).

We next assessed the effect of combining phthalocyanine treatment with viral infection (**Fig. 11** and **Fig. S9**). For this purpose, we used the previously mentioned oHSV1, a virus identical to oHSV1-KR but lacking the sequence encoding KR or any other fluorescent reporter. hGBM-82 cells were

infected at an MOI of 1 or mock infected, loaded with phthalocyanine or left untreated. Finally, half of the samples were photoactivated as described above. Twenty-four hours later, the samples were analyzed via microscopy (**Fig. S9**), and their viability was quantified via the CellMeter™ Colorimetric Cytotoxicity Assay (AAT Bioquest®), as MTT assays are unsuitable for use with phthalocyanine-treated cells [57]. Neither phthalocyanine nor photoactivation alone affected cell viability (**Fig. 11 A)**. In contrast, oHSV1 infection markedly reduced viability, and this effect was significantly enhanced when phthalocyanine loading was combined with photoactivation, indicating a marked enhancement and acceleration of oHSV1-mediated cytotoxicity following photodynamic activation.

Finally, we confirmed this combinatorial effect in hGBM-82-derived spheroids (**Fig. 11B, C**). Spheroids were infected with 20,000 oHSV1 particles, and after 72 hours, when morphological signs of infection became evident (e.g., rounded cells at spheroid edges), they were loaded with 10 µM phthalocyanine for 1 hour at 37°C (**Fig. 11C**) and photoactivated at room temperature for 20 minutes (fluence H = 60 J/cm^2). The same control groups as above were also included. Forty-eight hours later, cell viability was measured with the CellTiter-Glo® 3D Cell Viability Assay. Compared with all the other conditions, a significant decrease in viability was detected following combined virus plus phthalocyanine photoactivation (**Fig. 11 B**). Notably, oHSV1 infection or phthalocyanine photoactivation alone produced robust cytotoxicity, yet their combination further amplified this effect.

Collectively, these findings demonstrate that phthalocyanine acts as an effective ROS-generating chemical photosensitizer that can be combined with oHSV1 therapy to achieve stronger and faster GBM cell killing, supporting its potential for development in combinatorial photodynamic plus virotherapy strategies.

Together, these findings highlight complementary advantages of genetically encoded and chemical photosensitization strategies rather than establishing the superiority of one approach over the other.

Conclusions

In this study, we developed and characterized oHSV1-KR, a highly neuroattenuated oncolytic HSV1 platform designed to combine direct viral oncolysis with light-triggered photodynamic augmentation in GBM models. Across human and murine GBM cell lines, patient-derived GBM cells, and 3D spheroids, oHSV1-KR efficiently infected tumor cells, propagated within tumor-like structures, and reduced cell viability, confirming the robustness of the virolytic backbone.

We provide a mechanistic demonstration that memKR photoactivation is accompanied by substantial ROS generation in oHSV1-KR-infected GBM cells. Live-cell imaging with DCF revealed a marked photoactivation-dependent increase in oxidant-sensitive fluorescence in infected cells relative to irradiated non-infected controls, and this conclusion was independently supported by lipid peroxidation measurements with Liperfluo and by a fluorometric intracellular ROS assay. Together, these data indicate that the increased cytotoxicity observed after illumination reflects a genuine ROS-dependent photodynamic contribution superimposed on viral oncolysis.

We further show that this principle is not restricted to a genetically encoded photosensitizer. Aluminum phthalocyanine chloride also generated strong photoinduced oxidative signals and significantly potentiated oHSV1-mediated killing in both 2D cultures and tumor spheroids. Thus, the study supports a broader concept in which ROS-generating photodynamic modules—either virally encoded or chemically delivered—can be coupled to oncolytic HSV1 to enhance and accelerate antitumor activity.

Importantly, photodynamic augmentation remained compatible with monocyte-mediated viral delivery, suggesting that this strategy can be integrated with carrier-based approaches intended to overcome the practical limitations of direct intracranial virus administration. This expands the translational relevance of the platform beyond proof-of-principle local infection models.

Collectively, our findings identify photodynamic–virotherapy as a modular and controllable multimodal strategy against GBM, now supported not only by efficacy readouts but also by direct evidence of treatment-associated ROS production. At the same time, the present work remains

limited to advanced in vitro and ex vivo systems. Future studies in immune-competent in vivo models will be needed to define therapeutic windows, optimize light dosimetry and delivery, and assess how ROS-dependent cytotoxicity interacts with antiviral responses, neurotoxicity risk, and the tumor microenvironment.

Abbreviations

5-ALA: 5-aminolevulinic acid; BAC: Bacterial Artificial Chromosome; BBB: Blood–Brain Barrier; bFGF: Basic Fibroblast Growth Factor; CAM: Chorioallantoic Membrane; CMV: Cytomegalovirus; DCFH-DA: 2',7'-Dichlorofluorescein Diacetate; EGF: Epidermal Growth Factor; Fluc: Firefly Luciferase; GBM: Glioblastoma; GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; iPDT: Interstitial PDT; KR: KillerRed; LV: Lentiviral Vector; Mir-124: MicroRNA-124; MOI: Multiplicity of Infection; NCI: National Cancer Institute; NIR: Near-Infrared Region; oHSV1: Herpes Simplex type 1-based Oncolytic Virus; oHSV1-KR: Herpes Simplex virus type 1 expressing KillerRed protein; OV: Oncolytic Viruses;

p: P-value; Pen-Strep: Penicillin–Streptomycin; PDT: PhotoDynamic Therapy; PFU: Plaque Forming Units; PpIX: Protoporphyrin IX; PS: Photosensitizing Agent; PBMCs: Peripheral Blood Mononuclear Cells; ROS: Reactive Oxygen Species; SD: Standard Deviation; TAMs: Tumor-Associated Macrophages; T-VEC: Talimogene laherparepvec; ULA: Ultra-Low Attachment; VSV: Vesicular Stomatitis Virus.

Supplementary Information

Supplementary Materials 1: displays the Supplementary Figures and related legends.

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Author contributions

Conceptualization, A.C., F.M.; methodology, A.C., A.Ro., A.R., V.D., D.M., F.M.; formal analysis, A.Ro., A.R., M.P., V.D.; investigation, A.C., A.Ro., A.R., M.V.F., V.D., A.G.D.O.D.R., M.P., C.D.P., M.T., E.R., D.M., F.M.; resources, A.C., M.T.L.A.P., C.P., F.M.; data curation, A.Ro., A.R., V.D.; writing-original draft preparation, A.C., A.Ro., F.M.; writing-review and editing, A.C., A.Ro., A.R., F.M.; visualization, A.Ro., A.R.; supervision, A.C., F.M.; project administration, F.M.; funding acquisition, A.C., L.P., C.P., F.M. All authors have read and agreed to the published version of

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Data availability statement

All the data included in this study are available upon request by contacting the corresponding authors.

Consent for publication

All the authors agree to the publication of the article.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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Figure Legends

Fig. 1. oHSV1-KR efficiently replicates and kills human and murine GBM cells. (**A**) Human GBM cell lines (U87-MG, upper panel; LN-229, lower panel) and (**B**) murine GBM cell lines (CT-2A, upper panel; GL-261, lower panel) were infected with oHSV1-KR (MOI=1) and analyzed via fluorescence microscopy over the following days (scale bars: 500 µm). Representative images are shown. Cell viability was assessed 48 hours post-infection via the MTT assay. Each graph shows the percentage of viable cells (mean ± standard deviation (SD)) from at least three independent samples, calculated as the ratio of infected sample absorbance to the mean absorbance of the corresponding uninfected control. Statistical analysis was performed via Student's *t* test between the control and infected groups; $p < 0.05$ was considered statistically significant (exact *p* values are indicated above the bars). "Merged Fluo and B.F." represents the merging of the red fluorescence (Fluo) channel and bright field (B.F.).

Fig. 2. oHSV1-KR efficiently kills human and murine GBM cells over time. U87-MG and CT-2A cells were seeded in 96-well plates (20,000 cells/well) and infected 24 hours later with oHSV1-KR (MOI=1). Cell viability was evaluated on days 1, 2, 3, and 4 post infection via the Trypan blue exclusion assay. The graphs show the mean cell count (cells/ml ± SD; n = 3) for each time point.

Fig. 3. oHSV1-KR efficiently infects and kills patient-derived human GBM cells (hGBM-82). (**A-B**) Primary hGBM-82 cells were infected with oHSV1-KR at an MOI of 0.1 (**A**) or 1 (**B**) and monitored for 3 days via fluorescence microscopy (left panels). Representative images are shown. Scale bars: 500 µm. Cell viability was evaluated on days 3 and 7 post infection via the MTT assay (right panels). Each graph shows the percentage of viable cells (mean ± standard deviation (SD)) from at least three independent samples, calculated as the ratio of infected sample absorbance to the mean absorbance of uninfected controls. Statistical significance was determined by Student's *t* test;

$p < 0.05$ was considered significant (exact p values are shown above the bars). "Merged Fluo and B.F." represents the merging of the red fluorescence (Fluo) channel and bright field (B.F.).

Fig. 4. oHSV1-KR infects and kills murine and human GBM spheroids**.** (**A-C**) CT-2A (**A**), hGBM-82 (**B**), and hGBM-15 (**C**) spheroids were infected with oHSV1-KR (20,000 PFU/spheroid) or mock infected (Ctrl). Viral spread was monitored by fluorescence microscopy (left panels). Representative images are shown. Scale bars: 500 μm. Cell viability was assessed on days 3 and 7 post infection via the MTT assay (right panels). Each graph shows the percentage of viable cells (mean ± standard deviation (SD)) from at least three independent samples, calculated as the ratio of infected sample absorbance to the mean absorbance of uninfected controls. Statistical analysis was performed via Student's *t* test; $p < 0.05$ was considered significant (exact p values are shown above the bars). "Merged Fluo and B.F." represents the merging of the red fluorescence (Fluo) channel and bright field (B.F.).

Fig. 5. KR photoactivation enhances the cytotoxic effect of oHSV1-KR infection in human GBM cells. hGBM-82 cells were infected with oHSV1-KR at two different doses (MOI=0.1, upper panels; MOI=1, lower panels) or mock infected (Ctrl). Forty-eight hours post-infection (MOI=0.1) or 24 hours post-infection (MOI=1), the cells were photoactivated (Irr) under the same conditions as those previously described. Before photoactivation, KR expression was assessed via fluorescence microscopy (left panels, scale bar: 500 μm). Cell viability was measured via the MTT assay 24 hours after photoactivation. Each graph shows the percentage of viable cells (mean ± SD), which was calculated as the ratio of infected sample absorbance to the mean absorbance of uninfected controls. Statistical analysis was performed via two-sample Student's *t* test; $p < 0.05$ was considered statistically significant (exact p values are shown above the bars). B.F. stands for bright field. "Merged Fluo and B.F." represents the merging of the red fluorescence (Fluo) channel and bright field (B.F.).

Fig. 6. KR photoactivation promotes ROS production in oHSV1-KR-infected human GBM cells. (**A**) Representative fluorescence micrographs of oHSV1-KR-infected hGBM-82 cell cultures and corresponding non-infected controls (Ctrl), both loaded with DCF and irradiated with a pulsed 588 nm laser from t = 10 min to t = 45 min. Scale bars: 100 μm. (**B**) Median KR (left) and concurrent DCF (right) fluorescence traces (red thick lines), overlaid with individual cell responses (black light lines) from a representative experiment in oHSV1-KR-infected hGBM-82 cells (oHSV1-KR). (**C**) Median DCF fluorescence trace (red thick line), overlaid with individual cell responses (black light lines) from a representative experiment in non-infected hGBM-82 cells (Ctrl). (**D**) Dot-box plots comparing DCF fluorescence variation in oHSV1-KR and Ctrl cells; pooled data from n = 3 independent experiments in each condition. P-value (p) determined by Kruskal-Wallis test ($p < 0.05$ was considered significant).

Fig. 7. Photoactivation potentiates the oncolytic effect of oHSV1-KR in hGBM-82 GBM spheroids. hGBM-82 spheroids were infected with oHSV1-KR (20,000 PFU/spheroid) or mock infected (Ctrl) and monitored for KR expression over several days. Representative images taken at significant time points are depicted in the left panels. Scale bars: 500 μm. Three days post-infection, when KR fluorescence peaked, the spheroids underwent the first photoactivation (Irr) with pulsed 588 nm laser light lasting 40 minutes (fluence H = 144 J/cm^2). A second photoactivation was performed 24 hours later under identical conditions. Cell viability was measured one day after the second photoactivation using the CellTiter-Glo® 3D Cell Viability Assay (right panel). The graph on the right shows the percentage of viable cells (mean ± SD) from at least n = 3 independent samples, calculated as the ratio of sample luminescence to the mean luminescence of the uninfected control. Statistical significance was determined via two-sample Student's t test; $p < 0.05$ was considered significant (exact p values are shown above the lines). B.F. stands for bright field. "Merged Fluo and B.F." represents the merging of the red fluorescence (Fluo) channel and bright field (B.F.).

Fig. 8. Primary human monocytes loaded with oHSV1-mCherry migrate toward and transmit viral infection to human GBM cells. (**A**) Primary human monocytes were infected with oHSV1-mCherry at an MOI of 5 and allowed to migrate for 3 hours through a 5.0 μm pore filter toward serum-free medium (Ctrl) or medium conditioned by human GBM cells (LN229-c.m.; U87MG-c.m., respectively) as indicated. Migrated cells were quantified from at least n = 3 independent fields of view acquired in the lower chamber. The graph shows the mean number of monocytes (mean ± SD) from at least n = 3 independent samples. Statistical significance was determined via two-sample Student's *t* test; $p < 0.05$ was considered significant (exact *p* values are shown above the lines). (**B**) oHSV1-infected monocytes were co-cultured at a 1:1 ratio with confluent U87-MG or LN-229 cells, as indicated. The supernatants were collected at the specified time points, and the viral titers were determined via plaque assay in Vero cells. Each graph shows the mean PFU/ml (mean ± SD) from at least three independent samples. Statistical significance was determined via two-sample Student's *t* test; $p < 0.05$ was considered significant (exact *p* values are shown above the lines).

Fig. 9. Human primary monocytes efficiently delivered oHSV1-KR to hGBM-82-derived GBM spheroids. hGBM-82 spheroids were cocultured with human primary monocytes labeled with CellTracker™ Green fluorescent dye and subsequently infected with oHSV1-KR at an MOI of 3 (monocytes + oHSV1-KR). The spheroids were monitored over time via fluorescence microscopy. Representative images are shown in the left panels (scale bars: 500 μm). Cell viability was assessed at 7 days post-culture via the MTT assay (right panel). The graph shows the mean viability (percentage ± SD) of at least three independent samples, which was calculated as the ratio of sample absorbance to the mean absorbance of the uninfected control. Statistical analysis was performed via Student's *t* test; $p < 0.05$ was considered statistically significant (exact *p* values are shown above the lines). B.F. stands for bright field. "Merged Fluo and B. F" represents the merge of

the red/green fluorescence (Fluo) channels and bright field (B.F.). Ctrl stands for uninfected spheroids. oHSV1-KR stands for spheroids infected with free virus.

Fig. 10. Photoactivation enhances the cytotoxic effect of monocyte-delivered oHSV1-KR in hGBM-82 spheroids. hGBM-82 spheroids were cocultured with human primary monocytes (5,000 monocytes/spheroid) labeled with CellTracker™ Green fluorescent dye and subsequently infected with oHSV1-KR at an MOI of 3 (monocytes + oHSV1-KR). Three days post-infection, when KR fluorescence peaked, the spheroids underwent the first photoactivation (Irr) using pulsed 588 nm laser light for 40 minutes (fluence H = 144 J/cm^2), followed 24 hours later by a second photoactivation under identical conditions. The spheroids were analyzed via fluorescence microscopy 24 hours after the first and second photoactivations. Representative images are displayed in the left panels (scale bars: 500 μm). Cell viability was measured 24 hours after the second irradiation using the CellTiter-Glo® 3D Cell Viability Assay. The graph on the right shows the mean viability (percentage ± standard deviation (SD)) of at least n = 3 independent samples, calculated as the ratio of sample luminescence to the mean luminescence of the uninfected control. Statistical significance was determined via two-sample Student's t test; $p < 0.05$ was considered statistically significant (exact p values are shown above the lines). B.F. stands for bright field. "Merged Fluo and B. F." represents the merging of the green/red fluorescence (Fluo) channels and bright field (B.F.) images. Ctrl stands for uninfected spheroids.

Fig. 11. Photoactivation of phthalocyanine-loaded hGBM-82 cells under 2D and 3D culture conditions enhances the cytotoxic effect of oHSV1 infection. (**A**) hGBM-82 cells cultured in 2D media were infected with oHSV1 (MOI=1) or mock infected (Ctrl) and, 24 hours later, treated with phthalocyanine (Phthalo) and irradiated (Irr) for 10 minutes via a pulsed 660 nm LED (fluence H = 30 J/cm^2). Cell viability was measured 24 hours after photoactivation via the CellMeter™ Colorimetric Cytotoxicity Assay Kit (AAT Bioquest®). (**B**) hGBM-82 spheroids were infected with

oHSV1 (20,000 PFU/spheroid) or mock infected (Ctrl). Three days later, the spheroids were loaded with phthalocyanine (Phthalo) and subjected to photoactivation (100 mW, 500 ms pulsed light for 20 minutes). Spheroid viability was evaluated 48 hours after photoactivation (Irr) via the CellTiter-Glo® 3D Cell Viability Assay (Promega). Each graph shows the mean viability (percentage ± SD) of at least n = 3 independent samples, which was calculated as the ratio of sample luminescence to the mean luminescence of uninfected controls. Statistical significance was determined via two-sample Student's *t* test; $p < 0.05$ was considered statistically significant (exact *p* values are shown above the lines). (C) Representative images of Ctrl and oHSV1-infected tumor spheroids loaded with phthalocyanine and not irradiated.