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Starvation-Induced Differential Virotherapy Using an Oncolytic Measles Vaccine and Vaccinia Virus

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Content

1. Introduction

Starvation is probably as old as time itself. Rich food offer was often followed by food scarcity which is why a variety of adaption mechanisms evolved in the course of evolution to deal with extreme environmental conditions.

Cells that undergo fasting conditions divert energy from growth and reproduction to maintenance and repair systems and thereby increase cellular protection [1].

Studies in simple organisms led to the assumption that starvation may slow aging. For example, bacteria such as *E. coli* survive four times longer when medium is switched from calorie-rich to calorie-free medium. This effect is reversed by addition of calories, but not acetate which is a carbon source of diet associated with starvation [2].

Also in other laboratory model organisms like yeast cells, worms and mice dietary restriction induces an extended longevity and increases cellular protection against reactive oxygen species (ROS) [3-9].

This is mainly mediated by downregulation of nutrient sensing pathways and the activation of transcription factors dealing with stress resistance, yet all detailed mechanisms remain poorly understood [4]. Growth hormone receptor and insulin like growth factor-1 (IGF-1) deficiency in humans reduces age related diseases like diabetes and cancer [10].

Thus, reduction of food intake is a non-genetic way to modify cellular signaling pathways thereby extending healthy life span and delaying occurrence of neoplasia [11-13].

Chemotherapeutic drugs have been used as wide spectrum anticancer agents since the 1940s [14]. These substances allowed for the first time to treat hematological malignancies and disseminated solid tumors, but commonly induce a wide range of side effects including acute toxicities like leukopenia, hair loss, nausea and vomiting.

Moreover, other types of tumors (so-called secondary malignancies) may develop years after treatment, e. g. leukemia [15]. Unlike previously assumed, chemotherapy does not only destroy malignant cells but also normal tissues.

In cancer research the phenomenon of differential stress resistance (DSR) recently moved into the focus *given its potential for widespread clinical applications* [1, 16].

Cancer cells react differently to food deprivation than normal cells. In the absence of glucose and growth factors, normal cells are entering a standby mode and are getting more protected against extreme environmental conditions.

In the course of gaining numerous mutations, most tumor cells achieve the ability to proliferate independently of external growth factors and are thus prone to becoming more vulnerable to stress conditions, like chemotherapy, when deprived of nutrients [17, 18].

However, there are certain cancer cell lines that are resistant to anti-growth effects through food restriction because of phosphatidylinositol 3,4,5-kinase (PI3K) activation [19]. Nevertheless, starvation demonstrated to be a promising field in terms of cancer research.

In this context, the insulin-like growth factor-1 (IGF-1) pathway has been proposed to be growth-promoting and anti-apoptotic, thereby favoring carcinogenesis [20].

Fasting, which is defined as a short-term transient total absence of food, has shown evidence of being more efficient in reducing levels of IGF-1 and glucose than any prolonged caloric restriction and, furthermore, favors chronic weight loss that is highly deleterious to most cancer patients [21].

A recent study by Lee et al. [22] *revealed that 15 of 17 mammalian cancer cell lines could be sensitized to doxorubicin and cyclophosphamide (CP) by fasting 24 hours prior and 24 hours post chemotherapy* (Figure 1).

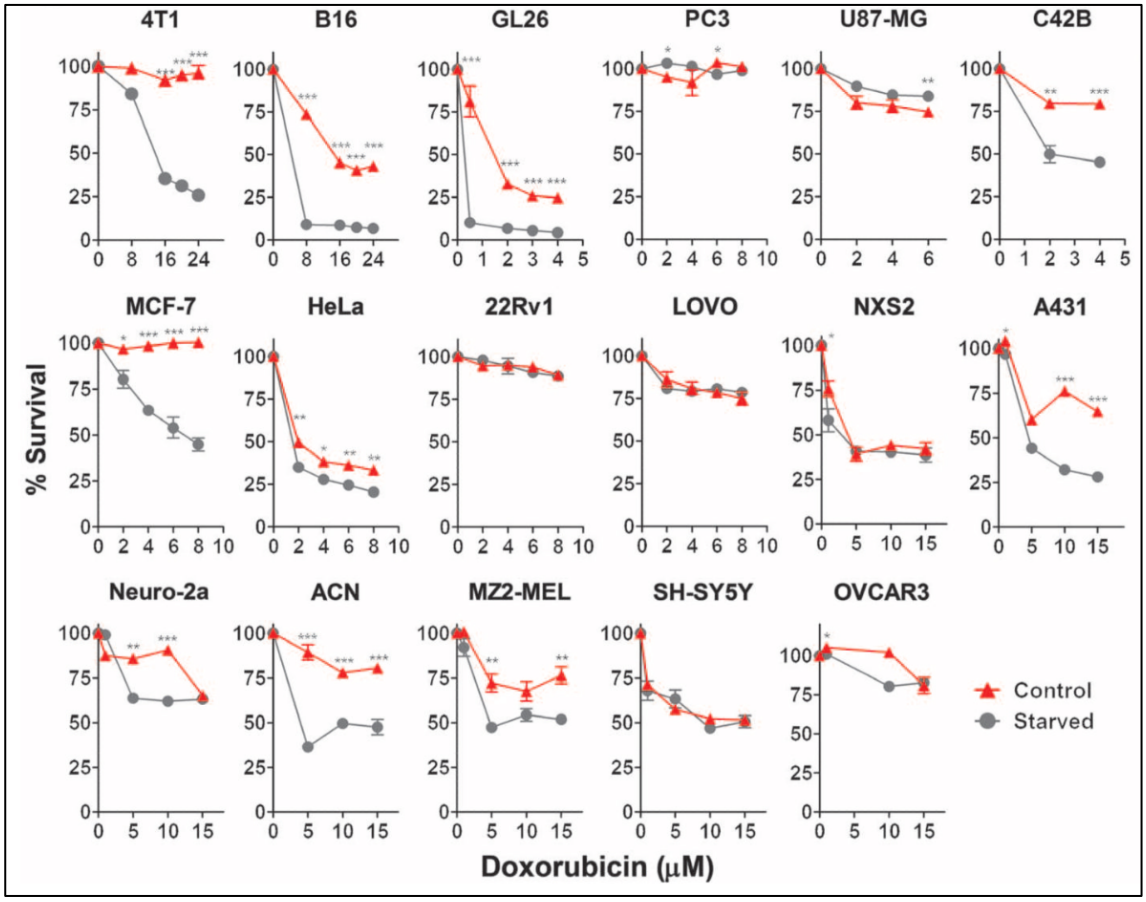


Figure 1: Effect of starvation on doxorubicin sensitivity of 17 different cancer cell lines in vitro.

Starvation was applied to cells 24 hours before and 24 hours during doxorubicin (DXR) treatment. Control groups were cultured in glucose (1.0 and 2.0 g/liter, for human and murine cells, respectively), supplemented with 10% fetal bovine serum (FBS). Starved groups were cultured in glucose (0.5 g/liter) supplemented with 1% FBS. Survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Data are from at least three independent experiments and shown as means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, Student's t test. Murine cells: 4T1, breast cancer; B16, melanoma; GL26, glioma; NXS2 and Neuro-2a, neuroblastoma. Human cells: PC3 and 22Rv1, prostate cancer; MCF-7 and C42B, breast cancer; U87-MG, glioblastoma; HeLa, cervical cancer; LOVO, colon cancer; ACN and SH-SY5Y, neuroblastoma; A431, epidermoid carcinoma; MZ2-MEL, melanoma; OVCAR3, ovarian cancer [22].

Next, when using a mouse tumor model, it could be shown that two cycles of fasting were as effective in the treatment of human metastatic cancer as two cycles of chemotherapy with cisplatin (Figure 2).

Interestingly, the combination of both, i.e. starvation plus chemotherapy, had been shown to be the most effective regimen which suggests that fasting has the potential to increase the efficacy of chemotherapy [22] and potentially, also, other non-genotoxic forms of cancer therapies.

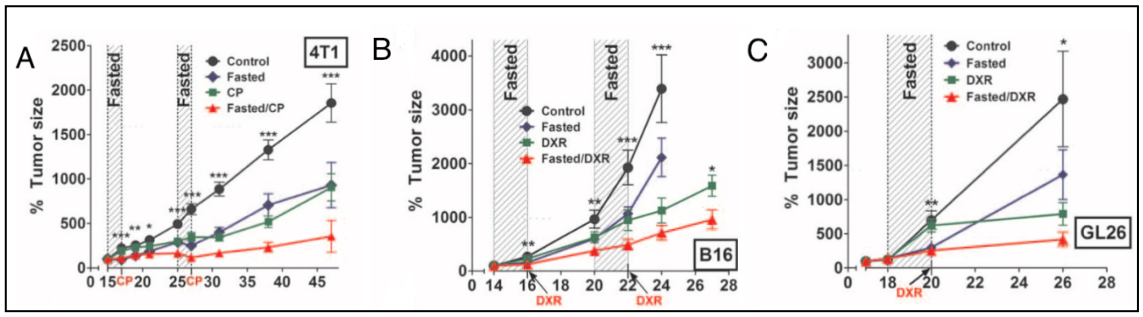


Figure 2: Effect of fasting on the sensitivity of allograft tumors to chemotherapeutic agents in mice.

Effect of fasting on tumor progression as percent of initial tumor size (A) in breast tumors (4T1; n=12), (B) in melanoma (B16; n = 11), and (C) in glioma (GL26; n = 8). Fasting in the glioma model was applied only once due to the rapid tumor growth in the control (ad lib, no chemotherapy) group. One-way ANOVA with Tukey's post test [Student's t test for (B) day 27)]. *P < 0.05; **P < 0.01; ***P < 0.001. All data are means ± SEM. DXR=doxorubicin; CP=cisplatin [22].

Even more puzzling, the starvation of tumor bearing-mice allowed researchers to increase the administered doses of chemotherapy with etoposide (ETO) up to three times the maximal dose approved in humans (Figure 3; [1]). *Whereas ETO at this concentration killed 43% of control mice, only 6% of the mice that were pre-starved died after ETO treatment* [1].

To date, only a small number of clinical trials have explored the effect of combined fasting and chemotherapy in patients [16, 24-26].

A case report on 10 patients suffering from different cancer entities showed a significant reduction of side effects, such as fatigue or weakness, when fasting was performed 48–140 h prior to and 5–56 h post-chemotherapy (Figure 4; [16]).

Another more recent clinical study evaluated the effects of short-term fasting on tolerance to adjuvant chemotherapy in HER-2-negative breast cancer patients [25]. *As a result, erythrocyte and thrombocyte counts post chemotherapy were higher in fasted patients* (Figure 5).

In 34 women with breast and ovarian cancer, 60 h fasting plus chemotherapy not only proved to be safe and feasible, but also improved the quality of life, well-being, and fatigue when compared to chemotherapy alone [26].

Accordingly, fasting was well-tolerated in cancer patients and has the potential to

reduce side effects, but there is a lack of data supporting that it can increase the efficiency of current anticancer therapies, as data from in vitro and animal studies has demonstrated.

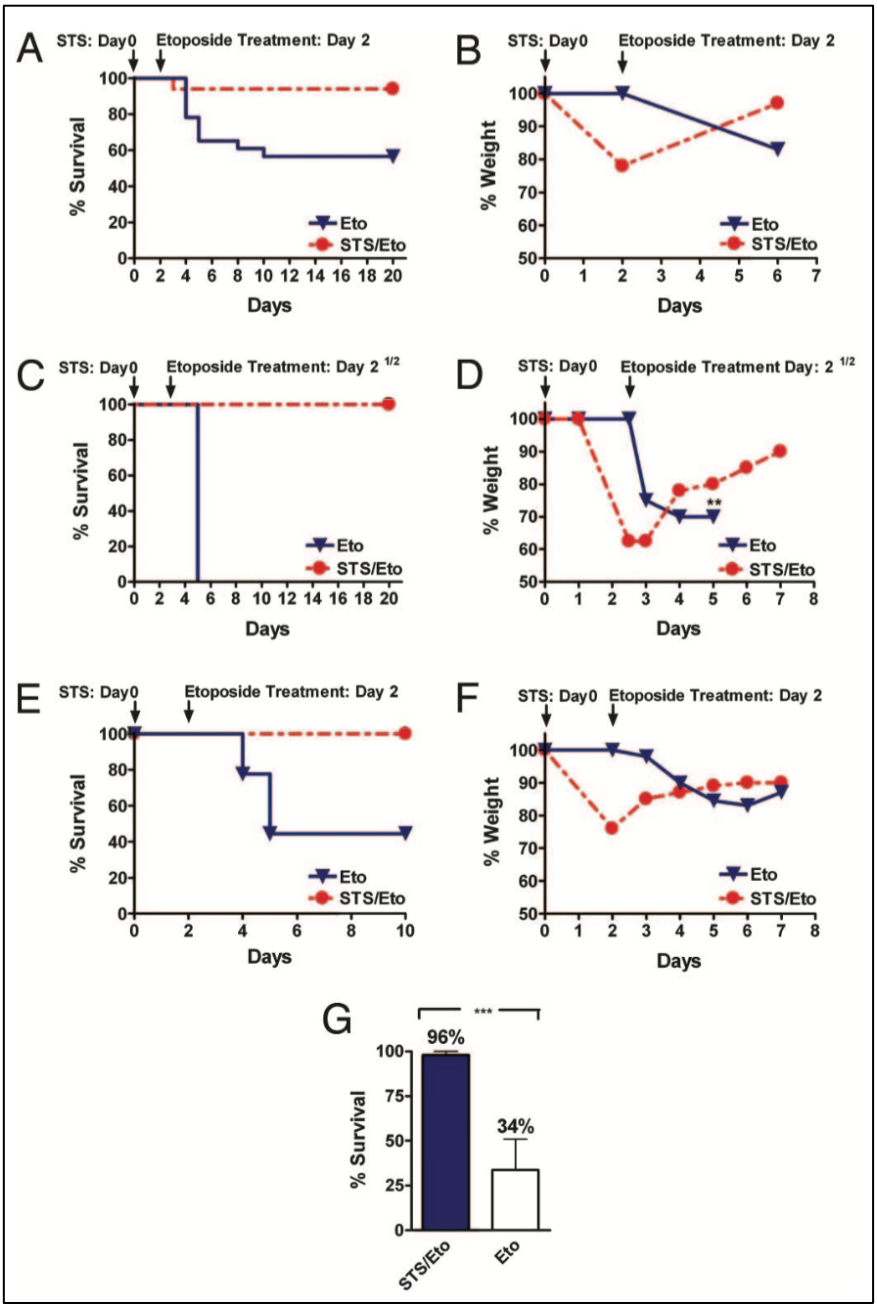


Figure 3: Short-term starvation (STS) protects against high-dose chemotherapy in vivo.

(A) A/J mice were treated (i.v.) with 80 mg/kg etoposide (Eto) with (STS/Eto, n=17) or without (Eto, n=23) a prior 48 hour starvation (STS). **(B)** Percent weight loss (a measure of toxicity) after Eto treatment in STS-treated (n=17) or untreated (n=23) A/J mice. **(C)** CD-1 mice were treated (i.v.) with 110 mg/kg Eto with (STS/Eto, n=5) or without (Eto, n=5) a 60 hour prior starvation. **(D)** Percent weight loss after Eto treatment in STS-treated (n=5) or untreated (n=5) CD-1 mice. Asterisks indicate the day at which all mice died of toxicity. **(E)** Athymic (Nude-nu) mice were treated (i.v.) with 100 mg/kg Eto with (STS/Eto, n=6) or without (Eto, n=9) a 48 hour prior starvation. **(F)** Percent weight loss after Eto treatment in the treated (STS/Eto, n=6) or untreated (Eto, n=9) athymic (Nude-nu) mice. **(G)** Comparison of survival of all of the mice that were either prestarved (STS/Eto) or not (Eto) before Eto injection. The survival of all STS-treated (n=28) and untreated (n=37) mice from all genetic backgrounds above (A/J, CD1, and Nude-nu) has been averaged (***, P 0.05) [1].

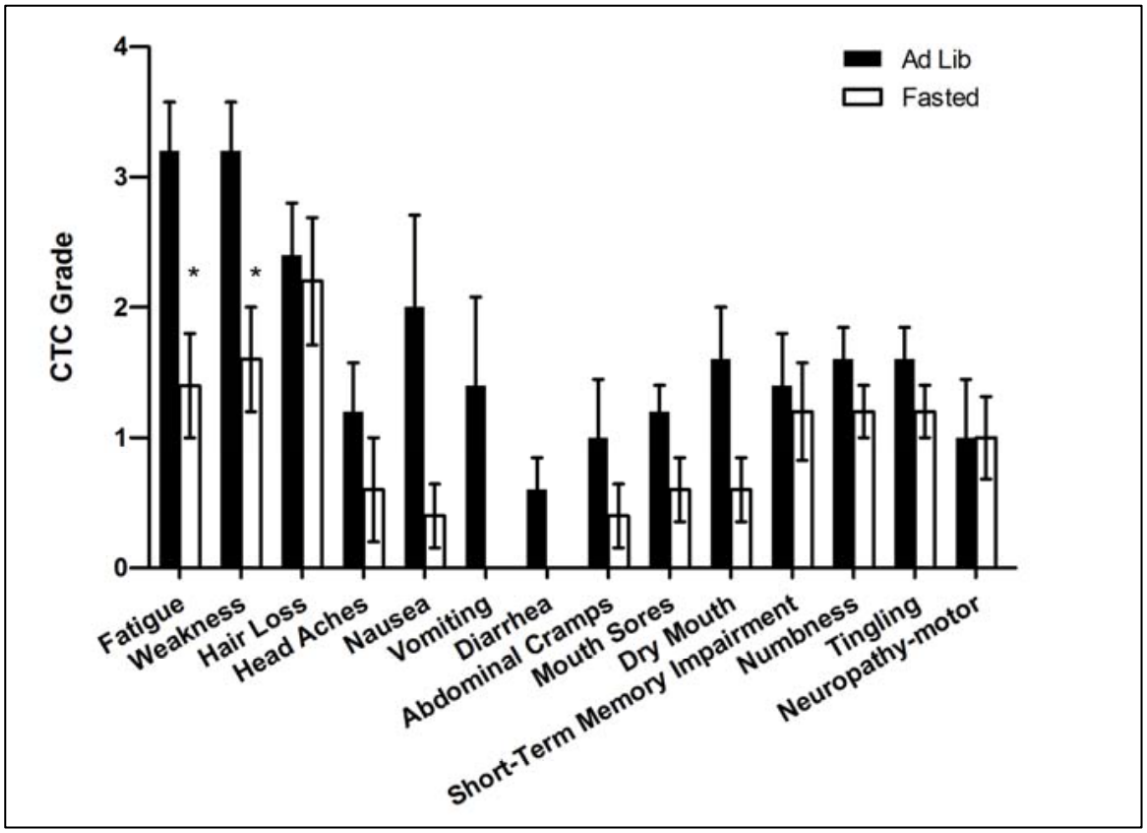


Figure 4: Average of self reported side effects after chemotherapy with or without fasting.

Data represent average of CTCAE grade from matching fasting and non fasting cycles (Ad Lib). 6 patients received either chemotherapy alone or combined chemo-fasting treatments. Self reported side effects from the closest two cycles were compared to one another. Statistic analysis was performed only from matching cycles. Data presented as standard error of the mean (SEM). P value was calculated with unpaired, two tail t test. (*, $P<0.05$) [16].

In conclusion, fasting proved to be safe and feasible and demonstrated the potential to

(i) sensitize tumor cells to chemotherapy *in vivo* (Figure 1),

(ii) retard tumor progression in mice to the same extent as chemo-therapy alone and even more effective in combination with cisplatin or DXR (Figure 2),

(iii) achieve tolerance to high-dose chemotherapy in mice (Figure 3),

(iv) reduce chemotherapy associated side effects in humans (Figure 4),

(v) and to prevent a decline of blood cell counts after chemotherapy treatment in cancer patients (Figure 5).

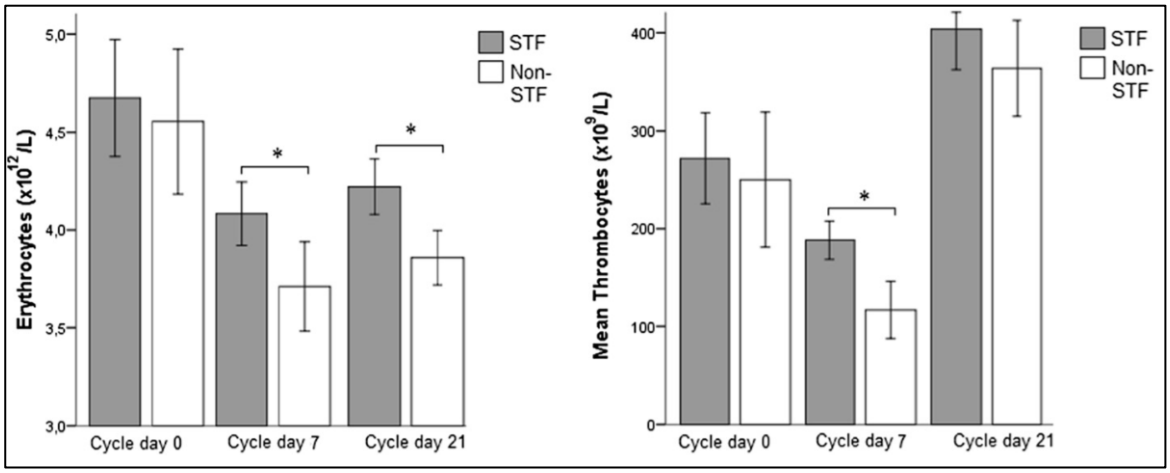


Figure 5: Hematologic parameters compared between both groups.

Values are measured on day 0 of cycle 1 immediately before the chemotherapy infusion, on day 7 of cycle 1-5 combined and day 21 of cycle 1-5 combined. * P value <0.05. STF; short-term fasting, Reference values: erythrocytes 4-5*10^{12}/L; thrombocytes 150-400*10^{9}/L [25].

Many larger clinical trials are currently ongoing to determine possible benefits of fasting regarding efficacy of treatment, adverse events, quality of life, weight changes, and changes in metabolic, hormone, and inflammatory response (NCT00936364, NCT01802346, NCT02710721, NCT03162289, NCT03340935, NCT03595540, NCT03709147, NCT03700437, NCT01175837; please note that the cited clinical studies are denoted by their ClinicalTrials.gov identifiers).

In an ongoing study at the University Hospital Tübingen (NCT02607826), short-term starvation for a timeframe beginning 24 h prior to chemotherapy administration and lasting until 6 h after administration is being tested in patients suffering from a wide variety of solid tumors, i.e. cholangiocarcinoma (CC), pancreatic ductal adenocarcinoma (PDAC), colorectal cancer (CRC), gastric cancer (GC)/ adenocarcinoma of the esophagogastreal junction (AEG) / esophagus cancer (EC).

According to current knowledge, patients could benefit from fasting for several reasons: (i) by reduction of chemotherapy associated side effects, (ii) by enhancing treatment efficacy and thereby halting tumor progression and (iii) by increasing the patients' self-efficacy or capability to fight with their own armament and means, a psychosomatic mechanism which should not be underestimated.

Moreover, fasting is a cheap and widely applicable way to improve conventional treatment regimens when compared e.g. to the costly new immunotherapies.

Oncolytic virotherapy has recently gained attention due to demonstration of the first cancer patient who experienced a complete remission that has been ongoing for more than five years now following treatment with a single high-dose shot of recombinant oncolytic measles vaccine virus expressing the sodium iodide symporter (MeV-NIS) [27]. *Hopes that this will not remain an isolated case have been encouraged by the recent approval of IMLYGIC™ (T-Vec; talimogene laherparepvec), the first oncolytic immunotherapeutic treatment for unresectable skin and lymph node lesions in patients with advanced melanoma* [28].

Numerous clinical trials have assessed the safety and effectiveness of oncolytic viruses [29, 30].

ONCOS-102, a granulocyte-macrophage colony-stimulation factor (GM-CSF)-encoding oncolytic adenovirus, has been demonstrated to induce infiltration of CD8+ T-lymphocytes into initially T-cell-negative mesothelioma in a 68-year-old patient [31]. *Accordingly, also poorly immunogenic tumors could be sensitized to immunotherapy by oncolytic viruses (OVs), which open up new possibilities for T-cell-based approaches in cancer treatment.*

Reovirus is another OV being evaluated in numerous tumor entities in phase II clinical trials in combination with conventional chemotherapy [32-37]. *REOLYSIN® , an isolate of reovirus type 3, was well-tolerated in patients, but did not improve progression-free survival (PFS) in metastatic colorectal carcinoma, non-small cell lung cancer (NSCLC), breast cancer, and prostate cancer compared to standard treatment arms; however, it was demonstrated to extend overall specific survival (OSS) specifically in breast cancer patients* [32, 33, 36, 37]. *Reovirus is currently being investigated in a phase I trial in combination with GM-CSF for the treatment of high-grade relapsed or refractory brain tumors (NCT02444546).*

Also, the use of recombinant tumor-targeting viruses, such as measles vaccine virus (MeV) and vaccinia virus (VACV), *has been demonstrated to be a safe and highly promising approach in cancer treatment* [38-49].

However, widespread resistance to oncolysis following MeV inoculation was observed when challenging the NCI-60 tumor cell panel with oncolytic MeV [50]. *This might be an important phenomenon that currently hinders the broader clinical success of virotherapy with basic, non-optimized, first generation vector types.*

Until now, only one study, published by Esaki et al., has investigated whether starvation may enhance the effectiveness of OVs [51]. *In this study, fasting was demonstrated to increase the replication and oncolytic activity of oncolytic herpes simplex virus (oHSV) in glioblastoma multiforme (GBM) cells, but not in human astrocytes. These results were confirmed in vivo, showing enhanced virus replication in starved mice* [51].

Oncogenes that prevent the starvation-induced cellular switch to a protected mode render the tumor cell vulnerable to stress such as chemotherapy and potentially also OVs. Consequently, this prompted our hypothesis that DSR could be a way to sensitize colorectal tumor cells to virotherapeutics while making healthy cells more robust against virus-mediated oncolysis.

1.1 Objectives

Preliminary work on characterizing the biochemical mechanisms of starvation has extended from investigating its role in aging to cancer research, establishing the starvation-induced differential stress resistance (DSR) model. Thus far, extensive studies have been conducted to evaluate the impact of starvation on chemotherapy-induced cytotoxicity and side effects revealing its potential to unshield cancer cells and protect normal cells to chemotherapeutic drugs.

Oncolytic virotherapy represents a novel approach in cancer therapy, but its wide clinical use remains limited due to various resistance phenomena demonstrating the need of new approaches to overcome these obstacles.

Against this background, this dissertation set out to assess starvation as an intervention to synergize with the oncolytic activity of virotherapy. In particular, it was investigated how various starvation regimens could impact on (i) tumor cell survival, (ii) virus mediated oncolysis by two different virotherapeutic vectors (i.e., measles vaccine virus (MeV) and vaccinia virus (GLV-1h68) based viro-therapeutics), and (iii) viral replication in three human colon carcinoma and two non-transformed primary human colon cell lines. To further specify nutrients causing possible effects, we investigated several starvation protocols, in detail, restriction of either glucose alone, serum alone, or both glucose and serum.

Since in vitro studies created the basis to translate the application of starvation-based chemotherapy into clinical trials, this thesis lays claim to generate first in vitro data on starvation based differential virotherapy in colorectal cancer and thereby implementing this approach in future biological cancer therapy regimes.

2. Materials and Methods

2.1 Cell Culture

Human colorectal cancer cell lines HT-29, HCT-15, and HCT-116 are cell lines from the U.S. National Cancer Institute's NCI-60 tumor cell panel and were purchased from Charles River Laboratories (Charles River Laboratories Inc., New York, NY, USA). Normal human cell lines CCD 18 Co (non-malignant fibroblastic colon cells) and CCD-841 CoN (non-malignant epithelial colon cells) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). African green monkey kidney (Vero) cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Tumor cells and Vero cells were maintained in high-glucose (4.5 g glucose per liter) Dulbecco's Modified Eagle's Medium (DMEM, D6429, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany). Normal human cell lines were grown in Alpha Modified Eagle's Medium (Alpha-MEM, BE12-169F, Lonza, Verviers, Belgium) enriched with 10 % FCS and 10 mM L-glutamine (Gibco, Paisley, Scotland, UK). Cell culture flasks were stored in humidified incubators at 37 °C under 5% CO_2.

When cells were confluent, they were washed with phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA), detached from the cell culture flask with Trypsin EDTA (Sigma-Aldrich, St. Louis, MO, USA) and diluted with culture medium. Cell suspension was then transferred into centrifugation vials and centrifuged (4 min; 1000 rpm, 22°C). Supernatants were decanted and cell pellets resuspended in cell culture medium.

To cryopreserve cells, the cell suspension was centrifuged (4 min; 1000 rpm) and the cell pellet was suspended in freezing medium (20 % FCS, 10 % Dimethylsulfoxide, 70 % DMEM/ Alpha MEM). Aliquots of 1 mL each of the cell suspension were dispensed into cryovials, which were placed in an isopropanol bath and cooled down slowly to -80°C and finally stored at -150°C.

To recultivate frozen cells, cryovials were immediately placed into a 37°C water bath and quickly removed after thawing (< 1 min). The cell suspension was transferred into centrifugation tubes containing prewarmed cell culture medium. After centrifugation (4 min; 1000 rpm; 22°C), supernatants were decanted, cell pellets diluted in cell culture medium + 10 % FCS and transferred into cell culture flasks and stored in incubators.

For cell counting, 10 µL of cell suspension was transferred into an Eppendorf cup prepared with 90 µL Trypan Blue (0.4 %; Sigma-Aldrich, St. Louis, MO, USA) and vortexed to get a homogeneous suspension. The improved Neubauer hemocytometer was cleaned with ethanol and covered with the coverslip. 10 µL of the cells were pipetted to the chamber and then four squares (1 mm^2) were counted under the 10x objective using the phase contrast. Trypan Blue allows the detection of viable cells that don't absorb the color in distinction to dead cells that stain blue. Number of cells per mL was estimated by multiplying the average cell count of one square with the dilution factor 10 and the conversion factor 10^4 (to convert the cell suspension volume above one square (0.1 cm x 0.1 cm x 0.01 cm = 10^{-4} mL) to 1 mL).

2.2 Experimental setup

2.2.1 Seeding of cells

Cells were seeded in 24-well plates (TPP, Trasadingen, Switzerland) in 0.5 mL standard medium/ well (see below) at the following densities at day 0:

MeV-GFP infection: HCT-15: 3×10^4; HCT-116: 2.5×10^4; HT-29: 4×10^4; CCD-18 Co: 2×10^4; CCD-841 CoN: 2×10^4 cells/ well

GLV-1h68 infection: HCT-15: 4×10^4; HCT-116: 3×10^4; HT-29: 4×10^4 cells/ well

Medium either remained in the well (see following point 2.2.3) or was switched to 0.5 mL starvation medium/ well (see following point 2.2.4) at day 1. After infection at day 2, cells were grown in standard or starvation medium until the endpoint assay was performed.

2.2.2 Standard cell culture medium

For tumor cells HT-29, HCT-15, and HCT-116 high-glucose DMEM (containing 4.5 g glucose per liter) supplemented with 10 % FCS was used. For non-malignant colon cells, CCD-18 Co and CCD841 CoN Alpha-MEM (containing 1 g glucose per liter) supplemented with 10 % FCS was used.

2.2.3 Standard regimen (no starvation)

2.2.3.1 Determination of cell line specific MOI resulting in approximately 25 %-oncolysis rate

Cells were seeded in standard medium at day 0. After infection with ascending MOIs at day 2 (see section virus infection), medium was replaced with standard medium. Remnant cell mass was determined by the sulforhodamine B (SRB) assay at 96 h post-infection (hpi) at day 6.

2.2.4 Starvation regimens

2.2.4.1 Short-term low-glucose, low-serum starvation (=short-term starvation)

Cells were cultured in serum- and glucose-restricted culture medium for 24 h before infection. At day 1, standard medium was replaced with low-glucose, low-serum medium (glucose concentrations of 0, 0.5, 1, 2, and 4.5 g/L supplemented with 1 % FCS each; control: standard medium). Starvation medium was prepared by mixing high-glucose DMEM (D6429, Sigma-Aldrich, St. Louis, MO, USA) + 1 % FCS and glucose-free DMEM (F0405, Biochrom, Berlin, Germany) + 1 % FCS.

At day 2, medium was removed, replaced with infection medium (see virus infection), and finally switched to standard medium in which cells were cultivated until the endpoint assay was performed (24 h starvation period).

2.2.4.2 Long-term low-glucose, low-serum starvation

Cells were cultured in serum and glucose-restricted culture medium for 24 h before and 96 h post-infection. Medium was switched to low-glucose, low-serum at day 1. After infection at day 2, the starvation medium was renewed until readout (120 h starvation period).

2.2.4.3 Long-term low-glucose, standard serum starvation

Cells were cultured in glucose-restricted culture medium (standard serum culture medium) for 24 h before infection and 96 h post-infection. Low-glucose, standard serum medium (glucose concentrations of 0, 0.5, 1, 2, and 3 g/L supplemented with 10 % FCS; control: standard medium) was applied at day 1. After infection at day 2, the starvation medium was renewed (120 h starvation period).

2.2.4.4 Long-term standard glucose, low-serum starvation

Cells were cultured in serum-restricted cell culture medium (standard glucose culture medium) for 24 h before and 96 h post-infection. Standard glucose, low-serum medium (glucose concentration of 4.5 g/L (tumor cells) and 1 g/L (normal colon cells), supplemented with 1 %, 2.5 %, 5 %, or 7.5 % FCS; control: standard medium) was applied at day 1. After infection at day 2, the starvation medium was renewed (120 h starvation period).

2.3 Oncolytic virotherapeutics

2.3.1 MeV-GFP: *A commercially available original monovalent vaccine batch of measles virus (MeV) strain Mérieux (Sanofi-Pasteur, Leimen, Germany) was modified by insertion of a gene encoding green fluorescent protein (MeV-GFP), as described* [52].

Frozen aliquots of this recombinant virus with defined viral titers (calculated by the method of Kärber and Spearman [53, 54]) were stored at -80°C.

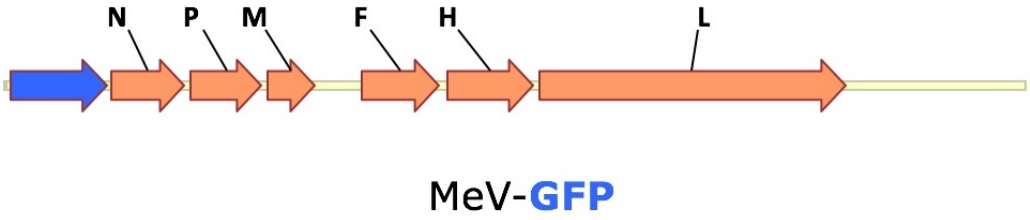


Figure 6: Schematic representation of the MeV-GFP genome.

The cDNA encoding green fluorescent protein (GFP) was inserted in front position upstream of the N gene. N, nucleocapsid gene; P, phosphoprotein gene; M, matrix protein gene; F, fusion protein gene; H hemagglutinin gene; L, large protein gene [41].

2.3.2 GLV-1h68: Replication competent genetically stable modified vaccinia virus (VACV) that also incorporates a gene for GFP was obtained by Genelux Corp., San Diego, California. Aliquots of VACV GLV-1h68 (GL-ONC1) were stored at -80°C.

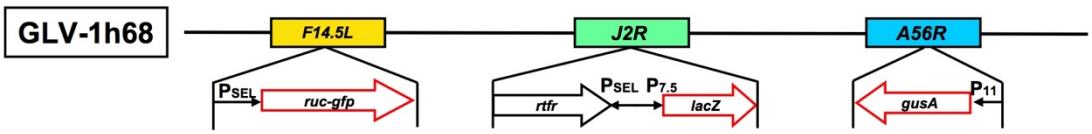


Figure 7: Schematic representation of the VACV GLV-1h68 genome

GLV-1h68 carries three gene cassettes inserted into the viral genome: a *Renilla* luciferase-green fluorescent protein (RUC-GFP) fusion cassette at the F14.5L locus, a reverse inserted human transferrin receptor and β-galactosidase cassette at the J2R locus (which encodes TK), and a β-glucuronidase cassette at the A56R locus (encoding hemagglutinin) [55].

2.4 Virus infection

Infection was performed at indicated multiplicities of infection (MOI) at day 2. MOCK treated cells (no virus, infection medium only) served as control.

2.4.1 MeV-GFP infection: *Cells were washed once with PBS and then treated with a distinct MOI of MeV-GFP diluted in 250 µL Opti-MEM (Opti-MEM + GlutaMAX Supplement, Gibco, Paisley, Scotland, UK). At 3 h post-infection (hpi), the inoculum was removed and changed to standard or starvation medium.*

2.4.2 GLV-1h68 infection: Frozen virus aliquot was thawed on ice and then sonified (Branson Sonifier 450, G. Heinemann, Schwäbisch Gmünd, Germany) for 30 s at 4°C. Cells were washed once with PBS. Infection was performed in 250 µL DMEM + 2 % FCS. At 1 hpi, the inoculum was removed and changed to standard or starvation medium

2.5 Sulforhodamine B (SRB) Cell Viability Assay

Cells were seeded in 24-well plates and infected 48 h later. At 96 hpi, cells were washed with ice-cold PBS, fixed with 10% trichloroacetic acid (TCA), and incubated at 4 °C for 30 min. Then, TCA was removed, and the fixed cells were washed with tap water and dried for 24 h. To stain cells, 250 µL of SRB dye (0.4 % dissolved in 1 % acetic acid) was added followed by incubation at RT for 10 min, and cells were washed with 1 % acetic acid and dried again. Protein-bound dye was dissolved in 10 mM Tris base for 10 min before measurement of optical density was performed in a 96-well microtiter plate reader (Tecan Genios Plus, Tecan Deutschland, Crailsheim, Germany) at a wavelength of 550 nm (reference wavelength of 620 nm). The SRB assay allows densitometric quantification of the total cellular protein mass after incubation of the cell mass of interest with cytotoxic substances for a distinct time range; thereby, a remnant cell mass is calculated which represents the cytotoxic effectiveness measured under the respective experimental conditions.

2.6 Lactate dehydrogenase (LDH) Assay

Cells were seeded in 24-well plates, starved starting from day 1, and infected with MeV-GFP at day 2 using the indicated MOIs. Values corresponding to medium without cells were subtracted as the blank for each starving condition. At 96 hpi, cell culture supernatant was transferred into new plates and cells were lysed in 0.1 % Triton X100 (dissolved in PBS) (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Supernatant, lysate, and medium without cells (10 µL each) were analyzed in a 96-well plate. The enzymatic reaction was started by adding 200 µL pyruvate/NADH reagent (LDH-Kit, Analyticon Biotechnologies, Lichtenfels, Germany). LDH release was quantified by photometric measurement of NADH decrease in a microtiter plate reader (Magellan, Tecan Deutschland, Crailsheim, Germany) at a wavelength of 340 nm.
The percentage of cell lysis was calculated as follows:

$$\%\ \text{cell lysis} = \frac{\text{LDH [supernatant]-LDH [medium]}}{\text{LDH [lysate] +(LDH [supernatant]-LDH [medium])}} \times 100$$

The LDH assay detects lactate dehydrogenase (LDH) activity, an enzyme that is released by loss of cell membrane integrity due to necrosis or toxic cell damage. It serves as a standard parameter for cell lysis taking place under the respective experimental conditions.

2.7 Viral growth curve

A total of 3 × 10^5 cells/well were seeded in a 6-well plate, starvation medium was applied at day 1 (1 mL/well), and MeV-GFP infection (MOI as indicated) was performed in 1 mL Opti-MEM at day 2. At 3 hpi, the infection medium was removed, and cells were washed three times with PBS before standard or starvation medium was renewed. Supernatants and cell lysates (scraped into 1 mL OptiMEM) were harvested at 3, 24, 48, 72, and 96 hpi and subjected to one freeze–thaw cycle. Viral titers of supernatants and cell lysates were quantified by

fluorescence microscopy of GFP on Vero cells and then calculated as total viral titer (supernatants + lysates).

In detail, Vero cells (5 × 10^4 cells/mL) were seeded in DMEM + 5 % FCS in 96-well plates (200 µL/well). The following day, samples were thawed, vortexed, and centrifuged (2 min, 3000 rpm, 22 °C). Supernatants were used to prepare a dilution series at concentrations ranging from 1 × 10^0 to 1 × 10^{-7} in DMEM + 5 % FCS of which 50 µL each were transferred onto Vero cells. At 96 hpi, fluorescence microscopy of GFP expression (IX50, Olympus, Tokyo, Japan; analySIS, Soft Imaging System, Münster, Germany) allowed for the calculation of viral titers according to the method of Kärber and Spearman [53, 54].

2.8 Statistics

The results of cell viability assays and viral growth curves are expressed as mean ± standard deviation (SD). Graphics were created using GraphPad Prism Software version 4.01 (GraphPad Software, La Jolla, CA, USA). Statistical analysis was conducted with JMP Software version 12.2.0 (SAS Institute Inc., Cary, NC, USA).

In order to make a statement about the influence of starvation on virus-mediated oncolysis, the ratios of uninfected (MOCK) versus virus test groups (VIRUS) were compared based on whether these groups were cultured under starving or standard medium conditions. For this purpose, the differences of logarithmized MOCK and VIRUS group cell mass values (quotient of nonlogarithmized values) (log10 MOCK − log10 VIRUS) were compared between starved groups and control groups for each run of the experiment by the Dunnett's multiple comparison test. P-values < 0.05 were considered as statistically significant.

For illustration purposes, we delogarithmized the data and termed the results quotient of geometric mean (QoGM).

$$QoGM = 10^{(log_{10} MOCK)-(log_{10} VIRUS)}$$

QoGM serves as a parameter for virotherapy efficacy. High values of QoGM in relation to control (no starvation) indicate an increase of virus-mediated oncolysis efficacy, whereas low values indicate a decrease.

3. Results

To comprehensively investigate starvation-induced differential virotherapy using an oncolytic measles vaccine virus, our study comprised a two-step approach for each cell line. At first, cells were grown in standard medium and treated with different virus concentrations to determine MOIs resulting in a "moderate" oncolysis of around 25 %, leading to a cell line-dependent adjustment of the respective virus dosages for each cell line. These MOIs then were used in subsequent experiments applying different starvation regimens investigating a combined starvation-based virotherapy. Cells were either starved for 24 h pre-infection, termed short-term starvation (Figure 8a) *or starved 24 h pre-infection and 96 h post-infection* (Figure 8b)*. For all regimens, cell viability assays were performed at 96 hpi.*

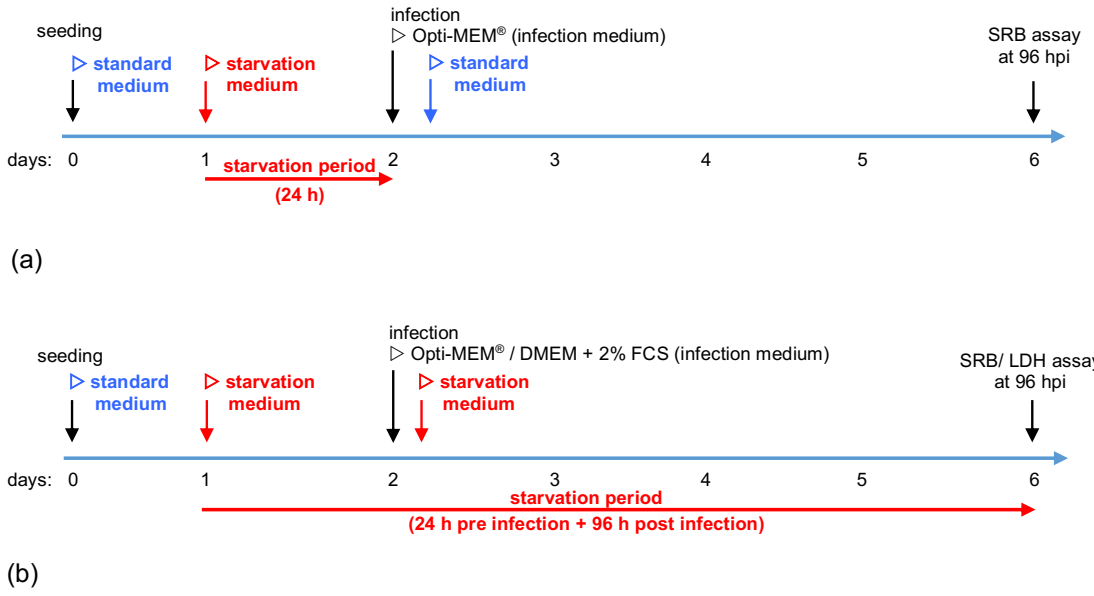


Figure 8. Illustration of starvation conditions.

(a) Short-term starvation: Cells were seeded in standard medium. On day 1, medium was changed to starvation medium (i.e., starvation medium with variations in contents of glucose and fetal calf serum (FCS)). On day 2, infection with MeV was performed in infection medium (Opti-MEM ®). At 3 h post-infection (hpi), infection medium was replaced with standard medium. At 96 hpi, the remaining tumor cell masses were determined by the sulforhodamine B (SRB) assay. (b) Long-term starvation: Cells were seeded in standard medium on day 0. Medium was changed to starvation medium on day 1. At 3 hpi, infection medium was replaced with the respective starvation medium. At 96 hpi, the remaining tumor cell masses were determined by SRB or lactate dehydrogenase (LDH) assay. [23]

3.1 Determination of Adjusted MOIs in Colon Carcinoma and Non-Malignant Colon Cell Lines.

First, we set out to analyze distinct malignant and non-malignant colon cells by SRB viability assays in an individual manner, in order to identify the distinct MOI leading to a cell mass reduction of about 25 % (Figure 9 and 10)*. Then, these cell line-adapted MOIs were used to investigate the influence of starvation on virotherapy-induced cytotoxicity in further experiments (please note that these suboptimal cell-killing MOIs were chosen in order to provide enough "space" to measure any additional cell-killing effects of virus-mediated oncolysis that were a result of starvation).*

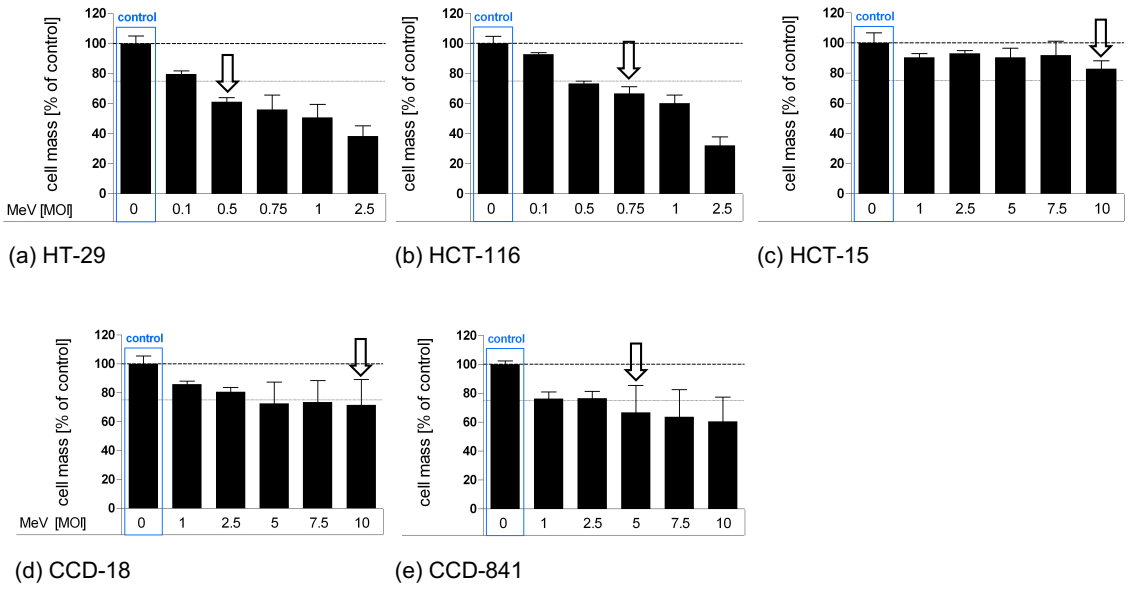


Figure 9. Susceptibilities of human colon carcinoma and normal human colon cells to MeV-mediated oncolysis.

Human colon carcinoma cell lines HT-29 (a), HCT-116 (b), and HCT-15 (c) and normal colon cell lines CCD-18 Co (d) and CCD-841 CoN (e) were cultured in standard medium and infected 48 h after seeding in Opti-MEM ® (infection medium) with ascending multiplicities of infection (MOIs as indicated above) of MeV, or remained uninfected (control). At 96 h post-infection (hpi), the remaining tumor cell masses were determined by SRB viability assay. White arrows indicate selected MOIs which were used in further experiments to investigate a combined starvation plus virus-induced tumor cell toxicity. Means and standard error of the means (SEM) of two independent experiments are shown. [23]

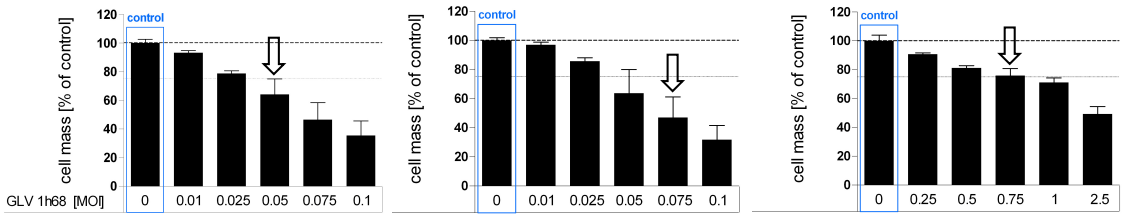
Based on this experimental design, we treated both malignant and non-malignant colon cell lines with ascending MOIs of the virotherapeutic vector MeV-GFP (Figure 9) and GLV-1h68 (Figure 10).

Remaining cell masses were determined by SRB viability assays at 96 hours post infection (hpi).

Among the tumor cells, HCT-15 served as a representative for a cell line knowing to be resistant towards MeV-based virotherapy, whereas HCT-116 and HT-29 represent cell lines knowingly being susceptible to MeV, as previously classified in the studies on the NCI-60 tumor cell panel by Noll et al., 2013 [50].

For colorectal cancer cell line HCT-15 (Figure 9c)*, only MOI 10 led to a marginal cell mass reduction of 17 % (please note that we did not apply MOIs higher than 10 since it is clinically challenging to achieve such virus concentrations in cancer patients). By contrast, human colon cancer cell lines HCT-116 (*Figure 9b*, MOI 0.75, cell mass reduction 33 %) and HT-29 (*Figure 9a*, MOI 0.5, cell mass reduction 39 %) were found to be more susceptible to MeV-GFP-mediated oncolysis.*

*MeV-GFP infection of non-malignant CCD-18-Co cells (*Figure 9d*), a human colon fibroblast cell line (MOI 10, cell mass reduction 29 %), and of non-malignant CCD-841-CoN cells (*Figure 9e*), a human colon epithelial cell line (MOI 5, cell mass reduction 33 %), showed patterns of virotherapy resistance similar to HCT-15.*



(a) HT-29 (b) HCT-116 (c) HCT-15

Figure 10. Susceptibilities of human colon carcinoma cells to vaccinia virus-mediated oncolysis.

Human colon carcinoma cell lines HT-29 (a), HCT-116 (b), and HCT-15 (c) were cultured in standard medium and infected 48 h after seeding in DMEM® + 2 % FCS (infection medium) with ascending multiplicities of infection (MOIs as indicated above) of the GFP marker gene encoding oncolytic vaccinia virus GLV-1h68, or remained uninfected (control). At 96 h post-infection (hpi), the remaining tumor cell masses were determined by SRB viability assay. White arrows indicate selected MOIs which were used in further experiments to investigate a combined starvation plus virus-induced tumor cell toxicity. Means and standard error of the means (SEM) of two independent experiments are shown.

As illustrated in Figure 10, application of VACV GLV-1h68 to human colon cancer cell lines HT-29 (Figure 10a, MOI 0.05, cell mass reduction 36 %), HCT-116 (Figure 10b, MOI 0.075, cell mass reduction 53 %), and HCT-15 (Figure 10c, MOI 0.75, cell mass reduction 24 %) demonstrated that lower amounts of virus were needed to achieve a comparable degree of cell mass reduction as with MeV-GFP, being in the range of factor 10 required less infectious viral particles.

Interestingly, characteristics of resistance or susceptibility to VACV GLV-1h68 remained the same as to MeV-GFP for each tested cell line. Accordingly, ten times more virus particles were needed for HCT-15 than for HCT-116 to achieve an equal degree of cell killing for both treatments with MeV-GFP and GLV-1h68.

3.2 Short-Term Starvation (24 h) Decelerated Tumor Cell Growth and Kept MeV-GFP-Mediated Oncolysis Intact.

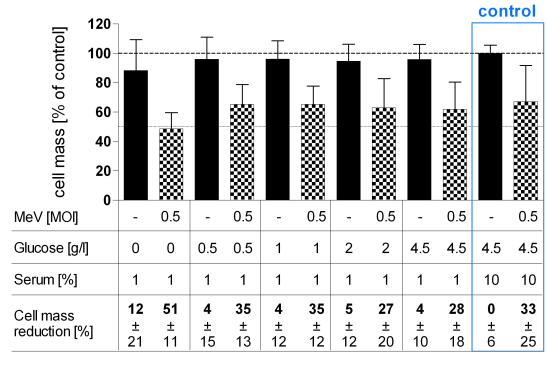
Next, combined starvation plus viral oncolysis settings were investigated. For this purpose, human colon carcinoma cell lines HT-29 (Figure 11a), *HCT-15* (Figure 11c), *and HCT-116* (Figure 11e) *were starved for 24 h pre-infection in low-glucose, low-serum medium. Then, these pre-starved malignant cells were either infected with the above determined MOIs of MeV-GFP or MOCK-infected. In each experiment, control group data (blue frames) are shown from cells that were cultivated in standard (non-starved) cell culture medium. Remnant cell mass was determined by SRB assay at 96 hpi and is illustrated using bar graphs.*

Remarkably and consistently for all cell lines, even a short period of 24 h "starvation only" (no infection) was found to be sufficient to reduce the tumor cell mass by 4 % (4.5 g Glc/L, 1 % FCS) up to ~10 % (0 g Glc/L, 1 % FCS). Accordingly, we observed a tendency for greater inhibition of tumor cell growth in cell lines HT-29 and HCT-15 the lower the levels of glucose and serum were (Figure 11a and 11c). *The extent of "starvation only"-induced cell mass reduction was found to be equal throughout all cell lines and independent of any virotherapy resistance characteristics.*

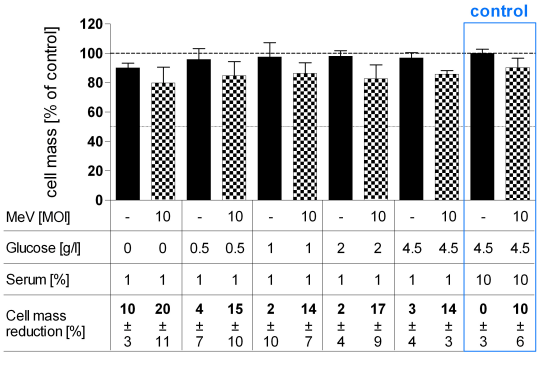
To better illustrate the efficacy of virus-mediated oncolysis under starvation conditions, we compared the ratios of uninfected (MOCK) and infected (VIRUS) cell mass groups between starving and control groups. Results are reported as "quotient of geometric mean" (QoGM) functioning as an indicator for virotherapy efficacy and are displayed in scatter dotplots (for details, see section "statistics"). For HT-29 cells, we observed a trend of increasing QoGM at 0 g Glc/L and 1 % FCS, however, values of QoGM were not found to significantly differ between starving and non-starving control groups (Figure 11b). *In summary, the efficacy of MeV-mediated oncolysis in HCT-15 and HCT-116 cells remained predominantly unaffected by short-term starvation, while in HT-29, an additional effect of starvation plus virotherapy could be observed.*

Table 1: Illustration of cell culture media used for short-term starvation experiments (infection with MeV-GFP)

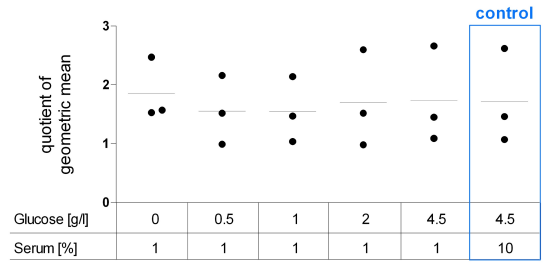
	FCS	Glc/L
Starvation culture conditions; short-term = 24 h period pre-infection	1 %	0 - 4.5 g
Infection culture conditions (Opti-MEM®; 3h period)	0 %	2.5 g
Standard culture conditions (control)	10 %	4.5 g



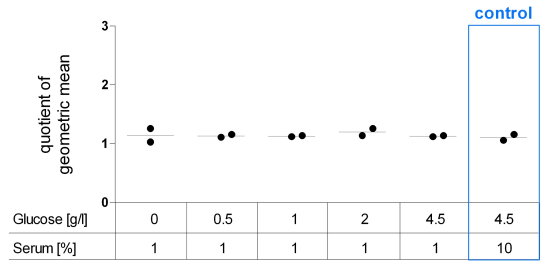
(a)



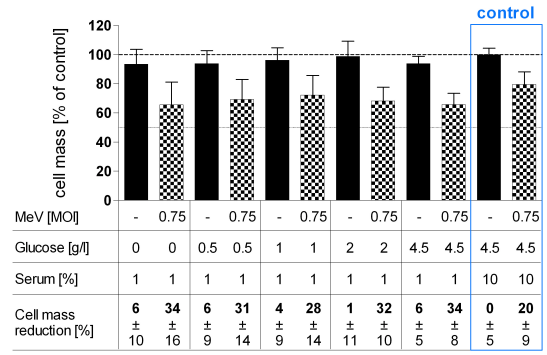
(c)



(b)

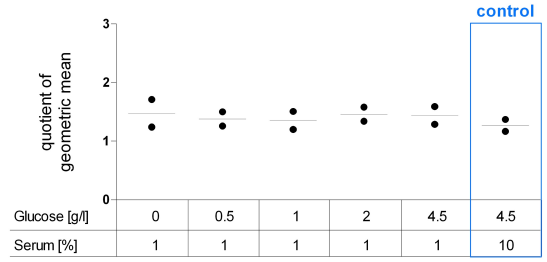


(d)



MeV [MOI]	-	0.75	-	0.75	-	0.75	-	0.75	-	0.75	-	0.75
Glucose [g/l]	0	0	0.5	0.5	1	1	2	2	4.5	4.5	4.5	4.5
Serum [%]	1	1	1	1	1	1	1	1	1	1	10	10
Cell mass reduction [%]	**6** ± 10	**34** ± 16	**6** ± 9	**31** ± 14	**4** ± 9	**28** ± 14	**1** ± 11	**32** ± 10	**6** ± 5	**34** ± 8	**0** ± 5	**20** ± 9

(e)



Glucose [g/l]	0	0.5	1	2	4.5	4.5
Serum [%]	1	1	1	1	1	10

(f)

Figure 11. Effect of short-term pre-infection starvation (24 h) on MeV-mediated oncolysis in HT-29 (a,b), HCT-15 (c,d) and HCT-116 (e,f) cells.

Short-term starvation and MeV infection of neoplastic HT-29, HCT-15, and HCT-116 cells (human colon carcinoma) were performed according to Figure 8a. At 96 hpi, the remaining tumor cell masses were determined by SRB assay. (a,c,e) Black bars represent MOCK-infected tumor cells (addition of Opti-MEM only, no infectious virus particles); checkered bars display tumor cells infected with MeV. Means and SEM of three or two independent experiments are shown; control: tumor cell cultures and infections performed under standard medium conditions (no starvation). (b,d,f) The impact of starvation on the virus-mediated oncolysis efficacy was evaluated by comparing the ratios of uninfected (MOCK) and virus-infected groups (VIRUS) between starving and standard medium conditions (control). Statistical analysis was performed using the Dunnett's multiple comparison test. Each dot represents one run of the experiment; horizontal lines represent means of quotient of geometric mean (QoGM). [23]

3.3 Long-Term Starvation Substantially Inhibited Tumor Cell Growth and Enhanced the Efficacy of MeV-GFP-Mediated Oncolysis for HT-29 Cells Cultured in Low-glucose, Low-serum but Not in Low-glucose, Standard Serum Medium.

To further intensify cytotoxic effects, we extended the fasting period to a total timespan of 120 h (Figure 12). *For this purpose, HCT-15, HCT-116, and HT-29 tumor cells were starved 24 h pre-infection and 96 h post-infection either in low-glucose, low-serum medium* (Figure 12a,c,e) *or at low-glucose, standard serum medium* (Figure 12b,d,f). *Cells were either infected with MeV-GFP or MOCK-infected before remnant cell masses were determined by SRB assay at 96 hpi.*

As shown by bar graphs, long-term "starvation only" (no infection) dramatically reduced tumor cell masses of all cell lines (black bars). Combining starvation with MeV-GFP treatment (checkered bars) resulted in measles virus-induced cell killing that was still effective: For HCT-15 cells, treatment with MeV-GFP led to a cell mass reduction of ~22 % under standard medium conditions (control) and to a proportional extent either during low-glucose, low-serum (Figure 12c)*, or low-glucose, standard serum starvation conditions* (Figure 12d)*. The same applies to HCT-116 cells, where a 35 % cell mass reduction under control conditions was similarly observed under starvation conditions* (Figure 12e)*. Exceptions apply to the low concentration ranges, especially in low-glucose, low-serum starvation, where remnant cell masses were nearly zero, and therefore no remarkable difference between "starved only" and "starved plus virotherapy" groups could be measured.*

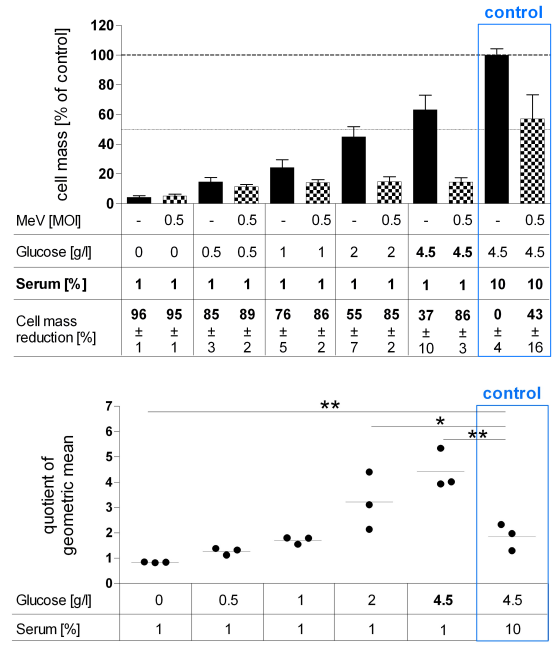
For HT-29 cells, the virus-mediated cell-killing rate under standard conditions correlates to the extent of low-glucose, standard serum conditions (Figure 12b)*. Notably, MeV-mediated oncolysis rate doubled under low-glucose, low-serum starvation (4.5 g Glc/L, 1 % FCS and 2 g Glc/L, 1% FCS), compared to control* (Figure 12a)*. These findings were confirmed when looking at our parameter QoGM indicating virotherapy efficacy: "QoGM" increased significantly for HT-29 cells when long-term starvation conditions were* applied (at 4.5 g and 2 g Glc/L,

1 % FCS compared to control). The decrease of QoGM at 0 g Glc/L, 1 % FCS can be attributed to the very low amount of remaining cell mass, where no significant differences between infected and uninfected groups could be measured. In summary, low-glucose, low-serum starvation has the potential to enhance MeV-mediated oncolysis efficacy in HT-29 CRC cells, in terms of a synergistic effect.

In HCT-15 and HCT-116 cells, values of QoGM stayed unchanged after long-term low-glucose, low-serum, as well as after low-glucose, standard serum starvation (Figure 12c–f)*, meaning that under long-term starvation, MeV-GFP-mediated oncolysis worked to the same extent as under standard conditions. Consequently, an additional cell-killing effect of starvation plus virotherapy could be observed for long-term starvation in HCT-15, HCT-116, and HT-29 CRC cells (glucose only starvation*).

Table 2: Illustration of cell culture media used for long-term low-glucose, low-serum and low-glucose, standard serum starvation experiments (infection with MeV-GFP)

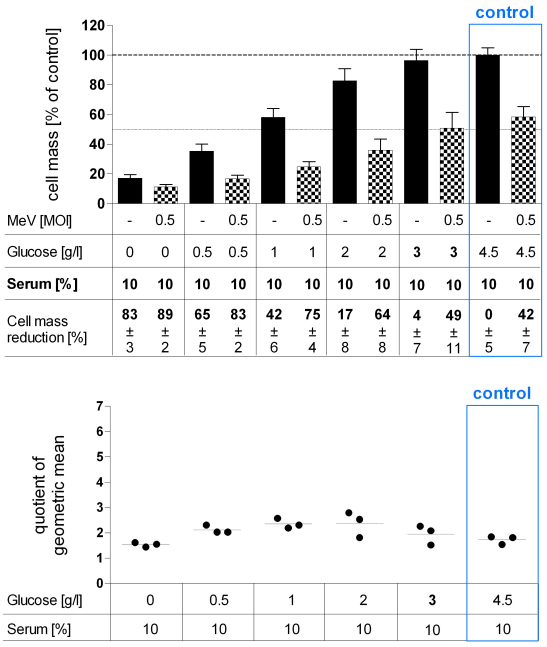
	FCS	Glc/L
Starvation culture conditions; long-term = 120 h period (24 h pre-infection + 96 h post-infection)		
LT low-glucose, low-serum starvation	1 %	0 - 4.5 g
LT low-glucose, standard serum starvation	10 %	0 - 3 g
Infection culture conditions (Opti-MEM®; 3h period)	0 %	2.5 g
Standard culture conditions (control)	10 %	4.5 g



MeV [MOI]	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5
Glucose [g/l]	0	0	0.5	0.5	1	1	2	2	**4.5**	**4.5**	4.5	4.5
Serum [%]	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**10**	**10**
Cell mass reduction [%]	**96** ± 1	**95** ± 1	**85** ± 3	**89** ± 2	**76** ± 5	**86** ± 2	**55** ± 7	**85** ± 2	**37** ± 10	**86** ± 3	**0** ± 4	**43** ± 16

Glucose [g/l]	0	0.5	1	2	**4.5**	4.5
Serum [%]	1	1	1	1	1	10

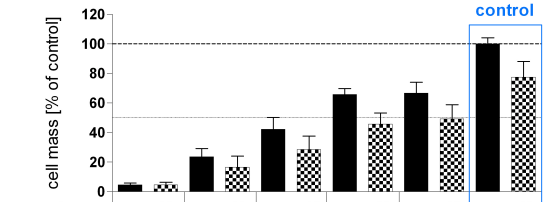
(a)



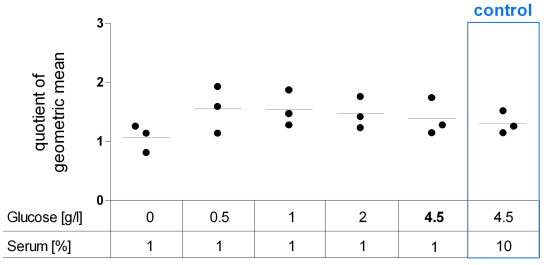
MeV [MOI]	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5
Glucose [g/l]	0	0	0.5	0.5	1	1	2	2	**3**	**3**	4.5	4.5
Serum [%]	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Cell mass reduction [%]	**83** ± 3	**89** ± 2	**65** ± 5	**83** ± 2	**42** ± 6	**75** ± 4	**17** ± 8	**64** ± 8	**4** ± 7	**49** ± 11	**0** ± 5	**42** ± 7

Glucose [g/l]	0	0.5	1	2	**3**	4.5
Serum [%]	10	10	10	10	10	10

(b)

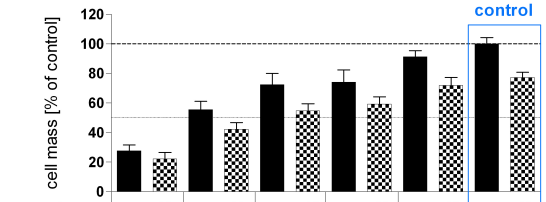


MeV [MOI]	-	10	-	10	-	10	-	10	-	10	-	10
Glucose [g/l]	0	0	0.5	0.5	1	1	2	2	**4.5**	**4.5**	4.5	4.5
Serum [%]	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**10**	**10**
Cell mass reduction [%]	**95** ± 1	**95** ± 2	**76** ± 6	**83** ± 8	**58** ± 9	**71** ± 9	**34** ± 4	**54** ± 8	**33** ± 7	**51** ± 10	**0** ± 4	**22** ± 11

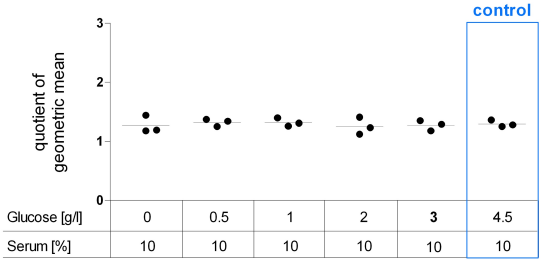


Glucose [g/l]	0	0.5	1	2	**4.5**	4.5
Serum [%]	1	1	1	1	1	10

(c)



MeV [MOI]	-	10	-	10	-	10	-	10	-	10	-	10
Glucose [g/l]	0	0	0.5	0.5	1	1	2	2	**3**	**3**	4.5	4.5
Serum [%]	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Cell mass reduction [%]	**72** ± 4	**78** ± 4	**44** ± 6	**58** ± 5	**28** ± 8	**45** ± 5	**26** ± 8	**41** ± 5	**9** ± 4	**28** ± 5	**0** ± 4	**23** ± 4



Glucose [g/l]	0	0.5	1	2	**3**	4.5
Serum [%]	10	10	10	10	10	10

(d)

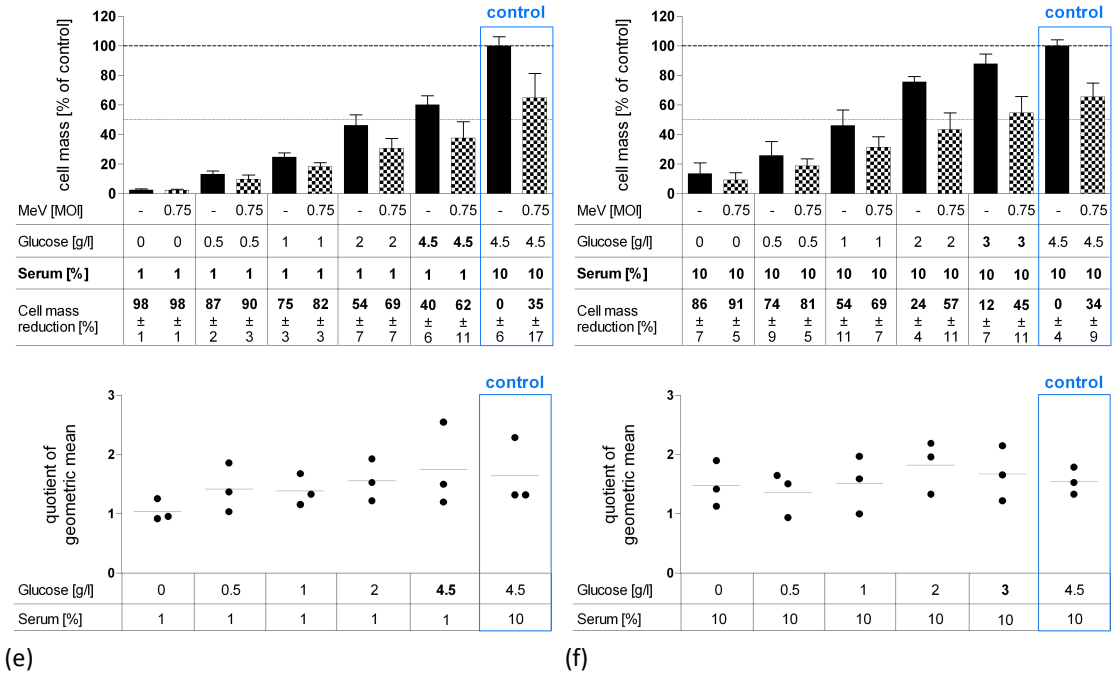


Figure 12: Effect of long-term starvation (120 h) on MeV-mediated oncolysis in HT-29 (a,b), HCT-15 (c,d) and HCT-116 (e,f) cells.

Long-term starvation of HT-29, HCT-15 and HCT-116 cells was performed according to Figure 8b. On day 1, medium was changed to low-glucose, low-serum medium (a,c,e) or low-glucose, standard serum medium (b,d,f). At 96 hpi, remaining tumor cell masses were determined by SRB assay. Differences were considered significant when P-values were < 0.05 (*) or < 0.01 (**). Differences in glucose and serum concentrations are pointed out in bold face. [23]

3.4 MeV-GFP Replication Was Impaired by Long-Term Low-Glucose, Low-Serum Starvation, but Widely Unaffected by Standard Glucose, Low-Serum Starvation, and Increased in Short-Term Low-Glucose, Low-Serum-Starved HT-29 Cells.

Since our results indicate that starvation decelerates tumor cell growth and may even increase the efficacy of MeV-GFP-mediated oncolysis in HT-29 cells, we wanted to investigate how starvation affects virus replication.

For this purpose, we generated virus growth curves (Figure 13)*. HT-29 cells were starved either 24 h pre-infection (short-term starvation) or 24 h pre-infection and 3–96 h post-infection (long-term starvation) in low-glucose, low-serum (red and green curves) or standard glucose, low-serum medium (black curve). A respective blue curve depicts the control group where cells were grown under*

standard conditions (no starvation). Infection with MeV-GFP was performed at an MOI of 0.5. Titration of HT-29 cell supernatants and lysates on recipient Vero cells delivered virus titers as plaque-forming units (PFU)/mL.

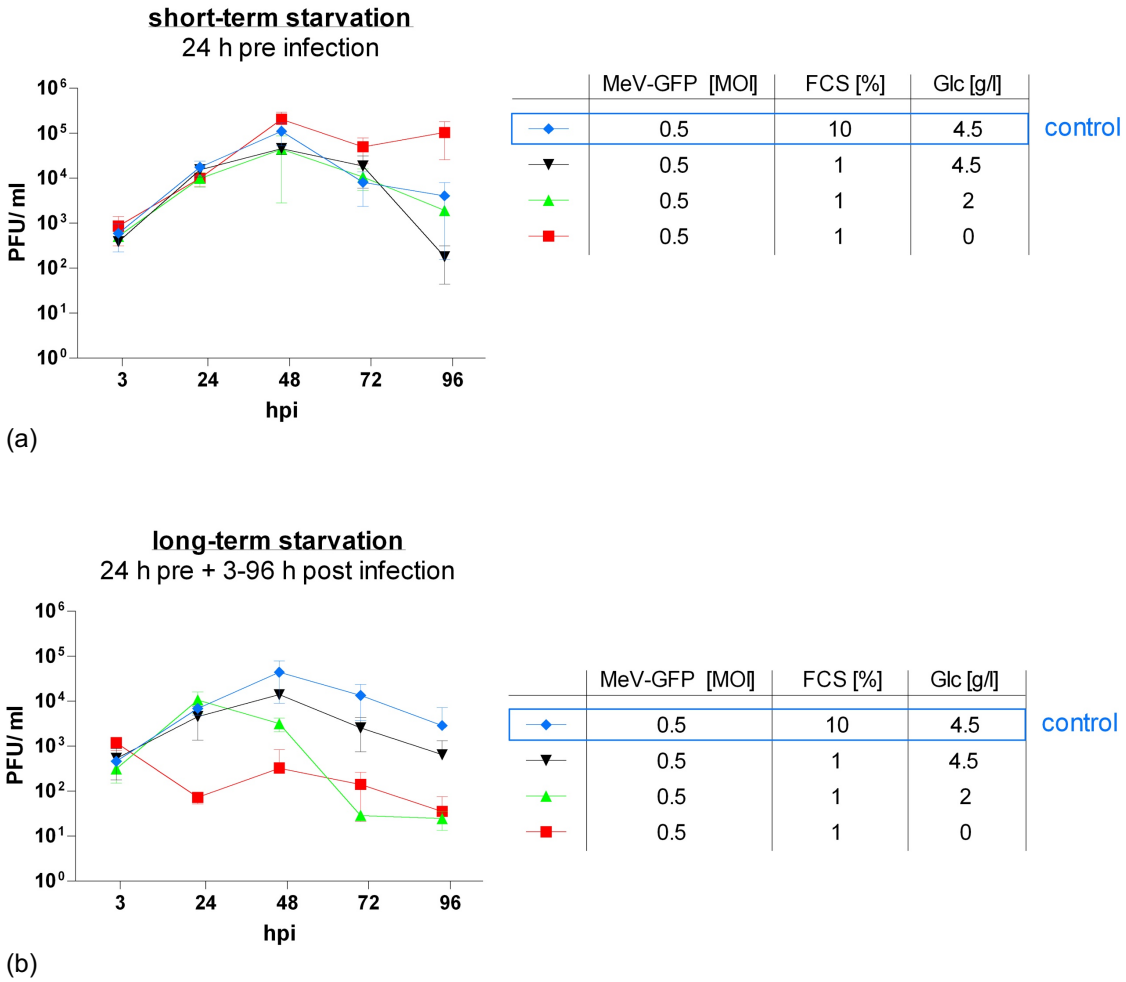


Figure 13: Viral replication under short-term (a) or long-term low-serum (b) starvation in HT-29 cells.

After seeding on day 0, HT-29 cells underwent starvation either for 24 h pre-infection ((a): short-term starvation; Figure 8a) or for both 24 h pre- and 3–96 h post-infection ((b): long-term starvation; Figure 8b). Infection with MeV (MOI 0.5) was performed on day 2. Supernatants and tumor cell lysates were harvested at 3, 24, 48, 72, and 96 hpi. Titrations were performed on Vero cells and calculated as total amount of plaque-forming units (PFU)/mL (comprising PFU in supernatants plus cell lysates); control: tumor cell cultures and infections performed under standard medium conditions (no starvation). Means and SD of three independent experiments are shown. [23]

Short-term starvation (Figure 13a) *at 0 g Glc/L, 1 % FCS increased the replication of oncolytic MeV-GFP in HT-29 cells. By contrast, short-term starvation at higher glucose concentrations did not alter viral replication compared to control.*

When cells were continuously starved after infection (long-term starvation, Figure 13b)*, virus replication was roughly proportionally diminished to the intensity of starvation, except for one outlier at 2 g Glc/L, 1 % FCS at 72 hpi. In detail, viral replication was notably lowered at glucose and serum restriction (red and green curve). Under serum restriction only (black curve), viral replication followed a similar pattern to that of not starved cells (blue curve) and exhibited that viral replication is widely unaffected by long-term standard glucose, low-serum starvation.*

3.5. Long-Term Serum Starvation Increased MeV-GFP-Mediated Oncolysis in Human Colon Carcinoma HT-29 Cells, But Not in Normal Human Colon Cells CCD-18 Co and CCD-841 CoN

Since our results revealed that especially long-term serum restriction favors MeV-GFP-mediated oncolysis in distinct cancer cells and, at the same time, impairs measles vaccine virus replication only marginally, we set out to investigate the effects of serum starvation more deeply. Further, we were interested in how normal human colon cells respond to nutrient restriction and virotherapy. As illustrated in Figure 14*, CCD-18 Co (human colon fibroblast cell line,* Figure 14a*)*, *CCD-841 CoN (human colon epithelial cell line,* Figure 14b*) and HT-29 (human colorectal carcinoma cell line,* Figure 14c*) cells underwent long-term standard glucose, low-serum starvation*. Cell lines were infected with MeV-GFP (checkered bars) or MOCK-infected (black bars), respectively, and remnant cell masses were determined by SRB assay.

"Starvation only" reduced cell masses in non-malignant cells CCD-18 Co (Figure 14a) *and in non-malignant CCD-841 CoN cells* (Figure 14b) *under descending FCS concentrations. In malignant HT-29 cells, lower concentrations of serum led to reduction of cell mass* (Figure 14c)*. Note that the standard medium for normal colon cells contains only 1 g Glc/L, whereas the standard medium for neoplastic cells contains 4.5 g Glc/L. Additional MeV-GFP treatment enhanced cell killing in HT-29 at all serum concentrations, with up to 89 % cell mass reduction for combined starvation and virotherapy. MeV-GFP-infection of CCD-18 Co and CCD-841 CoN cells reduced cell masses to a small extent at standard serum levels but did not lead to increased cell killing under descending FCS concentrations.*

However, it is important to mention that non-malignant colon cells were infected with a high MOI of either 10 or 5 (in contrast to MOI 0.5 which was used for HT-29 cancer cell infection). This was done in order to compensate for the much lower expression of the measles vaccine virus receptor (i.e., CD46) on all non-malignant cells.

As illustrated by scatter dotplot, values of QoGM indicate that MeV-mediated oncolysis efficacy in starved HT-29 cells was increased by up to three-fold at 1 % FCS (p = 0.023) (Figure 14c). *By contrast, starvation impaired virus-induced cell killing significantly in CCD-18 Co cells* (Figure 14a) *and slightly in CCD-841 CoN cells* (Figure 14b).

To distinguish whether cell mass reduction was caused by (i) inhibition of cell proliferation or (ii) direct cell lysis, LDH release was quantified as a marker of direct cell lysis (Figure 15a–c). *Values of "starvation only"-induced cell lysis (black bars) were at a modest level and only rose slightly with increasing starvation intensity at a range of 7 %–12 % for CCD-18 Co* (Figure 15a), *8 %-20 % for CCD-841 CoN cells* (Figure 15b)*, and 13 %-20 % for HT-29* (Figure 15c). *By contrast, infection of HT-29 cells with MeV-GFP (MOI 0.5) under serum restriction (checkered bars) approximately doubled the lysis rate compared to standard conditions (37 %-68 %). For CCD-18 Co and CCD-841 CoN cells, only a moderate increase was found after MeV-GFP infection. Taken together, our QoGM parameter for virotherapy efficacy showed an increase of cell lysis efficacy for HT-29 cells (p = 0.010), whereas QoGM remained unchanged for non-malignant CCD-18 Co and CCD-841 CoN cells.*

Table 3: Illustration of cell culture media used for long-term standard glucose, low-serum starvation experiments (infection with MeV-GFP)

	FCS	Glc/L
Starvation culture conditions; long-term = 120 h period (24 h pre-infection + 96 h post-infection) *LT standard glucose, low-serum starvation*	1 - 7.5 %	**4.5 g** (HT-29) **1 g** (CCD-18 Co, CCD-841 CoN)
Infection culture conditions (Opti-MEM®; 3 h period)	0 %	2.5 g
Standard culture conditions (control)	10 %	**4.5 g** (HT-29) **1 g** (CCD-18 Co, CCD-841 CoN)

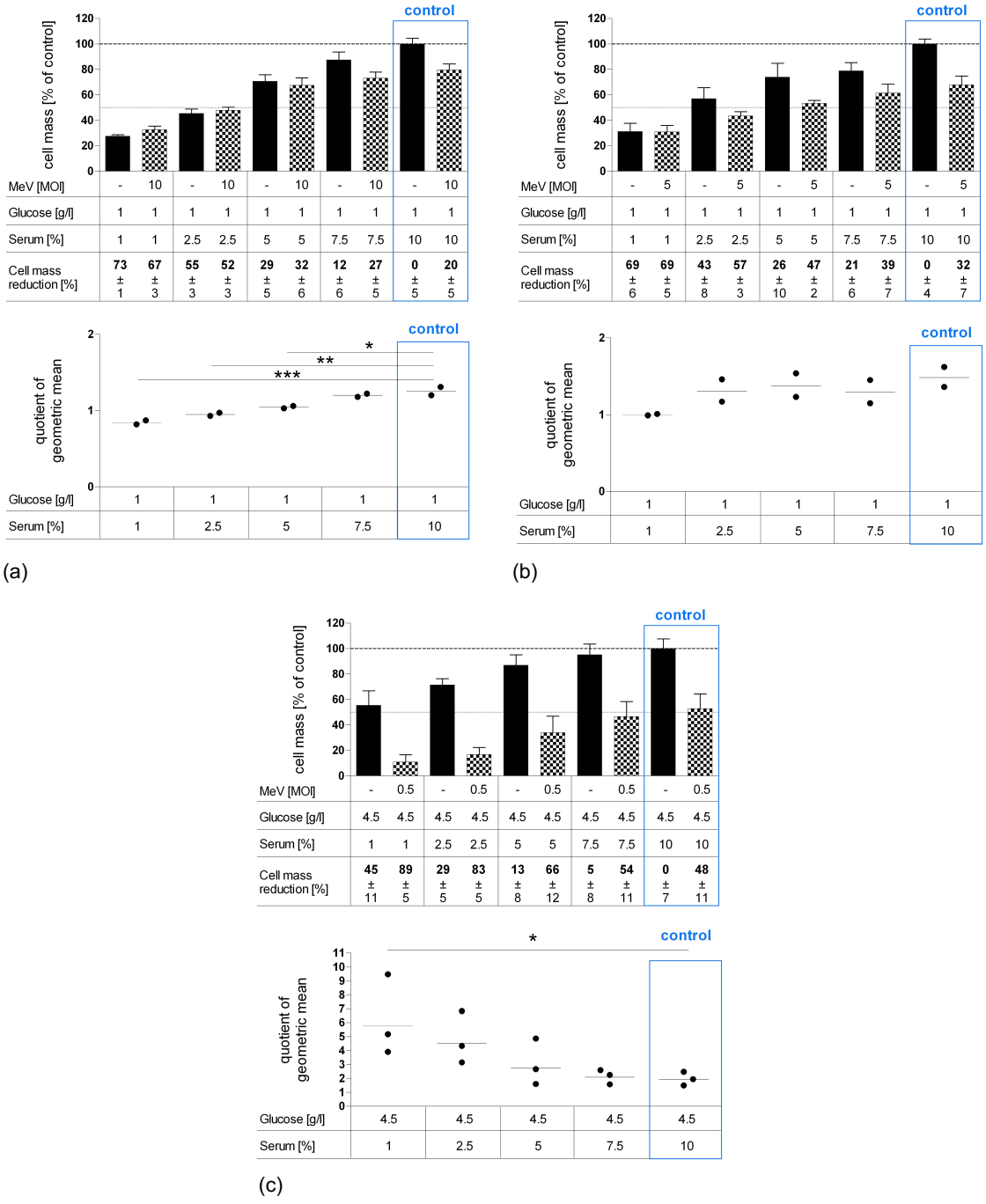


Figure 14. Effect of long-term standard glucose, low-serum starvation on MeV-mediated oncolysis in normal human colon fibroblast cell line CCD-18 Co (a) and epithelial cell line CCD-841 CoN (b) compared to neoplastic cell line HT-29 (c) as determined by SRB assay.

Long-term starvation and MeV infection of HT-29, CCD-18, and CCD-841 cells were performed according to Figure 8b. Starvation medium contained standard glucose, but low-serum. Note, standard medium in normal colon cells contained only 1 g Glc/L, whereas standard medium in neoplastic cell lines contained 4.5 g Glc/L. For non-malignant cell lines, much higher MOIs were used (MOI 10 and 5) compared to the malignant cell line (MOI 0.5). At 96 hpi, the remaining tumor cell masses were determined by SRB assay. Differences were considered significant when P-values were < 0.05 (*), < 0.01 (**), and < 0.0001 (***). [23]

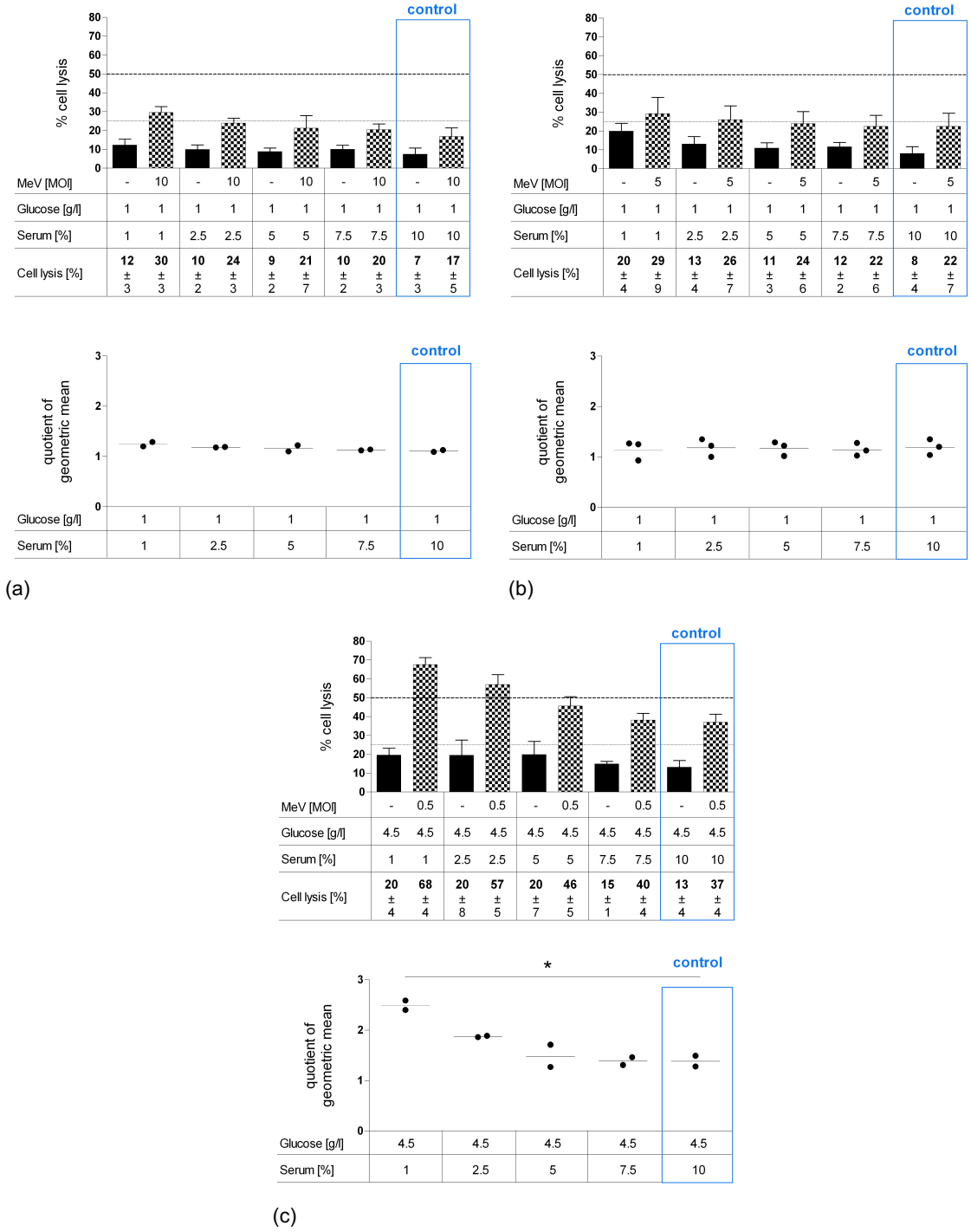


Figure 15. Effect of long-term standard glucose, low-serum starvation on MeV-mediated oncolysis in normal human colon fibroblast cell line CCD-18 Co (a) and epithelial cell line CCD-841 CoN (b) compared to HT-29 cells (c) determined by LDH assay.

Cell culture, starvation and infection were carried out as in Figure 14. At 96 hpi, an LDH assay was performed to determine cell lysis. Differences were considered significant when P-values were < 0.05 (*). [23]

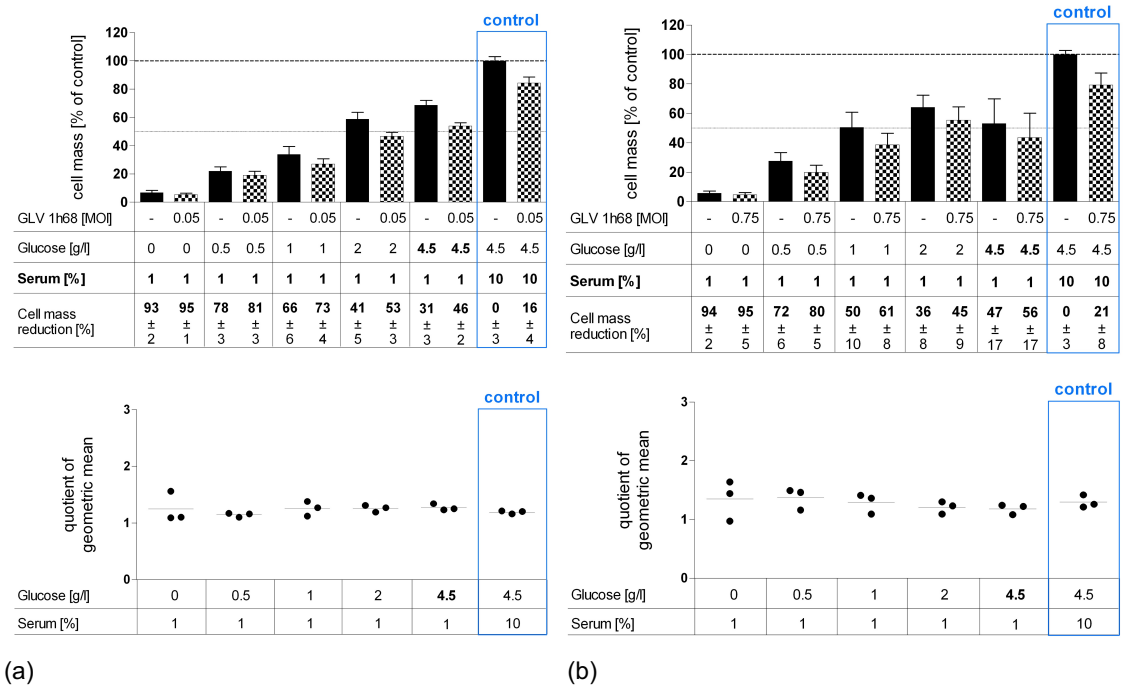
3.6 Infection with VACV GLV-1h68 (dsDNA Virus) at Long-Term Low-Glucose, Low-Serum Starvation Did Not Provide Additional Benefits Compared to MeV-GFP Infection.

To test whether a DNA virus may also take advantage of starvation conditions, we treated long-term low-glucose, low-serum starved cancer cells HT-29 (Figure 16a), HCT-15 (Figure 16b) and HCT-116 (Figure 16c) with the recombinant GFP marker gene encoding vaccinia virus vector GLV-1h68 [56] and compared the effects with MOCK infected control cells. Infection was performed in DMEM + 2 % FCS for 1 h at an MOI of 0.05 for HT-29 cells, an MOI of 0.75 for HCT-15 cells and an MOI of 0.075 for HCT-116 cells. Remaining cell mass was determined by SRB assay at 96 hpi.

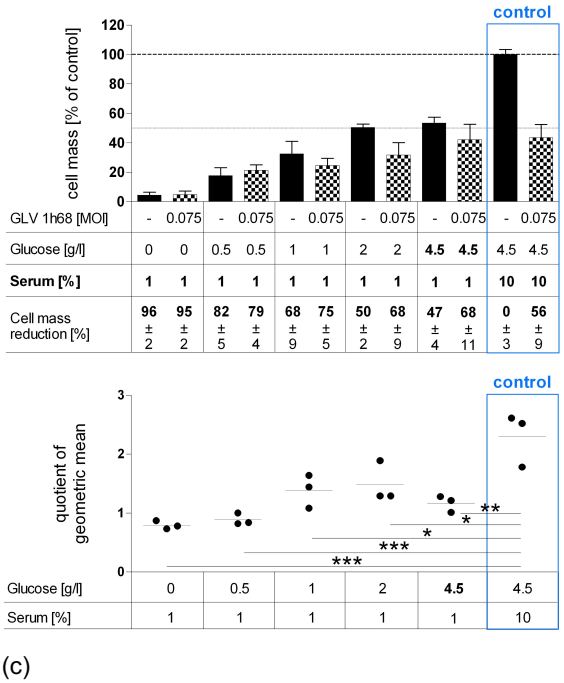
As outlined before, “starvation-alone” massively retarded tumor cell proliferation by up to 95 % (black bars). Although virotherapy with GLV-1h68 was found to additionally kill tumor cells, this effect was not enhanced by starvation. Focusing on HT-29 (Figure 16a), synergistic effects were observed for MeV-GFP infection and starvation at 4.5 g Glc/l and 1 % FCS. GLV-1h68 infection killed HT-29 cells to the same extent at 4.5 g Glc/l and 1 % as under standard conditions. These observations are supported by QoGM, our parameter for virotherapy efficacy, which was found to remain on the same level under starvation conditions. For HCT-15 cells (Figure 16b) results are similar to the one´s obtained with HT-29 cells. Virus-induced cell killing sufficiently worked but was not enhanced. GLV-1h68 treatment of HCT-116 (Figure 16c) cells exhibited that oncolysis efficacy can also be significantly impaired by starvation.

Table 4: Illustration of cell culture media used for long-term low-glucose, low-serum starvation experiments (infection with GLV-1h68)

	FCS	Glc/L
Starvation culture conditions; long-term = 120 h period (24 h pre-infection + 96 h post-infection) *LT low-glucose, low-serum starvation*	**1 %**	**0 - 4.5 g**
Infection culture conditions (DMEM® + 2 % FCS; 1 h period)	0 %	4.5 g
Standard culture conditions (control)	10 %	4.5 g



(a) (b)



(c)

Figure 16: Effect of long-term starvation (120 h) on vaccinia virus-mediated oncolysis in human colon carcinoma cells HT-29 (a), HCT-15 (b) and HCT-116 (c).

Long-term starvation of HT-29, HCT-15 and HCT-116 cells was performed according to Figure 8b. On day 1, medium was changed to low-glucose, low-serum medium. Differences were considered significant when P-values were < 0.05 (*), < 0.01 (**) or < 0.0001 (***). Differences in glucose and serum concentrations are pointed out in bold face.

4. Discussion

Even though much progress has been made in the prevention, screening, and treatment of colorectal carcinoma (CRC), it still remains today's third most common cause of cancer-related deaths worldwide [57].

The use of targeted therapies such as the VEGF-inhibitor Bevacizumab or the EGF-inhibitor Cetuximab alongside conventional chemotherapeutics have broadened treatment options with significant prolongation of overall survival of metastatic disease [58-60]. But for the treatment of stage II and III patients these antibodies deliver no additional benefit to chemotherapy alone [61].

Oncolytic virotherapy as an alternative treatment option is currently being investigated for various malignancies. OVs selectively infect, replicate in and lyse cancer cells where effective antiviral defense mechanisms are compromised due to various genetic mutations [62].

In addition to direct cell lysis, OVs may initiate a profound and long-lasting antitumoral immunogenicity [63, 64].

Infected cells that are undergoing cell death release immunogenic cellular debris that is taken up by antigen presenting cells (APC) leading to activation of CD8+ T-cells. Cytotoxic T-cells are activated via cross presentation, primed also against non-infected cancer cells and induce tumor cell apoptosis via perforin and granzyme release [63, 64]. Eliciting an innate anti-cancer immune response is regarded as an essential mechanism to evoke a sustaining, strong and systemic anti-tumor effect and is currently in the focus of cancer research.

A first phase 1b trial demonstrated an acceptable safety profile of intravenously administering oncolytic and immunotherapeutic vaccinia virus Pexa-Vec to CRC patients [65].

Given that nutritional depletion had been shown to modulate nutrient signaling pathways, sensitize cancer cells to chemotherapeutics, and protect normal cells [1], we sought to investigate the effects of nutrient restriction on oncolytic virotherapy with two virotherapeutic vectors, i.e. MeV (MeV-GFP, being of RNA origin) and VACV (GLV-1h68, being of DNA origin).

In the present study, we found that long-term starvation is capable of enhancing the oncolytic potential of MeV-GFP specifically in the human colon cancer cell line HT-29. Under standard conditions, all cell lines were lysed by our vector MeV-GFP, and the extent correlated with the employed MOI. We initially tested the impact of short-term starvation on virus-mediated cell killing. Colon cancer cells deprived of glucose and serum for 24 h pre-infection were reduced by up to 10 % in cell mass. Infection with our vector MeV-GFP further reduced tumor cell mass, however, without potentiating the effect. As expected, when the fasting period was extended to 24 h pre- and 96 h post-infection, cell masses were more dramatically reduced. Interestingly, our results delivered evidence that serum restriction in HT-29 cells enhanced the efficacy of MeV-GFP-mediated oncolysis, whereas a restriction in glucose had no effect. OV treatment of serum- and glucose-restricted HCT-15 and HCT-116 cells showed no significant increase in the respective oncolytic activities of MeV.

Low protein intake is associated with a major decrease in levels of IGF-1 [66]*, which we mimicked by serum restriction in the cell culture medium. Since the insulin-like-growth-factor receptor (IGF-1R) is overexpressed in different cancer cells* [67-69] *and the IGF-1 dependent pathway plays a major role in cancer cell proliferation, novel approaches aim to inhibit the IGF-1R in cancer treatment* [70]*. Beyond that, OV replication has shown to rely on distinct pathways, e.g. poxvirus JX-594 replication demonstrated to be limited to cells with activated EGFR/ Ras signaling pathways* [62, 71]*. These data suggest that oncolytic viruses may take advantage of starvation-driven alterations in the IGF-1 pathway.*

Specifically for MeV, it has been shown that MeV infection exploits autophagy for an optimal replication [72, 73]*. Normally, autophagy is a cell defense mechanism that activates the lysosomal pathway to fight intracellular pathogens, such as bacteria or viruses. Interestingly, MeV uses autophagy receptors, particularly NDP52 and T6BP, for replication during the course of infection* [74]*. Since autophagy is upregulated in response to extracellular or intracellular stress, such as starvation, growth factor deprivation, and pathogen infection, a link between autophagy and starvation has been demonstrated* [75-77]*. Since MeV replication*

depends on autophagy that is being upregulated by starvation, this phenomenon might contribute to MeV-mediated oncolysis.

To verify the mechanisms of increased oncolysis of MeV-GFP in HT-29 CRC cells, we assessed viral replication under starvation conditions. When these CRC cells were fasted 24 h pre-infection and medium was switched to standard medium after infection, viral replication was found to be increased for low-glucose, low-serum levels.

A different picture emerged when cells were also fasted after infection. Viral replication in long-term starved cells was diminished by up to 2-log under glucose and serum restriction, but only slightly decreased under serum restriction and standard glucose levels.

A recent study of HSV replication in GBM cells [51] *revealed that transient fasting before, but not after, infection raised the yield of HSV both in vitro and in vivo* (for comparison with this study see Table **5**). *Remarkably, this effect appeared specifically in cancer cells as there was no increase of viral replication in normal astrocytes* [51]. *These findings suggest the necessity of sufficient nutrient supply after infection for optimal virus replication, and led to the assumption that the switch from low to high nutrient availability favors cell metabolism for viral replication. This may be explained by the induction of autophagy following short-term starvation, favoring MeV replication* [77]*. Serum restriction alone seems to have only little impact on viral replication.*

Moreover, cell cytotoxicity was improved due to increased HSV replication. However, in our study, increased viral replication did not result in enhanced oncolytic activity. In fact, we observed the most significant enhancement of oncolysis under long-term standard glucose, low-serum starvation, where viral replication properties were – even if only slightly – impaired compared to standard cell culture conditions. Therefore, it can be assumed that improved oncolysis in the HT-29 colon carcinoma cell line may be due to mechanisms other than increased viral replication. Though, it would be of interest to further evaluate whether oncolytic vectors in other tumor entities could profit from increased replication in terms of improved cell killing.

Table 5: Comparison of eligible studies investigating starvation based oncolytic virotherapy [51]

This table compares the key points of the 2016 published study by Esaki, S. et al. "Transient fasting enhances replication of oncolytic herpes simplex virus in glioblastoma" with our own study "Starvation Based Differential Virotherapy". The green shaded fields contrast the basic features of the studies as the investigated cell lines, virotherapeutic vectors and standard cell culture medium. Different methods and starvation regimes having been applied are opposed in the blue shaded fields. Orange shaded fields present a comparative overview of the results and discussion for both studies.

	Esaki et al. 2016	Our study: "*Starvation-Induced Differential Virotherapy Using an Oncolytic Measles Vaccine and Vaccinia Virus"*
Viral vectors	HSV G47Δ, G47Δ-US11fluc	MeV-GFP, VACV GLV1h68
Cell lines	Patient-derived GBM cell lines MGG8F and MGG29F, normal human astrocytes	HCT-15, HCT-116, HT-29, CCD-18 Co, CCD-841 CoN
***In vitro* standard culture conditions (= no starvation)**	DMEM 4.5 g Glc/L + 10 % FCS	DMEM 4.5 g Glc/L + 10 % FCS (cancer cells) Alpha-MEM 1 g Glc/L, 10 % FCS (normal human colon cells)
***In vivo* standard conditions**	Ad libitum diet	-
***In vitro* virus late gene expression assay**	Bioluminescence measurement	-
***In vitro* starvation culture conditions**	DMEM 0.5 g Glc/L + 1 % FCS DMEM 4.5 g Glc/L + 1 % FCS DMEM 0.5 g Glc/L + 10 % FCS	-
MOI	1 (G47Δ-US11fluc)	-
Starvation regimes	Various sequences of standard and starvation medium 32 - 72 h pre- and 24 h post infection	-
Results	• Infection after 24 h starvation followed by 24 h normal medium provided increased virus late gene expression in GBM cells • Starvation ≥ 48 h pre-infection or 24 h post infection decreased levels of gene expression in GBM cells • Both glucose and serum starvation resulted in greatest increase of late gene expression • Starvation in normal astrocytes did not increase virus gene expression	-
Discussion	• The switch from starvation to normal nutritional conditions creates an intracellular environment optimal for HSV replication • Both glucose and serum restriction were needed for this effect • Effect of enhanced HSV replication appeared specifically in GBM cells, not in normal astrocytes	-
***In vitro* cytotoxicity assay**	• Cell count on hemocytometer of trypan blue stained cells 96 hpi	SRB assay, LDH assay 96 hpi

	• MS assay 48 and 96 hpi	
MOI	0.1, 1 (HSV G47Δ)	Adjusted to cell lines (MeV-GFP and VACV GLV-1h68)
***In vitro* starvation culture conditions**	DMEM 0.5 g Glc/L + 1 % FCS	• Low-glucose, low-serum: DMEM 0 - 4.5 g Glc/L + 1 - 10 % FCS • Standard glucose, low-serum: DMEM 4.5 g Glc/L + 1 - 7.5 % FCS • Low-glucose, standard serum: DMEM 0 - 3 g Glc/L + 10 % FCS
Starvation regimes	• TF (transient fasting): 24 h starvation medium → 24 h standard medium → infection → 96 h standard medium	• STS (short-term starvation): 24 h starvation medium → infection → 96 h standard medium • LTS (long-term starvation): 24 h starvation medium → infection → 96 h starvation medium
Results	• TF did not alter proliferation rate of MGG8F and MGG29F • HSV G47Δ-mediated oncolysis in GBM cells was significantly improved after TF	• STS and LTS decreased cell masses in a time and dose dependent fashion in all cell lines • Virus-mediated cytotoxicity was additively enhanced in cancer cell lines, whereas normal cell lines tended to become more robust to virus infection after starvation • MeV-GFP-mediated oncolysis was synergistically improved in HT-29 after LTS at 2 and 4.5 g Glc/L and 1 % FCS
Discussion	• Starvation sensitizes GBM cells to HSV-mediated oncolysis most likely due to increased viral replication	• Serum starvation differentially sensitizes HT-29 cancer cells to virotherapy whereas it makes normal cells more robust to infection • Enhanced oncolysis is not mediated by increased viral replication, but possibly by upregulation of mTOR signaling in cancer cells and downregulation in normal cells
Virus yield assay	Plaque assay on Vero cells 48 hpi	Plaque assay on Vero cells 3 - 96 hpi
MOI	1 (HSV G47Δ)	0.5 (MeV-GFP)
***In vitro* starvation culture conditions**	DMEM 0.5 g Glc/l + 1 % FCS	Low-glucose, low-serum: DMEM 0 - 4.5 g Glc/L + 1 - 10 % FCS
starvation regimes	TF	STS, LTS
Results	• TF increased G47Δ yield about two-fold in MGG8F and MG-G29F GBM, but did not alter it in normal human astrocytes	• STS (at 0 g Glc/L, 1 % FCS) increased MeV-GFP yield by 1.8-fold in HT-29 cells • LTS halved MeV-GFP yield at 0 and 2 g Glc/L + 1 % FCS, but did not significantly alter it at 4.5 g Glc/L + 1 % FCS
Discussion	• In response to a fasting period, normal cells shut down translational activities, whereas neoplastic cells upregulate their cellular metabolism which is exploited for replication • Normal medium is required for optimal replication	

	• Increased viral replication after TF is specific for cancer cells and leads to improved GBM oncolysis	• Long periods of glucose and serum depletion hinder viral replication, whereas long periods of serum depletion only have little impact on viral replication • Increased replication after STS does not result in improved HT-29 oncolysis
***In vivo* virus expression assay**	Bioluminescent imaging (BLI)	-
Virus concentration	1.5×10^6 PFU/3 µL (G47Δ-US11fluc)	
***In vivo* application**	Stereotactical implantation of glioma cells into the brain of SCID mice (n=8), 72 h later, stereotactical injection of virus into grown out brain tumors	-
***In vivo* starvation conditions**	Total food deprivation (no calories at all), free access to water	-
Starvation regime	TF: 48 h starvation → 24 h ad libitum diet → infection	-
Results	• Mice preconditioned with TF had almost 3-fold higher luciferase signals compared to non-starved mice 24 hpi • 96 hpi signals dropped in all mice, so there was no significant difference between control and starved group • Body weight was comparable between starved and unstarved mice	-
Discussion	• TF augments intratumoral HSV replication transiently • The decrease of virus yield after 96 h is possibly due to tumor destruction	-

Further on, we investigated whether the model of DSR [6] also applies for starvation-based virotherapy.

In response to nutritional depletion, normal cells protect themselves by reducing proliferative activities since they require external growth signals for cell proliferation, whereas cancer cells are proliferating self-sufficiently making cells more vulnerable to stress.

In this study, serum restriction evoked a differential response to virotherapy in normal and neoplastic colon cells. Our data show that long-term serum starvation increased MeV-GFP cell-killing efficacy in the malignant CRC cell line HT-29, whereas it decreased (CCD-18 Co) or was unaffected (CCD-841 CoN) in normal

colon cells. Thus, we provide new experimental evidence that differential stress resistance (DSR) plays a pivotal role in the differential biological response of normal and cancerous cells not only to chemotherapy but also to virotherapy.

Note that we used cell line-adjusted MOIs, since the range of oncolysis differs significantly (from 2 % to 90 % at an MOI of 1) when using the same MOI for the different cell lines of the NCI-60 tumor cell panel [50]*. Due to these large differences in oncolytic effectiveness, we used "adjusted"/cell line-specific MOIs for all further experiments to allow enough space to detect the additional cell mass reduction caused by starvation and the combination of both. Otherwise, MeV infection alone could lead to an almost complete destruction of the target cell mass, whereby additional cytotoxic effects possibly caused by our different starvation conditions could no longer be observed. However, the use of different MOIs makes the comparison between different cell lines more difficult, since the availability of glucose or serum per virus particle differs in this setup.*

Moreover, we used slightly different standard media containing either 4.5 g glucose/L for malignant cell lines or 1 g glucose/L for normal colon cell lines. This leads to a lower availability of glucose per MeV virus particle specifically in the normal cells CCD-18 Co and CCD-841 CoN, and impairs comparability to malignant cell lines. However, cancer cells consume more glucose when cultured in vitro due to their faster proliferation which, to some extent, compensates for the different glucose levels that were applied.

Most tumor cells obtain their increased energy demand from aerobic glycolysis, a significantly more inefficient way to generate ATP than through mitochondrial oxidative phosphorylation, the main ATP source for normal differentiated cells, which is commonly known as the Warburg effect [78, 79]*. In tumorigenesis, degenerated cells often undergo metabolic stress due to high proliferation rates, insufficient vascularization, and high overall energy consumption. As a consequence of hyperactivation of metabolic pathways, byproducts such as lactate during glycolysis or ROS during oxidative phosphorylation can accrue and damage the tumor cell* [80]*. Thus, tumor cells counter their increased nutritional demand through broadening the spectrum of energy sources by deriving ATP*

from fatty acid oxidation (FAO) [81]*. In addition, tumor cells can also utilize glutamine or one-carbon amino acids* [82, 83]*. These data suggest that starvation of glucose alone might not be exceedingly detrimental for tumor cells since they are able to reprogram their metabolic needs. This supports our observations that the combination of serum and glucose starvation reduced cell masses significantly compared to glucose starvation alone.*

Studies investigating the adaption mechanisms of viruses to metabolic stress showed that viral X protein in Hepatitis B infected cells is able to divert ATP synthesis from glycolysis and oxidative phosphorylation to FAO in glucose starvation conditions [84]. Furthermore, it has recently been reported that the Kaposi's sarcoma-associated herpesvirus (KSHV) inhibits aerobic glycolysis and oxidative phosphorylation under metabolic stress by downregulation of the glucose uptake transporters GLUT 1 and GLUT 3. Thereupon, the Akt-NF-κB pro-survival pathway is activated in response to a negative feedback loop mediated through downregulation of the glucose transporters [80].

These findings deliver evidence that viruses specifically modify metabolic pathways to ensure the homeostasis of cells in order to survive and maintain proliferation.

The PI3K/Akt/mTOR pathway and its downstream effectors S6K1 and 4E-BP1 are known to regulate protein synthesis, proliferation, differentiation, and cell survival in response to growth factors, nutrients, and stress (Figure 17) [85]*. High mTOR activity is common in various cancers, including CRC* [86].

Data from experimental studies indicate that features of elevated phosphorylation of Akt and S6K1 in starved breast cancer cells might constitute a possible mechanism of sensitizing cancer cells to chemotherapy [22]*. Downregulation of the mTOR pathway due to fasting, on the other hand, may be a contributing factor to the protection of normal cells* [51, 87, 88]*. mTOR inhibitors, such as rapamycin, can be used to mimic nutritional depletion in the cell. The combination of rapamycin with oncolytic VACV JX-594 promoted oncolysis and enhanced viral replication in glioma cells, suggesting a critical role of the mTOR pathway for the effectiveness of OVs* [89]*. Myxoma virus, a rabbit-specific poxvirus with tropism*

for human cancer cells, has evolved mechanisms to regulate the Akt pathway to establish an environment for optimal viral replication [90].

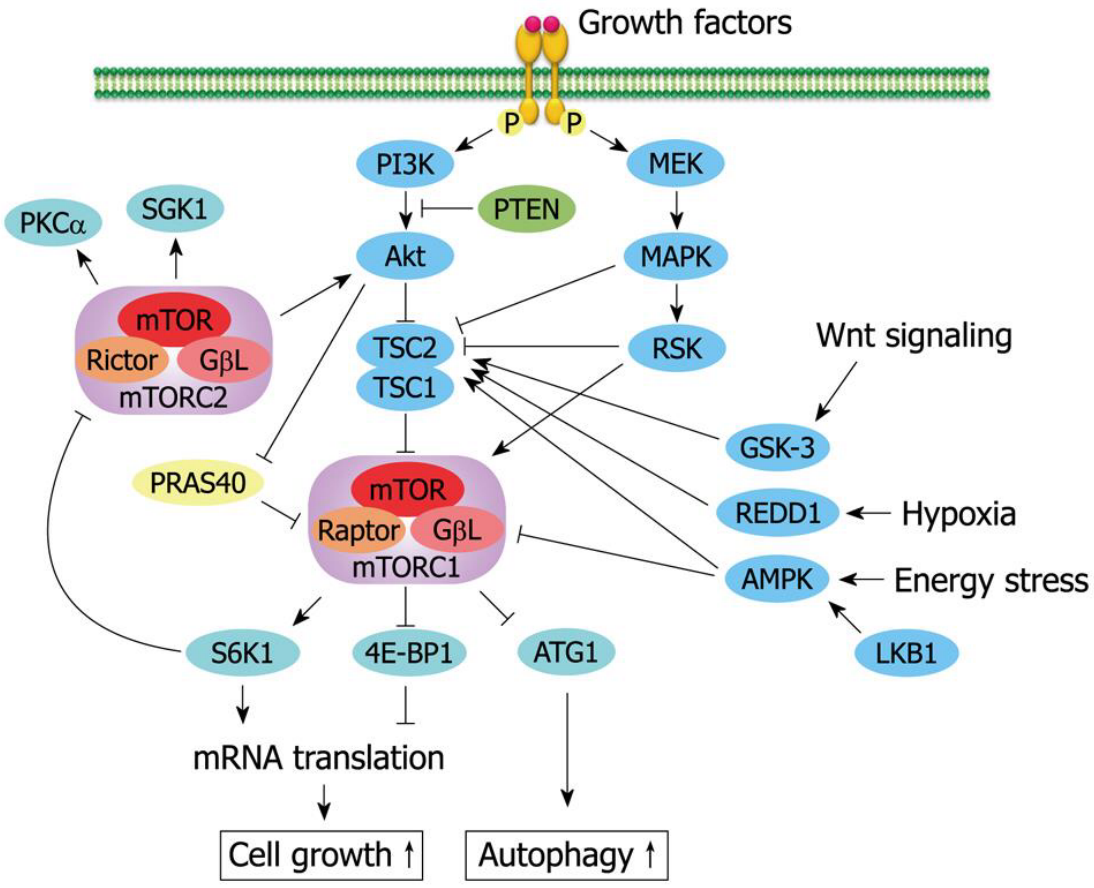


Figure 17: Key regulators of the mammalian target of rapamycin (mTOR) signaling pathway.

Mammalian target of rapamycin (mTOR) forms the catalytic core of two functionally distinct complexes, mTOR complexes 1 and 2 (mTORC1 and mTORC2), respectively. Diverse environmental factors, including growth factors (e.g. insulin, insulin-like growth factor 1 and epidermal growth factor) and stress events (e.g. hypoxia or energy) promote mTORC1-dependent cell growth and down-regulate autophagy. The negative feedback loop signals of mTORC1/p70-S6 kinase 1 (S6K1) suppress the mTORC2/Akt signaling pathway. PTEN: Phosphatase and tensin homolog deleted from chromosome 10; TSC: Tuberous sclerosis complex; Raptor: Regulatory-associated protein of mTOR; Rictor: Rapamycin-insensitive companion of mTOR; MEK: Mitogen-activated protein kinase; RSK: Ribosomal s6 kinase; PRAS40: Proline-rich PKB/ Akt substrate of 40 kDa; ATG1: Autophagy-related gene 1; PKCα: Protein kinase C α; SGK1: Serine/ threonine-protein kinase 1; GSK-3: Glycogen synthase kinase 3; REDD1: Regulated in development and DNA damage responses 1; AMPK: AMP-activated protein kinase; LKB1: Liver kinase B1; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3,4,5-kinase; P: Phosphate [91].

Since viral vectors rely on host cell metabolism, we speculate that starvation-induced modification of the nutrient-sensing PI3K/Akt/mTOR pathway may also alter the cellular environment in HT-29 cells, and may potentially render cancer cells more susceptible to MeV infection. However, mechanisms which prevent

synergistic effects between starvation and virus-mediated oncolysis in other cells, such as HCT-15 and HCT-116, need to be unraveled in further investigations. Both glucose and serum starvation alone were shown to have significant cytoreductive capacities. The detection of relatively unaffected cell lysis of HT-29 cells following long-term serum starvation without infection support the assumption that cell mass reduction is predominantly mediated through proliferation inhibition rather than cell lysis.

These findings match with observations that starvation shifts the cell cycle of pancreatic cancer cells from S phase into G1/G0 phase and thus hinders DNA replication [92].

In future, in vivo studies (both preclinical as well as clinical studies) are required to investigate the combination of starvation and virotherapy in order to determine optimal length of fasting, oncolytic activity, viral replication, and optimal administration of OVs (local vs. systemic treatment). A practical approach for an animal study could be to compare tumor-bearing mice treated with MeV that were either short-term- or long-term-starved with mice fed an ad lib diet. Interesting endpoints would be overall survival, weight development, and (starvation-associated) mortality. Furthermore, it would be of interest whether healthy mice respond differentially to starvation regarding weight loss and mortality when compared to tumor-bearing mice.

Furthermore, we assessed the oncolytic activity of VACV GLV1h68 in the setting of long-term low-glucose, low-serum starvation. Ottolino-Perry et al. found synergistic interactions between VACV expressing the human somatostatin receptor and red fluorescent protein (RFP) under control of the p7.5 and Psel promoters, respectively and chemotherapeutic drugs used for the treatment of CRC [93].

Yet, we could only find additive effects between starvation and virotherapy for the cell lines HCT-15 and HT-29 or even impaired GLV1h68-mediated oncolysis for HCT-116. Starvation-induced alterations in cell metabolism and signaling pathways cause a microenvironment in tumor cells whose implications on

oncolytic viruses seem to diverge. May one virus such as MeV-GFP benefit from these changes in certain tumor cells, another one such as GLV-1h68 may not.

Identifying tumors taking advantage of this combinatorial approach as well as characterizing possible resistance mechanisms will be the next step on implementing this treatment in future cancer therapy.

Fasting in patients, however, is a matter of debate. While it has been demonstrated to be well-tolerated in most patients with only mild side effects, such as weakness and short-term weight loss in humans [16]*, it is certainly not suitable for patients with cachexia, sarcopenia, and malnutrition* [94, 95]*. Current guidelines recommend increasing the intake of protein and fat in patients with cachexia* [96]. *On the other hand, fasting is easily conductible, able to reduce side effects, and potentially effective in a wide variety of tumors, although tumors with PI3K mutations might not be sensitive* [19]*. Thus, fasting may be limited to relatively stable patients either as a (neo)adjuvant therapy or as a chemotherapy-free approach.*

We conclude that starvation-based virotherapy could provide benefits for distinct cancer patients in terms of a personalized starvation-enhanced virotherapy. Despite the fact that fasting requires a lot of perseverance on the patient's side, most cancer patients have demonstrated a high motivation with regard to gaining the ability to personally contribute in the fight against their malignant disease.

5. Summary

Colorectal carcinoma (CRC) is today's third most common cause of cancer related death worldwide. Even though considerable advances in prevention, early detection and treatment options have been obtained in the last decades, metastatic CRC still implies very poor prognosis.

Starvation (Fasting) has been shown to sensitize tumor cells to chemotherapy whilst protecting normal cells at the same time [Differential Stress Resistance, (DSR)]. Upregulation of pro-survival and proliferation pathways due to various mutations prevents cancer cells from responding to external growth factors. Consequently, malignant cells, unable to adapt to extreme environmental conditions such as absence of nutrients, are becoming more vulnerable to stress. Normal cells, in contrast, enter a standby mode in response to starvation and are getting more protected.

The ability of oncolytic virotherapeutics (OVs) to selectively infect, replicate in and lyse cancer cells outlines a promising approach in cancer therapy. However, combinatorial concepts seem to be needed to achieve sustained anti-cancer effects, such as combination of OVs with chemotherapy or new immuno-modulatory drugs.

We hypothesized that starvation would increase the oncolytic potential of OVs in CRC cell lines and protect normal colon cells against virus-mediated cell lysis.

Three different human colon carcinoma cell lines (HT-29, HCT-15 and HCT-116) as well as two human normal colon cell lines (CCD-18 Co and CCD-841 CoN) were subjected to various starvation regimes in glucose and/or serum restricted cell culture medium and infected with two state-of-the-art OVs [i.e., measles vaccine virus (MeV) and vaccinia virus (GLV-1h68)].

Fasting regimes applied were either short-term starvation (24 h pre-infection) or long-term starvation (24 h pre- and 96 h post-infection).

We used cell viability assays to determine the cell killing capabilities of i) virotherapy, ii) starvation, and iii) the combination of these two. Virus growth curves were generated to assess the replication of MeV in starved and non-starved HT-29 cells.

As a result, starvation retarded cell growth in all cell lines in a time and concentration dependent manner. Infection of starved cancer cells exhibited additional oncolytic potential of virotherapy plus starvation for most combinations, indicating that virus-mediated oncolysis is sufficiently working under starvation conditions.

Remarkably, long-term standard glucose, low-serum starvation potentiated the efficacy of MeV-mediated cell killing in HT-29 cancer cells, whereas it was decreased in normal colon cells CCD-18 Co and CCD-841 CoN. Interestingly, viral replication of MeV in HT-29 was decreased in long-term starved cells, but was increased after short-term low-glucose, low-serum starvation.

We speculate that particular nutrient signaling pathways such as the PI3K/ Akt/ mTOR pathway are modified upon fasting depending on specific mutations in cancer cells resulting in a differential response of distinct CRC cells to OVs.

In conclusion, starvation based virotherapy could enhance the oncolytic effect on CRC in future anti-cancer therapy while protecting normal tissues from side effects.

6. Zusammenfassung

Das kolorektale Karzinom stellt heute die weltweit dritthäufigste Todesursache unter den bösartigen Tumoren dar. Obwohl in den letzten Jahrzehnten beachtliche Fortschritte in der Prävention, Früherkennung und Behandlung dieser Tumoren erzielt wurden, hat das metastasierte Kolonkarzinom nach wie vor eine schlechte Prognose.

In den letzten Jahren konnte gezeigt werden, dass Fasten das Potenzial besitzt Tumorzellen gegenüber Chemotherapie zu sensibilisieren, während gesunde Körperzellen zur gleichen Zeit robuster werden [Modell der differentiellen Stressresistenz (DSR)]. Krebszellen werden durch die Hochregulation von Überlebens- und Wachstums-Signalwegen aufgrund zahlreicher Mutationen in ihrer Fähigkeit eingeschränkt auf externe Wachstumsfaktoren zu reagieren. Daher werden maligne Zellen, welche unfähig sind sich an extreme Umwelt-bedingungen wie Nährstoffmangel anzupassen, vulnerabler gegenüber Stress. Im Gegensatz dazu treten normale Zellen in einen Ruhemodus ein, der sie deutlich widerstandsfähiger werden lässt.

Die Fähigkeit von onkolytischen Viren (OV) Krebszellen selektiv zu befallen, darin zu replizieren und sie schließlich zu lysieren stellt einen vielversprechenden Ansatz in der Tumortherapie dar. Jedoch scheinen Kombinationskonzepte von Nöten zu sein, um eine nachhaltige Anti-Tumor Wirksamkeit zu erreichen, wie die Kombination mit Chemotherapie oder immunmodulierenden Substanzen.

Wir stellten die Hypothese auf, dass Fasten die Effizienz der Zelllyse von onkolytischen Viren im kolorektalen Karzinom steigern kann und gleichzeitig normale Kolonzellen protektiert.

Um diese Hypothese zu testen, wurden drei verschiedene Kolonkarzinomzelllinien (HT-29, HCT-15 und HCT-116), sowie zwei normale Kolonzelllinien (CCD-18 Co und CCD-841-CoN) unterschiedlichen Fastenregimen in Glukose- und/oder Serum-restringiertem Zellkulturmedium unterzogen und mit zwei modernen onkolytischen Viren [Masernimpfvirus (MeV) und Vaccinia Virus (GLV-1h68)] infiziert.

Die Zellen durchliefen entweder eine Kurzzeit- (24 Stunden vor Infektion) oder Langzeitfastenperiode (24 Stunden vor- und 96 Stunden nach Infektion). In Zellviabilitätsassays untersuchten wir die Fähigkeit Zelltod zu induzieren von i) Virotherapeutika, ii) Fasten sowie iii) die Kombination beider Modalitäten. Viruswachstumskurven wurden erstellt, um die Replikation von MeV in gefasteten und nicht-gefasteten HT-29 Zellen zu bestimmen.

Die Ergebnisse zeigten, dass Fasten das Wachstum von allen Zelllinien zeit- und konzentrationsabhängig verlangsamte. Wurden gefastete Tumorzellen mit Virotherapeutika behandelt, addierte sich der onkolytische Effekt der Virotherapie mit der des Fastens für die meisten Kombinationen, was impliziert, dass die Virusvermittelte Onkolyse unter Fastenbedingungen suffizient funktioniert.

Hervorzuheben sind die Daten aus den Langzeit-Serumfastenversuchen, die eine gesteigerte Effizienz der MeV-vermittelten Onkolyse in HT-29 Tumorzellen zeigten, wohingegen die Lyseeffizienz in den normalen Kolonzellen CCD-18 Co und CCD-841 CoN vermindert war. Interessanterweise war die Virusreplikation von MeV jedoch in den Langzeit-gefasteten Zellen erniedrigt, während sie nach dem Kurzzeitzeitfasten gesteigert war.

Virale Onkolyse mit unserem Vektor GLV-1h68 unter Fastenbedingungen zeigte hingegen nur eine additive oder sogar verminderte Zelllyseaktivität, was auf ein unterschiedliches Ansprechen gefasteter Zellen auf die beiden getesteten Viren hindeutet.

Wir vermuten, dass durch Fasten bestimmte nährstoffabhängige Signalwege, wie u.a. der PI3K/ Akt/ mTOR Signalweg, abhängig von spezifischen Mutationen in Tumorzellen modifiziert werden. Möglicherweise resultiert diese Modifikation dann in der differentiellen Antwort der verschiedenen kolorektalen Krebszellen auf die onkolytischen Viren.

Zusammenfassend weisen diese Ergebnisse darauf hin, dass eine Fasten-basierte Virotherapie den onkolytischen Effekt im kolorektalen Karzinom in zukünftigen Tumortherapien verbessern und normale Gewebe vor Nebenwirkungen schützen könnte.

7. Appendix

7.1 List of Figures

7.2 List of Tables

7.3 Index of abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
AEG	Adenocarcinoma of the esophagogastreal junction
Alpha-MEM	Alpha Modified Eagle's Medium
APC	Antigen presenting cell
ATCC	American Type Culture Collection
ATP	Adenosine tri-phosphate
BLI	Bioluminescent imaging
CC	Cholangiocarcinoma
$CD8^+$	Cluster of differentiation 8 positive
cDNA	Complementary deoxyribonucleic acid
CP	Cyclophosphamide
CRC	Colorectal carcinoma
CTC	Common Toxicity Criteria
CTCAE	Common Terminology Criteria of Adverse Events
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DSR	Differential stress resistance
DSS	Differential stress sensitization
DXR	Doxorubicin
E. coli	Escherichia coli

EC	Esophagus cancer
EDTA	Ethylenediaminetetraacetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ETO	Etoposide
FAO	Fatty acid oxidation
FBS	Fetal bovine serum
FCS	Fetal calve serum
fluc	Firefly luciferase
G0 phase	Gap 0 phase
G1 phase	Gap 1 phase
GBM	Glioblastoma multiforme
GC	Gastric cancer
GFP	Green fluorescent protein
GLUT	Glucose transporter
HER-2	Human endothelial growth factor receptor 2
hpi	Hours post infection
HSV	Herpes simplex virus
IGF-1	Insulin-like growth factor
KSHV	Kaposi’s sarcoma-associated herpesvirus
LDH	Lactate dehydrogenase
LT	Long-term
LTS	Long-term starvation
MeV	Oncolytic measles vaccine virus

MeV-GFP	Oncolytic measles vaccine virus encoding for green fluorescent protein
MeV-NIS	Measles Virus-sodium iodide symporter
MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
mTORC	Mammalian target of rapamycin complex
NADH	Nicotinamide adenine dinucleotide
NCI-60	National Cancer Institute cancer cell line panel
OV	Oncolytic virus
PBS	Phosphate-buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PFU	Plaque forming units
PI3K	Phosphatidylinositol 3,4,5-kinase
QoGM	Quotient of geometric mean
R0 resection	No cancer cells microscopically detectable at the resection margin
RFP	Red fluorescent protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RUC-GFP	Renilla luciferase-green fluorescent protein
S-phase	Synthesis phase
S6K1	Ribosomal protein S6 kinase
SCID	Severe combined immune deficiency
SD	Standard deviation

SEM	Standard error of the mean
SRB	Sulforhodamine B
ST	Short-term
STF	Short term fasting
STS	Short-term starvation
T-Vec	Talimogene laherparepvec
TCA	Trichloroacetic acid
TF	Transient fasting
VACV	Oncolytic vaccinia virus
VEGF	Vascular endothelial growth factor

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9. Publikationen mit eigener (Co-)Autorschaft

Calvo-Castro LA, Burkard M, Sus N, **Scheubeck G**, Leischner C, Lauer UM, Bosy-Westphal A, Hund V, Busch C, Venturelli S., Frank J., *The Oral Bioavailability of 8-Prenylaringenin from Hops (Humulus Lupulus L.) in Healthy Women and Men is Significantly Higher than that of its Positional Isomer 6-Prenylnaringenin in a Randomized Crossover Trial.* Mol Nutr Food Res. 2018 Jan 23. doi: 10.1002/mnfr.201700838.

Scheubeck G, Bechtold S, Smirnow I, Schenk A, Beil J., Lauer U. M *Starvation-Induced Differential Virotherapy Using an Oncolytic Measles Vaccine Virus.* Viruses. 2019. **11**(7).

10. Erklärung zum Eigenanteil der Dissertationsschrift

Die Arbeit wurde in der Medizinischen Universitäts- und Poliklinik, Abteilung für Innere Medizin VIII, Klinische Tumorbiologie, in der Arbeitsgruppe Virotherapie unter Betreuung von Professor Dr. med. Ulrich M. Lauer durchgeführt.

Die Konzeption der Studie erfolgte in Zusammenarbeit mit Professor Dr. med. Ulrich M. Lauer und Dr. med. Susanne Berchtold (Laborleiterin und wiss. Mitarbeiterin).

Sämtliche Versuche wurden nach Einarbeitung durch die Labormitglieder Frau Dr. med. Susanne Berchtold und Frau Irina Smirnow (MTA) eigenständig von mir durchgeführt.

Die statistische Auswertung erfolgte durch mich (nach einer entsprechenden Beratung durch das Institut für Klinische Epidemiologie und angewandte Biometrie unter Anleitung von Professor Dr. Martin Eichner).

Ich versichere, das Manuskript selbstständig (nach Anleitung durch Frau Dr. med. Susanne Berchtold und Professor Dr. med. Ulrich M. Lauer) verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Teile der Dissertationsschrift wurden bereits in der Publikation „Scheubeck G, Bechtold S, Smirnow I, Schenk A, Beil J., Lauer U. M *Starvation-Induced Differential Virotherapy Using an Oncolytic Measles Vaccine Virus.* Viruses. 2019. **11**(7)“ veröffentlicht. Wörtliche Übernahmen sind mittels *Kursivdruck* kenntlich gemacht.

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Gabriel Scheubeck

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