Aus der Medizinischen Universitätsklinik und Poliklinik Tübingen

Abteilung Innere Medizin VIII, Klinische Tumorbiologie

Overcoming of primary & secondary chemotherapy resistance of advanced staged pancreatic cancer by additive virotherapy

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

Binz, Eike Hendrik

2018

Dekan: Professor Dr. I.B. Autenrieth

1. Berichterstatter: Professor Dr. U.M. Lauer
2. Berichterstatter: Professor Dr. F. Stubenrauch
3. Berichterstatter: Privatdozent Dr. D. Nettelbeck

Tag der Disputation: 27.02.2018

Table of content

1 Introduction

1.1 Pancreatic cancer

1.1.1 Epidemiology

Pancreatic cancer, although in total numbers an infrequent tumor entity, represents the tumor disease with the worst prognosis of patient survival. In Germany, pancreatic cancer mainly occurs in the elderly population with a median age of diagnosis of 71 among men and 75 among women (1). In 2012, according to the German cancer statistic approx. 16700 newly diagnosed cases of pancreatic cancer were reported (1), making it the tenth most common tumor entity among men and the sixth most common among women in terms of incidence (Figure 1). Globally, incidence rates of pancreatic cancer are both higher among men and in developed countries (2).

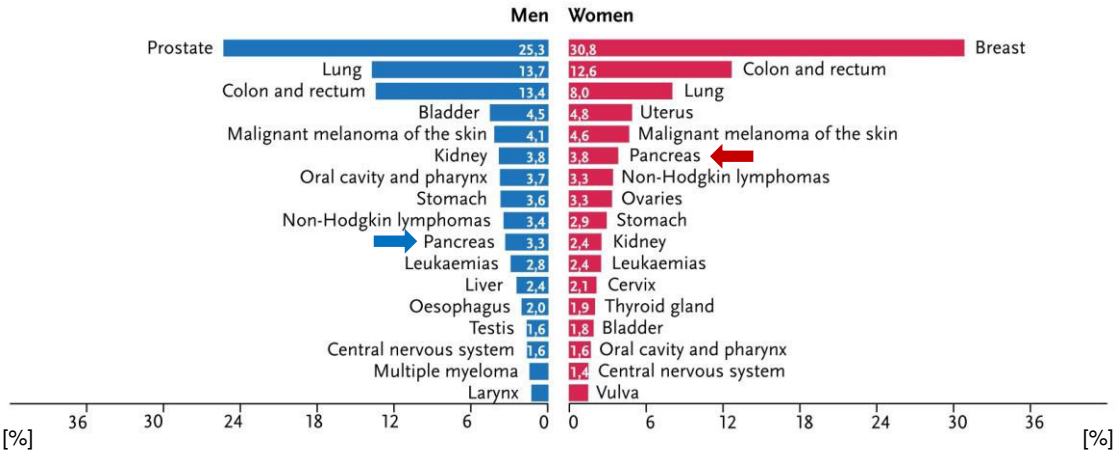

Figure 1 *Most frequent tumor sites as a percentage of all new cases in Germany 2012 (not including non-melanoma skin cancer). Taken and modified from the German cancer statistic 2011/2012 (1).*

Despite intensive research on more effective treatment modalities the therapy outcome of pancreatic cancer remains very poor. In Germany, 5 years after initial diagnosis only 7 % of the patients remain alive, representing the lowest rate of patient survival among all types of cancer. In total numbers, in 2012 approx. 16100 deaths were caused by this fatal tumor disease accounting for 6.6 % and 8.1 % of cancer-related death among men and women respectively (1). Thus, pancreatic cancer is the fourth leading cause of cancer-related mortality both in Germany (Figure 2) and the US (3). Globally seen it is estimated to be the seventh leading cancer-related cause of death in both men and women (2).

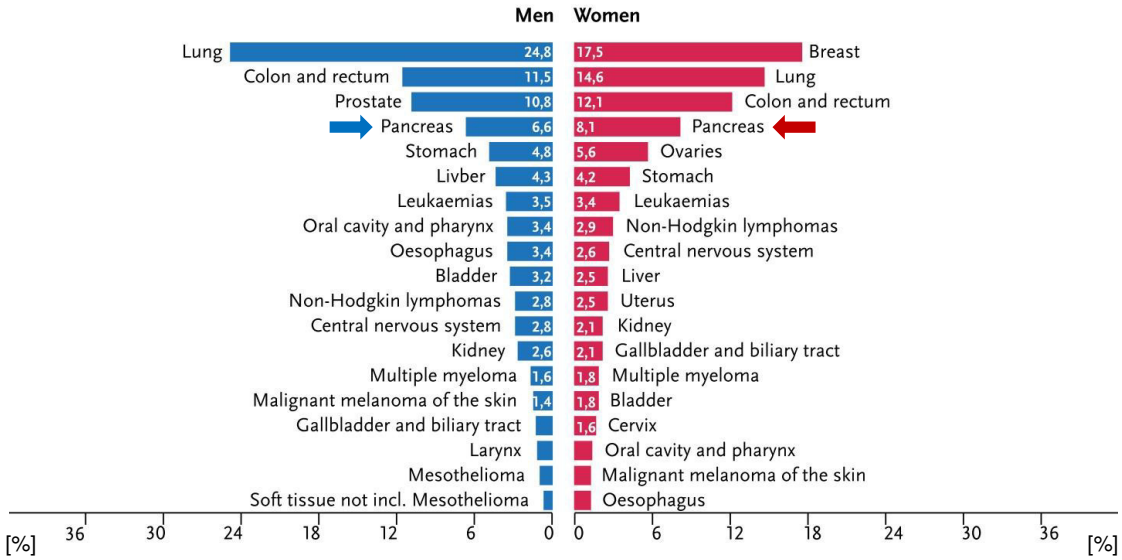

Figure 2 *Most frequent tumor sites when cancer was the cause of death in Germany 2012. Taken and modified from the German cancer statistic 2011/2012 (1).*

Generally speaking the term 'pancreatic cancer' refers to exocrine tumors of the pancreas, mainly represented by the pancreatic ductal adenocarcinoma (PDA).

1.1.2 Therapeutic guidelines

As in the treatment of other tumor entities, therapy of pancreatic cancer depends on the localization and dimension of the primary tumor (T) as well as on potential metastatic lesions in lymph nodes (N) or secondary organs (M), the so-called TNM stadium. The currently valid S3-guideline for the treatment of pancreatic ductal adenocarcinoma (4) has just been updated in 2013 to align clinical practice to recent findings (5).

To date, surgical resection still represents the only potentially curative treatment method for pancreatic cancer. Thereby, it is broadly agreed that a resection margin within healthy tissue without any tumorous cells (R0 resection) is associated with a better treatment outcome and prognosis of the patients, compared to surgery in which not all tumor tissue could be resected and the resection margin includes tumorous cells (R1 resection) (6). However, real survival benefit even after R0 resection was shown to be associated with a histopathologically measured distance of tumor cells from the resection margin greater than 1 mm (median survival ranging from 35 to 16 to 14 months after R0-wide, R0 or R1 resection, respectively) (7). Accordingly, surgical treatment is only indicated for patients with

a high chance for R0 resection and an acceptable risk profile of perioperative morbidity and mortality. Possibility of surgical removal is therefore decided on a case by case basis and depends on the respective tumor localization, distant metastases and the involvement of local vasculature (Figure 3) (8). In case of a non-metastatic but unresectable tumor, individually depending on the tumor localization and health status of the patient, a neoadjuvant (radio-)chemotherapy can be performed in the hope of reaching resectability and therefore a better prognosis. By all means surgery should be followed by an adjuvant chemotherapy with 5-fluorouracil (5-FU) or gemcitabine in monotherapy for 6 months (4).

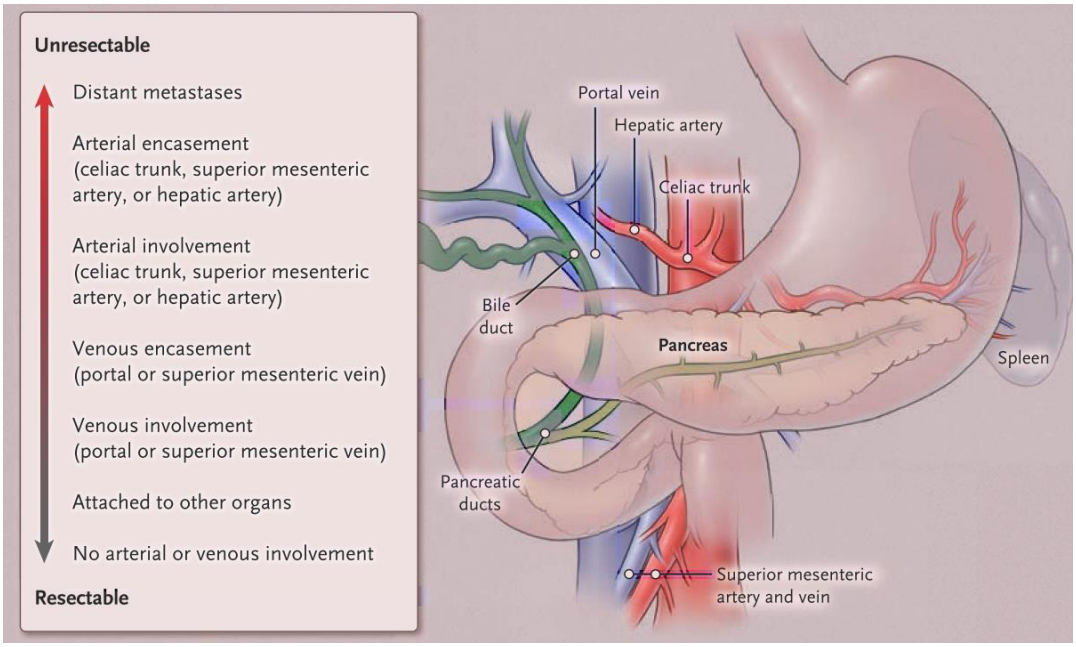

Figure 3 *Anatomy and surgical resectability of pancreatic cancer. Pancreatic cancers are categorized on a continuum from resectable to unresectable according to the involvement of adjacent structures and the presence of distant metastases. Taken from Ryan et al. (8).*

In case of unresectable or metastatic pancreatic cancer only palliative treatment modalities remain, aiming at improving the health-related quality of life as well as potentially prolonging the survival of the individual patient. Currently, for this indication different first-line chemotherapy protocols are available. Due to its favorable toxicity profile and feasibility the preferred and today most commonly performed chemotherapy is a monotherapeutic treatment with gemcitabine. However, clinical data have proven that under certain conditions combinations with other chemotherapeutic agents result in a superior treatment outcome and higher patient survival (Table 1) (9). Results of a phase III trial combining gemcitabine

with the epithelial growth factor receptor (EGFR) targeting tyrosine kinase inhibitor erlotinib have demonstrated a significant increase of overall survival compared to gemcitabine monotherapy (10). However, therapeutic benefit of this regimen was linked to the appearance of a skin rash, a typical side-effect of anti-EGFR treatment, within 8 weeks after initiation of treatment (Table 1). Another combination, the so-called FOLFIRINOX regimen (a combination of folinic acid (leucovorin), 5-FU, irinotecan and oxaliplatin) represents a highly tumoricidal but likewise (hemato-)toxic chemotherapy protocol for advanced pancreatic cancer. Therefore, although significantly prolonging overall survival compared to gemcitabine monotherapy (11), it should only be applied in a subgroup of patients below the age of 75 with a good performance status.

Besides these protocols and the dual chemotherapy *nab*-paclitaxel + gemcitabine, so far, no further combinations of chemotherapeutic agents or targeted therapies are approved or recommended as first-line treatment for advanced pancreatic cancer. In case of disease progression under first-line monotherapy with gemcitabine, the combination 5-FU + oxaliplatin should be given as second-line treatment. If the FOLFIRINOX regimen fails, a second-line monotherapy with gemcitabine can be performed.

Table 1 *Recent randomized and controlled trials on combination chemotherapy for advanced pancreatic cancer showing a survival advantage over gemcitabine monotherapy in the palliative setting.*

Treatment regimen	N	Progression-free survival	Overall survival	1-year survival rate	EBM grade
Erlotinib + gemcitabine versus gemcitabine (10)	569	3.75 versus 3.55 months (HR 0.77, p = 0.004)	6.24 versus 5.91 months (HR 0.82, p = 0.038)*	23 % versus 17 % (p= 0.023)*	Ib
FOLFIRINOX versus gemcitabine (11)	342	6.4 versus 3.3 months (HR 0.47, p < 0.001)	11.1 versus 6.8 months (HR 0.57, p < 0.001)	48 % versus 21 % (p < 0.001)	Ib
Nab-paclitaxel + gemcitabine versus gemcitabine (12)	861	5.5 versus 3.7 months (HR 0.69, p < 0.001)	8.5 versus 6.7 months (HR 0.72, p < 0.001)	35 % versus 22 % (p < 0.001)	Ib

* In the subgroup analysis of overall survival for patients with rash ≥ grade 2: Overall survival 10.5 months (HR 0.74, p = 0.037) and 1-year overall survival 43 % (p < 0.001). EBM, evidence based medicine; HR, hazard ratio. Taken and modified from Seufferlein et al. (5).

Recent data from a randomized multicenter phase III trial demonstrated the superior response of patients with metastatic pancreatic cancer to the dual chemotherapy *nab*-paclitaxel + gemcitabine compared to gemcitabine monotherapy (12). Thereby, the outcome of the dual chemotherapy was not quite as beneficial as the combination of gemcitabine with erlotinib or after application of the FOLFIRINOX regimen, in which more potent increases of overall survival were seen in

the individual patient subgroups compared to gemcitabine monotherapy (Table 1). However, the rate of serious adverse effects in patients receiving the combination *nab*-paclitaxel + gemcitabine was comparable to gemcitabine monotherapy, although reversible side effects like myelosuppression and peripheral neuropathy occurred slightly more often in the combination group (12).

These results led to clinical approval of Abraxane® (*nab*-paclitaxel) + gemcitabine by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) as first-line treatment protocol for metastatic pancreatic cancer in 2013 (13). Since treatment outcome of this combination so far isn't known to be linked to any patient or tumor characteristics, in this indication it might even replace gemcitabine monotherapy as standard of care first-line chemotherapy protocol.

1.1.3 Rationale for the development of new treatment protocols

Reasons for the poor outcome of pancreatic cancer mainly originate from its unfavorable tumor biology (recently reviewed by Hidalgo (14)). Due to the central retroperitoneal localization of the pancreas, symptoms of the tumor disease are mostly unspecific and mimic diseases of other abdominal organs. In addition, they often manifest only in an advanced tumor stage. If not detected accidentally in the context of otherwise performed imaging of the abdomen, this often leads to a late diagnosis of the tumor disease when the primary tumor can no longer be surgically resected and/or has already spread to other parts of the body. Unfortunately, pancreatic cancer spreads early to lymph nodes and secondary organs (mainly liver, lung and bone marrow), excluding surgical treatment modalities and leaving only options of palliative care.

Although various palliative treatment protocols exist, their influence on the prognosis of PDA in matters of long-term survival so far is strongly limited by its complex and heterogeneous tumor microenvironment (Figure 4). PDA formation is characterized by a desmoplastic reaction in which pancreatic tissue is reorganized to a dense and highly fibrotic stroma. This stromal reconstruction, associated with an abnormal vasculature and the creation of an immunosuppressive microenvironment locally restraining antitumor immunity (14), seems to be involved with enhanced tumor progression and early metastasis (15). Progression

of other tumor diseases in the majority of cases depends upon an extensive tumor vascularization, supplying tumor growth with therefore required nutrients. In case of PDA, tumor cell survival and growth are adapted to its specific hypovascular and hypoxic microenvironment, rendering it mostly insensitive to antiangiogenic agents (16). Taken together, both desmoplastic stroma and poor vascularization of PDA form a substantial barrier for the effective delivery of any systemically applied cytotoxic agent.

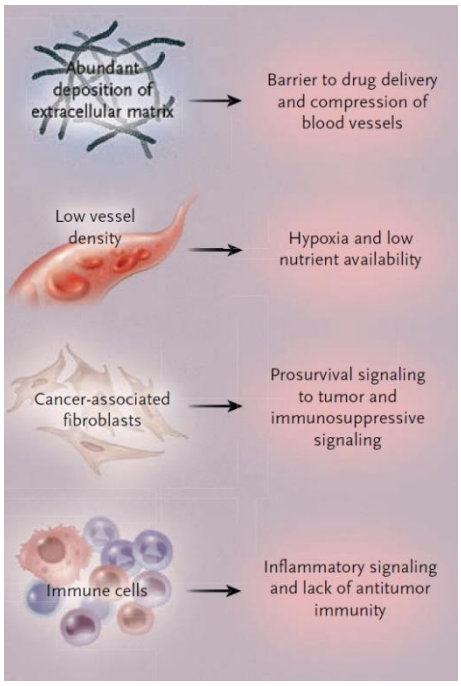

Figure 4 *Microenvironment of pancreatic adenocarcinoma. Taken and modified from Ryan et al. (8).*

PDA formation is known to be linked to a successive accumulation of gene mutations in an early stage of tumor development (17). Similar to the adenoma-to-carcinoma sequence in colon cancer, PDA typically derives from distinct precursor lesions such as Pancreatic Intraepithelial Neoplasia (PANIN) (8). Recurring intrinsic genetic mutations within the premalignant lesion ultimately result in the formation of PDA. The rapid progression of PDA is thereby based on its highly instable genome, leading to a constant process of ever-evolving tumor cell subpopulations not only in the primary but secondary tumor lesions as well. Key mutations, originating from the primary tumor and present in every tumor cell inde-

pendently of its respective localization, are paralleled by further genomic rearrangements creating a complex inter- and intratumoral genomic heterogeneity. De-novo-mutations in metastatic lesions not only render them genetically different from the primary tumor but also adapt their cellular metabolism to the tissue-specific microenvironment of the newly-affected organ. The individual tumor cell subpopulations thereby differ in their susceptibility to cytotoxic agents, leading to a natural selection of tumor cells with primary or secondary acquired resistance mechanisms in the course of antitumor treatment. Moreover, tumor cells with stem cell-like features for self-renewal and cell migration have been found to play an important role in tumor invasion and metastasis while exhibiting considerable primary resistance against standard chemotherapy protocols (18).

It seems logical that any attempt of complete tumor eradication without relapse of the tumor disease has to address the specific but highly heterogeneous tumor biology of pancreatic cancer. Current regimens of cytotoxic treatment (mainly represented by single or combined chemotherapeutic agents) so far fail in this regard and are therefore unable to effectively stabilize the tumor disease. Considering the devastating prognosis and depressing survival of patients with pancreatic cancer, new treatment protocols incorporating novel agents with distinct modes of action are desperately needed.

1.2 Oncolytic virotherapy

1.2.1 Basic principles

Oncolytic virotherapy represents one of the currently most promising novel treatment regimens, utilizing wild-type or genetically modified viruses for targeted antitumor therapy (19). Oncolytic viruses (OVs), as stated in the so-called oncolytic virotherapy paradigm (20), specifically target and infect tumor cells followed by exploitation of the host cell metabolism for efficient viral replication (Figure 5) (21). This process ultimately leads to massive tumor cell lysis (so-called oncolysis) and the release of progeny virus particles able to infect yet uninfected neighboring or distant tumor cells. Subsequent oncolytic cycles promote cumulative tumor eradication until no further tumor cells exist. Concomitantly the release of both viral as well as tumor antigens results in the induction of innate and adaptive immune

responses directed against the tumor cells even if they aren't infected by the OV. For this reason oncolytic virotherapy is believed not only to result in a potent tumor eradication in the short-term (within weeks or months) but also in the long-term immunotherapeutic process of arousing antitumor immunity and creating a lasting antitumor memory (22).

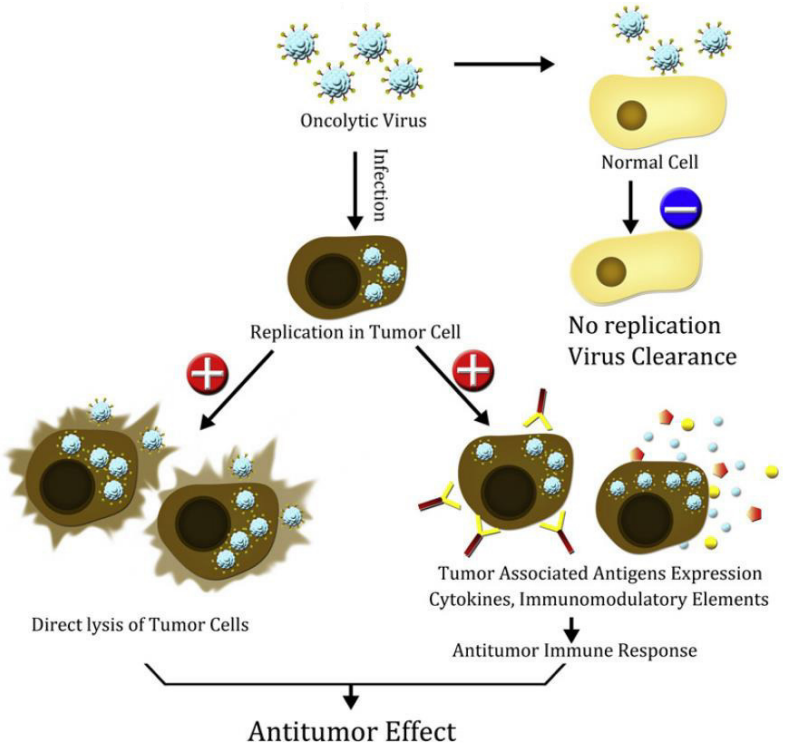

Figure 5 *Oncolytic virotherapy - Mechanism of action. Taken and modified from Xu et al. (21).*

To assure the specific targeting of tumor cells has been (and still is) one of the field's major challenges. Generally speaking tumor cells generate a local immunosuppressive microenvironment to circumvent tumor-directed immune responses. One of such mechanisms is the downregulation of interferon-dependent pathways, normally inducing a response of the innate and adaptive immune system. Since the interferon pathway also plays a key role in the defense of virus-infected cells, in return, such conditions facilitate viral infection and render tumor cells a favorable host for the viral life cycle (23).

Some naturally occurring viruses such as Reolysin® (Reovirus type 3 Dearing strain, ReoT3D) (recently reviewed by Clements et al. (24)) have an intrinsically

strong tropism for tumor cells. Others, such as adeno-, herpes-, measles- or vaccinia viruses have to be genetically modified to prevent infection and oncolysis of healthy tissues, the prerequisite for their safe clinical application with minimal toxicity. Basic strategies include the deletion of virus encoded virulence genes such as the E1B 55-kDa protein in the adenoviral mutants ONYX-015 (25) and H101 (26), their translational development marking an important milestone in the more recent history of oncolytic virotherapy.

In the course of adenoviral infection, the viral E1B protein prevents infected cells from undergoing apoptosis (a well-known mechanism of the antiviral host response) by inhibition of p53-regulated genes normally activating pro-apoptotic signaling pathways. Since in many tumor cells p53 is mutated, leading to aberrant and dysfunctional apoptotic pathways, E1B-deleted adenoviral mutants unable to overcome such physiologic countermeasures are constricted to the tumor microenvironment for their viral life cycle to take place. However, whether viral replication of ONYX-015 depends on the status of p53 exclusively remains controversial (27). Other strategies focus on the deletion of virus encoded enzymes such as herpes- or vaccinia virus encoded thymidine kinase needed to enlarge the intracellular nucleotide pool for sufficient viral replication (28,29). Rapidly proliferating tumor cells, which also require an adequate environment for excessive DNA replication, possess their own enzymatic facilities (often overexpressed cellular homologues of the virus encoded proteins) to satisfy their need for genetic raw material.

Those mechanisms were among the first to be developed and are exemplary for the understanding of how viruses can be modified to achieve tumor selectivity. Over the years many more strategies have been developed such as inserting foreign gene expression cassettes in nonessential gene loci (30) or placing the expression of essential viral genes under the control of tumor-specific promoter regions (31). Often different strategies have been combined to generate an even more tumor/replication-restricted OV. Generally speaking such modified OVs are not intrinsically selective for tumor cells but their ability to efficiently replicate in healthy tissues has been severely restrained.

But while safety concerns regarding the replication restriction of OVs have to be addressed in the process of successfully bringing oncolytic virotherapy from the bench to the bedside, the manipulation of genes essential for the viral life cycle may have its downside. Since the genetic backbone of naturally occurring viruses is adjusted for efficient replication while simultaneously circumventing antiviral host responses, along with the engineering of the viral genome to accomplish optimal tumor selectivity comes the attenuation of viral replication not only in normal but in tumor cells as well. Thus, achieving optimal efficacy of a selected viral vector is a process of delicate balance between reaching adequate tumor selectivity (dampening upcoming safety concerns) without attenuating viral replication as key element determining its oncolytic and immunotherapeutic potency (30).

1.2.2 History and development – What has been learned?

OVs have been part of clinical investigations for over a century now (32). Although knowledge about virus biology was greatly limited in the beginnings of virotherapeutic research, early case reports of tumor shrinkage in the course of natural virus infections promoted further investigations. Over the years, this led to ever evolving insights not only in the proceeding of OVs but also in basic characteristics of the human immune response and tumor biology itself. However, due to a lack of proper understanding and overambitious efforts disregarding vital safety concerns, at times this process was overshadowed by severe incidents, some of them flawing the faith in oncolytic virotherapy to this very day. Nevertheless, overwhelming progress has been made and groundbreaking achievements on genetic research in the 1990s have marked the beginning of a new era of virotherapeutic research, resulting for example in the construction of the very first recombinant OVs encoding marker and/or therapeutic transgenes.

Case reports of tumor remission during naturally occurring virus infections (the first dating back to the 19th century) mostly included patients with hematological malignancies. One of the most recent (from 1971) refers to the complete tumor regression of an 8-year old boy with Burkitt's lymphoma while showing clinical signs of a concomitant measles virus infection (33). In a follow-up examination 4

months after virus infection the boy remained tumor-free which exceeded previous reports of only short-lived tumor remissions (1-2 months). Ultimately, such reports led to the conclusion that some naturally occurring viruses possess intrinsic antitumor activity (especially in immunocompromised patients as is the case in patients suffering from hematological malignancies) and established the research on oncolytic virotherapy.

Though few clinical applications of naturally occurring viruses were already performed in the beginnings of the 20th century, back then basic knowledge about virus and tumor biology was almost nonexistent and their setup was rather highly experimental than science-based. For first groundbreaking achievements, the field had to wait until the 1950s when *ex vivo* culturing of human cells and rodent cancer models had just been developed. The first proof of principle of oncolytic virotherapy as a novel treatment regimen was thereby contributed by Alice Moore in 1949 when she was able to demonstrate that treatment of mouse sarcoma 180 with Russian Far East encephalitis virus led to complete tumor regression in some animals, if the viral agent had been given in sufficiently high enough doses (34,35).

More than 20 years later Teruo Asada successfully used non-attenuated mumps virus to treat a variety of tumor entities (36). Of 90 terminal cancer patients 37 were reported to respond drastically with complete or extensive tumor regression while observing only minimal toxicity. However, since established experimental models for high-dose virus production were still missing at that time, the used strains of mumps virus were obtained from several sources and unfortunately have been lost after the work was discontinued (20).

In the 1970s and 1980s the field of virotherapeutic research became rather quiet until being reignited by the development of recombinant DNA techniques in the 1990s, now enabling to address emerging safety concerns by focusing on the construction of attenuated and more tumor-selective OVs (32). The new technology also allowed the development of standardized cell culture systems from which the newly-constructed OVs could be obtained in much higher doses than before. Since then, the safety of OVs in the human host has been successfully

ascertained by a multitude of clinical trials employing OVs for the treatment of almost every tumor entity (37).

Among the first virus strains to be genetically 'optimized' were adeno- and herpesviruses, eventually culminating in the construction of ONYX-015 and its derivative variant H101, both being E1B 55-kDa deleted adenoviruses (the importance of the E1B protein during adenoviral infection has already been illustrated in section 1.2.1, p. 7). ONYX-015 soon reached phase III status but partly due to monetary reasons further clinical development of this agent was stopped in 2003. However, in 2005 the combination of H101 with the chemotherapeutic agents cisplatin and 5-fluorouracil (5-FU) received approval for the treatment of patients with head and neck cancer in the People's Republic of China (38) after previous phase II/III trials (39,40) had demonstrated an enhanced tumor response to the combination compared to standard chemotherapy with cisplatin and 5-FU alone. But as gratifying this development was for the field of oncolytic virotherapy, approval for this protocol in the western hemisphere still failed due to methodological flaws in collecting evidence for clinical benefit and enhanced overall survival (38).

1.2.3 Current state of virotherapeutic research – Present challenges and strategies

Over the last 30 years, due to enormous achievements in genetic recombination techniques the design of ever more refined OVs has been facilitated. Today, aiming at maximizing their oncolytic and immunotherapeutic potential while generating tumor/replication-restricted OVs, viral genomes can be sequenced and manipulated more easily than ever before. Nonetheless, clinical trials applying OVs in monotherapy in the majority of cases only have been able to report single case success (41) and after the initial approval of H101 in combination with cisplatin and 5-FU in the People's Republic of China for a long time no further virotherapeutic has found its way into clinical routine. Only now, as the first of its kind the GM-CSF expressing HSV mutant talimogene laherparepvec (T-VEC, IMLYGIC™) (42), has been approved by the FDA and the EMA for the treatment of metastatic melanoma (43). It is now up to this particular agent to prove therapeutic benefit of oncolytic immunotherapy in clinical routine, to dampen still prevailing safety

concerns and to pave the way for further oncolytic agents. Much work has yet to be done to ultimately establish oncolytic virotherapy at the bedside and to accomplish the lofty goal of finding a virotherapeutic cure for the masses of different tumor entities. As in the development and subsequent (pre-)clinical testing phase of any therapeutic agent, many emerging challenges had to and yet have to be faced, an ongoing process not only consuming enormous resources but also requiring a vast quantity of time.

Being biologic agents with a complex mode of action, the efficacy of oncolytic viruses depends on a multitude of parameters (Figure 6). Key factors especially determining the success of oncolytic virotherapy are believed to be the successful virus delivery to the tumor and highly efficient inter- and intratumoral spread after primary infection. Both issues have been addressed by the development of many strategies circumventing restraining host factors such as initially unfavorable antiviral innate and adaptive immune responses (the latter following vaccinations in the youth), virus sequestration in liver and spleen, neutralization by serum factors, deficient virus extravasation or a dense tumor stroma by arming OVs with costimulatory molecules, shielding them by using virus-infected cell carriers or applying them in combination with other drugs, e.g. chemotherapeutic compounds or novel checkpoint inhibitors (20). Additionally, high-throughput screenings have been conducted, aiming at finding new therapeutic targets, circumvent host cell response mechanisms and in consequence further refine oncolytic viruses, boosting their oncolytic potency (44).

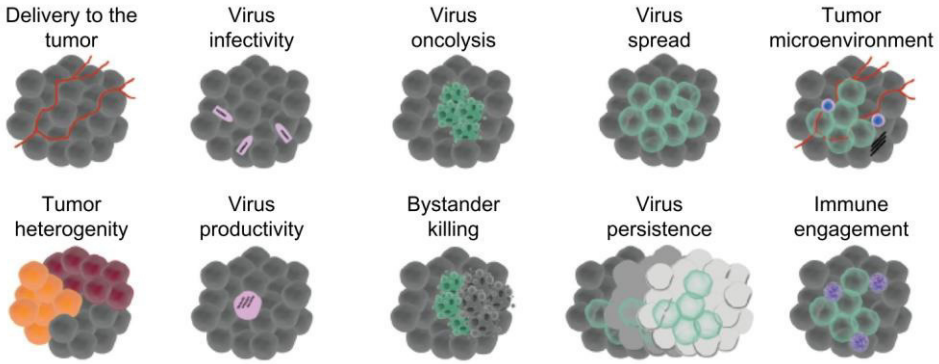

Figure 6 *Key factors affecting oncolytic virus therapeutic efficacy. Taken from Allan et al (44).*

However, oncolysis-mediated tumor debulking has proven to be insufficient in creating a lasting tumor response and significant benefit for patient survival. Accordingly, in recent years the focus has been broadened to likewise promote the aspect of oncolytic immunotherapy effectively inducing tumor-directed innate and, more importantly, adaptive immune responses. Recent results from OPTiM, a phase III trial of T-VEC, have revealed the immunological properties of oncolytic virotherapy for the first time in a larger patient cohort (n=291) (45). The intralesional application of T-VEC to patients with injectable advanced-stage melanoma (≥ stage IIIb) thereby resulted in durable responses (≥ 6 months) both in injected and uninjected lesions. Patients 'only' receiving subcutaneous GM-CSF served as comparison group in this trial.

Since i) T-VEC expresses the immunostimulatory molecule GM-CSF, ii) melanoma is known to be highly susceptible to immunotherapy and iii) durable responses were seen in uninjected lesions as well, it is highly probable that the therapeutic success of T-VEC depends on the successful activation of antitumor immunity. Of especial interest are also the facts that response rates were greater in patients receiving T-VEC as first-line therapy and that differences in overall survival between both treatment groups were higher in patients with skin, subcutaneous, or nodal disease only than in patients with metastases in the lung or other visceral organs.

Assured by these data, T-VEC has finally achieved FDA and EMA approval for the treatment of metastatic melanoma on October 27 and December 17, 2015, respectively (43). But although such results greatly encourage boosting the immunological properties of OVs to achieve a lasting immune-mediated tumor response in the long-term, an initially strong antiviral host response might impede primary viral infection and replication, the key for (immuno-)therapeutic success.

OVs are biological agents constantly changing their dose after primary infection. In the context of evaluating their safety as well as their infection and replication efficacy (especially under the influence of the host immune system) it has become instrumental to monitor viral spread after primary application by inserting different

reporter genes into their genetic backbone. Strategies thereby include virus encoded serum markers (46), the construction of light-emitting viruses (47,48) or radiologic strategies such as viral expression of the *thyroidal sodium iodide symporter (hNIS)* enabling the tumor-specific uptake of radioisotopes (49). Such tumor-imaging OVs represent both promising therapeutic agents which additionally can be used as a diagnostic tool not only in the beginnings after initiation of the virotherapeutic treatment but also in a more advanced state of cancer therapy. Therefore these OVs belong to a group of agents typically referred to as 'theranostics' (50).

Detecting virus particles after their systemic administration also seems to be crucial in consideration of reaching sufficient doses for efficient delivery to their site of action. Recent results from a phase I trial intravenously infusing the vaccinia virus JX-594 (Pexa-Vec) demonstrated that, similar to other systemic agents, successful viral extravasation and migration to the tumor is a dose-dependent process (51). In tumor biopsies collected 8-10 days after virotherapeutic treatment, intravenously infused JX-594 was only detectable when applied in doses of 10^9 infectious units or higher. This led to the conclusion that doses of systemically applied virotherapeutic agents have to exceed a 'viremic threshold' to successfully reach the tumor site.

High-dose applications of many OVs are primarily limited by so far insufficient manufacturing procedures being unable to yield extra high viral titers in most of the currently used virotherapeutic systems. However, since the field is progressing and more efficient fabrication processes are likely to be developed, reemerging safety issues have to be considered. Although the clinical tolerability of novel tumor/replication-restricted OVs has been ensured, extensively higher doses of a therapeutic agent are also supposed to result in a less favorable toxicity profile. Adverse effects typically seen shortly after virotherapeutic treatment are low-grade flu-like symptoms (including fever, chills, myalgia and/or asthenia). In the process of administering OVs in higher doses and/or in combination with other drugs, safeguard strategies like potent antiviral drugs have to be available to intercept excessive toxicity.

One of the biggest challenges of oncolytic virotherapy still remains the choice of the ‘right’ virus for distinct tumor indications. Although almost every OV can be genetically modified to replicate only in a certain tissue with maximal oncolytic efficacy, different virus families are defined by their distinct biological features. Based on basic parameters such as particle structure and life cycle, each OV is adapted intrinsically to a certain host range in which efficient infection, potent replication, strategies counteracting antiviral host responses, oncolysis and virus spread operate best. By ‘improving’ their genetic configuration each of these factors can be influenced, both positively and negatively, implying that for each tumor entity it has to be elucidated which OV may cause the best tumor response.

For that matter, current preclinical cell or animal models are still highly insufficient. Virus-induced immune responses can only be investigated in immunocompetent animals which render human tumor xenografts infeasible and therefore so far fail to simulate human conditions properly. In addition, some OVs (such as measles vaccine virus-based virotherapeutics) are constricted to the human host, making their preclinical evaluation only possible in cell culture or human xenografts in immunodeficient animals. Clinical trials, early phases I/II primarily designed to ensure the agent’s safety, are very time-consuming and costly. Furthermore, at the time at which a particular OV has finally reached phase III stadium (after several years) often, due to ‘simple’ genetic recombination, another - more promising - OV is already on its way.

Additionally, the importance of the route of administration of the respective virotherapeutics still remains obscure (52). Intratumoral injection of OVs probably reaches the highest oncolytic efficacy and typically is associated with lower rates of systemic toxicity. However, systemic application regimens are generally favored to also cover microscopic metastatic lesions now being accessible for direct virus-induced destruction. Although intratumorally injected OVs to a lesser extent will also spread over the body and a tumor-directed immune response might also eradicate distant tumor lesions, the high-dose systemic application of OVs promises to be more effective. In addition, only a subset of tumor entities can be reached successfully by intralesional injection. However, the efficacy of

intravenous spread depends upon different factors and, due to their specific virus biology, not all virus families may equally be suited for systemic infusion (53). Other possible application routes include intraperitoneal/intrapleural administration in case of peritoneal/pleural tumor spread or intrathecal delivery to achieve higher treatment doses for tumors of the central nervous system. To profit from the benefits of different administration routes combinations thereof also might be justified.

In conclusion, todays biggest challenges of oncolytic virotherapy indeed remain finding, constructing and further optimizing the 'right' viral agent for a distinct tumor indication before applying it via the most effective route of administration. Vice versa, in view of personalized medicine and complex immunologic heterogeneity in the human population, it may be of even more importance to select the OV on the basis of human host factors such as the 'right' genetic tumor cell and immune cell setting or a sufficient and therefore promising initial tumor response. However, despite intensive research over the last decades, mostly only limited (mono-)virotherapeutic success has been achieved so far. Supported by recent trial data it has become evident that new protocols such as combining OVs with other treatment regimens have to be developed to overcome their to date only limited clinical efficacy, finally aiding oncolytic virotherapy to unfold its whole potential of targeted antitumor therapy.

1.2.4 Chemovirotherapy

Usually the efficacy of monotherapeutic regimens is limited by an insufficient tumor response and the induction of resistance mechanisms against the applied agent (as illustrated more detailed for the case of pancreatic cancer in section 1.1.3, p. 5). Therefore, the combination of agents with distinct mechanisms of action promises to be highly effective not only in eradicating the initial tumor mass but also in preventing the induction of resistance mechanisms. Accordingly, the combination of OVs with chemotherapeutic agents (so-called chemovirotherapy) is of major interest. But before successfully applying theory into praxis, several parameters have to be considered in order to fully assess both possible benefits and threats of such a combined, i.e. chemovirotherapeutic, treatment.

Untargeted cytostatics, such as most of the currently clinically applied chemotherapeutic agents, affect every proliferating cell of the human body independently of their malignant potential. Since this mode of action is rather unspecific, the main barrier for their high-dose clinical application is their unfavorable toxicity profile. Based on the general idea that tumor cells naturally proliferate more strongly than their physiological counterparts, they are supposed to also be more affected by systemically applied chemotherapeutics. But while higher doses may result in a more profound response of the tumor cells, other fast proliferating cell populations such as mucous membranes and hematopoietic stem cells will be affected as well. Adverse effects such as anemia, leukopenia and mucositis are therefore typical consequences in the course of chemotherapeutic treatment regimens. Therefore, the important lesson was learned that individualized anticancer treatment not only includes choosing the 'right' agents but also to carefully balance their risk-benefit profile towards a more effective antitumor response while preserving an acceptable health-related quality of life.

Moreover, the cytotoxic effect of chemotherapy depends nearly entirely on operating cell death mechanisms such as apoptosis. By interfering with key functions of cellular metabolism, chemotherapeutics shift the delicate balance from anti- to pro-apoptotic signaling pathways ultimately leading to apoptotic cell death. But in the process of tumorigenesis, tumor cells, whose process of accumulating genetic alterations naturally constitutes a potent pro-apoptotic stimulus, often circumvent physiologic signaling pathways by overexpressing anti- and downregulating pro-apoptotic gene products. This severely impedes the success of chemotherapeutic treatment regimens and often makes is necessary to (i) apply high-dose chemotherapy (also involving high toxicity), (ii) combine different chemotherapeutic agents (mostly with overlapping toxicity profiles) or (iii) apply them in combination with other antitumor agents with different sites of action.

Replication-restricted OVs in contrast are engineered for their viral life cycle to take place in tumor cells only while impeding viral replication in healthy tissues. Shortly after viral infection, tumor cells are actively killed by oncolysis to release the progeny virus particles independently of physiologic cell death mechanisms.

In addition, the combination of immunogenic OVs and tumor antigen release is proposed to stimulate antitumor immunity. This kind of antitumor treatment therefore mostly faces immunologic resistance mechanisms such as interferon-mediated host cell responses (23) or immune cell-based resistance mechanisms such as tumor-associated granulocytic myeloid-derived suppressor cells (G-MDSCs) which induce a immunosuppressive tumor cell phenotype thereby limiting the desired immunotherapeutic effect (54). Tumor cells may also be intrinsically resistant against virotherapeutic treatment if the OVs can't properly infect and exploit their cellular metabolism for efficient replication. However, innate immune responses are supposed to be a more prominent primary resistance mechanism against oncolytic virotherapy which has to be overcome to reach sufficient virus doses at the tumor site. Adaptive immune responses on the other hand will be triggered in response to primary (and any subsequent) infection with the oncolytic agent. Though utilizing this response for arousing antitumor immunity is greatly desired, a too strong preliminary viral clearance may prevent tumor cell infection and viral spread and pose a highly potent acquired resistance mechanism, especially in the long-term of oncolytic virotherapy and particularly when the viral agent is applied at multiple times.

The mechanisms of action and resistance profiles of these two approaches chemo- and virotherapy are therefore entirely different, in fact considerably more dissimilar than those of different chemotherapeutics (whereupon multimodal chemotherapy protocols are widely used in the clinic). Additionally, due to the virus-mediated induction of antiviral host responses such as apoptosis, infected tumor cells even with dysfunctional cell death pathways are likely to be more prone to chemotherapy-induced cell death.

Virotherapy typically leads to flu-like symptoms (as illustrated previously in section 1.2.3, p. 12) whereas chemotherapy typically results in anemia, leucopenia and mucositis (as illustrated above). The condition that the toxicity profiles of both approaches don't overlap is therefore of utmost importance. This matter is further

affirmed by the fact that potential synergistic interactions between distinct therapeutic approaches not only would result in a more potent tumor response but also permit dose reductions of the applied agents to a less toxic degree.

Moreover, although virus-mediated oncolysis can result in a significant tumor reduction, the main effect of oncolytic virotherapy is believed to be a tumor-directed response of the innate and more importantly the adaptive immune system. However, this process requires time that cancer patients, being mostly diagnosed in advanced stages of the tumor disease, usually don't have. Harnessing the immunotherapeutic potential of oncolytic virotherapy therefore depends on an effective stabilization of the tumor disease in the first place. For most tumor entities chemotherapy represents the standard of care (both in the (neo-)adjuvant as well as in the palliative setting) and therefore provides the opportunity to gain the time required for slower, but long-lasting antitumor effects such as profound immune responses. Furthermore, curbing tumor cell growth in the short-term will likely be more successful when combining the cytotoxic effect of chemotherapeutics with the oncolytic effect of OVs.

Preclinical evidence for potential clinical benefit of the chemovirotherapeutic approach is overwhelming (55). OVs have been combined with a multitude of different chemotherapeutic agents and synergistic interactions with each of the mainly used virus families adenovirus (56), herpes simplex virus (57), reovirus (24) and vaccinia virus (58) (and other virus families as well) have been found. However, every genetic modification of a given oncolytic vector may profoundly influence its biology and for this reason also its interaction with chemotherapeutic agents. Therefore, every chemovirotherapeutic protocol, even if incorporating only a slightly dissimilar OV, has to be tested and its potential clinical benefit evaluated. Since with today's facilities the engineering of a new viral vector is a rather easy procedure, this poses a Sisyphus work and (pre-)clinical testing of all potential combinations will not be possible. Further understanding of the complex interactions between OVs and chemotherapeutic agents is therefore desperately needed.

The fact that chemotherapy represents the standard of care for many tumor entities also opens up new possibilities in the clinical setting. Given that replication-restricted OVs primarily had to ease upcoming safety concerns, trials so far mostly included patients at their end-stage of the disease (after all other treatment modalities had failed to achieve the desired tumor response). Accordingly, it has to be assumed that this subset of patients exhibits rather unfavorable conditions for oncolytic virotherapy to unfold its whole potential. Tumor cells having survived treatment with a plurality of different anticancer agents, have to be regarded as highly resistant, capable of evading cell death via a multitude of different mechanisms. Although OVs operate in a completely different manner than chemotherapeutics, it seems unlikely that such highly aberrant cells will be killed before they become resistant anew, this time against the applied OV as well. More importantly, the immune system of palliative cancer patients is likely to be enfeebled and might not have the ability to mount a strong immune response. Future clinical applications of OVs in combination with chemotherapeutic agents therefore provide the opportunity to address earlier tumor stages as well, i.e. in the setting of first-line chemovirotherapeutic regimens.

Based on the promising preclinical data an array of clinical trials incorporating chemovirotherapeutic protocols has already been started (59). The few clinical data which so far have been published thereby identified no enhanced toxicity after chemovirotherapeutic treatment (even when the agents were given concomitantly) and therefore validated the safety of the combinatorial approach. The currently most advanced OV in monotherapy, the herpes virus-based T-VEC, has recently also been combined with the checkpoint inhibitor ipilimumab. Preliminary results from a phase Ib trial in patients with advanced melanoma thereby have demonstrated an even stronger response to the chemovirotherapeutic combination than to either agent alone (60) (results from the phase III trial in monotherapy have been illustrated in section 1.2.3, p.12). The phase II part of this trial is currently ongoing (NCT01740297) and its results are eagerly awaited.

The first chemovirotherapeutic protocols which lately have reached phase III testing incorporated the naturally occurring reovirus Reolysin® and the GM-CSF expressing vaccinia virus JX-594. The combination of intravenously administered Reolysin® with carboplatin and paclitaxel in patients with head and neck cancer has recently been completed and was able to demonstrate a significant improvement of overall survival by the chemovirotherapeutic combination compared to the dual chemotherapy alone (61). However, published data of this study (designated as REO 018) are still awaited. The phase II combination of JX-594 with the oral multiple kinase inhibitor sorafenib in treatment-refractory patients with hepatocellular carcinoma (HCC) not only was found to be safe but also enhanced the therapeutic effect (62). These data led to the recent design of a phase III trial this time employing the chemovirotherapeutic combination as first-line treatment for patients with advanced HCC (NCT02562755) (63).

Combining OVs with established chemotherapeutic agents, preferably in a first-line chemovirotherapeutic treatment scenario, is therefore based on various good reasons. Not only has been proven that the two different approaches can interact synergistically in eradicating the tumor disease, but they might also provide enough time for a strong response of the adaptive immune system. Since chemotherapeutics already represent the standard of care in first- and second-line treatment protocols, their combination with OVs might finally establish oncolytic virotherapy at the clinical bedside and improve current treatment outcomes.

1.3 Virotherapy with the oncolytic vaccinia virus GLV-1h68

1.3.1 Structure and life cycle of vaccinia viruses

Vaccinia virus belongs to the family of poxviruses (*Poxviridae*). Due to its intertwined history with the smallpox causing variola virus during smallpox eradication, it is also its most extensively investigated representative (64). Accordingly, today's understandings about poxvirus biology are mainly based on the research on vaccinia virus.

The viral particle (virion) of vaccinia virus consists of a linear double-stranded DNA molecule enveloped by different numbers of lipid bilayer membranes (Figure 7). Its entire life cycle takes place in the host cell's cytoplasm and thereby mostly

relies on its own encoded proteins. Along with many other mechanisms, the resulting minimal interaction with proteins of the host cell enables vaccinia virus to infect a wide range of tissues while simultaneously avoiding antiviral host responses. Unlike variola virus, vaccinia virus is not restricted to the human host but can also infect some animals.

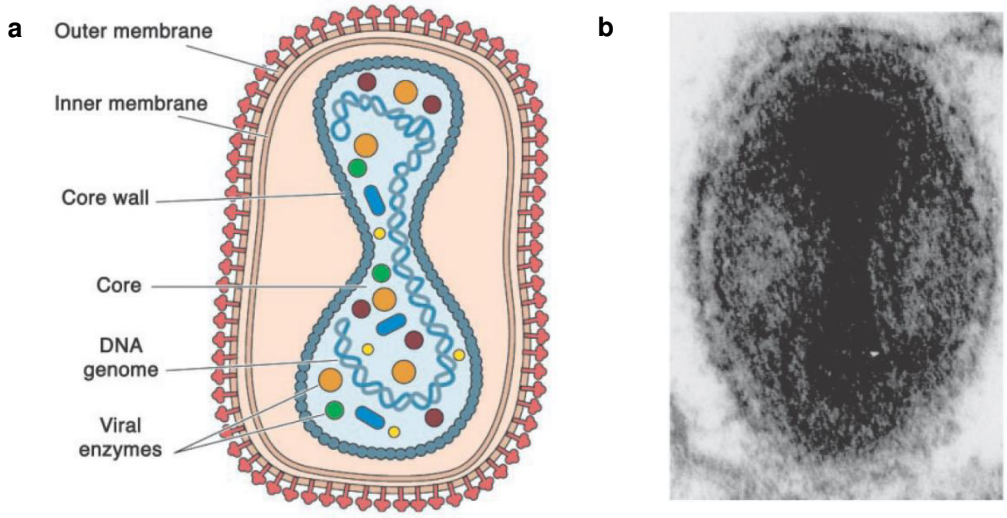

Figure 7 *Virion structure of vaccinia virus (a) schematic and (b) microscopic. Taken and modified from Harrison et al. (64)*

The exact process of how vaccinia virus enters the host cell still is not well understood. For cell entry vaccinia virus thereby seems to exploit endocytotic mechanisms of its target cells (65). However, in contrast to adenovirus or measles virus which, if not specifically engineered otherwise, utilize receptors such as CAR (coxsackie and adenovirus receptor) or CD46 for their cell entry, no such receptor has yet been found for vaccinia virus. Further understanding is additionally hindered by the fact that vaccinia virus produces three forms of infectious particles: intracellular mature virus (IMV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV).

Newly produced virions first form IMV particles (depicted in Figure 7a) which mainly remain in the host cell until lysis. However, a subset of IMVs dissociate from their host cell into the local vasculature now spreading as EEVs over the body. CEVs in contrast penetrate surrounding cells and enable direct cell-to-cell spread of vaccinia virus. In the process of exiting the host cell both CEVs and EEVs acquire an additional lipoprotein bilayer that surrounds the IMV particle. In

case of EEV this outer envelope is equipped with complement control proteins protecting the EEV from complement activation (66).

Those three forms, IMV, CEV and EEV, not only are constructed differently but also fulfill different tasks in the strategy of vaccinia virus to reach maximal viral spread. IMVs represent by far the majority of formed virions but are only released in the final stage of virus infection when the host cell undergoes virus-induced lysis. CEVs and EEVs are released much earlier and are therefore essential for rapid short- and long-range virus spread. Although EEVs constitute only a minor fraction of formed virus particles (< 10 %), vaccinia mutants unable to produce them in sufficient numbers were shown to be significantly attenuated by inefficient virus dissemination (67).

1.3.2 Discovery of vaccinia virus and its role during smallpox eradication

Vaccinia virus also represents the virus with the longest history of clinical use in humans (68). In 1798, the English doctor Edward Jenner was able to show that people who had previously suffered from cowpox were resistant to the similar but significantly worse smallpox disease with a lethal outcome of up to 40 %. In his case study describing the first successful vaccination, Jenner gathered pus from a cowpox infected milkmaid and injected it into the arm of an 8-year old boy who subsequently developed light symptoms of the disease (69). After a second injection 1.5 months later, this time with pus from a smallpox patient, the boy remained healthy even when being rechallenged with smallpox several months later. With this experiment, Jenner unknowingly was able to show for the first time that infection with a similar but less virulent pathogen can immunize against the more virulent disease and create a lasting immunological memory. Appropriately the term ‘vaccination’ originates from ‘*vacca’* the latin word for cow. Edward Jenner and Louis Pasteur who later described the underlying mechanisms of this process are therefore often recognized as the ‘fathers of immunology’ (70).

Word about vaccination spread fast in the Western hemisphere which led to its frequent use during the next century (68). However, in the beginnings of the 20th century when basic knowledge about virus biology just had started to emerge, it

was noticed that the virus strain used at that time differed from the original cowpox virus. Due to its favorably milder vaccination reaction this now called 'vaccinia virus' was further used for subsequent smallpox vaccination though its exact origin remains obscure. In the following years, owing to the global usage of vaccinia virus in smallpox vaccination and laboratory research, different new virus strains evolved 'locally' such as the *Western Reserve*, *Wyeth*, *Copenhagen* or *Lister* strains.

Clinical use of vaccinia virus finally culminated in the global Smallpox Eradication Program (SEP) (71). Lasting from 1966 to 1980 the program addressed especially regions in Africa and Asia where smallpox was still endemic at that time. At its end in 1980 the World Health Organization (WHO) declared smallpox finally being eradicated globally. Today stocks of variola virus still remain in the US and Russia, though - unknowingly - further stocks might be stashed elsewhere (64).

1.3.3 Rationale for using vaccinia virus in oncolytic virotherapy

Primarily used as potent vaccine, in recent years the application range of vaccinia virus has broadened. Being extensively studied, vaccinia virus exhibits several unique features that make it a promising virotherapeutic agent (72).

For starters vaccinia virus is known for its wide host range not only in humans but animals as well. Being able to infect almost every tissue allows for a wide-spread clinical use against many tumor entities and suggests efficacy across tumor cell subpopulations with distinct genetic configurations. It also facilitates indispensable preclinical testing since established animal models can be easily used without viral constraint to the human host. Moreover, compared to other OVs vaccinia virus infection has the advantage of being independent of the cellular expression of viral entry receptors.

Secondly, replication and viral spread of vaccinia virus are performed both rapidly and highly efficiently. The entire viral life cycle, from the moment of infecting the host cell until its lysis, lasts only 24 hours und culminates in the release of a vast amount of infectious particles. Viral spread is maximized by the formation of 3 different infectious particles that spread and enter host cells differently. CEV and

EEV, responsible for efficient short- and long-range spread, are released even earlier than IMV approximately 6 hours after infection and additionally accelerate virus dissemination (68).

Another favorable property of vaccinia virus is its large genome of approx. 192 kb that can additionally accommodate foreign DNA sequences of at least 25 kb (73). By comparison, other OVs such as oncolytic adenoviruses can accommodate considerably less foreign DNA. This renders vaccinia virus a suitable gene vector for the insertion of reporter genes, tumor-associated antigens, therapeutic transgenes or immunomodulatory molecules. As a result, many vaccinia-based gene vector systems encoding a multitude of transgenes have been developed (58).

Since vaccinia virus encodes its own enzymatic facilities, no integration of the viral genome into the host cell's genome takes place. Moreover, viral replication is localized in cytosolic 'virus factories' independent of the host cell's nucleus (64). Vaccinia virus therefore not only represents a potent stand-alone virotherapeutic but also interacts minimally with the host cell genome, both being preferable prerequisites for its safe clinical application.

Due to its successful use as vaccine against smallpox, vaccinia virus is also known to be highly immunogenic (74). It would be even more so if it did not encode several proteins to circumvent antiviral host responses (75,76). The deletion of virulence genes in the quest of engineering a tumor-selective vaccinia-based agent therefore might also augment its immunological potential. Since OVs are mainly believed to be immunotherapeutics in nature, using vaccinia virus for the treatment of cancer is of great interest (58). Thus, it is hardly surprising that the currently clinically most advanced vaccinia virus JX-594, a derivative of the *Wyeth* strain, encodes the proinflammatory cytokine GM-CSF (77).

Only recently a phase II trial employing JX-594 to patients with advanced hepatocellular carcinoma (HCC) was completed (results from another phase II trial of JX-594 in combination with sorafenib have been illustrated in section 1.2.4, p. 17). Intralesionally infused JX-594 thereby was shown to induce a polyclonal humoral

immune response leading to antibody-mediated complement-dependent cytotoxicity (78). Moreover, tumor responses were seen both in injected and non-injected distant tumor lesions. Additionally, the baseline presence/absence of neutralizing antibodies against vaccinia virus (possibly impeding viral replication and spread) did not correlate with therapeutic efficacy. The most crucial parameter determining therapeutic benefit was rather the applied viral dose of JX-594, overall survival thereby ranging from 13.6 months (high-dose group) to 4.3 months (low-dose group).

In terms of safety no other virus has been longer in clinical use or more extensively been studied than vaccinia virus (68). Adverse effects following vaccination were typically mild and included local pain at the injection site as well as mild flu-like symptoms such as headache, myalgia, chills, nausea and fatigue (79). Moderate or severe complications were extremely rare (1-250 / million primary vaccinations) and included eczema vaccinatum, generalized or progressive vaccinia, myopericarditis or encephalitis. For this reason, application of vaccinia virus in humans so far has been remarkably safe. Furthermore, for the unlikely case of uncontrolled virus infection a number of different antiviral agents have already been developed (64). Currently the only approved agent is intravenous vaccinia immune globuline (VIGIV) (80) while the nucleotide analogue cidofovir (81) is reserved for second-line anti-vaccinia therapy. Other agents such as the egress inhibitor tecovirimat (aka ST-246) (82) and certain tyrosine kinase inhibitors (83) are indeed rather experimental but have shown their efficacy against vaccinia virus. Of note, this includes the inhibitory effect of sorafenib on the replication of vaccinia viruses leading to the necessity for a sequential application of first sorafenib and then (after a 14 day pause) the onset of vaccinia virotherapy (84).

In summary vaccinia virus is not only able to infect almost any cell type but also to transport and express large amounts of foreign genetic material while being independent of host cell nuclear transcription processes. Extensive clinical experience has certified its safe application and for the rare case of excessive toxicity potent antivirals are ready to hand. Future clinical applications of vaccinia virus

should mainly focus on harnessing its oncolytic but even more its immunotherapeutic potential to prove real benefit of this agent in anticancer therapy.

1.3.4 Construction of GLV-1h68

The oncolytic vaccinia virus GLV-1h68 (aka GL-ONC1) used as virotherapeutic agent in this thesis has previously been constructed by insertion of three expression cassettes in nonessential gene loci of the parental *Lister* strain vaccinia virus (Figure 8) (46):

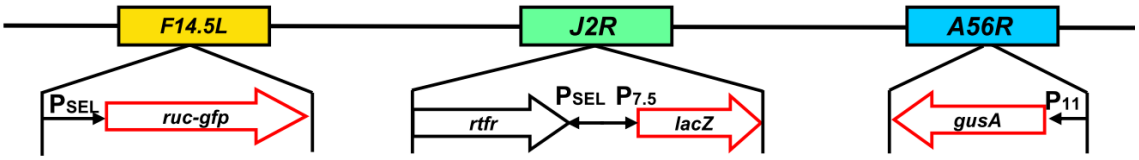

Figure 8 *Genetic construct of GLV-1h68. Taken and modified from Zhang et al. (46).*

- F14.5L locus → expression cassette encoding *Renilla luciferase-Aequorea green fluorescent protein fusion* (GFP) *[ruc-gfp]*
- J2R locus (encoding viral thymidine kinase) → expression cassette encoding *β-galactosidase* (*β-gal*) *[lacZ]*
- A56R locus (encoding viral hemagglutinin) → expression cassette encoding *β-glucuronidase (β-gluc) [gusA]*

Inactivation of the well-known genes for thymidine kinase (its importance during viral infection has already been illustrated in section 1.2.1, p. 7) and hemagglutinin as well as the less studied F14.5L gene product led to considerable viral attenuation in breast tumor-bearing nude mice whereas virus colonization was largely restricted to tumor tissues. In comparison to the parental *Lister* strain virus and single- or double-mutant derivatives, not only the level of attenuation increased with each gene inactivation (30) but also its oncolytic potency (46). Additionally, it was assumed that, due to a higher translational burden, the insertion of foreign gene expression cassettes into nonessential gene loci attenuated the OV even more than simply disrupting the corresponding virulence genes (85). Infection with GLV-1h68 *in vivo* also led to a latent upregulation of genes mostly matching functions of the innate immune response (46). It was therefore hypoth-

esized that this GLV-1h68-mediated activation of the immune system is a response to potent viral replication, steadily cumulating oncolysis and the concurrent release of proinflammatory molecules.

Due to the expression of serum and tissue markers (*β-gluc, β-gal*) as well as its capability of luminescent and fluorescent light emission (GFP), GLV-1h68 unifies both therapeutic and diagnostic properties enabling real-time monitoring of its therapeutic efficacy (86) (more detailed information about 'theranostics' are specified in section 1.2.3, p. 12). Initial treatment with GLV-1h68 was highly efficient and led to complete tumor elimination in over 95 % of breast tumor-bearing nude mice which was linked to 100 % survival at the end of the study (130 days post infection) (46). In addition, tumor regression was paralleled by gradual extinction of GFP fluorescence and decreasing β-galactosidase activity, as shown by immunohistochemical staining of tumor sections. 56 days after virus injection, along with complete tumor remission in most of the mice, neither GFP expression nor β-galactosidase could be detected.

1.3.5 Virotherapeutic research with GLV-1h68

Based on these promising initial results a multitude of further investigations on GLV-1h68 and its potential role in future virotherapeutic application regimens has been conducted. Screening the NCI-60 cancer cell lines for their susceptibility to GLV-1h68 infection has shown that GLV-1h68 is able to efficiently infect and replicate within a wide range of tumor tissues (87). Generally, viral infection seemed to be favored by overexpression of cellular components involved in cell movement and adhesion as well as the tumor cell-mediated suppression of the innate immune response. GFP expression of GLV-1h68 correlated positively with viral replication and was not tumor- but rather cell line-dependent. Furthermore, GLV-1h68 application has been confirmed to be safe and highly effective in different animal models of human cancer (46,85,88-105).

Continuing research on GLV-1h68 also gave further insights into the mechanisms determining therapeutic success of this particular agent. Although GLV-1h68 exhibits anti-vascular (89) and proinflammatory properties (90-92,106), a strong viral replication of GLV-1h68 seems to be the most crucial predictive marker for

potent antitumor efficacy (46,85,89,90,92-94,107). However, the degree to which the different interactions contribute to the oncolytic potency of GLV-1h68 was demonstrated to depend on the particular tumor microenvironment (89).

Strong viral replication of GLV-1h68 was also shown to be essential for the activation of the innate immune system. In nude mice GLV-1h68 shifted the chronic inflammation process of rejecting the human xenograft to an acute and destructive one (92). In another immunocompromised model tumor remission after intravenous injection of the viral agent was accompanied by high numbers of macrophages and NK cells at the tumor site (90). GLV-1h68 induced intratumoral infiltration of myeloid cells could also be detected by ^{19}F-magnetic resonance imaging (MRI) (108).

However, responses of the innate immune system are mostly unspecific and, in one case, were shown to be directed against GLV-1h68 and not against the tumor tissue (93). Since the adaptive immune system also possesses antiviral properties and modulates the activity of innate immune responses, it is likely to affect replication and spread of GLV-1h68 as well. Nevertheless, proof for its beneficial or detrimental effect on the therapeutic efficacy of GLV-1h68 from human patients or immunocompetent animal models is still missing.

Additionally, GLV-1h68 was shown to be able to eradicate tumor cells with stem-cell like features (95) as well as preferentially colonizing lymph node metastases in a prostate cancer model in nude mice (96). The latter has been contributed to an increased vascular permeability and a higher number of immune cells in lymph node metastases in comparison to the primary tumor. Successful tracking of lymph node metastases by GLV-1h68 has also been shown in an immunocompetent animal model (97). In this regard, localized virus-mediated GFP expression not only is of essential prognostic relevance but also enables real-time detection of affected lymph nodes during surgery. To enhance viral delivery, GLV-1h68 has been shielded with silk-elastin-like protein polymers (SELPs) in a mouse model of incomplete tumor resection (98). Shielding of the viral particles resulted in an enhanced transgene expression and increased tumor shrinkage of the residual tumor cells. Therefore, the administration of GLV-1h68 to the

postsurgical resection bed was shown to be another potential use of this viral agent.

In vitro, markers of early gene expression like β-gal, luciferase and GFP expression correlated well with the susceptibility of different tumor cell lines to GLV-1h68 mediated oncolysis (99,106). This may be of significant prognostic value for predicting its therapeutic potency *in vivo*. But whether high susceptibility to GLV-1h68 *in vitro* is associated with high oncolytic efficacy in animal models or human patients remains controversial. *Ex vivo* assays measuring early gene products of GLV-1h68 indeed have been proposed as being useful in predicting the therapeutic success in an animal model (100). Additionally, the comparison of GLV-1h68 with some less-attenuated and therefore more strongly replicating derivatives has demonstrated a clear correlation between enhanced viral replication *in vitro* and more rapid tumor shrinkage *in vivo* (85). However, although the less-attenuated derivatives caused an increased tumor response, in some of the cases this came at the price of higher toxicity and lesser survival of the mice. In another study two pancreatic cell lines differing in their susceptibility to GLV-1h68 mediated oncolysis *in vitro* were found to respond similarly *in vivo* (101). On the contrary, some well responding cell lines *in vitro* demonstrated less therapeutic efficacy *in vivo* which was associated with lower viral titers (92). In summary, these data clearly suggest that *in vivo* additional factors beyond the baseline genetic configuration of tumor cells influence viral replication and treatment outcome.

Human genes possibly predicting the effective viral replication of vaccinia-based agents in the human host have already been found (109). However, in view of future 'personalized' clinical applications of GLV-1h68 it would not only be essential to include tumor patients with the 'right' genetic setting but also to evaluate as early as possible whether the virotherapeutic treatment is beneficial and results in the desired tumor response. In this regard, the monitoring of viral replication and therapeutic efficacy by measuring viral expression of β-gal, β-gluc, luminescence and fluorescence seems to be crucial to distinguish responders from non-responders.

To refine its therapeutic and diagnostic properties GLV-1h68 has also served as parental virus for the development of new vaccinia virus constructs. Similarly to the previously constructed measles virus encoding the *human sodium iodide symporter* (MV-NIS) (49) the oncolytic vaccinia virus GLV-1h153 has been constructed by replacing the β-glucuronidase encoding *gusA* cassette of GLV-1h68 with a *hNIS* gene cassette (110). Likewise, another derivative, GLV-1h99, was constructed by replacing the *ruc-gfp* gene cassette with a gene cassette containing the *human norepinephrine transporter gene (hNET)* (111). Due to the tumor-specific uptake of different radiotracers, detectable by imaging modalities such as positron emission tomography, both GLV-1h99 and GLV-1h153 enable deep-tissue-imaging of tumors while monitoring viral spread and therapeutic efficacy (112,113). Other constructs encoding the anti-VEGF single chain antibody *GLAF-1* (114) or the essential cell cycle protein *Cdc6* (115) have demonstrated the therapeutic benefit of inserting therapeutic transgenes. Moreover, the hyper-IL-6 expressing GLV-1h90 was able to additionally reduce chemotherapy-induced thrombocytopenia in nude mice while remaining as potent as its parental virus GLV-1h68 (116).

GLV-1h68 has also been successfully combined with other treatment regimens. Radiation has been shown to sensitize tumor cells for subsequent GLV-1h68 treatment in human melanoma and glioma xenografts in nude mice (94,102) as well as enhancing the levels of apoptotic cell death in sarcoma cell lines when given adjuvantly (117). Combination of GLV-1h68 with cyclophosphamide resulted in an enhanced tumor growth inhibition compared to treatment with either agent alone (118). Utilizing GLV-1h68 mediated expression of β-galactosidase to convert a prodrug *seco*-analogue of the cytotoxic antibiotic duocarmycin SA to its toxic compound led to the induction of apoptosis and enhanced oncolysis (119). Moreover, GLV-1h68 has been combined successfully with chemotherapy applied via isolated limb perfusion, radiation and surgery in a orthotopic model of advanced extremity sarcoma (120).

Of particular importance for this thesis was the combination of GLV-1h68 with the chemotherapeutic agents cisplatin or gemcitabine in 2 different cell lines of human pancreatic adenocarcinoma *in vitro* and *in vivo* (101). Both combination protocols resulted in an acceleration of tumor shrinkage and increased therapeutic efficacy, though treatment with GLV-1h68 alone was able to cause the same tumor response as the gemcitabine combination group when only given sufficient time.

Based on these data, few clinical trials incorporating GLV-1h68 (referred to as GL-ONC1 when used in clinical trials) have been performed. In patients with peritoneal carcinomatosis intraperitoneal infusion of GLV-1h68 not only was shown to be safe and well tolerated but to result in a dramatic decline of tumor cells in the ascitic fluid (121). Another phase I trial investigating the combination of GLV-1h68 with the platinum compound cisplatin and radiation in patients with locoregional advanced head and neck cancer has recently been completed. According to preliminary data the triple combination was found to be safe and a follow-up phase II trial is planned (122). Final data of this study are still pending. Currently, clinical trials employing GLV-1h68 in patients with ovarian cancer (NCT02759588) or malignant pleural effusion (NCT01766739) and its combination with the complement inhibitor eculizumab (NCT02714374) are under way.

In conclusion, GLV-1h68 application in humans or tumor-bearing animals so far has been safe and highly efficient while viral replication was constrained to tumor tissues. Therapeutic efficacy could be measured non-invasively and correlated well with the decrease of the inserted reporter genes. However, (pre-)clinical results from combination protocols with chemotherapy and/or radiation have already indicated the additional therapeutic value of combining GLV-1h68 with other treatment regimens.

1.4 Objectives

Due to its unfavorable tumor biology, pancreatic adenocarcinoma is one of the deadliest types of cancer. Unfortunately, pancreatic ductal adenocarcinoma (PDA) is mostly diagnosed in a locally advanced or metastatic stage when curative surgical treatment is no longer possible and current treatment regimens in

form of multimodal chemotherapy protocols can't effectively stabilize the tumor disease. Key determinants that contribute to its considerable primary and secondary resistance against chemotherapeutic treatment are mostly its i) genetic instability (generating a manifold of genetically different tumor cell subpopulations), ii) desmoplastic stroma (impeding any systemic treatment modalities) and iii) local immunosuppressive microenvironment.

Oncolytic virotherapy on the other side is assumed to benefit from such conditions. Tumor cells with highly aberrant and dysfunctional cell-death pathways are suitable host cells for oncolytic viruses (OVs) that are engineered for being unable to overcome physiologic antiviral host responses. Furthermore, tumor-mediated immunosuppression not only favors initial viral infection and intratumoral spread but also represents a therapeutic target that might finally be overridden by the immunotherapeutic properties of oncolytic virotherapy.

Accordingly, pancreatic cancer has been the focus of many (pre-)clinical investigations, most thereof employing monovirotherapeutic regimens (21,123). Since recent data indicated therapeutic benefit of chemovirotherapy, oncolytic virotherapy preferably being an add-on component to already established treatment modalities, pancreatic cancer has also been treated with different (mainly gemcitabine-based) combination protocols in the preclinical setting (101,124-137). However, current chemovirotherapeutic trials only incorporate adeno- or reovirus-based regimens (59).

Here, in this thesis, the oncolytic vaccinia virus GLV-1h68 was tested in combination with various chemotherapeutic compounds on 4 well-characterized cell lines of pancreatic adenocarcinoma. This *in vitro* investigation aimed at providing a first preclinical basis for future chemovirotherapeutic regimens putatively improving the treatment outcome of PDA.

Initially, it had to be evaluated whether the pancreatic cancer cell lines differ in their susceptibility to different chemotherapeutic agents (such as standard of care gemcitabine and others) or the virotherapeutic agent GLV-1h68 in monotherapy and to which degree resistance is already preexistent.

Based on these data distinct chemovirotherapeutic protocols had to be devised in which the different agents were administered in tumor cell line-adjusted subtherapeutic doses. Primary aim of these protocols was to overcome any therapy-impeding resistance and achieve therapeutic benefit by inducing a more potent antitumor response. Of especial interest were so far uninvestigated chemovirotherapeutic combinations such as GLV-1h68 applied together with the recently approved dual chemotherapy *nab*-paclitaxel + gemcitabine.

Since it was speculated that success of any combination therapy not only depends on the applied agents but also their dose and administration sequence, the influence of these parameters on the particular chemovirotherapeutic protocols and their therapeutic benefit had to be assessed as well. Thus, the combination partners had to be administered either concurrently or one of the agents had to be applied first while the other was delayed. In line with this consideration it was of interest whether a specific application setting could be identified which would lead to an enhanced cytotoxicity in all of the tested tumor cell lines.

Additionally, it was assumed that a potent viral replication would constitute an important prerequisite for a strong immunotherapeutic effect. Since chemotherapeutics are known to potentially influence the viral life cycle, potential chemotherapy-mediated reductions of viral replication had to be investigated in any chemovirotherapeutic protocol. Accordingly, the monitoring of viral titers and of viral gene expression under the influence of chemotherapy constituted another vital part of this investigation.

In the end, these investigations aimed at developing novel highly potent GLV-1h68-based chemovirotherapeutic protocols which then had to be tested in further work in animal models as well as later on in clinical trials.

2 Material and Methods

2.1 Material

2.1.1 Consumables

Cell scrapers	Corning Inc.
CELLSTAR® Conical-bottom tube 15 ml	Greiner Bio One
CELLSTAR® Conical-bottom tube 50 ml	Greiner Bio One
Combitips 2.5 ml, 12.5 ml	Eppendorf
Cryotubes 1ml	Corning Inc.
Pasteur pipettes, 230 mm long size	WU Mainz
Pipettes 5 ml, 10 ml, 25 ml, 50 ml	Corning Inc.
Pipette tips 10 µl, 100 µl, 200 µl, 1000 µl	Biozym / Peqlab
Reaction tubes 1.5 ml, 2.0 ml	Eppendorf
Reaction tubes 1.5 ml, 2.0 ml (amber)	Eppendorf
Tissue culture flask 75 cm^2	Greiner Bio One
Tissue culture plate 24 well	TPP
Tissue culture plate 96 well	TPP / Corning Inc.

2.1.2 Chemicals

5-fluorouracil (stored light-protected)	Pharmaceutical Department, University Hospital Tübingen
Acetic Acid	Merck KGaA
CellTiter-Blue® (CTB) Reagent	Promega
CMC	Sigma-Aldrich
Crystal violet	Carl Roth
Descosept	Dr. Schuhmacher GmbH
DMSO	AppliChem
Erlotinib	ChemieTek
Gemcitabine	LC Laboratories
Hydrochloric Acid	Merck KGaA
Hydrochloric Acid (fuming)	Merck KGaA
Irinotecan	Sigma-Aldrich
Isopropanol (70 %)	SAV Liquid Production
Nab-paclitaxel	Pharmaceutical Department, University Hospital Tübingen
Oxaliplatin	Sigma-Aldrich
Sekusept® Extra N	ECOLAB Healthcare
Sulforhodamine B (SRB)	Sigma-Aldrich
Thyazolyl BlueTetrazolium Bromide (MTT)	Sigma-Aldrich
Trichloroacetic acid	Carl Roth
TRIS	Carl Roth
Trypan blue	Sigma-Aldrich

2.1.3 Media, Sera and Buffer

DMEM	Biochrom
DMEM (colorless)	Biochrom
Gibco® Antibiotic-Antimycotic (penicillin, streptomycin, amphotericin B)	Life Technologies
Gibco® Fetal Calf Serum	Life Technologies
PBS (cell culture use)	PAA Laboratories
RPMI 1640 (colorless)	PAA Laboratories
Trypsin/EDTA	Lonza

Self-prepared solutions:

CMC solution	DMEM	500 ml
	FCS	25 ml
	CMC	7.5 g
	AB/AM	5 ml
DMEM + 2 % FCS (infection medium)	DMEM	500 ml
	FCS	10 ml
DMEM + 10 % FCS (growth medium)	DMEM	500 ml
	FCS	50 ml
MTT dye (stored light-protected)	MTT	2.5 mg/ml (1.25 g)
	RPMI (colorless)	500 ml
MTT Solubilization/Stop Solution	HCl (37 %)	10 ml
	Isopropanol	90 ml
PBS (non cell culture use)	NaCl	137 mM (8 g)
	KCl	2.7 mM (0.2 g)
	Na_2HPO_4	10 mM (1.44 g)
	KH_2PO_4	1.8 mM (0.24 g)
	H_2O_{dd}	filled up to 1 l
SRB dye (0.4 % in 1 % acetic acid)	SRB	4 g
	Acetic acid	10 ml
	H_2O_{dd}	filled up to 1 l
TCA solution (10 %)	TCA	100 g
	H_2O_{dd}	filled up to 1 l
Tris base	TRIS	10 mM (1.21 g)
	H_2O_{dd}	filled up to 1 l
	pH	10.5

2.1.4 Cell lines

AsPc-1	Human pancreatic adenocarcinoma cells. Taken from the ascites of a 62-year-old woman with metastatic adenocarcinoma in the head of the pancreas. Producing both abundant mucin and carcinoembryonic antigen (CEA). Purchased from Sigma-Aldrich.
BxPc-3	Human pancreatic adenocarcinoma cells. Taken directly from the primary tumor of a 61-year-old woman with adenocarcinoma in the body of the pancreas. Producing CEA, human pancreas cancer-associated antigen (PCAA), human pancreas-specific antigen and traces of mucin. Purchased from ATCC.
MIA PaCa-2	Human pancreatic adenocarcinoma cells. Taken directly from the primary tumor of a 65-year-old man with adenocarcinoma in the body and tail of the pancreas infiltrating the periaortic area. Purchased from ATCC.
Panc-1	Human pancreatic adenocarcinoma cells. Taken directly from the primary tumor of a 56-year-old man with a metastatic adenocarcinoma in the head of the pancreas invading the duodenal wall. Purchased from Sigma-Aldrich.
CV-1	African green monkey kidney cells. CV-1 cells were provided by the Genelux Corp.

2.1.5 Oncolytic virus

GLV-1h68 (aka GL-ONC1)	Genelux Corp.

2.1.6 Laboratory Equipment

Assistent® Hemocytometer	Glaswarenfabrik Karl Hecht
Autoclave 3850 EL	Systec
Branson Sonifier S-450A analog ultrasonic cell disruptor	Emerson Electric Company
Centrifuge	Eppendorf / Heraeus
CK40 phase contrast inverted microscope	Olympus
F-View II FireWire fluorescence camera	Soft Imaging System
Fluorescence microscope IX50	Olympus

Freezer (-20 °C, -80 °C, -145 °C)	Liebherr
GENios Plus Multifunction Fluorescence Microplate Reader	Tecan
HandyStep® S	Brand
Incubator	Heraeus / INTEGRA / Memmert
HERAsafe® Laminar Flow Workbench	Thermo Electron Corp.
Multichannel pipette	Eppendorf
Multitemp II Thermostatic Circulator 2219	LKB Bromma
Pipette Boy	INTEGRA
Pipettes	BIOHIT HealthCare / Eppendorf
Rotational Vacuum Concentrator	Christ
Synergy HT Multi-Mode Microplate Reader	BioTek
Vortexer	IKA® Werke
Water bath 3042 (37 °C)	Köttermann

2.2 Methods

2.2.1 Safety

The laboratory, in which this research took place, is certified as Biosafety Level 2 according to the *Directive 2000/54/EC – biological agents at work* released by the European Parliament in 2000 (138). Therefore, all experiments imbedding (potentially or actually) infectious or hazardous substances were performed under a laminar flow workbench. Additionally, if any experiments were performed on substances containing highly infectious poxvirus, protective glasses were worn. Afterwards, all materials were disinfected accurately, irradiated with UV-light for at least 15 minutes and ultimately autoclaved.

2.2.2 Microscopy

To guarantee sterile and proper conditions as well as to assess the performance of cell culture or after treatment, the cells were continuously examined under the CK40 phase contrast inverted microscope from Olympus. If any fluorescence had to be detected or visualized, the fluorescence microscope IX50 from Olympus was used.

2.2.3 Cell culture

2.2.3.1 General cell culture

All 4 pancreatic adenocarcinoma cell lines, both immortalized and adherent, were cultivated in growth medium (DMEM + 10 % FCS) in tissue culture flasks with filter screw caps. The flasks and tissue culture plates, in which the tumor cells

were seeded for treatment, were incubated at 37 °C in a humid atmosphere containing 5 % CO_2. All treatment steps were performed under sterile conditions in a laminar flow workbench. Media and PBS were prewarmed to 37 °C before use.

2.2.3.2 Passaging cells

In order to split or harvest the tumor cells, they were washed first with warm PBS and then detached by adding trypsin/EDTA. BxPc-3 cells were treated twice with trypsin, due to their high level of adherence to the flask's surface. Detached tumor cells were diluted in fresh growth medium, inactivating trypsin by its supplementation with FCS, while single cell suspensions were generated by gently resuspending the tumor cell suspension a few times with a pipette. Afterwards, the suspension was split and spread in new culture flasks or stored temporarily in 15 or 50 ml tubes to be counted and later seeded in tissue culture plates for further treatment (see section 2.2.3.3, p. 40).

2.2.3.3 Cell counting and seeding

Before the tumor cells could be seeded in tissue culture plates, their number per ml tumor cell suspension had to be determined using an improved Neubauer hemocytometer.

To distinguish between viable and dead cells, cells were stained with *trypan blue* before counting. Viable cells don't absorb *trypan blue* whereas it can pass the cell membrane of dead cells, staining the cellular proteins. Unstained viable cells, appearing brighter than their surroundings, now could be counted using a microscope with a 10x objective.

The counting chamber of the improved Neubauer hemocytometer is defined by the actual hemocytometer, a thick glass microscope slide with rectangular indentations parceled out by squares of different size (Figure 9a), and its cover glass. For counting, the 4 outer squares, each subdivided into 16 lesser squares, were used. The dimensions of the outer squares are 1 x 1 mm, while the distance between the microscope slide and the properly fixed cover glass is 0.1 mm. This results in a total capacity of 100 nl per outer square.

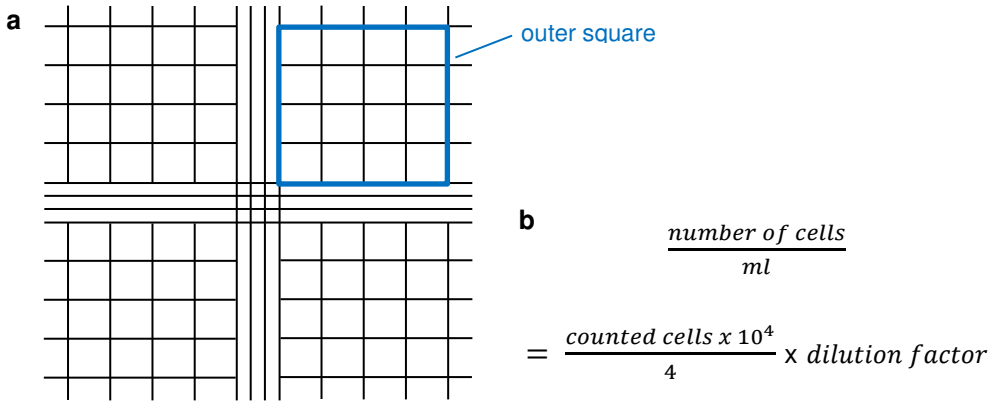

Figure 9 *Functionality of an improved Neubauer hemocytometer. (**a**) Schematics of the counting grid. (**b**) Calculation of cell concentration.*

First, the counting chamber was prepared by rubbing the cover glass carefully along two elevated glass edges on the microscope slide, until so-called Newton rings appeared, indicating the proper fixation. Subsequently, stained tumor cell suspension was administered to the margin of the cover glass, utilizing the capillary force to soak it into the counting chamber. Viable cells in all 4 outer squares were counted and their mean value was multiplied with 10^4 to calculate the number of cells per ml stained solution. Considering the thinning caused by staining with *trypan blue* the actual number of cells per ml cell suspension was calculated accurately (Figure 9b).

For treatment, tumor cells were seeded in 24-well plates at a density of 4 x 10^4 (BxPc-3, MIA PaCa-2, Panc-1) or 5 x 10^4 (AsPc-1) cells per well. The chosen densities resulted from the prior observation of approx. 90 - 100 % confluence after 24 hours incubation, at which time the tumor cells should be treated.

2.2.3.4 Cryopreservation and thawing

To stock the tumor cells for a longer time period, they were cryopreserved in a freezing cabinet at -145 °C. Tumor cells were first harvested (see section 2.2.3.2, p. 40) and transferred into 15 ml tubes. The cell suspension then was centrifuged for 3 minutes at 1200 rpm (rounds per minute) and room temperature. After removing the supernatant, the remaining tumor cell pellet was gently resuspended in cryo medium (90 % (DMEM + 20 % FCS), 10 % DMSO). One ml aliquots were transferred into cryotubes, which were packed in an isopropanol bath to be cooled

down slowly to -80 °C overnight. The next day, the frozen cryotubes were relocated to a freezing cabinet with -145 °C.

To recultivate the stocked tumor cells, the cryotubes were thawed in a water bath at 37 °C. In order to prevent DMSO from killing the tumor cells, the solution was diluted immediately in 5 ml growth medium and transferred into a 15 ml tube to be centrifuged as described above. The supernatant was removed and the cell pellet was gently resuspended in growth medium. The generated single cell suspension ultimately was cultivated in a new tissue culture flask. On the following day, the medium was replaced with new growth medium to finally remove any remnants of DMSO.

2.2.4 Virotherapeutic treatment

2.2.4.1 (Mono-)virotherapy

On the day of infection, previous to the actual virotherapeutic treatment, the GLV-1h68 solution was prepared. Frozen virus solution was thawed carefully and sonicated for 30 s at 4°C. Depending on the needed virus dose (MOI, multiplicity of infection), the dispersed viral particles then were diluted in DMEM supplemented with 2 % FCS (infection medium).

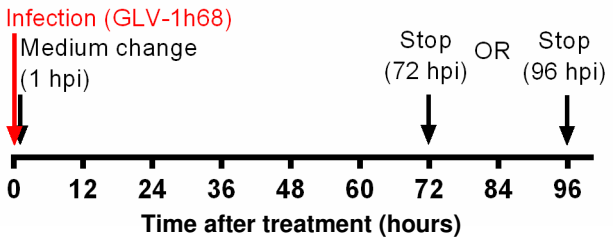

Figure 10 *Setting (mono-)virotherapy. hpi, hours post infection. Schematics for 72 h incubation after infection published in (139).*

Approx. 24 hours after seeding, tumor cells were washed with warm PBS and 250 µl infection medium, containing different MOIs of infectious GLV-1h68, were administered into each well (Figure 10). During the following hour, plates were swayed every 15 – 20 minutes to ensure that all tumor cells were exposed to the infectious viral particles and therefore had a chance of being infected successfully. At 1 hour post infection (hpi), the infection medium was replaced with 500 µl growth medium. Tumor cells were further incubated at 37 °C. At 72 and 96 hpi,

the remaining tumor cell mass after virotherapy was measured by SRB assay (see section 2.2.7.1, p. 48).

2.2.4.2 Virus growth curves

To assess the influence of the chemotherapeutics *nab*-paclitaxel and/or gemcitabine on the viral replication of GLV-1h68, virus growth curves were generated, comparing the viral replication of GLV-1h68 in cells that were treated with one, both or without the combination partners.

Tumor cells were seeded in 24-well plates and, on the following day, infected with GLV-1h68 according to the normal virotherapy scheme (as described above in section 2.2.4.1, p. 42). The following treatment steps belong to the chemovirotherapeutic triple-therapy and are described more detailed in section 2.2.6.4. At 1 hpi, the infection medium of the tumor cells which were treated with the combination therapy was replaced with *nab*-paclitaxel-containing growth medium. Half an hour later (1.5 hpi), gemcitabine-containing growth medium was added. If only *nab*-paclitaxel or gemcitabine was given, instead of the other agent normal growth medium was added. Tumor cells which were treated solely with GLV-1h68 received MOCK treatment by replacing the infection medium with normal growth medium and later adding more growth medium. Tumor cells were further incubated at 37 °C.

Samples of 4 wells per treatment group were taken at different time points after the primary infection (1.5 hpi, 24 hpi, 48 hpi, 72 hpi, 96 hpi). First, the tumor cells were scraped from the bottom into the medium, after which the suspension was resuspended thoroughly. Then, the cell suspensions of each 4 wells were pooled in test tubes and frozen at -80 °C without adding anti-freezing agent. Thus, the tumor cells burst, releasing the intracellular GLV-1h68 particles.

For the following virus titration a virus plaque assay was performed. CV-1 cells were seeded in 24-well plates and, by reaching 100 % confluence, infected with the collected virus samples. Therefore, the test tubes were thawed in a water bath at 37 °C and sonicated as described above (see section 2.2.4.1, p. 42). Thereafter, a 1:10 serial dilution of the virus samples (10^{-1} to 10^{-6}) was generated

by adding 100 µl virus sample to 900 µl infection medium, subsequently transferring 100 µl of the resulting dilution to another 900 µl infection medium and repeating this transfer 4 more times. Each generated solution was vortexed thoroughly before the next degree of dilution was produced. Two wells of the seeded CV-1 cells then were infected with 250 µl of the respective dilutions and incubated at 37 °C. After swaying the plates every 20 minutes, at 1 hpi, each well was overlaid with 1 ml CMC solution. Finally, the plates were incubated for 2 days at 37 °C.

To visualize the virus plaques, cells were stained by adding 250 µl *crystal violet* to each well. Subsequently, the plates were incubated for 4 hours at room temperature, after which the supernatants were removed. Tumor cells were washed 2 times with tap water and the plates were irradiated with UV-light for at least 15 minutes. Stained virus plaques of each well were counted after which the corresponding virus titers [PFU/ml, plaque forming units per ml] of each treatment group were calculated accurately in consideration of the particular dilution factors and the amount of infection medium administered in the plaque assay (Figure 11).

$$viral\ titer = \frac{mean\ value\ of\ the\ duplicates}{0.25\ ml\ (infection\ volume)}\ x\ dilution\ factor$$

Figure 11 *Virus plaque assay - Calculation of the virus titer.*

2.2.5 Chemotherapeutic treatment

2.2.5.1 Monochemotherapy

Approx. 24 hours after tumor cell seeding, medium was removed and 500 µl growth medium containing different concentrations of the respective chemotherapeutic agent were administered (Figure 12). Treatment with the light sensitive agent 5-FU was performed under dimmed light. Tumor cells were further incubated at 37 °C. At 48 and 72 hours post treatment (hpt), the remaining tumor cell mass was measured by SRB assay (see section 2.2.7.1, p. 48).

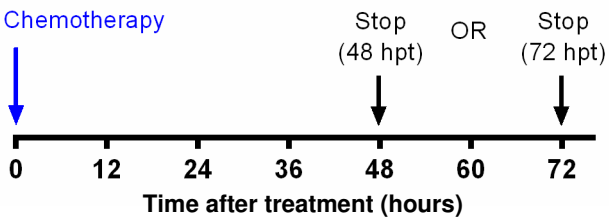

Figure 12 *Setting chemotherapy. hpt, hours post treatment.*

2.2.5.2 Dual chemotherapy

Approx. 24 hours after tumor cell seeding, medium was removed and 500 µl growth medium containing different concentrations of combined *nab*-paclitaxel and gemcitabine were administered. The concentrations of both chemotherapeutic agents were based on the respective treatment doses for combinatorial treatment, resulting alone in a remaining tumor cell mass of ≈ 75 % at 72 hours post treatment (designated as a so-called 25 % lethal dose, 'LD25 dose'). After 72 hours of dual chemotherapy the remaining tumor cell mass was measured by SRB assay (see section 2.2.7.1, p. 48). 3 treatment groups with distinct dose variations were differentiated:

1. Ratio of combined *nab*-paclitaxel and gemcitabine remained constant but two-fold dilutions were generated (Table 2).

Table 2 *Dual chemotherapy - Constant ratio of combined nab-paclitaxel + gemcitabine*

Agents	**Doses**						
***nab*-PTX**	LD25	0.5 x LD25	0.25 x LD25	0.125 x LD25	0.0625 x LD25	0.03125 x LD 25	0.015625 x LD25
Gem	LD25	0.5 x LD25	0.25 x LD25	0.125 x LD25	0.0625 x LD25	0.03125 x LD 25	0.015625 x LD25

Abbreviations: Gem, gemcitabine; LD25, 25 % lethal dose; *nab*-PTX, *nab*-paclitaxel.

2. Treatment doses of *nab*-paclitaxel remained constant at 50 % of the LD25 dose while the doses of gemcitabine were varied (Table 3).

Table 3 *Dual chemotherapy - Variation of the gemcitabine doses*

Agents	**Doses**						
***nab*-PTX**	0.5 x LD25	0.5 x LD25	0.5 x LD25	0.5 x LD25	0.5 x LD25	0.5 x LD25	0.5 x LD25
Gem	LD25	0.75 x LD25	0.5 x LD25	0.25 x LD25	0.1 x LD25	0.075 x LD 25	0.05 x LD25

Abbreviations: Gem, gemcitabine; LD25, 25 % lethal dose; *nab*-PTX, *nab*-paclitaxel.

3. Treatment doses of gemcitabine remained constant at 50 % of the LD25 dose while the doses of *nab*-paclitaxel were varied (Table 4).

Table 4 *Dual chemotherapy - Variation of the nab-paclitaxel doses*

Agents	Doses						
***nab*-PTX**	LD25	0.75 x LD25	0.5 x LD25	0.25 x LD25	0.1 x LD25	0.075 x LD 25	0.05 x LD25
Gem	0.5 x LD25	0.5 x LD25	0.5 x LD25	0.5 x LD25	0.5 x LD25	0.5 x LD25	0.5 x LD25

Abbreviations: Gem, gemcitabine; LD25, 25 % lethal dose; *nab*-PTX, *nab*-paclitaxel.

2.2.6 Chemovirotherapeutic treatment

2.2.6.1 Chemotherapy 1 hour post infection

Approx. 24 hours after seeding, tumor cells were infected with GLV-1h68 according to the normal virotherapy scheme (specified in section 2.2.4.1, p. 42). At 1 hpi, the infection medium was replaced with 500 µl growth medium containing the LD25 dose of the respective chemotherapeutic agent given in combination (Figure 13). Tumor cells were further incubated at 37 °C. At 72 hpi, the remaining tumor cell mass after combinatorial treatment was measured by SRB assay (see section 2.2.7.1, p. 48).

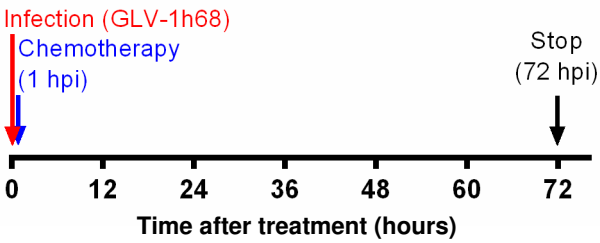

Figure 13 *Setting chemovirotherapy 1 hpi (hour(s) post infection). Figure published in (139).*

2.2.6.2 Chemotherapy 24 hours post infection

Approx. 24 hours after seeding, tumor cells were infected with GLV-1h68 according to the normal virotherapy scheme (see section 2.2.4.1, p. 42). At 24 hpi, the normal growth medium was replaced with 500 µl growth medium which contained the LD25 dose of the respective chemotherapeutic agent given in combination (Figure 14). Tumor cells were further incubated at 37 °C. At 72 hpi, the remaining tumor cell mass after combinatorial treatment was measured by SRB assay (see section 2.2.7.1, p. 48).

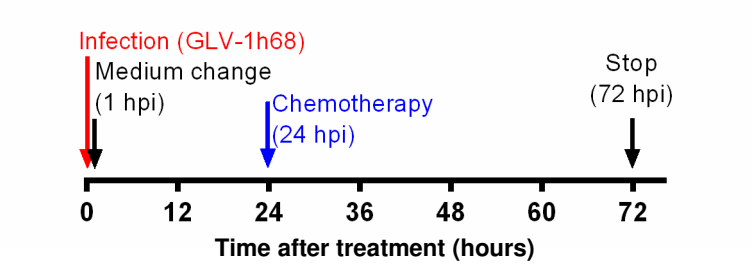

Figure 14 *Setting chemovirotherapy 24 hpi (hour(s) post infection). Figure published in (139).*

2.2.6.3 Chemotherapy 24 / 48 hours prior to infection

Approx. 24 hours after tumor cell seeding, medium was replaced with 500 µl growth medium containing the LD25 dose of the respective chemotherapeutic agent given in combination (Figure 15). 24 or 48 hours later, the tumor cells were infected with GLV-1h68 according to the normal virotherapy scheme (see section 2.2.4.1, p. 42) and further incubated at 37 °C. Because the tumor cells were seeded more than 24 hours prior to the virotherapeutic treatment, samples of each treatment group were counted before virotherapy in order to calculate the proper MOI. At 72 hpi, the remaining tumor cell mass after combinatorial treatment was measured by SRB assay (see section 2.2.7.1, p. 48).

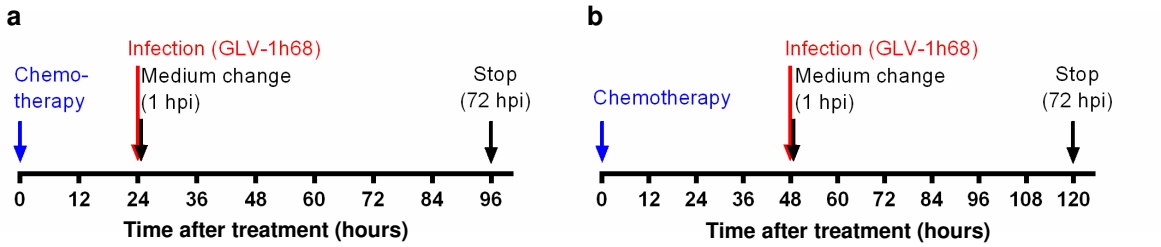

Figure 15 *Settings chemovirotherapy -24 / -48 hpi (hour(s) post infection). (**a**) Setting chemovirotherapy -24 hpi. (**b**) Setting chemovirotherapy -48 hpi.*

2.2.6.4 Chemovirotherapeutic triple-therapy

Approx. 24 hours after seeding, tumor cells were infected with GLV-1h68 according to the normal virotherapy scheme (see section 2.2.4.1, p. 42). At 1 hpi, the infection medium was replaced with 490 µl *nab*-paclitaxel-containing growth medium (Figure 16). Half an hour later (1.5 hpi), 10 µl gemcitabine-containing growth medium were added. The concentrations of *nab*-paclitaxel (in 490 µl growth medium) and gemcitabine (in 10 µl growth medium) were calculated to result in the proper (for triple-therapy adjusted) LD25 doses of both agents in 500 µl growth medium. Tumor cells were further incubated at 37 °C. At 72 hpi, both remaining

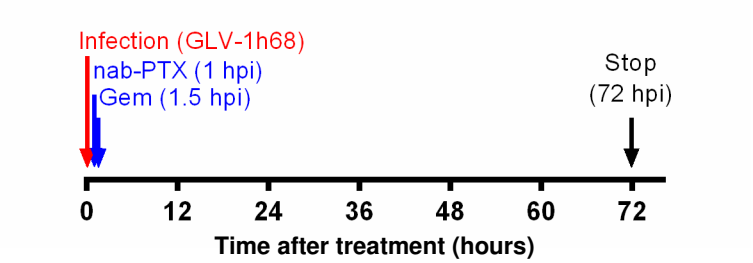

Figure 16 *Setting chemovirotherapeutic triple-therapy. hpi, hour(s) post infection. Figure published in (139).*

cell mass and cell viability after combinatorial treatment were measured by SRB, CellTiter-Blue® and MTT assays (see sections 2.2.7.1 - 2.2.7.3, p. 48 - 50), respectively.

2.2.7 Cell viability assays

2.2.7.1 Sulforhodamine B Assay

The sulforhodamine B (SRB) assay was the primary assay performed to quantify the remaining tumor cell mass after treatment in relation to the untreated control (MOCK). Basic principle of this colorimetric assay is the unspecific conjugation of SRB dye to protein components of cells, directly proportional to the cell mass (140). By solubilizing the protein-bound dye the resulting absorbance can be measured with a photospectrometer.

First, tumor cells were seeded in 24-well plates. 24 hours later the respective chemo- and/or virotherapeutic treatment was performed. After a defined incubation time, depending on the respective treatment setting, tumor cells were washed with cold PBS and fixated by administrating 250 µl of cold 10 % TCA solution to each well. After 30 minutes incubation at + 4 °C, TCA was removed and cells were washed 3 times with tap water. In case of being treated with GLV-1h68, cells were additionally irradiated with UV-light for at least 15 minutes to ensure all infectious viral particles being inactivated. Finally, the plates were dried in a drying chamber at 40 °C.

Cell protein staining was performed by adding SRB dye for 10 minutes. Excess dye then was washed out with 1 % acetic acid several times until all unbound dye was removed. Afterwards, the plates were dried another time.

To quantify the protein-bound dye per well, it was solubilized in 10 mM Tris base. Depending on the staining intensity 1-2 ml Tris base were administered. 160 µl solution of each well then were spread equally in 2 wells of a 96-well plate (pipet control, 2 x 80 µl). Thereafter, the optical density (OD) of the solutions was measured at a wave length of 550 nm using the GENios Plus Multifunction Fluorescence Microplate Reader (Tecan). If the OD values of the pipet control differed more than 0.1, the respective samples had to be transferred and analyzed again.

Individual OD values of each treatment group were correlated with the mean value of the untreated control (MOCK), which was set at 100 % of remaining tumor cell mass.

2.2.7.2 CellTiter-Blue® Assay

The CellTiter-Blue® (CTB) assay, measuring the metabolic capacity of cells as indicator for cell viability, was performed to confirm the data obtained with the SRB assay, thereby validating the effectivity of antitumor treatment with the triple-therapy. It is based on the potency of viable cells to reduce the dark-blue dye *resazurin* (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide), which possesses only little intrinsic fluorescent activity, to the pink and highly fluorescent compound *resorufin* (7-Hydroxy-3*H*-phenoxazin-3-one). The resulting change of fluorescence and absorbance, directly proportional to the number of viable cells, can be measured with a fluorometer or photospectrometer respectively.

Since seeding and treatment conditions of the tumor cells remained the same as for the SRB assay (specified in section 2.2.7.1, p. 48), the manufacturer's protocol (141) had to be slightly adapted to 24-well plates. At 72 hpi, 100 µl medium of each well were replaced with the same amount of CTB Reagent. Tumor cells were further incubated at 37 °C for 1 up to 4 hours, the particular time period was empirically determined depending on the individual metabolic rate of each cell line. End-point fluorescence was quantified at an excitation wavelength of 550 nm by measuring the resulting emission at 595 nm with the GENios Plus Multifunction Fluorescence Microplate Reader (Tecan).

Individual fluorescence values of each treatment group were correlated with the mean value of the untreated control (MOCK), which was set at 100 % of cell viability.

2.2.7.3 MTT Assay

To verify the collected data of treating pancreatic adenocarcinoma cells with the triple-therapy, the MTT assay, another colorimetric assay measuring the metabolic capacity of cells, was performed (142). Viable cells retain their potency to reduce the light sensitive tetrazolium dye MTT to the insoluble agent *formazan*, which accumulates as a precipitate inside the cells. By solubilizing the intracellular formazan the resulting absorbance, directly proportional to the number of viable cells, can be measured with a photospectrometer.

Seeding and treatment conditions of the tumor cells remained the same as for the SRB assay (specified in section 2.2.7.1, p. 48). At 72 hpi, the tumor cells were washed with 500 µl warm PBS, after which 250 µl MTT dye were added to each well under dimmed light. In succession, the plates were incubated for 2 hours at 37 °C.

To quantify the metabolized MTT, the excess dye then was replaced with 1 ml solubilization/stop solution (10 % HCl in isopropanol). The plates were swayed another 10 minutes, resulting in a yellow dilution of solubilized *formazan*. 200 µl of each well were transferred to a 96-well plate and their OD values were measured at the wave length of 570 nm using the Synergy HT Multi-Mode Microplate Reader (BioTek).

Individual OD values of each treatment group were correlated with the mean value of the untreated control (MOCK), which was set at 100 % of cell viability.

2.2.8 Software

The GENios Plus Multifunction Fluorescence Microplate Reader (Tecan) and the Synergy HT Multi-Mode Microplate Reader (BioTek) were run with the *XFluor4* and *Gen5* software, respectively. Microscopic images were taken with *analySIS*

V3.2 (Soft Imaging System). Basic calculations were executed with *Microsoft Excel 2013*. Visualization and further statistical evaluation of the data were performed with *GraphPad Prism V6* (GraphPad Software).

3 Results

In order to establish a novel chemovirotherapeutic combination therapy potentially improving the treatment outcome of pancreatic adenocarcinoma, a combinatorial treatment of 4 established and well-characterized pancreatic adenocarcinoma cells lines with the genetically modified vaccinia virus GLV-1h68 and various chemotherapeutic agents was investigated *in vitro*.

Preliminary to the actual chemovirotherapy, the treatment doses of the individual chemo- and virotherapeutic agents had to be determined under monotherapeutic conditions in a case-by-case approach. For this purpose, pancreatic adenocarcinoma cells were treated first with ascending concentrations of each agent in monotherapy (shown in section 3.1). After analyzing the cytotoxic effect of the individual mono(viro-/chemo-)therapies, 'adjusted' doses of each and every single agent were determined for subsequent combination therapy approaches. Here, the proper concentration of each combination partner when used alone (i.e., under monotherapeutic conditions) should result in a remaining tumor cell mass of ≈ 75 % after 72 hours of treatment (designated as a so-called 25 % lethal dose, 'LD25 dose'). Higher cytotoxicity might disguise any additional effect otherwise seen after combinatorial treatment when cytotoxicities would sum up too close to 100 % of tumor cell destruction thereby hindering any read-out of putative combinatorial effects.

Secondly, the actual chemovirotherapy, combining the vaccinia virus GLV-1h68 with each of the chosen chemotherapeutic agents separately, was performed and its cytotoxicity analyzed (shown in section 3.2). Since in combination therapy all combination partners interact with each other, the concentrations of the single agents are not the only important factor determining the best possible treatment outcome. Hence, the influence of different application sequences (i.e., the order of which compound was given first, followed by a defined schedule of the other combinatorial partners) was the main focus of these experiments.

Thirdly, based on the already collected data, pancreatic adenocarcinoma cells were treated with the chemovirotherapeutic triple-therapy, a combination of the

vaccinia virus GLV-1h68 with the approved dual chemotherapy *nab*-paclitaxel + gemcitabine (shown in section 3.3). After analyzing its cytotoxic effect, the influence of the dual chemotherapy and the respective chemotherapeutic agents on viral gene expression and replication of GLV-1h68 was investigated with the visualization of viral GFP expression and generation of virus growth curves, respectively.

3.1 Mono(viro-/chemo-)therapy – Dose-finding for combination therapy

3.1.1 Virotherapy with the oncolytic vaccinia virus GLV-1h68

To determine proper doses of GLV-1h68 for chemovirotherapy, as well as to analyze their susceptibility to viral infection and oncolysis, each of the 4 pancreatic adenocarcinoma cell lines was infected with individually ascending virus concentrations. The MOI (multiplicity of infection) thereby represents the ratio of infectious virus particles per tumor cell (a MOI of 0.1 accordingly describes a propor

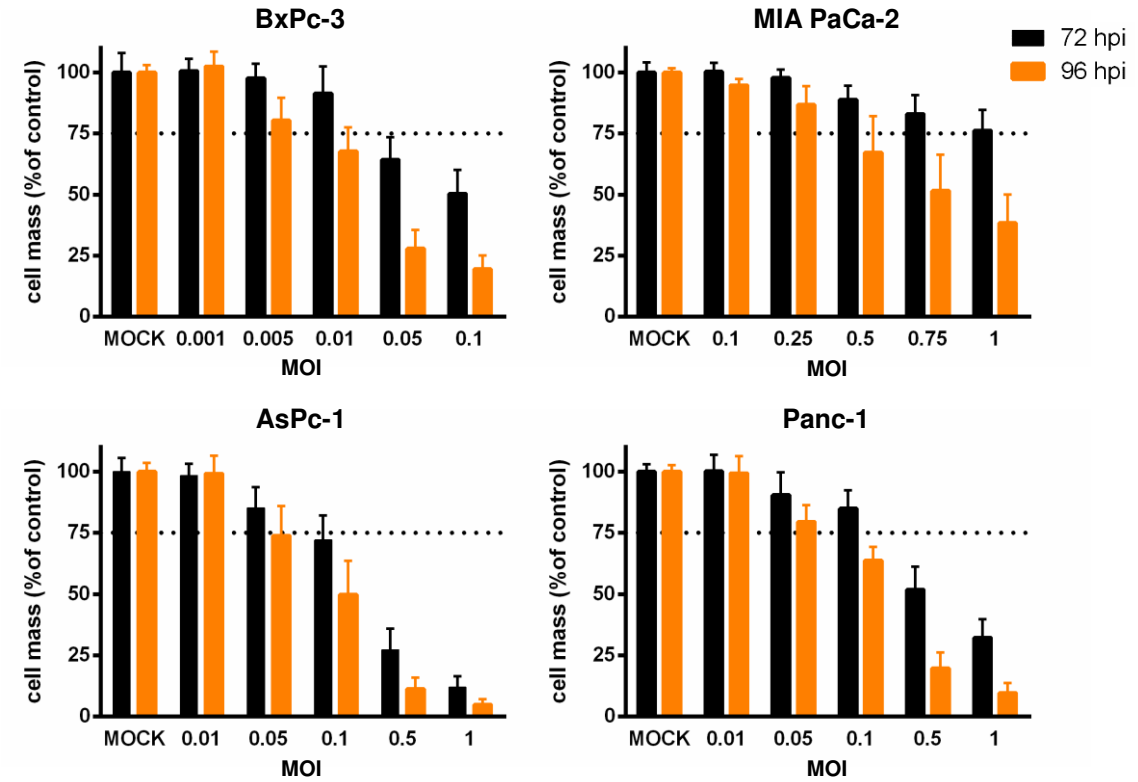

Figure 17 *Virotherapy with ascending doses of GLV-1h68 in four different human pancreatic tumor cell lines for 72 h (depicted in black bars) or 96 h (depicted in orange bars) in vitro. Tumor cells were first infected with the oncolytic vaccinia virus GLV-1h68. One hour post infection (hpi) the inoculum was removed and normal growth medium was added. Remaining tumor cell mass was analyzed by sulforhodamine B (SRB) assay (n ≥ 3, mean and standard deviation are shown). MOCK, untreated control. MOI, multiplicity of infection. Figure of 72 hpi values published in (139).*

tion of 1 infectious virus particle per 10 tumor cells). Tumor cells were further incubated for 72 or 96 hours, after which the remaining cell masses were analyzed by SRB assay (Figure 17). Additionally, dose-dependent GFP expression was visualized in Panc-1 cells (Figure 18).

It could be shown that the 4 tumor cell lines varied considerably in their response to the virotherapeutic treatment with the oncolytic vaccinia virus GLV-1h68. Though GLV-1h68 demonstrated a dose-dependent oncolytic effect in all 4 tumor cell lines, proving the feasibility of virotherapy in general, the viral doses of GLV-1h68 needed for potent tumor cell killing differed greatly. Compared to the untreated control (MOCK), especially the tumor cell line BxPc-3 showed an enhanced response to lower concentrations of GLV-1h68. Moreover, comparing the remaining cell masses at 72 and 96 hours post infection (hpi), a longer incubation time with GLV-1h68 after primary infection also resulted in an enhanced tumor cell killing. In conclusion, the therapeutic response to virotherapeutic treatment with GLV-1h68 not only depended on the administered virus dose, but also on the treated tumor cell line and the duration of virotherapy.

Based on the remaining cell masses at 72 hpi, the following MOIs of GLV-1h68 were selected for chemovirotherapeutic treatment in the particular tumor cell lines (Table 5).

Table 5 *Partial ('adjusted') doses of GLV-1h68 chosen for chemovirotherapy*

Tumor cell lines	Partial doses
AsPc-1	MOI 0.1
BxPc-3	MOI 0.01
MIA PaCa-2	MOI 0.5
Panc-1	MOI 0.1

Abbreviations: MOI, multiplicity of infection.

The dose-dependent character of viral infection was similarly illustrated by visualization of viral GFP expression in Panc-1 cells (Figure 18). At the lowest MOI of 0.01 only single cell clusters showed signs of viral infection. With ascending

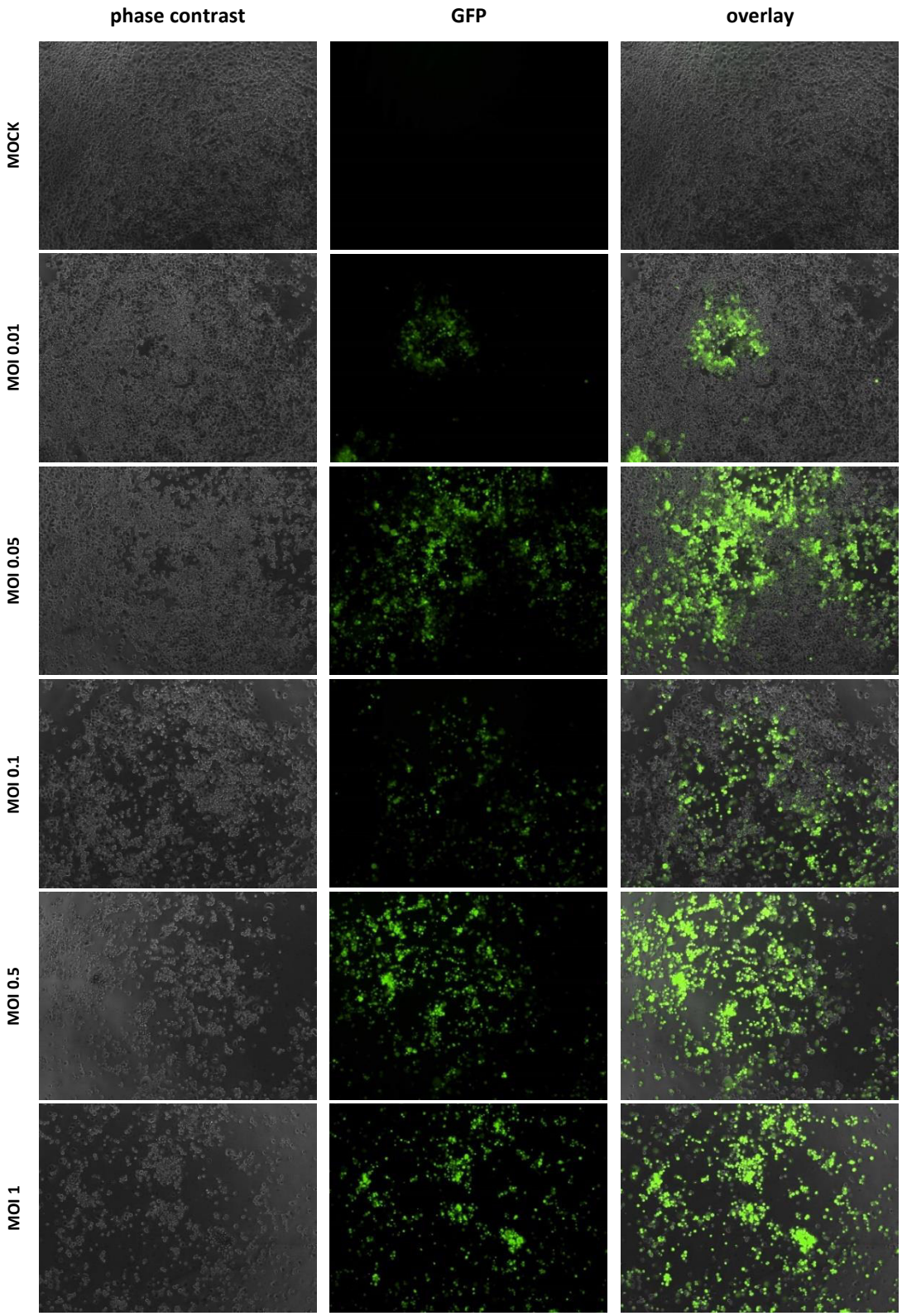

Figure 18 *Visualization of GLV-1h68 infection in Panc-1 cells at 72 hpi. Tumor cells were infected with ascending doses (MOIs) of GLV-1h68. At 1 hpi, the medium was changed. Expression of green fluorescent protein (GFP) indicating viral infection (central panels), corresponding phase contrast pictures (left panels) and the respective overlay images (right panels) are shown (scale 1:200). hpi, hour(s) post infection. MOCK, untreated control. MOI, multiplicity of infection.*

MOIs, i.e. rising ratios of infectious virus particles per tumor cell, also fluorescence levels increased until at the highest MOI of 1 nearly all tumor cells were stained by viral GFP expression. These findings were paralleled by a visible decrease of the number of tumor cells being remnant at 72 hpi in the phase contrast images which additionally demonstrated the character of virus-mediated oncolysis. Viral infection spreading from the primary infection site to neighboring tumor cells was followed by likewise outwards spreading oncolysis, leaving tumor-free gaps in the cell layer. Accordingly, the seemingly low GFP signal at the MOI of 0.1 originated from the initial oncolytic effect of GLV-1h68.

3.1.2 Chemotherapy with the nucleoside analogue 5-fluorouracil (5-FU)

In order to find a suitable combination partner for GLV-1h68 and to determine its proper concentration for chemovirotherapy, pancreatic adenocarcinoma cell lines were treated with different chemotherapeutic agents.

The first agent applied in (mono-)chemotherapy was the nucleoside analogue 5-fluorouracil (5-FU). In doing so, all 4 pancreatic adenocarcinoma cell lines were

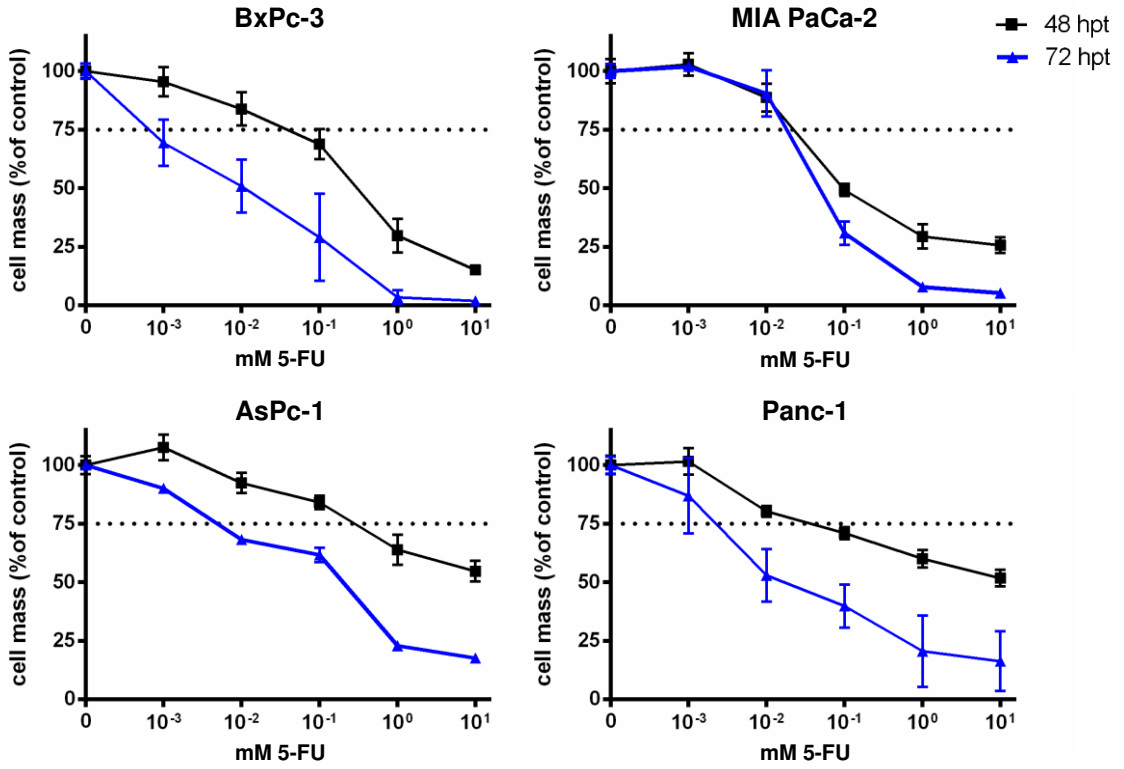

Figure 19 *Chemotherapy with ascending concentrations of the nucleoside analogue 5-fluorouracil (5-FU) in four different human pancreatic tumor cell lines for 48 h (depicted as black line) or 72 h (depicted as blue line) in vitro. Remaining tumor cell mass was analyzed by sulforhodamine B (SRB) assay (n=2, mean and standard deviation are shown). hpt, hour(s) post treatment.*

treated with ascending concentrations of 5-FU (0.001, 0.01, 0.1, 1 and 10 mM). Tumor cells were further incubated for 48 or 72 hours, after which the remaining cell masses were analyzed by SRB assay (Figure 19).

All 4 tumor cell lines responded to the chemotherapy with 5-FU in a dose-dependent fashion while, likewise, indications for different levels of resistance to the cytotoxic agent could be observed. At 48 hpt, BxPc-3 and MIA PaCa-2 cells were the first showing any considerable response to 5-FU at concentrations of 0.01 mM and higher. The tumor cell killing seen in the other 2 cell lines (AsPc-1 and Panc-1) remained poor even when higher doses of 5-FU were administered. When treated for 72 hours 3 of the 4 tumor cell lines (AsPc-1, BxPc-3 and Panc-1) demonstrated a considerably enhanced tumor cell killing at the whole range of concentrations. MIA PaCA-2 cells, however, didn't seem to benefit greatly from a longer incubation time with 5-FU, being indicated by the lesser gain of cytotoxicity at 72 hpt versus 48 hpt.

Based on the remaining cell masses at 72 hpt, these findings resulted in the selection of the following concentrations of 5-FU for chemovirotherapeutic treatment in the particular tumor cell lines (Table 6).

Table 6 *Partial ('adjusted') doses of 5-fluorouracil chosen for chemovirotherapy*

Tumor cell lines	Partial doses
AsPc-1	1 x 10^{-2} mM
BxPc-3	1 x 10^{-3} mM
MIA PaCa-2	1 x 10^{-2} mM
Panc-1	5 x 10^{-3} mM

3.1.3 Chemotherapy with the nucleoside analogue gemcitabine

The second chemotherapeutic agent applied in (mono-)chemotherapy was gemcitabine, another nucleoside analogue. Thereby, all 4 pancreatic adenocarcinoma cell lines were treated with ascending concentrations of gemcitabine (0.01, 0.1, 1, 10 and 100 µM). Tumor cells were further incubated for 48 or 72 hours, after which the remaining cell masses were analyzed by SRB assay (Figure 20).

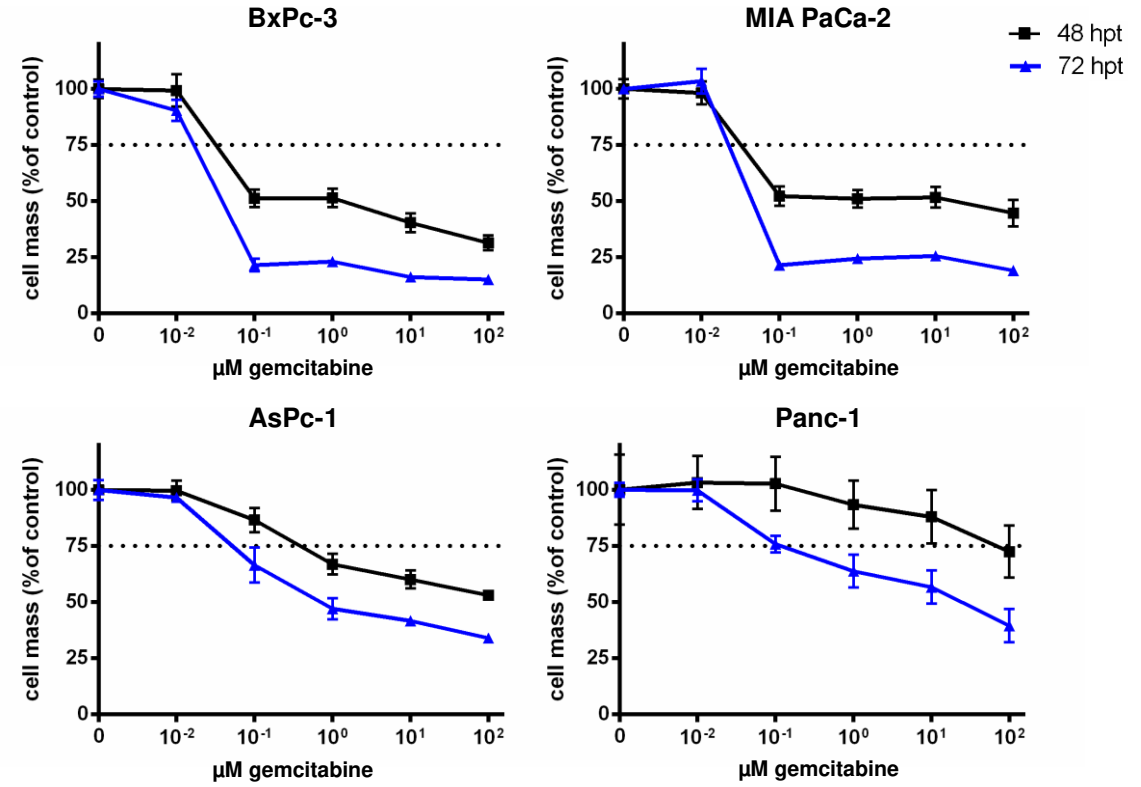

Figure 20 *Chemotherapy with ascending concentrations of the nucleoside analogue gemcitabine in four different human pancreatic tumor cell lines for 48 h (depicted as black line) or 72 h (depicted as blue line) in vitro. Remaining tumor cell mass was analyzed by sulforhodamine B (SRB) assay (n=3, mean and standard deviation are shown). hpt, hour(s) post treatment. Figure of 72 hpt values published in (139).*

As a result, all 4 tumor cell lines responded poorly to the chemotherapy with gemcitabine, indicating resistance to this cytotoxic agent. While in comparison with the other 2 tumor cell lines BxPc-3 and MIA PaCa-2 cells already responded to lower doses of gemcitabine, the observed tumor cell killing in these cell lines seemed to persist when higher doses were administered, a dose increase of gemcitabine resulting in no or only a moderate increase of cytotoxicity. However, when comparing the remaining cell masses at 48 and 72 hpt an enhanced tumor

Table 7 *Partial ('adjusted') doses of gemcitabine chosen for chemovirotherapy*

Tumor cell lines	Partial doses
AsPc-1	1 x 10^{-1} µM
BxPc-3	2 x 10^{-2} µM
MIA PaCa-2	3 x 10^{-2} µM
Panc-1	7.5 x 10^{-2} µM

cell killing after prolongation of the chemotherapy could be seen in all 4 tumor cell lines.

Based on the remaining cell masses at 72 hpt, these findings resulted in the selection of the following concentrations of gemcitabine for chemovirotherapeutic treatment in the particular tumor cell lines (Table 7).

3.1.4 Chemotherapy with the mitotic inhibitor *nab*-paclitaxel

The next chemotherapeutic agent applied in (mono-)chemotherapy was the mitotic inhibitor *nab*-paclitaxel, clinically approved for the treatment of advanced pancreatic adenocarcinoma only in combination with gemcitabine. Following the scheme of the previously performed experiments, all 4 pancreatic adenocarcinoma cell lines were treated with ascending concentrations of *nab*-paclitaxel. Due to the lesser experience with this relatively new agent a greater range of concentrations (0.0001, 0.001, 0.01, 0.1, 1, 10 and 100 µM) was surveyed. Tumor cells were further incubated for 48 or 72 hours, after which the remaining tumor cell masses were analyzed by SRB assay (Figure 21).

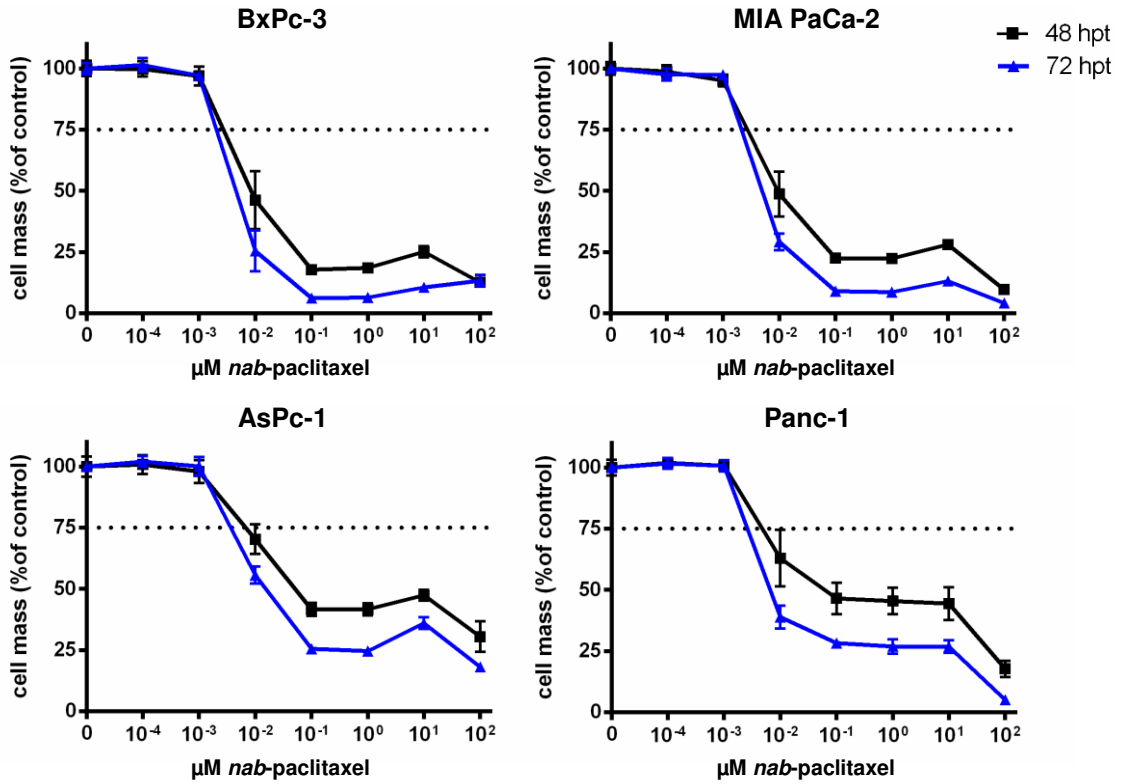

Figure 21 *Chemotherapy with ascending concentrations of the mitotic inhibitor nab-paclitaxel in four different human pancreatic tumor cell lines for 48 h (depicted as black line) or 72 h (depicted as blue line) in vitro. Remaining tumor cell mass was analyzed by sulforhodamine B (SRB) assay (n ≥ 3, mean and standard deviation are shown). hpt, hour(s) post treatment. Figure of 72 hpt values published in (139).*

All 4 tumor cell lines responded similarly to the chemotherapy with *nab*-paclitaxel. 0.01 µM *nab*-paclitaxel thereby seemed to be a critical concentration at which all 4 tumor cell lines first showed a considerable response to the chemotherapeutic agent. Yet, when higher doses than 0.1 µM *nab*-paclitaxel were applied, most notably in AsPc-1 and Panc-1 cells, no further reduction of tumor cell mass was seen, an effect only overcome after administration of 100 µM *nab*-paclitaxel, the highest concentration administered in this setting. This condition was apparent independently of the duration of treatment, although in all 4 tumor cell lines a longer treatment with *nab*-paclitaxel also resulted in a slightly enhanced tumor cell killing.

Based on the remaining cell masses at 72 hpt, these findings resulted in the selection of the following concentrations of *nab*-paclitaxel for chemovirotherapeutic treatment in the particular tumor cell lines (Table 8).

Table 8 *Partial ('adjusted') doses of nab-paclitaxel chosen for chemovirotherapy*

Tumor cell lines	Partial doses
AsPc-1	1 x 10^{-2} µM
BxPc-3	1 x 10^{-2} µM
MIA PaCa-2	5 x 10^{-3} µM
Panc-1	5 x 10^{-3} µM

3.1.5 Chemotherapy with the platinum compound oxaliplatin

Other chemotherapeutic agents, usually not part of monochemotherapeutic first-line protocols in the treatment of advanced pancreatic adenocarcinoma, were surveyed in the preliminary testing phase as well. After *nab*-paclitaxel the second of this group of chemotherapeutic agents was the platinum compound oxaliplatin, clinically used only as part of the FOLFIRINOX regimen (folinic acid (leucovorin) + 5-fluorouracil + irinotecan + oxaliplatin) (first-line) or in combination with gemcitabine (second-line). Pursuing the schematics of the already performed (mono-)chemotherapies all 4 pancreatic adenocarcinoma cell lines were treated with ascending concentrations of oxaliplatin (0.01, 0.1, 1, 10 and 100 µM). Tumor

cells were further incubated for 48 or 72 hours, after which the remaining cell masses were analyzed by SRB assay (Figure 22).

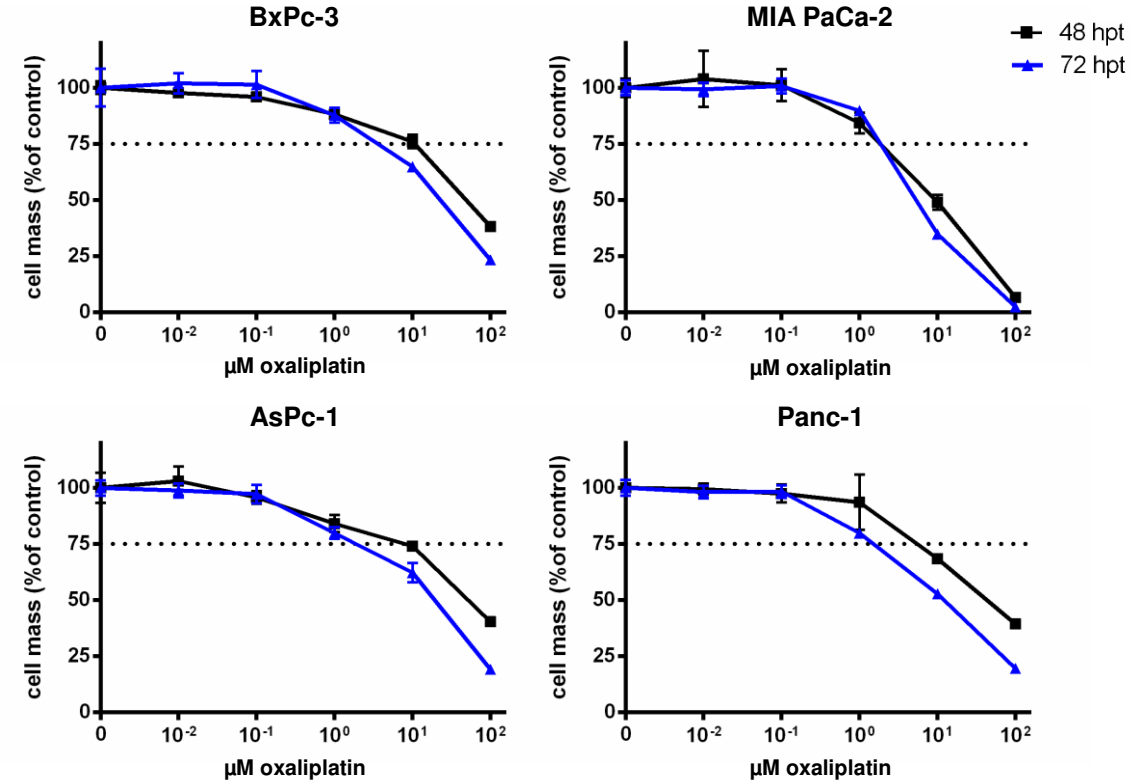

Figure 22 *Chemotherapy with ascending concentrations of the platinum compound oxaliplatin in four different human pancreatic tumor cell lines for 48 h (depicted as black line) or 72 h (depicted as blue line) in vitro. Remaining tumor cell mass was analyzed by sulforhodamine B (SRB) assay (n ≥ 1, mean and standard deviation are shown). hpt, hour(s) post treatment.*

As can be seen, all 4 tumor cell lines showed only poor response rates to the chemotherapy with oxaliplatin at the applied range of concentrations. MIA PaCa-2 cells, in comparison with the other 3 tumor cell lines, indeed responded best, but considerable tumor cell killing was seen only at concentrations of 10 µM oxaliplatin and higher. However, in none of the 4 tumor cell lines a substantial difference between the treatment outcomes after 48 or 72 hours chemotherapy could be seen. These findings clearly indicate resistance to oxaliplatin, all 4 tumor cell lines responding only after high concentrations of the chemotherapeutic agent were administered, finally overcoming cellular resistance mechanisms.

Based on the remaining cell masses at 72 hpt, these findings resulted in the selection of the following concentrations of oxaliplatin for chemovirotherapeutic treatment in the particular tumor cell lines (Table 9):

Table 9 *Partial ('adjusted') doses of oxaliplatin chosen for chemovirotherapy*

Tumor cell lines	Partial doses
AsPc-1	1 x 10^{-2} µM
BxPc-3	1 x 10^{-2} µM
MIA PaCa-2	5 x 10^{-3} µM
Panc-1	1 x 10^{-3} µM

3.1.6 Chemotherapy with the topoisomerase I inhibitor irinotecan

Another chemotherapeutic agent usually not applied in monochemotherapy for the treatment of advanced pancreatic adenocarcinoma is the topoisomerase I inhibitor irinotecan, representing another component of the FOLFIRINOX regimen (folinic acid (leucovorin) + 5-fluorouracil + irinotecan + oxaliplatin). Thereby, all 4 pancreatic adenocarcinoma cell lines were treated with ascending concentrations of irinotecan (0.01, 0.1, 1, 10 and 100 µM). Tumor cells were further incubated for 48 or 72 hours, after which the remaining cell masses were analyzed by SRB assay (Figure 23).

At the applied range of concentrations, all 4 tumor cell lines demonstrated an overall poor response to chemotherapy with irinotecan. Mirroring the results seen after chemotherapy with oxaliplatin, in comparison with the other 3 tumor cell lines, MIA PaCa-2 cells responded best, but only when concentrations of 10 µM irinotecan and higher were administered. Furthermore, comparing the remaining cell masses at 48 and 72 hpt, a prolongation of treatment for 24 hours also led to a slightly enhanced tumor cell killing, but only at higher concentrations trespassing a critical concentration at which an initial response was seen already after 48 hours of treatment.

Based on the fact that irinotecan in the clinic is usually applied only in combination with several other chemotherapeutic agents, chemovirotherapeutic protocols incorporating irinotecan were not further developed during this investigation. The obtained results, however, can be seen as a basis for possible combination regimens in the future.

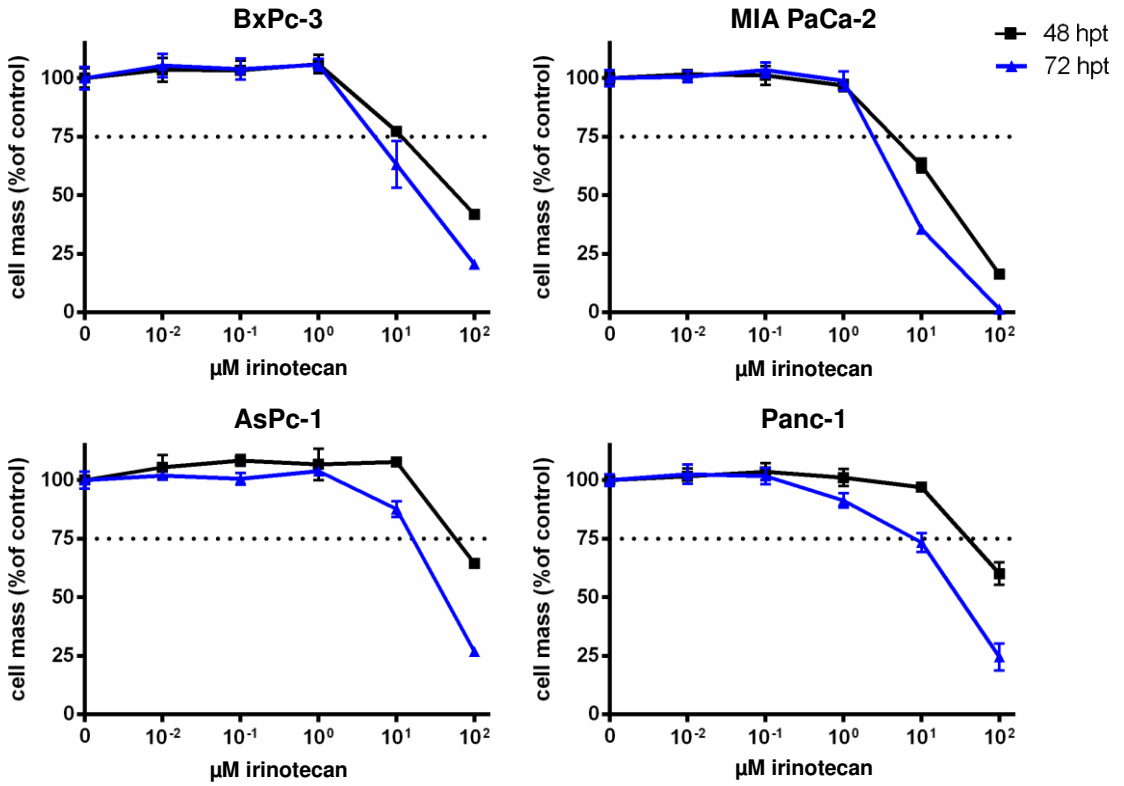

Figure 23 *Chemotherapy with ascending concentrations of the topoisomerase I inhibitor irinotecan in four different human pancreatic tumor cell lines for 48 h (depicted as black line) or 72 h (depicted as blue line) in vitro. Remaining tumor cell mass was analyzed by sulforhodamine B (SRB) assay (n ≥ 1, mean and standard deviation are shown). hpt, hour(s) post treatment.*

3.1.7 Tyrosine kinase inhibitor therapy with EGFR targeting erlotinib

The last agent surveyed in (mono-)chemotherapy as possible combination partner for GLV-1h168 was the tyrosine kinase inhibitor erlotinib, clinically approved for the treatment of advanced pancreatic adenocarcinoma only in combination with gemcitabine. As performed in the previous experiments, all 4 pancreatic adenocarcinoma cell lines were treated with ascending concentrations of erlotinib (0.01, 0.1, 1, 10 and 100 µM). Tumor cells were further incubated for 48 or 72 hours, after which the remaining tumor cell masses were analyzed by SRB assay (Figure 24).

As could be shown, all 4 tumor cell lines responded poorly to the tyrosine kinase inhibitor therapy with erlotinib, though to a varying extent. While AsPc-1 and BxPc-3 cells responded to dose increases of erlotinib with an enhanced tumor cell killing at the whole range of concentrations, Panc-1 cells showed only a very

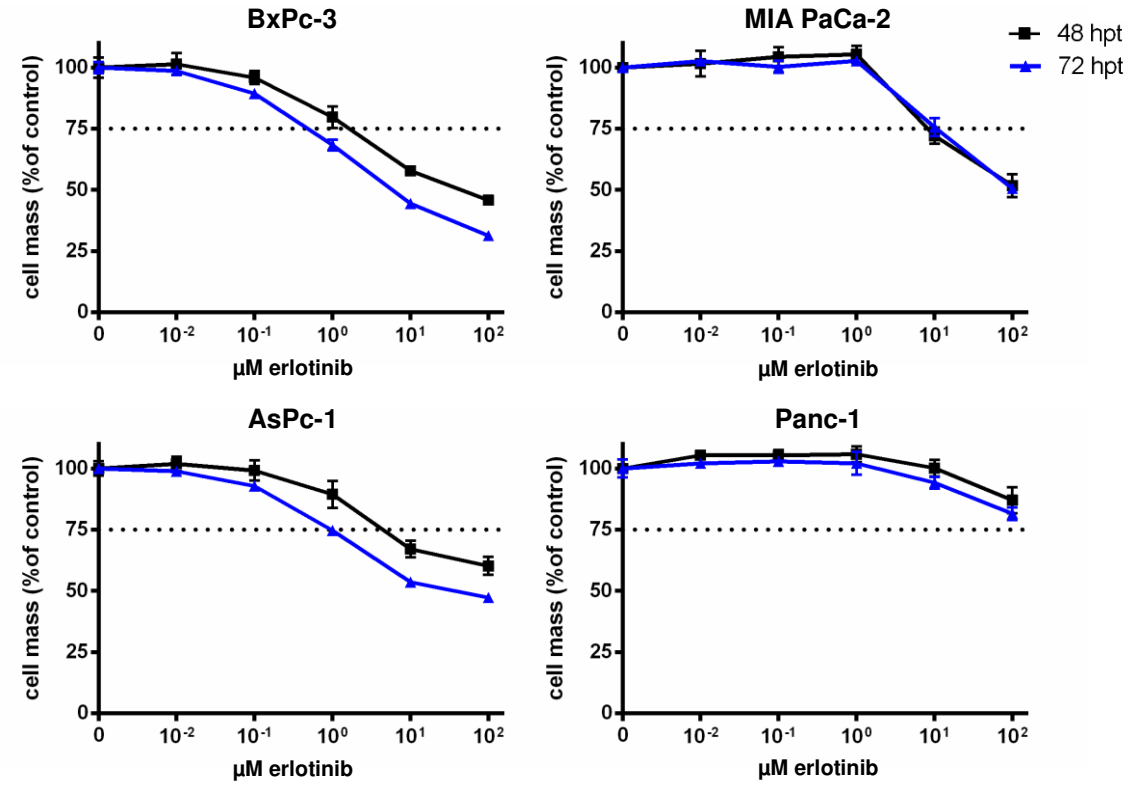

Figure 24 *Therapy with ascending concentrations of the tyrosine kinase inhibitor erlotinib in four different human pancreatic tumor cell lines for 48 h (depicted as black line) or 72 h (depicted as blue line) in vitro. Remaining tumor cell mass was analyzed by sulforhodamine B (SRB) assay (n ≥ 1, mean and standard deviation are shown). hpt, hour(s) post treatment*

moderate response even at the highest concentrations administered in this setting. MIA PaCa-2 cells only responded to concentrations of 10 µM erlotinib and higher. Comparing the remaining cell masses after 48 and 72 hours of tyrosine kinase inhibitor therapy, the better responding cell lines AsPc-1 and BxPc-2 showed at least a moderately increased tumor cell killing, whereas MIA PaCa-2 and Panc-1 cells didn't seem to respond better after a prolongation of treatment for 24 hours at all.

Due to the fact that erlotinib is only approved in combination with gemcitabine for the treatment of advanced pancreatic adenocarcinoma and only in case the patients show an initial dermatologic response to the combination therapy, no chemovirotherapeutic protocol imbedding erlotinib was developed during this investigation. Similar to the results obtained after chemotherapy with irinotecan, these data can be seen as a basis for possible combination regimens in the future.

3.2 Chemovirotherapy – Finding the right combinatorial sequence

3.2.1 Combination of GLV-1h68 with 5-fluorouracil (5-FU)

After performing the initial monotherapies and selecting suitable partial 'adjusted' doses of each agent, the first devised chemovirotherapeutic protocol was the combination of GLV-1h68 with the nucleoside analogue 5-FU.

Not knowing in which way the two agents would interact and in consideration that the most feasible administration sequence would be a concomitant application of both agents, 5-FU was administered directly after primary infection with GLV-1h68 at 1 hpi. Both agents were thereby applied in their particular LD25 doses (see above). Tumor cells were further incubated until, at 72 hpi, the cytotoxicity of the chemovirotherapy was analyzed by SRB assay (Figure 25).

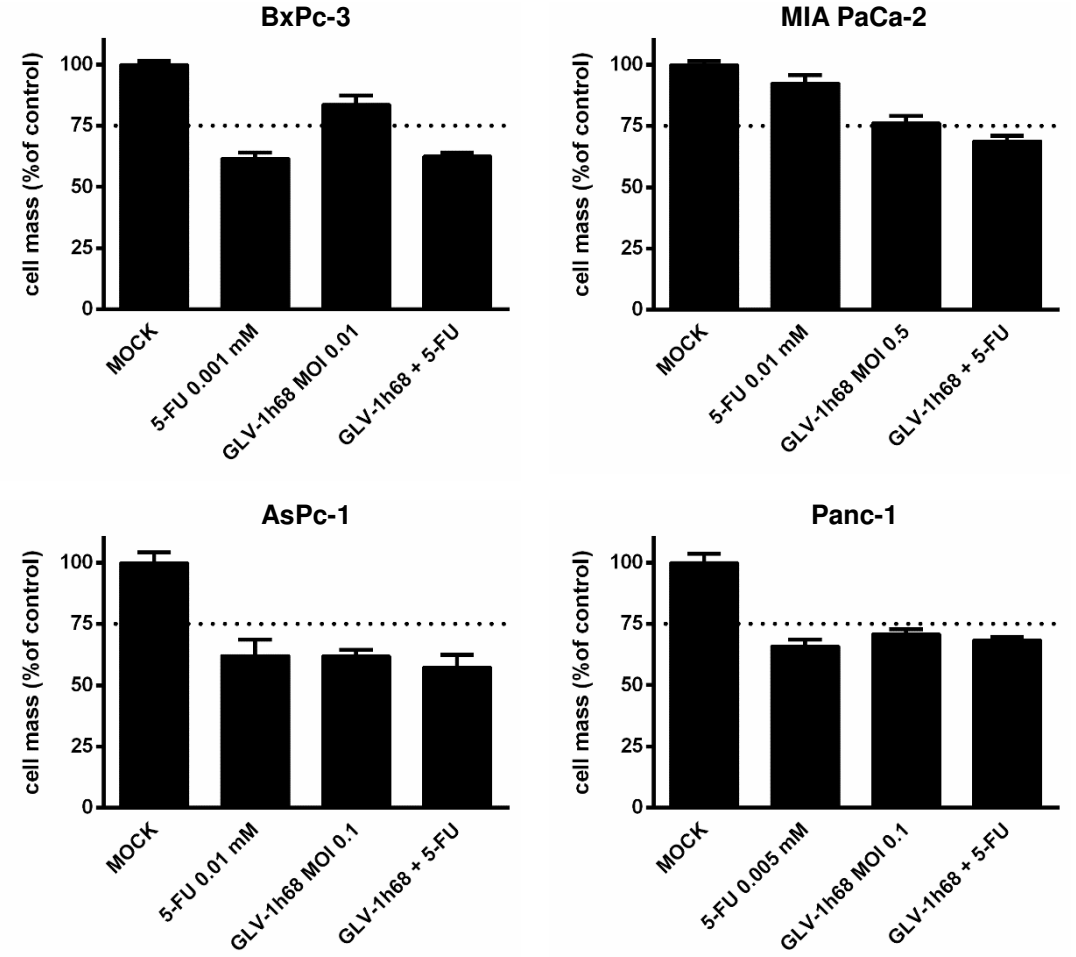

Figure 25 *Chemovirotherapy with GLV-1h68 and 5-fluorouracil (5-FU) in four different human pancreatic tumor cell lines in vitro. 5-FU was added after infection with GLV-1h68 at 1 hpi. Remaining tumor cell mass was analyzed at 72 hpi by sulforhodamine B (SRB) assay (n=1, mean and standard deviation are shown). hpi, hour(s) post infection. MOCK, untreated control. MOI, multiplicity of infection.*

To assess the results in the correct manner, by looking at the remaining cell masses of both monotherapies it has to be reviewed first if suitable partial doses of each agent were chosen. Secondly, to determine if and to which extent an enhanced or reduced cytotoxic effect of the chemovirotherapeutic protocol can be seen, the remaining cell mass after chemovirotherapeutic treatment has to be compared to both of the respective monotherapies separately.

In doing so, all 4 tumor cell lines responded to both monotherapeutic treatments within the acceptable limits of variation from the target value of 75 % remaining cell mass after 72 hours of treatment (Figure 25), confirming the previously collected data. However, no substantial difference of cytotoxicity between the chemovirotherapeutic combination protocol and the respective monotherapies could be seen in neither of the 4 tumor cell lines. Tumor cell loss after combination therapy within a tumor cell line corresponded at least with the outcome seen after single viro- or chemotherapy, initially excluding any potential antagonistic effects.

3.2.2 Combination of GLV-1h68 with gemcitabine

To analyze whether the missing additional effect of the previously investigated chemovirotherapeutic protocol with 5-FU was linked to its properties as nucleoside analogue, GLV-1h68 was combined next with gemcitabine, a different nucleoside analogue of the here screened array of chemotherapeutic agents.

Going beyond the previous administration setting, additionally, the chronological order of the single agents was varied. Gemcitabine not only was administered concomitantly (1 hpi), but also 24 hours after (24 hpi) or even 48 hours prior (-48 hpi) to virotherapeutic treatment with GLV-1h68. Independently of the particular administration sequence and the resulting differences in the duration of treatment the remaining cell masses were analyzed at 72 hpi by SRB assay (Figure 26).

When evaluating the results of tumor cell loss with regard to the three different administration sequences, additionally to the prior mentioned criteria other variables have to be considered as well. Although the duration of virotherapy within each setting always was the same (72 h), the duration of chemotherapy (CTX) varied from 48 (CTX 24 and -48 hpi) to 71 hours (CTX 1 hpi). Moreover, when

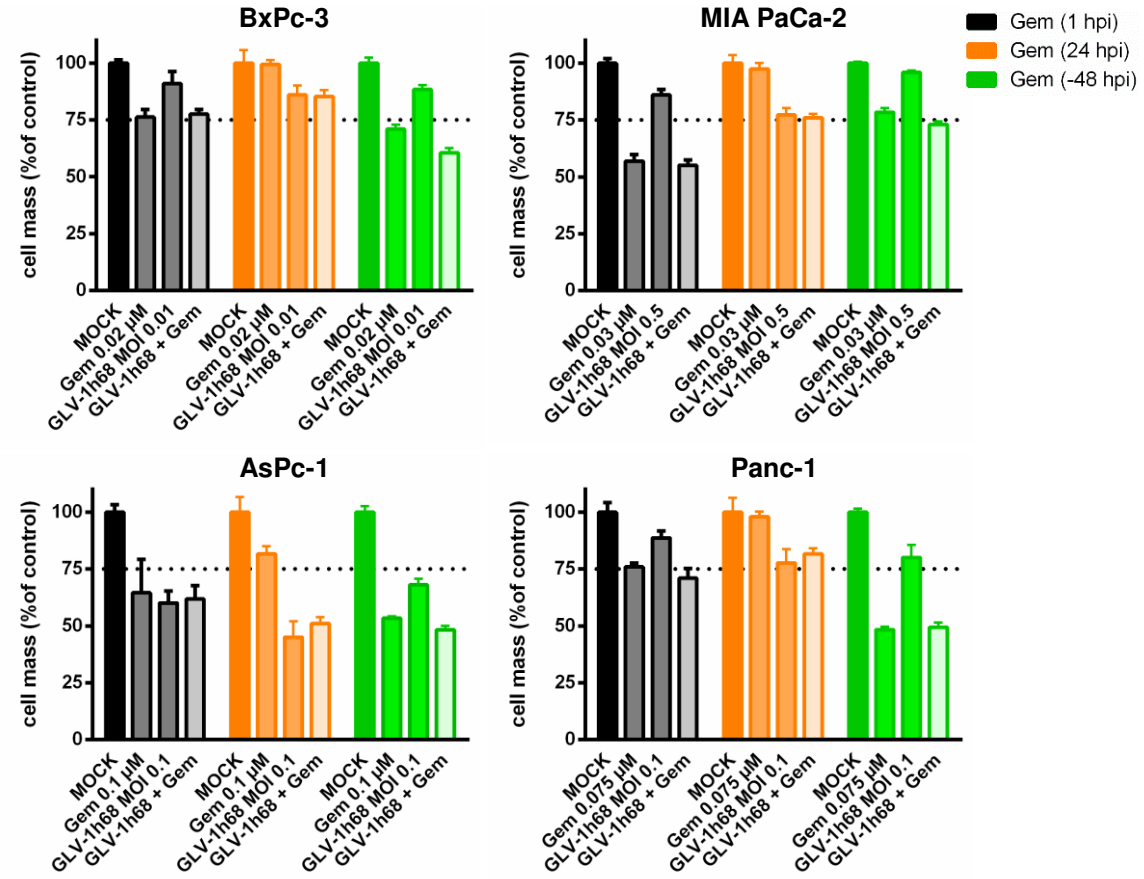

Figure 26 *Chemovirotherapy with GLV-1h68 and gemcitabine (Gem) in four different human pancreatic tumor cell lines in vitro. Gem was added either after infection with GLV-1h68 at 1 hpi (depicted in black bars) and 24 hpi (depicted in orange bars) or prior to infection at -48 hpi (depicted in green bars). Remaining tumor cell mass was analyzed at 72 hpi by sulforhodamine B (SRB) assay (n=1, mean and standard deviation are shown). hpi, hour(s) post infection. MOCK, untreated control. MOI, multiplicity of infection. Figure of Gem (1 hpi) and Gem (-48 hpi) values published in (139).*

the chemotherapeutic agent was administered prior to the virotherapeutic treatment (CTX -48 hpi), after viral infection the tumor cells were incubated for another 72 hours without addition of any chemotherapeutic agent. Thus, with reference to the different durations of treatment, the remaining cell masses after chemotherapy within a single tumor cell line have to be correlated with each other. Moreover, and more importantly, when considering the effectivity of chemovirotherapeutic treatment, not only within a single tumor cell line but also in comparison with the others, it has to be checked whether a pattern can be identified in which a particular application setting results in an enhanced cytotoxic effect in all or at least the majority of treated tumor cell lines.

Looking at the loss of the tumor cell mass after 71 hours of chemotherapy with gemcitabine (Gem 1 hpi) suitable concentrations of gemcitabine were chosen

(Figure 26). As expected, when compared with the remaining cell masses after only 48 hours incubation with gemcitabine (Gem 24 hpi) all 4 tumor cell lines responded more strongly to the longer lasting chemotherapy. Interestingly, when treated first with gemcitabine (Gem -48 hpi), the initial response of AsPc-1 and Panc-1 cells to the monochemotherapy seemed to outweigh the longer incubation time without gemcitabine. Whether gemcitabine additionally might have induced a persisting growth inhibition couldn't be determined on the basis of these data. In the end, no antagonistic effects of the combination could be seen in neither of the 4 tumor cell lines but when compared to both respective monotherapies no combination protocol led to a considerably enhanced cytotoxic effect neither.

3.2.3 Combination of GLV-1h68 with oxaliplatin

Since chemovirotherapy with nucleoside analogues didn't result in any noteworthy increase of cytotoxicity in the 4 pancreatic adenocarcinoma cell lines, the next investigated chemovirotherapeutic protocol should incorporate a chemotherapeutic agent with a different mode of action. Accordingly, GLV-1h68 was combined with the platinum compound oxaliplatin. Thereby, the administration sequence of both agents remained the same. Oxaliplatin was administered either concomitantly (1 hpi), 24 hours after (24 hpi) or 48 hours prior (-48 hpi) to primary infection with GLV-1h68. At 72 hpi, the remaining cell masses were analyzed by SRB assay (Figure 27).

While in AsPc-1 cells the chemovirotherapeutic treatment with GLV-1h68 and oxaliplatin led to a clear increase of cytotoxicity independently of the chronological order of the agents, in the other 3 tumor cell lines (BxPc-3, MIA PaCa-2 and Panc-1) this effect could only be seen in a few settings and then only to a lesser extent. In BxPc-3 cells the response after chemovirotherapy invariably was the same as after monochemotherapy with oxaliplatin alone, whereas MIA PaCa-2 and Panc-1 cells somewhat responded better to the combination when oxaliplatin was given 24 hours after infection with GLV-1h68 (Ox 24 hpi). Looking at the remaining cell masses of the 71-hour monochemotherapy with oxaliplatin (Ox 1 hpi) in all 4 tumor cell lines proper concentrations of the chemotherapeutic agent

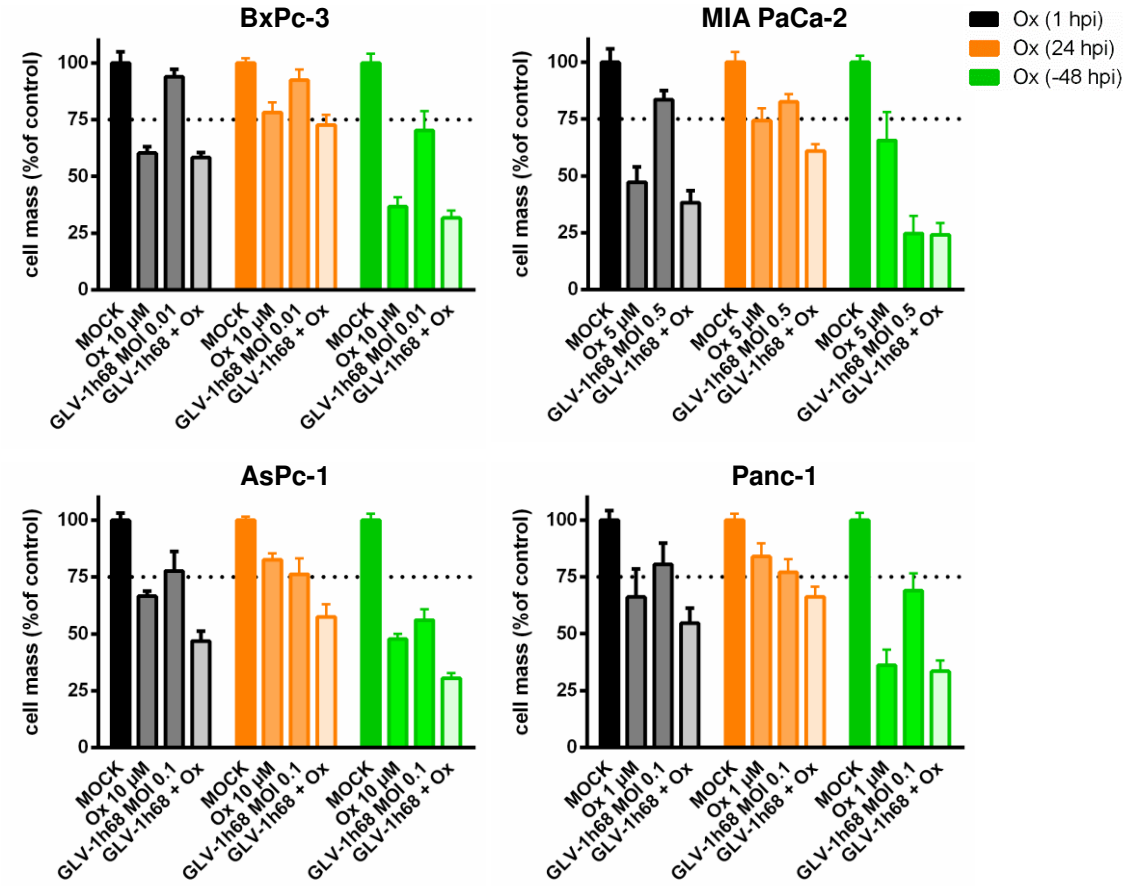

Figure 27 *Chemovirotherapy with GLV-1h68 and oxaliplatin (Ox) in four different human pancreatic tumor cell lines in vitro. Ox was added either after infection with GLV-1h68 at 1 hpi (depicted in black bars) and 24 hpi (depicted in orange bars) or prior to infection at -48 hpi (depicted in green bars). Remaining tumor cell mass was analyzed at 72 hpi by sulforhodamine B (SRB) assay (n=3, mean and standard deviation are shown). hpi, hour(s) post infection. MOCK, untreated control. MOI, multiplicity of infection.*

were chosen. Furthermore, when oxaliplatin was applied prior to the virotherapeutic treatment (Ox -48 hpi) all 4 tumor cell lines, but especially BxPc-3 and Panc-1 cells, demonstrated signs of a notable initial tumor cell killing in the monochemotherapeutic treatment arm.

3.2.4 Combination of GLV-1h68 with *nab*-paclitaxel

Encouraged by the results seen after chemovirotherapy with oxaliplatin, another chemotherapeutic protocol imbedding the mitotic inhibitor *nab*-paclitaxel was designed. Moreover, based on published data from Huang et al. (143), in which a derivative of the *Western Reserve* strain of vaccinia virus was combined successfully with paclitaxel *in vitro*, the parameters of the administration sequence were altered slightly. *Nab*-paclitaxel was added either concomitantly (1 hpi), 24 hours

after (24 hpi) or prior (-24 hpi) to the virotherapeutic treatment. As before, independently of the individual duration of treatment, the remaining cell masses were analyzed at 72 hpi by SRB assay (Figure 28).

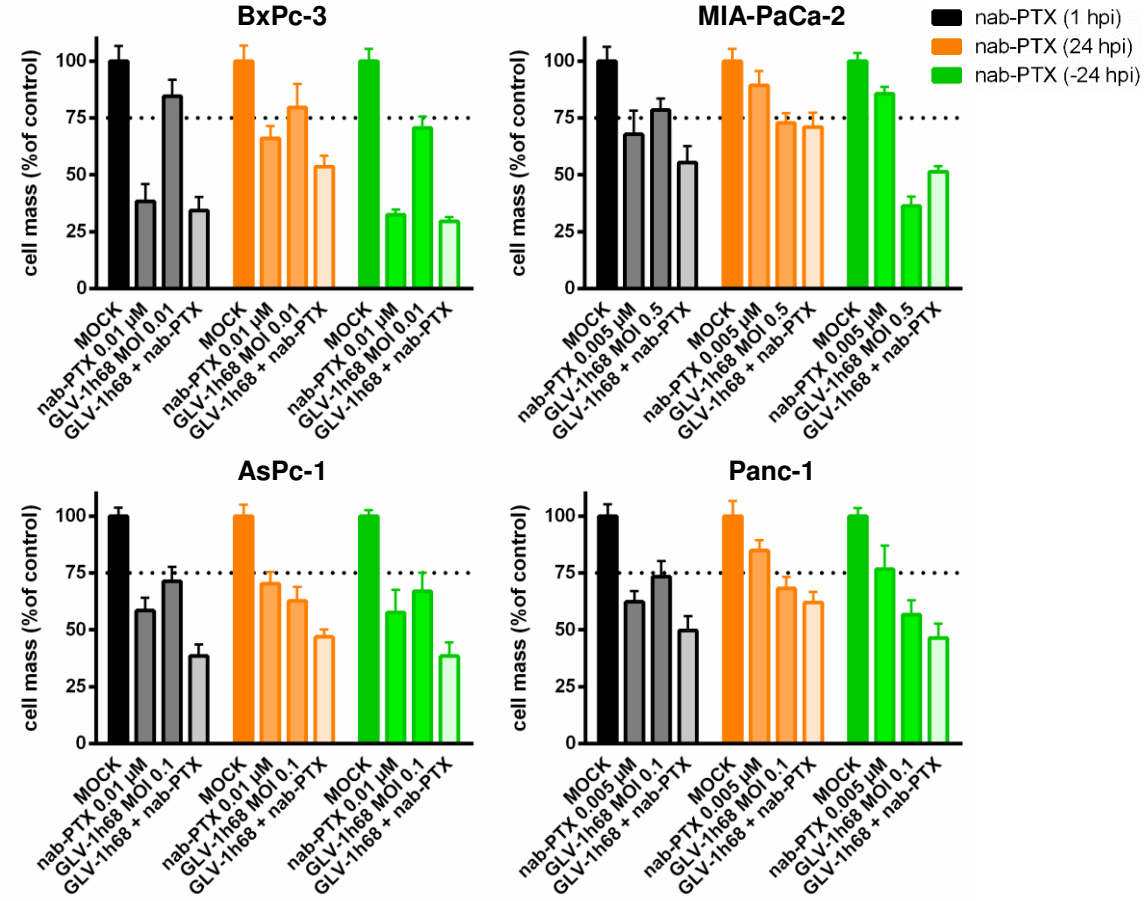

Figure 28 *Chemovirotherapy with GLV-1h68 and nab-paclitaxel (nab-PTX) in four different human pancreatic tumor cell lines in vitro. Nab-PTX was added either after infection with GLV-1h68 at 1 hpi (depicted in black bars) and 24 hpi (depicted in orange bars) or prior to infection at -24 hpi (depicted in green bars). Remaining tumor cell mass was analyzed at 72 hpi by sulforhodamine B (SRB) assay (n=3, mean and standard deviation are shown). hpi, hour(s) post infection. MOCK, untreated control. MOI, multiplicity of infection. Figure of nab-PTX (1 hpi) and nab-PTX (24 hpi) values published in (139).*

In contrast to the previously obtained results, the chemovirotherapeutic combination of GLV-1h68 with *nab*-paclitaxel led to a slightly increased tumor cell loss in the majority of different combination protocols. However, in MIA PaCa-2 cells an antagonistic effect of the combination therapy could be seen when *nab*-paclitaxel was administered 24 hours prior to the virotherapeutic treatment (*nab*-PTX -24 hpi). In 3 out of 4 tumor cell lines (AsPc-1, MIA PaCa-2 and Panc-1) proper concentrations of *nab*-paclitaxel were chosen, whereas in BxPc-3 cells the monochemotherapy alone lead to a remaining tumor cell mass below 50%. As seen

before, all 4 tumor cell lines responded better to the 24-hour-longer chemotherapy with *nab*-paclitaxel while in the monochemotherapeutic pretreatment arm (*nab*-PTX -24 hpi) the observed reductions of tumor cell masses were the result of the initial chemotherapy.

Nonetheless, the increase of therapeutic efficacy seen in AsPc-1 cells was independent of the chronological order of the agents. In the other 3 tumor cell lines (BxPc-3, MIA PaCa-2 and Panc1) this response was inconsistent across the different settings of administration and then could be seen only to a lesser extent. Therefore, no pattern evolved in which a single chemovirotherapeutic protocol led to a superior tumor cell loss in all 4 tumor cell lines when compared with the respective monotherapies.

3.3 Chemovirotherapeutic triple-therapy

Although the most promising results were seen in the chemovirotherapeutic combination of GLV-1h68 with *nab*-paclitaxel, for the treatment of pancreatic adenocarcinoma this chemotherapeutic agent isn't approved in monotherapy. Ideally, if inhibitory effects of GLV-1h68 on its combination partner could be excluded, GLV-1h68 should be given as add-on to established treatment protocols which already have proven their therapeutic value in clinical studies by improving the therapeutic outcome and prolonging patient survival. This consideration resulted in the final design of a chemovirotherapeutic protocol incorporating the vaccinia virus GLV-1h68 in combination with the approved dual chemotherapy *nab*-paclitaxel + gemcitabine.

3.3.1 Dual chemotherapy with *nab*-paclitaxel and gemcitabine

Given that the former LD25 doses of the chemotherapeutic agents were determined by their tumor cell killing after monotherapy, previously to the actual triple-therapy the partial doses of *nab*-paclitaxel and gemcitabine had to be adjusted to prevent the dual chemotherapy alone from resulting in a deceptive high cytotoxicity. Accordingly, the dual chemotherapy was conducted by varying the concentrations of both agents based on the respective LD25 doses in each tumor cell

line. Thereby, 3 different treatment groups with distinct dose variations were differentiated (specified in detail in section 2.2.5.2, p. 45). 72 hpt, the remaining cell masses were analyzed by SRB assay (Figure 29 and Figure 30).

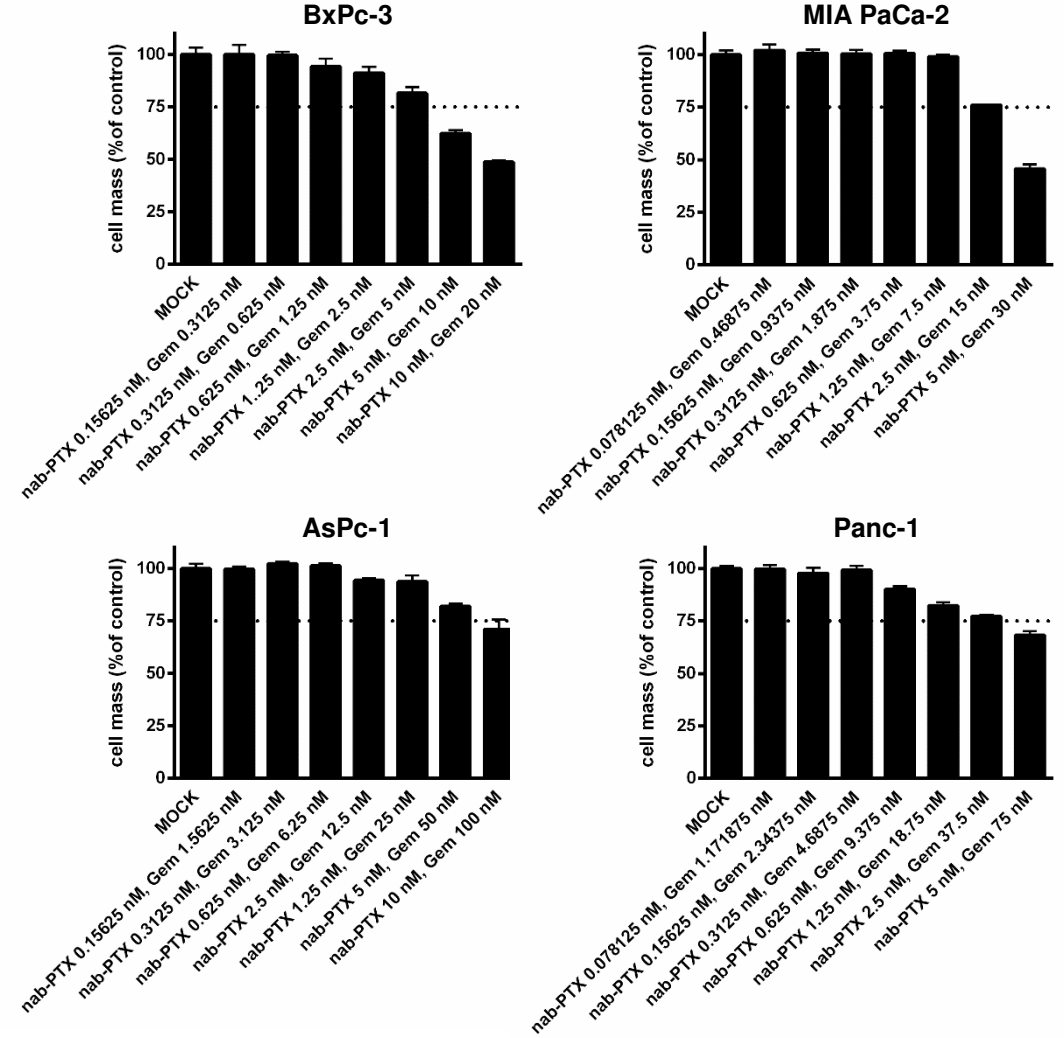

Figure 29 *Dual chemotherapy with nab-paclitaxel and gemcitabine in four different human pancreatic tumor cell lines in vitro. Starting from their previously determined LD25 doses a constant ratio of the cytotoxic agents was maintained by generating twofold dilutions (specified in detail in Table 2, p. 45). Remaining tumor cell mass was analyzed at 72 hours post treatment by sulforhodamine B (SRB) assay (n=1, mean and standard deviation are shown). Gem, gemcitabine. nab-PTX, nab-paclitaxel. MOCK, untreated control.*

When increasing the concentrations of both agents to the same degree, all 4 tumor cells responded dose-dependently to the dual chemotherapy. Thereby, a definite concentration threshold had to be trespassed before any cytotoxic effect could be seen. This condition was mainly apparent in MIA PaCa-2 cells, in which concentrations almost reaching the respective LD25 doses had to administered, while AsPc-1, BxPc-3 and Panc-1 cells already responded to lower doses of the

dual chemotherapy, although the initial response was poor. Interestingly, in AsPc-1 and Panc-1 cells the application of both agents in the full LD25 doses merely led to a tumor cell killing comparable to the results seen after monotherapy alone, whereas BxPc-3 and MIA PaCa-2 cells responded more strongly when both agents were combined in their respective LD25 doses. Yet, as concluded before, in BxPc-3 cells the LD25 dose of *nab*-paclitaxel may have been too high, therefore potentially mistaking the apparently strong response as additional effect of the dual chemotherapy. Eventually, in reference to the results after monotherapy, 1 out of 4 tumor cell lines demonstrated a definitely enhanced cytotoxic effect of the dual chemotherapy with *nab*-paclitaxel and gemcitabine.

Based on these results the following concentrations of *nab*-paclitaxel and gemcitabine were chosen for the chemovirotherapeutic triple-therapy (Table 10).

Table 10 *Partial ('adjusted') doses of nab-paclitaxel and gemcitabine for the triple-therapy*

Tumor cell lines	Partial doses	
AsPc-1	*nab*-PTX: 10 nM	Gem: 100 nM
BxPc-3	*nab*-PTX: 2.5 nM	Gem: 5 nM
MIA PaCa-2	*nab*-PTX: 2.5 nM	Gem: 15 nM
Panc-1	*nab*-PTX: 2.5 nM	Gem: 37.5 nM

Abbreviations: Gem, gemcitabine; *nab*-PTX, *nab-paclitaxel*.

In the other 2 treatment groups, in which the concentrations of one chemotherapeutic agent were varied while the other was administered constantly at 50% of its LD25 dose, differences in the influence of *nab*-paclitaxel and gemcitabine on the tumor cell lines could be seen (Figure 30). Panc-1 cells indeed responded dose-dependently to gemcitabine but already in 50% of the LD25 dose gemcitabine led to a considerable tumor cell killing virtually independent of any further dose increases of *nab*-paclitaxel. In MIA PaCa-2 cells constant doses of *nab*-paclitaxel and gemcitabine both were sufficient to induce a primary response to the dual chemotherapy which could be further enhanced by increasing the dose of the combination partner. Thereby, dose increases of *nab*-paclitaxel resulted in an enhanced tumor cell killing at the whole range of concentrations, while the

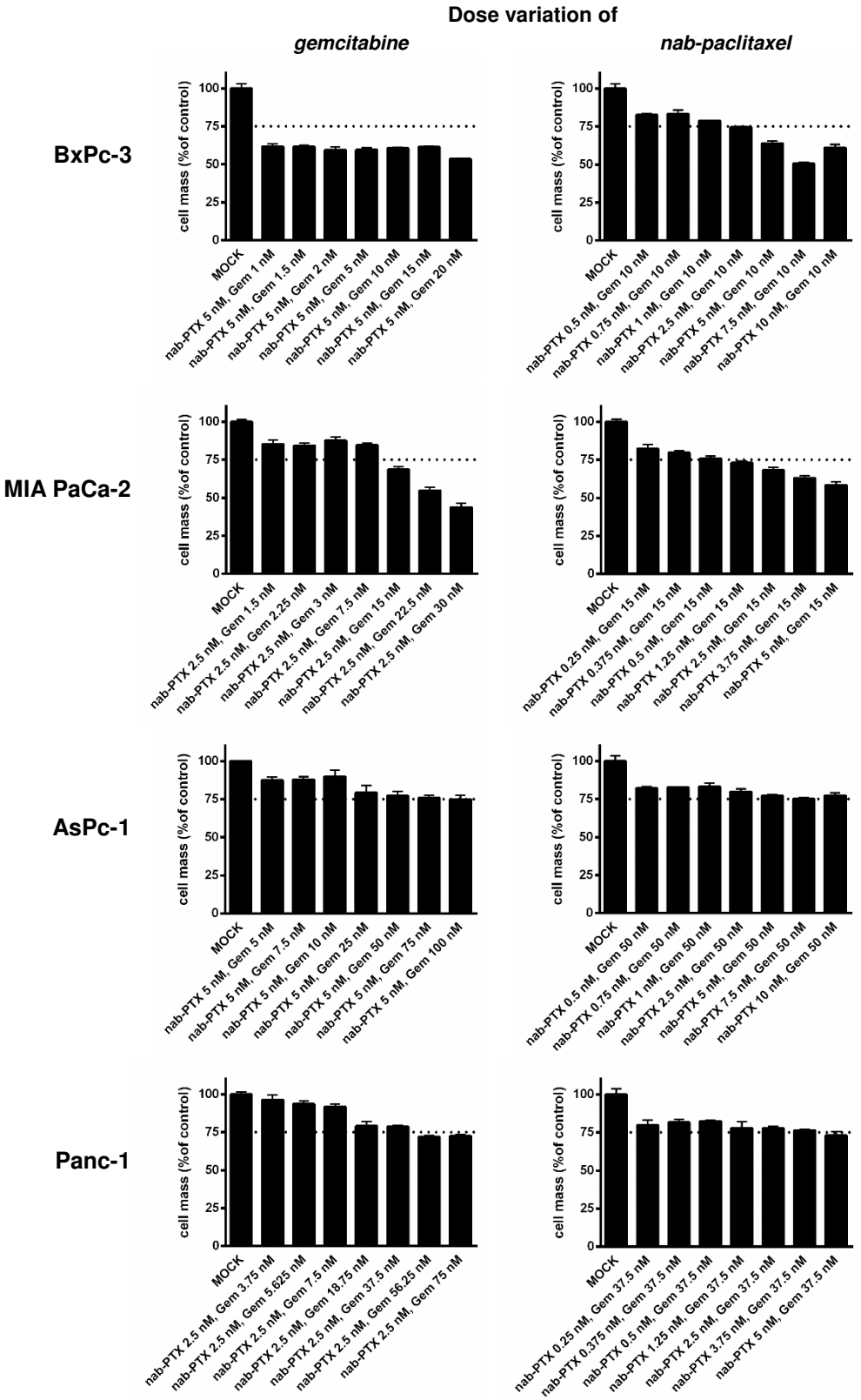

Figure 30 *Dose variations of the dual chemotherapy in four different human pancreatic tumor cell lines in vitro. Treatment doses of gemcitabine (left panel) or nab-paclitaxel (right panel) were varied while the combination partner was constantly applied in 50 % of its previously determined LD25 dose (specified in detail in Table 3, p. 45 and Table 4, p. 46 respectively). Remaining tumor cell mass was analyzed at 72 hours post treatment by sulforhodamine B (SRB) assay (n=1, mean and standard deviation are shown). Gem, gemcitabine. nab-PTX, nab-paclitaxel. MOCK, untreated control.*

dose of gemcitabine had to trespass a certain concentration threshold. A similar primary response also was seen in BxPc-3 cells but only when the gemcitabine concentration remained constant. In the other treatment group, the application of constant doses of *nab*-paclitaxel resulted in a considerable tumor cell loss independently of the concentration of gemcitabine. However, as concluded before, the chosen LD25 doses of *nab*-paclitaxel might have been too high and therefore might have disguised an additional effect of gemcitabine. AsPc-1 cells responded similarly to both variation settings, demonstrating a primary response which could be slightly enhanced by dose increases of the combination partner.

Although in these experiments, by varying the concentration of the combined agents and analyzing their effect on different tumor cell lines, only two parameters of combination therapy were investigated, it has to be reasoned clearly that even in case of only two agents being combined rather complex interactions can be observed.

3.3.2 Combination of GLV-1h68 with the dual chemotherapy

After reviewing and considering the results of the previously performed combination therapies, a final chemovirotherapeutic protocol imbedding GLV-1h68 in combination with *nab*-paclitaxel + gemcitabine was devised.

Since in the previous settings no application sequence was found to result in a superior or inferior cytotoxicity, the dual chemotherapy was administered concomitantly to the virotherapeutic treatment. Thereby, adapting the approved and actually performed clinical protocol of the dual chemotherapy, *nab*-paclitaxel was applied first (1 hpi) while gemcitabine was added half an hour later (1.5 hpi). Moreover, after reviewing the obtained results after monovirotherapy, GLV-1h68 was administered in three different virus doses (MOIs), one being the previously determined LD25 dose whereas the other two resembled similar virus doses. At 72 hpi, the remaining cell masses and cell viabilities were analyzed by SRB, CTB and MTT assay respectively (Figure 31).

Additionally to the previously applied evaluation criteria, it now had to be verified whether the results of the 3 different assays were consistent or whether various

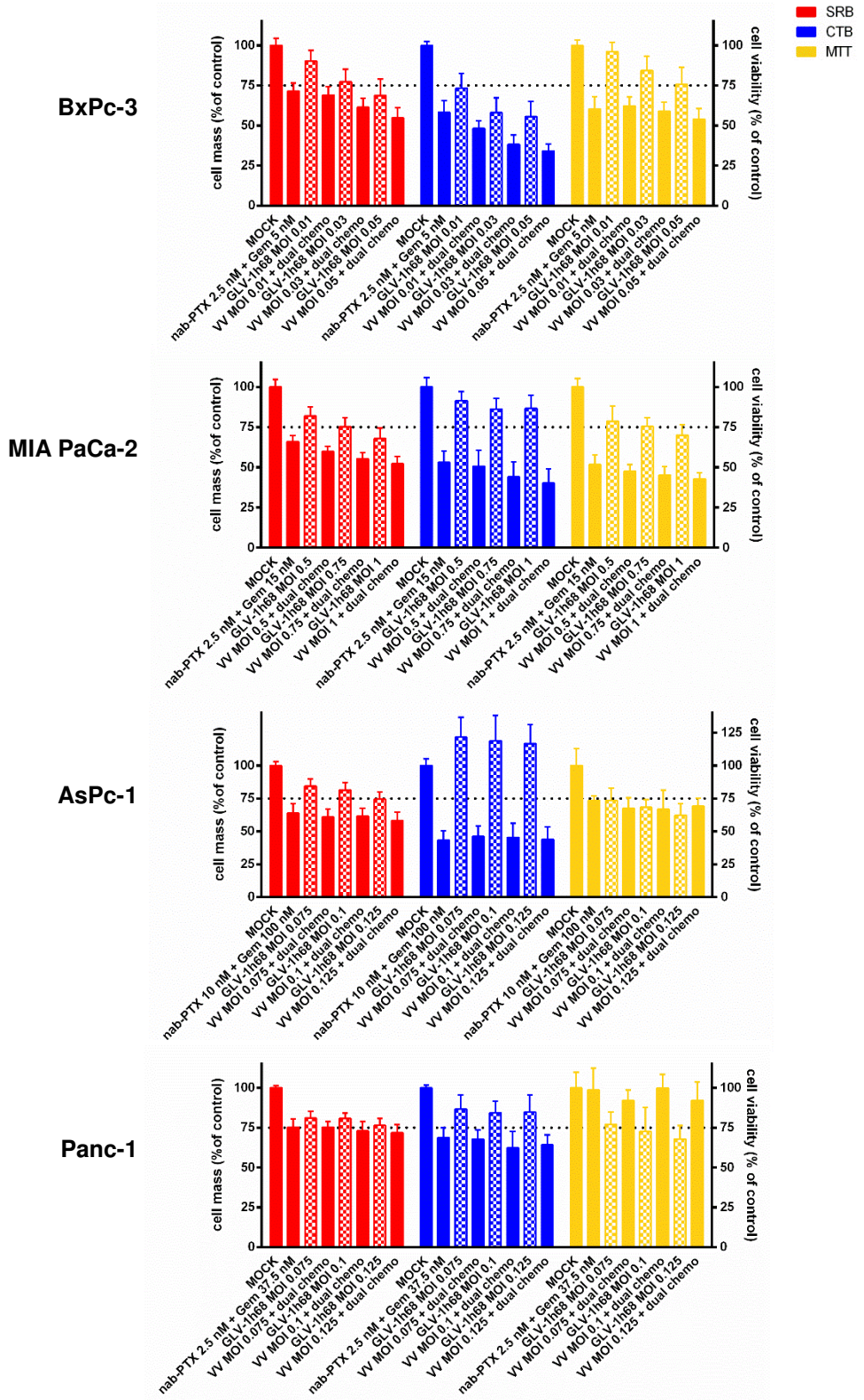

Figure 31 *Triple-therapy with GLV-1h68 and the dual chemotherapy nab-PTX (added at 1 hpi) + Gem (added at 1.5 hpi) in four different pancreatic tumor cell lines. Remaining cell mass and cell viability after 72 h treatment at increasing doses of GLV-1h68 were analyzed by the sulforhodamine (SRB) assay (depicted in red bars), the CellTiter-Blue® (CTB) assay (depicted in blue bars) and the MTT assay (depicted in yellow bars), respectively (n ≥ 3, mean and standard deviation are shown). nab-PTX, nab-paclitaxel. Gem, gemcitabine. hpi, hour(s) post infection. MOCK, untreated control. MOI, multiplicity of infection. VV, vaccinia virus GLV-1h68. Figure of SRB and CTB values published in (139).*

conclusions could be drawn from the individual testing method. In doing so, all 3 assays agreed that in BxPc-3 and MIA PaCa-2 cells the response after chemovirotherapy was clearly enhanced compared to the respective monotherapies. In the same 2 tumor cell lines, an increase of the virus dose also led to an increase of tumor cell killing, not only in the monotherapeutic but in the chemovirotherapeutic treatment arm as well. On the contrary this condition was barely apparent in AsPc-1 and Panc-1 cells. Considering the remaining cell masses of the particular 'monotherapies' in all 4 tumor cell lines suitable concentrations of the chemo- and virotherapeutic agents were chosen.

Compared to the untreated control (MOCK), in AsPc-1 cells the CTB assay even demonstrated an increased cell viability after monovirotherapeutic treatment, which could be strongly reduced in combination with the dual chemotherapy. These data disagree with the otherwise measured cell viability in the MTT assay which demonstrated that virotherapy with GLV-1h68 didn't increase the cell metabolism of AsPc-1 cells. Whether this condition in AsPc-1 cells was restricted to the metabolism of *resazurin* to *resorufin* only (similar metabolic pathways might be involved as well), or whether cell metabolism was enhanced in general while the metabolism of MTT to *formazan* remained unaffected by virotherapeutic treatment with GLV-1h68, can't be determined on the basis of the current data.

On the contrary, although the MTT assay validated the results in the better responding BxPc-3 and MIA-Paca-2 cells, in Panc-1 cells an antagonistic response of the triple-therapy was seen. With the exception of one setting (GLV-1h68 + *nab*-PTX -24 hpi), all of the previous results had indicated that the outcome after combination therapy at least resembled the better outcome seen after single viro- or chemotherapy. Now, Panc-1 cells indeed responded dose-dependently to the monovirotherapeutic treatment with GLV-1h68 but in combination with the dual chemotherapy cell viability rose again to the inferior outcome seen after dual chemotherapy alone. Considering the results obtained with the SRB assay, in which the dual chemotherapy alone as well as in combination with GLV-1h68 led to a substantial decrease of tumor cell mass, these data suggest an enhanced

cell metabolism of the remaining tumor cells, at least with regard to the metabolism of MTT to *formazan*.

Along with the previously illustrated response of AsPc-1 cells to virotherapeutic treatment, these data clearly disagree with the values of cell viability measured in the CTB assay. In these 2 tumor cell lines this fact therefore indicates a diversified influence of viro- and/or chemotherapeutic treatment on varying domains of cellular activity and not on cell metabolism in general.

Regardless of these differences, compared to the outcome after chemo- or virotherapy alone, all 3 assays agree on the central finding that in BxPc-3 and MIA PaCa-2 cells the triple-therapy was beneficial whereas in the other 2 cell lines it was not. Moreover, the combination of GLV-1h68 with *nab*-paclitaxel and gemcitabine caused the greatest increase of therapeutic efficacy seen during this investigation.

3.3.3 Influence of the dual chemotherapy on the replication of GLV-1h68

With regard to parameters potentially constraining the outcome of chemovirotherapy it has to be considered that any chemotherapeutic agent might interfere with the replication of the viral agent given in combination. Independently of long-term consequences this initially would lead to a considerable repression of virus-mediated oncolysis. Signs of detrimental interactions between the agents were thereby first seen in a cell line-dependent reduction of viral GFP expression after adding the dual chemotherapy (Figure 32). Interestingly, this was only the case in AsPc-1 and Panc-1 cells in which the triple-therapy had caused no further cytotoxicity compared to the respective monotherapies. In the superior responding BxPc-3 and MIA PaCa-2 cells viral GFP expression remained strong.

To fully assess the influence of the dual chemotherapy *nab*-paclitaxel + gemcitabine on viral replication of GLV-1h68, virus growth curves were generated. Given that an actual beneficial response after treatment with the triple-therapy was seen only in 2 of the 4 tumor cell lines it was of especial interest whether this condition was linked to an altered viral replication of GLV-1h68. Therefore, according to the schedule of the triple-therapy, GLV-1h68 was administered with or without the

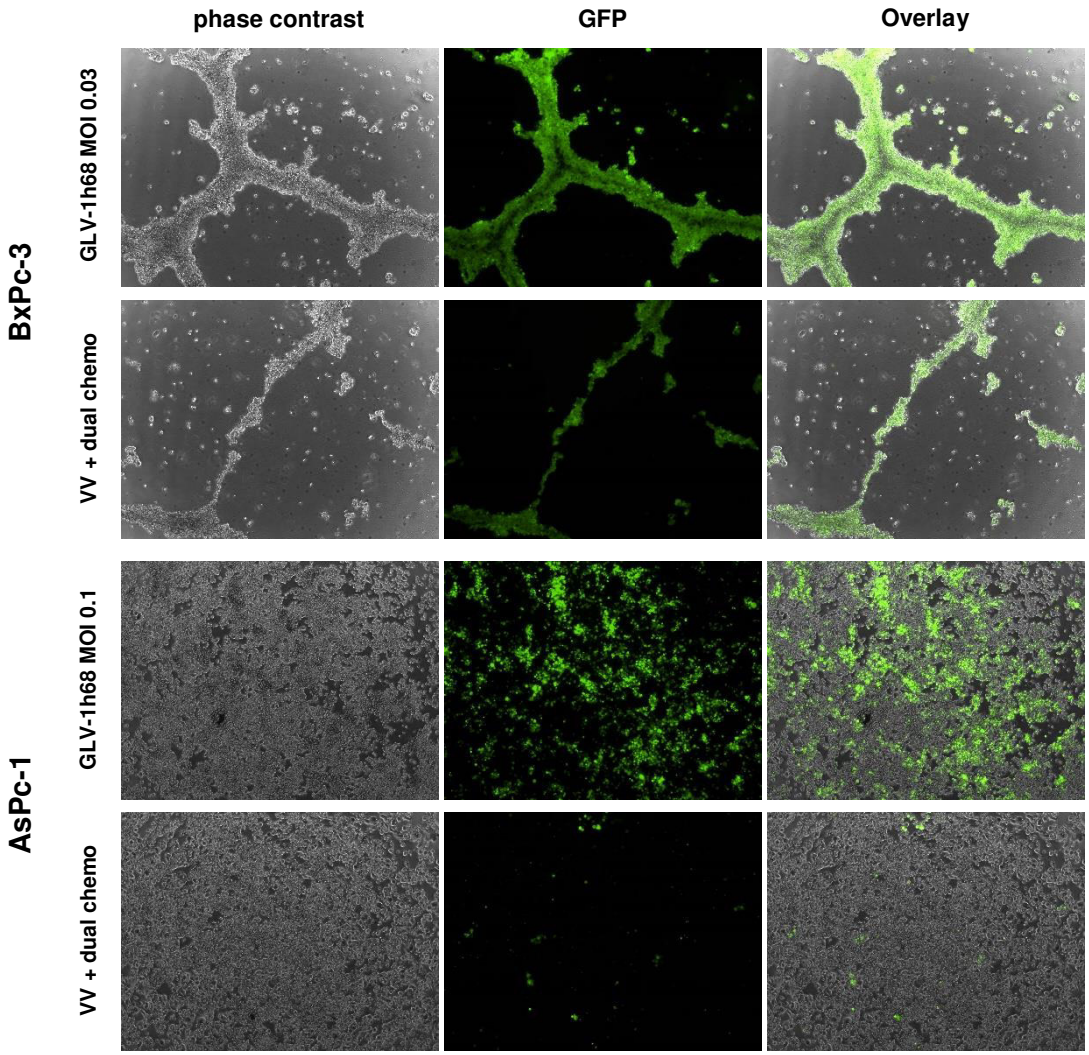

Figure 32 *Viral GFP expression after the triple-therapy at 72 hpi in BxPc-3 and AsPc-1 cells infected with the medium viral dose previously administered in the triple-therapy setting (Figure 31). Expression of green fluorescent protein (GFP) indicating viral infection (central panels), corresponding phase contrast pictures (left panels) and the respective overlay images (right panels) are shown (scale 1:190). hpi, hour(s) post infection. MOI, multiplicity of infection. VV, vaccinia virus GLV-1h68.*

dual chemotherapy. At certain time points post infection (1.5 hpi, 24 hpi, 48 hpi, 72 hpi and 96 hpi) the corresponding virus titer was quantified by virus titration (Figure 33). Tumor cells thereby were infected with the highest virus doses administered previously.

As a matter of fact, among the 4 tumor cell lines notable differences regarding the viral replication were seen. Considering the substantial increase of virus titer over time in the monovirotherapeutic treatment arm, potent viral replication was seen in all 4 tumor cell lines, proving that each tumor cell line could be successfully infected with GLV-1h68. Yet, in AsPc-1 and Panc-1 cells the addition of *nab*-

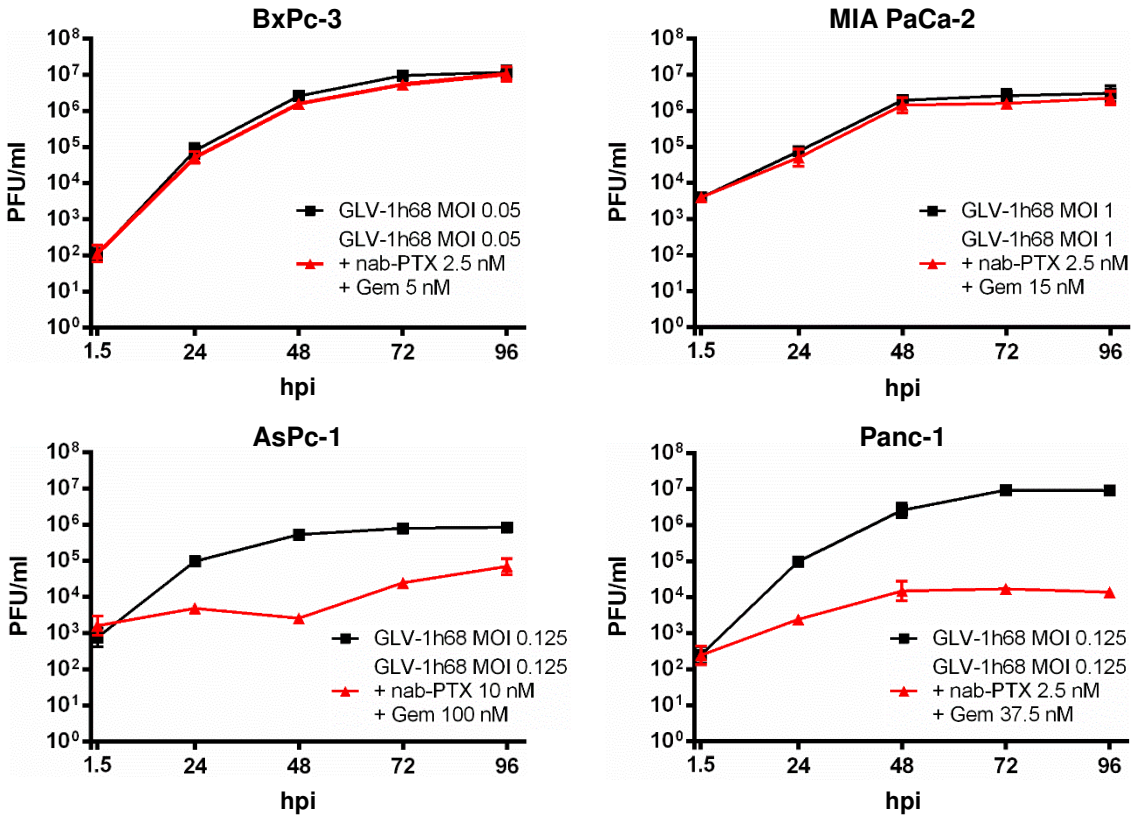

Figure 33 *Effect of the dual chemotherapy nab-paclitaxel + gemcitabine on the viral replication of GLV-1h68 in four different pancreatic tumor cell lines. Tumor cells were treated according to the previously described combination setting with the highest used MOI in either cell line (Figure 31). At five given time points (1.5, 24, 48, 72, 96 hpi) tumor cells were harvested, followed by virus titer determination [PFU - plaque forming units] (n=3, mean and standard deviation are shown). nab-PTX, nab-paclitaxel. Gem, gemcitabine. hpi, hour(s) post infection. MOI, multiplicity of infection. Figure modified from this version published in (139).*

paclitaxel + gemcitabine led to a considerable reduction of viral replication paralleling the previously observed decrease of viral GFP expression. On the contrary, in BxPc-3 and MIA PaCa-2 cells, viral replication of GLV-1h68 remained potent and resembled the virus titers after monovirotherapy. Therefore, the negative influence of the dual chemotherapy on viral replication of GLV-1h68 was found to be directly linked to a loss of therapeutic benefit otherwise seen after the triple-therapy.

In conclusion, although no definite statement can be made without further knowledge of other parameters potentially determining success or failure of the triple-therapy, these data suggest that an unhindered virus replication represents a mandatory requirement for a possible success of this chemovirotherapeutic protocol.

3.3.4 Influence of *nab*-paclitaxel on viral replication of GLV-1h68

It was assumed that the observed reduction of viral titers in AsPc-1 and Panc-1 cells was the result of overdosing one or even both chemotherapeutic agents. To assess the influence of each of the chemotherapeutic agents on viral replication of GLV-1h68 separately, first, virus growth curves in combination with *nab*-paclitaxel were generated. For this investigation, the responsive cell line BxPc-3 was selected to evaluate whether the unfavorable environment for viral replication in AsPc-1 and Panc-1 cells could be emulated by administering higher doses of the chemotherapeutic agent.

According to the schedule of the triple-therapy BxPc-3 cells were treated first with GLV-1h68 and at 1 hpi with ascending doses of *nab*-paclitaxel while viral titers were determined as previously (Figure 34). Additionally, viral GFP expression in the corresponding tumor cells was visualized at 72 hpi (Figure 35).

Compared to the viral titer seen after GLV-1h68 treatment alone, *nab*-paclitaxel was found not to influence viral replication in BxPc-3 cells, even in the highest administered concentration of 25 nM (Figure 34). Since an unchanged viral replication had previously been found to determine the therapeutic benefit of the triple-therapy it was hypothesized that *nab*-paclitaxel didn't negatively influence the outcome in AsPc-1 and Panc-1 cells.

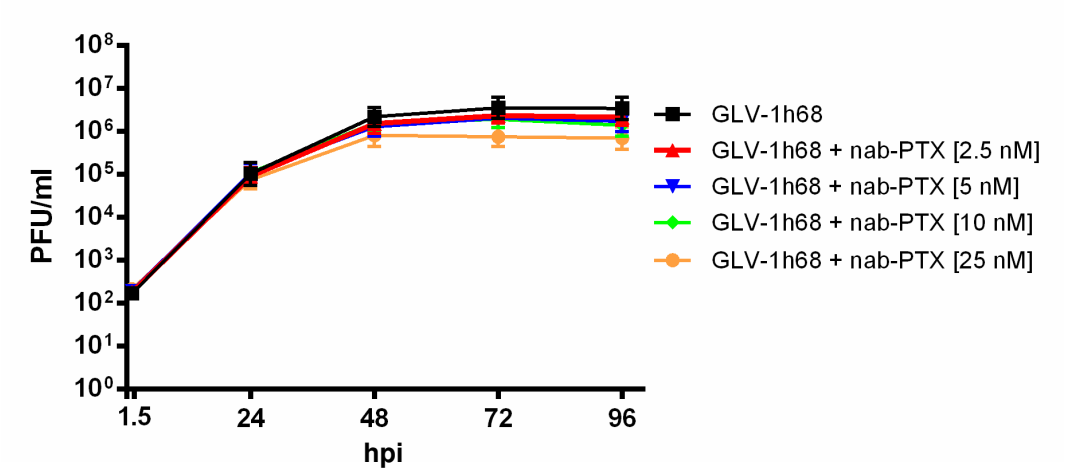

Figure 34 *Viral titers of GLV-1h68 under the influence of nab-*paclitaxel *(nab-PTX) in the pancreatic tumor cell line BxPc-3. Tumor cells were infected with GLV-1h68 (MOI 0.05). At 1 hpi, the inoculum was removed and medium containing nab-PTX was added. At five given time points (1.5, 24, 48, 72, 96 hpi) tumor cells were harvested, followed by virus titer determinations [PFU, plaque forming units] (n=3, mean and standard deviation are shown). hpi, hour(s) post infection.*

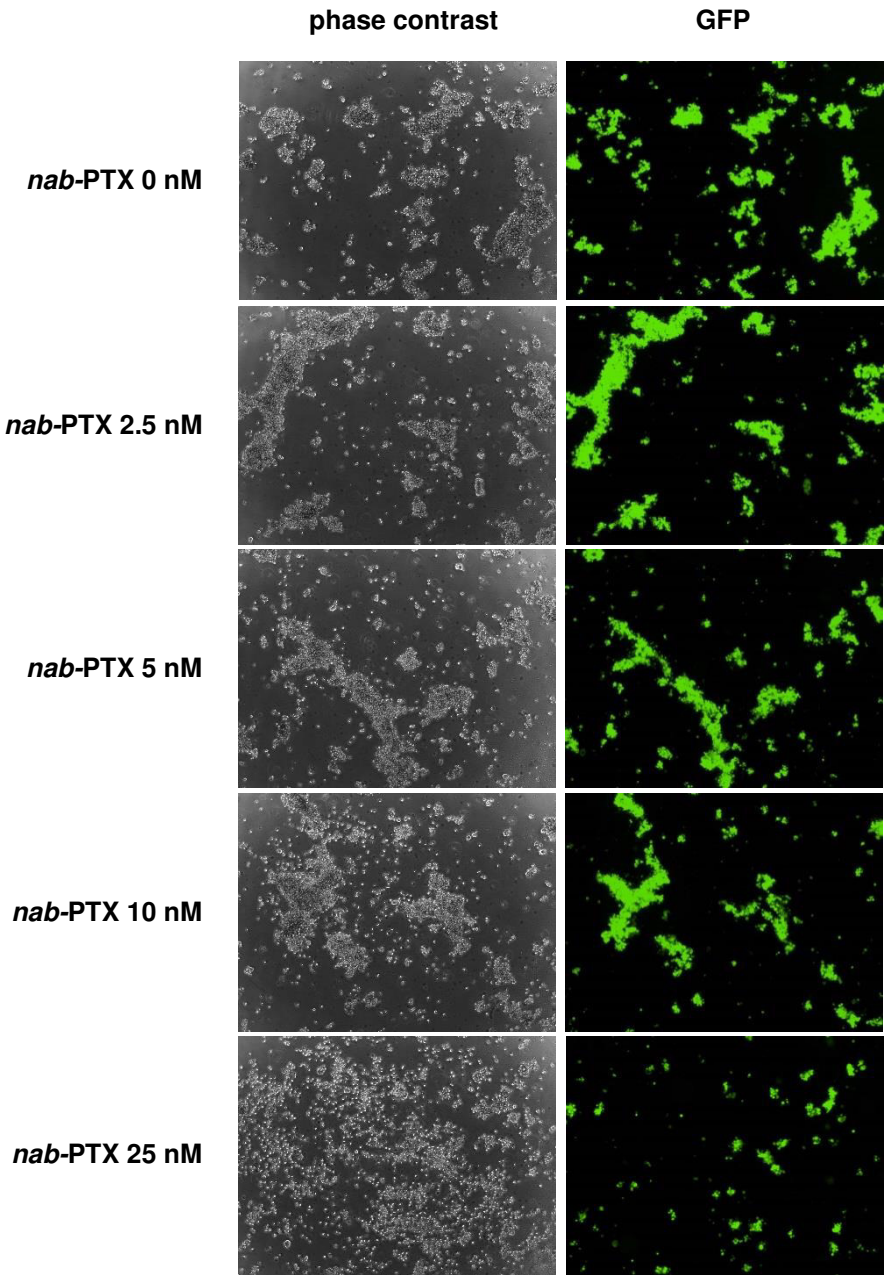

Figure 35 *Effect of nab-*paclitaxel *on viral infection in BxPc-3 cells analog to the previously measured viral titers at 72 hpi (Figure 34). Expression of green fluorescent protein (GFP) indicating viral infection (right panels) and corresponding phase contrast pictures (left panels) are shown (scale 1:190). hpi, hour(s) post infection. nab-PTX, nab-*paclitaxel.

On the contrary in the associated microscopic images it seemed that the levels of GFP expression decline when *nab*-paclitaxel was administered in a concentration of 25 nM (Figure 35). Moreover, the remaining tumor cell mass in the corresponding phase contrast images appears to increase with ascending doses of *nab*-paclitaxel. However, as visualized previously (Figure 18) virus-mediated oncolysis kills cells differently than chemotherapeutic agents. OVs spread outwards

from the primary infection site leaving only strongly GFP expressing tumor cell clusters at the margin whereas chemotherapy kills all tumor cells homogenously. The latter results in a thin cell layer of evenly spread tumor cells which only suggests a weak GFP expression. Since viral infection could clearly be detected at all administered doses of *nab*-paclitaxel this clarified a strong viral replication of GLV-1h68 in BxPc-3 cells additionally undergoing chemotherapy with *nab*-paclitaxel.

3.3.5 Influence of gemcitabine on viral replication of GLV-1h68

In a next step, the effect of gemcitabine on viral titers of GLV-1h68 was determined. Similarly to the previous setting, viral growth curves were generated by treating BxPc-3 cells first with GLV-1h68 and at 1.5 hpi with ascending doses of gemcitabine (Figure 36). As before, GFP expression of GLV-1h68 was visualized in the corresponding tumor cells at 72 hpi (Figure 37).

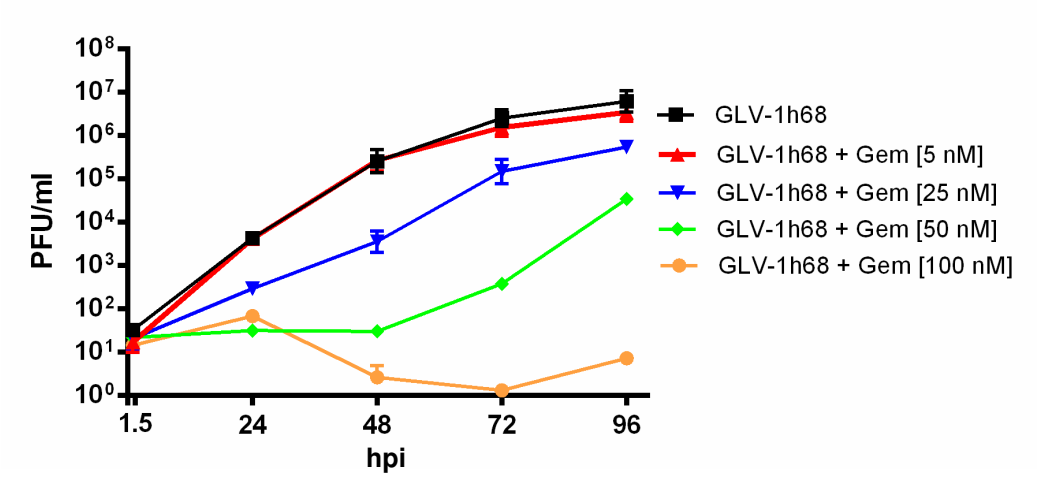

Figure 36 *Viral titers of GLV-1h68 under the influence of gemcitabine (Gem) in the pancreatic tumor cell line BxPc-3. Tumor cells were infected with GLV-1h68 (MOI 0.05). At 1 hpi, the inoculum was removed and normal growth medium was added. Half an hour later (1.5 hpi), gemcitabine was added. At five given time points (1.5, 24, 48, 72, 96 hpi) tumor cells were harvested, followed by virus titer determinations [PFU, plaque forming units] (n=3, mean and standard deviation are shown). hpi, hour(s) post infection.*

This time, higher doses of the chemotherapeutic agent, i.e. gemcitabine concentrations ≥ 25 nM, positively correlated with a significant decline of viral titers (Figure 36). Accordingly, viral replication in BxPc-3 cells could be suppressed to a similar level as seen before in AsPc-1 and Panc-1 cells after adding the dual chemotherapy. Gemcitabine concentrations of 100 nM even were able to reduce

viral titers below their baseline level at 1.5 hpi. Such high-dose administration of gemcitabine therefore nearly completely shut down viral replication.

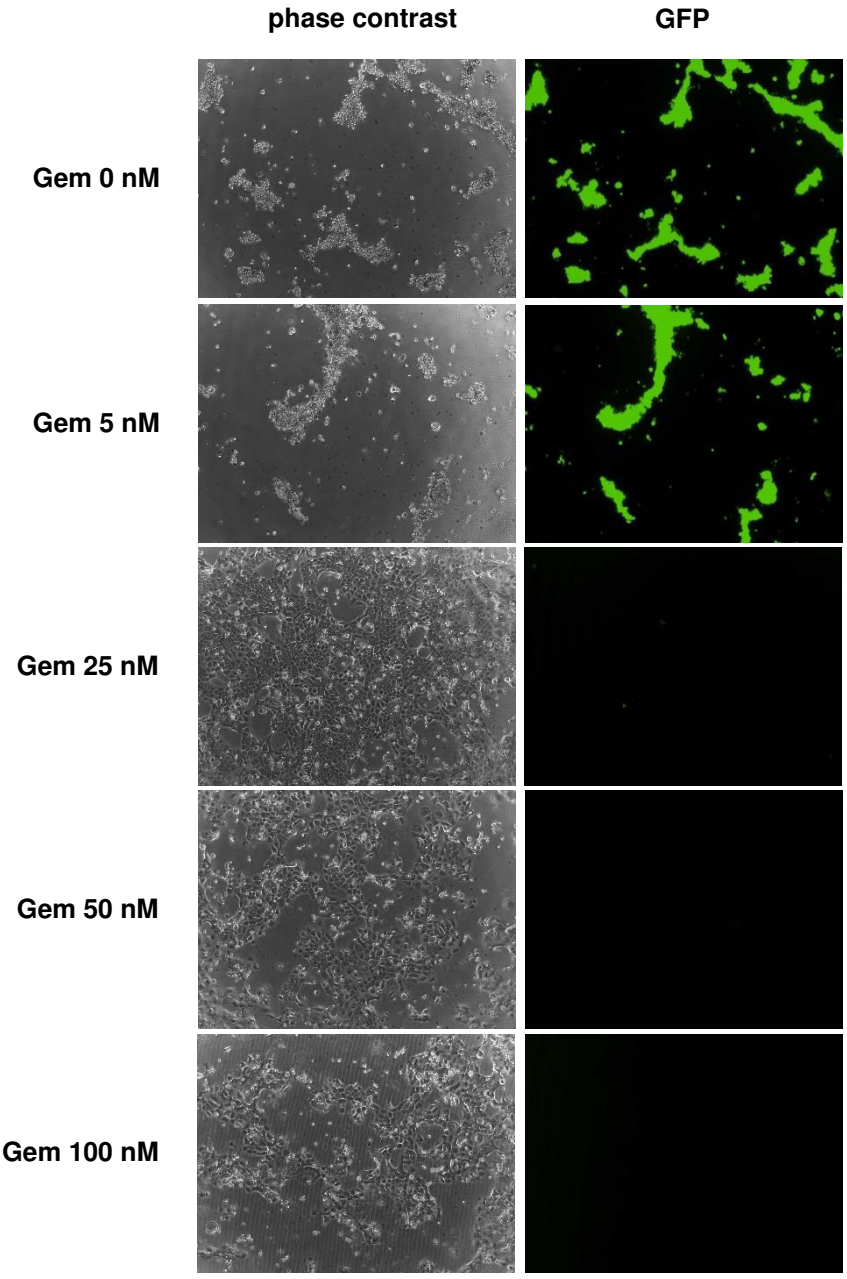

Figure 37 *Effect of gemcitabine on viral infection in BxPc-3 cells analog to the previously measured viral titers at 72 hpi (Figure 36). Expression of green fluorescent protein (GFP) indicating viral infection (right panels) and corresponding phase contrast pictures (left panels) are shown (scale 1:190). hpi, hour(s) post infection. Gem, gemcitabine.*

These findings were also reflected in a dose-dependent cut-off of viral GFP expression visualized at 72 hpi (Figure 37). While low doses of gemcitabine (< 25 nM) neither influenced viral titers nor viral GFP expression, concentrations of 25 nM and higher significantly decreased viral titers and completely prevented viral

GFP expression. The phase contrast images thereby confirm the abrupt loss of oncolytic efficacy. Only at higher gemcitabine concentrations the cytotoxic effect of the chemotherapy was able to catch up with the oncolytic effect of GLV-1h68.

In a final step, it was assessed whether a delayed administration of gemcitabine would give GLV-1h68 enough time to reach sufficient viral titers able to outweigh the poor performance of viral replication. Accordingly, the timing of gemcitabine administration was varied (1.5 h or 24 hpi) and its influence on GFP expression visualized at 72 hpi (Figure 38).

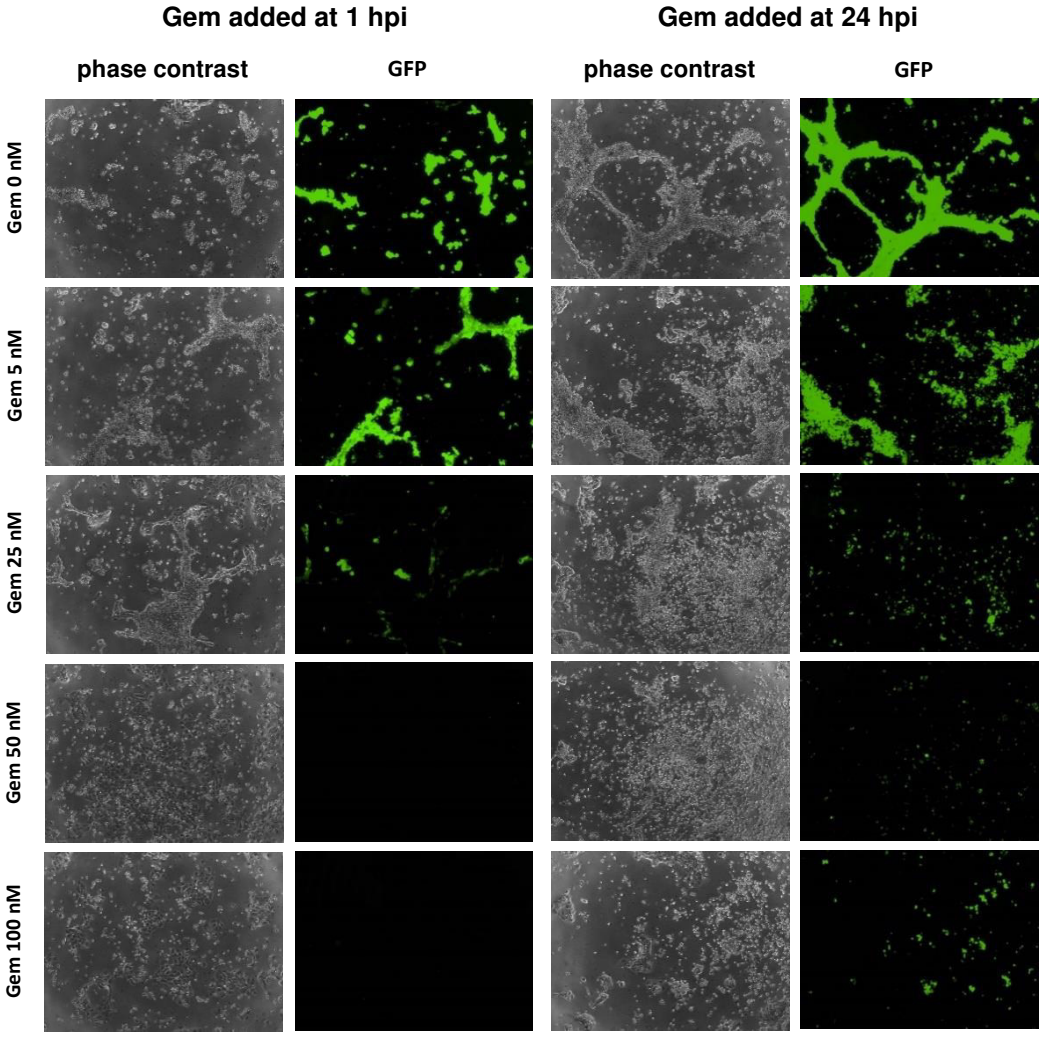

Figure 38 *Time-dependent effect of gemcitabine on viral infection in BxPc-3 cells at 72 hpi. Tumor cells were infected with GLV-1h68. At 1 hpi, the medium was changed while Gem was added either at 1 or 24 hpi. Expression of green fluorescent protein (GFP) indicating viral infections (right panels) and corresponding phase contrast pictures (left panels) are shown (scale 1:140). Gem, gemcitabine. hpi, hour(s) post infection.*

As a result, viral GFP expression was similarly influenced independently of the point in time when gemcitabine was administered (1 hpi or 24 hpi). Although the cut-off of fluorescence at concentrations of 25 nM wasn't as evident as before, a severe decrease of GFP expression could be seen at this dose in both settings. However, when gemcitabine was administered at 24 hpi, fluorescence could be visually detected even in high-dose (> 25 nM) regimens. These findings indicate that a delayed administration of gemcitabine provides time for viral replication to somewhat compensate chemotherapy-related detrimental decreases of the viral titer.

In conclusion, gemcitabine was demonstrated to severely restrain viral replication and gene expression of GLV-1h68 in the pancreatic tumor cell line BxPc-3. This was not only a dose-dependent effect but was also influenced by the point in time when gemcitabine was added. Since concurrent *nab*-paclitaxel administration was demonstrated to leave viral titers unchanged it has to be assumed that in the cell lines AsPc-1 and Panc-1 too high doses of gemcitabine were the reason for the negative influence on viral replication thereby impeding therapeutic benefit of the triple-therapy.

4 Discussion

To date, despite intensive research over the past decades, so far only one single virotherapeutic protocol has received approval for oncologic treatment in the western hemisphere. As the first of its kind the GM-CSF expressing oncolytic herpes virus talimogene laherparepvec (T-VEC, IMLYGIC™) only recently achieved FDA and EMA approval for the monotherapeutic treatment of metastatic melanoma in 2015 (43). However, both preclinical and clinical data of the last decade indicate that only in combination with already approved treatment modalities such as chemotherapy, the true therapeutic potential of oncolytic virotherapy may be unlocked. Basic considerations for a possible success of this chemovirotherapeutic approach include a more potent initial response of tumor cells to a combination therapy, leading to a stabilization or even regression of the tumor disease, combined with a virus-mediated induction of a strong antitumor immune response in the long-term.

Prerequisites for a successful combination therapy involve basic parameters such as concentrations and the administration sequence of the single agents. Possible dose-dependent interactions between the combination partners might affect the treatment outcome and therefore the success of combinatorial treatment. In case of chemovirotherapy, while the initial cytotoxic effect of the combination can be measured and quantified by means of approved testing methods both *in vitro* and *in vivo*, it is still difficult and nearly impracticable to prove possible immune-mediated long-term consequences. This would require not only extensive and time-consuming experiments in immunocompetent animals or human patients, but also the design of reliable but so far unfortunately only unstandardized testing methods. In contrast, approaches measuring the viral replication as surrogate parameter for potent initial and long-term efficacy of virotherapy have been developed and are aiming at giving evidence in this complex matter.

In this thesis, the chemovirotherapeutic combination of oncolytic vaccinia virus GLV-1h68 with various chemotherapeutic agents as a novel treatment option for advanced pancreatic adenocarcinoma was investigated *in vitro*. Based on promising intermediate results and in accordance with currently valid guidelines for the

treatment of locally advanced / metastatic pancreatic cancer, a triple-therapy protocol incorporating GLV-1h68 and the clinically approved dual chemotherapy *nab*-paclitaxel + gemcitabine was devised. Finally, the influence of the dual chemotherapy on viral replication of GLV-1h68 was analyzed.

4.1 Resistance of pancreatic cancer to chemo- and virotherapy in vitro

Main issue in developing more effective treatment modalities for pancreatic ductal adenocarcinoma (PDA) is the overcoming of primary and (therapy-induced) secondary resistance mechanisms. In the clinical context, the resistance of PDA to chemotherapy is mediated by multiple factors: i) the intrinsic chemoresistance of tumor (stem) cells, ii) its hypoxic and highly immunosuppressive desmoplastic microenvironment, iii) drug-inhibiting matrix components and iv) a high interstitial pressure (144). Unfortunately, *in vitro* such conditions can't be reproduced adequately. Analyzing the cytotoxic effect of different agents on long-term cultivated cell lines of pancreatic adenocarcinoma therefore mainly focuses on evaluating their individual sensitivity/resistance to chemo- and/or virotherapy.

To clearly differentiate between sensitive and resistant tumor cell lines, it has to be elucidated first at which point a tumor cell line has to be regarded as resistant. Clinically, treating a cancer patient with a cytotoxic agent the tumor is resistant against would result in the same survival outcome as 'doing nothing specific' (i.e., best supportive care) and unnecessarily expose the patient to drug-induced toxicity. However, *in vitro* even resistant tumor cells will be killed if only the concentrations reach levels sufficient enough to outweigh the diverse resistance mechanisms. Since high-dose application in the clinic is undeniably paralleled by an increase of toxicity, a tumor cell line *in vitro* could therefore be considered resistant if it doesn't respond to concentrations that are achievable *in vivo* (145). The fact that even *in vitro* highly-complex bioinformatic analyses are necessary to identify tumor-specific gene profiles and protein signatures that correlate with resistance to anticancer agents underlines the difficulty of this matter (146).

In more complex biologic systems, i.e. in animals or humans, therapeutic agents are subject to diverse degradation processes. Accordingly, the effective dose which mediates the tumor response at the tumor site will be much lower than the

originally applied dose and dose-dependent effects may differ from the *in vitro* results. The assignment of concentrations determined *in vitro* to the *in vivo* setting and vice versa is therefore a rather imprecise matter. Based on these considerations the monotherapeutic treatment of 4 established and well-characterized cell lines of PDA with different cytotoxic/oncolytic agents not only was a means of finding suitable doses for chemovirotherapy but also an evaluation of cell line-dependent levels of sensitivity/resistance to the respective single agents *in vitro*.

In line with previous reports (133), low sensitivity to standard of care gemcitabine has been found in Panc-1 cells when compared to the better responding BxPc-3 and MIA PaCa-2 cells (Figure 20). However, in the latter the increase of gemcitabine concentrations to levels higher than 0.1 µM barely enhanced the therapeutic effect. In these particular tumor cell lines, high-dose chemotherapy therefore may have been unable to further enhance the levels of cell death already induced by lower doses of gemcitabine. Prolongation of the chemotherapy on the other hand, may have provided the necessary time for more tumor cells to undergo chemotherapy-induced cell death independently of the applied concentration of the cytotoxic agent.

Panc-1 cells (and to a lesser extent AsPc-1 cells) were also shown to be among the least sensitive cell lines for 5-FU treatment correlating with the expression of a drug efflux transporter (147). Here, AsPc-1 cells outmatched the capability of Panc-1 cells to withstand high 5-FU concentrations (Figure 19). In this context it is of especial interest that AsPc-1 cells originate from a patient who underwent 5-FU-based chemotherapy previous to the process of tumor material sampling and establishment of this tumor cell line (148). Reason for the observed survival advantage might therefore be a secondary acquired resistance to 5-FU.

The collected data also indicate resistance of all 4 tumor cell lines both to the platinum compound oxaliplatin and the topoisomerase I inhibitor irinotecan. Initial signs of cytotoxicity were seen only at concentrations of 1 µM oxaliplatin whereas even higher concentrations of this cytotoxic agent had to be administered to achieve considerable therapeutic efficacy (Figure 22). Clinically, although not directly compared to each other, with an overall survival of 3.4 months the outcome

after monotherapy with oxaliplatin (investigated in a rather small number of patients, n=17) (149) was even worse than after standard of care gemcitabine treatment (ranging from 5.91 to 6.8 months in recent trials) (Table 1). Previous reports also confirmed the low sensitivity to irinotecan *in vitro*, AsPc-1 and Panc-1 cells thereby being the least sensitive tumor cell lines (Figure 23) (128). These data are further confirmed by the fact that for the first-line treatment of advanced PDA both oxaliplatin and irinotecan are only used as part of the multidrug FOLFIRINOX regimen (4).

Similarly poor results were obtained with the EGFR-targeting tyrosine kinase inhibitor erlotinib (Figure 24). Initially, it was unclear whether the observed meager therapeutic outcome was the result of a low or missing cellular EGFR expression. However, previous investigations had shown that in many tumor cell lines of pancreatic cancer the EGF receptor is highly conserved: In Panc-1 cells a mutated EGFR gene is expressed whereas AsPc-1 and MIA PaCa-2 cells express the receptor in its wildtype form (150,151). Clinically, though, levels of EGFR expression didn't correlate with the survival benefit after combination therapy with gemcitabine and erlotinib (10). Predictive marker for therapeutic benefit was rather the development of a skin rash shortly after initiation of treatment. Thus, given that more reliable predictive markers are still missing, this therapeutic regimen in the clinic so far is performed on a 'trial and error' basis and was not further pursued during this investigation.

Nab-paclitaxel has only recently been approved for the treatment of advanced pancreatic cancer in combination with gemcitabine, after a phase III trial was able to demonstrate increased survival after the dual chemotherapy compared to standard of care gemcitabine (12). Moreover, *nab*-paclitaxel was found to deplete the desmoplastic stroma partly contributing to the substantial chemoresistance of PDA as well as to increase intratumoral levels of gemcitabine by 2.8-fold in tumor-bearing mice (152). The latter has been attributed to a *nab*-paclitaxel-mediated decrease of tumoral cytidine deaminase, mainly expressed in tumor epithelial cells and degrading gemcitabine into its therapeutically less active metabolites

(153). Here, however, such conditions can't be reproduced adequately with the selected array of tumor cell lines *in vitro*.

In this investigation, all 4 tumor cell lines responded similarly to a monotherapeutic treatment with *nab*-paclitaxel with a therapeutic cut-off at concentrations of 0.01 µM, showing a dramatic decline of remaining tumor cell masses (Figure 21). Moreover, in 1 out of 4 tumor cell lines (MIA PaCa-2), its combination with gemcitabine definitely resulted in an enhanced therapeutic response, whereas in BxPc-3 cells this may have been the result of overdosing *nab*-paclitaxel (Figure 29). Then again, the responses of the tumor cells to dose variations of one agent while administering its combination partner at 50 % of its respective LD25 dose were rather inconsistent (Figure 30). Dose increases of one cytotoxic agent either directly correlated with an additional reduction of tumor cell masses (MIA PaCa-2 / Panc-1: Gem ↑; BxPc-3 / MIA PaCa-2: *nab*-PTX ↑) or caused almost no further enhancement of tumor cell killing (BxPc-3 / AsPc-1: Gem ↑; AsPc-1 / Panc-1: *nab*-PTX ↑). Unfortunately, general statements on the particular importance of either *nab*-paclitaxel or gemcitabine on therapeutic success of the dual chemotherapy can't be made on the basis of these data.

Choosing the vaccinia virus GLV-1h68 for treatment of pancreatic adenocarcinoma was based on several good reasons. Even though *in vitro* conditions can't properly address this issue, the importance of the special tumor microenvironment of pancreatic cancer (specified more detailed in section 1.1.3, p. 5) is crucial in consideration for further research potential. In PDA, cancer-associated fibroblasts, playing an important role in mediating resistance to chemotherapeutic agents, are present in high numbers and have been shown to promote oncolytic virus infection (154). Speaking in favor of using a *Lister* strain derivative was the fact that, although the hypoxic conditions of PDA represent a considerable barrier for efficient virus infection and replication, neither virus protein expression nor viral titers of *Lister* strain derivatives of vaccinia virus were found to be reduced under such hypoxic conditions (155).

Screening the NCI-60 cancer cell lines uncovered different cell line-dependent levels of permissivity to GLV-1h68 infection (87). Although the pancreatic cancer

cell lines MIA PaCa-2 and Panc-1 were not among the most sensitive tumor cell lines for viral infection, therapeutic benefit after virotherapy with GLV-1h68 nonetheless has clearly been proven in such tumor-bearing mice (101). Coincidentally, *in vitro,* different levels of susceptibility to GLV1h68-mediated oncolysis were found. In accordance with these data, here, MIA PaCa-2 cells only responded to high viral doses while in BxPc-3 cells significantly lower viral doses were sufficient to achieve a potent oncolytic effect (AsPc-1 and Panc-1 cells ranging in between) (Figure 17). Moreover, since GLV-1h68 represents a biological agent which constantly changes its dose as a result of continuous viral replication and subsequent oncolytic cycles, it was hardly surprising that prolongation of the virotherapy led to a dramatic decline of tumor cell numbers. GLV-1h68 had more time to amplify its viral dose, to lyse its host cells and to spread further to yet uninfected tumor cells thereby starting the oncolytic cycle anew.

In conclusion, the collected evidence confirmed the generally low susceptibility of pancreatic cancer cells to cytotoxic agents *in vitro*. A poor therapeutic outcome was seen especially after chemotherapy with oxaliplatin, irinotecan or erlotinib which might be caused by an intrinsic resistance of pancreatic adenocarcinoma against these agents. Moreover, different tumor cell line dependent levels of susceptibility to the chemotherapeutic agents 5-FU, *nab*-paclitaxel and/or gemcitabine as well as to the virotherapeutic agent GLV-1h68 were found. Based on these data, different ('dose adjusted') chemovirotherapeutic protocols were devised, focusing on increasing the so far poor therapeutic outcome while also analyzing the interactions depending on the application sequence of chemo- and virotherapeutics.

4.2 Response of pancreatic cancer to chemovirotherapy

Initially, after combining GLV-1h68 with the nucleoside analogue 5-FU, oncolysis was not substantially changed in neither of the 4 tumor cell lines (Figure 25). Although antagonistic effects of this particular setting could therefore be excluded, the lack of therapeutic benefit stands in contrast to previous reports of successfully treating pancreatic adenocarcinoma with 5-FU and other viral constructs. *In vitro,* the combination of 5-FU with herpes virus-based agents potentiated the

antitumor response, which in one case was attributed to an enhanced viral replication (137), in another to an increase of apoptosis (135). The latter may be of especial interest since the deletion of an anti-apoptotic gene in the corresponding herpes virus L1BR1 may have partly contributed to the therapeutic effect. Administration of a parvovirus encoding a prodrug convertase (cytosine deaminase) in combination with its prodrug 5-FC two days after inoculation of AsPc-1 cells into the peritoneal cavity of nude mice completely prevented peritoneal dissemination (125). When administered two weeks after tumor inoculation the combination of virus and prodrug decreased CEA levels and significantly increased survival of tumor-bearing mice. *In vitro,* the conversion of 5-FC to its cytotoxic compound 5-FU caused a strong bystander effect. Interestingly, *in vivo* the therapeutic benefit after combining a conditionally replicating adenovirus with either 5-FU or gemcitabine wasn't influenced by the administration sequence although sequence-dependent synergism and antagonism had been seen *in vitro* before (131).

Gemcitabine, another nucleoside analogue, represents the current standard of care for advanced pancreatic cancer and has been combined with many viral constructs. Its combination with the oncolytic herpes virus AV25CDC *in vivo* not only strongly reduced the tumor weight of subcutaneously established and orthotopic tumors in nude mice but also in an immunocompetent Syrian hamster model (124). In other immunocompetent models gemcitabine-based combination protocols were shown to prolong tumor growth inhibition (126) and animal survival (133), while in the latter synergism of the parvovirus H-1PV and gemcitabine had previously been observed in BxPc-3, MIA PaCa-2 and Panc-1 cells *in vitro*. Clinically, systemic gemcitabine infusion has been successfully combined with intratumorally injected ONYX-15 in a phase I/II trial (156). However, although chemovirotherapy was found to be well tolerated, the treated cohort of 18 patients was too small for the read-out of potentially meaningful therapeutic benefit. Unfortunately, since further research on ONYX-015 was stopped in 2003, these investigations led down a blind alley and no other chemovirotherapeutic protocol addressing pancreatic cancer has yet reached phase III. Currently, trials incorporating the hyaluronidase expressing adenoviral vector VCN-01 or the naturally occurring reovirus Reolysin® are under way (59).

Gemcitabine has also been previously combined with GLV-1h68 *in vivo*. Chemovirotherapy accelerated tumor shrinkage and inhibited the growth of subcutaneous Panc-1 tumors in nude mice (101). However, unlike the combination of GLV-1h68 with cisplatin, after which complete remissions were seen in 7 of 8 tumor-bearing mice, the gemcitabine combination protocol wasn't able to completely eradicate the tumor disease. In addition, monotherapy with GLV-1h68 alone was able to catch up with the therapeutic outcome when only given sufficient time. In this study, chemovirotherapeutic treatment with GLV-1h68 and gemcitabine *in vitro* did not result in any considerable increase of cytotoxicity in neither of the administration settings (Figure 26), paralleling the previously obtained results in combination with 5-FU. Reason for the lack of therapeutic benefit may be that both chemotherapeutic agents are nucleoside analogues. Given that the main mechanism of action of such agents is the interference with cellular DNA replication, unintentionally, the replication of the DNA virus GLV-1h168 may have been inhibited as well, thereby reducing its oncolytic potency. Factors possibly mediating the difference between the *in vitro* and *in vivo* results may have been the greater delay of chemotherapy *in vivo* (15 days after single virus injection) and its intraperitoneal administration possibly reducing the effective dose of gemcitabine at the tumor site.

Chemovirotherapeutic combinations incorporating oxaliplatin have not yet been part of (pre-)clinical investigations addressing pancreatic cancer. Nonetheless, therapeutic value might be assumed to originate from its capability to induce immunogenic cell death, dying tumor cells thereby additionally triggering a long-term antitumor response (157). Given that such an immunotherapeutic effect is also believed to be the main property of OVs, incorporating agents such as oxaliplatin into chemovirotherapeutic protocols is of great interest. *In vitro,* treatment of colorectal cancer with a combination of oxaliplatin and Reolysin® has been reported to reduce the numbers of viable tumor cells to a greater degree than either of the two monotherapies (158). In the same tumor entity, cell line-dependent levels of synergy between oxaliplatin and the *Western Reserve* strain vaccinia virus vvDD ('double deleted' virus: thymidine kinase and vaccinia growth factor) were found (159). Here, only 1 out of 4 tumor cell lines (AsPc-1) demonstrated a

clear increase of cytotoxicity after combination therapy with GLV-1h68 and oxaliplatin, compared to the respective monotherapies independently of the administration sequence (Figure 27). In the other 3 cell lines, therapeutic benefit was seen only inconsistently in specific settings. However, main challenge in proving benefit of such immunotherapeutic regimens still remains the establishment of suitable tumor models and *in vitro* approaches so far aren't able to sufficiently address this issue.

Given its only recent development and approval for i) metastatic breast cancer, ii) locally advanced or metastatic non-small-cell lung cancer and iii) metastatic pancreatic cancer (160), *nab*-paclitaxel has only once been combined with viral agents (161). Solvent-based paclitaxel (Taxol®) on the other hand has been part of many chemovirotherapeutic protocols. *In vitro,* the combination with an adenoviral vector was highly synergistic in treating non-small cell lung cancer cells (162) and even paclitaxel-resistant cell lines were more strongly killed by the combination of paclitaxel and Reolysin® (163). Furthermore, *in vitro* synergism in breast cancer cell lines could be translated to *in vivo* efficacy (164) and near to complete tumor eradication was seen in nude mice bearing xenografts of ovarian and prostate cancer (165,166). In an immunocompetent model the combination of an oncolytic adenovirus with paclitaxel restored its oncolytic potency to similar levels as previously seen in athymic mice, possibly due to the chemotherapy-mediated inhibition of initial viral clearance by the immune system (167). Combining paclitaxel with the 'double deleted' vaccinia virus vvDD potently curbed tumor growth in colorectal cancer-bearing nude mice and significantly increased animal survival (143). Similarly, tumor growth of MIA PaCa-2 xenografts was more strongly inhibited by the combination of paclitaxel with the oncolytic adenovirus Ad-Delo3-RGD (130). Clinically, only recently a phase II trial has found the combination of paclitaxel, carboplatin and Reolysin® to be safe but failed to prove a beneficial effect on progression-free survival for patients with metastatic pancreatic adenocarcinoma (168).

Compared to the widely used solvent-based paclitaxel, *nanoparticle albumin-bound* paclitaxel (Abraxane®) has demonstrated both pharmacokinetic and therapeutic advantages (169). It primarily was designed to compensate for the unfavorable toxicity profile of Cremophor EL, the solvent used to solubilize highly water-insoluble paclitaxel. Accordingly, *nab*-paclitaxel can be administered in higher effective doses with less systemic toxicity. Even more, the albumin-bound agent was found to accumulate more potently in tumor tissues which was hypothesized to be the result of various mechanisms such as an enhanced endothelial transcytosis (170) and the prevalence of albumin-binding proteins at the tumor site (171). Clinical benefit of *nab*-paclitaxel was finally proven in a phase III trial addressing patients with metastatic breast cancer (172). Compared to solvent-based paclitaxel, treatment with *nab*-paclitaxel demonstrated both a more favorable toxicity profile and a higher tumor response rate. However, such factors might be of more relevance in the *in vivo* setting and *in vitro* the outcome after *nab*-paclitaxel or solvent-based paclitaxel treatment may be similar.

Here, chemovirotherapeutic treatment with GLV-1h68 and *nab*-paclitaxel led to a slightly increased treatment outcome in the majority of cell lines and settings tested (Figure 28). However, only in AsPc-1 cells a potent effect was seen (independently of the administration order) and in MIA PaCa-2 cells for the first time during this investigation antagonism after chemovirotherapy was observed when the chemotherapeutic was administered first (*nab*-PTX -24 hpi). In addition, therapeutic benefit couldn't be pinned down to a specific administration setting. In consideration of the so far collected evidence this led to the conclusion that GLV-1h68 can be combined with chemotherapeutic agents such as *nab*-paclitaxel, gemcitabine or oxaliplatin in a more flexible time pattern. Clinically, cancer therapy usually depends upon both patient-related factors, such as health-related quality of life and morbidity, as well as work-related parameters such as aligning strict therapy protocols with unpredictable clinical routine. Being able to unravel a therapy protocol without the fear of greatly losing its therapeutic effect therefore makes it more feasible in the clinic.

For the treatment of metastatic pancreatic cancer however *nab*-paclitaxel is only approved in combination with gemcitabine. Given that the majority of chemovirotherapeutic regimens aim at adding the oncolytic and potentially immunotherapeutic properties of OVs to currently established treatment protocols, a triple-therapy protocol incorporating GLV-1h68, *nab*-paclitaxel and gemcitabine was devised. The addition of the approved dual chemotherapy *nab*-paclitaxel + gemcitabine to an oncolytic adenovirus has only recently been proven to be superior to chemotherapy or virotherapy alone in an *in vivo* model of pancreatic cancer (161). A similar protocol combining Reolysin® with carboplatin and paclitaxel, the dual chemotherapy being approved for the treatment of advanced head and neck cancer, has only recently been tested in the REO 018 phase III trial and was able to demonstrate therapeutic benefit by improving patient survival (61).

“Here, in this work, as a matter of fact, in 2 cell lines of PDA (BxPc-3 and MIA PaCa-2) the triple-therapy resulted in a considerable increase of tumor cell killing, whereas in the other 2 cell lines (AsPc-1 and Panc-1) the response after triple-therapy resembled the response after single viro- or dual chemotherapy alone” (139) (Figure 31). Importantly, these results were validated by 3 differently operating cell viability assays. Moreover, in the tumor cell lines which demonstrated no increase of cytotoxicity after the triple-therapy, viral titers were shown to be drastically reduced under the influence of the dual chemotherapy (Figure 33). Accordingly, it has to be assumed that a potent viral replication of GLV-1h68 is necessary for a therapeutic benefit of the triple-therapy *in vitro*.

4.3 Viral replication is influenced by chemotherapeutic agents

“Main focus of chemovirotherapeutic regimens lies in harnessing the oncolytic and, more importantly, immunotherapeutic potential of the applied viral agents. Since both parameters are presumed to depend on a strong viral replication, it has to be ascertained that the application of chemotherapeutic agents does not interfere with viral replication and spread of infectious progeny virus particles in a negative manner. Such interactions depend not only on the chosen agents but

also on their dosages and the order of administration of the respective compounds, basic determinants that have to be considered for the design of any successful combination therapy." (139)

"Thus far, therapeutic benefit after chemovirotherapy both in vitro and in vivo has been shown to be linked to an unchanged or even enhanced viral replication in most cases." (139) The latter has mainly been described in adenoviruses or herpes viruses although the underlying mechanisms could not always be elucidated. "In some cases, chemotherapy-induced DNA damage resulted in a cellular overexpression of GADD34 or ribonucleotide reductase (RR) which led to an increased replication of herpes virus-based agents if homologous viral gene products had previously been deleted (137,173-175). Similarly, chemotherapy was shown to increase the levels of E1A, an early expressed adenoviral gene product that not only regulates a multitude of both cellular and viral genes to initiate the adenoviral replication cycle but is also known for its chemosensitizing effects (167,176). The mitotic inhibitor paclitaxel was found to increase adenoviral assembly and subsequent release from the host cell while leaving DNA synthesis unaffected (162)." (139) As another example, increased titers of myxoma virus or the vaccinia virus vvDD were observed after combination with rapamycin, which correlated with therapeutic benefit both *in vitro* and *in* vivo (177,178). "Furthermore, it was shown that chemotherapy-induced senescence promoted replication of a measles vaccine virotherapeutic virus and led to increased tumor cell killing (179). However, in most cases it remains unclear whether an enhanced viral replication constitutes the main determinant for therapeutic efficacy." (139) This would suggest that the respective chemo- and virotherapeutics operate independently, at which the tumor cells are killed either by the cytotoxic effect of the chemotherapy or viral-mediated oncolysis. Consequently, if therapeutic benefit is mainly caused by a chemotherapy-induced augmentation of viral replication, in other chemovirotherapeutic protocols a similar condition could be recreated by administrating higher viral doses.

"In contrast, it was previously shown that chemotherapeutics such as 5-FU or irinotecan induce an unfavorable environment for viral replication (180)" (139),

depending on the respective genetic setting of the oncolytic agent (134). “SN-38, an active metabolite of clinically used irinotecan, was found to inhibit replication of the HSV-1 vector G47Δ, which decreased the therapeutic benefit otherwise seen in combination with etoposide where viral replication was not influenced (181). Interestingly, therapeutic benefit could be observed despite reduced viral titers. Enhanced levels of apoptosis in response to chemovirotherapeutic treatment were found to result in enhanced therapeutic efficacy and therefore outweighed detrimental decreases of the effective viral dose (159,182,183). Prodrug converting strategies led to a powerful bystander effect, although viral replication was inhibited by the converted cytotoxic compound 5-FU (184,185).” (139) However, the *in vitro* and *in vivo* results were not always conclusive. While gemcitabine was found to severely impede adenoviral replication in cells of pancreatic cancer *in vitro*, potent expression of early and late viral genes could be detected *in vivo*, leading to the assumption that the corresponding viral gene products were the mediator for the enhanced therapeutic effect (132).

Other data have made aware the importance of monitoring viral replication for a sufficient time period. Although diverse chemotherapeutics were found to severely decrease early replication of adenovirus, viral titers were found as high as after virotherapy alone when only given sufficient time (186,187). Moreover, different kinds of infectious particles may be affected differently by chemotherapeutic agents. Vaccinia virus for instance forms 3 types of infectious particles which fulfill distinct tasks in its strategy to maximize viral spread. While numbers of intracellular mature virus, representing the majority of formed particles that are only released during oncolysis, were found to be strongly reduced by the mitotic inhibitor paclitaxel, levels of extracellular enveloped virus, which is released early during the viral life cycle and is therefore responsible for long-range viral spread, remained nearly unchanged (143).

So far in most chemovirotherapeutic protocols an additive or synergistic cytotoxic effect was accompanied by an unaltered viral replication. Combined treatment with chemo- and virotherapeutics thereby often led to increases in the proportion of apoptotic tumor cells (31,188-191). Furthermore, such interactions were found

to depend on the influence of the viral agent on the cell cycle. Addition of the HSV-1 agent G47Δ increased the potency of paclitaxel to arrest cells in G2/M-phase ultimately followed by apoptosis (164). Treatment of lung cancer cells with the adenovirus OBP-301 led to an accumulation in S-phase which potentiated gemcitabine-mediated cytotoxicity and enhanced therapeutic benefit (192). G2/M-phase arrest, which is typically induced by temozolomide treatment and renders cells more resistant to the chemotherapeutic agent, was overridden after addition of the adenovirus Δ-24-RGD (193).

However, although complementing mechanisms of the applied chemo- and virotherapeutic agents, such as induction of cell death, are very likely, the possibility remains that both approaches act independently of each other which would suggest 'only' additional cell killing. Nonetheless, such a chemovirotherapeutic protocol not only would improve initial tumor eradication and therefore more effectively stabilize the tumor disease in the first place but also promote an immunotherapeutic effect in the long-term.

"The here collected evidence demonstrates that the therapeutic benefit of triple-therapy with GLV-1h68 + *nab*-paclitaxel and gemcitabine in treating different pancreatic cancer cell lines depends on an unaltered viral replication in vitro. These findings are of special interest, since, in view of future clinical applications, GLV-1h68 provides the possibility to non-invasively monitor viral replication as a surrogate marker for an (immuno)therapeutic effect in animal models or human patients." (139) However, chemotherapeutics have been shown to affect various forms of vaccinia virus differently and the numbers of extracellular enveloped virus (EEV) might be unchanged under the influence of the dual chemotherapy which would ensure a sufficient long-range spread of GLV-1h68, most notably in the *in vivo* setting.

4.4 Sequence-dependency of chemovirotherapy

Interactions between the applied agents are likely to depend on their treatment order. "Generally, three distinct administration sequences can be differentiated, pretreatment with either i) the chemo- (C) or ii) the virotherapeutic (V) agent

(C→V / V→C) or iii) their concurrent administration (C⇔V).” (139) In case of prodrug converting strategies the rationale for a pretreatment with the OV followed by the respective prodrug seems rather obvious. Nonetheless, the exact timing of prodrug administration has to be elucidated. In some cases, the greatest therapeutic benefit was seen when the prodrug was administered early, i.e. 1 day after the last virotherapeutic treatment (194,195). On the other hand, 5-FU, which inhibits the replication of HSV, was found to cause the strongest bystander effect when its prodrug 5-FC was given at the peak viral titer 3 days after OV administration (184). Accordingly, although based on supposedly simple considerations, prodrug converting strategies illustrate the complex matter of combining chemo- and virotherapeutic agents. “Chemovirotherapeutic strategies are still experimental, rather than based on a profound understanding of the underlying mechanisms, and sequence-dependent interactions are not easy to predict. Of special interest are synergistic interactions independently from the treatment order but mediated via different antitumoral mechanisms. Huang et al. (143) postulated that pretreatment with paclitaxel (C→V) induced a cell cycle arrest of colorectal cancer cells in the G2/M phase, which rendered them more susceptible to vaccinia virus infection. Pretreatment with the virotherapeutic (V→C), was shown to sensitize for an adjacent chemotherapy by the release of cellular danger signals.” (139) Therefore, to more accurately predict possible interactions, many more parameters will have to be considered.

For starters, “hypothetic considerations of one therapeutic approach sensitizing for the other hint at superior treatment outcomes after sequential administration of the agents (C→V / V→C) and to a lesser extent, in the concurrent setting (C⇔V) as well. Any therapeutic effect based on an augmented viral replication would therefore suggest a benefit of administering chemotherapeutics first (174,196).” (139) Indeed, the highest titers of reovirus were reached when it was applied without further delay than 24 hours after paclitaxel administration (197). However, adjusting the timely pattern of a chemovirotherapeutic protocol to a small therapeutic window might be a central issue. Besides, “similar increases of adenoviral replication have been found to be independent of the treatment order (C→V/ V→C) (198).” (139)

“Then again, the requirement of a strong viral replication (or rather a high number of viral gene products) possibly sensitizing for adjacent chemotherapy suggests a therapeutic benefit if tumor cells are pretreated with the virotherapeutic agent (V→C) (199). Nevertheless, some of the synergistic interactions associated with a potent viral replication have been found to be sequence-independent (166,200). In contrast, although gemcitabine negatively influenced the viral life cycle of parvovirus H-1PV in the concurrent setting, it was found to prolong survival of tumor-bearing rats when its administration took place much earlier (i.e., 2 weeks) (C→V) (133). Such combination protocols demonstrate the possibility to employ more flexible time patterns between chemo- and virotherapy in case of detrimental interactions in the concurrent setting.” (139)

Other sequence-dependent interactions such as exploiting cell death have been proposed as well. In the context of chemovirotherapy, apoptosis primarily represents the mode of action of chemotherapeutic agents which usually depend on the induction of physiologic cell death mechanisms. Viral infection on the other hand is a trigger for apoptosis as part of the antiviral host response, independently of its actual oncolytic effect. Since in the process of tumorigenesis proapoptotic signaling pathways are typically downregulated (rendering chemotherapy less effective while facilitating viral infection), OVs (or rather their gene products) are believed to sensitize tumor cells for chemotherapy induced apoptosis. In line with this consideration, pretreatment with the OV (V→C) or concurrent treatment (C⇔V) were shown to result in enhanced tumor cell killing owing to an increased induction of apoptosis (128,159,201). However, similarly increased apoptosis was also found in paclitaxel-pretreated cells followed by treatment with an oncolytic adenovirus (C→V) (202). Even more, chemotherapy-induced apoptosis has been shown to facilitate initial viral penetration and promote viral spread (C→V) (203).

The complexity of this matter is further pronounced by the fact that the administration sequence in some cases might be cell type-dependent. In the human PDA cell line Hs766T the combination of myxoma virus with gemcitabine *in vitro* was shown to be beneficial only when gemcitabine was applied first (C→V),

whereas in murine Pan02 cells only pretreatment with the OV (V→C) achieved therapeutic benefit (204). Similar effects were seen in the immunocompetent Pan02 model *in vivo*, implying that current syngeneic immunocompetent models may not be sufficient to fully evaluate sequence dependent effects and translate this findings to human patients.

Nonetheless, investigations identifying underlying mechanisms of sensitization to chemo- or virotherapy have to address sequence-dependent effects. “Unfortunately, the experimental settings of investigations addressing sequence-dependent effects are not always conclusive. In one case, both concurrent and delayed administration of cisplatin (C⇔V / V→C) resulted in a similar therapeutic benefit; however, the increase of cisplatin-induced apoptosis was only investigated in the former and therefore might not contribute to the therapeutic success in the sequential administration setting (205). Similarly, although pretreatment with either agent (C→V / V→C) often results in superior treatment outcomes, the effect of the chemotherapeutic agents on viral replication is frequently measured only in the concurrent setting. Depending on the respective treatment order, chemotherapeutics are known to also affect other parameters determining the cellular environment for viral replication such as the induction of cell cycle arrest (186) or changes in gene expression (137,173-175). Therefore, although chemotherapeutics may directly interfere with the viral life cycle (which is measured in the concurrent setting) it would be shortsighted to extrapolate such results for the sequential setting.” (139)

Here, “the chronological order of the viro- and chemotherapeutic agents (GLV-1h68 + *nab*-paclitaxel, gemcitabine” (139) or oxaliplatin) “did not influence the therapeutic effect in either of the four tumor cell lines. As a result, it was concluded that chemo- and virotherapy could be administered in a more flexible time pattern and a triple-therapy protocol was devised in which *nab*-paclitaxel and gemcitabine were added directly after the initial virus infection (at 1 + 1.5 hpi). Moreover, analyses of the antitumor effect as well as of the influence of the dual chemotherapy on viral replication of GLV-1h68 were performed under similar conditions. Therapeutic success of the triple-therapy in the concurrent setting was therefore

clarified to depend on an effective chemotherapy in addition to an unchanged viral replication." (139)

4.5 Dose-dependent effects between chemo- and virotherapeutic agents determine the therapeutic outcome

Therapeutic success of combination therapy may also be somewhat dose-dependent, the positive/negative influence of one agent on the other thereby correlating with the administered concentrations. Moreover, "possible synergistic interactions may intensify therapeutic success and allow for dose reductions of the applied agents to a less toxic degree (164,206,207). High-dose combination therapy was actually unable to further increase the levels of tumor cell death already being induced by low-dose chemovirotherapy (208,209). Furthermore, virus-mediated chemosensitization was shown to be powerful enough to render chemotherapy-resistant tumor cells sensitive for low-dose chemotherapy (210,211)." (139)

"Since some chemotherapeutic agents are known to directly interfere with the viral life cycle, dose-dependent relations in this regard have been in the focus of diverse investigations. Although application of high-dose mitomycin C was found to severely reduce replication of an oncolytic herpes simplex virus, viral titers were found to be unchanged when administered in lower and thereby 'beneficial' doses (212). Furthermore, low-dose chemotherapy was found to increase viral titers to a greater extent than its high-dose application (174,198)." (139) Therefore, the therapeutic window for optimal augmentation of viral replication which is already dependent on the application sequence narrows down even more.

"The applied dose of an agent in combination therapy is usually determined by its cytotoxic effect in monotherapy. However, to demonstrate therapeutic benefit of the combination, suitable doses of single agents have to be chosen carefully. If doses are too high, single agents will be too 'successful' in killing tumor cells on their own and the readout of potential combinatorial therapeutic benefits could be threatened. Moreover, even if the chemotherapy does not directly interfere with the viral life cycle, high concentrations would be immediately cytotoxic and therefore prevent effective viral replication by killing tumor cells, which function

as hosts for replicating virotherapeutics (132).” (139) This was suspected to be the case in pancreatic cancer cell lines where different levels of sensitivity to gemcitabine were shown to predict the therapeutic outcome of the chemovirotherapeutic protocol (128). “Higher gemcitabine doses in gemcitabine-insensitive cell lines were assumed to cause a greater inhibition of viral replication and in accordance to also prevent therapeutic benefit.” (139)

“In line with these considerations, here the chemovirotherapeutic protocols were devised by carefully adjusting concentrations of the respective compounds, ensuring remaining tumor cell masses of ≈ 75 % after 72 hours of chemo- or virotherapeutic treatment in monotherapy (designated as a so-called LD25). By doing so, the primary aim was to prevent excessive tumor cell killing possibly disguising additional effects of the chemovirotherapeutic combination. Nonetheless, reductions in the viral titers in tumor cell lines that had been non-responsive to the triple chemovirotherapy still could be the result of overdosing chemotherapy. On closer inspection, AsPc-1 and Panc-1 tumor cells indeed received higher concentrations of *nab*-paclitaxel and/or gemcitabine than the triple-chemovirotherapy-responsive tumor cell lines BxPc-3 and MIA PaCa-2. Therefore, chemotherapeutic doses still might have been adjusted in a too high range could potentially have negatively influenced viral replication as a result.” (139)

Further investigations revealed that in BxPc-3 cells even high-dose administration of *nab*-paclitaxel didn’t negatively influence viral replication whereas higher gemcitabine concentrations actually reduced viral titers in a dose-dependent manner (Figure 34 and Figure 36). Moreover, viral GFP expression could be somewhat conserved if gemcitabine administration was delayed (Figure 38). Hence, 2 separate assumptions can be made on the basis of these data. Either would it be beneficial to reduce the concentration of the chemotherapeutic agent to a degree at which viral replication remains strong. However, whether less cytotoxic doses would likewise result in a beneficial treatment outcome as in the responding cell lines would have to be elucidated *in vitro*. Given the fact that the so far collected evidence indicates a rather additive character of the therapeutic

agents in independently killing the tumor cells, this conclusion seems rather dubious. In contrast, it appears to be more promising to unravel the schedule of the treatment protocol and apply GLV-1h68 and the dual chemotherapy separately in their effective doses. Thus, detrimental interactions of the therapeutic agents and chemotherapy-mediated decreases of the viral titer, especially in the initial oncolytic cycles, could be circumvented. Unraveling the therapy protocol would also beneficially lessen therapy-induced adverse effects and safeguard overlapping toxicity which to date is frequently used in multimodal chemotherapy protocols.

In accordance with this consideration the combination of gemcitabine with 2-weeks-delayed parvovirus H-1PV (C→V) was shown to prolong the survival of immunocompetent tumor-bearing rats, although in the concurrent setting the viral life cycle was negatively influenced (133). Of even more relevance was the successful combination of GLV-1h68 with 15-days-delayed gemcitabine (V→C) which significantly improved the response of pancreatic cancer xenografts in nude mice (101).

Of significance may also be the fact that a multitude of chemotherapeutics has been shown to exhibit immunotherapeutic properties on their own (213). Contrary to the wide-spread belief that chemotherapy in general acts rather immunosuppressive, in specific settings humoral and cellular antitumor immune responses can be beneficially influenced. This is of especial interest since the field of virotherapeutic research currently considers the combination of oncolytic viruses with checkpoint inhibitors to have the potential for groundbreaking immunotherapeutic success. The combinations of T-VEC with ipilimumab or pembrolizumab, both monoclonal antibodes targeting and blocking the immunosuppressive CTLA-4 or PD-1 receptor respectively, are currently under evaluation in a phase II and phase III trial (NCT01740297, NCT02263508) addressing melanoma patients and will be instrumental in assessing this hypothesis. Immunovirotherapeutic success with *Western Reserve* strain derivatives of vaccinia virus was seen *in vivo* after combining them with antibodies against CTLA-4 (214,215) or PD-1 (215,216)

which not only significantly decreased tumor burden and improved survival of tumor bearing mice but in some cases also drastically delayed tumor growth after rechallenging the mice with the same tumor tissue, indicating the presence of a systemic anti-tumor immunity.

Both chemotherapeutic agents of the here established novel triple-therapy, paclitaxel and gemcitabine, have been shown to exhibit immunotherapeutic properties on their own. Paclitaxel was shown to induce the maturation of dendritic cells and increase antigen presentation *in vitro* (217,218). Gemcitabine, while exhibiting detrimental effects on humoral immune responses, was shown to leave antigen-specific cellular immunity unaffected (219). Since chemovirotherapy mainly focuses on harnessing the immunotherapeutic potential of an adaptive immune response while initially circumventing innate antiviral host responses, this condition could enhance both oncolytic and immunotherapeutic potential of such a combination. In patients with advanced pancreatic cancer, gemcitabine did not severely deplete immune cells (220). Decreases of T lymphocytes and NK cells were only transient and the numbers of dendritic and antigen-presenting cells were even increased (221).

The combination of gemcitabine with an immune-activating anti-CD-40 antibody was synergistic *in vivo* and led to an infiltration of CD4+ and CD8+ positive T-cells to the tumor tissue. Moreover, long-surviving mice resisted rechallenge with the same tumor (222). The combination of myxoma virus with gemcitabine in an intraperitoneal dissemination model of pancreatic resulted in 100 % long term survivors at the end of the study, but only in immunocompetent mice. In the immunodeficient model no such effects were seen (204). Gemcitabine was also shown to create favorable conditions for OV-induced antitumor immunity by overriding reovirus induced recruitment of immunosuppressive MDSCs and accelerating tumor-specific T-cell responses (223).

However, a recent phase III trial treating metastatic pancreatic cancer with a combination of gemcitabine, capecitabine (prodrug of 5-FU), a telomerase peptide vaccine and the proinflammatory cytokine GM-CSF wasn't able to prove therapeutic benefit of this chemoimmunotherapeutic regimen (224). Given that i) *nab-*

paclitaxel probably has similar immunotherapeutic properties as solvent-based paclitaxel and ii) was shown to increase intratumoral gemcitabine levels whereas iii) the vaccinia virus GLV-1h68 has the immunotherapeutic potential of more strongly inducing a profound antitumor immune response, the triple-therapy protocol holds promise for further (pre-)clinical investigations.

In conclusion, the triple-therapy combining the oncolytic vaccinia virus GLV-1h68 with the clinically approved dual chemotherapy *nab*-paclitaxel + gemcitabine has demonstrated therapeutic benefit for treating pancreatic adenocarcinoma *in vitro* when viral replication, the key for a potent oncolytic and immunotherapeutic effect, wasn't influenced.

Based on these and previous findings, unraveling the treatment protocol by administering the chemo- and virotherapeutic agents separately has been proposed to further augment therapeutic efficacy and circumvent potentially detrimental interactions between the applied agents. "Any approach trying to prove therapeutic benefit of such a regimen besides investigating its cytotoxic effect would have to focus especially on viral replication and its consequences on antitumor immunity." (139) "However, when employing human pancreatic ductal adenocarcinoma (hPDA) cell lines as investigated in this work (AsPc-1, BxPc-3, MIA-PaCa-2, Panc-1) such experiments only could be performed in xenograft animal models (e.g., in nude or SCID mice). Unfortunately, these immunodeficient mice are lacking important features of the adaptive immunity. As an alternative, usage of humanized mice with a partially or nearly fully reconstituted immune system could provide insights on (i) how this triple therapeutic regimen would affect anti-tumor immunity, (ii) how immune checkpoint inhibitors could be placed on top, and (iii) how means aiming at a depletion of the immunosuppressive phenotypes of human pancreatic cancer could be made successful; however, proper answers to these highly interesting questions only can be provided by future clinical trials. Of further interest are investigations on how the triple chemovirotherapy regime with GLV-1h68 plus *nab*-paclitaxel + gemcitabine would affect the dense stroma being associated with hPDA. Again, xenograft mouse models are not suitable for such

investigations due to the fact that hPDA cells cannot be mixed with human pancreatic stromal cells for a remodeling of the specific histological features of hPDA. As an alternative, organotypic culture models have emerged as tractable systems to recapitulate the complex three-dimensional organization of hPDA (225) and could be implemented for such analyses in the future. Such hPDA organoids also would be highly instrumental for further investigations on the mechanistic effects of the triple chemovirotherapy regime with GLV-1h68 plus *nab*-PTX + Gem." (139) Full insight in this complex matter will only be provided by treating human patients in well documented clinical trials in the long-term.

Summary

Oncolytic virotherapy utilizes naturally occurring or genetically modified viruses for targeted cancer treatment. Such oncolytic viruses (OVs) are designed to specifically infect and replicate in tumor cells ultimately leading to their lysis (so-called oncolysis). Additionally, the release of both tumor antigens and immunogenic viral particles is believed to strongly induce antitumor immunity. However, in line with the consideration that multimodal cancer therapy is likely to be more effective than monotherapeutic treatment protocols, chemovirotherapy focuses on adding the oncolytic and immunotherapeutic potential of OVs to already established chemotherapeutic treatment protocols.

Especially for pancreatic ductal adenocarcinoma (PDA) chemotherapy still fails to considerably improve patient survival. Additionally to PDA's substantial intrinsic resistance to chemotherapeutic agents the dense and highly immunosuppressive tumor microenvironment impedes the efficacy of current chemotherapy protocols. Then again, such conditions have been shown to favor oncolytic virotherapy.

This thesis therefore focused on the design of a novel chemovirotherapeutic protocol to improve the currently poor treatment outcome of pancreatic cancer. For this purpose, 4 established and well-characterized tumor cell lines of pancreatic adenocarcinoma (AsPc-1, BxPc-3, MIA PaCa-2, Panc-1) were treated *in vitro* with the oncolytic vaccinia virus GLV-1h68 in combination with selected chemotherapeutic agents. Moreover, the influence of different administration sequences on the therapeutic outcome of chemovirotherapy was analyzed. Cytotoxicity after treatment was measured by sulforhodamine B (SRB) assay and confirmed by CellTiter Blue (CTB) and MTT assays, respectively. Due to its insertion of *green fluorescent protein* (GFP) GLV-1h68 additionally enabled the non-invasive visualization of viral gene expression and replication.

In a first step, for each agent doses having only subtherapeutic tumoricidal effects had to be determined. Accordingly, all 4 tumor cell lines were treated with as-

cending doses of the respective chemo- or virotherapeutic agents in monotherapy. As a result, drug- and dose-dependent antitumor responses were seen indicating resistance against some cytotoxic agents.

In a next step, the actual chemovirotherapy was performed. Combinations of GLV-1h68 with either 5-fluorouracil (5-FU), gemcitabine or oxaliplatin were found to result only in a moderate increase of cytotoxicity regardless of their administration sequence. Only the combination with the mitotic inhibitor *nab*-paclitaxel showed promising signs of potent tumor cell killing. However, given the fact that for metastatic pancreatic cancer *nab*-paclitaxel is only approved in combination with gemcitabine a triple-therapy protocol combining GLV-1h68 with the dual chemotherapy *nab*-paclitaxel + gemcitabine was devised. Interestingly, this triple-therapy resulted in 2 out of 4 tumor cell lines (BxPc-3, MIA PaCa-2) in a strongly improved treatment outcome. Notably, in the other tumor cell lines in which no enhanced response was seen after the triple-therapy (AsPc-1, Panc-1) viral titers were found to be considerably reduced under the influence of the dual chemotherapy. Thus, therapeutic success of this therapeutic regimen was linked to an unaltered viral replication of GLV-1h68 and - *vice versa* - failure to a missing suppression of fabricating the progeny GLV-1h68 particles.

Further investigations indicated that the interference with viral replication specifically was the result of overdosing gemcitabine. When delaying gemcitabine administration viral GFP expression could be somewhat conserved. Similarly, previous reports had demonstrated the benefit of unraveling a chemovirotherapeutic treatment protocol by administering the chemo- and virotherapeutic agents separately. Thus, the next step of promoting the combination of GLV-1h68 with *nab*-paclitaxel and gemcitabine would be to investigate the influence of such measures on therapeutic success, at best in an immunocompetent animal model while non-invasively monitoring viral replication and therapeutic efficacy.

Zusammenfassung

Onkolytische Virotherapie setzt natürlich vorkommende oder genetisch modifizierte Viren zur spezifischen Krebstherapie ein. Solche onkolytischen Viren (OVs) wurden konstruiert, um spezifisch Tumorzellen zu infizieren, in deren Zellinnerem zu replizieren und sie letztlich zu lysieren (sogenannte Onkolyse). Zusätzlich verspricht man sich von der gleichzeitigen Freisetzung von Tumorantigenen und immunogenen Viruspartikeln die Induktion einer starken tumorgerichteten Immunantwort. Der Fokus der Chemovirotherapie liegt – gemäß der Überlegung, dass eine multimodale Krebstherapie eine vermeintlich stärkere Wirkung besitzt als monotherapeutische Behandlungsschemata – folglich darauf, das onkolytische und immuntherapeutische Potential von OVs zu bereits etablierten Chemotherapie-Protokollen hinzuzufügen.

Vor allem im Falle des Pankreaskarzinoms führen aktuelle Chemotherapie-Protokolle noch immer nicht zu einer wesentlichen Verbesserung des Patientenüberlebens. Die therapeutische Wirkung wird dabei nicht nur von einer erheblichen intrinsischen Resistenz gegenüber Chemotherapeutika behindert, sondern auch durch ein gegenüber zahllosen Krebstherapeutika undurchlässiges und in hohem Maße immunsupprimiertes Tumormilieu. Demgegenüber konnte jedoch gezeigt werden, dass ein eben solches Umfeld Virotherapie günstig beeinflusst.

Ziel der hier vorgestellten Promotionsarbeit war daher die Entwicklung eines neuartigen Chemovirotherapie-Protokolls zur Verbesserung der Behandlung des Pankreaskarzinoms zunächst unter *in vitro* Bedingungen. In diesem Zuge wurden 4 etablierte und gut charakterisierte Pankreasadenokarzinom-Zelllinien (AsPc-1, BxPc-3, MIA PaCa-2, Panc-1) mit dem Pockenimpfvirus GLV-1h68 und ausgewählten Chemotherapeutika behandelt. Zusätzlich wurde der Einfluss von verschiedenen Applikationsschemata auf den Behandlungserfolg nach Chemovirotherapie untersucht. Das Therapieansprechen wurde dabei zunächst primär mittels des Sulforhodamine B (SRB) Assays bestimmt und später zusätzlich mithilfe der Cell-TiterBlue (CTB) und MTT Assays validiert. GLV-1h68 bot zudem – aufgrund seiner genetischen Ausstattung mit *grün fluoreszierendem Protein* (GFP)

– die Möglichkeit, die virale Genexpression und damit auch die Effizienz viraler Replikation nicht-invasiv zu überwachen.

Zu Beginn der Arbeit mussten zunächst subtherapeutisch wirksame Dosen jedes einzelnen Therapeutikums bestimmt werden. Dafür wurden die 4 Tumorzelllinien mit aufsteigenden Konzentrationen des jeweiligen Chemo- oder Virotherapeutikums behandelt. Ein sowohl von der Wahl des Therapeutikums als auch von dessen Konzentration abhängiges Therapieansprechen wies dabei auf bereits ausgansmäßig vorliegende bzw. erworbene Resistenzen gegenüber manchen Zytostatika hin.

Schließlich wurde die eigentliche Chemovirotherapie durchgeführt. Die Kombination aus GLV-1h68 mit 5-Fluorouracil (5-FU), Gemcitabine oder Oxaliplatin resultierte dabei jedoch lediglich in einer geringfügigen Verbesserung des Behandlungserfolges, unabhängig von deren zeitlichen Abfolge. Einzig die Kombination mit dem Mitosehemmer *nab*-Paclitaxel versprach ein bedeutsam gesteigertes Therapieansprechen. Zur Behandlung des metastasierten Pankreaskarzinoms ist *nab*-Paclitaxel jedoch ausschließlich in Kombination mit Gemcitabine zugelassen. Dies führte letztlich zum Entwurf eines vollkommen neuartigen Triple-Therapie Protokolls, in dessen Zuge GLV-1h68 mit der dualen Chemotherapie aus *nab*-Paclitaxel und Gemcitabine kombiniert wurde. Tatsächlich führte dieser Ansatz in 2 der 4 Tumorzelllinien (BxPc-3, MIA PaCa-2) zu einem erhöhten Therapieansprechen. Gleichzeitig waren in den nicht in erhöhtem Maße ansprechenden Tumorzelllinien (AsPc-1, Panc-1) die viralen Titer unter Einfluss der dualen Chemotherapie deutlich verringert. Damit hing der Behandlungserfolg der Triple-Therapie ganz offensichtlich direkt mit einer effizienten viralen Replikation zusammen bzw. im Umkehrschluss mit einer fehlenden Hemmung der Bildung von viralen Nachkommen des Pockenimpfvirus GLV-1h68.

Weitere Untersuchungen wiesen darauf hin, dass die Hemmung der viralen Replikation die direkte Folge von diesbezüglich überdosiertem Gemcitabine war. Zudem konnte die virale GFP Expression bei zeitlich verzögerter Gabe von Gemcitabine zu einem gewissen Grad erhalten werden. In ähnlicher Weise konnte bereits in vorausgegangenen Untersuchungen der Vorteil eines zeitlich “entzerrten”

Therapieschemas durch die separate Gabe von Chemo- und Virotherapeutika gezeigt werden.

Basierend auf diesen Erkenntnissen besteht der nächste Schritt zur Weiterentwicklung der Kombination aus GLV-1h68 mit *nab*-Paclitaxel und Gemcitabine in einer systematischen Untersuchung der Auswirkung solcher Maßnahmen auf den Behandlungserfolg, bestenfalls in einem immunkompetenten Tiermodell unter nicht-invasiver Überwachung der viralen Replikation und therapeutischen Wirksamkeit.

Appendix

List of figures

List of tables

Index of abbreviations

5-FU	5-fluorouracil
AB/AM	Antibiotic-Antimycotic
aka	also known as
approx.	approximately
β-gal	β-galactosidase
β-gluc	β-glucuronidase
CAR	coxsackie and adenovirus receptor
CEA	carcinoembryonic antigen
CEV	cell-associated enveloped virus
CMC	carboxymethylcellulose
CTB	CellTiter-Blue®
CTX	chemotherapy
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EEV	extracellular enveloped virus
EGFR	epithelial growth factor receptor
EMA	European Medicines Agency
FCS	fetal calf serum
FDA	US Food and Drug Administration
Gem	gemcitabine
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
h	hour(s)
HCC	hepatocellular carcinoma
HCl	hydrochloric acid
hNET	human norepinephrine transporter
hNIS	human sodium iodide symporter
hPDA	human pancreatic ductal adenocarcinoma
hpi	hour(s) post infection
hpt	hour(s) post treatment
HSV	herpes simplex virus
i.e.	id est – that is
IMV	intracellular mature virus
kb	kilobase
LD25	25 % lethal dose
MOCK	untreated control
MOI	multiplicity of infection (ratio of virus particles per tumor cell)
MRI	magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nab-PTX	*nab*-paclitaxel
NCT	National Clinical Trial number
OV	oncolytic virus
Ox	oxaliplatin
rpm	rounds per minute
PANIN	Pancreatic Intraepithelial Neoplasia

PBS	phosphate buffered saline
PCAA	human pancreas cancer-associated antigen
PDA	pancreatic ductal adenocarcinoma
PFU	plaque forming unit
SELP	silk-elastin-like protein polymer
SEP	smallpox eradication program
SRB	sulforhodamine B
TCA	trichloroacetic acid
TRIS	tris(hydroxymethyl)aminomethane
US	United States of America
UV	ultraviolet
VIGIV	intravenous vaccinia immune globuline
VV	vaccinia virus
WHO	World Health Organization

Literature

1: Robert Koch Institute (ed.) and the Association of Population-based Cancer Registries in Germany (ed.) (2016) Cancer in Germany 2011/2012, *10th edition*. Berlin, 2014.

2: Torre LA et al. (2015) Global cancer statistics, 2012. *CA Cancer J Clin 65(2):87-108.*

3: Siegel RL et al. (2016) Cancer statistics, 2016. *CA Cancer J Clin 66(1):7-30.*

4: Seufferlein T et al. (2013) [S3-guideline exocrine pancreatic cancer]. *Z Gastroenterol 51(12):1395-440.*

5: Seufferlein T et al. (2014) Ductal Pancreatic Adenocarcinoma: Surgery, Pathology Work-up, and Neoadjuvant, Adjuvant, and Palliative Treatments. *Dtsch Arztebl International 111(22):396-402.*

6: Hartwig W et al. (2011) Pancreatic cancer surgery in the new millennium: better prediction of outcome. *Ann Surg 254(2):311-9.*

7: Konstantinidis IT et al. (2013) Pancreatic ductal adenocarcinoma: is there a survival difference for R1 resections versus locally advanced unresectable tumors? What is a "true" R0 resection? *Ann Surg 257(4):731-6.*

8: Ryan DP et al. (2014) Pancreatic adenocarcinoma. *N Engl J Med 371(22):2140-1.*

9: Chan K et al. (2014) A Bayesian Meta-Analysis of Multiple Treatment Comparisons of Systemic Regimens for Advanced Pancreatic Cancer. *PLoS ONE 9(10):e108749.*

10: Moore MJ et al. (2007) Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol 25(15):1960-6.*

11: Conroy T et al. (2011) FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med 364(19):1817-25.*

12: Von Hoff DD et al. (2013) Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med 369(18):1691-703.*

13: Celgene (2014) ABRAXANE® Plus gemcitabine Receives European Marketing Authorization for First-Line Treatment of Patients with Metastatic Pancreatic Cancer [Press Release January 7, 2014]. Summit: Celgene; Available from: http://ir.celgene.com/releasedetail.cfm?releaseid=821049. Accessed April 9, 2018.

14: Hidalgo M (2012) New insights into pancreatic cancer biology. *Ann Oncol 23 Suppl 10:x135-8.*

15: Mahadevan D and Von Hoff DD (2007) Tumor-stroma interactions in pancreatic ductal adenocarcinoma. *Mol Cancer Ther 6(4):1186-97.*

16: Erkan M et al. (2009) Cancer-stellate cell interactions perpetuate the hypoxia-fibrosis cycle in pancreatic ductal adenocarcinoma. *Neoplasia 11(5):497-508.*

17: Campbell PJ et al. (2010) The patterns and dynamics of genomic instability in metastatic pancreatic cancer. *Nature 467(7319):1109-13.*

18: Rao CV and Mohammed A (2015) New insights into pancreatic cancer stem cells. *World J Stem Cells 7(3):547-55.*
19: Russell SJ and Peng KW (2017) Oncolytic Virotherapy: A Contest between Apples and Oranges. *Mol Ther 25(5):1107-1116.*
20: Russell SJ et al. (2012) Oncolytic virotherapy. *Nat Biotechnol 30(7):658-70.*
21: Xu C et al. (2013) Viral therapy for pancreatic cancer: tackle the bad guys with poison. *Cancer Lett 333(1):1-8.*
22: Keller BA and Bell JC (2016) Oncolytic viruses-immunotherapeutics on the rise. *J Mol Med (Berl) 94(9):979-91.*
23: Ebrahimi S et al. (2017) Interferon-Mediated Tumor Resistance to Oncolytic Virotherapy. *J Cell Biochem*
24: Clements D et al. (2014) Reovirus in cancer therapy: an evidence-based review. *Oncolytic Virother 2014:3:69-82.*
25: Kirn D (2001) Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? *Gene Ther 8(2):89-98.*
26: Yu W and Fang H (2007) Clinical trials with oncolytic adenovirus in China. *Curr Cancer Drug Targets 7(2):141-8.*
27: Chen GX et al. (2014) Clinical utility of recombinant adenoviral human p53 gene therapy: current perspectives. *Onco Targets Ther 7:1901-9.*
28: Martuza RL et al. (1991) Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science 252(5007):854-6.*
29: Puhlmann M et al. (2000) Vaccinia as a vector for tumor-directed gene therapy: biodistribution of a thymidine kinase-deleted mutant. *Cancer Gene Ther 7(1):66-73.*
30: Zhang Q et al. (2009) The highly attenuated oncolytic recombinant vaccinia virus GLV-1h68: comparative genomic features and the contribution of F14.5L inactivation. *Mol Genet Genomics 282(4):417-35.*
31: Wirth T et al. (2005) Telomerase-dependent virotherapy overcomes resistance of hepatocellular carcinomas against chemotherapy and tumor necrosis factor-related apoptosis-inducing ligand by elimination of Mcl-1. *Cancer Res 65(16):7393-402.*
32: Kelly E and Russell SJ (2007) History of oncolytic viruses: genesis to genetic engineering. *Mol Ther 15(4):651-9.*
33: Bluming AZ and Ziegler JL (1971) Regression of Burkitt's lymphoma in association with measles infection. *Lancet 2(7715):105-6.*
34: Moore AE (1949) The destructive effect of the virus of Russian Far East encephalitis on the transplantable mouse sarcoma 180. *Cancer 2(3):525-34.*
35: Moore AE (1951) Inhibition of growth of five transplantable mouse tumors by the virus of Russian Far East encephalitis. *Cancer 4(2):375-82.*
36: Asada T (1974) Treatment of human cancer with mumps virus. *Cancer 34(6):1907-28.*
37: Pol J et al. (2016) Trial Watch-Oncolytic viruses and cancer therapy. *Oncoimmunology 5(2):e1117740.*
38: Garber K (2006) China approves world's first oncolytic virus therapy for cancer treatment. *J Natl Cancer Inst 98(5):298-300.*

39: Lu W et al. (2004) Intra-tumor injection of H101, a recombinant adenovirus, in combination with chemotherapy in patients with advanced cancers: a pilot phase II clinical trial. *World J Gastroenterol 10(24):3634-8.*
40: Xia ZJ et al. (2004) [Phase III randomized clinical trial of intratumoral injection of E1B gene-deleted adenovirus (H101) combined with cisplatin-based chemotherapy in treating squamous cell cancer of head and neck or esophagus]. *Ai Zheng 23(12):1666-70.*
41: Russell SJ et al. (2014) Remission of disseminated cancer after systemic oncolytic virotherapy. *Mayo Clin Proc 89(7):926-33.*
42: Kohlhapp FJ and Kaufman HL (2016) Molecular Pathways: Mechanism of Action for Talimogene Laherparepvec, a New Oncolytic Virus Immunotherapy. *Clin Cancer Res 22(5):1048-54.*
43: Greig SL (2016) Talimogene Laherparepvec: First Global Approval. *Drugs 76(1):147-54.*
44: Allan KJ et al. (2016) High-throughput screening to enhance oncolytic virus immunotherapy. *Oncolytic Virother 5:15-25.*
45: Andtbacka RH et al. (2015) Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma. *J Clin Oncol*
46: Zhang Q et al. (2007) Eradication of solid human breast tumors in nude mice with an intravenously injected light-emitting oncolytic vaccinia virus. *Cancer Res 67(20):10038-46.*
47: Yu YA et al. (2004) Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins. *Nat Biotechnol 22(3):313-20.*
48: LaRocca CJ and Davydova J (2016) Oncolytic Virotherapy Increases the Detection of Microscopic Metastatic Disease at Time of Staging Laparoscopy for Pancreatic Adenocarcinoma. *EBioMedicine 7:15-6.*
49: Dingli D et al. (2004) Image-guided radiovirotherapy for multiple myeloma using a recombinant measles virus expressing the thyroidal sodium iodide symporter. *Blood 103(5):1641-6.*
50: Rojas JJ and Thorne SH (2012) Theranostic potential of oncolytic vaccinia virus. *Theranostics 2(4):363-73.*
51: Breitbach CJ et al. (2011) Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature 477(7362):99-102.*
52: Bell J and McFadden G (2014) Viruses for tumor therapy. *Cell Host Microbe 15(3):260-5.*
53: Fukuhara H et al. (2016) Oncolytic virus therapy: A new era of cancer treatment at dawn. *Cancer Sci 107(10):1373-1379.*
54: Hou W et al. (2016) Oncolytic Virus-Mediated Targeting of PGE2 in the Tumor Alters the Immune Status and Sensitizes Established and Resistant Tumors to Immunotherapy. *Cancer Cell 30(1):108-19.*
55: Wennier ST et al. (2012) Bugs and drugs: oncolytic virotherapy in combination with chemotherapy. *Curr Pharm Biotechnol 13(9):1817-33.*
56: Bressy C and Benihoud K (2014) Association of oncolytic adenoviruses with chemotherapies: an overview and future directions. *Biochem Pharmacol 90(2):97-106.*

57: Kanai R et al. (2010) Oncolytic herpes simplex virus vectors and chemotherapy: are combinatorial strategies more effective for cancer? *Future Oncol 6(4):619-34.*
58: Badrinath N et al. (2016) Viruses as nanomedicine for cancer. *Int J Nanomedicine 11:4835-4847.*
59: Binz E and Lauer UM (2015) Chemovirotherapy: combining chemotherapeutic treatment with oncolytic virotherapy. *Oncolytic Virother 2015:4:39-48.*
60: Puzanov I et al. (2016) Talimogene Laherparepvec in Combination With Ipilimumab in Previously Untreated, Unresectable Stage IIIB-IV Melanoma. *J Clin Oncol 34(22):2619-26.*
61: Oncolytics Biotech (2014) Oncolytics Biotech Inc. Announces Additional Data from REO 018 Randomized Study of REOLYSIN in Head and Neck Cancers [Press Release April 8, 2014]. Calgary: Oncolytics Biotech; Available from: https://www.oncolyticsbiotech.com/press-releases/detail/308/oncolytics-biotech-inc-announces-additional-data-from. Accessed April 9, 2018.
62: Heo J et al. (2013) Phase II trial of Pexa-Vec (pexastimogene devacirepvec; JX-594), an oncolytic and immunotherapeutic vaccinia virus, followed by sorafenib in patients with advanced hepatocellular carcinoma (HCC). J Clin Onc 31:4122 (Suppl; ASCO Meeting Abstract).
63: Transgene (2015) Agreement with FDA Announced for Special Protocol Assessment for Upcoming Phase 3 Pexa-Vec Trial in Advanced Liver Cancer [Press Release April 16, 2015]. Strasbourg: Transgene; Available from: http://www.transgene.fr/wp-content/uploads/2015/04/20150416-SPA-release-version-EN-clean.pdf. Accessed April 9, 2018.
64: Harrison SC et al. (2004) Discovery of antivirals against smallpox. *Proc Natl Acad Sci U S A 101(31):11178-92.*
65: Schmidt FI et al. (2012) Poxvirus host cell entry. *Curr Opin Virol 2(1):20-7.*
66: Vanderplasschen A et al. (1998) Extracellular enveloped vaccinia virus is resistant to complement because of incorporation of host complement control proteins into its envelope. *Proc Natl Acad Sci U S A 95(13):7544-9.*
67: Payne LG (1980) Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. *J Gen Virol 50(1):89-100.*
68: Shen Y and Nemunaitis J (2005) Fighting cancer with vaccinia virus: teaching new tricks to an old dog. *Mol Ther 11(2):180-95.*
69: Jenner E (1798-1800) The Three Original Publications on Vaccination Against Smallpox. The Harvard Classics. New York: P.F. Collier & Son 1909-14; Published April 25, 2001 by Bartleby.com. Available from: http://www.bartleby.com/38/4/1.html. Accessed April 9, 2018.
70: Smith KA (2012) Louis Pasteur, the Father of Immunology? *Frontiers in Immunology 3:68.*
71: Fenner F et al. (1988) Smallpox and its Eradication. Geneva: World Health Organization; Available from: http://www.who.int/iris/handle/10665/39485. Accessed April 9, 2018.

72: Kirn DH and Thorne SH (2009) Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nat Rev Cancer 9(1):64-71.*
73: Smith GL and Moss B (1983) Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. *Gene 25(1):21-8.*
74: Thorne SH (2011) Immunotherapeutic potential of oncolytic vaccinia virus. *Immunol Res 50(2-3):286-93.*
75: Perdiguero B and Esteban M (2009) The interferon system and vaccinia virus evasion mechanisms. *J Interferon Cytokine Res 29(9):581-98.*
76: Taylor JM et al. (2006) The vaccinia virus protein F1L interacts with Bim and inhibits activation of the pro-apoptotic protein Bax. *J Biol Chem 281(51):39728-39.*
77: Kim JH et al. (2006) Systemic armed oncolytic and immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF. *Mol Ther 14(3):361-70.*
78: Heo J et al. (2013) Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. *Nat Med 19(3):329-36.*
79: Jacobs BL et al. (2009) Vaccinia virus vaccines: past, present and future. *Antiviral Res 84(1):1-13.*
80: Wittek R (2006) Vaccinia immune globulin: current policies, preparedness, and product safety and efficacy. *Int J Infect Dis 10(3):193-201.*
81: De Clercq E (2002) Cidofovir in the therapy and short-term prophylaxis of poxvirus infections. *Trends Pharmacol Sci 23(10):456-8.*
82: Yang G et al. (2005) An orally bioavailable antipoxvirus compound (ST-246) inhibits extracellular virus formation and protects mice from lethal orthopoxvirus Challenge. *J Virol 79(20):13139-49.*
83: Reeves PM et al. (2005) Disabling poxvirus pathogenesis by inhibition of Abl-family tyrosine kinases. *Nat Med 11(7):731-9.*
84: Heo J et al. (2011) Sequential therapy with JX-594, a targeted oncolytic poxvirus, followed by sorafenib in hepatocellular carcinoma: preclinical and clinical demonstration of combination efficacy. *Mol Ther 19(6):1170-9.*
85: Chen NG et al. (2011) Replication efficiency of oncolytic vaccinia virus in cell cultures prognosticates the virulence and antitumor efficacy in mice. *J Transl Med 9:164.*
86: Frentzen A et al. (2015) Use of GLV-1h68 for Vaccinia Virotherapy and Monitoring. *Methods Mol Biol 1317:225-37.*
87: Ascierto ML et al. (2011) Permissivity of the NCI-60 cancer cell lines to oncolytic Vaccinia Virus GLV-1h68. *BMC Cancer 11:451.*
88: Pugalenthi A et al. (2015) Recombinant vaccinia virus GLV-1h68 is a promising oncolytic vector in the treatment of cholangiocarcinoma. *Cancer Gene Ther 22(12):591-6.*
89: Gentschev I et al. (2011) Efficient colonization and therapy of human hepatocellular carcinoma (HCC) using the oncolytic vaccinia virus strain GLV-1h68. *PLoS One 6(7):e22069.*
90: Ehrig K et al. (2013) Growth inhibition of different human colorectal cancer xenografts after a single intravenous injection of oncolytic vaccinia virus GLV-1h68. *J Transl Med 11:79.*

91: Gentschev I et al. (2010) Regression of human prostate tumors and metastases in nude mice following treatment with the recombinant oncolytic vaccinia virus GLV-1h68. *J Biomed Biotechnol 2010:489759.*
92: Worschech A et al. (2009) Systemic treatment of xenografts with vaccinia virus GLV-1h68 reveals the immunologic facet of oncolytic therapy. *BMC Genomics 10:301.*
93: Weibel S et al. (2011) Viral-mediated oncolysis is the most critical factor in the late-phase of the tumor regression process upon vaccinia virus infection. *BMC Cancer 11:68.*
94: Advani SJ et al. (2012) Preferential replication of systemically delivered oncolytic vaccinia virus in focally irradiated glioma xenografts. *Clin Cancer Res 18(9):2579-90.*
95: Wang H et al. (2012) Oncolytic vaccinia virus GLV-1h68 strain shows enhanced replication in human breast cancer stem-like cells in comparison to breast cancer cells. *J Transl Med 10:167.*
96: Donat U et al. (2012) Preferential colonization of metastases by oncolytic vaccinia virus strain GLV-1h68 in a human PC-3 prostate cancer model in nude mice. *PLoS One 7(9):e45942.*
97: Kelly KJ et al. (2009) Real-time intraoperative detection of melanoma lymph node metastases using recombinant vaccinia virus GLV-1h68 in an immunocompetent animal model. *Int J Cancer 124(4):911-8.*
98: Price DL et al. (2014) Silk-elastin-like protein polymer matrix for intraoperative delivery of an oncolytic vaccinia virus. *Head Neck*
99: Yu Z et al. (2009) Oncolytic vaccinia therapy of squamous cell carcinoma. *Mol Cancer 8:45.*
100: Kelly KJ et al. (2008) Novel oncolytic agent GLV-1h68 is effective against malignant pleural mesothelioma. *Hum Gene Ther 19(8):774-82.*
101: Yu YA et al. (2009) Regression of human pancreatic tumor xenografts in mice after a single systemic injection of recombinant vaccinia virus GLV-1h68. *Mol Cancer Ther 8(1):141-51.*
102: Kyula JN et al. (2014) Synergistic cytotoxicity of radiation and oncolytic Lister strain vaccinia in (V600D/E)BRAF mutant melanoma depends on JNK and TNF-alpha signaling. *Oncogene 33(13):1700-12.*
103: Chernichenko N et al. (2013) Oncolytic vaccinia virus therapy of salivary gland carcinoma. *JAMA Otolaryngol Head Neck Surg 139(2):173-82.*
104: He S et al. (2012) Effective oncolytic vaccinia therapy for human sarcomas. *J Surg Res 175(2):e53-60.*
105: Lin SF et al. (2008) Oncolytic vaccinia virotherapy of anaplastic thyroid cancer in vivo. *J Clin Endocrinol Metab 93(11):4403-7.*
106: Gentschev I et al. (2010) Significant Growth Inhibition of Canine Mammary Carcinoma Xenografts following Treatment with Oncolytic Vaccinia Virus GLV-1h68. *J Oncol 2010:736907.*
107: Lin SF et al. (2007) Treatment of anaplastic thyroid carcinoma in vitro with a mutant vaccinia virus. *Surgery 142(6):976-83; discussion 976-83.*
108: Weibel S et al. (2013) Imaging of intratumoral inflammation during oncolytic virotherapy of tumors by 19F-magnetic resonance imaging (MRI). *PLoS One 8(2):e56317.*

109: Reinboth J et al. (2012) Correlates between host and viral transcriptional program associated with different oncolytic vaccinia virus isolates. *Hum Gene Ther Methods 23(5):285-96.*
110: Haddad D et al. (2011) Insertion of the human sodium iodide symporter to facilitate deep tissue imaging does not alter oncolytic or replication capability of a novel vaccinia virus. *J Transl Med 9:36.*
111: Chen N et al. (2009) A novel recombinant vaccinia virus expressing the human norepinephrine transporter retains oncolytic potential and facilitates deep-tissue imaging. *Mol Med 15(5-6):144-51.*
112: Brader P et al. (2009) Imaging a Genetically Engineered Oncolytic Vaccinia Virus (GLV-1h99) Using a Human Norepinephrine Transporter Reporter Gene. *Clin Cancer Res 15(11):3791-801.*
113: Haddad D et al. (2012) Imaging characteristics, tissue distribution, and spread of a novel oncolytic vaccinia virus carrying the human sodium iodide symporter. *PLoS One 7(8):e41647.*
114: Frentzen A et al. (2009) Anti-VEGF single-chain antibody GLAF-1 encoded by oncolytic vaccinia virus significantly enhances antitumor therapy. *Proc Natl Acad Sci U S A 106(31):12915-20.*
115: Hofmann E et al. (2011) Vaccinia virus GLV-1h237 carrying a Walker A motif mutation of mouse Cdc6 protein enhances human breast tumor therapy in mouse xenografts. *Int J Oncol 38(3):871-8.*
116: Sturm JB et al. (2012) Functional hyper-IL-6 from vaccinia virus-colonized tumors triggers platelet formation and helps to alleviate toxicity of mitomycin C enhanced virus therapy. *J Transl Med 10:9.*
117: Wilkinson MJ et al. (2016) Oncolytic vaccinia virus combined with radiotherapy induces apoptotic cell death in sarcoma cells by down-regulating the inhibitors of apoptosis. *Oncotarget 7(49):81208-81222.*
118: Hofmann E et al. (2014) Combination treatment with oncolytic Vaccinia virus and cyclophosphamide results in synergistic antitumor effects in human lung adenocarcinoma bearing mice. *J Transl Med 12:197.*
119: Seubert CM et al. (2011) Enhanced tumor therapy using vaccinia virus strain GLV-1h68 in combination with a beta-galactosidase-activatable prodrug seco-analog of duocarmycin SA. *Cancer Gene Ther 18(1):42-52.*
120: Wilkinson MJ et al. (2016) Isolated limb perfusion with biochemotherapy and oncolytic virotherapy combines with radiotherapy and surgery to overcome treatment resistance in an animal model of extremity soft tissue sarcoma. *Int J Cancer 139(6):1414-22.*
121: Lauer UM et al. (2013) Phase I/II clinical trial of a genetically modified oncolytic vaccinia virus GL-ONC1 in patients with unresectable, chemotherapy-resistant peritoneal carcinomatosis. J Clin Onc 31:3098 (May 20 Suppl; ASCO Meeting Abstract).
122: Mell LK et al. (2015) Phase I trial of intravenous attenuated vaccinia virus (GL-ONC1) with concurrent chemoradiotherapy (CRT) for locoregionally advanced head and neck carcinoma. *Journal of Clinical Oncology 33(15_suppl):6026-6026.*
123: Khan ML et al. (2014) Immunotherapeutic and oncolytic viral therapeutic strategies in pancreatic cancer. *Future Oncol 10(7):1255-75.*

124: Weber HL et al. (2015) A Novel CDC25B Promoter-Based Oncolytic Adenovirus Inhibited Growth of Orthotopic Human Pancreatic Tumors in Different Preclinical Models. *Clin Cancer Res 21(7):1665-74.*
125: Rejiba S et al. (2013) Oncosuppressive suicide gene virotherapy "PVH1-yCD/5-FC" for pancreatic peritoneal carcinomatosis treatment: NFkappaB and Akt/PI3K involvement. *PLoS One 8(8):e70594.*
126: Hastie E et al. (2013) Oncolytic vesicular stomatitis virus in an immunocompetent model of MUC1-positive or MUC1-null pancreatic ductal adenocarcinoma. *J Virol 87(18):10283-94.*
127: Kangasniemi L et al. (2012) Effects of capsid-modified oncolytic adenoviruses and their combinations with gemcitabine or silica gel on pancreatic cancer. *Int J Cancer 131(1):253-63.*
128: Cherubini G et al. (2011) The oncolytic adenovirus AdDeltaDelta enhances selective cancer cell killing in combination with DNA-damaging drugs in pancreatic cancer models. *Gene Ther 18(12):1157-65.*
129: Bossow S et al. (2011) Armed and targeted measles virus for chemovirotherapy of pancreatic cancer. *Cancer Gene Ther 18(8):598-608.*
130: Rognoni E et al. (2009) Adenovirus-based virotherapy enabled by cellular YB-1 expression in vitro and in vivo. *Cancer Gene Ther 16(10):753-63.*
131: Nelson AR et al. (2009) Combination of conditionally replicative adenovirus and standard chemotherapies shows synergistic antitumor effect in pancreatic cancer. *Cancer Sci 100(11):2181-7.*
132: Leitner S et al. (2009) Oncolytic adenoviral mutants with E1B19K gene deletions enhance gemcitabine-induced apoptosis in pancreatic carcinoma cells and anti-tumor efficacy in vivo. *Clin Cancer Res 15(5):1730-40.*
133: Angelova AL et al. (2009) Improvement of gemcitabine-based therapy of pancreatic carcinoma by means of oncolytic parvovirus H-1PV. *Clin Cancer Res 15(2):511-9.*
134: Watanabe I et al. (2008) Effects of tumor selective replication-competent herpes viruses in combination with gemcitabine on pancreatic cancer. *Cancer Chemother Pharmacol 61(5):875-82.*
135: Kasuya H et al. (2007) Suitability of a US3-inactivated HSV mutant (L1BR1) as an oncolytic virus for pancreatic cancer therapy. *Cancer Gene Ther 14(6):533-42.*
136: Hoffmann D and Wildner O (2006) Enhanced killing of pancreatic cancer cells by expression of fusogenic membrane glycoproteins in combination with chemotherapy. *Mol Cancer Ther 5(8):2013-22.*
137: Eisenberg DP et al. (2005) 5-fluorouracil and gemcitabine potentiate the efficacy of oncolytic herpes viral gene therapy in the treatment of pancreatic cancer. *J Gastrointest Surg 9(8):1068-77; discussion 1077-9.*
138: European Parliament and Council (2000) Directive 2000/54/EC - biological agents at work. Bilbao, European Agency for Safety and Health at Work; Available from: https://osha.europa.eu/en/legislation/directives/exposure-to-biological-agents/77. Accessed April 9, 2018.
139: Binz E et al. (2017) Chemovirotherapy of Pancreatic Adenocarcinoma by Combining Oncolytic Vaccinia Virus GLV-1h68 with nab-Paclitaxel Plus Gemcitabine. *Mol Ther Oncolytics 6:10-21.*

140: Skehan P et al. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst 82(13):1107-12.*
141: Promega (2013) CellTiter-Blue® Cell Viability Assay [Protocol revised 8/2013]. Fitchburg: Promega; Available from: https://www.promega.de/resources/protocols/technical-bulletins/101/celltiter-blue-cell-viability-assay-protocol/. Accessed April 9, 2018.
142: Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods 65(1-2):55-63.*
143: Huang B et al. (2011) Synergistic anti-tumor effects between oncolytic vaccinia virus and paclitaxel are mediated by the IFN response and HMGB1. *Gene Ther 18(2):164-72.*
144: Schober M et al. (2014) Desmoplasia and chemoresistance in pancreatic cancer. *Cancers (Basel) 6(4):2137-54.*
145: Sirot J et al. (1996) Definition and determination of in vitro antibiotic susceptibility breakpoints for bacteria. *Clin Microbiol Infect 2 Suppl 1:S5-s10.*
146: Moghaddas Gholami A et al. (2013) Global proteome analysis of the NCI-60 cell line panel. *Cell Rep 4(3):609-20.*
147: Nambaru PK et al. (2011) Drug efflux transporter multidrug resistance-associated protein 5 affects sensitivity of pancreatic cancer cell lines to the nucleoside anticancer drug 5-fluorouracil. *Drug Metab Dispos 39(1):132-9.*
148: Chen WH et al. (1982) Human pancreatic adenocarcinoma: in vitro and in vivo morphology of a new tumor line established from ascites. *In Vitro 18(1):24-34.*
149: Ducreux M et al. (2004) Randomized phase II study evaluating oxaliplatin alone, oxaliplatin combined with infusional 5-FU, and infusional 5-FU alone in advanced pancreatic carcinoma patients. *Ann Oncol 15(3):467-73.*
150: Tzeng CW et al. (2007) Epidermal growth factor receptor (EGFR) is highly conserved in pancreatic cancer. *Surgery 141(4):464-9.*
151: Durkin AJ et al. (2003) Defining the role of the epidermal growth factor receptor in pancreatic cancer grown in vitro. *Am J Surg 186(5):431-6.*
152: Von Hoff DD et al. (2011) Gemcitabine plus nab-paclitaxel is an active regimen in patients with advanced pancreatic cancer: a phase I/II trial. *J Clin Oncol 29(34):4548-54.*
153: Frese KK et al. (2012) nab-Paclitaxel potentiates gemcitabine activity by reducing cytidine deaminase levels in a mouse model of pancreatic cancer. *Cancer Discov 2(3):260-9.*
154: Ilkow CS et al. (2015) Reciprocal cellular cross-talk within the tumor microenvironment promotes oncolytic virus activity. *Nat Med 21(5):530-6.*
155: Hiley CT et al. (2010) Lister strain vaccinia virus, a potential therapeutic vector targeting hypoxic tumours. *Gene Ther 17(2):281-7.*
156: Hecht JR et al. (2003) A phase I/II trial of intratumoral endoscopic ultrasound injection of ONYX-015 with intravenous gemcitabine in unresectable pancreatic carcinoma. *Clin Cancer Res 9(2):555-61.*

157: Vacchelli E et al. (2014) Trial Watch: Chemotherapy with immunogenic cell death inducers. *Oncoimmunology 3(1):e27878.*
158: Smakman N et al. (2006) KRAS(D13) Promotes apoptosis of human colorectal tumor cells by ReovirusT3D and oxaliplatin but not by tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res 66(10):5403-8.*
159: Ottolino-Perry K et al. (2015) Oncolytic vaccinia virus synergizes with irinotecan in colorectal cancer. *Mol Oncol*
160: Kundranda MN and Niu J (2015) Albumin-bound paclitaxel in solid tumors: clinical development and future directions. *Drug Des Devel Ther 9:3767-77.*
161: Mato-Berciano A et al. (2017) A NOTCH-sensitive uPAR-regulated oncolytic adenovirus effectively suppresses pancreatic tumor growth and triggers synergistic anticancer effects with gemcitabine and nab-paclitaxel. *Oncotarget 8(14):22700-22715.*
162: AbouEl Hassan MA et al. (2006) Paclitaxel and vincristine potentiate adenoviral oncolysis that is associated with cell cycle and apoptosis modulation, whereas they differentially affect the viral life cycle in non-small-cell lung cancer cells. *Cancer Gene Ther 13(12):1105-14.*
163: Sei S et al. (2009) Synergistic antitumor activity of oncolytic reovirus and chemotherapeutic agents in non-small cell lung cancer cells. *Mol Cancer 8:47.*
164: Zeng WG et al. (2013) An oncolytic herpes simplex virus vector, G47Delta, synergizes with paclitaxel in the treatment of breast cancer. *Oncol Rep 29(6):2355-61.*
165: Ingemarsdotter CK et al. (2010) Low-dose paclitaxel synergizes with oncolytic adenoviruses via mitotic slippage and apoptosis in ovarian cancer. *Oncogene 29(45):6051-63.*
166: Yu DC et al. (2001) Antitumor synergy of CV787, a prostate cancer-specific adenovirus, and paclitaxel and docetaxel. *Cancer Res 61(2):517-25.*
167: Cheong SC et al. (2008) E1A-expressing adenoviral E3B mutants act synergistically with chemotherapeutics in immunocompetent tumor models. *Cancer Gene Ther 15(1):40-50.*
168: Noonan AM et al. (2016) Randomized Phase 2 Trial of the Oncolytic Virus Pelareorep (Reolysin) in Upfront Treatment of Metastatic Pancreatic Adenocarcinoma. *Mol Ther 24(6):1150-1158.*
169: Yardley DA (2013) nab-Paclitaxel mechanisms of action and delivery. *J Control Release 170(3):365-72.*
170: Desai N et al. (2006) Increased antitumor activity, intratumor paclitaxel concentrations, and endothelial cell transport of cremophor-free, albumin-bound paclitaxel, ABI-007, compared with cremophor-based paclitaxel. *Clin Cancer Res 12(4):1317-24.*
171: Desai N et al. (2009) SPARC Expression Correlates with Tumor Response to Albumin-Bound Paclitaxel in Head and Neck Cancer Patients. *Transl Oncol 2(2):59-64.*

172: Gradishar WJ et al. (2005) Phase III trial of nanoparticle albumin-bound paclitaxel compared with polyethylated castor oil-based paclitaxel in women with breast cancer. *J Clin Oncol 23(31):7794-803.*
173: Aghi M et al. (2006) Effect of chemotherapy-induced DNA repair on oncolytic herpes simplex viral replication. *J Natl Cancer Inst 98(1):38-50.*
174: Adusumilli PS et al. (2006) Cisplatin-induced GADD34 upregulation potentiates oncolytic viral therapy in the treatment of malignant pleural mesothelioma. *Cancer Biol Ther 5(1):48-53.*
175: Petrowsky H et al. (2001) Functional interaction between fluorodeoxyuridine-induced cellular alterations and replication of a ribonucleotide reductase-negative herpes simplex virus. *J Virol 75(15):7050-8.*
176: Cheng PH et al. (2013) Combination of autophagy inducer rapamycin and oncolytic adenovirus improves antitumor effect in cancer cells. *Virol J 10:293.*
177: Zemp FJ et al. (2013) Treating brain tumor-initiating cells using a combination of myxoma virus and rapamycin. *Neuro Oncol 15(7):904-20.*
178: Lun XQ et al. (2009) Efficacy of systemically administered oncolytic vaccinia virotherapy for malignant gliomas is enhanced by combination therapy with rapamycin or cyclophosphamide. *Clin Cancer Res 15(8):2777-88.*
179: Weiland T et al. (2014) Enhanced killing of therapy-induced senescent tumor cells by oncolytic measles vaccine viruses. *Int J Cancer 134(1):235-43.*
180: Kulu Y et al. (2013) Concurrent chemotherapy inhibits herpes simplex virus-1 replication and oncolysis. *Cancer Gene Ther 20(2):133-40.*
181: Cheema TA et al. (2011) Enhanced antitumor efficacy of low-dose Etoposide with oncolytic herpes simplex virus in human glioblastoma stem cell xenografts. *Clin Cancer Res 17(23):7383-93.*
182: Zhong S et al. (2010) An armed oncolytic adenovirus ZD55-IL-24 combined with ADM or DDP demonstrated enhanced antitumor effect in lung cancer. *Acta Oncol 49(1):91-9.*
183: Maliandi MV et al. (2015) AduPARE1A and gemcitabine combined treatment trigger synergistic antitumor effects in pancreatic cancer through NF-kappaB mediated uPAR activation. *Mol Cancer 14:146.*
184: Yamada S et al. (2012) Oncolytic herpes simplex virus expressing yeast cytosine deaminase: relationship between viral replication, transgene expression, prodrug bioactivation. *Cancer Gene Ther 19(3):160-70.*
185: Foloppe J et al. (2008) Targeted delivery of a suicide gene to human colorectal tumors by a conditionally replicating vaccinia virus. *Gene Ther 15(20):1361-71.*
186: Ma G et al. (2010) Combinatory cytotoxic effects produced by E1B-55kDa-deleted adenoviruses and chemotherapeutic agents are dependent on the agents in esophageal carcinoma. *Cancer Gene Ther 17(11):803-13.*
187: Raki M et al. (2005) Combination of gemcitabine and Ad5/3-Delta24, a tropism modified conditionally replicating adenovirus, for the treatment of ovarian cancer. *Gene Ther 12(15):1198-205.*

188: Roulstone V et al. (2013) Synergistic cytotoxicity of oncolytic reovirus in combination with cisplatin-paclitaxel doublet chemotherapy. *Gene Ther 20(5):521-8.*
189: Jiang G et al. (2013) A dual-regulated oncolytic adenovirus expressing interleukin-24 sensitizes melanoma cells to temozolomide via the induction of apoptosis. *Tumour Biol 34(2):1263-71.*
190: Lin SF et al. (2008) Synergy of a herpes oncolytic virus and paclitaxel for anaplastic thyroid cancer. *Clin Cancer Res 14(5):1519-28.*
191: Schache P et al. (2009) VSV virotherapy improves chemotherapy by triggering apoptosis due to proteasomal degradation of Mcl-1. *Gene Ther 16(7):849-61.*
192: Liu D et al. (2009) Preclinical evaluation of synergistic effect of telomerase-specific oncolytic virotherapy and gemcitabine for human lung cancer. *Mol Cancer Ther 8(4):980-7.*
193: Alonso MM et al. (2007) Adenovirus-based strategies overcome temozolomide resistance by silencing the O6-methylguanine-DNA methyltransferase promoter. *Cancer Res 67(24):11499-504.*
194: Ungerechts G et al. (2007) Lymphoma chemovirotherapy: CD20-targeted and convertase-armed measles virus can synergize with fludarabine. *Cancer Res 67(22):10939-47.*
195: Kaufmann JK et al. (2013) Chemovirotherapy of malignant melanoma with a targeted and armed oncolytic measles virus. *J Invest Dermatol 133(4):1034-42.*
196: Li H et al. (2007) Coadministration of a herpes simplex virus-2 based oncolytic virus and cyclophosphamide produces a synergistic antitumor effect and enhances tumor-specific immune responses. *Cancer Res 67(16):7850-5.*
197: Kottke T et al. (2011) Precise scheduling of chemotherapy primes VEGF-producing tumors for successful systemic oncolytic virotherapy. *Mol Ther 19(10):1802-12.*
198: Quirin C et al. (2007) Combining adenoviral oncolysis with temozolomide improves cell killing of melanoma cells. *Int J Cancer 121(12):2801-7.*
199: Heise C et al. (2000) Efficacy with a replication-selective adenovirus plus cisplatin-based chemotherapy: dependence on sequencing but not p53 functional status or route of administration. *Clin Cancer Res 6(12):4908-14.*
200: Fujiwara T et al. (2006) Enhanced antitumor efficacy of telomerase-selective oncolytic adenoviral agent OBP-401 with docetaxel: preclinical evaluation of chemovirotherapy. *Int J Cancer 119(2):432-40.*
201: Tobias AL et al. (2013) The timing of neural stem cell-based virotherapy is critical for optimal therapeutic efficacy when applied with radiation and chemotherapy for the treatment of glioblastoma. *Stem Cells Transl Med 2(9):655-66.*
202: Kondo N et al. (2010) Antitumor effects of telomelysin in combination with paclitaxel or cisplatin on head and neck squamous cell carcinoma. *Oncol Rep 23(2):355-63.*

203: Nagano S et al. (2008) Cancer cell death enhances the penetration and efficacy of oncolytic herpes simplex virus in tumors. *Cancer Res 68(10):3795-802.*
204: Wennier ST et al. (2012) Myxoma virus sensitizes cancer cells to gemcitabine and is an effective oncolytic virotherapeutic in models of disseminated pancreatic cancer. *Mol Ther 20(4):759-68.*
205: Moehler M et al. (2011) Activation of the human immune system by chemotherapeutic or targeted agents combined with the oncolytic parvovirus H-1. *BMC Cancer 11:464.*
206: Ma B et al. (2015) Synergistic suppression effect on tumor growth of hepatocellular carcinoma by combining oncolytic adenovirus carrying XAF1 with cisplatin. *J Cancer Res Clin Oncol 141(3):419-29.*
207: Shen W et al. (2010) Oncolytic adenovirus mediated Survivin RNA interference and 5-fluorouracil synergistically suppress the lymphatic metastasis of colorectal cancer. *Oncol Rep 24(5):1285-90.*
208: Rein DT et al. (2012) Combination of a MDR1-targeted replicative adenovirus and chemotherapy for the therapy of pretreated ovarian cancer. *J Cancer Res Clin Oncol 138(4):603-10.*
209: Oberg D et al. (2010) Improved potency and selectivity of an oncolytic E1ACR2 and E1B19K deleted adenoviral mutant in prostate and pancreatic cancers. *Clin Cancer Res 16(2):541-53.*
210: Takakura M et al. (2010) Intraperitoneal administration of telomerase-specific oncolytic adenovirus sensitizes ovarian cancer cells to cisplatin and affects survival in a xenograft model with peritoneal dissemination. *Cancer Gene Ther 17(1):11-9.*
211: Chen G et al. (2006) Oncolytic adenovirus-mediated transfer of the antisense chk2 selectively inhibits tumor growth in vitro and in vivo. *Cancer Gene Ther 13(10):930-9.*
212: Toyoizumi T et al. (1999) Combined therapy with chemotherapeutic agents and herpes simplex virus type 1 ICP34.5 mutant (HSV-1716) in human non-small cell lung cancer. *Hum Gene Ther 10(18):3013-29.*
213: Bracci L et al. (2014) Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. *Cell Death Differ 21(1):15-25.*
214: Rojas JJ et al. (2015) Defining Effective Combinations of Immune Checkpoint Blockade and Oncolytic Virotherapy. *Clin Cancer Res 21(24):5543-51.*
215: Fend L et al. (2017) Immune checkpoint blockade, immunogenic chemotherapy or IFN-alpha blockade boost the local and abscopal effects of oncolytic virotherapy. *Cancer Res*
216: Liu Z et al. (2017) Rational combination of oncolytic vaccinia virus and PD-L1 blockade works synergistically to enhance therapeutic efficacy. *Nat Commun 8:14754.*
217: Shurin GV et al. (2009) Chemotherapeutic agents in noncytotoxic concentrations increase antigen presentation by dendritic cells via an IL-12-dependent mechanism. *J Immunol 183(1):137-44.*

218: Tanaka H et al. (2009) Classification of chemotherapeutic agents based on their differential in vitro effects on dendritic cells. *Cancer Res 69(17):6978-86.*
219: Nowak AK et al. (2002) Gemcitabine exerts a selective effect on the humoral immune response: implications for combination chemo-immunotherapy. *Cancer Res 62(8):2353-8.*
220: Plate JM et al. (2005) Effect of gemcitabine on immune cells in subjects with adenocarcinoma of the pancreas. *Cancer Immunol Immunother 54(9):915-25.*
221: Soeda A et al. (2009) Regular dose of gemcitabine induces an increase in CD14+ monocytes and CD11c+ dendritic cells in patients with advanced pancreatic cancer. *Jpn J Clin Oncol 39(12):797-806.*
222: Nowak AK et al. (2003) Synergy between chemotherapy and immunotherapy in the treatment of established murine solid tumors. *Cancer Res 63(15):4490-6.*
223: Gujar SA et al. (2014) Gemcitabine enhances the efficacy of reovirus-based oncotherapy through anti-tumour immunological mechanisms. *Br J Cancer 110(1):83-93.*
224: Nowak AK and Lesterhuis WJ (2014) Chemoimmunotherapy: still waiting for the magic to happen. *Lancet Oncol 15(8):780-1.*
225: Baker LA et al. (2016) Modeling pancreatic cancer with organoids. *Trends Cancer 2(4):176-190.*

Publication list

Publications in peer-reviewed journals

1. Binz E and Lauer UM (2015). **Chemovirotherapy: combining chemotherapeutic treatment with oncolytic virotherapy**. *Oncolytic Virother 2015:4:39-48. (REVIEW)*

2. Binz E, Berchtold S, Beil J, Schell M, Geisler C, Smirnow C and Lauer UM (2017). **Chemovirotherapy of Pancreatic Adenocarcinoma by Combining Oncolytic Vaccinia Virus GLV-1h68 with *nab*-Paclitaxel Plus Gemcitabine**. *Mol Ther Oncolytics 6:10-21. (Publikation ausgewählter Originaldaten der hier vorgelegten Promotionsarbeit)*

Abstracts/Posters

1. Binz E, Berchtold S, Geisler C, Smirnow I, Malek NP and Lauer UM (2015). **Chemovirotherapy – Improving the clinical outcome of advanced pancreatic adenocarcinoma by combining the oncolytic vaccinia virus GLV-1h68 with the dual chemotherapy *nab*-Paclitaxel + gemcitabine**. *Z Gastroenterol 53: KG065. (Präsentation auf der Jahrestagung der Deutschen Gesellschaft für Verdauungs- und Stoffwechselerkrankungen (DGVS) 2015)*

2. Binz E, Berchtold S, Geisler C, Smirnow I, Malek NP and Lauer UM (2014). **Improvement of therapeutic anti-cancer strategies by combinations of oncolytic vaccinia virus GLV-1h68 with chemotherapeutics (*nab*-Paclitaxel + gemcitabine)**. (*Poster Präsentation beim Forschungskolloqium der Medizinischen Fakultät Tübingen)*

Erklärung zum Eigenanteil

Erklärungen zum Eigenanteil der Publikationen

1. Binz E and Lauer UM (2015). **Chemovirotherapy: combining chemotherapeutic treatment with oncolytic virotherapy**. *Oncolytic Virother 2015:4:39-48.*

Herr Prof. Dr. Ulrich M. Lauer war federführend an der Konzeption der Arbeit beteiligt, in die Ausarbeitung und Ausgestaltung der Abbildungen involviert und hat das Manuskript im Überarbeitungsprozess fortwährend korrigiert.

Herr Binz übernahm die Literaturrecherche sowie das Zusammentragen der chemovirotherapeutischen Studien, erstellte aus den gewonnenen Informationen selbstständig Übersichtsabbildungen sowie den ersten Entwurf des Manuskripts, übernahm die gewünschten Korrekturen des Manuskriptes bis zur finalen Fassung und reichte es schlussendlich zur Veröffentlichung ein.

2. Binz E, Berchtold S, Beil J, Schell M, Geisler C, Smirnow I and Lauer UM (2017). **Chemovirotherapy of Pancreatic Adenocarcinoma by Combining Oncolytic Vaccinia Virus GLV-1h68 with *nab*-Paclitaxel Plus Gemcitabine**. *Mol Ther Oncolytics 6:10-21.*

Frau Dr. Susanne Berchtold war an der Konzeption der Arbeit beteiligt, betreute sie und übernahm Korrekturen des Manuskriptes. Frau Dr. Berchtold war an der Durchführung der Virusreplikations-Kurven beteiligt, die in Abbildung 7 gezeigt sind.

Frau Dr. Julia Beil sowie Frau Dr. Martina Schell waren an der Konzeption der Arbeit beteiligt, betreuten sie und übernahmen Korrekturen des Manuskripts.

Frau Geisler (MTA) sowie Frau Smirnow (MTA) waren verantwortlich für die Durchführung der Virusreplikations-Kurven, die in Abbildung 7 gezeigt sind.

Herr Prof. Dr. Ulrich M. Lauer war federführend an der Konzeption der Arbeit beteiligt, betreute sie und übernahm Korrekturen des Manuskripts.

Herr Eike Hendrik Binz hat die Versuche 1, 2, 3, 4, 5, 6 sowie deren Auswertung durchgeführt und die dazugehörigen Abbildungen (zudem Abbildung 7 und Supplementary figure 1) erstellt. Herr Binz war an der Konzeption der Arbeit beteiligt, führte die Literaturrecherche durch, verfasste selbstständig den ersten Entwurf

des Manuskripts, übernahm die gewünschten Korrekturen des Manuskriptes bis zur finalen Fassung und reichte es schlussendlich zur Veröffentlichung ein.

Erklärungen zum Eigenanteil der Dissertationsschrift

Die vorliegende Arbeit wurde in der Medizinischen Universitätsklinik, Abteilung für Innere Medizin VIII, unter Betreuung von Herrn Prof. Dr. Ulrich M. Lauer durchgeführt.

Die Konzeption der Studie erfolgte federführend durch Herrn Prof. Dr. Lauer in Zusammenarbeit mit Frau Dr. Berchtold, Frau Dr. Beil und Frau Dr. Schell (allesamt wissenschaftliche Mitarbeiterinnen).

Sämtliche Versuche – sofern nachfolgend nicht anderweitig aufgeführt – wurden (nach Einarbeitung durch Frau Smirnow, MTA) von mir selbständig durchgeführt.

Die Erstellung der Virusreplikations-Kurven (abgebildet in den Figures 33, 34, 36) sowie die Dokumentation des dosis- und zeitabhängigen Einflusses der Chemotherapeutika *nab*-Paclitaxel oder Gemcitabine auf die virale GFP Expression (abgebildet in den Figures 35, 37, 38) erfolgte zu Teilen durch Frau Geisler, Frau Smirnow (beide MTA) und Frau Dr. Berchtold (wissenschaftliche Mitarbeiterin).

Die statistische Auswertung aller Versuche erfolgte selbstständig durch mich.

Ich versichere hiermit das Manuskript, nach Anleitung durch Frau Dr. Berchtold und Herrn Prof. Dr. Lauer, selbstständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den 09.04.18

Eike Hendrik Binz