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Chemovirotherapy employing Gemcitabine together with Oncolytic Measles Vaccine Viruses as a new Option for the Treatment of Pancreatic Cancer

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May, Verena Larissa Josefine Ilse

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Dekan: Professor Dr. B.J. Pichler

1. Berichterstatter: Professor Dr. U. Lauer
2. Berichterstatter: Professor Dr. F. Stubenrauch

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Abbreviations

5-FC	5-Fluorocytosine
5-FU	5-Fluoroururacil
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
FDA	USA Food and Drug Administration
F-Protein	fusion protein
FBS	fetal bovine serum
H-Protein	hemagglutinin protein
hpi	hours post infection
L-Protein	large protein
M-Protein	matrix protein
MeV	measles virus
MOI	multiplicity of infection (ratio infectious particles / cell)
OV	oncolytic virus
P-Protein	phosphoprotein
PBS	phosphate buffered saline
PFU	plaque forming unit
RNA	ribonucleic acid
rpm	rounds per minute
SA-β-gal	senescence associated β-galactosidase
SLAM	signalling lymphocyte activation molecule
TCA	trichloroacetic acid
TIS	therapy induced senescence
TRIS	tris(hydroxymethyl)-aminometha

1. Introduction

1.1. Pancreatic cancer

1.1.1. Epidemiology

In economically developed countries, cancer is the leading cause of death and the second leading cause in developing countries. Globally, about 337,000 people are being diagnosed with pancreatic cancer yearly [1]. Among cancer, pancreatic cancer remains a very aggressive entity as - referring to statistics from the US - only 7 % of patients survive past 5 years and most die within the first year after diagnosis (Siegel et al., [2, 3]). Beneath the poor prognosis its incidence is quite high (about 49,000 patients estimated diagnosed in 2015, [2]) and constantly rising (53,670 in 2017, [3]). Both contribute to pancreatic cancer being the fourth-leading cause of cancer related deaths (all numbers refer to statistics from the United States). The minority of patients (9-10 %) presents in a resectable stage; 5-year survival rate among those patients is only 26 % [2]. Symptoms occur rarely in early stages, that is why more than 80 % of patients are diagnosed with locally advanced or metastatic disease, which are both unresectable. Unfortunately, unresectable disease is associated with an expected survival of 6 months [4, 5]. Constant increase in incidence is related to cigarette smoking, obesity and diabetes type II. Chronic inflammation and insulin resistance are major risk factors for the development of pancreatic cancer. Besides, advanced age, inherited familial cancer syndromes, Ashkenazi Jewish heritage and dietary factors raise the individual risk for the development of pancreatic cancer [1].

1.1.2. Tumor Biology and Microenvironment

Current research describes oncogenic transformation in the pancreas as a multistage process including activation of oncogenes and loss of tumor suppressor genes [5]. Mostly, activating mutation in the *KRAS* gene followed by a mutation in one or more of the tumor suppressor genes *TP53, p16/CDKN2A*

and *SMAD4* leads to the development of pancreatic cancer [6]. Both activation of *KRAS* and loss of tumor suppressor gene function seem to be indispensable for the development of pancreatic cancer, as mice with a single point mutation in the *KRAS* oncogene only developed pancreatic preneoplastic lesions [7].

Microenvironment constitutes - besides the mostly late diagnosis - another important reason for the poor prognosis. Delivery of systemically administered chemotherapy is impaired as, due to the dense extracellular matrix, configuration of blood and lymphatic vessels in pancreatic cancer exhibits an abnormal structure which inhibits perfusion resulting in an impeded delivery of chemotherapeutic drugs [8]. Therefore, novel therapy options aim at overcoming that barrier which seems to protect tumor cells [9]. Besides the unfavorable stromal matrix, pancreatic cancer exhibits a large amount of inflammatory cells within the tumor. Those inflammatory cells reinforce the protumorigenic surrounding. This long-term inflammation contributes to the development of pancreatic cancer [10]. Additionally, inflammatory cells are mostly immunosuppressive cells instead of cytotoxic T-cells [11]. The advantage of cytotoxic T-cells, especially regarding virotherapy, would be the immunotherapeutic effect on infected cancer cells which is an efficient way to raise potency of oncolytic virotherapy [5]. Concerning propensity to metastases, a mechanism called epithelial to mesenchymal transition (EMT) plays an important role. EMT is a mechanism, which is originally crucial for differentiation of tissues and organs in development and includes the loss of E-Cadherin. In a malign setting, EMT is associated with metastases and worse prognosis [12]. Especially for pancreatic cancer, EMT was shown to correlate with systemic aggressiveness and resistance towards chemotherapy [13].

1.1.3. Therapy

As described above, resection is only an option in about 20 % of all cases, although systemic recurrence constitutes a major problem [14]. If pancreatic cancer is already locally advanced or metastatic, a palliative chemotherapy has been the only option so far. Since 1997, gemcitabine (Gemzar, Eli Lilly,

Indianapolis, IN), a nucleoside analogue was standard first-line therapeutic [15]. Therapy with gemcitabine leads to a 5-year survival of only 2 %, 1-year survival is between 17 and 23 % [15-17]. Add-on of further chemotherapeutic drugs was mostly inefficient and did not result in any benefit concerning progression-free survival or long-term survival. Two important exceptions to mention are a combination of gemcitabine with erlotinib, which targets an epidermal growth factor tyrosine kinase (TKI-EGFR) with an improvement of overall survival of 2 weeks [16] and add-on of nab-paclitaxel (Abraxane®, Celgene) [18]. Nab-paclitaxel, which was approved by the US FDA in 2013, consists of albumin-bound paclitaxel, a microtubule inhibitor, and was reported to work synergistically with gemcitabine in a murine model [19]. In a multinational phase III study, nab-paclitaxel in combination with gemcitabine prolonged median overall survival significantly in patients with metastatic pancreatic cancer [20]. Another therapy regimen, which was established in 2011, constitutes a combination of three chemotherapeutic drugs and one adjuvant: FOLFIRINOX, including 5-fluorouracil plus leucovorin, irinotecan and oxaliplatin (FOL= Leucovorin Calcium (Folinic Acid); F = Fluorouracil; IRIN = Irinotecan Hydrochloride; OX = Oxaliplatin). However, this regimen is reserved for patients with good performance status, as the side effects are quite severe. Overall survival improved compared to gemcitabine alone about 4.3 months [17]. Expectedly, combination therapy with more than one chemotherapeutic agent led to more severe side effects, more precisely a greater risk for grade 3-4 toxicities than gemcitabine alone [21].

1.2. Gemcitabine

1.2.1. Chemotherapy with Gemcitabine

Gemcitabine and its mode of action has been described first by Huang et al. in 1991 [22]. In his work, Huang described the influence of *2',2'-difluoro-deoxycytidine* - which describes the chemical structure of gemcitabine - on

DNA-synthesis. Gemcitabine is an analogue of the nucleoside deoxycytidine, which explains its efficacy as antimetabolite. As gemcitabine is incorporated as ‘wrong’ metabolite during DNA-synthesis, DNA-synthesis comes to a pause leading to cell death. Additionally, the enzyme ribonucleotide reductase (RNR), which is essential for DNA replication, is inhibited by one of the metabolites of gemcitabine [23, 24].

In the case of pancreatic cancer, gemcitabine has shown to be one of the most efficient chemotherapeutics as 1-year survival was about 18 % in comparison to therapy with 5-fluorouracil (5-FU), which led to a 1-year survival of only 2 % [25].

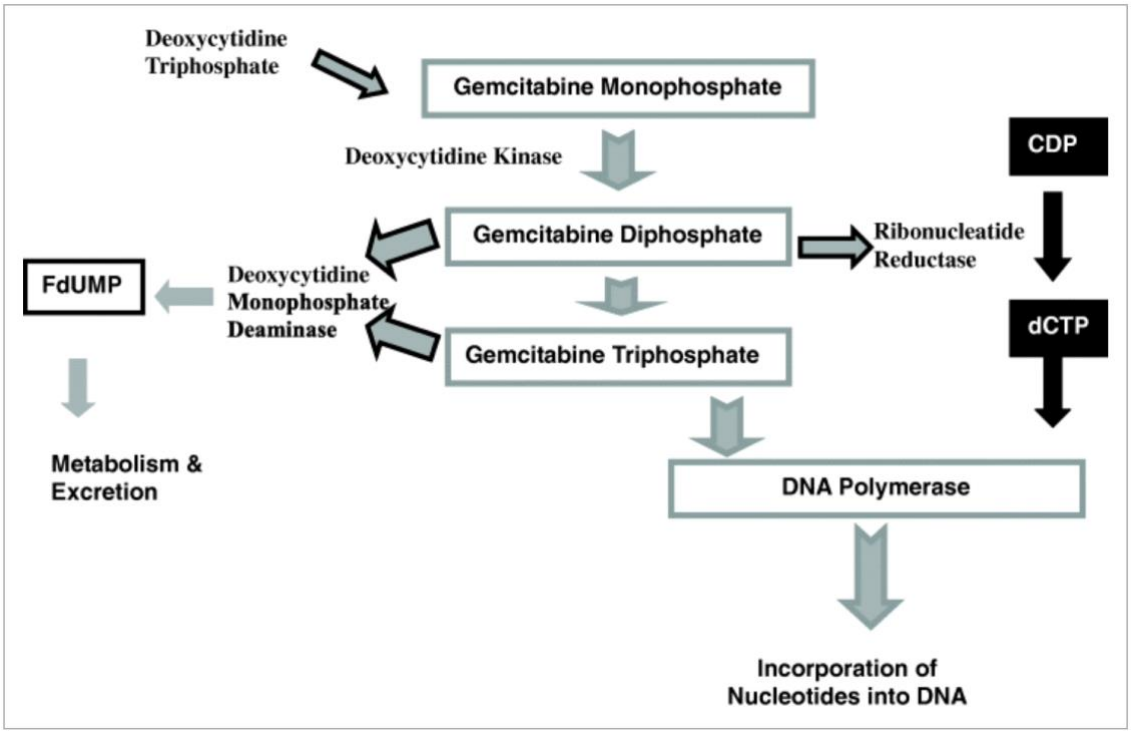


Figure 1: Metabolism and mechanisms of action of gemcitabine according to Shore et al. [24]. Incorporation of nucleotides into DNA leads to a pause of DNA synthesis and consequently to cell death. Inhibition of the enzyme ribonucleotide reductase leads to an inhibition of DNA polymerase.

Figure: Shore et al.: Review article: Chemotherapy for pancreatic cancer.

1.2.2. Therapy induced senescence

Gemcitabine is capable not only to kill cells but also to bring cells in a state, which can be compared to hibernation. This state is referred to as ‘senescence’, which originates from the Latin verb ‘senescere’, which means ‘aging’. Senescence has firstly been described in cell culture by Hayflick et al. in 1964 [26].

Characterization of senescence includes a permanent growth arrest and a distinct cellular morphology [27]. Senescent cells appear flattened and enlarged, the cytoplasmic granularity is enhanced and the nucleus prominent. Moreover, senescent cells express senescence-associated β-galactosidase [28] as a correlative of increased lysosomal mass [29].

Senescence can be triggered by numerous mechanisms, including loss of telomeres or harboring of genetic damage, which both result in a DNA damage response (DDR), initiating senescence [30]. DDR itself reinforces senescence as it is linked to so-called DNA-SCARS (DNA segments with chromatin alterations reinforcing senescence). These are chromatin alterations, which lead to secretion of DDR proteins. Moreover, DDR leads to a distinct senescence associated secretory phenotype (SASP), including growth factors, proteases and cytokines [31, 32]. Thus, senescent cells still have paracrine and autocrine functions, although they are not able to replicate.

1.3. Oncolytic Virotherapy

1.3.1. History and development

Oncolytic virotherapy (OV) has been detected “accidentally” as spontaneous tumor regression has been reported in the course of naturally occurring virus infections. The earliest reports go back to the late 19th century, mostly in leukemia or - more recently published - also in lymphoma. However, tumor regression was only short term, lasting 1 or 2 months [33-38]. Of note, those

findings were preferentially made in young patients with a suppressed immune system as they suffered from either leukemia or lymphoma [39]. In the beginning of the 20th century, ideas for the exploitation of viruses as cancer therapeutics arose; yet first important approaches, using for example Hepatitis B virus or Mumps virus were made starting in 1949 [40, 41]. The focus on patient's safety was minor than nowadays, as viruses were not attenuated and unpurified body fluids were either injected or inhaled. Consequently, development of hepatitis, also leading to death, was among the side effects. After 1949, many different viruses have been tested preclinically and clinically – with a special focus on the Egypt 101 isolate of the West Nile Virus. In the course of those experiments, researchers found out about most disadvantages of OV: in patients with an intact immune system, viruses were often ineffective. If patients were immunosuppressed, as the malignancy involved the immune system, side effects were too severe if not life threatening or fatal [39, 42, 43]. The abandoning of those ineffective or unsafe viruses resulted in a convergence of interest towards more effective viruses for OV such as adeno-viruses, herpesviruses, picornaviruses, paramyxoviruses and poxviruses. The named viruses were mostly used in an attenuated version due to the pathogenicity of wild type viruses. Besides human pathogenic viruses, which are either attenuated or quite mild concerning their symptoms, animal viruses without human pathogenicity were tested as OVs. One important example is the Newcastle Disease Virus, an avian virus [44], which continues to be used in cancer therapy with good remission rates [45]. In November 2005, a breakthrough in the field of OV was achieved: China was the first country to approve an OV for cancer therapy. A genetically engineered adenovirus called H101 was approved for the treatment of head and neck cancer [46]. In 2015, a engineered herpes simplex virus (T-VEC) has been approved for the treatment of malignant melanoma in the US and Europe [47, 48]

1.3.2. Mechanism of viral infection in tumors and oncolysis

The main principle of oncolytic virotherapy is the exploitation of a natural phenomenon, as viruses are commonly parasites in infected cells. Some viruses exhibit a natural tropism for cancer cells – the mechanisms of such a tumor-selectivity are not always known. Concerning measles virus, viral entry is granted by an overexpression of the CD46 receptor in tumor tissues [49]. Defects in the IFN signaling pathway facilitate viral replication. Mostly, engineering is needed to grant tumor selectivity [50].

After infection, three steps are very important for effective operation of oncolytic virotherapy: (i) first of all, the direct cytotoxicity of viruses towards their host cells; host cell machinery is exploited until destruction to allow the virus to replicate; (ii) in the course of destruction, viral particles are released, which infect further tumor cells [50]; (iii) in a living organism, the immune system is activated by the stimulus of the viral infection. As the focus is within the infected tumor, the immune system will attack preferentially tumor cells and consequently, virus induces an immune response to the tumor [5]. Russel and Peng call the described mechanisms of OV “The oncolytic virotherapy paradigm” (Fig. 2) which provides a good review on the involved components.

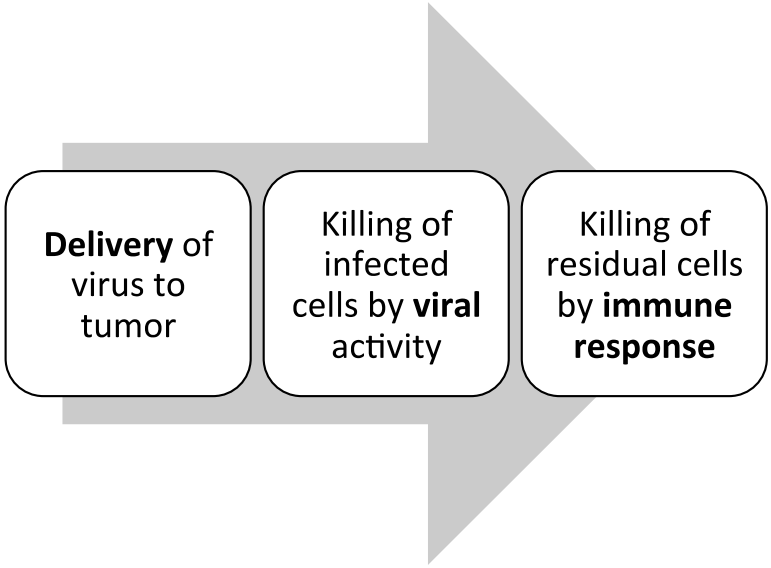


Figure 2: The oncolytic virotherapy paradigm according to Russel and Peng [51]. For efficient OV, three components are indispensable. (i) Delivery of the virus to the tumor has to be granted. Therefore, virus must be targeted to tumor cells and overcome immunologic barriers. (ii) Killing of infected cells by viral toxicity. Viral replication within the tumor cells is critical to ensure further infection and spread of viral particles. (iii) As long-term effect, stimulation of the immune system is eligible. Remaining tumor cells are targeted leading to remission without relapse.

1.3.2.1. Advantages and disadvantages

As oncolytic virotherapeutics are rather different from other anticancer drugs, they include special advantages and disadvantages. The first unique feature of OVs is their ability to replicate. Thus, if infection and replication are efficient, an augmentation of the administered viral load is taking place [52]. Another advantage of that mechanism is the automatized ‘dose finding’. As only cancer cells allow viral particles to replicate, replication is self-limiting as soon as all cancer cells are lysed. Consequently, side effects are rather soft or missing, as the therapeutic is quite targeted and only cancer cells are affected. One important example to mention is the overexpression of CD46 in cancer cells, which leads to a facilitated viral entry of MeV [52]. The stimulation of the immune system enhances the therapeutic effect by targeting cancer cells,

which are under normal conditions capable to evade the immune system [53, 54].

Yet oncolytic virotherapy also meets obstacles. As mentioned above, the immune system plays an important role for virotherapeutical success. Nevertheless, the immune system is also the predominant obstacle to overcome [55]. To minimize side effects and to ensure that virotherapy is safe (see 1.3.1. History and development), only attenuated viruses or viruses with a low pathogenicity are used for OV. Consequently, viruses are rarely challenging opponents for the immune system. In the worst case, viruses are detected and destroyed directly after application. Immunosuppression is not a definite solution for this problem – as symptoms of the viral infection are likely to get out of control. To overcome the neutralizing effect of the immune system towards administered viruses, different ways of application were investigated. Intravenous injection is quite disadvantageous if the immune system is familiar with the applied virus [56]. This precondition is common, as patients are either vaccinated (e.g. measles virus) or have been infected with the virus before.

Another possibility – though not always easy to realize – is intratumoral application [57]. The advantage of this method is obviously the localized rather high concentration including the prevention of systemic side effects. For peritoneal carcinomatosis, intraperitoneal application is also under clinical investigation by our group as it targets not only primary tumor but also intraperitoneal dissemination [58]. In our phase I/II study we were able to show, that intraperitoneal application does not lead to severe side effects when a recombinant vaccinia virus was administered in patients with metastatic gastric cancer or ovarian cancer or mesothelioma. Tumor cells in ascites were shown to decrease in numbers by only a single dose of vaccinia virus.

However, intratumoral and intraperitoneal application are limited as not every cancer entity can be reached by those methods. Moreover, one advantage of intravenous administration is, that not only the primary tumor is targeted but also possible metastases [59].

1.3.2.2. Enhancing oncolytic effects

To improve oncolytic effects, the main goal is to overcome the obstacles described before. Simplified, that means to ensure delivery and replication of the virus and to stimulate the immune system for a ‘second hit’ of the tumor. The different ways of delivery have been described before; emphasizing that intravenous injection is the method of choice to grant delivery to all tumor sides and possible metastases. For that purpose, the immune system has to be ‘compassed’.

The main ‘enemies’ for successful delivery are (i) liver and spleen, where viruses are cleared by the mononuclear phagocytic system (MPS) and (ii) serum factors such as antibodies or complement, which neutralize viruses [59]. To minimize sequestration by liver and spleen, one option is to modify viral coat proteins to avoid opsonization and subsequent phagocytosis by the MPS [60]. Two modifications of the viral coat proteins for prolonged circulation time are polyethylene glycol (PEG) and N-(2-hydroxypropyl)methacrylamide (HPMA) [61, 62] which has been investigated for example for VSV [63] and adenovirus type 5 [64]. To evade neutralization by serum factors, an efficient way is to ‘hide’ the virus inside of carrier cells such as tumor cells [65] or normal primary cells with the natural ability to home to tumor beds [66]. Of course, carrier cells should not be killed by the virus before the tumor has been reached [59]. Especially for mesenchymal stem cells (MSCs), dendritic cells and T cells investigations concerning delivery of the virus to the tumor are quite promising and constitute an interesting approach [67-69].

Further key points for successful delivery include optimal targeting of the tumor which means (i) tumor blood vessels must be permeable for virus and (ii) tumor vessel endothelium must be targeted by the virus itself [59].

1.4. Measles Virus

1.4.1. Introduction, Epidemiology and History

Measles virus is a leading cause of death in children in developing countries, even though a cost-effective vaccine exists [70]. Measles is one of the most contagious diseases and transmitted by aerosols. Almost every infection becomes clinically manifest and can include severe complications; especially in developing countries, malnutrition raises lethality [71]. According to the WHO, in 2015 there were 134,200 measles deaths globally. Even though those numbers seem to be enormous, efforts to raise the vaccination coverage have led to a drop of measles deaths of 79 % worldwide between 2000 and 2015 [70], after the WHO had launched a worldwide program to eliminate measles [71]. In 1954, John F. Enders and colleagues firstly isolated the virus in cell culture, followed by the development of the live attenuated measles vaccine in 1963 [72].

1.4.2. Classification

According to the International Committee on Taxonomy of Viruses (ICTV), measles virus belongs to the order mononegavirales, the family paramyxo-viridae and the genus morbillivirus; measles virus is one of 7 species of the genus morbillivirus, like rinderpest or canine distemper virus [73].

1.4.3. Structure, Pathology and Infection

As measles virus belongs to the Paramyxoviridae, it is a negative-stranded RNA-virus with an envelope [74]. The two envelope proteins are the hem-agglutinin (H) and the fusion (F) protein, which are responsible for membrane attachment and cell fusion [72]. As depicted in Figure 3, the mentioned proteins are two of a total of six viral proteins (F, H, L, M, N, P), which are encoded in

the RNA genome [75]. The receptor protein on the host side for wild type virus is the signaling lymphocyte activation protein (SLAM / CD150), which is mainly expressed by immune cells, leading to lymphotropism and immune suppression. Measles vaccine strains use CD46 as a receptor. CD46 is ubiquitously expressed on human cells except for erythrocytes and frequently overexpressed on malignant cells [52, 72]. The interaction of the mentioned receptors with the envelope proteins leads to viral entry. Usually, virions are transmitted via aerosol droplets [72], leading to a primary infection of the respiratory tract [75]. The primary infection is either mediated by the receptor protein SLAM or a newly discovered epithelial receptor called Nectin-4, which is an adherence-junction protein. Nectin-4 can be found on polarized epithelial surfaces, including those of the airways and adenocarcinoma. Concerning oncolytic virotherapy, the manner of application (e.g. intravenous, intratumoral, intraperitoneal) influences primary infection, as there is no transmission via aerosol droplets. The CD46 receptor, which is overexpressed in malignant cells leads to a preferred infection within the tumor [52].

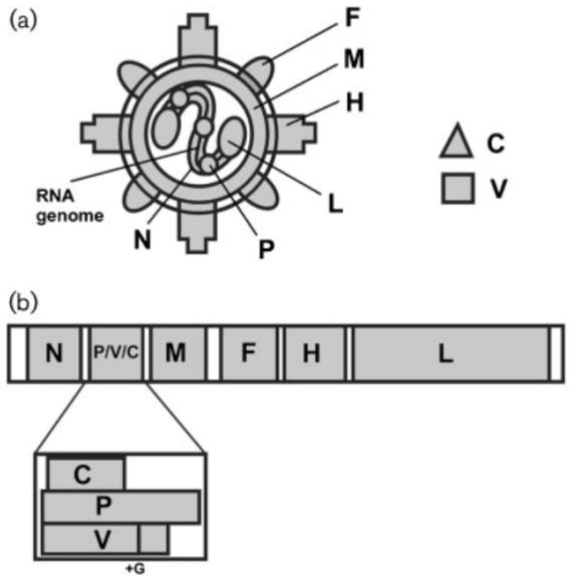


Figure 3. Schematic diagram of measles virion and genome
(a) Depicted are the RNA genome and the six viral proteins (F, H, L, M, N, P). H and F are associated very closely within the envelope.
(b) The RNA genome of MeV contains six genes and about 16 kB. The function of the C and V proteins, which are non-structural and encoded by the P gene, is not fully understood but related to interferon antagonism

Figure:
Yanagi et al.: Measles virus, cellular receptors, tropism and pathogenesis

1.4.4. Vaccination and use as OV

In 1957, John F. Enders and Thomas C. Peebels described for the first time the isolation of measles virus. As the 11-year-old child, from whom they isolated the virus, was called David Edmonston, they called it *"Edmonston strain"* [76]. Having achieved that hallmark, the next logical consequence was the development of a vaccine. The isolated virus was attenuated by infection of chick embryo cells, leading to the license of the vaccine in 1965 [72]. Since then, the vaccine was used in more than a billion patients and has never happened to retrieve its former pathogenic abilities [77].

The safety and stability of the vaccine is a very important aspect concerning oncolytic virotherapy as the vaccine is used for virotherapeutic approaches. So far, the vaccines, which have been derived from the Edmonston vaccine strains are able to selectively infect, replicate within and lyse cancer cells without significantly harming benign ones [77].

Considerations concerning viruses as cancer therapeutics base upon numerous case reports, which describe cancer remission after viral infection (see chapter 1.3.1.). Concerning measles, Bluming and Ziegler described in 1971 the case of an eight-year-old boy suffering from Burkitt's lymphoma, who showed a remission after a natural measles infection [37].

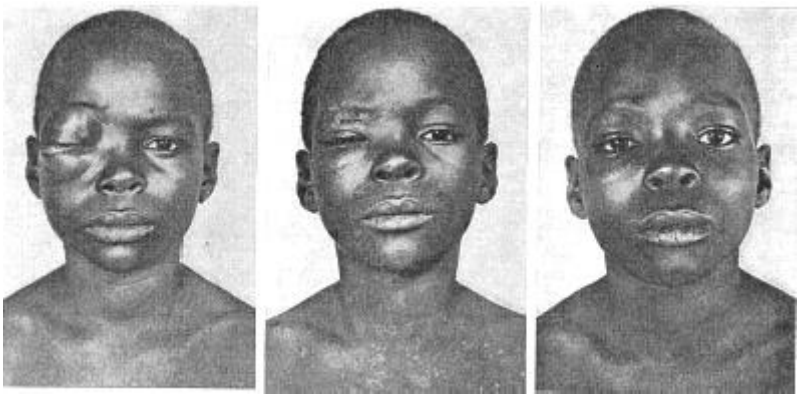


Figure 4. Remission of Burkitt's Lymphoma after Measles Infection.
The picture on the left was taken on the first of December in 1970 showing a swelling of the right eye socket, which was histologically diagnosed as a Burkitt's lymphoma. After the picture was taken, the boy was infected by wild type measles, leading to a decline of the tumor (picture in the middle). The picture on the right was taken on the 6th of January in 1971; no signs of tumor or measles exanthema are visible anymore. Taken from Bluming and Ziegler: Regression of Burkitt's Lymphoma in association with Measles Infection [37].

In the following years, measles virus as oncolytic agent has been investigated more intensely. As the side effects of a measles infection can be quite severe and life-threatening [70], the opportunity of a safe vaccine with oncolytic potency as present in the case of measles is enormous. So far, measles vaccine virus has been shown to be efficient in various preclinical works [52, 78], but also *in vivo*, for example when administered intraperitoneally in patients with ovarian cancer, the treatment with measles vaccine virus showed promising results [79]. Besides, measles vaccine virus is currently under investigation in patients suffering from recurrent glioblastoma multiforme and recurrent or refractory multiple myeloma [52].

To improve therapeutic success, measles vaccine virus has been genetically engineered in different ways. In this study, a vaccine strain armed with a fusion protein consisting of cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT), called Supercytosinedeaminase (SCD) was used (MeV-SCD). SCD catalyzes the deamination of cytosine to uracil [80], an enzymatic step, which is not performed in normal human cells [81]. For oncolytic purposes, the prodrug 5-fluorocytosine (5-FC) can be added, leading to severe cytotoxic

effects as the SCD fusion protein catalyzes the deamination to 5-FU [81]. The application of 5-FC and consequently the efficiency of arming measles virus with SCD is not subject of this work but has been investigated intensively by our group [82]. Other examples for engineered measles vaccine viruses are focus on the usage of tracers to visualize infection. In this work, MeV-GFP, encoding a green fluorescent protein, has been used to depict infection. In clinical studies, tracers are used to monitor infection and cancer progress or remission and to administer additional radiation very precisely. For example, sodium iodide symporter gene leads to a facilitated localization and offers the opportunity for tumor radiovirotherapy [79].

Another possibility that can be employed to amplify the efficiency of oncolytic virotherapy is the induction of anticancer immunity. Immunologic effects can theoretically be achieved by every oncolytic therapy approach, as the infection leads to local inflammation, activating the immune system [83]. To enhance that effect, viruses can be armed with granulocyte-macrophage colony-stimulating factor (GM-CSF), for example, leading to immune cell recruitment, going along with clinical benefits [57, 84].

Even though all those important hallmarks have been achieved, primary resistance phenomena of cancer cells towards measles virus are still an obstacle to overcome. Analyzing the NCI-60 tumor cell panel, only 50 % of all cell lines are susceptible towards MeV-SCD [78]. Our group was able to show, that primary resistances could be resolved either by increasing the multiplicity of infection (MOI) of MeV-SCD or combining MeV-SCD with 5-FC, exploiting the SCD suicide gene function [78].

To accomplish this work, further combinatorial options have to be investigated to overcome resistance phenomena, for example tumor-specific chemotherapy as depicted in this work.

1.5. Preexisting findings for virotherapeutic approaches in combination with gemcitabine

As a background for our work, there are two elementary publications to mention, dealing with oncolytic virotherapy in combination with gemcitabine or senescence. In both works, measles vaccine virus was used as oncolytic agent. Table 1 sums up the work of Bossow et al. [85], investigating the susceptibility of gemcitabine-resistant pancreatic cancer cell lines towards measles vaccine virus infection. Facilitated, the results show, that there is no difference in gemcitabine-resistant pancreatic cancer cell lines and naïve ones concerning infection of measles vaccine virus, cell viability and prodrug activation (as an armed virus was used). For translation into clinical practice, those findings are rather important, as resistances of pancreatic cancer cell lines towards chemotherapy are a major obstacle to overcome.

cell line all gemcitabine-resistant, incubated > 200 d, n=nM gemcitabine	BxPC-3/ Gmc-100	Capan-1/ Gmc-50	T3M4/ Gmc-100	Mia PaCa-2 / Gmc-25
comparison with naive cell line	BxPC-3	Capan-1	T3M4	Mia PaCa-2
susceptibility to MeV-infection: formation of syncytia	=	=	=	=
susceptibility to MeV-infection: cell viability	=	=	=	=
efficacy of bystander killing by the prodrug fludarabine	=	=	=	=

Table 1. Summary of the results of Bossow et al. "*Armed and targeted measles virus for chemovirotherapy of pancreatic cancer*" (facilitated) investigation of four pancreatic cancer cell lines showed, that a resistance towards chemotherapy (gemcitabine) does not influence susceptibility towards a MeV infection. The virus in this work was armed with a prodrug convertase purine nucleotide phosphorylase,

promoting the prodrug fludarabine. Efficacy of the prodrug was not impaired by gemcitabine-resistance of the cell lines.

Weiland et al. [27] were able to show that senescent cancer cells are more efficiently infected and lysed than there non-senescent counterparts. Table 2 provides a facilitated summary of the findings in the publication: senescence was induced using several agents (doxorubicin, taxol, gemcitabine) in cancer cell lines originating from different organs (hepatoblastoma, mammary gland, pancreas). In comparison to non-senescent cancer cells of the same kind, oncolysis was more efficient in senescent cell lines, demonstrated by reduced cell mass in the SRB assay. As explanation for that phenomenon, CD-46 upregulation and interferon-beta release were investigated in the senescent cells. So far, no pattern for the described findings could be detected.

cell line (all human origin) / results	HepG2 Hepatoblastoma (Senescence induced by doxorubicine, taxol, gemcitabine)	MCF7 Mammary gland (Senescence induced by taxol)	MIA PaCa-2 Pancreatic (Senescence induced by gemcitabine)
Reduced cell mass (SRB)*	+	+	+
Senescent cellular phenotype	+		
GFP-expression (ubiquitous)	+		
Formation of syncytia	+		
Increased viral titers*	+		
Accelerated cell lysis*	+		
CD-46 upregulation*	-		
Interferon-beta release°	ø	🡻	(+/-)

* in comparison to each treatment (senescence-inducing chemotherapeutic drug or MeV-infection) alone
° Senescence induced by gemcitabine in all three cell lines; IFN-beta release measured after viral infection

Table 2. Summary of the results of Weiland et al. "*Enhanced killing of therapy-induced senescent tumor cells by oncolytic measles vaccine viruses*" The results show, that MeV-infection and oncolysis is more efficient in senescent cells than in untreated cancer cells. For HepG2, further dimensions of viral infection such as GFP-expression (a recombinant MeV carrying green fluorescent marker protein was used), formation of syncytia and viral titers have been investigated. As likely explanations for

either a facilitated viral entry caused by CD-46 upregulation or an increased viral replication caused by decreased interferon-beta release as a result of senescence-inducing drugs are to be mentioned. No general pattern for those two factors as reason for facilitated viral entry or increased viral replication could be detected in the mentioned cell lines.

1.6. Objective

Due to the poor prognosis of patients who suffer from pancreatic cancer, it is very important to overcome therapy resistance. Gemcitabine is known to be effective in pancreatic cancer; however, it only is capable to delay tumor progression for a few weeks. Dose-dependent side effects limit the application additionally. Therefore, it is necessary to counteract pancreatic cancer on another target to make therapy with gemcitabine more efficient and to reduce the dosage being required.

Measles vaccine virus (MeV) has shown to replicate within pancreatic cancer cells and to have a lytic potential. Thus, it seemed a convenient therapeutic to combine MeV with gemcitabine. The question coming up with this approach is the mutual influence of each therapeutic on the other and the joint effect. As MeV usually replicates in pancreatic cancer and gemcitabine induces senescence, it was important to find out, whether either of these two effects was influenced in the combinatorial context.

2. Material and Methods

2.1. Material

2.1.1. Consumables

Cell scrapers	Corning Inc.
Combitips 2.5 ml, 12.5 ml	Eppendorf
Conical-bottom tube 5 ml	BD Falcon
Conical-bottom tube 15 ml	Greiner Bio One
Conical-bottom tube 50 ml	BD Falcon
Cryotubes 1 ml	Corning Inc.
Pasteur pipettes, 230 mm long size	WU Mainz
Pipettes 5 ml, 10 ml, 25 ml, 50 ml	Corning Inc.
Pipette tips 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 cm², 150 cm²	Greiner Bio One
Tissue culture plate 6 well	Corning Inc.
Tissue culture plate 12 well	Corning Inc.
Tissue culture plate 24 well	TPP
Tissue culture plate 96 well	TPP / Corning Inc.

2.1.2. Chemicals

β-Galactosidase Reporter Gene Staining Kit	SIGMA
5-Fluorouracil	Pharmaceutical Department, Universitätsklinikum Tübingen
Acetic Acid	Merck
Descosept	Dr. Schuhmacher GmbH
DMSO	AppliChem

Erlotinib	Roche
Gemcitabine	LC laboratories
Hydrochloric Acid	S3 Chemicals
Irinotecan	Fresenius Kabi
Isopropanol (70%)	SAV Liquid Production
Nab-Paclitaxel	Celgene
Oxaliplatin	Fresenius Kabi
Paraformaldehyde (PFA), 4.0 %	Otto Fischar GmbH
Secusept	ECOLAB
Sulforhodamine B	Sigma Aldrich
Thyazolyl BlueTetrazolium Bromide (MTT staining)	Sigma Aldrich
Trichloroacetic acid	Carl Roth
TRIS	Carl Roth
Trypan blue	SIGMA

2.1.3. Antibodies

Alexa Fluor® 546	Invitrogen
Goat Anti Mouse IgG (H+L), A11003	Thermo Fisher Scientific
anti-MeV-NP	Creative Biolabs

2.1.4. Media, Sera and Buffer

DMEM	BIOCHROME
DMEM (colorless)	BIOCHROME
EDTA Trypsin	Lonza
Fetal Bovine Serum	Gibco
Opti-MEM	Gibco / Life Technologies
PBS (cell culture use)	PAA
Tween-20	Carl Roth

Self-prepared solutions:

MTT staining solution	MTT	2.5 g / ml
	Colorless DMEM	
MTT solvent	HCl (fuming)	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
10 x TBS (Tris-buffered saline)	NaCl	1.5 M (438.3 g)
	TRIS	0.5 M (302.85 g)
	pH	7.4, adjusted with HCl
	H_2O_{dd}	filled up to 5 l
TBS-Tween (0.02 %)	Tween-20	5 ml of 20 %
	10 x TBS	500 ml
	H_2O_{dd}	filled up to 5 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

2.1.5. Cell lines

BxPC-3, MIA PaCa-2, PANC-1, Vero	German Collection of Micro-organisms and Cell Cultures (DSMZ, Braunschweig, Germany)

2.1.6. Virus

MeV-GFP	Wolfgang Neubert
MeV-SCD	Wolfgang Neubert

2.1.7. Laboratory Equipment

Autoclave 3850 EL	Systec
Centrifuge	Eppendorf, Heraeus
Fluorescence microscope	Olympus
Haemocytometer	Hecht Assistant
HPLC	Merck Hitachi
Incubator	Heraeus / Integra / Memmert
Laminar Flow Work Bench	Heraeus
Light microscope	Olympus
Multichannel pipette	Eppendorf
Handystep	Brand
Photometer Genios Plus	Tecan
Pipette Boy	Integra
Pipettes	BioHit / Eppendorf
Refrigerator (-18 °C, -80 °C, -120 °C)	Liebherr
Rotational Vacuum Concentrator	Christ
Vortexer	Janke + Kunkel IKA Labortechnik
Water bath 3042 (37 °C)	Köttermann

2.2. Methods

2.2.1. Cell culture

2.2.1.1. General cell culture

All cell lines were kept in Tissue culture flasks with vented caps and cultured in DMEM supplemented with 10% FBS. Flasks and plates were stored in an incubator at 37 °C in a humidified atmosphere containing 5 % CO_2. Treatment was performed under sterile conditions in a laminar flow workbench. When reaching confluence, cells were washed once with sterile PBS (37 °C) and removed from culture vessel with trypsin/EDTA. After removal, cells were diluted in fresh medium and seeded partly in new flasks and partly on tissue culture plates.

2.2.1.2. Determination of cell number in solution

Before seeding cells on tissue culture plates, the number of cells was determined using an improved Neubauer haemocytometer. The counting chamber of the improved Neubauer haemocytometer is divided into squares, which are divided in 16 smaller squares to facilitate counting. One large square measures 1 mm² and the distance between the counting chamber and the cover glass is 0.1 mm. Thus, the volume contained by one square is 100 nl. Cells in 4 squares were counted and the average of 4 squares was multiplied with 10,000, which produces the number of cells per ml. To discriminate dead cells from living ones, cells were diluted in trypan blue. Trypan blue only stains dead cells, which effects that living cells are brighter than the surrounding. The thinning caused by the trypan blue solution was considered in calculation.

2.2.1.3. Cryopreservation of cells

For long-term storage, cells had to be frozen at -80 °C. To isolate cells from medium, cell suspension was centrifuged at 1200 rounds per minute for 3 minutes at room temperature. Afterwards, medium was removed and cells were re-suspended in cryo medium (DMEM with 20% FBS and 10% DMSO) to a concentration of circa 1000 000 cells per ml. 1 ml of the suspension was pipetted in cryo tubes which were put into a cryo freezing container and frozen slowly over night at -80 °C.

To re-cultivate the cells, the cryo tubes were thawed in a 37 °C water bath in order to fasten the process and immediately diluted with DMEM containing 10% FBS. To remove DMSO, the cell suspension was centrifuged as described above and diluted in new medium. Cell suspension was cultivated in a cell culture flask.

2.2.2. Cell viability assays

2.2.2.1. Determination of cell mass / sulforhodamine b assay

To quantify the remaining tumor cell mass after treatment with chemo-therapeutics and/or MeV, a sulforhodamine b assay (SRB assay) was performed [86].

Cells were seeded on 24 well plates at a cell number of 40 000 cells per well. After seeding the cells, plates were panned to spread cells evenly within one well. Afterwards, cells were incubated overnight to allow them to adhere. The next day, the actual experiment including either chemotherapeutics or MeV or both was performed. After a defined incubation time, cells were washed with 500 µl cold PBS and afterwards fixed with 250 µl cold trichloroacetic acid (TCA, 10 % w/v). After 30 minutes incubation at 8 °C, TCA was removed and plates were washed twice with tab water. Plates were dried overnight in a heating

cabinet (40 °C). To stain the fixed cells, plates were stained with 250 µl / well SRB staining solution. After an incubation time of 30 minutes, SRB staining solution was removed and cells were washed with 1% acidic acid until all unbound solution was removed. The plates were again dried in a heating cabinet and stored in dark until measurement (not longer than one week). For measurement, the stain was solubilized in tris (10 mM, pH 10). For average color intensity, 1 ml tris per well was chosen; if the stain was very intensive due to a high number of cells, the amount of tris was raised to 1.5 ml per well. After SRB was solubilized, 80 µl of the solution was transferred twice in a 96 well plate. Optical density was measured at a wave length of 550 nm in a micro titer plate reader. Density results of treated cells were related to the results of mock-treated cells.

2.2.2.2. Determination of cellular enzyme activity / MTT assay

To review the accurateness of the SRB assay, another assay called MTT assay was performed. The difference in comparison to the SRB-Assay is that MTT assay measures the activity of cellular enzymes, thus the activity of living cells.

The substance used in MTT assay is the yellow-colored tetrazole (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide which is reduced to formacan (purple color) by living cells [87].

Seeding of cells and experiment were performed as described above.

Before staining, cells were washed with warm PBS (500 µl per well). After that, cells were stained with 250 µl per well MTT staining solution. After the staining solution was added, plates were incubated at 37 °C for 2 hours. After incubation time, the staining solution was removed and plates were frozen at -20 °C.

For measurement, the stained cells were solubilized in MTT solvent. 200 µl of the solution of each well was transferred in a 96 well plate and optical density was measured at a wave length of 570 nm and 650 nm in a micro plate reader.

2.2.3. Virological methods

2.2.3.1. Virus infection

To infect the cells with MeV-SCD, opti-MEM was used as Medium. Infection was performed after washing the cells with warm PBS. To infect 24 well plates, 250 µl medium was added per well, in 6 well plates 1 ml. To make sure that the virus was able to infect, plates were panned every 15 to 20 minutes during 3 hours of incubation time. After 3 hours, medium was changed and DMEM containing 10 % FBS or 5 % FBS (in the case of a virus growth curve) was added. When treated additionally with chemotherapeutics, the drugs were added when medium was changed.

2.2.3.2. Virus growth curves

To determine the influence of chemotherapeutics on virus growth, it was necessary to generate virus growth curves. Virus replication in cells was compared between cells, which were treated with chemotherapeutics and cells, which were not. Cells were seeded one day before infection on a six well plate. The number of cells seeded per well was 1×10^4. As first step, cells were infected with MeV-SCD as described above. As reference, one well per plate remained uninfected. After 3 hours, medium was removed and cells were washed three times with warm PBS. After that, 1 ml DMEM containing 5 % FBS and either chemotherapeutic or not was added on each well. The supernatant of one well per plate was removed and frozen at -80 °C in a test tube. 1 ml Opti-MEM was added on the same well and cells were scraped into the medium. Medium and contained cells were also frozen at -80 °C. Removal of supernatant and scraping of cells was repeated every 24 hours post infection (hpi) until 96 hours hpi. Harvested wells and space between the wells were filled with PBS to avoid drying of the plates.

When all wells had been harvested, titration was started. One day before the start of titration, Vero cells (kidney epithelial cells extracted from an African

green monkey) were plated in 96 well plates. The cell number in each well was 10 000, diluted in 200 µl DMEM + 5 % FBS. When Vero cells had attached to the culture dish and looked healthy the next day, titration could be started.

For titration, samples were thawed in a 37 °C water bath for 2 minutes, vortexed for 15 seconds and centrifuged in a table top centrifuge at 3000 rounds per minute (rpm) for 2 minutes.

For serial dilution of the samples, a 96 well plate was used. For one sample, eight degrees of dilution were provided. The first well of a 96 well plate was left empty, the following 7 wells were filled with 270 µl DMEM + 5 % FBS. 300 µl of the supernatant of a centrifuged sample was then added into the first well. 30 µl of the first well was pipetted into the second well and re-suspended several times. This step was repeated from the second to the third well and so on using a new pipet tip for each well. 50 µl of each degree of dilution were then transferred to the Vero cells. The transfer step was repeated another three times to improve accurateness.

The read out was performed after an incubation time of 96 hours. Before fixation and staining, plates were examined microscopically to detect signs of viral infection. When at least one syncytium could be found in a well it was considered as positive for viral infection. To make sure that all infected wells were stained, the following three wells of a positive one in the serial dilution were included in staining. After examination, medium was removed and plates were washed with 200 µl PBS per well. Then, cells were fixed with 50 µl of 4% formaldehyde and incubated for 10 minutes at room temperature. After fixation washing was repeated two more times. Plates can be stored with PBS at 4 °C for several days.

To stain cells with antibodies and detect viral spread via immunofluorescence, samples were blocked for 30 minutes with TBS-Tween containing 1% FBS. Cell membranes were permeabilized by tween-20, which is contained in TBS-tween.

The permeabilization is necessary to enable antibodies to bind also to intracellular proteins.

After blocking, the solution was replaced by the primary antibody (anti-MeV-NP; 1:1000 in TBS-tween), which was allowed to incubate for 30 minutes. To remove free antibody, plates were washed three times with TBS-tween and afterwards, the secondary antibody (goat anti-mouse; 1:1000 in TBS-tween) was applied. Plates were incubated for another 30 minutes in the darkness to preserve the fluorescence. After incubation, plates were washed another three times with TBS-Tween. PBS was added in each well for storage and microscopy.

2.2.4. Senescence

2.2.4.1. Detection of Senescence / senescence-associated β-galactosidase activity (SA-β-gal)

Gemcitabine is known to induce senescence, which is defined as an irreversible cell cycle arrest in cancer cells such as HepG2 (human hepatoblastoma), MIA PaCa-2 and MCF7 (human mammary gland adenocarcinoma). This phenomenon has been investigated in combination with MeV by our group [27]. To find out, whether gemcitabine induces senescence in the concentrations used in this work and in combination with MeV-infected cells, a senescence assay was performed.

MIA PaCa-2 was the cell line used for this assay. To facilitate counting of cells, cell number seeded per well was decreased to 2×10^4 cells per well in a six well plate. Infection with MeV-SCD and treatment with gemcitabine were performed analogical to the experiments described to determine cell viability to make results comparable. For infection, 1 ml Opti-MEM was added and incubated for three hours. After infection, medium was removed and replaced by 2 ml DMEM containing gemcitabine. To obtain a positive control, one well per plate remained uninfected and was treated with 100 µM gemcitabine. The

negative control was represented by a MOCK treated well. After an incubation time of 72 hours, plates were washed two times with 2 ml PBS per well. For fixation and staining, a staining kit (Sigma Aldrich) was used. After washing, 1.5 ml fixation buffer was added on each well and incubated for 10 minutes at room temperature. After fixation, plates were washed another three times with PBS. Then, 1 ml staining solution was added in each well. Plates with staining solution were incubated for 12 to 16 hours at 37 °C in a carbon dioxide depleted atmosphere. Incubation time was considered adequate, when the majority of cells of the positive control were stained blue and the cells of the negative control remained unstained. Staining solution was removed and plates were washed two times with PBS. To store and count cells, 1 ml glycerol in distilled water was added.

2.2.5. Microscopy

Cells were continuously examined under the microscope to guarantee sterile and proper conditions. Therefore, the phase contrast microscope CK40 from Olympus was used. If any fluorescence had to be detected, the fluorescence microscope IX50 from Olympus was used.

2.2.6. Safety

The laboratory where the experiments of this thesis were performed is classified as Biosafety Level 2 of the Directive 2000/54/EC – biological agents at work from the European Parliament from the year 2000. Therefore, all experiments containing hazardous or potentially hazardous substances were performed under a laminar flow workbench. All materials were disinfected afterwards, irradiated with UV-light for at least 15 minutes and autoclaved.

2.2.7. Software

For calculations, Microsoft Excel 2003 was used. Data was analyzed and pictured by Graph Pad Prism 4.0.

3. Results

The aim of this thesis was to investigate the mutual influence between measles vaccine virus (MeV-SCD) and chemotherapeutic compounds (such as gemcitabine) on pancreatic cancer cell lines when administered simultaneously. Thus, it first was necessary to find out, how cellular growth was inhibited by both substances in comparison to treatment with one substance alone. In this context, two different cell viability assays were performed to determine either remaining tumor cell masses (by the SRB assay) or remaining activities of cellular enzymes (by the MTT assay). Another interesting issue was the influence of gemcitabine on viral replication. Hence, virus growth curves were generated to compare viral replication in pancreatic cancer cell lines infected with MeV-SCD alone or in combination with gemcitabine. As gemcitabine is a strong inductor of senescence, an irreversible cell cycle arrest, it was important to figure out, whether the infection of cells with MeV-SCD had an influence on that phenomenon. Therefore, a senescence assay (senescence-associated β-galactosidase, SA-β-Gal assay) was performed. To illustrate contemporaneous presence of senescence and viral infection in the same cell, pictures of MeV infected and SA-β-Gal positive cells were taken.

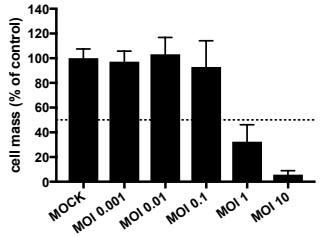
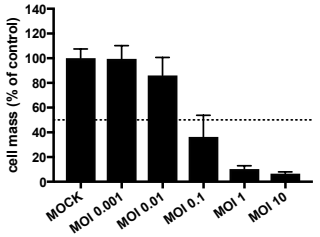
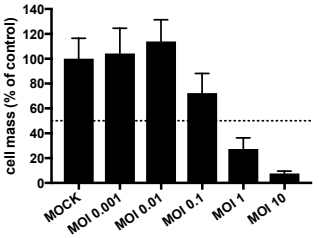
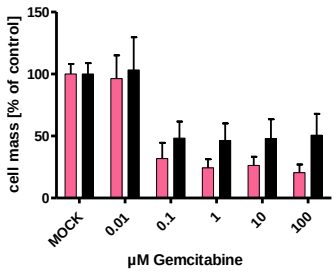
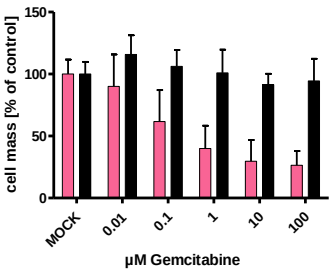
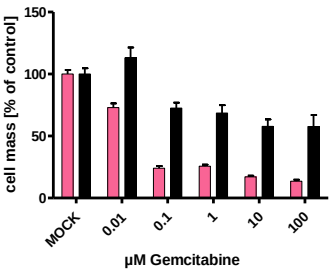
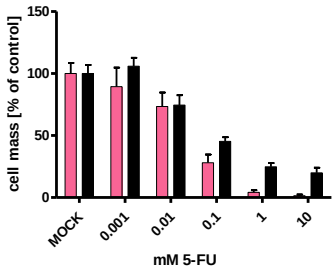
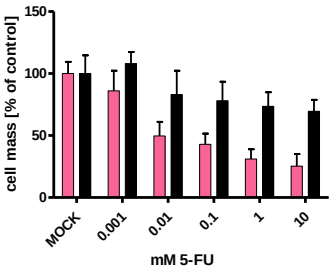
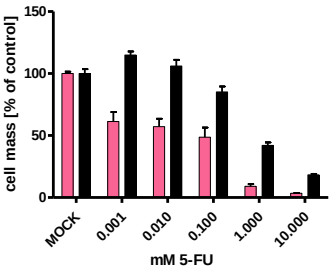
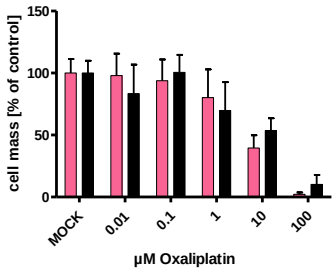
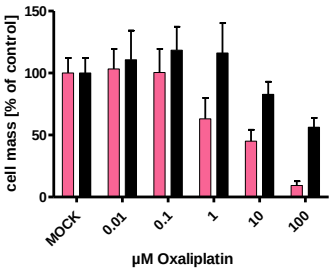
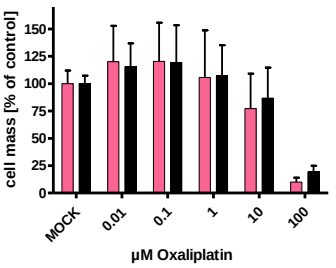
3.1. Introductory experiments

3.1.1. Sensibility of pancreatic cancer cell lines for MeV-SCD and chemotherapeutic drugs

As first step, pancreatic cancer cell lines MIA PaCa-2, PANC-1 and BxPC-3 were treated with five different multiplicities of infection (MOI) of MeV-SCD ranging between 0.001 and 10 and incubated for 72 hours to investigate sensibility of cell lines to MeV-mediated cytotoxicity (Fig. 5, upper panels). Cell viability was determined by the SRB assay. It was necessary to determine a distinct MOI for each cell line, which showed an oncolytic effect but did not

reduce the tumor cell mass by more than 50 %. For the tumor cell line MIA PaCa-2, an MOI of 0.1 was the lowest concentration tested that revealed an oncolytic effect of about 10 %. Remaining cell viability for an MOI of 1 was about 25 %. In PANC-1, an MOI of 0.01 exhibited a slight oncolytic effect; cell viability for an MOI of 0.1 was about 40 %. In BxPC-3, an MOI of 0.1 resulted in a cell viability of about 75 %. Approaching an MOI with an oncolytic activity that did not undercut 50 % cell viability for each individual tumor cell line, the previous data were important to find a frame for experiments with further MOIs.

The same applied for chemotherapy concentrations. Tumor cell sensibilities were tested for different concentrations of various chemotherapeutic drugs (Fig. 5) including gemcitabine (0.01 µM – 100 µM), 5-Fluorouracil (5-FU, 0.001 mM – 10 mM), oxaliplatin (0.01 µM – 100 µM), irinotecan (0.01 µM – 100 µM), nab-paclitaxel (0.0001 – 100 µM) and erlotinib (0.01 µM – 100 µM). Incubation time of 72 hours (pink bars) was compared with incubation time of 48 hours (black bars). Gemcitabine and 5-FU revealed a significantly lower efficiency when incubation time was 48 hours in all tested cell lines. The same pattern was detectable for nab-paclitaxel but not as explicit as for the first two. In MIA PaCa-2 and BxPC-3 cells, no difference in cytotoxicity between incubation times of 48 and 72 hours for oxaliplatin and irinotecan could be found. In PANC-1, an incubation time of 72 hours showed a significantly higher cytotoxicity than 48 hours. Erlotinib did not cause cell death in the used concentrations in PANC-1 at all, whereas an effect was detectable in the other two cell lines beginning at 10 µM. As an incubation time of 72 hours revealed a significantly higher cytotoxicity for chemotherapeutics in most cell lines, 72 hours were chosen as incubation time for the following experiments. Thus, it was nearby to administer MeV-SCD and chemotherapy as contemporaneously as possible. As described in 2.2.3.1., virus infection had to be performed in a different medium (opti-mem), which was changed three hours post infection (hpi) and replaced by normal medium. Therefore, the best way to converge incubation time of MeV-SCD and chemotherapy was to administer chemotherapy simultaneously with the change of medium 3 hpi (Figure 8A, scheme for experiments combining MeV-SCD with gemcitabine).

MIA PaCa-2 MeV-SCD	PANC-1 MeV-SCD	BxPC-3 MeV-SCD
		
MIA PaCa-2 chemotherapy	**PANC-1 chemotherapy**	**BxPC-3 chemotherapy**
		
		
		

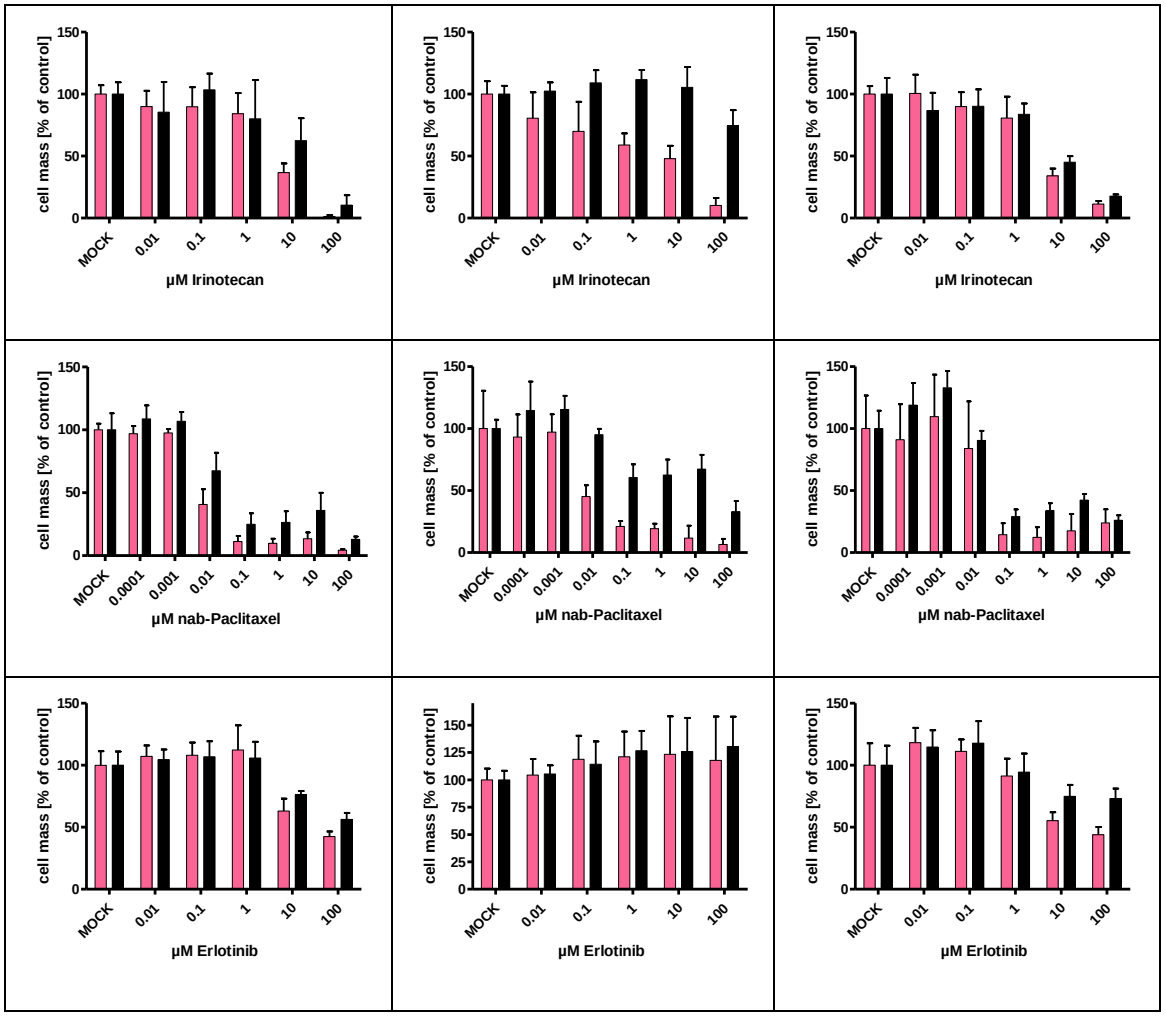


Figure 5. ***Upper panels (Figure and corresponding description published in [88]):*** **"*Susceptibility of pancreatic cancer cell lines to MeV-mediated oncolysis*.** *Cells were infected with oncolytic measles vaccine virus MeV-SCD at the indicated MOIs. At 72 h post infection the remaining cell masses were determined by Sulforhodamin B viability assays; 50% threshold is marked by a dotted line. Data are presented as the mean ± standard deviation of three independent experiments. MeV, measles vaccine virus, MOIs, multiplicities of infection*." ***Lower panels:*** **Susceptibility of pancreatic cancer cell lines for chemotherapeutic drugs**. Incubation time was either 72 hours (pink bars) or 48 hours (black bars) for chemotherapeutic drugs. Mean and standard deviation of three independent experiments are shown. Cell viability was measured using an SRB assay and normalized to a MOCK-treated control (MOCK = 100 %).

3.1.2. Chemotherapeutic drugs suitable for combination with MeV-SCD

Subsequently, distinct chemotherapeutics were chosen for testing in combination with MeV-SCD.

As the efficacy of erlotinib was either rather low in the concentrations used for the cell lines MIA PaCa-2 and BxPC-3 or non existing for the cell line PANC-1, no further combinatorial experiments with erlotinib were performed.

The same applied for irinotecan and oxaliplatin as there were too high meanderings concerning sensibility for those chemotherapeutics between the three cell lines.

Nab-paclitaxel revealed some issues concerning drug quality. Results were hard to reproduce, which was also illustrated by large error bars, as depicted in Figure 5. Approved therapeutic regimens for the mentioned chemotherapeutic compounds are mostly multidrug therapies consisting of distinguished combinations of the mentioned compounds – those regimens have not been investigated in this work, which may have an impact on the described results.

As a result of this selection process, chemotherapeutic drugs chosen for combination experiments were gemcitabine and 5-FU.

3.1.3. Identification of required multiplicities of infection of MeV-SCD and chemotherapy concentrations

For combination experiments, it was necessary to investigate different MOIs of MeV-SCD in combination with different gemcitabine and 5-FU concentrations, which were within a rather small range as very low concentrations were used and it was indispensable to reach a certain cytotoxic effect. Cell viability was determined using the SRB assay.

Accordingly, three MOIs of MeV-SCD and three different gemcitabine / 5-FU concentrations within a small range (table 3 for gemcitabine and table 4 for 5-FU) were combined bailing out all 9 possibilities for combination settings (Fig. 6 for gemcitabine and Fig. 7 for 5-FU). In the case of gemcitabine, the final MOIs / concentrations were chosen looking for an only moderately cytotoxic effect when used as a single agent and highlighted within the table / figure (adjusted to remnant tumor cell viabilities between 50 and 80 %).

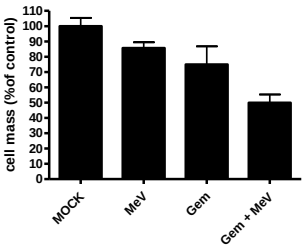
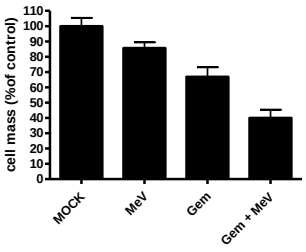
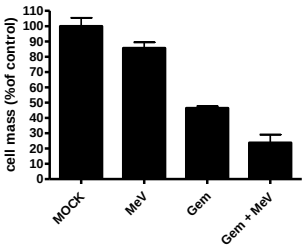
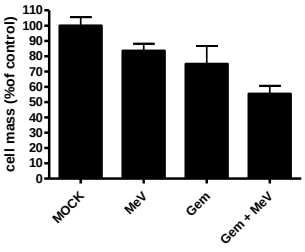
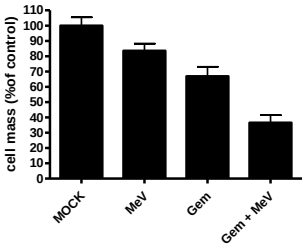
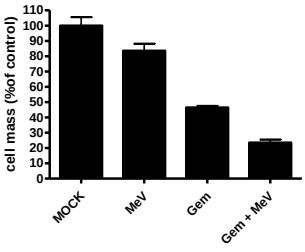
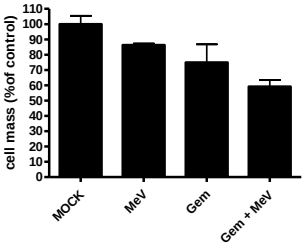
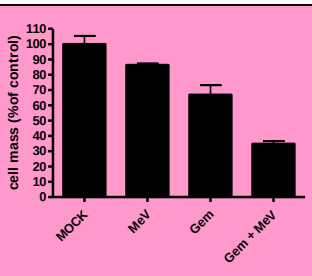
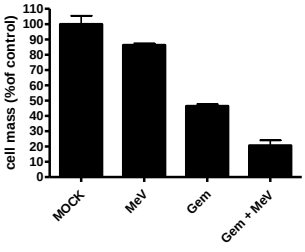
3.1.3.1. Gemcitabine and MeV-SCD in combination

In MIA PaCa-2, the lowest concentration of gemcitabine (0.02 µM) was not efficient enough to undercut 50 % cell viability when combined with MeV-SCD. However, the highest concentration (0.04 µM) already caused cytotoxicity higher than 50 % when applied alone and was consequently too high for combination experiments. Gemcitabine concentration of 0.03 µM resulted in a cell viability of about 65 % when applied as a single agent and less than 50 % when combined with MeV-SCD for all used MOIs. The chosen MOI was 0.4 (Fig. 6). In PANC-1, all listed concentrations of gemcitabine and all MOIs of MeV-SCD resulted in a cell viability of higher than 50 % when used alone and lower than 50 % when used in combination. Chosen concentration for further experiments was 0.075 µM gemcitabine and an MOI of 0.075 (Fig. 6). In BxPC-3 only a gemcitabine concentration of 0.02 µM was low enough and did not undercut 50 % when used alone. In combination with MeV-SCD, remaining cell mass was less than 50 % for combination therapy for all MOIs. The MOI for further experiments was 0.125 (Fig. 6).

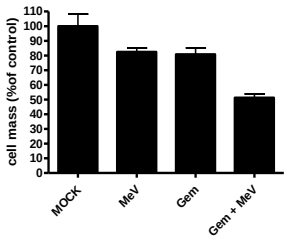
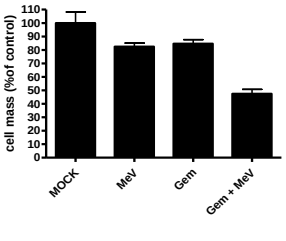
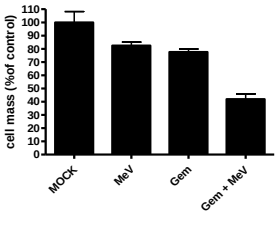
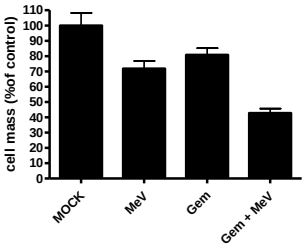
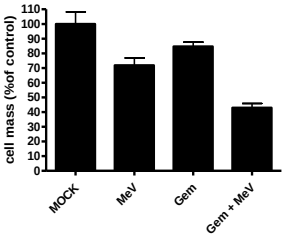
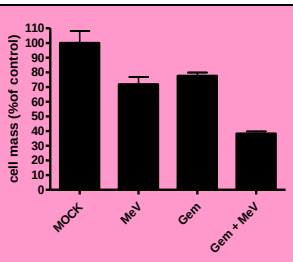
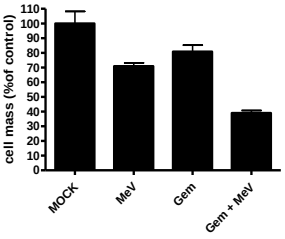
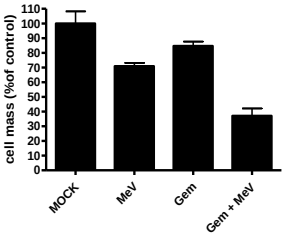
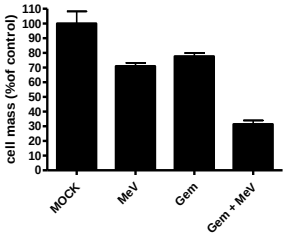
Cell line	**MeV-SCD (MOI)**	**gemcitabine (µM)**
MIA PaCa-2	0.3	0.02
	0.35	0.03
	0.4	0.04
PANC-1	0.05	0.025
	0.075	0.05
	0.1	0.075
BxPC-3	0.075	0.02
	0.1	0.03
	0.125	0.04

Table 3. Listing of all MOIs of MeV-SCD and all concentrations of gemcitabine investigated to find the accurate dose for combination treatment. Every MOI of MeV-SCD was combined with every concentration of gemcitabine, resulting in nine possibilities for combination in each cell line. Colored concentrations denominate concentrations used for further experiments. For results see Figure 6.

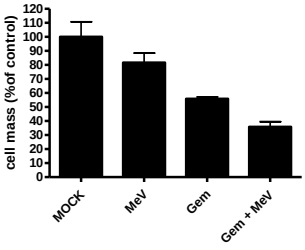
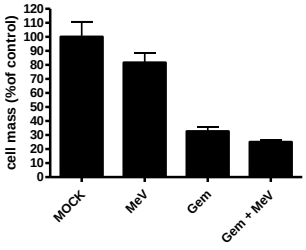
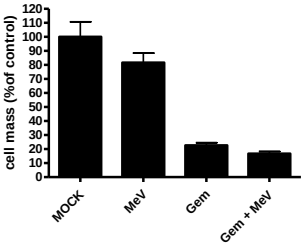
A. MIA PaCa-2

MOI	0.02 µM gemcitabine	0.03 µM gemcitabine	0.04 µM gemcitabine
0.3			
0.35			
0.4			

B. PANC-1

MOI	0.025 µM gemcitabine	0.05 µM gemcitabine	0.075 µM gemcitabine
0.05			
0.075			
0.1			

C. BxPC-3

MOI	0.02 µM gemcitabine	0.03 µM gemcitabine	0.04 µM gemcitabine
0.075			

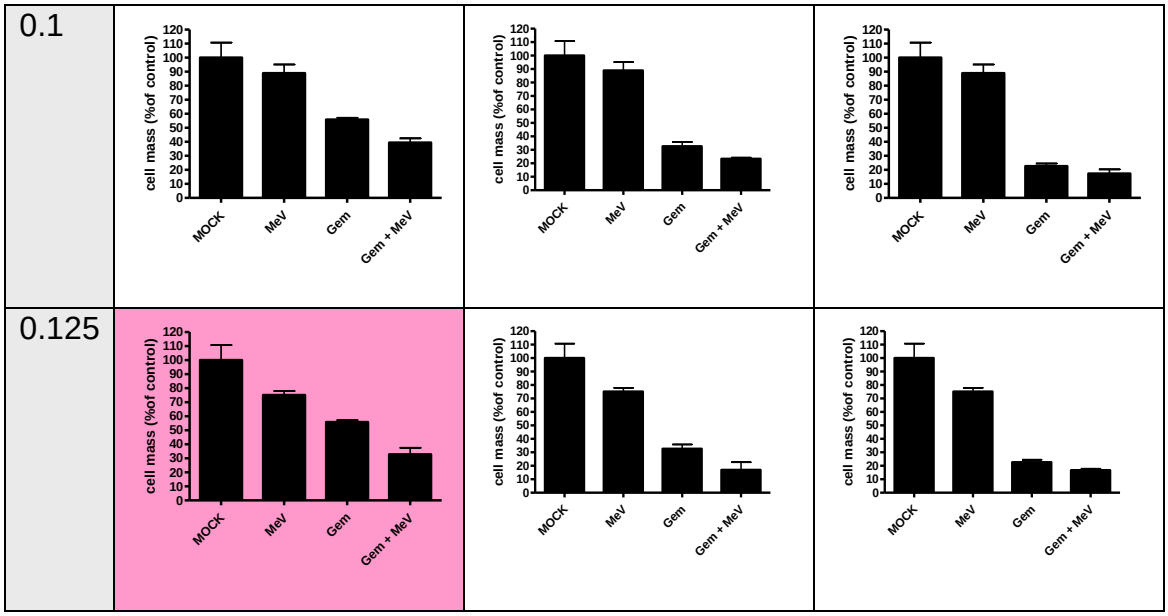


Figure 6. Preliminary experiments investigating the efficacy of the combination of MeV-SCD and gemcitabine for each cell line (A = MIA PaCa-2, B = PANC-1, C = BxPC-3). Three individual MOIs of MeV-SCD and three concentrations of gemcitabine were combined (Table 3), resulting in nine graphs for each cell line. The MOIs / concentrations for each cell line were chosen looking for a moderate cytotoxic effect when used as a single agent (aiming at tumor cell viabilities in the range of 50-80 %). The best fitting combination of both (indicated by colored graphs) was chosen to perform further experiments. MOCK = control, MeV = Measles vaccine virus, MeV-SCD, Gem = gemcitabine.

3.1.3.2. 5-FU and MeV-SCD in combination

In general, combination treatment with 5-FU and MeV-SCD was not as efficient as combination of gemcitabine and MeV-SCD as tumor cell viabilities were less frequently found to undercut 50 % and never undercut 40 %. Moreover, differences concerning cell viability between treatment with one agent alone and the combination of both were less significant. In MIA PaCa-2, best effects were shown for the combination of 12.5 µM 5-FU with an MOI of 0.35 as cell viability was between 70 and 80 % for both agents when applied alone and about 45 % when combined.

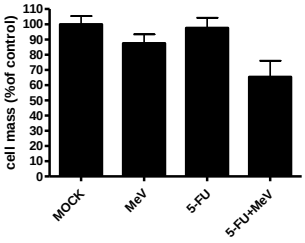
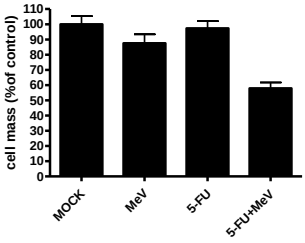
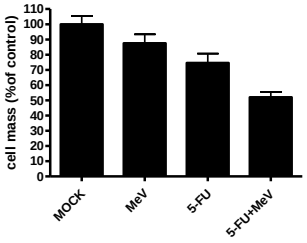
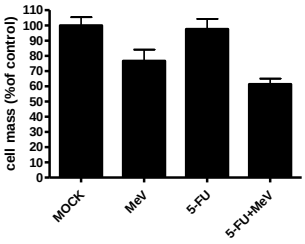
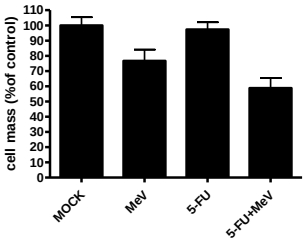
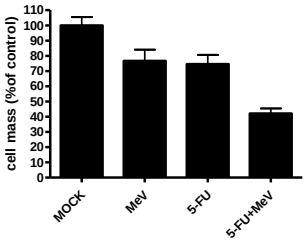
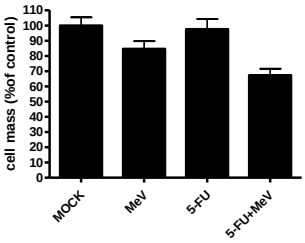
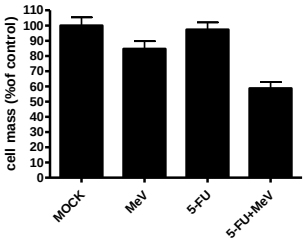
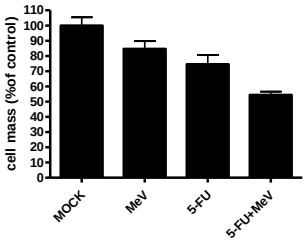
In PANC-1, cell viability for combination treatment was in all cases between 45 and 60 % and did not show high meanderings. Best effects were visible for an MOI of 0.075 and a 5-FU concentration of 2.5 µM.

In BxPC-3, best effects were shown combining 2.5 µM 5-FU with an MOI of 0.125 of MeV-SCD. Cell viability was between 70 and 80 % for both agents when applied alone and about 45 % when combined. All mentioned results depicted in Figure 7.

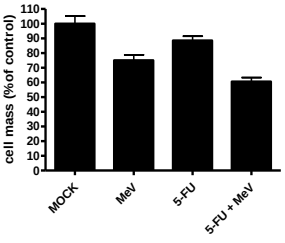
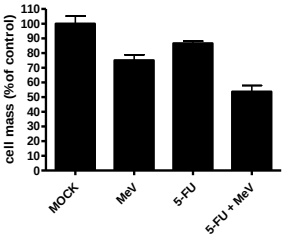
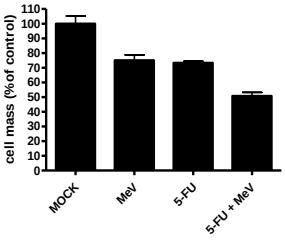
Cell line	**MeV-SCD (MOI)**	**5-FU (µM)**
MIA PaCa-2	0.3	7.5
	0.35	10.0
	0.4	12.5
PANC-1	0.05	1.25
	0.075	2.5
	0.1	5.0
BxPC-3	0.075	2.5
	0.1	5.0
	0.125	7.5

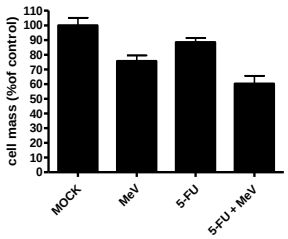
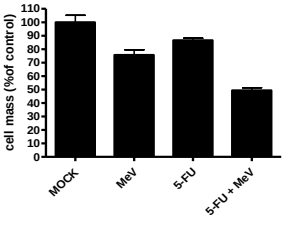
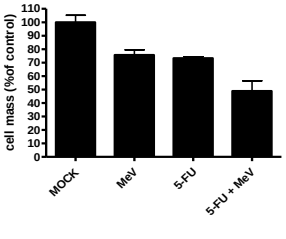
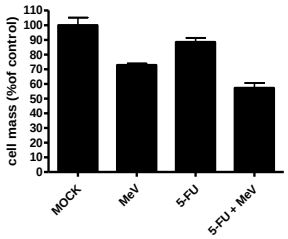
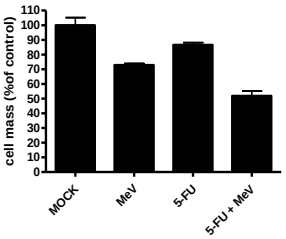
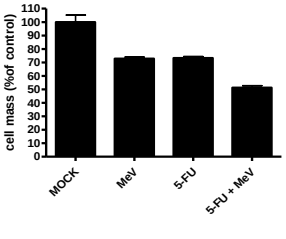
Table 4. Listing of all MOIs of MeV-SCD and all concentrations of 5-FU investigated to approach an adequate dose for combination treatment. Every MOI of MeV-SCD was combined with every concentration of 5-FU, resulting in nine possibilities for combination in each cell line. For results see Figure 7.

A. MIA PaCa-2

MOI	7.5 µM 5-FU	10 µM 5-FU	12.5 µM 5-FU
0.3			
0.35			
0.4			

B. PANC-1

MOI	1.25 µM 5-FU	2.5 µM 5-FU	5 µM 5-FU
0.05			

0.075			
0.1			

C. BxPC-3

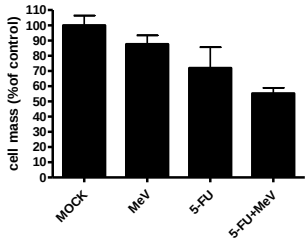
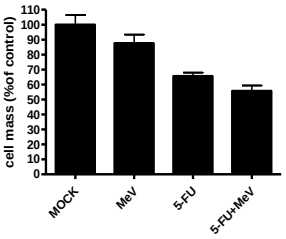
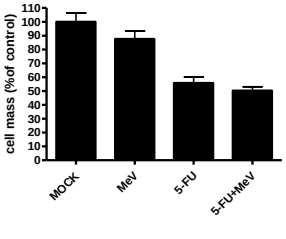
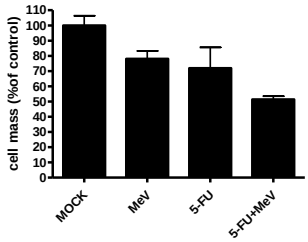
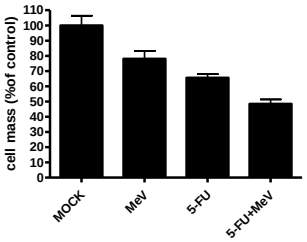
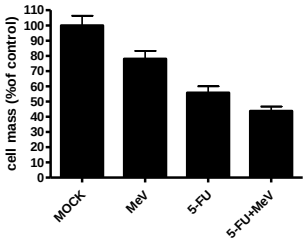
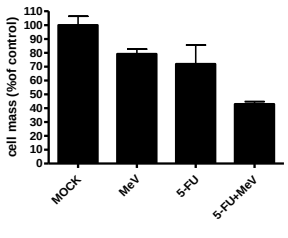
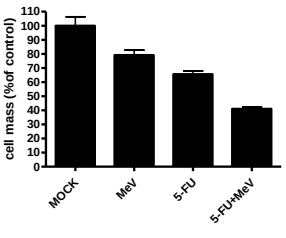
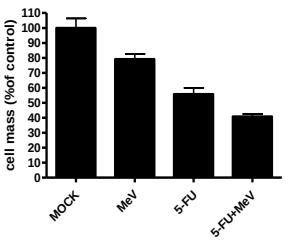
MOI	2.5 µM 5-FU	5 µM 5-FU	7.5 µM 5-FU
0.075			
0.1			
0.125			

Figure 7. Preliminary experiments investigating the efficacy of the combination of MeV-SCD and 5-Fluorouracil (5-FU) for each cell line (A = MIA PaCa-2, B = PANC-1, C = BxPC-3). Three individual MOIs of MeV-SCD and three concentrations of 5-FU were combined (Table 4) resulting in nine graphs for each cell line. The MOIs / concentrations for each cell line were chosen looking for a moderately cytotoxic effect when used as a single agent resulting in a remaining cell mass of about 50-80 %. MOCK = control, MeV = Measles vaccine virus, MeV-SCD

3.2. Combination of MeV and Gemcitabine

As gemcitabine has been *first-line* chemotherapeutic agent in pancreatic cancer for a long time and still plays an important role, performing experiments combining it with OV in more detail seems to be a reasonable approach. Moreover, it is the only drug that is approved as single-agent therapy and is known to induce senescence (therapy induced senescence = TIS). Including all those considerations and the high efficacy that was shown for combination with MeV-SCD in the previous experiments, it was self-evident to perform further experiments concerning interaction between gemcitabine and MeV-SCD. All following experiments were performed with the MOIs of MeV-SCD and the gemcitabine concentrations listed in table 5.

Cell line	MOI of MeV-SCD	Gemcitabine (µM)
MIA PaCa-2	0.4	0.03
PANC-1	0.075	0.075
BxPC-3	0.125	0.02

Table 5. MOIs of MeV-SCD and concentrations of gemcitabine chosen for final experiments for each cell line.

3.2.1. Comparison of SRB assay and MTT assay

After the identification of the accurate doses of gemcitabine and MeV-SCD, results of the SRB assay were confirmed using another cell viability assay, the MTT assay. The SRB assay measures the remaining cell mass after the

fixation of cells whereas the MTT assay measures the activity of cellular enzymes, thus the rate of living cells. Results were summarized in one graph to compare results of SRB assay and MTT assay directly. Mostly, results only varied slightly, which imply that both SRB and MTT assay are adequate for the determination of cell viability in these cell lines. All results depicted in Figure 8B.

Cell viability undercut 50 % in all cell lines when MeV-SCD and gemcitabine were combined which constitutes a considerable cytotoxic effect in contrast to the rather low efficacy of the therapeutics when used alone.

In MIA PACa-2, cell viability was reduced to less than 30 % for combination therapy. Using the same concentrations of MeV-SCD (MOI 0.4) and gemcitabine (0.03 µM) as single agent, cell viability did not undercut 70 % for MeV-SCD and 55 % for gemcitabine (Fig. 8B).

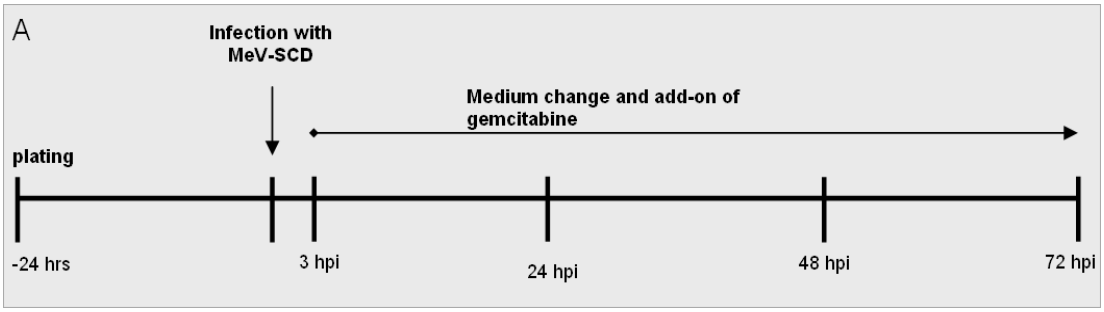
In PANC-1, cell viability was higher than 65 % (MeV-SCD, MOI 0.075) and higher than 75 % (0.075 µM gemcitabine) for single agent treatment and 40-45 % for the combinatorial approach.

In BxPC-3, cell viability for MeV-SCD (MOI 0.125) alone was about 80 % and 65 % for gemcitabine (0.02 µM) alone. Combination of both resulted in a cell viability of less than 50 %.

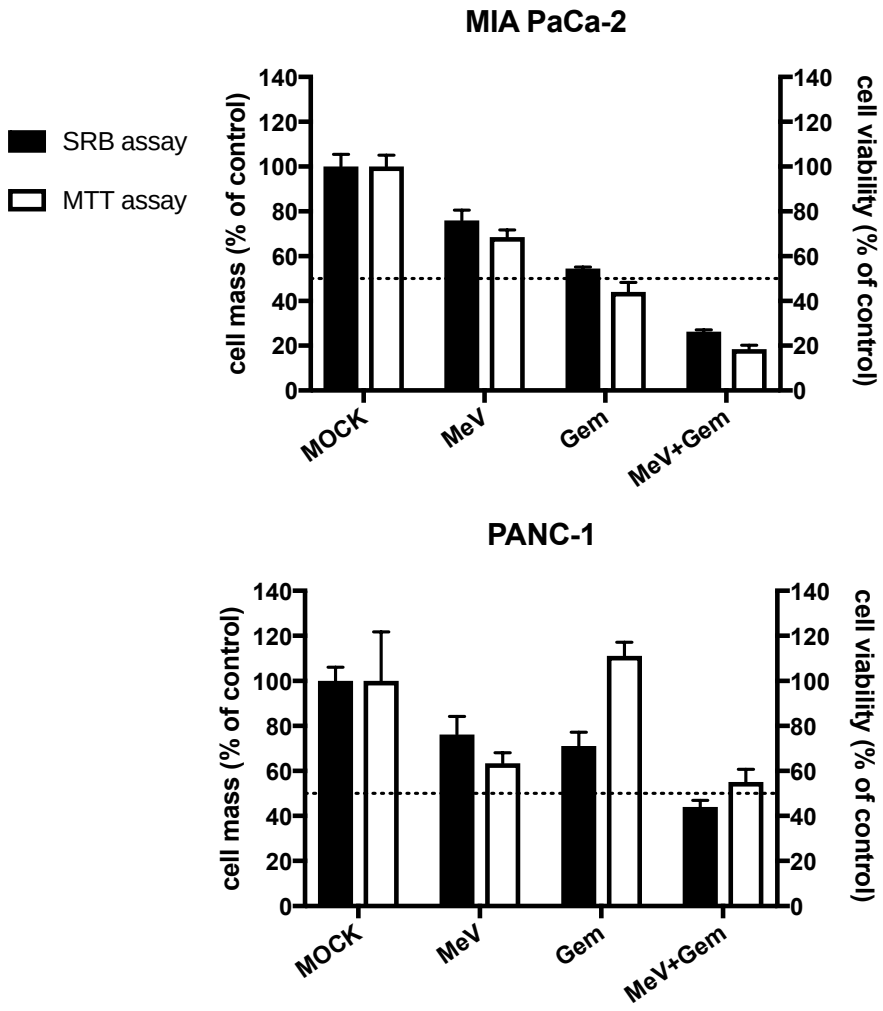
Of notice, in PANC-1 cells, a significant difference between both assays for the treatment with gemcitabine alone was measured (Fig. 8B). Cell viability in the MTT assay was about 100 % whereas cell mass in the SRB assay was measured as less than 80 %. A likely explanation for that difference is the senescence-inducing potency of gemcitabine. Cell metabolism in senescent cells remains active, which is a parameter measured by the MTT assay. Interestingly, cell viability of the combination of both MeV-SCD and gemcitabine was not found to differ between both assays.

Comparing combination therapy with single-agent therapy, significantly reduced cell viability was found in all three cell lines for the combinatorial approach. Consequently, it became obvious that both agents did not influence the

cytotoxic potency of each other in pancreatic cancer cell lines. To confirm that theory, viral replication of MeV-SCD as well as therapy-induced senescence (TIS) caused by gemcitabine was tested for combination therapy.



B



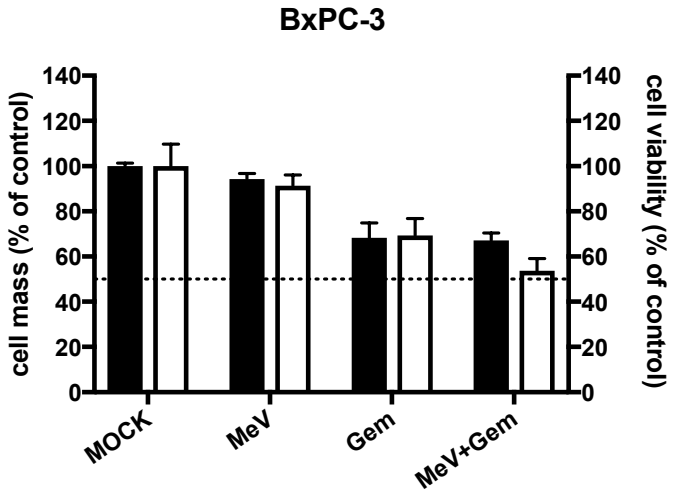


Figure 8. ***Chemovirotherapy employing gemcitabine (GEM) together with MeV-SCD in three pancreatic cancer cell lines. [88]*** (A) Setting: tumor cells were infected 24 hours after plating. Add-on of gemcitabine was performed 3 hours post infection (hpi) when medium was changed. Total incubation time of virus was 72 hours. (B) ***(Figure and corresponding description published in [88])*** *"Cell viability was measured using two different assays [SRB (black bars) and MTT (white bars) assays, respectively] and normalized to an uninfected (MOCK-treated) control (set to 100% cell viability). MOIs of MeV and Gem concentrations were chosen at low enough levels to reduce tumor cell masses <50% when used as a single compound. When used in combination, the remaining tumor cell mass was found to be <5% in all three tumor cell lines. For MeV, MOIs of 0.4 (MIA PaCa-2), 0.075 (PANC-1) and 0.125 (BxPC-3) were chosen, respectively. For Gem, concentrations of 0.03 µM (MIA PaCa-2), 0.075 µM (PANC-1) and 0.02 µM (BxPC-3) were used, respectively. Data are presented as the mean ± standard deviation of three independent experiments. GEM, gemcitabine; hpi, hours post infection; MOIs, multiplicities of infection; MeV, measles vaccine virus; SRB, Sulforhodamin B."*

3.3. Virus growth curves

Virus growth curves were generated to measure viral replication within a cell line over a period of 96 hours (hours post infection = hpi). Virus was quantified in both cell suspension (cell associated virus) and supernatant (released virus) of infected cells. MOI and gemcitabine concentration was equal to SRB and MTT assays.

3.3.1. Influence of gemcitabine on viral replication

Viral replication within infected cancer cells constitutes one of the most important modes of action of oncolytic virotherapy (OV), as it is indispensable for lysis and further infection.

To elicit the influence of gemcitabine, viral replication was investigated in the absence or presence of gemcitabine. Virus growth curves were generated for viral replication within cells (Fig. 9, “cell associated virus”, continuous graphs on the left) and released virus in supernatant (dotted graphs on the right).

To compare viral replication of MeV-SCD alone with the combination of MeV-SCD and gemcitabine, virus growth curves for MeV-SCD alone and for combination therapy were generated under the same conditions. MOI of MeV-SCD and concentrations of gemcitabine were the same as applied for SRB and MTT assay.

In general, viral replication was rather similar for both conditions. For two cell lines (MIA PaCa-2 and BxPC-3) viral replication was slightly inhibited by the additional treatment with gemcitabine.

For cell associated virus in MIA PaCa-2, viral titers 72 hpi were about 8×10^6 plaque forming units (PFU) for MeV-SCD alone and 1×10^6 PFU for the combination.

In BxPC-3, cell associated virus 48 hpi was 1.6×10^3 PFU for MeV-SCD and 0.17×10^3 PFU for combination.

It must be pointed out that cell viability was very likely to be reduced by adding gemcitabine (the MOI of MeV-SCD was the same for both conditions). Thus, the most probable reason for the diminished viral replication was the reduced cell viability and not any mechanism of direct inhibition of viral replication caused by gemcitabine. Furthermore, viral replication in BxPC-3 was rather low compared to the other two cell lines and did not exceed 10^4 PFU. To investigate that phenomenon more precisely, cell number seeded per well was raised to 2 x 10^4 cells per well and 3 x 10^4, respectively (for results see 3.3.2.).

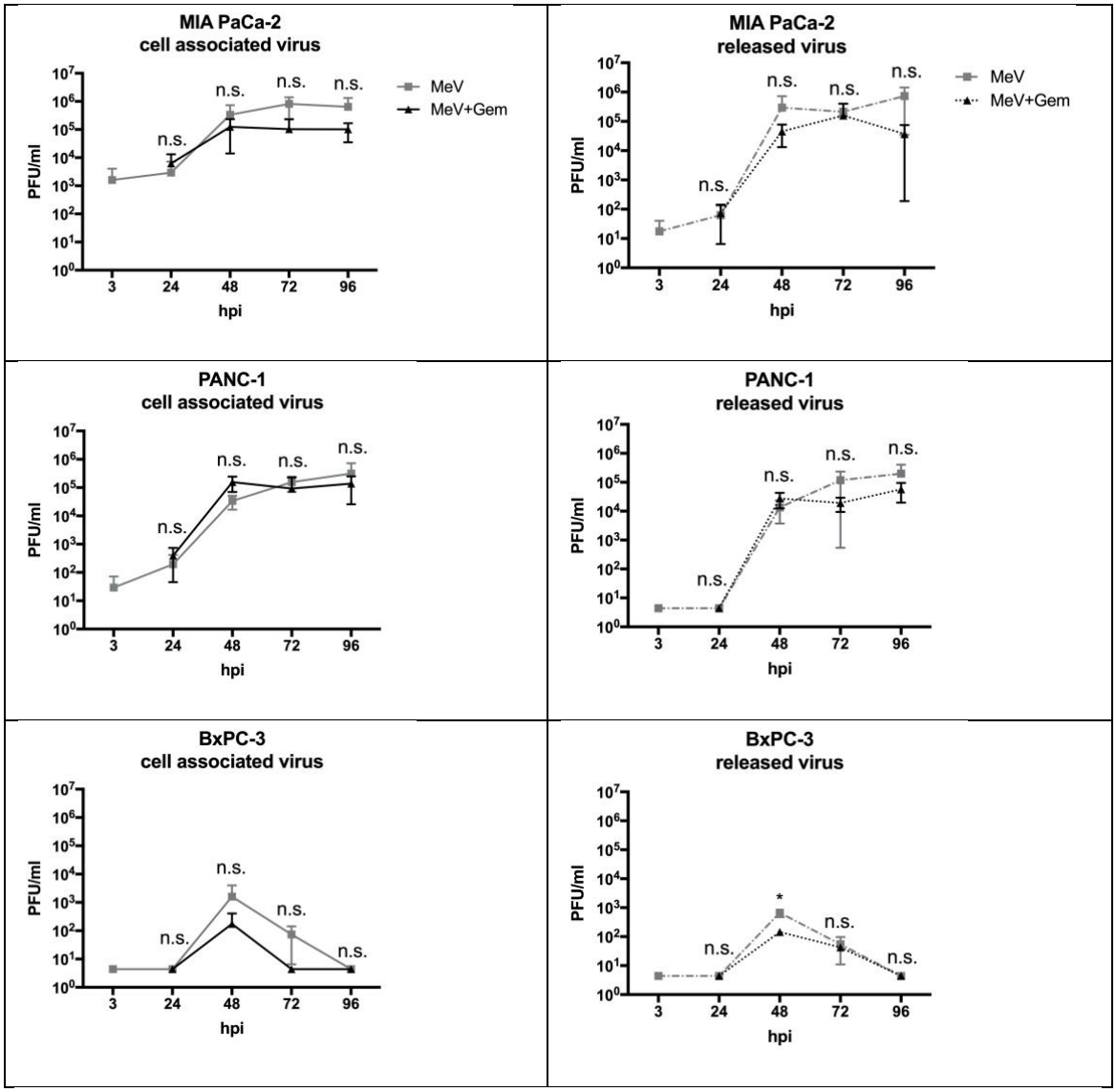


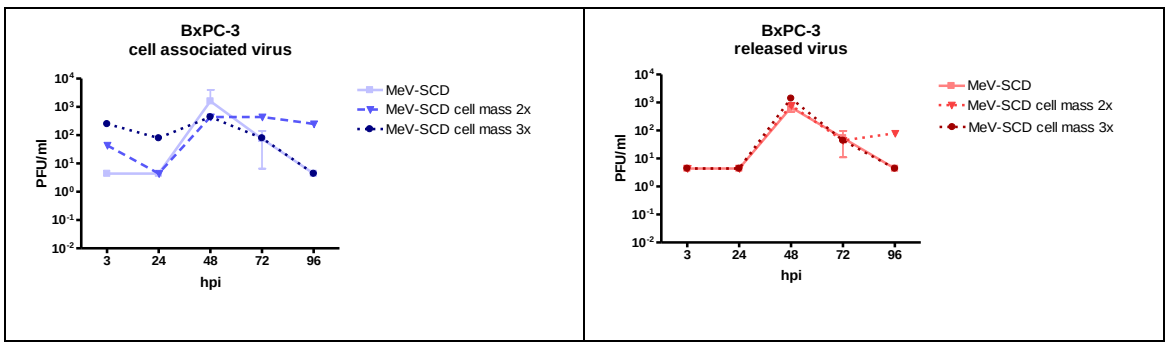
Figure 9. ***(Figure and corresponding description published in [88]) "Virus growth curves illustrating the course of viral replication in pancreatic cancer cells infected with oncolytic MeV.*** *Virus growth of MeV-SCD (here denoted as MeV) was*

*determined both in cell suspensions (continuous line graphs, cell associated virus) as well as in tumor cell culture supernatants (dotted line graphs, released virus). Viral replication was compared between: i) Single treated, i.e., 'only' infected tumor cells (MeV; mono-virotherapy, grey graphs); and ii) chemovirotherapeutic treated tumor cells, being infected first and then treated additionally with gemcitabine at 3 hpi (MeV+Gem, black graphs). Notably, tumor-cell specific multiplicities of infection of MeV and concentrations of Gem were used equal to the concentrations employed before in the viability assays (Sulforhodamin B and MTT). Except for the 48-h-value of released virus in the cell line BXPC-3, there was no statistically significant difference between the values of viral growth with or without Gem. All statistical analyses were conducted with Bonferroni's multiple comparison test. *P<0.05 vs. MeV-Gem. PFU, plaque forming units; n.s., not significant; hpi, hours post infection; GEM, gemcitabine; MeV, measles vaccine virus."*

3.3.2. Increase of seeded cell number (BxPC-3)

As mentioned in section 3.4.1. and shown in Figure 9, viral replication of MeV-SCD in the tumor cell line BxPC-3 was rather low compared to the other two cell lines. To investigate that phenomenon in more detail, the cell number seeded per well was raised. Cell mass was duplicated (2 x 10^5) and triplicated (3 x 10^5). Experiments were performed exactly as previous ones. To facilitate comparing the differences of the different cell numbers, replication curves of one entity (e.g. cell associated virus of MeV-SCD without gemcitabine) were visualized in one graph.

As a result, viral titers were again in all cases found to be lower than 1 x 10^4 PFU and compared to the original cell number no significant differences in viral replication were found.



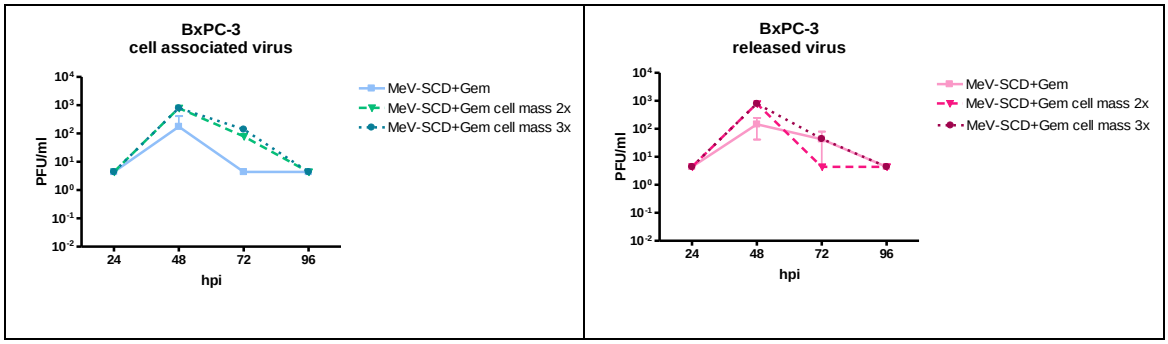


Figure 10. Comparison of viral replication quantities using different cell numbers in the cell line BxPC-3. Viral replication was not increased by the duplicated and triplicated cell number in comparison to the original experiments. Viral peak titers always remained below 10^4 PFU/ml independently of cell number or presence of gemcitabine.

3.4. Induction of senescence

As mentioned before, gemcitabine is able to induce senescence (therapy induced senescence = TIS) in viable cells, which is defined as a permanent cell cycle arrest. The interesting question concerning combination therapy was, if senescence can be induced by gemcitabine in MeV-SCD infected cells. Obviously, results had to be surveyed considering whether viral replication was possible in remaining tumor cells, although they were senescent. Another interesting aspect was to investigate in more detail the mutual influence of both therapeutics when administered simultaneously.

3.4.1. Influence of MeV-SCD infection on therapy-induced senescence

Light microscopically, all cell lines revealed signs of senescence, which are for example an enlarged and flattened phenotype. To detect senescent cells, the senescence-associated β-galactosidase (SA-β-Gal) assay was used. The chosen cell line for the SA-β-Gal assay was MIA PaCa-2 as this method hardly works in the other two cell lines. Senescent cells were stained blue (β-galactosidase positive cells) and counted normalized to the total number of cells. As positive control, 100 µM gemcitabine was used. To make sure that the

concentration of gemcitabine (0.03 µM) that was used for previous experiments was able to induce senescence, the assay was performed with cells treated with 0.03 µM gemcitabine alone and with a combination of gemcitabine and an MOI of 0.4 of MeV-SCD.

As a result, both gemcitabine alone and the combination revealed the same potency to induce senescence as the positive control, which was about 70 to 80 % (Fig. 11A). As negative control, MOCK-treated cells were investigated for senescence. Furthermore, cells were treated with an MOI of 0.4 of MeV-SCD alone. Cells were tested negatively for senescence, as β-galactosidase positive cells were less than 15 % (Fig.11A).

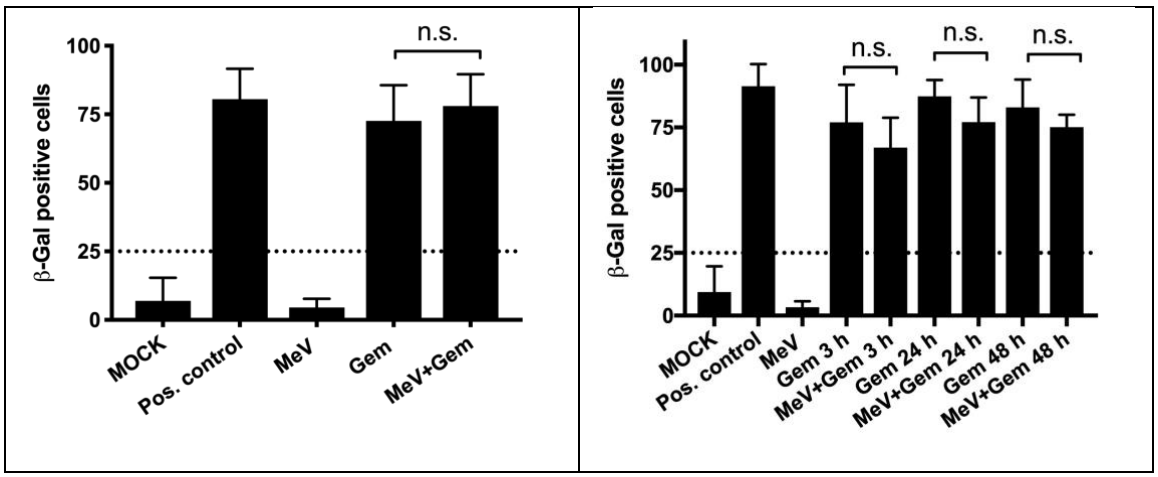


Figure 11. ***(Figure and corresponding description published in [88]) "SA-β-Gal assay illustrating therapy-induced senescence in MIA PaCa-2 cells.*** *(A) Tumor cells were either infected with the oncolytic MeV-SCD (here denoted as MeV) or treated with Gem at 3 hpi, and underwent combined chemovirotherapeutic treatment (MeV+Gem) or were left untreated (MOCK); pos. control: Gem 100 µM; then, expression of SA-β-gal was determined 72 h later. Statistical analyses were conducted with an unpaired t-test, confidence interval 95% and two-tailed. (B) Time dependency of senescence induction. Gem (0.03 µM) was added either at 3, 24 or 48 hpi. Expression of SA-β-gal was determined again at 72 hpi. There was no statistically significant impairment of induction of senescence by the presence of virus. Statistical analyses were conducted with Bonferroni's multiple comparison test. hpi, hours post infection; SA-β-Gal, senescence-associated β-galactosidase; Gem, gemcitabine; hpi, hours post infection; pos. control, positive control; n.s., not significant; MeV, measles vaccine virus."*

3.4.2. Time-dependency of senescence induction

For the experiments described in 3.4.1., gemcitabine was added 3 hours post infection (hpi), which constitutes the same pattern as in the previous experiments. Another interesting issue was whether the time point of the add-on of gemcitabine after the virus infection with MeV-SCD had any influence on the potency of gemcitabine to induce senescence. The later the add-on of gemcitabine was performed, the more virus particles were allowed to replicate before induction of senescence took place.

To investigate if the time point of the addition of gemcitabine had any influence on its efficacy concerning senescence induction, gemcitabine was either added 24 hpi or 48 hpi. Incubation time was 72 hours after the add-on of gemcitabine to ensure the same incubation time for gemcitabine as in previous experiments.

As shown in Figure 9, viral replication either reaches a peak (cell line BxPC-3) or a plateau (cell lines MIA PaCa-2 and PANC-1) 48 hpi. Thus, it was not necessary to investigate later time points after viral infection. Again, gemcitabine was also added without infection of MeV-SCD at the same time points (24 h, 48 h) to ensure senescence-inducing potency was present. MOI of MeV-SCD and gemcitabine concentrations were the same as in previous experiments. The same applied for positive and negative control.

Taken together, the time point of the add-on of gemcitabine did not alter the efficacy of senescence induction. For both time points, more than 75 % of cells were found to be senescent, as depicted in Figure 11B.

3.4.3. Visualization of a contemporaneous presence of senescence and MeV-infection in the same cells

To reassure that senescence and viral infection did not only occur as side-by-side phenomena but also simultaneously in the same tumor cells, β-galactosidase positive MIA PaCa-2 cells were analyzed microscopically. For this purpose MeV-GFP was used, a measles vaccine virus encoding a green

fluorescent marker protein, which can be detected by fluorescence microscopy. Senescence is easy to visualize not only by an enlarged and flattened phenotype of the cell, but also by the light blue color in the SA-β-Gal assay. Therefore, cells were infected and treated as in previous experiments with the only difference that MeV-GFP was used instead of MeV-SCD.

At 72 hours post infection, green fluorescent cells were detected by fluorescence microscopy (Figure 12B and 12E). The SA-β-Gal assay was performed subsequently as fluorescence cannot be detected in cells, which had been fixed and stained before.

As a result, SA-β-Gal positive cells showing blue staining were identified by bright field microscopy of the same area (Figure 12A and 12D). Additionally, light microscopy revealed the enlarged and flattened phenotype being typical of senescence (Fig. 12C and 12F). As a further result, Figure 12 shows that MeV infected cells showing green fluorescence are able to undergo senescence (Fig. 12A-C). Higher magnification revealed that also a syncytium being characteristic for MeV infection displayed a senescent phenotype (Fig. 12D-F).

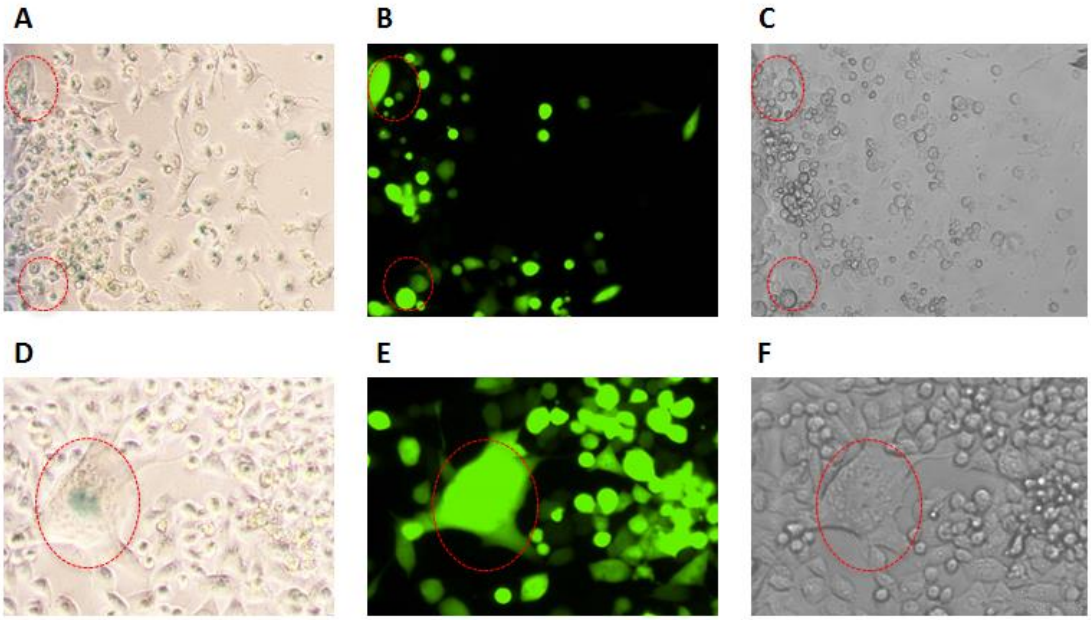


Figure 12. ***(Figure and corresponding description published in [88]) "Senescence patterns induced by gemcitabine in pancreatic cancer cells infected with oncolytic MeV.*** *Upper panel (all magnification, ×4): (A) MIA PaCa-2 tumor cells infected with the GFP marker gene encoding oncolytic MeV (MeV-GFP) exhibited blue staining of senescence-associated β-galactosidase, detected by bright field*

microscopy. (B) Visualization of MeV-GFP infected tumor cells by fluorescence microscopy. (C) Light microscopy of the same sector exhibiting an enlarged and flattened phenotype of tumor cells, being characteristic for the induction of therapy-induced senescence. Red dotted circles indicate examples of MeV-GFP infected senescent tumor cells. Lower panel (all magnification, ×10): (D) Higher magnification of a MeV (MeV-GFP) induced syncytium of MIA-PaCa-2 tumor cells exhibiting a blue colored (β-galactosidase positive) senescent phenotype. (E) Proving infection with MeV-GFP, this syncytium (encircled in red) exhibited a strong GFP-mediated fluorescence signal. (F) Light microscopy depicted the multinucleated phenotype being typical for MeV-induced syncytia. Tumor cells (MIA PaCa-2) were treated with the respective concentrations used in the combination experiments (MOI of MeV: 0.4; concentration Gem: 0.03 µM), pictures were taken at 72 hpi. Gem, gemcitabine; hpi, hours post infection; MeV, measles vaccine virus; GFP, green fluorescent protein.“

4. Discussion

Oncolytic virotherapy provides a new perspective concerning the therapy of cancer. Within the numerous oncolytic viruses which are currently investigated for their efficacy in cancer cells, oncolytic Measles vaccine viruses (MeV) constitute a novel approach to overcome therapeutic resistance of pancreatic cancer, yet resistances against virotherapy exist or arise [78]. Therefore, novel combination strategies to treat pancreatic cancer have to be developed.

As gemcitabine constitutes the *first-line* therapeutic for the treatment of locally advanced or metastatic pancreatic cancer, we investigated whether the combination of MeV with that very agent showed superior effects when compared to either agent alone. The same was performed using 5-Fluorouracil (5-FU) in combination with MeV. As our virotherapy regimen including gemcitabine showed more promising results than the combination with 5-FU, viral replication and the influence of virus on senescence, caused by gemcitabine, were also investigated.

Cell viability assays showed a superior cytotoxic effect for the combination of MeV and gemcitabine when compared to both therapeutics administered as single agents. These results are also interesting for a possible transfer to *in vivo* treatment considering that lower drug concentrations lead to lower side-effects, as already shown in combination of gemcitabine with myxoma virus [89]. Two different assays were performed: SRB assay measuring cell mass and MTT assay measuring metabolic activity of cells. For the pancreatic cancer cell lines MiaPaCa-2 and BxPC-3 results were quite similar. In contrast, for PANC-1 tumor cells, when treated solely with gemcitabine, we observed a significant difference between the results of the SRB and the MTT assay (see Fig. 8B; third pair of bars from the left): cell viability in the MTT assay was about 100% whereas cell mass in the SRB assay was measured as less than 80%. A likely explanation for that difference is the senescence-inducing potency of gemcitabine. Cell metabolism in senescent cells remains active [90]. The MTT

assay measures cell viability via the activity of cell metabolism, which can lead to varieties concerning assay results [91]. Interestingly, cell viability of the combination of both MeV and gemcitabine was not found to differ between both assays, both cell mass and cell metabolism were diminished to a similar amount. Thus, no disadvantages due to the “side effect” senescence were detectable concerning efficiency of the combination therapy, which was also verified by virus growth curves.

The combination of 5-FU and MeV showed better cytotoxic effects on the investigated cancer cell lines than each agent alone. Thus, it can be assumed, that there is no reciprocal impairment of the efficacy of both agents, when combined. However, results imply (see Fig. 7 in comparison to Fig. 6), that the efficacy of the combination therapy succumbs the combination of MeV and gemcitabine. Moreover, 5-FU alone usually is not used as chemotherapy in pancreatic cancer in a palliative setting. For those reasons, in this work, the combination of gemcitabine and MeV has been studied in more detail. The influence of MeV on senescence or the influence of gemcitabine and senescence on viral growth was investigated. As a perspective for future experiments, another pragmatic chemovirotherapeutic approach containing 5-FU could be the combination of MeV with FOLFIRINOX, which consists of leucovorin, 5-FU, irinotecan and oxaliplatin. FOLFIRINOX is another therapeutic regimen used in advanced pancreatic cancer. As there are three chemotherapeutic drugs included, side effects are more severe ([17], see introduction). Consequently, it is applicable only for the fitter patients. If the results for chemovirotherapy with FOLFIRINOX would be similar to the ones with gemcitabine or 5-FU, combination therapy could also be a future perspective to lower the chemotherapy dosage and hence the mentioned side effects.

The phenomenon of senescence is a highly discussed mechanism and intensely researched, as its role in the development and suppression of cancer is still not certain [30, 92]. Several drugs - including gemcitabine - are able to induce senescence in cancer cells resulting in a permanent cell cycle arrest

and consequently maintaining cells in a less malignant state [27]. As 5-FU is also capable to induce senescence [93], the question might arise, why senescence has not been investigated in our work. As 5-FU mainly induces senescence via reactive oxygen species (ROS), mostly endothelial cells are being converted in a senescent state when treated with 5-FU [93]. The cells being used in this work reveal rather epithelial and mesenchymal characteristics [94], which is a likely explanation for the absence of senescence in these cases.

As the induction of senescence is not necessarily irreversible, therapeutic approaches have to be developed, which efficiently eliminate senescent cells. In a previous study, Weiland et al. from our group could show that MeV can infect, replicate within and lyse senescent cells including pancreatic cancer cells even more efficiently than non-senescent cells [27]. *In vivo*, it is almost impossible to ensure the sequence of application to every single cell, if a combination therapy is aspired. Therefore, we wanted to complement the previously mentioned finding and investigated if senescence can be induced in MeV-infected cells. For this purpose, we first infected pancreatic cancer cells with MeV and then added gemcitabine at several different time points up to 48 hours post infection with MeV. The results point out that senescence-induction is not altered by viral infections independently of the time point of the add-on of gemcitabine.

In summary these findings point out that senescence and MeV infection are not inconsistent cellular mechanisms when cytotoxicity is aimed at. No matter which mode of application was chosen concerning sequence of administration, induction of senescence led to an increased oncolytic cell death when compared to MeV infection alone. Similar results were obtained for the combination of the oncolytic coxsackievirus A21 in combination with the senescence inducing agent doxorubicine hydrochloride in a work published by Skelding et al. in 2012. Simultaneous application of virus and drug as well as infection and addition of the chemotherapeutic 24 h later showed synergistic

effects concerning cytotoxicity. No influence of doxorubicine on viral replication was observed [95].

Gemcitabine has already been co-administered with different oncolytic viruses. Wennier et al. treated pancreatic carcinoma cell lines with gemcitabine in combination with myxoma virus (MYXV). The drug was found to inhibit viral gene expression upon simultaneous administration. Sequential treatment, however, resulted in a striking decrease in cell viability when compared to the respective mono-therapies. Interestingly, the optimal sequence (drug first or virus first) was dependent on the cell line [89]. Gemcitabine was also shown to increase the oncolytic efficiency of the rat parvovirus H-1PV in pancreatic carcinoma cells when administered 24 h before the virus [96].

In our study we could show that (1) MeV and the chemotherapeutic compounds gemcitabine and 5-FU did not impair the efficacy of each other when administered together. (2) As many other virochemotherapy regimens, which have been tested so far, the combination of MeV and gemcitabine showed no reduced efficacy, no matter what mode and sequence of administration was chosen. Nevertheless, it is always important to consider the tumor entity, as differences of therapeutic efficacy exist depending on the sequence of application.

To take a closer look at the impact of gemcitabine on MeV, the viral replication behavior of MeV in the presence of gemcitabine has been investigated as well. Viral replication is a central mechanism for the efficacy of OV as it ensures a multiplication of the oncolytic potency as well as further spreading of viral particles [97]. Thus, the influence of gemcitabine on viral replication was an important aspect to investigate. As illustrated in Fig. 9, we observed a slight decrease in viral replication when gemcitabine was administered as an add-on. The lowest viral titers and the most distinct difference concerning viral growth with or without gemcitabine were detected in the cell line BxPC-3. Therefore, the experiment was repeated using two- and three-fold numbers of seeded cells to investigate whether it had an influence on the results. The intention was also to see whether a putative cytotoxicity of gemcitabine had a major impact

on BxPC-3 tumor cells and, as a result, viral growth was diminished due to decreased tumor cell number. As visible in the results, increase of seeded cell number had no overwhelming influence on viral growth. It is most likely, that viral growth is more active in the other two cell lines than in BxPC-3. That result is also mirrored by the decreased cytotoxicity in the combination therapy of gemcitabine and MeV in BxPC-3 in comparison to the other two cell lines. Eisenberg et al. were able to show that replication of oncolytic herpes virus NV1066 increases in the presence of gemcitabine [98]. In this study, a different application scheme was used with gemcitabine being added 6 h prior to infection. The reduced viral replication depicted in our work might be caused by the reduced cell number due to the cytotoxic effect of gemcitabine. As the presence of vital cells is crucial for the replication of MeV, fewer cells result in lower viral titers.

Direct alteration of viral replication by gemcitabine is also very likely as gemcitabine was originally developed as antiviral therapy and shows antiviral activity against RNA and DNA viruses [99, 100]. However, viral replication was only decreased but not suppressed and cytotoxicity was significantly higher with MeV than with gemcitabine alone. In numerous other works, a potentiation of viral replication was described when combined with chemotherapy or radiation [98, 101, 102]. The current explanation for that phenomenon is, in a simplified view, the host cellular stress response caused by chemotherapy or radiation, which facilitates viral entry, replication and lysis, as the host cell is weakened.

Summarizing all those findings, we can deduce that a certain impairment of viral replication either caused by the reduced cell number or direct antiviral activity or both can be detected. As gemcitabine was administered only at distinct concentrations, it cannot be entirely excluded, that viral replication might be impaired to a larger extent in vivo. To answer that question, similar experiments should be performed using cell lines being more resistant towards gemcitabine, as the cytotoxicity of gemcitabine in higher concentrations would be lethal for the cell lines used in this work. Enhanced viral replication caused

by the additional cellular stress due to gemcitabine with simultaneously reduced cell number was neither proved nor disproved. Regardless of those considerations, viral replication was rather active and not suppressed at all by gemcitabine and showed a significant impact concerning cytotoxicity.

Combination of gemcitabine and MeV constitutes a reasonable new approach to overcome therapeutic resistance of pancreatic cancer cells *in vitro*. We were able to show that a combination of rather low concentrations of both therapeutics which were clearly suboptimal in terms of cytotoxicity led to a significant increase in cytotoxicity in three cell lines when compared to single agent treatment. It remains to be determined, whether there is a distinct molecular pathway that leads to the observed combinatorial effect. However, both therapeutics were shown to work well together and did not alter significantly efficacy of each other. For further research, it is indispensable to transfer our findings to *in vivo* to find out about possible obstacles and the most advantageous way of therapeutic application. For example, the investigation of viral replication behavior in combination with gemcitabine within a solid tumor would constitute an important approach to find out more about possible mutual interactions. Moreover, besides virotherapy, there are other novel therapeutics with the potency to revolutionize cancer therapy. An example to mention is the epidermal growth factor receptor tyrosine kinase inhibitor (EGFR TKI) erlotinib, which constitutes an alternative treatment strategy in combination with gemcitabine in advanced pancreatic cancer [16, 103], but also epigenetic drugs being currently under investigation for a combination treatment with virotherapeutics in cancer [104, 105] or the ataxia telangiectasia mutated protein (ATM), which potentiated the replication of reovirus in canine melanoma cell lines [106]. Another strategy investigated in glioblastoma cells is the application of chemotherapy in combination with Measles Vaccine Virus and radiotherapy resulting in synergistic therapeutic effects and a pro-inflammatory phenotype *in vitro* [107]. It is still to consider, whether the add-on of further cancer therapeutics will lead to further benefit in the outcome.

Concerning application of the therapeutics, it is very important to optimize delivery of therapeutics especially in pancreatic cancer, as it is hard to reach systemically due to its scarce vascularization and thus hypoxia as described before.

In general, as virotherapy has not entered a status of widespread clinical routine, many questions remain to be answered concerning application, dosage and frequency, which are currently under investigation [108]. Therefore, alternative modes of application should be tested *in vivo,* e.g. intratumoral, intraperitoneal (i.p.) [109] or even locoregional, e.g. by hepatic arterial infusion [110].

New findings concerning tumor biology should be taken into consideration searching for other therapeutic options. It is very important to analyze different therapeutic regimens [82], and the influence of the immune system on virotherapy - considering both “negative” aspects as it weakens viral infection and “positive” aspects as the activation of the immune system leads to antitumor immune response [111, 112]. Addressing this predominantly unsolved issue, three strategies have been described regarding oncolytic measles virotherapy: (1) the suppression of antiviral immunity in patients or (2) shielding of viral particles from detection of the immune system or (3) enhancing immune evasion of virus [113]. One approach to suppress antiviral immunity is the coadministration of cyclophosphamide to avoid antibody-mediated immune-response [114]. Concerning shielding of viral particles, carrier cells infected with virus, such as T-cells, monocytes or mesenchymal stem cells are under investigation [115] [113, 116].

Other approaches exploit the interaction of the virus with the immune system, such as the genetically modified herpes simplex virus type 1, known as talimogene laherparepvec (T-VEC), which was approved by the U.S. Food and Drug Administration (FDA) for the treatment of malignant melanoma in 2015 [47]. In the randomized open label phase-III OPTiM-trial, patients with un-resectable IIIB-IVM1c-stage malignant melanoma received T-VEC intratumoral. T-VEC is attenuated, as herpes neurovirulence viral genes are replaced by the

coding sequence for granulocyte-macrophage colony-stimulating factor (GM-CSF) to enhance T-cell responses. A control group received GM-CSF alone. The application of T-VEC resulted in better durable response rates (DRR) and overall survival (OS) without fatal adverse events reported [48, 117, 118].

As checkpoint inhibitors constitute a revolutionary finding in the field of cancer immunotherapy leading to profound antitumor immune response [119, 120], combination with oncolytic virotherapy appears as a rather worthwhile investigation. In their work, Engeland et al. [121] were able to show, that combination of CTLA-4 and PD-L1 inhibitors and measles virus resulted in delayed tumor progression and prolonged median overall survival. Interestingly, in this work, measles virus was used as vector for antibodies against CTLA-4 and PD-L1. An immunocompetent murine model with subcutaneous melanoma was treated intratumoral, leading to an increase of CD3+ and partly also CD8+ T-cells. A similar approach was performed by Dias et al. [122], using adeno-virus as a vector to encode an anti-CTLA-4 monoclonal antibody (mAb), resulting in higher intratumoral concentrations of the mAb than in plasma. Both works constitute interesting approaches for combination regimens, leading to a more targeted and more efficient checkpoint inhibition and thus to less side effects. Another interesting approach in the field of combination of OV and checkpoint inhibitors is a work by Mullins-Dansereau et al., exploring OV as a neoadjuvant therapeutic regimen in a murine model of breast cancer with post-operative re-challenge of tumor and treatment with immune checkpoint inhibitor at relapse [123]. Mice treated with OVs showed a higher rate of cure and smaller secondary tumors and less metastases. The effect was only observed in a immunocompetent model, suggesting, that the result was immune-mediated [123].

In a recently published review the necessity of a very profound investigation and understanding of tumor biology was pointed out as well [124]. Moreover, it was accented, that identification of synergistically working chemovirotherapy is urgently needed - aiming not only for an exploitation of cytotoxicity but also for

an alteration of the immunosuppressive microenvironment of pancreatic cancer [121, 125].

5. Summary

Current therapeutic options for locally advanced or metastatic pancreatic cancer still are not able to improve the prognosis of patients long-lasting. Thus, it is more than urgent to find new strategies to overcome therapeutic resistance and inefficiency, respectively, by establishing novel combinatorial approaches encompassing new therapeutic principles.

“Oncolytic virotherapy with vaccine viruses employs replicative vectors, which quite selectively infect tumor cells leading to massive virus replication followed by subsequent profound tumor cell death (oncolysis). Measles vaccine virus (MeV) has already shown great oncolytic activity against different types of cancers, including pancreatic cancer. Gemcitabine is a first line chemo-therapeutic drug used for pancreatic cancer in palliative treatment plans. Furthermore, this drug can be used to induce senescence, a permanent cell cycle arrest, in tumor cells. In our preclinical work, three well-characterized immortalized human pancreatic cancer cell lines were used to investigate the combinatorial effect of MeV-based virotherapy together with the chemo-therapeutic compound gemcitabine. Viability assays revealed that the combination of only small amounts of MeV together with subtherapeutic concentrations of gemcitabine resulted in a tumor cell mass reduction of > 50%. To further investigate the replication of the oncolytic MeV vectors under these distinct combinatorial conditions, viral growth curves were generated. As a result, viral replication was found to be only slightly diminished in the presence of gemcitabine. As gemcitabine induces senescence, the effect of MeV on that phenomenon was explored using a senescence-associated β-galactosidase assay. Notably, gemcitabine-induced tumor cell senescence was not impaired by MeV. Accordingly, the chemovirotherapeutic combination of gemcitabine plus oncolytic MeV constitutes a novel therapeutic option for advanced pancreatic carcinoma that is characterized by the mutual improvement of the effectiveness of each therapeutic component.“ [88]

5.1 Zusammenfassung

Zur Zeit sind die für das lokal fortgeschrittene oder metastasierte Pankreaskarzinom verfügbaren therapeutischen Optionen noch nicht in der Lage, die Langzeit-Prognose der Patienten zu verbessern. Die onkolytische Virotherapie mit Impfviren stellt eine neuartige Therapieoption dar, wobei replizierende Vektoren mit einer überwiegenden Selektivität für maligne Zellen Tumorgewebe infizieren, was zu einer massiven Tumorzell-gestützten Virusreplikation und darauf folgendem Tumor-Zelltod führt. Für Masern-Impfviren (MeV) konnte schon eine sehr gute onkolytische Aktivität bei verschiedenen Krebsarten nachgewiesen werden. Gemcitabine stellt eine etablierte Chemotherapie bei Pankreaskarzinom dar. Darüber hinaus kann dieses Medikament verwendet werden, um Seneszenz, einen permanenten Zellzyklusarrest, zu induzieren. Für unsere präklinische Arbeit wurden drei humane Pankreaskarzinom-Zelllinien verwendet, um den Kombinations-Effekt von MeV als Virotherapeutikum mit Gemcitabine zu untersuchen. In Zellviabilitäts-Assays konnte gezeigt werden, dass die Kombination aus einer geringen Anzahl an infektiösen MeV-Viruspartikeln (unterschwellig lytisch) zusammen mit sub-therapeutischen Konzentration des Chemotherapeutikums Gemcitabine zu einer Reduktion der Tumor-Zellzahlen von über 50 % führte. Um die Replikation von MeV unter diesen speziellen ‘Kombinations-Bedingungen’ weiter zu untersuchen, wurden Virus-Wachstumskurven erstellt. Es zeigte sich, dass die Virusreplikation leicht vermindert war, verglichen mit der Replikation in Abwesenheit von Gemcitabine. Da Gemcitabine Seneszenz induzieren kann, wurde der Einfluss von MeV auf dieses Phänomen mit Seneszenz-assoziierter β-Galactosidase (SA-β-gal) untersucht. Interessanterweise wurde die von Gemcitabine verursachte Tumorzell-Seneszenz nicht unterbunden durch die Anwesenheit von MeV. Zusammenfassend stellt die Chemovirotherapie bestehend aus einer Kombination von Gemcitabine mit Masern-Impfviren eine neuartige Therapieoption dar, die sich darin auszeichnet, dass sich beide Therapeutika in Kombination gegenseitig verstärken können.

6. Appendix

6.1. References

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Erklärung zum Eigenanteil der Dissertationsschrift

Die Arbeit wurde zunächst in der Medizinischen Universitätsklinik, Abteilung für Innere Medizin I, begonnen und dann später in der Abteilung für Innere Medizin VIII unter Betreuung von Herrn Prof. Dr. U.M. Lauer durchgeführt.

Die Konzeption der Studie erfolgte durch Herrn Prof. Dr. U.M. Lauer in Zusammenarbeit mit Frau Dr. S. Berchtold (wiss. Mitarbeiterin und Laborleiterin).

Sämtliche Versuche wurden (nach Einarbeitung durch Frau I. Smirnow, MTA) von mir eigenständig durchgeführt. Die Einarbeitung in die Methodik des SA-β-Gal assay sowie die Mitbetreuung erfolgte von Drs. S. Venturelli, A. Berger und Dipl. Biochem. C. Leischner.

Die statistische Auswertung erfolgte in Zusammenarbeit mit den Drs. S. Venturelli und M. Burkard

Ich versichere, das Manuskript selbständig (nach Anleitung durch Frau Dr. S. Berchtold und Prof. U.M. Lauer) verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Ergebnisse der Dissertationsschrift wurde im September 2019 *online* und im November 2019 gedruckt im Journal „Oncology Letters“ des Spandidos-Verlages unter dem Titel „Chemovirotherapy for pancreatic cancer: Gemcitabine plus oncolytic measles vaccine virus”, doi: 10.3892/ol.2019.10901 veröffentlicht. Die Publikation ist als vollständiger Text *online* einzusehen. Jegliche in der Doktorarbeit verwendete Abbildungen und Texte sind dementsprechend in der Beschreibung oder als Direktzitat kenntlich gemacht.

Weitere Präsentationen der Ergebnisse der Doktorarbeit fanden bei folgenden Kongressen statt:

- Posterpräsentation beim AEK-Kongress in Heidelberg 27.02.-01.03.2019 unter dem Titel „Chemovirotherapy for Pancreatic Cancer: Gemcitabine plus Oncolytic Measles Vaccine Virus”
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