"PULSED" versus "CONTINUOUS" application of the prodrug 5-FC for enhancing oncolytic effectiveness of a measles vaccine virus armed with a suicide gene

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1. Introduction

1.1. Gastrointestinal cancer

1.1.1. Introduction

According to the World Health Organisation (WHO), in 2012, there were 8.2 million people worldwide who died from cancer. It is estimated that the number will even rise to 13.1 million in 2030.

Of 8.2 million deaths in 2008, liver cancer accounts for approximately 746,000, colorectal cancer for 694,000 and gallbladder cancer for 143,000 deaths (Ferlay *et al*, 2012).

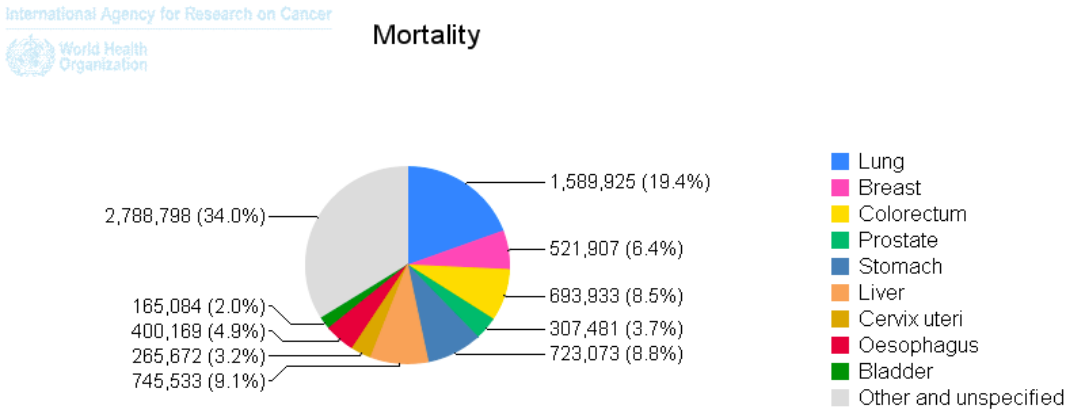


Figure 1.1. Cancer frequency in both sexes. Taken from (Ferlay *et al*, 2012).

1.1.2. Hepatocellular carcinoma

Of all cancer deaths world wide, liver cancer is the second leading cause (Ferlay, 2012) and numbers are constantly increasing (Maluccio & Covey, 2012).

There are many types of liver cancer, of which hepatocellular carcinoma (HCC) accounts for up to 70 - 85 % of all liver cancers worldwide (Perz *et al*,

2006). Major risk factors are aflatoxin B1 exposure (Ming *et al*, 2002), hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, to which 78 % of global HCC incidences are attributable (Perz *et al*, 2006). This also explains high rates of HCC in parts of Asia and Africa, where prevalences of chronic hepatitis B infections of up to 8 % are quite common (Jemal *et al*, 2011). In Western countries, which are at a rather low risk for HCC, factors such as alcohol-related cirrhosis and nonalcoholic steatohepatitis (NASH) mainly contribute to the development of HCCs (Jemal *et al*, 2011; Maluccio & Covey, 2012).

The only curative options for HCC still remain liver transplantation and surgical resection, both in quite early stages of the disease. Patients who meet with Milan criteria (basically HCC with one nodule < 5 cm or up to 3 with each < 3 cm) for liver transplantation can experience a 4-year overall survival of 85 % (Mazzaferro *et al*, 1996). However, as HCCs are mostly advanced and disseminated when diagnosed, curative treatment is mostly not possible. The current ten-year-survival rate after resection is 7.2 % (Gluer *et al*, 2012). With the introduction of a first systemic therapy with Sorafenib, median survival could be prolonged by 3 months (Llovet *et al*, 2008), but due to development of multidrug resistances, “conventional” chemotherapy is only temporarily effective (Li *et al*, 2001). These circumstances are also reflected in the almost identical numbers of incidence and mortality, indicating that most HCC patients cannot be “rescued” and then die of their disease (Ferlay *et al*, 2012; Maluccio & Covey, 2012).

1.1.3. Cholangiocarcinoma

Cholangiocarcinoma is an adenocarcinoma of the extra- and intrahepatic bile ducts (Lau & Lau, 2012; Pattanathien *et al*, 2013). Compared to other malignancies, numbers of Cholangiocarcinoma incidences are relatively low. However, high rates are found in parts of Eastern Asia and especially Thailand, which appears to be mainly due to infections with liver flukes (Shin *et al*, 2010). Chronic biliary inflammation in general is considered as a risk factor including primary sclerosing cholangitis (Claessen *et al*, 2009), hepatolithiasis and bile-

duct cysts (Tyson & El-Serag, 2011). In general, therapy of Cholangiocarcinoma is known to be highly difficult and challenging. Total surgical excision is still the only curative approach, since Cholangiocarcinomas hardly respond to neither radiation nor chemotherapy. If not resectable or already metastasized, patients are treated in a palliative manner (Lau & Lau, 2012). One study from Thailand provides median survival data of 15 months and 5-year survival rates of 10.8 % after surgical treatment (Pattanathien *et al*, 2013).

1.1.4. Colorectal carcinoma

Colorectal cancer (CRC) is the fourth leading cause of cancer deaths worldwide (Ferlay *et al*, 2012). Incidences are higher in industrialized countries and in men. It is assumed to be mainly due to dietary factors (red meat, alcohol, little intake of fibre), obesity, physical inactivity, diabetes mellitus and smoking (Jemal *et al*, 2011). In contrast, preventive screening programs for early detection of premalignant adenomas and localized cancer were shown to significantly decrease incidence and mortality of colorectal cancer (Atkin *et al*, 2010).

Surgery remains the basis of curative approaches and is combined, if necessary, with radiation and chemotherapy. If possible, total resection of the tumor should be aspired. Relapse after surgery mostly becomes manifest in liver and lung metastases (Cunningham *et al*, 2010). According to the EUROCARE-4 data between 2000 and 2002, colorectal cancer patients in Europe have a 5-year survival rate of 56.2 % (Verdecchia *et al*, 2007).

1.2. 5-FU and latest therapeutical state of the art

1.2.1. Antineoplastic therapy

Today's therapeutic options for cancer basically consist of chemotherapy, surgical resection, radiation therapy as well as novel biological anti-cancer therapies.

Chemotherapy has its origin in World War I and II, when autopsies of people poisoned by mustard gas revealed severe bone marrow depressions. Further investigations showed that the effect correlated with the proliferative activity of tissues. In 1942, its antineoplastic potential was discovered and mustard gas derivatives were thus introduced as first chemotherapeutic drugs (Papac, 2001).
Development of other alkylating drugs (e.g. cyclophosphamide) followed and with the finding of aminopterin as a folic acid antagonist, therapy with anti-metabolites was initiated (Farber & Diamond, 1948). Since that time, the search for new and efficient anti-neoplastic drugs has become a major field in tumor therapy.

However, there are severe disadvantages of chemotherapy: as already mentioned the anti-tumor properties of chemotherapy depend on the proliferative activity of cells. High proliferation rates are not only a characteristic of cancer cells, but also of hematopoietic cells and many epithelial tissues, which explains common toxic side effects of chemotherapy. This results in a dose-limiting toxicity and eventually in a less effective therapy (Moolten *et al*, 1990).
Furthermore, tumors often show decreasing response to drugs during therapy and acquire resistances. Cancer stem cells, which are hardly affected by common therapies, are thought to be responsible for the initiation of resistance and relapse after initially successful therapy (Cripe *et al*, 2009). Moreover, tumor cells develop mechanisms to inactivate drugs or induce production of

membrane transporters, which either take up less or export more of the chemotherapeutic drug (Zahreddine & Borden, 2013).

Taken together, despite continuous improvements in cancer therapy and prolonged survival of treated patients, complete remissions and cure of cancer are rare and anti-cancer drugs, which selectively affect tumor cells whilst sparing normal cells, are still being searched extensively.

1.2.2. 5-FU

5-fluorouracil (5-FU) is an anti-cancer drug, which has been introduced in 1957 by Charles Heidelberger (Heidelberger *et al*, 1957). Since that time, it has been used especially for palliation, treatment of metastasized breast and colon cancer (Heidelberger & Ansfield, 1963) and also for genito-urinary, gastric, liver, pancreas and head and neck cancer (Kanai *et al*, 1998).

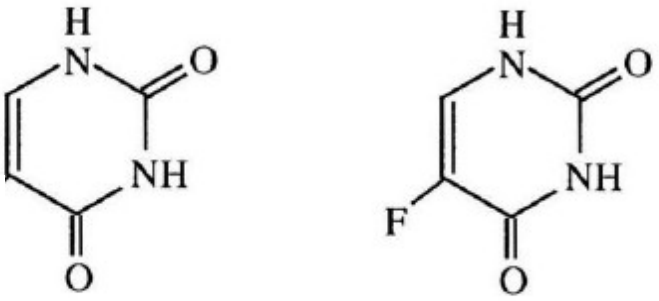


Figure 1.2. Chemical structure of Uracil (left) and 5-Fluorouracil/5-FU (right). Taken and modified from (Vermes et al, 2000).

5-FU is a pyrimidine anti-metabolite, as it very much resembles the pyrimidine uracil, but is fluorine-substituted at position 5. In the pyrimidine ring of thymine, to which uracil is converted by thymidylate synthase (TS), there is a methyl group at position 5. Conversion of uracil to thymine by TS was therefore supposed to be blocked (Heidelberger *et al*, 1957), thus inhibiting DNA synthesis, as TS is the only source of newly produced thymidine (Danenberg, 1977).

In fact, the toxic effect is directed to both, DNA (Cohen *et al*, 1958; Heidelberger & Ansfield, 1963) and RNA (Cory *et al*, 1979; Glazer & Peale, 1979; Glazer & Lloyd, 1982; Piper & Fox, 1982). The mechanism of action looks as follows: in the first step, 5-FU is converted to 5-fluorouridine-5'-monophosphate (5-FUMP). This happens either directly by orotate phosphoribosyltransferase (OPRT) or in two steps by uridine phosphorylase to intermediate 5-fluoro uridine and by uridine kinase to 5-FUMP (Cohen *et al*, 1958; Piper & Fox, 1982).

In the second step, 5-FUMP is further metabolized by cellular nucleotide kinases to 5-fluorouridine-5'-diphosphate (5-FUDP). 5-FUDP may then be toxified to the final, cytotoxic metabolites by (i) further phosphorylation by nucleotide kinases to 5-fluorouridine-5'-triphosphate (5-FUTP), which directly interferes with production and function of all RNA classes (Wilkinson & Pitot, 1973; Glazer & Peale, 1979; Glazer & Lloyd, 1982). 5-FUDP can also be converted by (ii) ribonucleotide reductase to 5-fluoro-2'-deoxyuridine-5'-diphosphate (5-FdUDP) and then to 5-FdUMP which irreversibly suppresses TS and therefore DNA synthesis (Cohen *et al*, 1958). A third possibility is that (iii) 5-FdUDP is converted to 5-FdUTP, which is incorporated into DNA and hence damages the DNA. Extensive base-excision repair causes DNA fragmentation and therefore cell death (Thorn *et al*, 2011).

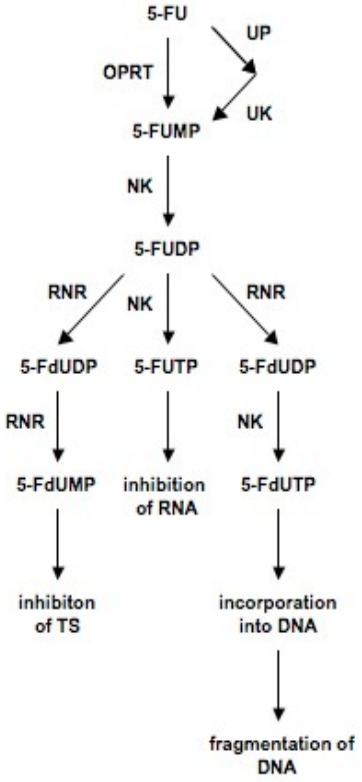


Figure 1.3 5-FU activation and mechanisms of action.

5-FU = 5-fluorouracil, 5-FUMP = 5-fluorouridine 5‘ monophosphate, 5-FUDP = 5-fluorouridine 5‘ diphosphate, 5-FUTP = 5-fluorouridine 5‘ triphosphate, NK = nucleotide kinase, OPRT = orotate phosphoribosyltransferase, RNR = ribonucleotide reductase, TS = thymidylate synthase, UK = uridine kinase, UP = uridine phosphorylase. Figure based on (Cory *et al*, 1979; Piper & Fox, 1982; Valeriote & Santelli, 1984; Thorn *et al*, 2011).

However, as 5-FU is an anti-metabolite, its effect at concentrations that are safe to administer systemically is dependent on cell proliferation. It is therefore assumed, that insufficient therapy with 5-FU is due to the fact that only 10 % of all carcinoma cells are cycling at the same time (Akbulut *et al*, 2004). Doses to also kill slowly or non-proliferating cells would cause unacceptable toxic effects.

Such toxic side effects depend on the method of administration (continuous therapy, daily or weekly bolus therapy) and are typically myelo-suppression with neutropenia, stomatitis, mucositis, diarrhea, dermatitis and conjunctivitis (Weh *et al*, 1994; de Gramont *et al*, 1997).

1.3. Oncolytic virotherapy

Oncolytic virotherapy is a new approach to treat cancer, which is currently under preclinical and clinical investigation. The idea is basically that a replication-competent virus selectively infects tumor cells, replicates within them to produce progenies and eventually destroys its host cells. Ideally, those viruses do not affect untransformed, non-malignant tissues and have therefore limited or even no toxic side effects.

In the following chapter, the discovery of oncolytic viruses, their principle of operation, advantages and disadvantages as well as further improvements to enhance the oncolytic effect will be explained.

1.3.1. History and finding of oncolytic viruses

The discovery of viruses as novel cancer therapeutics was rather coincidental. There are numerous case reports about tumor patients who occasionally developed a viral infection which led to an (although mostly partial) remission of their neoplastic malignancy (Kelly & Russell, 2007). Regressing leukaemias (Gross, 1971; Pasquinucci, 1971), Burkitt's lymphomas (Bluming & Ziegler, 1971), and Hodgkin's diseases (Mota, 1973) after infections with wild-type measles virus (MeV) are only few examples. Similar observations were made with cases of acute leukaemia after glandular fever infections (Taylor, 1953) and chicken pox (Moore, 1954). However, these reports were not limited to natural infections only, but also attenuated viruses used for vaccinations were able to cause tumor regressions. Repeated smallpox vaccination caused a complete remission for 3 years in a patient suffering from untreated chronic lymphocytic leukaemia (Hansen & Libnoch, 1978). A case of cervix cancer and few of melanoma were noted to improve after rabies vaccination (Moore, 1954).

Subsequently, viruses and their vaccines were investigated as totally new approaches for the therapy of cancer. Although some of those case reports were already presented much earlier, oncolytic virotherapy with wild-type viruses started in the 1950s. Further developments of the advanced viro-

therapeutic agents, being derived from genetic engineering, began in the 1990s (Liu *et al*, 2007).

1.3.2. Advantages and disadvantages

Broadening the options for the treatment of cancer, oncolytic viruses have some advantages over common therapies. First of all, viruses were evolutionarily adapted to efficiently infect and replicate within their host cells. They exploit the host to produce further progenies, which in turn are able to infect neighbouring cells (Msaouel *et al*, 2009). Secondly, oncolytic viruses selectively infect and replicate in tumor cells, leading to apoptosis or infection-related lysis. Consequently, non-transformed cells are not affected and systemic toxicity is therefore limited (Li *et al*, 2001; Liu *et al*, 2007). Thirdly, even tumor cells being apoptosis- or chemotherapy-resistant can be killed by viruses, demonstrating that there is no cross-resistance (Coukos *et al*, 2000; Khuri *et al*, 2000; Reid *et al*, 2002; Richard *et al*, 2007; Cattaneo *et al*, 2008). Also tumor stem cells, which are hardly affected by common chemotherapeutics, show susceptibility towards virotherapy (Cripe *et al*, 2009; Mahller *et al*, 2009). Beyond that, oncolytic viruses have been proved to be safe in all clinical trials that have been performed to date, generally having caused not more than any flu-like symptoms (Khuri *et al*, 2000; Kirn, 2001; Reid *et al*, 2002; Heinzerling *et al*, 2005; Liu *et al*, 2007; Galanis *et al*, 2010). Overall, safety has already been affirmed for at least six different virus species, administered to over 800 patients (Liu & Kirn, 2008).

However, there are also disadvantages of this novel therapeutic approach. The immune system not only supports oncolysis by identifying and killing infected cells, but also recognizes the viral particles as foreign and hence eliminates a major percentage before they can efficiently infect the tumor (Breitbach *et al*, 2007; Cattaneo *et al*, 2008; Liu & Kirn, 2008; White *et al*, 2008; Prestwich *et al*, 2009). In fact, relatively low antibody titers are already able to hugely neutralize the systemically delivered viruses (Ong *et al*, 2007). Repeated applications induce increasing titers of neutralizing antibodies and thus lessen

the effect (White *et al*, 2008). Even alternatives to circumventing intravenous application by, for example, directly injecting the viruses into the tumor, are insufficient (Russell *et al*, 2012). But not only antibodies, complement or phagocytes hinder the delivery, there are also mechanic barriers like fibrosis, necrosis and elevated interstitial pressure within the tumor complicating the infection (Jain, 2001). The reticuloendothelial system in organs like spleen and liver is also responsible for virus clearance (Prestwich *et al*, 2008; Russell *et al*, 2012).
Despite the promising oncolytic potency, a single therapy won't be sufficient and has therefore to be further investigated and combined with other therapeutic options (Liu *et al*, 2007; Cattaneo *et al*, 2008).

1.3.2. Mechanism of oncolysis

Tumor cells are characterized by their genetic mutations and genomic instability. Proliferation is associated with further genetic alterations and defects and may, by chance, result in new capabilities and growth advantage. However, those mutations are not directional and cancer cells not only acquire new features, but also lose preexisting abilities (Hanahan & Weinberg, 2011).

One example is mutation within interferon (IFN) mediated pathways (Colamonici *et al*, 1992; Sun *et al*, 1998), which normally protect cells from viral infections, inhibit cellular proliferation and activate apoptotic signals (Clemens & McNurlan, 1985; Stark *et al*, 1998). Being resistant towards those signals, cancer cells are protected from IFN mediated clearance (Stojdl *et al*, 2000; Naik & Russell, 2009; Lech & Russell, 2010). At the same time however, they become susceptible to viral infections when losing their innate antiviral protection, whilst untransformed cells turn into an antiviral state. This accounts, amongst other reasons, for the cancer-selectivity of oncolytic viruses (Stojdl *et al*, 2000; Haralambieva *et al*, 2007).

In addition to directly lysing the tumor, the virus serves as an activator of the immune system (Prestwich *et al*, 2008; Boisgerault *et al*, 2010). Migrating immature cells of the immune system such as dendritic cells detect viral and

tumor-associated antigens produced by infected tumor cells and become activated (Moehler *et al*, 2005; Greiner *et al*, 2006). In turn, they recruit further immune cells (especially CD8+ T cells and NK cells), which attack the tumor cells, induce production of IFN and other signaling molecules (Errington *et al*, 2008; Lech & Russell, 2010).

This fact was also confirmed by Diaz et al, when tumor bearing mice, lacking either CD8+ T cells or NK cells, were treated with oncolytic vesicular stomatitis virus (VSV). Both led to decreased oncolytic efficacy (Diaz *et al*, 2007), compared to fully immune competent mice. Activation of the adaptive immune system therefore contributes an important part to the oncolytic virotherapy.

1.3.4. Enhancing oncolytic effects

As already explained, a monotherapy with oncolytic viruses is insufficient and has to be enhanced (Liu *et al*, 2007). For this purpose, there are several approaches:

Combination with drugs is one possibility. With regard to virus clearance by the immune system, immunosuppressive drugs are being investigated. One of them is cyclophosphamide (CPA), a DNA-alkylating and immunosuppressive drug (Cattaneo *et al*, 2008). It has been shown that the combination with oncolytic viruses is synergistic. This is thought to be due to the fact that CPA reduces the concentration of antibodies (especially IgG), eliminates and inhibits proliferation of B- and T-lymphocytes and represses cellular innate antiviral immunity (Ikeda *et al*, 1999; Fulci *et al*, 2006; Qiao *et al*, 2008; Kottke *et al*, 2009; Lun *et al*, 2009).

With respect to mechanic barriers hindering the delivery of drugs and also oncolytic viruses to the tumor site, there are approaches to increase vascular permeability and lower interstitial pressure within the tumor (Jain & Stylianopoulos, 2010). Administration of VEGF-repressor derivatives were shown to increase vascular permeability and hence reovirus infection of tumor

endothelium (Kottke *et al*, 2010). This could also be demonstrated by applying IL-2 in a mouse model of lung metastases, treated with VSV virotherapeutics (Kottke *et al*, 2008).
A combination of virotherapy with histone deacetylase inhibitors (HDACi) to decrease response of tumor cells to IFN and therefore increase susceptibility to viral infection also showed promising results (Nguyen *et al*, 2008).

Targeting is a concept that aims to direct viruses even more precisely to cancerous tissues. Due to either expression of different or overexpression of common proteins, tumor cells can be addressed more specifically. Using this basic principle, viruses can be modified to target such differentially expressed proteins. For example, Jing et al modified MeV's H-protein to bind to urokinase-type plasminogen activator receptor (uPAR), which is frequently overexpressed in breast cancer, and could show a prolonged survival in a xenograft breast cancer model when treating with a targeted virus (Jing *et al*, 2009).

Another concept is to arm viruses by genetic manipulation (also called "arming"). Prodrug convertases, for example, can be transferred to the virus, which in turn brings the enzymes into the selectively infected tumor cells. The general idea is that these enzymes locally convert non-toxic to highly toxic substances (Knox & Connors, 1997; Cattaneo *et al*, 2008).

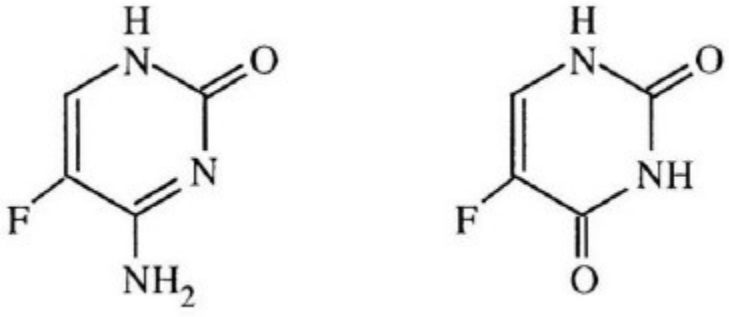


Figure 1.4. Chemical structure of 5-Fluorocytosine (5-FC) (left) and 5-Fluorouracil (5-FU) (right). Taken from (Vermes et al, 2000).

One system, which also has been used in this thesis, works with a fusion protein consisting of the enzymes cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT), both derived from certain bacteria (*E. coli*) or fungi (*Saccharomyces cerevisiae*). CD catalyzes deamination of cytosine to uracil or 5-fluorocytosine to 5-fluorouracil, respectively (Kilstrup *et al*, 1989). In contrast, neither do mammalian cells contain those enzymes, nor do they metabolize cytosine to uracil (Mullen *et al*, 1992). UPRT catalyzes the conversion of uracil to uracil monophosphate (UMP) and of 5-FU to 5-FUMP, which is an intermediate step in the utilization of 5-FU (Kanai *et al*, 1998). This extra enzyme has been introduced because of the observation that some 5-FU resistant tumor entities revealed mutations downstream in the pathway of 5-FU. With UPRT, this intermediate step and simultaneously this cause of 5-FU resistance can be evaded (Kanai *et al*, 1998; Erbs *et al*, 2000). Richard et al could also demonstrate that the intracellular prodrug conversion leads to far higher intracellular concentrations of 5-FU than achievable by systemic administration. Therefore even slowly dividing tumor cells could be killed efficiently (Richard *et al*, 2007). The complete activation pathway of 5-FU is described in section 1.2.2.

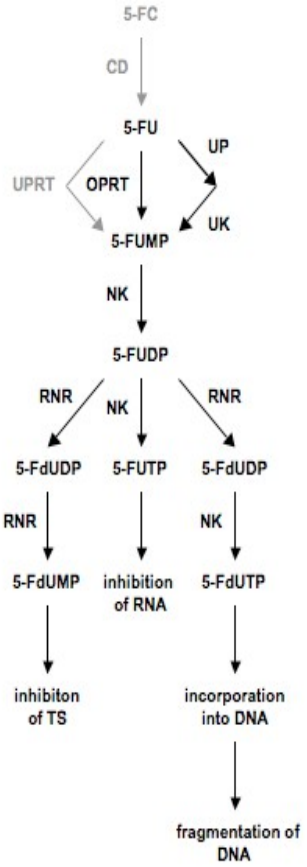


Figure 1.5 5-FC activation and mechanisms of action.
5-FC = 5-fluorocytosine, 5-FU = 5-fluorouracil, 5-FUMP = 5-fluorouridine 5‘ monophosphate, 5-FUDP = 5-fluorouridine 5‘ diphosphate, 5-FUTP = 5-fluorouridine 5‘ triphosphate, CD = cytosine deaminase, NK = nucleotide kinase, OPRT = orotate phosphoribosyltransferase, RNR = ribonucleotide reductase, TS = thymidylate synthase, UK = uridine kinase, UP = uridine phosphorylase, UPRT = uracil phosphoribosyltransferase. Figure based on (Cory *et al*, 1979; Piper & Fox, 1982; Thorn *et al*, 2011; Valeriote & Santelli, 1984).

These effects are highly toxic to mammalian cells but only occur in prodrug convertase-bearing cells. Those not being transformed escape the cytotoxicity (Mullen *et al*, 1992).
A chimeric protein, developed by Erbs et al, shows the activity of both enzymes and proved to be more efficient than the single enzymes (Erbs *et al*, 2000). Encoded by a replication-competent adenovirus (AdV) in combination with 5-FC, treatment of breast, pancreas and colon cancer cells revealed to be efficient (Erbs *et al*, 2000; Chung-Faye *et al*, 2001).

A similar approach was undertaken with vaccinia virus to investigate the effect on HCC, liver metastases, breast, pancreas, colon and lung cancer and was also confirmed to show a potent enhancement of oncolysis (Erbs *et al*, 2008; Foloppe *et al*, 2008).
Additionally, 5-FU is highly diffusible and can kill non-infected surrounding cells ("bystander effect"). Huber et al could show that significant regressions in xeno-graft models can be observed when as few as 2 % of all tumor cells express the prodrug convertases (Huber *et al*, 1994).
In conclusion oncolysis can be strongly enhanced by local prodrug toxification and diffusion of the toxified product to neighboring cells. This has already been confirmed in several *in vitro* and *in vivo* studies (Huber *et al*, 1994; Kanai *et al*, 1998; Topf *et al*, 1998; Erbs *et al*, 2000).

A second example is a thymidine kinase-system. Thymidine kinase (TK) catalyzes phosphorylation of guanosine analogs like aciclovir (ACV) or ganciclovir (GCV) to the correspondent monophosphates. Cellular kinases further phosphorylate those resulting in metabolites, which then inhibit DNA synthesis (Moolten *et al*, 1990; Knox & Connors, 1997). When Boviatsis et al treated gliosarcoma-bearing rats with herpes simplex virus (HSV) with or without TK and added GCV, they could significantly prolong survival of those treated with TK and GCV (Boviatsis *et al*, 1994).

With the purpose to avoid early viral clearance and directing the virus to the tumor site, there are approaches to use carriers that naturally migrate to the tumor (so-called cell-mediated virus shielding approaches). This process is called "tumor cell homing" and can be used to deliver the virus to the tumor more efficiently, simultaneously protecting the virus from early clearance by the immune system. Therefore, suitable carriers (e.g. dendritic cells, leukocytes or even tumor cells) are infected *ex vivo* and administered before expression of viral antigens on their cell surface so that they can accumulate at the tumor site and locally produce high numbers of progenies (Harrington & Vile, 2006; Power & Bell, 2008; Ilett *et al*, 2011).

Cytokine-induced killer cells, for example, can be obtained from the patient, infected with the oncolytic virus and then be reinfused. This principle was shown to be synergistic when treating tumor-bearing mice (Thorne *et al*, 2006). Another example is the usage of lethally irradiated myeloma cells, which were able to carry and protect MeV in mice exhibiting anti-measles antibodies (Liu *et al*, 2010). Similar results could be obtained with activated T-cells as carriers of MeV (Ong *et al*, 2007).

There are also approaches to lower the clearance by the reticuloendothelial system by simply increasing the dose of administered virus to saturate uptake or by shielding it with polymer coating (Fisher *et al*, 2001; Green *et al*, 2004; Manickan, 2006; Manickan *et al*, 2006). Depletion by monocytes could be decreased by simultaneous therapy with anticoagulants, clodronate, gadolinium and IgG (Ziegler *et al*, 2002; Shashkova *et al*, 2008). Additionally, monocyte receptors may be saturated by preincubation with polyinosinic acid (Haisma *et al*, 2008).

1.3.5. Clinical trials

The first clinical trials employing viruses to treat cancer began in the 1950s. One of them was based on case reports about remission of Hodgkin's disease after onset of viral hepatitis. 21 patients were treated with sera and tissues extracts containing hepatitis virus and examined up to 12 months. However, it was concluded that "[…] the induction of viral hepatitis at the present time cannot be recommended as a therapeutic measure and should not be used as a substitute for x-ray or nitrogen mustard therapy […]" (Hoster *et al*, 1949).

In contrast to such dissatisfying trials, results of current investigations are much more promising.

So far, numerous viruses have been under investigation and administered to patients suffering from various tumor entities. Most importantly, latest trials

demonstrated acceptable, minimal or even no toxicity of any applied virus and safety could therefore be confirmed. According to Russell et al., AdV, Coxsackievirus, HSV, MeV, New castle disease virus, parvovirus, poliovirus, reovirus, Seneca valley virus, retrovirus, different vaccinia viruses and VSV are currently being investigated in phase I, II or III trials for the treatment of various solid tumors (Russell *et al*, 2012).
In the following, selected clinical trials are presented in short.

Onyx-015 is a modified AdV that selectively replicates in and kills cells which harbour p53 mutations, which is often the case in tumor cells. It was the first engineered virus to be used in phase I and II clinical trials for the treatment of various tumors. In fact, therapy was very well tolerated in all cases. However, efficacy of single-agent therapy was disappointing, whereas response to chemotherapy could be enhanced in combination with Onyx-015 (Kirn, 2001; Liu *et al*, 2007). Another variant of AdV named H101 was approved for the treatment of head and neck cancer in combination with chemotherapy by Chinese authorities in 2005 (Garber, 2006). Tumor response could be significantly increased by combinational treatment with viruses and chemo-therapy (Liu *et al*, 2007).

In a phase II clinical trial, patients with unresectable metastatic melanoma were treated with oncolytic HSV type 1. The virus had been genetically engineered to block antigen presentation, to express GM-CSF (vector depicted as T-Vec or Talimogene laherparepvec) in order to enhance the immune response and to selectively replicate in tumor cells. Final data clearly confirmed effectiveness with a 26 % objective response rate and an overall 1-year survival rate of 40 %. Therefore, a phase III study is already ongoing (Senzer *et al*, 2009) and approval of T-Vec by FDA and EMA as a first “Western“ virotherapeutic is to be awaited for 2015.

20 patients with primary or metastatic liver cancer were treated in a phase I trial with a modified vaccinia virus Ankara, bearing prodrug convertases CD and

UPRT (MVA-FCU1). The virus was injected intratumorally with doses of 10^7 to 4 x 10^8 into the tumor and subsequently the prodrug 5-FC was administered. Tumor response and the maximal tolerated dose should be investigated. However, study results have not yet been published (http://www.transgene.fr).

1.4. Armed oncolytic measles viruses

1.4.1. Introduction

In 1971, Bluming and Ziegler from the “Lymphoma Treatment Center of the Uganda Cancer Institute” published a case report on an eight year old boy suffering from a histologically diagnosed Burkitt’s lymphoma at the right orbita. Before any antineoplastic therapy could have been started, the patient had been naturally infected with wild-type MeV. Surprisingly, the orbital swelling disappeared and the boy was in total remission for at least four months after infection (Bluming & Ziegler, 1971).

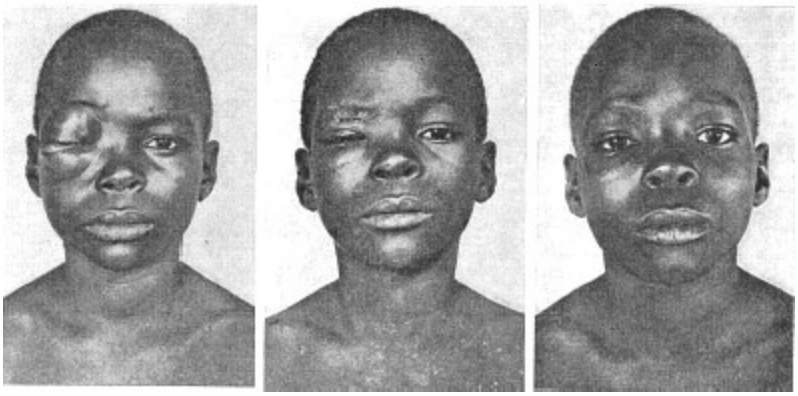


Fig. 1.6 Wildtype measles infection associated with remission of Burkitt’s lymphoma. The first picture was taken on the 1st of December 1970 showing a swelling of the right orbita, histologically diagnosed as Burkitt’s lymphoma. Before the second picture has been taken on the 21st of December 1970, the boy had undergone an infection with wildtype MeV and had then shown a detumescence. On the third picture, taken on the 6th of January 1971, there was neither a sign of the measles exanthema nor of the tumor. Taken from (Bluming & Ziegler, 1971).

However, wildtype measles is still a dangerous disease and may cause severe complications (Schneider-Schaulies & ter Meulen, 2002). In contrast, the vaccine strains are safe (Liu *et al*, 2007; Msaouel *et al*, 2012), but show similar anti-tumor activity though (Anderson *et al*, 2004).

This also applies to the MeV Edmonston vaccine strain, which already revealed to specifically infect and kill tumor cells, while hardly affecting normal

cells making the virus a tumor-selective agent (Anderson *et al*, 2004). Additionally, the vaccine strain indicated to be safe and to be efficiently oncolytic when treating first tumor patients in phase I / II clinical trials (Liu & Kirn, 2008; Galanis *et al*, 2010; Msaouel *et al*, 2012). Patients with subcutaneous T-cell lymphomas (Heinzerling *et al*, 2005) and patients with ovarian carcinoma (Galanis *et al*, 2010) were successfully treated. Therapy of further entities, such as glioblastoma multiforme and multiple myeloma, are under clinical investigation (Lech & Russell, 2010).

1.4.2. Measles virus

Measles is a highly contagious viral disease that is caused by the homonymous measles virus. Before the successful vaccination was introduced, the virus had been responsible for approximately 2.6 million deaths per year (WHO, 2015).

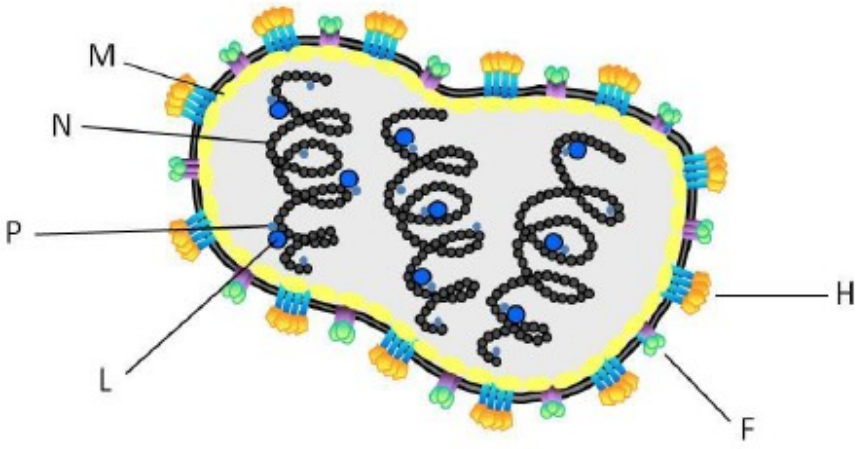


Fig 1.7 Schematic structure of measles virus. Directly underneath the lipid envelope (dark grey) with its embedded F- and H-proteins, the M-protein (yellow) surrounds the ribonucleoparticle, consisting of the P- and L-proteins (both blue) and the negative (-) stranded viral RNA (not shown), associated with the N-protein (black). Modified from (Springfeld, 2005).

The single virions are between 100 and 300 nm of size. From the outside to the center of the virion, there is a phospholipid bilayer envelope, which is derived from budding off the host's cell, containing hemagglutinin (H-protein) and the fusion (F-protein) protein. These integrated proteins are about 9 - 15 nm long (Lamb and Kolakofsky, 2001). The H-protein, a type II glycoprotein, binds to cellular receptors SLAM, Nectin-4 and CD46 and it, together with the F-protein, mediates membrane fusion for primary infection and formation of syncytia (Nussbaum *et al*, 1995).

At the inside of the envelope, the matrix protein (M-protein) surrounds the helical nucleocapsid, which consists of the nucleoprotein (N-protein), phospho-protein (P-protein) and the large protein (L-protein). Together with the MeV nonsegmented, single stranded and negatively orientated RNA, the nucleo-

capsid forms the ribonucleoparticle (RNP) (Griffin, 2001). Proteins V and C are non-structural and are not required for replication (Radecke & Billeter, 1996; Schneider *et al*, 1997).

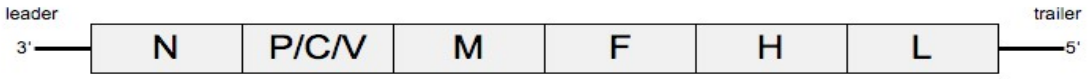


Fig 1.8 Schematic diagram of measles virus' genomic structure. The genes are shown in their exact order, but the sizes are not drawn to scale.

Measles mostly occur during winter and spring and are spread via droplet infection. This highly contagious disease takes about 10 - 14 days of incubation, until symptoms like cough, coryza, fever, a typical maculopapular rash and red spots at the buccal mucosa, called *Koplik's spots*, emerge (Griffin, 2001). The severity of the disease and its symptoms is very much determined by the patient's health status, age and immunocompetence (Schneider-Schaulies & ter Meulen, 2002).

In 1954, T.C. Peebles (1921 - 2010) and J.F. Enders (1897 - 1985) were able to grow MeV on human kidney cell cultures from whole blood and throat washings of an infected 11 years old child called David Edmonston (Enders *et al*, 1957; Griffin, 2001; Msaouel *et al*, 2012). This isolated virus was then named *"Edmonston strain"* or MeV-Edm. By transferring the virus to foreign hosts like chicken embryo cells, it slowly adapted to the new conditions and therefore became attenuated and able to be used as a live vaccine (Griffin, 2001; Msaouel *et al*, 2012), not giving any cause of concern about its safety (Schneider-Schaulies & ter Meulen, 2002; Liu *et al*, 2007). The strain being mostly used for vaccination all over the world obtained licence in 1965 (Griffin, 2001). A reversal so that the virus retrieves its former pathogenic abilities, for example to spread among other individuals, has never been observed or reported so far (Msaouel *et al*, 2012). Moreover, the measles vaccine has been used for about one billion people during the last 40 years, confirming its harmlessness (Msaouel *et al*, 2012). Only patients with a severe immuno-

deficiency (e.g. AIDS) may develop a serious infection and should therefore not be vaccinated (Kaplan *et al*, 1992; Lech & Russell, 2010).

The Edmonston vaccine strain is also very suitable for genetical manipulation, as it can accept huge genetic insertions (> 6 kb) and maintain stability *in vitro* as well as *in vivo*.

1.4.3. Oncolytic characteristics of measles vaccine virus

Malignant cells have defects in their innate antiviral immunity, making them susceptible for viral infections (see section 1.3.3.). In return, MeV-Edm is attenuated in its ability to suppress the IFN induced antiviral state in healthy cells and is therefore easily repelled by untransformed cells. In contrast, tumor cells are just lacking these characteristics und are unable to establish an anti-viral state, which makes them an ideal target for measles vaccine virus.

Another feature of measles vaccine virus is its receptor tropism. CD46 serves as the main receptor for infection and it has been found that, compared to normal cells, most tumor cells overexpress CD46. This might be due to the protective effect of CD46 against mechanisms of complement-mediated lysis (Fishelson *et al*, 2003; Anderson *et al*, 2004), constituting a clear advantage for malignant over normal cells.

This difference in CD46 receptor density makes cancer tissues a suitable target for MeV therapy, in particular for the Edmonston vaccine lineage with its distinct receptor tropism. This way, cancer cells can be selectively infected and lysed, whereas non-malignant cells are relatively unaffected, showing only little and negligible signs of infection (Anderson *et al*, 2004).

Moreover, not only the primary infection rate is increased, but also viral spread via formation of syncytia is enhanced significantly. Neighboring tumor cells which are not infected primarily are then likely to be gathered into cytopathic syncytia, which is also due to overexpression of CD46 (Anderson *et al*, 2004).

Certainly, measles vaccine virus has a clear disadvantage. In contrast to other oncolytic viruses, almost everyone has already had contact to measles (mostly the vaccine) and is therefore likely to have preexisting antibodies. Even low titers would intercept systemically applied virus thus lowering or even completely eliminating the therapeutic agent.

1.4.4. Arming of oncolytic measles vaccine virus

To enhance the oncolytic activity of measles vaccine virus, the virus can be modified genetically. Importantly, the genetic modification of the virus doesn't impair its oncolytic activity (Msaouel *et al*, 2012).

One approach is to modify measles vaccine virus with a gene encoding the soluble marker protein CEA. Measuring its levels can therefore monitor real-time viral gene expression kinetics.

Another possibility to trace local viral spread and replication is to deliver the thyroidal sodium iodide symporter (NIS), a membrane ion channel that transports radioisotopes into cells. Isotopes can therefore be used for detection and also for therapeutical local radiation therapy approaches (Msaouel *et al*, 2009).

In this thesis, an armed MeV virotherapeutic vector encoding a combinational fusion protein of CD and UPRT, called super cytosine deaminase (SCD), was used. (The mechanism of action has already been explained in section 1.3.4.) The gene encoding the SCD is expressed from the leader position at the 3' ending within the measles genome.

Fig. 1.9 Genome of engineered measles vaccine virus MeV-SCD. The gene encoding for the fusion protein super cytosine deaminase (SCD), which has a combined and enhanced activity of cytosine deaminase and uracil phosphoribosyltransferase, is expressed from the leader position at the 3' ending.

1.4.5. Clinical trials with oncolytic measles virus

In the first phase I clinical trial with measles vaccine virus, 5 patients with cutaneous T-cell lymphoma (CTCL) received intratumoral injections of an unmodified measles Edmonston-Zagreb vaccine strain after pretreatment with IFN-γ. CTCL are known to have defects in their IFN signaling and were therefore considered as an ideal target for the oncolytic virotherapy. Pretreatment with IFN-γ should avoid uncontrolled virus spread.
In addition to demonstrating tolerance and safety, four tumors showed a partial response whereas one lesion even resulted in total remission (Heinzerling *et al*, 2005).

In another phase I clinical trial, for the first time an engineered measles vaccine virus was administered. 21 patients with refractory and peritoneally metastasized ovarian cancer were treated with MeV expressing the soluble marker protein CEA (MeV-CEA). The virus was injected intraperitoneally and viral gene expression could be monitored by measuring CEA levels. Safety was confirmed, expression of the CEA marker gene could be detected in some patients and expected median survival has been doubled (Galanis *et al*, 2010).

MeV-CEA is currently also applied in patients with recurrent glioblastoma multiforme. The virus is either administered directly after surgical resection of the tumor (Group A) or 5 days before surgery with an implanted catheter (Group B). Tumors of group B are then resected and virus is readministered. The study is still running and has not yet been completed (http://www.clinicaltrials.gov/ct2/show/NCT00390299).

Patients with refractory or recurrent multiple myeloma are currently treated with MeV-NIS (encoding for a thyroidal sodium iodide symporter) with or without addition of CPA in a clinical phase I trial. Imaging by ^{123}I administration is performed to observe biodistribution and kinetics of viral spread (http://www.clinicaltrials.gov/ct2/show/NCT00450814). In 2014, Russell and

colleagues reported on a durable complete remission of a heavily pretreated multiple myeloma patient after a single-shot intravenous infusion of MeV-NIS (Russell *et al*, 2014). This was the first documented in-human case to prove the so called “oncolytic paradigm” (Russell *et al*, 2012) that systemically administered oncolytic viruses selectively infect disseminated sites of cancer with subsequent tumor-destruction and further detectable replication as self-amplifying biologicals. This hallmark case documents the possibility to cure cancer patients with a single infusion of MeV-based, but circumstances for this specific patient obviously were rather exceptional than being the rule for other cancer patients. For example, multiple myeloma showed to be a tumor entity being very well qualified for oncolytic measles vaccine therapy due to its distinct tumor biology and the patient described in the study has been seronegative for anti-measles antibodies at the onset of virotherapeutic treatment, thereby likely avoiding any rapid neutralization of infused measles vectors.

1.5. Objective

Oncolytic virotherapy with measles vaccine virus has already been demonstrated to be safe. However, early clinical results pointed out the necessity for an enhancement of oncolytic effectiveness of MeV-based virotherapeutics.

In our work, we are developing an armed measles vaccine virus MeV-SCD encoding a suicide fusion gene of yeast cytosine deaminase and uracil phosphoribosyltransferase, converting the non-toxic prodrug 5-FC to the chemotherapeutic drug 5-FU (European patent EP2605783 granted for MeV-SCD on 2015-03-25).

To preclinically investigate how an optimal prodrug-assisted therapeutic regimen could look like, we added 5-FC at different time points after infection with MeV-SCD and either let the prodrug remain in the tumor cell culture medium continuously for different time periods (“continuous” 5-FC application) or applied it only temporarily for defined shorter time periods (“pulsed” 5-FC application). We also varied the time point at which 5-FC was added after infection with MeV-SCD.

To be able to compare direct treatment with 5-FU to our SCD-armed virotherapeutic approach (based on enzymatic conversion of 5-FC to 5-FU), all tumor cell lines were also tested for their “base-line” sensitivity to 5-FU.

In addition, we examined the influence of prodrug addition on viral (MeV-SCD) replication. Treatment with cytostatic compounds such as 5-FU could not only kill tumor cells, but possibly influence replication of virotherapeutic vectors as well. In order to find out whether 5-FC prodrug conversion compounds might exert an impact on virotherapeutic replication, viral growth curves were performed.

2. Material and Methods

2.1. Material

If not described in any other way, all mentioned materials have been used in the highest achievable purity. These were either directly obtained in a sterile state from the cited companies or treated by autoclaving at 2 bar pressure and 121 °C for 20 minutes.

Only water that has been deionized and filtered (H_2O_{dd}) was used for experiments, unless declared differently.

2.1.1. Consumables

Cell scrapers	Corning Inc.
Cell strainer 40 µm	BD Falcon
Combitips 2.5 ml, 12.5 ml	Eppendorf
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, 1250 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 24 well	TPP
Tissue culture plate 96 well	TPP / Corning Inc.

2.1.2. Chemicals

5-Fluorocytosine	Roche
5-Fluorouracil	Pharmaceutical Department, Universitätsklinikum Tübingen
Acetic Acid	Merck
Descosept	Dr. Schuhmacher GmbH
DMSO	AppliChem
Isopropanol (70%)	SAV Liquid Production
Paraformaldehyde (PFA), 4.0 %	Otto Fischar GmbH
Secusept	ECOLAB
Sulforhodamine B	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	
MeV N-Protein NP clone 120 Mouse IgG2	ECACC

2.1.4. Media, Sera and Buffer

DMEM	BIOCHROME
EDTA Trypsin	Lonza
Fetal Calf Serum	Gibco
Opti-MEM	Gibco / Life Technologies
PBS (cell culture use)	PAA
RPMI 1640	PAA
Tween-20	Carl Roth

self-made solutions:

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

COLO205	colorectal adenocarcinoma (Duke's Type D) with ascites metastases and CEA production, taken from a 70 year-old male of Caucasian origin, who has been treated with 5-FU 4-6 weeks prior to cell rescue. Purchased from ATCC (NCI).
HCC2998	colorectal adenocarcinoma
HCT116	colorectal adenocarcinoma
HCT15	colorectal adenocarcinoma
Hep3B	hepatocellular carcinoma, containing hepatitis B virus genome and producing hepatitis B surface antigen (HBsAg) therefore requiring laboratory safety level 2. Cells taken from an 8 year-old, coloured boy purchased from ATCC (NCI).
HT29	colorectal adenocarcinoma, grade 3 taken from a 44 year-old woman
HuCCT-1	human bile duct carcinoma, producing CA19-9 and with ascites metastases taken from a 56 year-old male purchased from Riken Biosource Center
KM12	colorectal adenocarcinoma
SW620	colorectal adenocarcinoma, with metastases, taken

	from a 51 year-old male
Vero	african green monkey kidney cells purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany)

2.1.6. Virus

MeV-SCD	Wolfgang Neubert

2.1.7. Laboratory Equipment

Autoclave 3850 EL	Systec
Centrifuge	Eppendorf, Heraeus
Fluorescence microscope	Olympus
Haemocytometer	Hecht Assistant
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. Safety

The laboratory, in which all experiments have been performed, complies with Biosafety Level 2 of the Directive 2000/54/EC – biological agents at work from the European Parliament from the year 2000. Therefore, working with potentially infectious or hazardous biological particles was performed under a laminar flow hood (HERAsafe®). Afterwards, all materials were thoroughly desinfected, irradiated with UV-light for at least 15 minutes and additionally autoclaved.

2.2.2. Software

Calculations were done with Microsoft Excel 2003 and afterwards depicted and statistically analyzed with GraphPad Prism4 and GraphPad Prism6 (GraphPad Software).

2.2.3. Cell culture

Cells were cultivated in either DMEM or RPMI medium, supplemented with 5% or 10 % fetal calf serum (FCS) and kept in 75 cm^2 tissue culture flasks with vented caps. Flasks were stored in incubators at 37 °C in a humid atmosphere, containing 5 % CO_2. Treatment was done under sterile conditions in a HERAsafe® workbench.

2.2.3.1. General cell culture

To harvest or split cells, they were first washed with warmed (37 °C) PBS and then incubated with EDTA-Trypsin, until the cells detached from the bottom. Trypsin was inactivated with FCS-supplemented medium. Single cell suspensions were generated by properly resuspending with a pipette. If

necessary, the suspension was additionally filtered through a 40 µm cell strainer.

After harvesting the required amount of cells, fresh medium was added and the flask was again stored in the incubator.

2.2.3.2. Counting cells with a haemocytometer

In order to determine the concentration of cells in a suspension, a haemocytometric technique was used. The improved Neubauer counting chamber has engraved squares of different sizes. A large square, consisting of 16 smaller squares, measures 1 mm^2.

To use the Neubauer haemocytometer, the covering glass was put onto the moistened haemocytometer and rubbed carefully along the two elevated glass edges, until so called "Newton rings" appeared. These colours of interference indicate that between the glass edges and the counting chamber, there's only enough space for the light to pass through, which means that this distance can be neglected, whereas the gap between the coverglass and the counting chamber is exactly 0.1 mm big.

When the coverglass was properly fixed, a small amount (approximately 10 µl) was pipetted closely beside the coverglass. The capillary force then soaked the liquid just into the gap.

Using a microscope with a 10x objective, it was possible to count the cells in each large square. The number of cells counted in one square is diluted in a defined volume of 0.1 µl, which results from the size of the square (1 mm^2) and the height of the gap (0.1 mm). The calculated volume equates to exactly 0.1 mm^3, which is 0.1 µl.

Additionally, a colouring chemical could be added to exclude dead cells from the counting. Trypan blue (also Benzamineblue or 3,3′-Dimethyl-4,4′-bis(5-amino-4-hydroxy- 2,7-disulfonaphtyl-3-azo)-[1,1′-biphenyl]) is a diazo dye. Trypan blue allows distinguishing between dead and living cells, as the intact membrane of living cells doesn't let the chemical pass whereas it can enter the

dead cells and colour their protein. Under the microscope, dead cells appear dark blue.

Using this technique, the factor of dilution was considered when counting.

2.2.3. Cryoconservation and thawing

To store cells for a longer time they were cryoconserved at -145 °C. Therefore, the harvested and medium-suspended cells were sedimented by centrifuging at 1200 rpm for 2 minutes. The supernatant was removed and the remaining cell pellet was resuspended in cryomedium (90% RPMI (+20%FCS), 10% dimethyl sulfoxide (DMSO)). After apportioning to several cryotubes of 1 ml each, everything was packed into a styrofoam box or an isopropanol bath to assure a slow cool-down to -80 °C. The following day, the frozen cryotubes were transferred to a -145 °C freezing cabinet.

When thawing single cryotubes to recultivate the frozen cells, they were thawed in a water bath. Cells were added to 9 ml prewarmed medium and centrifuged at 1200 rpm for 2 minutes to remove toxic DMSO. After resuspending the cell pellet in fresh and warmed medium, the cells were transferred into a new tissue culture flask.

2.2.4. Virological methods

2.2.4.1. Infection of cells and application of 5-FC

Cells were seeded in 24 well plates at a density of 3 x 10^4 cells per well and allowed to adhere overnight. On the infection day, the virus was thawed carefully, vortexed and, depending on the needed MOI (multiplicity of infection), diluted in room-tempered Opti-MEM.

The medium was then removed and cells were washed with warmed PBS once. After that, the virus was added to the cells and incubated for 3 hours

at 37 °C. Every 20 minutes, the plates were carefully swayed in order to ensure a sufficient contact of virus to cells.

Three hours post infection (hpi), the inoculum was removed and replaced by warmed medium. If intended, the prodrug 5-FC was either added to the medium before pipetting into each well or directly pipetted into the wells. Importantly, the factor of dilution was considered.

If the prodrug should be added only for a certain period of time (e.g. 6 hour pulse) and taken off afterwards, the medium from the respective well was transferred to an empty plate, which was also stored and incubated under same conditions for the time of prodrug application. When the time had passed, the prodrug containing medium was removed and replaced by the medium which had been set aside.

The advantage of this technique compared to replacing the prodrug-containing medium by fresh medium was clearly to observe as the medium was preserved in each state of consumption and hence not induced proliferation as much as fresh medium does. In addition, no virus was lost.

As 5-FC is light sensitive, the light was switched off while working with the substance.

2.2.4.2. Creating a virus growth curve

In order to determine the viral growth kinetics in an experimental approach, samples were taken at certain time points post infection. Therefore, cells were seeded in 6 well plates at a density of 1 x 10^5 cells per well in 2 ml RPMI + 10 % FCS. On the following day they were infected at an MOI of 0.03 or 0.3 in 1 ml Opti-MEM. 3 hpi, the inoculum was removed and cells were washed three times with warmed PBS to remove all unbound virus. Then 1.5 ml RPMI + 5 % FCS was added. Notably, the medium contained only 5 % FCS, as cellular proliferation shouldn’t be induced too much.

At different time points post infection, samples were taken.
For this purpose, first the supernatant of a suitable well was transferred to a 2 ml reaction tube, then 1 ml room-tempered Opti-MEM was added to gather cells

and nascent virus when scraping the cells off the well's ground. After resuspending properly, the solution was transferred into another 2 ml reaction tube. Samples were then frozen at -80 °C without adding any anti-freezing agent to make the cells burst open and release the intracellular virus.

2.2.4.3. Titration of virus

One day prior to titration, Vero cells were plated in a 96 well plate at a density of 1 x 10^4 cells per well in 200 µl DMEM supplemented with 5 % FCS. If the Vero cells looked healthy and properly attached to the well's ground titration of virus was performed. For this purpose, the samples obtained as described above (2.2.7.2.) were thawed in a waterbath at 37 °C, vortexed for at least 15 seconds and then centrifuged for 2 minutes at 3000 rpm. The supernatant was then used as the virus-containing sample that should be examined.

To identify an unknown concentration of virus, basically a serial of 1:10 dilution of the undefined virus sample is used to infect a for the virus optimal system (Vero cells in case of measles). To prepare the dilution serial, 300 µl supernatant of a centrifuged reaction tube was pipetted to the first well of each 8 well row of an empty 96 well plate. The following 7 wells were filled with 270 µl DMEM + 5 % FCS. By transferring 30 µl from one well to the following, suspending properly and changing pipette tips after each step, a 1:10 serial dilution of each aliquot with 8 different concentrations was obtained.

With a multichannel pipette, each row of dilution was transferred to four rows of a 96-well Vero cell plate with 50 µl for each well. That way, each aliquot with 8 different dilutions was given to prepared Vero cells, so that, if containing virus at its state of dilution, it was able to infect the Vero cells.

After 96 hours of incubation cells were first washed with 200 µl PBS once and then fixed with 50 µl 4 % paraformaldehyde, dissolved in PBS and incubated for ten minutes at room temperature. Following fixation, the plate was again washed with 200 µl PBS twice.

The next step was to block with 100 µl 1 % FCS in TBS-Tween to avoid unspecific binding. This was followed by incubation with an anti-MeV-N protein

antibody, diluted 1:1000 in TBS-Tween for 30 minutes. After 3 steps of washing with TBS-Tween, the secondary antibody goat anti-mouse, also diluted 1:1000 in TBS-Tween, was added. The 30 minutes of incubation with the secondary antibody were performed in the dark to preserve the fluorescent potential and were then followed by another 3 steps of washing with TBS-Tween. At last, the wells were filled up with 100 µl PBS each to avoid drying out.

Using a fluorescence microscope, replication competent MeV infection could be detected and all measles-positive wells identified. As soon as there were more than single cells showing fluorescence, the well was determined as “positive”, whereas isolated single fluorescent signals were seen as replication incompetent measles and therefore excluded. The extent of viral spread and cytopathic effect was not considered.

With these information, the titer could be expressed with the “tissue culture infective dose 50”, describing the amount of virus causing 50 % positive inoculations of all (Condit, 2001). To make the unit more descriptive, it was transformed to pfu / ml. The titer was calculated by the Spearman-Kärber Method (Spearman, 1908; Kärber, 1931).

2.2.5. Microscopy

To control a certain treatment or culturing of cells, these 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 has been used.

2.2.6. Sulforhodamine B Assay

The sulforhodamine B assay (SRB-assay) is a technique to quantify the amount of cells by staining cellular protein unspecifically. Therefore, it is

possible to monitor the cytotoxicity of a given drug or intervention when comparing mock-treated with drug or virus treated cells.

SRB, an aminoxanthene dye with two sulfonic groups, attaches to basic amino acids of cellular proteins under mild acidic conditions.
When dissolved, the coloured cells can be measured photometrically. The colorimetric extinction is linear to the amount of cellular protein (Skehan, 1990).

In order to prepare cells for analysis via SRB-Assay, the medium was removed, the cells were washed with cold PBS (4 °C) and covered with cold TCA. After incubating for at least 30 minutes at 4 °C, the fixed cells were rinsed with tap water four times and dried overnight at 40 °C.
Then cells were stained with SRB solution for 10 minutes and subsequently washed with 1 % acetic acid until the washout became colourless. The plates were again dried and thereafter dissolved in 1000 µl Tris base.
From each well, 80 µl were transferred in duplicates to a 96-well plate. The photometrical extinction was then measured in a microtiter plate reader (Tecan Genios Plus) at a wavelength of 564 nm. Treated cells were compared to the corresponding mock-treated and the relation was given in percent.

3. Results

In order to establish an optimized application scheme for the prodrug 5-FC to treat tumor cells of gastrointestinal origin, which have been infected with an armed oncolytic measles vaccine virus, the following data were collected.

First of all, it was necessary to determine suitable dosages of infections, i.e. multiplicities of infection (MOIs), and appropriate prodrug concentrations for each and every tumor cell line. The two lowest MOIs that were just not able to kill the cells in the course of sole infections (threshold MOIs) were chosen. Additionally, also a threshold concentration of 5-FC moderately enhancing the oncolytic effect was identified. With these information, shown in section 3.1., it was possible to compare different approaches for prodrug application. Basically, a continuous versus a pulsed addition was examined, which is illustrated in sections 3.2. and 3.3. (Yurttas *et al*, 2014).

Secondly, the sensitivity to 5-FU was surveyed, so that differences in response to chemotherapy with 5-FU alone and virotherapy with prodrug conversion could be identified, shown in part 3.4. (Yurttas *et al*, 2014).

Thirdly, the effect of prodrug conversion not only on the tumor cells, but also on virus replication was examined. For this purpose, virus growth curves (section 3.5.) were performed (Yurttas *et al*, 2014).

3.1. Identification of threshold MOIs

To find out which two MOIs of MeV-SCD were suitable for further experiments with each tumor cell line, each of them was infected with ascending virus concentrations (MOIs 0.01, 0.1, 1, and 10). At 3 hours post infection (hpi) medium alone or medium supplemented with 5-FC (0.1 or 1 mM) was added.

At 144 hpi, all tumor cells were fixed for analysis via SRB viability assay.

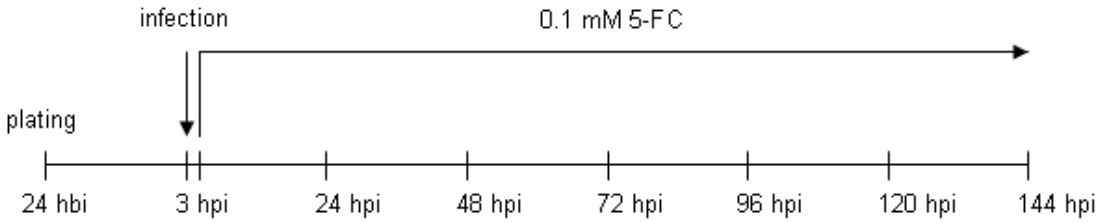


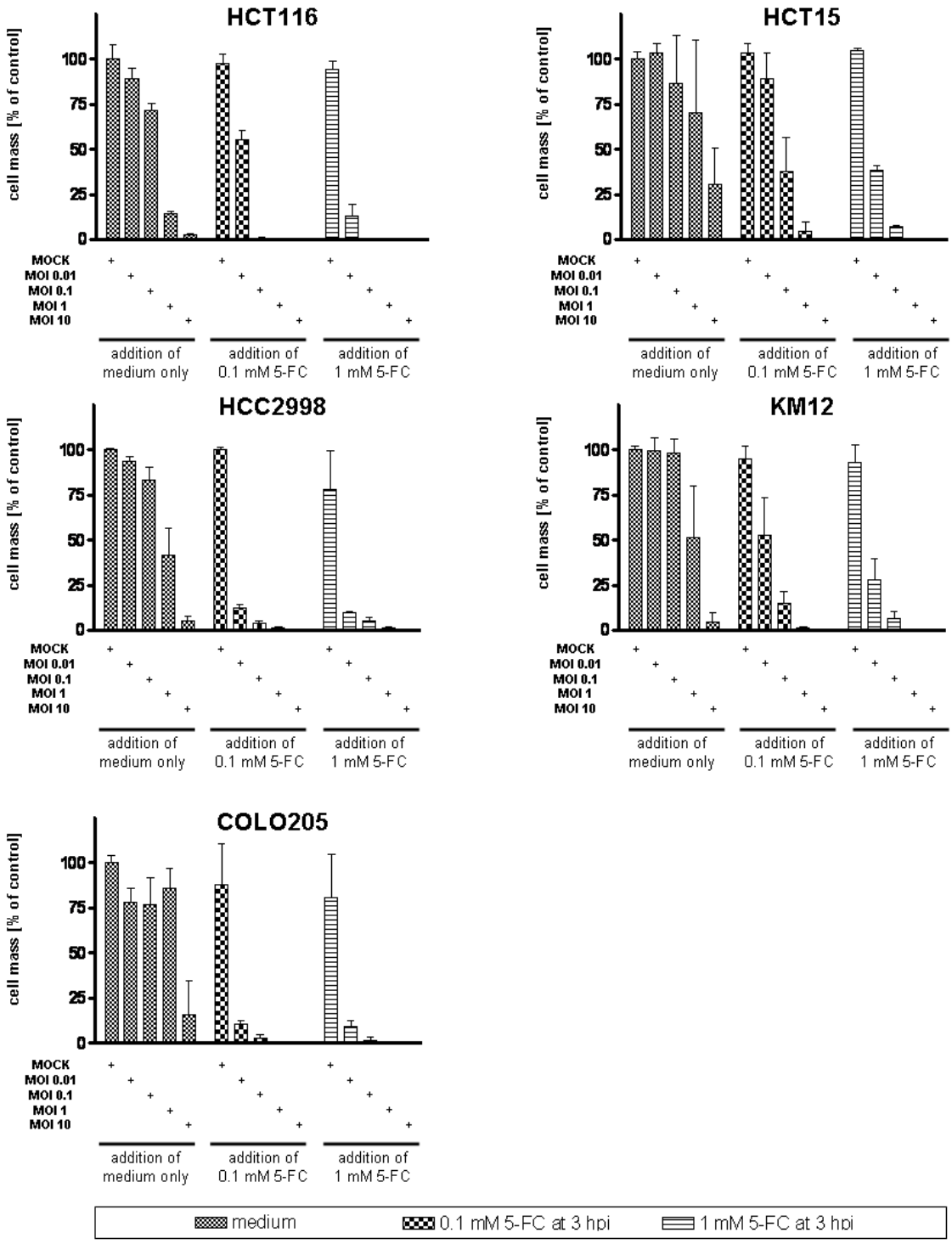
Figure 3.1. Application scheme for the determination of suitable (threshold) MOIs for each tumor cell line. Tumor cells were plated and infected or mock treated the following day. Additional medium with or without 0.1 mM 5-FC was added at 3 hours post infection (hpi) and remained there until the end of the experiment at 144 hpi, followed by quantification of remnant tumor cell masses by SRB viability assay. mM = millimolar, 5-FC = 5-fluorocytosine, hbi = hours before infection, hpi = hours post infection.

As a result, the oncolytic effect of the viral infections was found to be increased when using higher MOIs. Most of the tumor cell lines (except for SW620 and Hep3B) showed little response to the lowest MOI of 0.01. Whereas SW620 and Hep3B cells were almost completely eradicated with increasing MOIs, the other tumor cell lines were less sensitive.

However, addition of the prodrug 5-FC significantly enhanced oncolysis. At a concentration of 1 mM 5-FC the tumor cell mass was reduced already too much, so that even at the lowest MOIs tumor cells were hardly found left over at the end of the experiment. Therefore 0.1 mM 5-FC was used for all further tests.

By purpose, two MOIs to be used in further experiments were defined for each tumor cell line. For tumor cell lines HCT116, HT29, SW620, HCC2998, Hep3B, HuCCT-1, and COLO205, infection with MOI 0.01 alone showed little response but was already effectively killing most tumor cells when adding 0.1 mM 5-FC. For this reason, although not tested in this setting, MOI 0.001 was chosen as the second MOI to be used in the following investigations.

For KM12, MOIs 0.01 and 0.1 were determined and for HCT15, being the least responsive tumor cell line, MOIs 0.1 and 1 were found to be most adequate. All given MOIs and 5-FC concentration are listed in table 3.1.


HCT116
HCT15
HCC2998
KM12
COLO205
cell mass [% of control]
0
25
50
75
100
MOCK
MOI 0.01
MOI 0.1
MOI 1
MOI 10
addition of medium only
addition of 0.1 mM 5-FC
addition of 1 mM 5-FC
medium
0.1 mM 5-FC at 3 hpi
1 mM 5-FC at 3 hpi

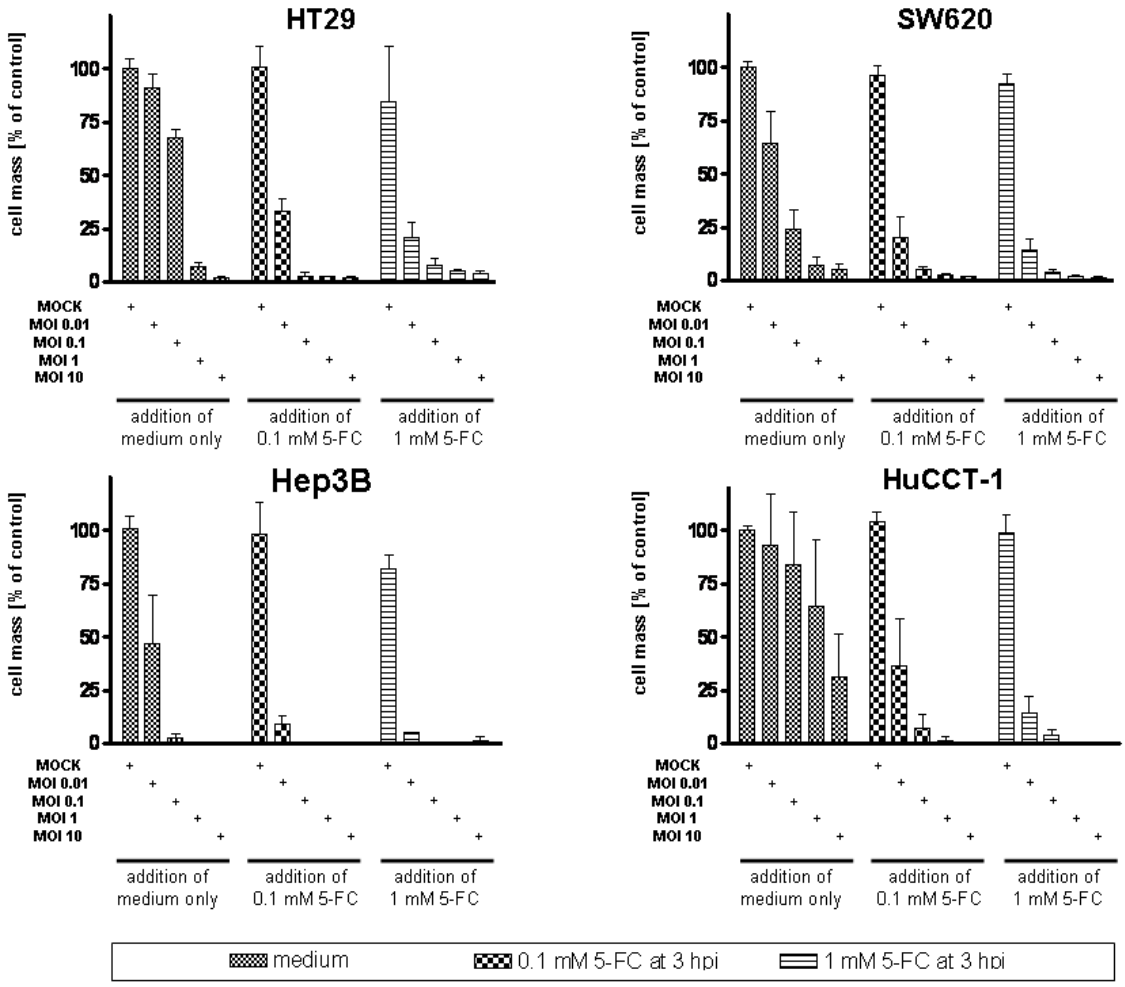


Figure 3.2. Determination of suitable MOIs for each tumor cell line. Tumor cells were mock treated or infected with MeV-SCD at MOI 0.01, 0.1, 1, and 10. Additional medium with or without 0.1 mM 5-FC was added at 3 hpi and remained there until the end of the experiment at 144 hpi; then, all remaining tumor cells were fixed with TCA. Remnant tumor cell mass was quantified via SRB assay. Values: mean of three independent experiments performed in triplicates. Error bars: SEM.

Table 3.1. Results of the pre-test with two suitable MOIs and 5-FC concentrations for each tumor cell line. MOI = multiplicity of infection, 5-FC = 5-fluorocytosine, mM = millimolar.

cell line	MOIs (virus / cell)		5-FC concentration (mM)
HCT116	0.001	0.01	0.1
HT29	0.001	0.01	0.1
HCT15	0.1	1	0.1
HCC2998	0.001	0.01	0.1
COLO205	0.001	0.01	0.1
SW620	0.001	0.01	0.1
KM12	0.01	0.1	0.1
Hep3B	0.001	0.01	0.1
HuCCT-1	0.001	0.01	0.1

3.2. Continuous application of 5-FC

With the findings from the pre-testing depicted in section 3.1., different approaches for the prodrug application could be investigated next. Each tumor cell line was infected with the two afore ascertained MOIs and 0.1 mM 5-FC was added either at 3 hpi, 24 hpi, 48 hpi or 72 hpi. At 144 hpi, all cells were fixed with TCA for analysis. This way, the time point post infection at which prodrug application shows the most effective oncolysis should be identified (Yurttas *et al*, 2014).

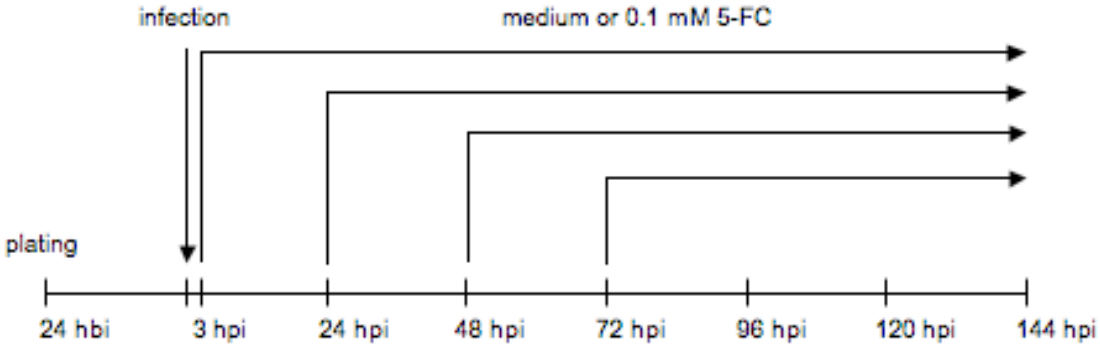
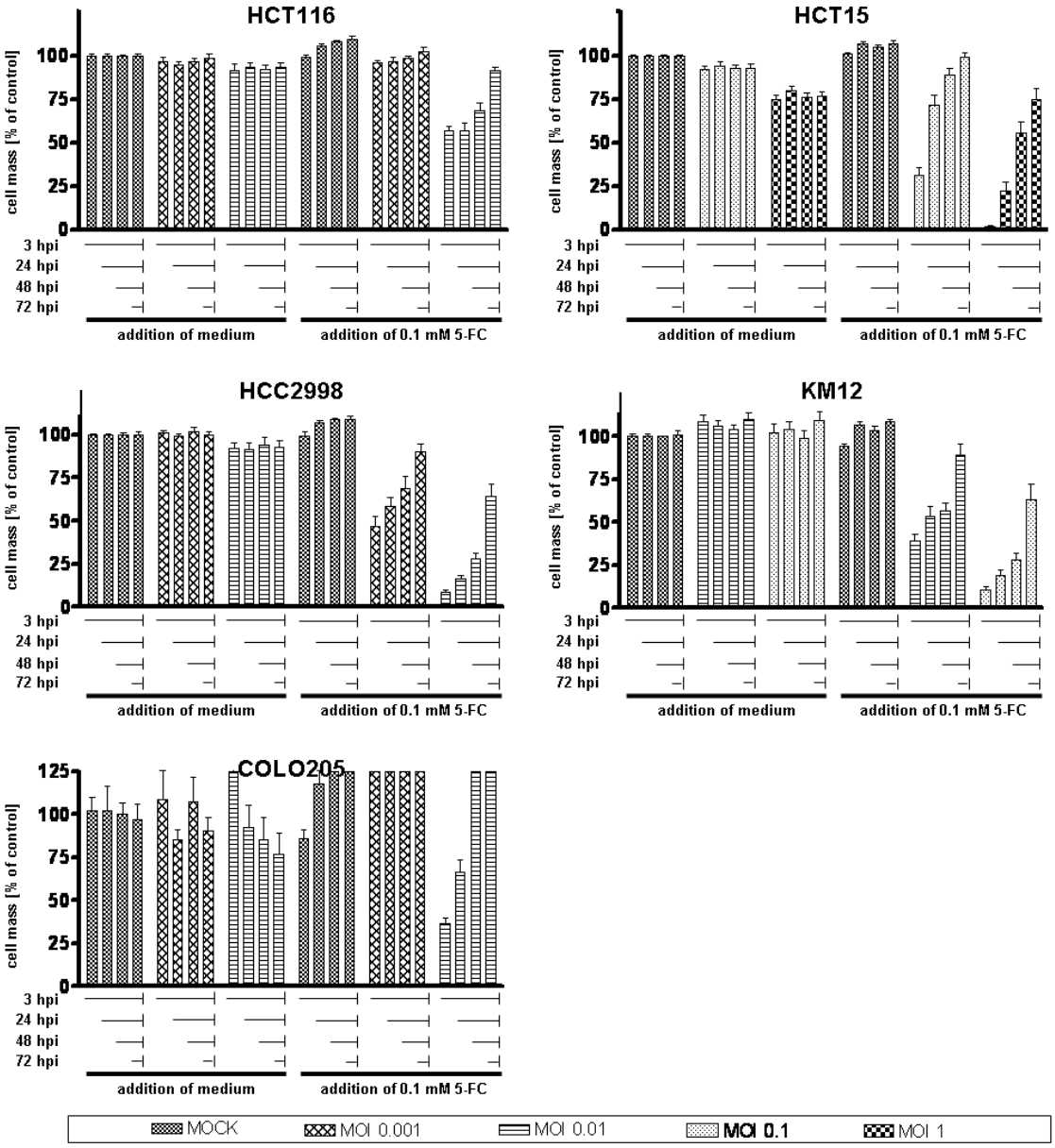


Figure 3.3. Application scheme for the continuous prodrug treatment of MeV-SCD infected tumor cells. Tumor cells were plated and infected or mock treated the following day. Additional medium with or without 0.1 mM 5-FC was added at 3 hpi, 24 hpi, 48 hpi or 72 hpi, respectively, and remained there until the end of the experiment at 144 hpi, followed by quantification of remnant tumor cell masses.


HCT116
HCT15
HCC2998
KM12
COLO205
cell mass [% of control]
3 hpi
24 hpi
48 hpi
72 hpi
addition of medium
addition of 0.1 mM 5-FC
MOCK
MOI 0.001
MOI 0.01
MOI 0.1
MOI 1

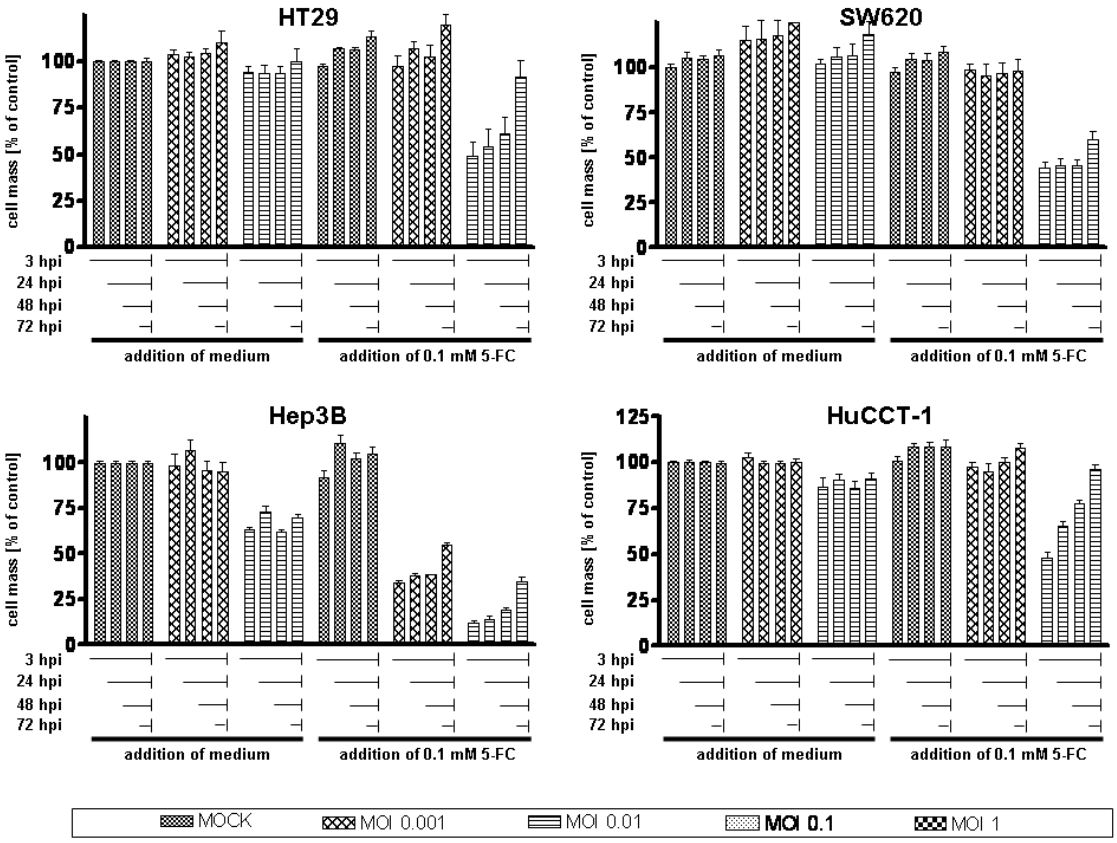


Figure 3.4. Effect of continuous prodrug application. Cells were mock treated or infected with MeV-SCD at MOI 0.01 or 1, respectively. Additional medium with or without 0.1 mM 5-FC was added at 3 hpi, 24 hpi, 48 hpi or 72 hpi (from left to right) and remained there until the end of the experiment at 144 hpi; then, all remaining cells were fixed with TCA. Remnant tumor cell mass was quantified via SRB assay. Values: mean of three independent experiments performed in triplicates. Error bars: SEM. (Yurttas *et al*, 2014)

Each cell line was treated in 6 different groups. The first group, represented by the first 4 columns in each graph, was mock treated and received extra medium without 5-FC at 3 hpi, 24 hpi, 48 hpi or at 72 hpi, respectively. The second and third group were infected with the two MOIs, which had been defined for each cell line. The fourth to sixth group were treated in the same way as the first to third, but received medium containing 0.1 mM 5-FC at 3 hpi, 24 hpi, 48 hpi, or 72 hpi.

The mock treated first group was set to 100 % cell mass. Groups two and three, which were infected with the two corresponding MOIs only, differed

only slightly from the mock treated, which complies with the defined conditions from the pre-test.

Group four was given extra medium with 0.1 mM 5-FC at the mentioned time points only, demonstrating no cytotoxicity. Quite the contrary, most cell lines proliferated marginally.

In contrast, virus and prodrug treated groups five and six clearly demonstrated enhanced oncolysis, compared to the corresponding groups two and three, which were infected without addition of 5-FC. In fact, cell lines HCT116, HT29, COLO205, SW620 and HuCCT-1 hardly responded to infection with corresponding lower MOI and prodrug addition at all, but HCT15, HCC2998, Hep3B and KM12 were already killed efficiently.

Infection with the higher MOI, combined with prodrug application, demonstrated oncolytic activity in all cell lines. Clearly, the time point post infection when 5-FC was added made a difference. The earlier and longer 5-FC was appended, the less the remaining cell mass 144 hpi, compared to mock treated. Whereas treatment of SW620 exhibited to be the least time-dependent, all other cell lines confirmed the observation (Yurttas *et al*, 2014).

However, it was not possible to determine, whether the enhanced effect was due to the duration of incubation with 5-FC only, the time point of prodrug addition only or because of both of them. As all cells were fixed at 144 hpi, 5-FC was not only added at different points of time, but also for variable time periods. As the mock treated wells were already overgrown at 144 hpi and the medium therefore almost used up, it was impossible to prolong incubation periods beyond 144 hpi. To circumvent this problem, a new approach was chosen.

As treatment with the corresponding higher MOI and prodrug addition 3 hpi and 24 hpi showed best results, only these conditions were further investigated. Also, only cell lines HCT116 and HCT15 were used for the following experiments. The cells were infected and 3 hpi or 24 hpi, 5-FC was added. Starting 24 hours after application of the prodrug, every 24 hours until 144 hpi one sample was fixed and analysed by SRB-Assay. This way, a kinetic of the remaining cell mass could be created (Yurttas *et al*, 2014).

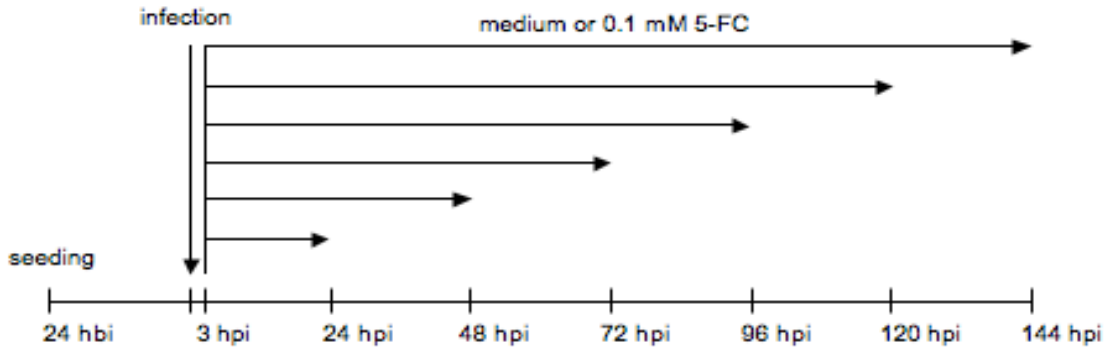


Figure 3.5. Application scheme for the time-dependent effect of continuous prodrug treatment at 3 hpi. Tumor cells were plated and infected or mock treated the following day. Additional medium with or without 0.1 mM 5-FC was added at 3 hpi and remained until the end of the experiment at 24, 48, 72, 96, 120 and 144 hpi. Remnant tumor cell masses were quantified by SRB assay.

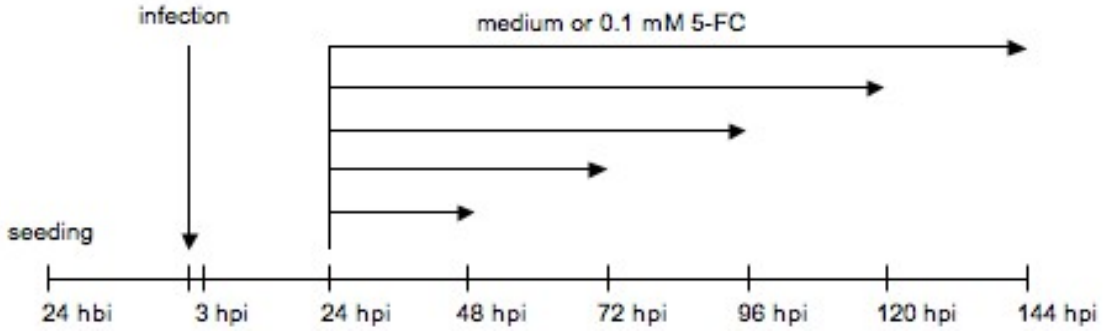


Figure 3.6. Application scheme for the time-dependent effect of continuous prodrug treatment at 24 hpi. Tumor cells were plated and infected or mock treated the following day. Additional medium with or without 0.1 mM 5-FC was added at 24 hpi and remained until the end of the experiment at 48, 72, 96, 120 and 144 hpi. Remnant cell masses were quantified by SRB assay.

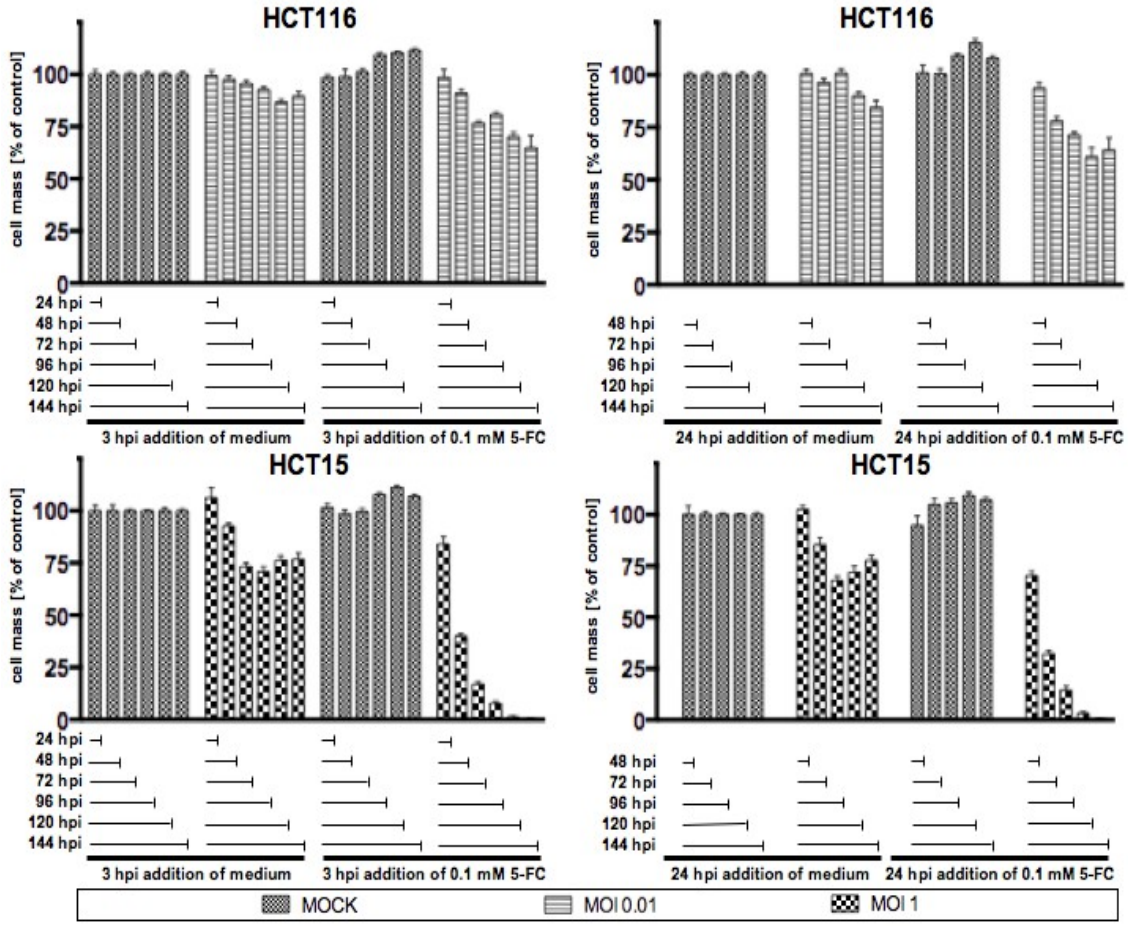


Figure 3.7. Time-dependent effect of continuous prodrug application. Cells were mock treated or infected with MeV-SCD at MOI 0.01 or 1, respectively. Additional medium with or without 0.1 mM 5-FC was added either at 3 hpi (panels to the left) or at 24 hpi (panels to the right) and remained on target cells continuously. Incubations were stopped at 24 hpi (panels to the left: only when 5-FC was added already 3 hpi), or at 48, 72 , 96, 120 and 144 hpi (all panels); remaining cells were fixed with TCA and remnant cell masses were determined by SRB assay. Values: mean of three independent experiments performed in triplicates. Error bars: SEM. (Yurttas *et al*, 2014)

The columns of each group show the remaining cell mass in percent of mock treated developing along the time up to 144 hpi. At every time point, the control was set to 100 %. Virus infection alone caused a constantly decreasing cell mass in HCT116, whereas HCT15 reached a minimum approximately at 72 hpi to 96 hpi, hereon showing increasing cell mass.

Addition of 5-FC at 3 hpi or 24 hpi to mock infected control cells resulted in slightly increased cell mass, as already noticed.

Infected and prodrug treated cells again revealed enhanced oncolysis. Whereas cell mass of HCT116 was constantly reduced to roughly 75 % until 144 hpi, the cellular mass of HCT15 mainly decreased shortly after prodrug addition. Besides, application of 5-FC at 24 hpi led to a comparable effect after 144 hpi as seen when given 3 hpi. The incubation period for 5-FC added at 24 hpi was indeed shorter; however, the relative reduction of cellular mass per 24 hours until 144 hpi was larger, seen especially shortly after addition (Yurttas *et al*, 2014).

To contemplate time points of prodrug application in an isolated manner, data from cells, which were treated with 5-FC for an equal time period, but where treatment was started at different time points (3 hpi, 24 hpi, 48 hpi, or 72 hpi) were compared (Yurttas *et al*, 2014).

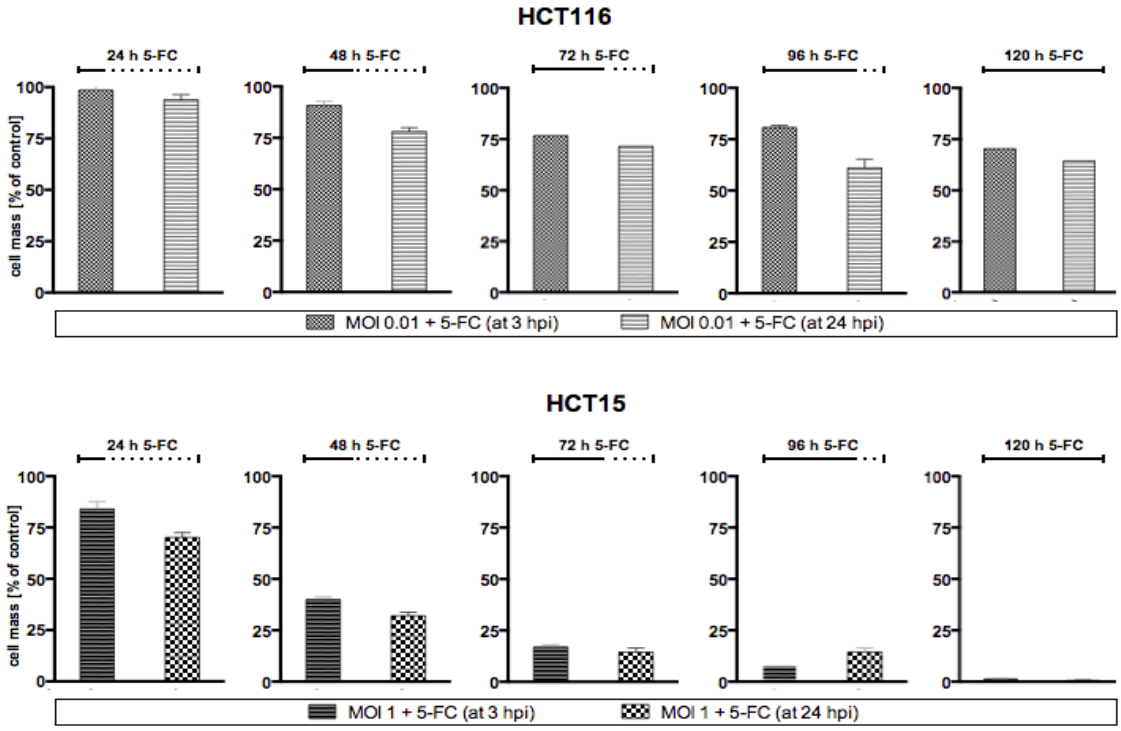


Figure 3.8. Comparison of the oncolytic effectiveness of MeV-SCD infected tumor cells after early (3 hpi) or later (24 hpi) addition of the prodrug 5-FC. 0.1 mM 5-FC was added either at 3 hpi (bars to the left) or at 24 hpi (bars to the right) and incubated for 24, 48, 72, 96 and 120 h. Remnant tumor cell masses were quantified by SRB assay. Values: mean of three independent experiments performed in triplicates. Error bars: SEM. (Yurttas *et al*, 2014)

The cell mass in percent of mock treated of both cell lines was decreased by infection and prodrug application. It was again possible to observe that the longer the incubation period and therefore 5-FC availability, the more striking the oncolytic effect.

However, when comparing different time points of prodrug addition post infection (3 hpi, 24 hpi, 48 hpi and 72 hpi), in case of HCT116, the remaining cell mass of those, which were treated with 5-FC at 24 hpi was always the lowest. HCT15 showed similar results when 5-FC was present for 24, 48 or 72 hours. After 96 and 120 hours, addition of 5-FC at 3 hpi led to a lower cell mass than any other (Yurttas *et al*, 2014).

Taken together, 5-FC seems to be most effective when being available as long as possible. Moreover, prodrug addition at 24 hpi was found to be most effective. When the presence of prodrug was limited to a defined duration, addition at 24 hpi seemed to work best. If there is no time restriction to prodrug availability, the results imply to apply 5-FC earlier and hence longer (Yurttas *et al*, 2014).

3.3. Pulsed application of 5-FC

There were also other options how 5-FC could be administered. One approach was to add 5-FC only for a certain period of time and to remove it afterwards, so that the tumor cells can proliferate and make themselves therefore more susceptible for chemotherapy. Also, periods in which there is no 5-FU available might support viral spread by not killing too many (progeny virus producing) tumor cells in the first place. Possible inhibition of viral replication by 5-FU could be altered by pulsed applications as well (Yurttas *et al*, 2014).

To examine single and multiple pulses of 5-FC, HCT116 and HCT15 tumor cells were treated as described in the following:

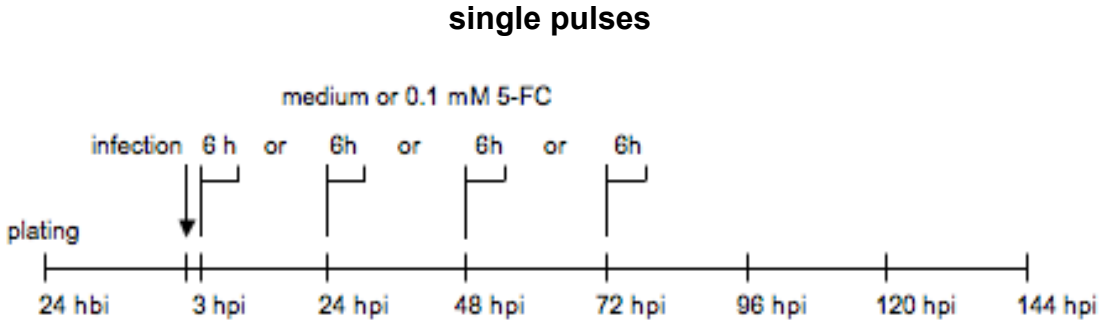


Figure 3.9. Application scheme for single pulsed prodrug treatment of MeV-SCD infected tumor cells. Tumor cells were plated and infected or mock treated the following day. Medium with or without 0.1 mM 5-FC was added at 3 hpi or 24 hpi or 48 hpi or 72 hpi and remained for a defined period of 6 h each. Then, at 144 hpi remaining cells were fixed with TCA. Remnant tumor cell masses were quantified by SRB assay.

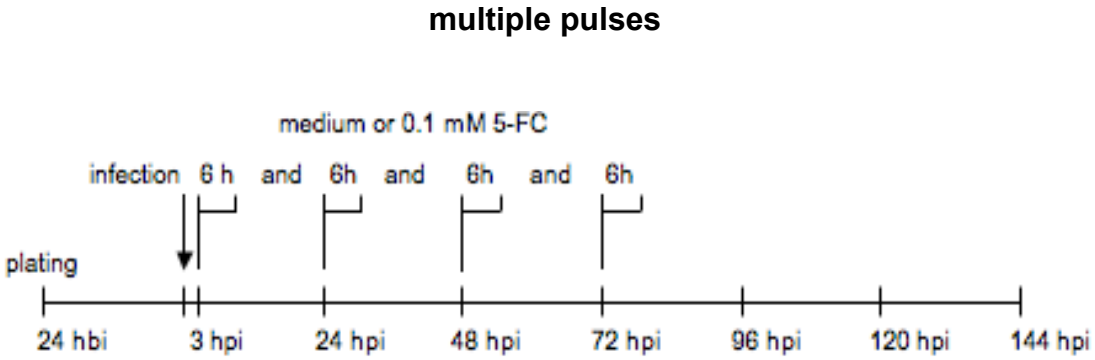


Figure 3.10. Application scheme for multiple pulsed prodrug treatment of MeV-SCD infected tumor cells. Tumor cells were plated and infected or mock treated the following day. Medium with or without 0.1 mM 5-FC was added (i) at 3, 24, 48, and 72 hpi, (ii) at 24, 48, and 72 hpi, (iii) at 24 and 72 hpi, or (iv) at 3 and 48 hpi; each 5-FC pulse remained there for a defined period of 6 h. Then, at 144 hpi remaining cells were fixed with TCA. Remnant cell masses were quantified by SRB assay.

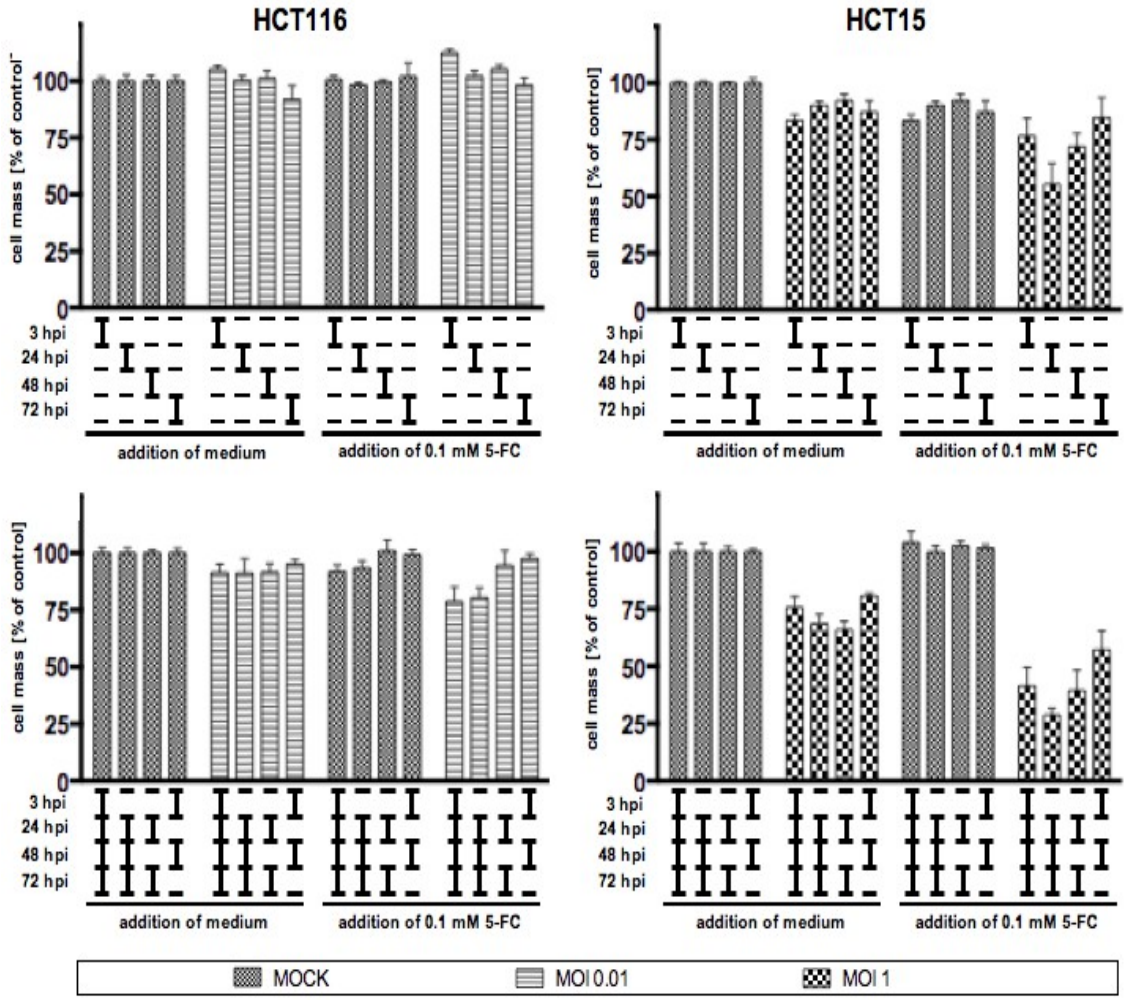


Figure 3.11. Effect of pulsed prodrug application. Tumor cells were mock treated or infected with MeV-SCD at MOI 0.01 or 1, respectively.

Single pulses (upper panels): medium with or without 0.1 mM 5-FC was added at 3 hpi or 24 hpi or at 48 hpi or 72 hpi (from left to right) and remained for a defined period of 6 h each.

Multiple pulses (lower panels): medium with or without 0.1 mM 5-FC was added (i) at 3, 24, 48, and 72 hpi, (ii) at 24, 48 and 72 hpi, (iii) at 24 and 72 hpi, or (iv) at 3 and 48 hpi; each 5-FC pulse remained there for a defined period of 6 h. Then, at 144 hpi remaining cells were fixed with TCA. Remnant tumor cell masses were quantified by SRB assay. Values: mean of three independent experiments performed in triplicates. Error bars: SEM. (Yurttas *et al*, 2014)

Similar to the continuous application of 5-FC as described in section 3.3., the pulsed application consisted of 6 groups. The four columns of each group describe the time points, at which 5-FC has been administered for 6 hours. From left to right, single pulses were added at 3 hpi, 24 hpi, 48 hpi or 72 hpi respectively. Multiple 6 h pulses were applied at 3 hpi, 24 hpi, 48 hpi and 72 hpi

in the first group, in the second at 24 hpi, 48 hpi and 72 hpi. The third group received 6 h of 5-FC at 24 hpi and 72 hpi and the fourth at 3 hpi and 48 hpi (Yurttas *et al*, 2014).

Mock treated cells in group one were set to 100 %. Viral infections with addition of 5-FC free medium showed only slight differences compared to the mock treated control.

Administration of single or multiple pulses of 5-FC to uninfected cells caused no measurable effect. Also, single pulses had no effect on HCT116 tumor cells being infected at MOI 0.001 and 0.01. Even multiple pulses led to only little oncolysis. Merely three or four 6 hour-pulses were able to reduce the cell mass in the range of about 20 %.

In contrast, HCT15 showed more response. Single pulses applied to infected cells (MOI 1) caused a significant tumor cell killing, which was most effective with a 6 hours 5-FC pulse applied at 24 hpi. Multiple pulses to MOI 1 infected HCT15 cells caused even more killing. The effect of four pulses with one pulse each at 3 hpi, 24 hpi, 48 hpi, and 72 hpi led to the same result as three pulses with one pulse each at 24 hpi, 48 hpi, and 72 hpi or 2 pulses given at 24 hpi and 72 hpi. However, two pulses with one being applied at 3 hpi and one at 48 hpi was not as effective as the other approaches.

Taken together, 5-FC pulsing for periods of 6 hours did not show any advantage in reducing the cell mass compared to a continuous application of 5-FC, neither single pulses nor multiple pulses with up to four repeats.

3.4. Sensitivity to 5-FU

To compare single treatment with 5-FU to armed virotherapy with prodrug addition, all tumor cell lines were tested for their sensitivity to the chemotherapeutic drug. Therefore, ascending concentrations from 10^{-6} to 1 mM 5-FU were administered for 72 h, 96 h and 120 h (Yurttas *et al*, 2014).

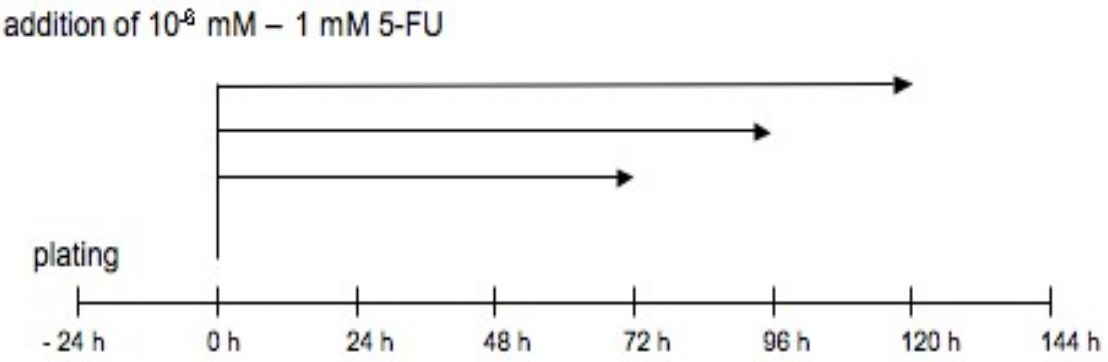
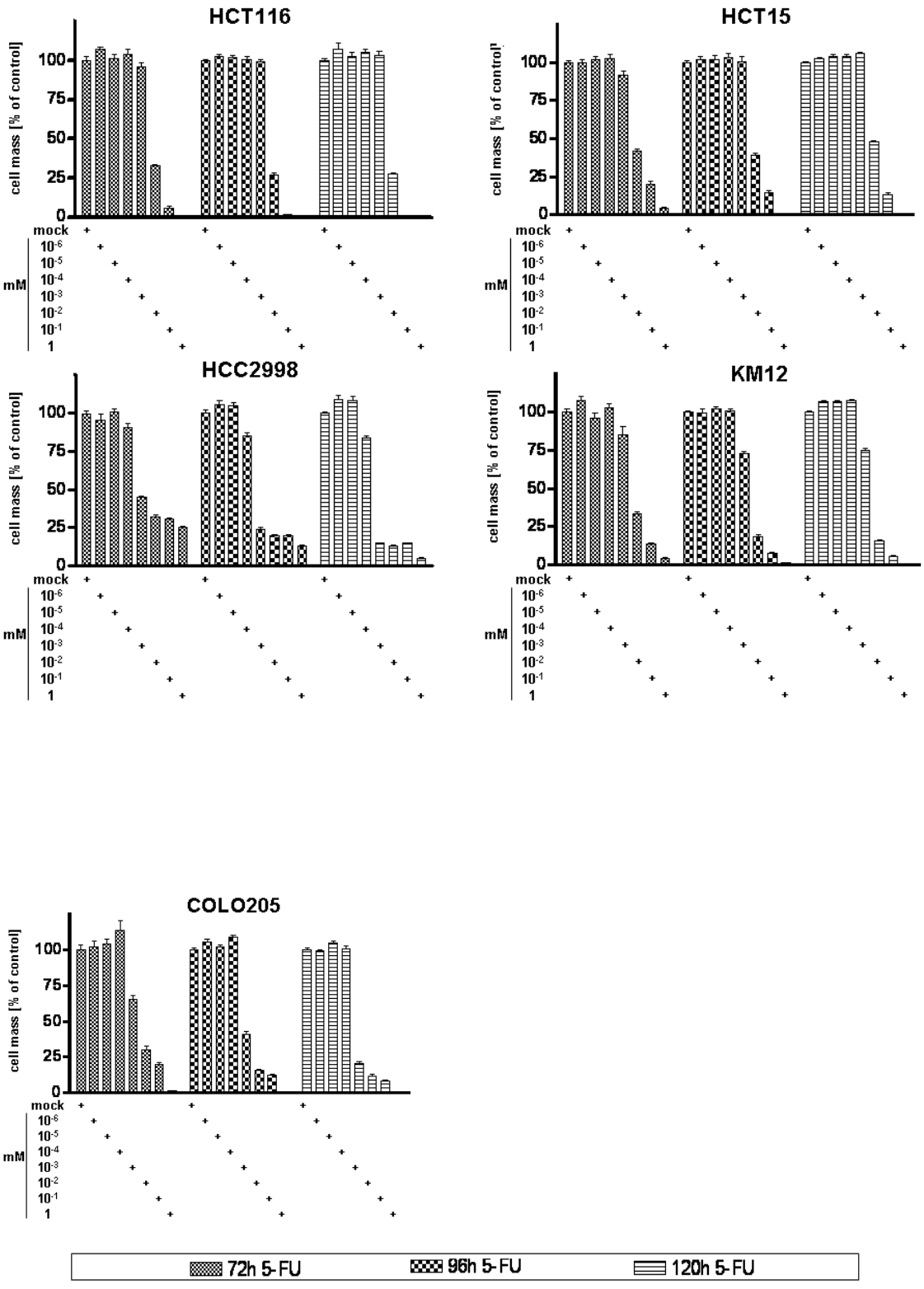


Figure 3.12. Time and dose dependent effects of 5-FU on tumor viabilities. Tumor cells were mock treated or with rising concentrations of 5-FU (10^{-6} - 1 mM, from left to right) and incubated for defined time periods of 72, 96 and 120 h. Remaining tumor cell masses were determined by SRB assays. Values: mean of three independent experiments performed in triplicates. Error bars: SEM.


HCT116
HCT15
HCC2998
KM12
COLO205
cell mass [% of control]
mM
mock
72h 5-FU
96h 5-FU
120h 5-FU

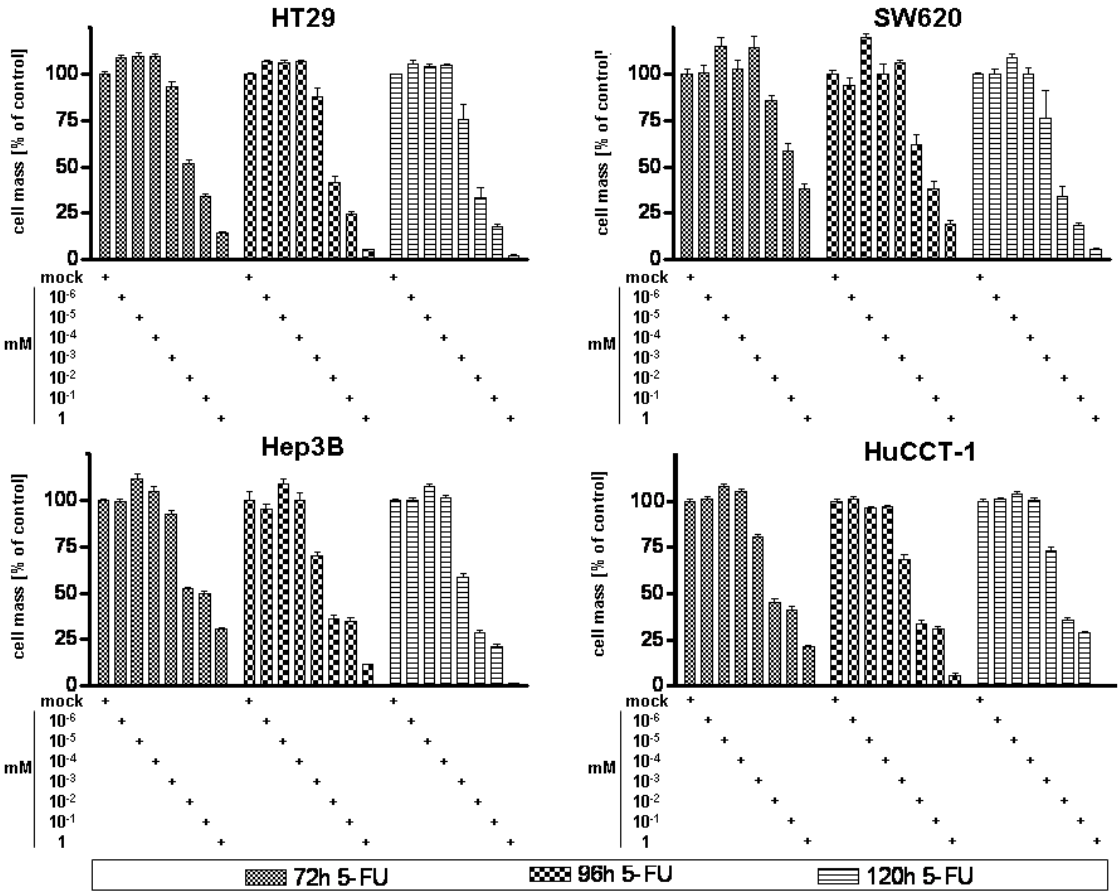


Figure 3.13. Time and dose dependent effects of 5-FU on tumor cell viabilities. Tumor cells were mock treated or with rising concentrations of 5-FU (10^{-6} - 1 mM, from left to right) and incubated for defined time periods of 72, 96 and 120 h. Remaining tumor cell masses were determined by SRB assays. Values: mean of three independent experiments performed in triplicates. Error bars: SEM. (Yurttas *et al*, 2014)

In general, concentrations up to 10^{-4} mM 5-FU only had little or even no effect at all. After 72 h of treatment with 10^{-3} mM, tumor cell lines COLO205, HCC2998, KM12 and HuCCT-1 began to respond to the drug. For cell lines Hep3B, it took 96 h of incubation with 10^{-3} mM 5-FU to cause an effect and 120 h for HT29 and SW620. When adding 5-FU in a concentration of 10^{-2} mM for 72 h, a reduction of cell mass was visible in all cell lines. However, not all of them could be killed more efficiently by extending the duration of treatment significantly. This was for example the case for HCT116 and HCT15. In contrast, all other cell lines did react to a prolongation. By all means, higher

concentrations enhanced cytotoxicity so that 1 mM 5-FU killed tumor cells almost completely (Yurttas *et al*, 2014).

In the following table, for each tumor cell line and for each duration of 5-FU treatment, the lowest concentration of 5-FU is given, which was able to reduce the tumor cell mass to less than 50 %.

Table 3.2. Results of the dose and time dependent effect of 5-FU on tumor cell viability. Shown are the concentrations of 5-FU causing a tumor cell mass reduction of > 50 % when appended for the durations of time given at the top of each column. 5-FU = 5-fluorouracil, mM = milimolar, h = hours.

	concentration of 5-FU (mM) causing cell reduction of >50 %		
	72 h	**96 h**	**120 h**
cell line			
HCT116	10^{-2}	10^{-2}	10^{-2}
HT29	10^{-1}	10^{-2}	10^{-2}
HCT15	10^{-2}	10^{-2}	10^{-2}
HCC2998	10^{-3}	10^{-3}	10^{-3}
COLO205	10^{-2}	10^{-3}	10^{-3}
SW620	1	10^{-1}	10^{-2}
KM12	10^{-2}	10^{-2}	10^{-2}
Hep3B	1	10^{-2}	10^{-2}
HuCCT-1	10^{-2}	10^{-2}	10^{-2}

3.5. Virus growth curves

The cytostatic compound 5-FU possibly not only kills tumor cells, but also could have an influence on viral replication. In order to investigate whether the converted prodrug might inhibit virus production, viral growth curves were performed with cell lines HCT116, HCT15, Hep3B and HuCCT-1. Cells were infected with MOIs of 0.03 and 0.3 respectively and 0.1 mM 5-FC or medium without 5-FC was added at 3 hpi or 24 hpi (Yurttas *et al*, 2014).

The following application scheme explains how the experiment was performed in detail.

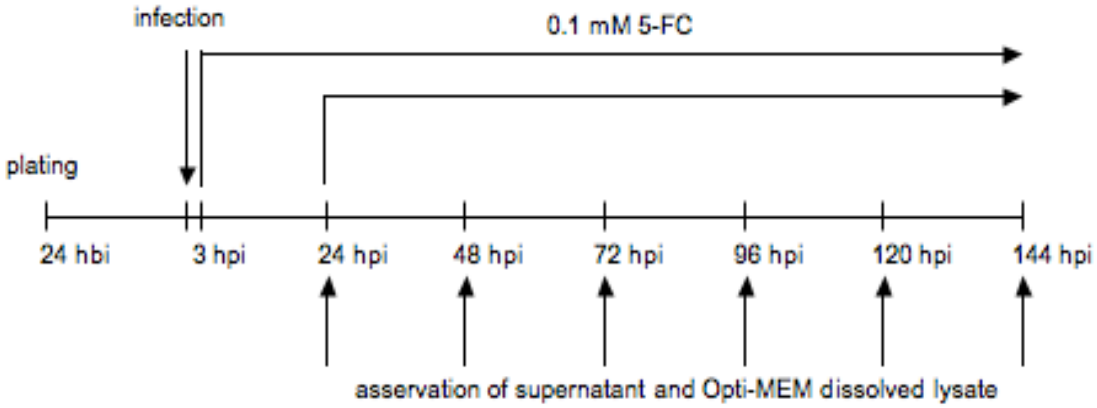


Fig 3.14. Application and harvesting scheme for virus growth curves of MeV-SCD with prodrug addition. Tumor cells were plated and infected the following day. Additional medium with or without 0.1 mM 5-FC was added at 3 hpi or at 24 hpi and remained there until sample taking. Samples of supernatant and Opti-MEM dissolved lysate were taken at the time points shown below the time bar.

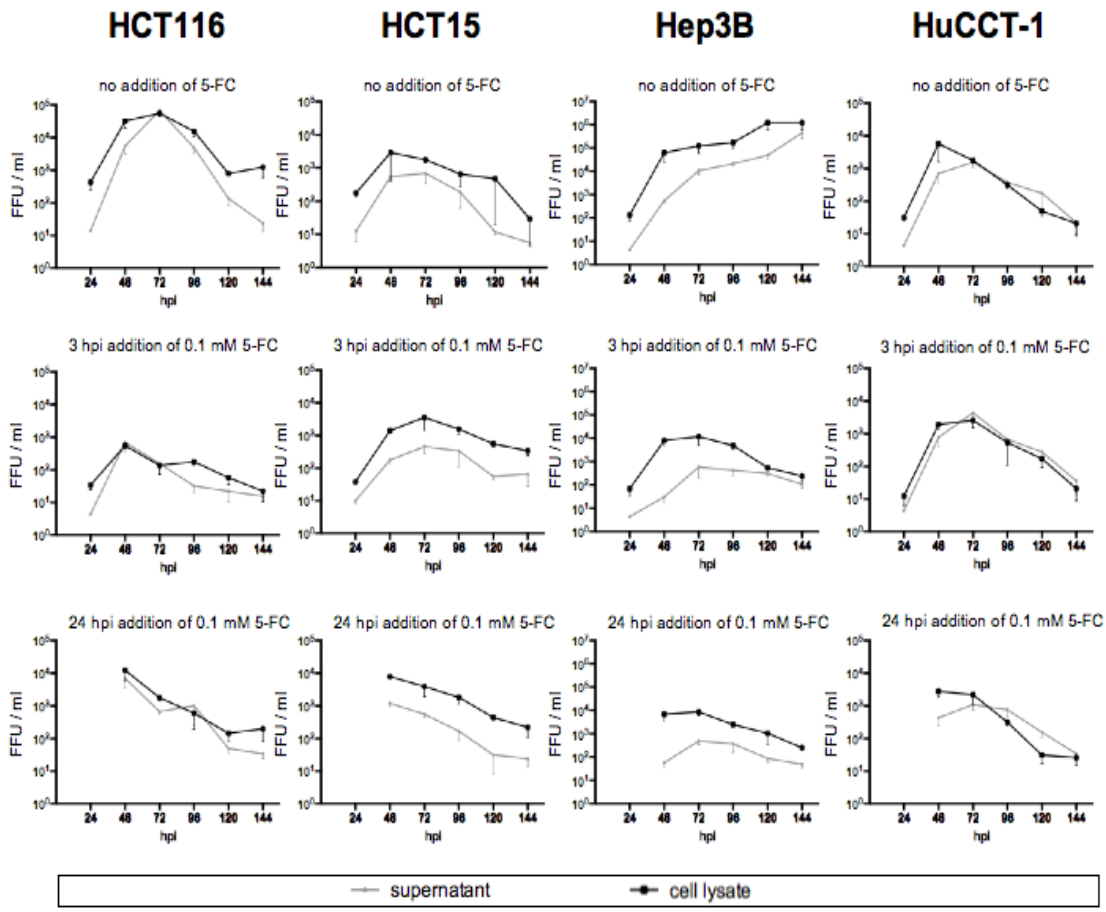


Figure 3.15. Virus growth behavior in dependence of prodrug 5-FC incubation onset and duration. Cells were infected with MeV-SCD at MOI 0.03 (HCT116, Hep3B, HuCCT-1) or 0.3 (HCT15), respectively. Additional medium with or without 0.1 mM 5-FC was added at 3 hpi or at 24 hpi and remained until the end of the experiment at 144 hpi. At 24, 48, 72, 96, 120, and 144 hpi, samples of supernatants and cell lysates were taken and analysed by titration on Vero cells for quantification of the amounts of newly produced progeny virus particles. FFU = fluorescence forming units. Values: mean of three independent experiments. Error bars: SEM. (Yurttas *et al*, 2014)

In all approaches, virus concentration increased in the first 48 hours, in supernatants as well as in the cell lysates.

With regard to results from HCT116, the titer reached a maximum of more than 10^4 pfu / ml in the supernatant and lysate without addition of 5-FC. Hereon after, the titer constantly decreased. Compared to the results in the presence of prodrug, the concentration was partly 10- to 100-fold higher. Whereas the addition of 5-FC at 3 hpi led to lower titers from 24 hpi on, 5-FC

addition at 24 hpi caused a virus reduction at 48 hpi. In general, the concentrations of infectious virus particles being detected within the supernatant and in tumor cell lysates were found to be similar (Yurttas *et al*, 2014).

In contrast, viral titers in the supernatant in all approaches with HCT15 were constantly about 10-fold lower than those in cell lysates. Without addition of 5-FC, the concentrations reached a maximum of 10^3 pfu / ml in the lysate and 10^2 pfu / ml in the supernatant at 48 hpi and later on were found to decrease. The addition of the prodrug at 3 hpi resulted in quite similar viral titers, except for the maximum at 72 hpi and a less abrupt drop then on. The highest viral titers at 48 hpi were reached when 5-FC was added at 24 hpi, consequently ending up in comparable titers to the approach with prodrug addition 3 hpi.

The highest viral titers overall were reached with the tumor cell line Hep3B without addition of 5-FC. The titer constantly increased to a maximum of 10^6 pfu / ml in both, supernatant and lysate at 144 hpi. Although the concentration was about 10-fold lower in all supernatant samples before 144 hpi, final data/yields were found to be almost identical. In combination with 5-FC, regardless of addition at 3 or 24 hpi, the titer was always lower than 10^4 pfu / ml. Concentration within the tumor cell lysate temporarily reached a maximum of 10^4 pfu / ml at 72 hpi and subsequently decreased. Titers in the supernatants were about 10- to 100-fold lower at early time points (24 hpi, 48 hpi, and 72 hpi), but converged at later time points (Yurttas *et al*, 2014).

Viral growth curves of HuCCT-1 were all found to be quite similar. Addition of 5-FC, whether at 3 or at 24 hpi, had no obvious effect on the titer compared to the approach without 5-FC application. Besides, the curves of supernatant and cell lysate are almost identical in all growth curves from HuCCT-1 (Yurttas *et al*, 2014).

3.6. Summary

Taken together, a continuous and early application of 5-FC was found to be most effectively enhancing the oncolytic effect, especially when compared to an intermittent treatment. This was despite the observation, that the prodrug addition caused an inhibitory effect on the generation of infectious progeny virus particles.

4. Discussion

Oncolytic virotherapy is a novel approach for the therapy of cancer. The employed viruses selectively infect and replicate in tumor cells and have therefore no effect on non-malignant cells (Russell *et al*, 2007). Measles vaccine virus also meets with these criteria (Msaouel *et al*, 2009). However, virotherapy when given alone has been shown to be mostly insufficient so far, so that current treatment modalities have to be improved (Liu *et al*, 2006). There are many approaches to enhance the oncolytic effect of administered viruses. One is based on the suicide gene therapy technology, which employs one or more viral, bacterial or fungal genes which allow the conversion of non-toxic compounds (prodrugs) into lethal drugs when being encoded in the respective virus genome (Lal *et al*, 2000). Due to the selective infection of tumor cells, the toxification only takes place within infected tumor cells (Cattaneo *et al*, 2008). Although suicide gene therapy has been used in the context of solid tumors in a large number of preclinical and also some clinical studies (mostly employing adenoviral or vaccinia virotherapeutics in solid cancer patients such as NCT00415454, NCT00583492, NCT00844623, NCT00964756 and NCT00978107), its application to cancer patients so far has not reached any clinical significance (Duarte *et al*, 2012).

One of the current limitations can be referred to the missing knowledge on how to optimally apply the respective prodrugs following application of viro-therapeutic vectors encoding distinct suicide genes. Of note, when looking at the literature, there are not many investigations so far which aim at dissecting which timing of prodrug addition would be optimal in an *in vivo* setting or in a clinical trial when making use of a vector encoded suicide gene function by systemic application of a respective prodrug. However, in order to design a clinical study employing MeV-SCD, an effective dosing scheme for prodrug 5-FC is required and also claimed by German authorities (Paul-Ehrlich Institute, Langen, Germany). Therefore, the aim was to investigate and compare different prodrug application schemes for the treatment of MeV-SCD infected tumor cells and to determine the most effective one.

Accordingly, this *in vitro* study has set out to provide a systematic data basis for the design of potential future prodrug application regimens, focussing on the time for our prototypic measles vaccine virus vector MeV-SCD which encodes a suicide fusion protein consisting of CD and UPRT (Erbs *et al*, 2000; Berchtold *et al*, 2013). Notably, we particularly looked for potential interferences of the prodrug being enzymatically converted into nucleoside analogs thereby potentially impairing virotherapeutic replication.

From the sketch book, there are several possibilities for the design of clinical prodrug application regimens: (i) to start with the prodrug application quite early after the application of the virotherapeutic vector, or (ii) to first let the virotherapeutic vectors replicate for a defined time span before initiating a prodrug-mediated enhanced killing of tumor cells (which unavoidably includes killing of vector producing tumor cells), (iii) to apply the prodrug continuously intravenously, thereby being able to ensure constant plasma levels of the prodrug over time, or (iv) to apply the prodrug repeatedly orally, mostly resulting in a noncontinuous plasma level of the prodrug (e.g., due to permanent renal elimination of the prodrug).
In order to simulate these scenarios *in vitro*, we initiated application of the prodrug 5-FC at different time points post infection and we either let the prodrug remain in the tumor cell culture medium continuously for different time spans (“continuous” 5-FC application) or applied it temporarily for defined shorter periods of time (“pulsed” 5-FC application), using changes of the culture medium at defined time points (Yurttas *et al*, 2014).

1. The enhanced oncolytic effect by prodrug addition is dependent on the time point and duration of prodrug application

Numerous studies dealt with the enhancement of oncolytic effectiveness by arming viruses with prodrug convertases. Especially AdV, HSV, VV and MeV were equipped with CD, UPRT, CD in combination with UPRT, TK or purine nucleoside phosphorylase (PNP). All of these studies reported an improved rate

of oncolysis both under *in vitro* and *in vivo* conditions. At the same time however, their results posed the question at which time prodrug addition might yield the best anti-tumoral effects.
Wildner et al treated mice bearing s.c. melanoma or cervical carcinoma with an armed AdV. Mice received intratumoral injections of AdV-TK, followed by 7 days of GCV application. Their survival was prolonged significantly, but the outcome of GCV addition being initiated at 24 hpi or at 72 hpi differed largely, showing that prodrug application beginning at 72 hpi was far more effective (Wildner *et al*, 1999).
Another group studied the ability of AdV, armed with UPRT, to infect and replicate in mice xenograft models of peritoneally disseminated biliary cancer. Treatment with 5-FU was found to be beneficial when administered 10 or 15 days after infection. Any earlier addition erased the virus and in consequence did not prolong survival. It was thus concluded that prodrug timing is a highly crucial factor (Seo *et al*, 2005).
Similar observations were made by Ungerechts et al, who armed MeV with PNP to convert fludarabine phosphate into its active metabolite 2-fluoroadenine, which interferes with DNA, RNA and protein synthesis. In a lymphoma xenograft model, MeV-PNP effectively infected tumor cells. Prodrug addition however only enhanced tumor cell killing when added not too early after infection (< 24 hpi), otherwise viral replication and spread were found to be inhibited significantly (Ungerechts *et al*, 2007).
These authors assumed that prodrug addition at the peak of convertase expression would be most beneficial. However, in a clinical scenario it would not be easy to determine the peak of convertase expression in a non-invasive manner.

To determine an optimal prodrug application scheme, in this thesis multiple tumor cell lines were infected with MeV-SCD and the prodrug 5-FC was added at different time points (3 hpi, 24 hpi, 48 hpi or 72 hpi) and remained continuously until the end of the experiment at 144 hpi. As a result, this experimental setting showed much higher effectiveness the earlier the prodrug

was applied (Yurttas *et al*, 2014). However, it was not possible to ascribe the effects to the earlier administration only as prodrug availability differed as well. Therefore, it was necessary to examine the conditions separately. Cell lines HCT116 and HCT15 were infected and treated with 5-FC from 3 or 24 hpi on and incubated for 24, 48, 72, 96 or 120 h. The results of prodrug addition at 3 or 24 hpi but for the same time periods were compared. As a result, incubation with 5-FC from 24 hpi on proved to be more effective compared to application from 3 hpi on (Yurttas *et al*, 2014).

A specific investigation of prodrug timing in coordination with viral gene expression was also performed by Yamada et al. HSV-1 was armed with pro-drug convertases CD and UPRT and used to infect human and mouse colon cancer cell lines *in vitro* and in a xenograft mouse colon cancer model. The *in vitro* data showed that tumor cell killing was the more effective the earlier 5-FC was administered after infection.
These data were quite similar to the results of the continuous prodrug application in this thesis. The most effective time point for addition of 5-FC was found to be the earliest as well (Yurttas *et al*, 2014). However, in the experimental approach of Yamada et al, it was not possible to distinguish whether the effect was due to an earlier or longer availability of the prodrug or a combination of both. These authors determined tumor cell killing at 5 days post infection. In contrast, in this thesis, tumor cell killing was determined at 6 days after infection, in order to provide more time for the read out of the different effects finally leading to tumor cell killing (i.e. high level replication of the study virus in tumor cells as well as suicide gene-mediated effects).
To separately examine the effect of prodrug addition at different time points in this thesis, the incubation period after prodrug application was kept the same. With this experimental setting, it became obvious that the increased effectiveness was due to a longer, but not to an earlier availability of the prodrug 5-FC. Tumor cell killing after prodrug addition at 24 hpi compared to addition at 3 hpi was found to be more effective, when comparing the same duration of prodrug availability (Yurttas *et al*, 2014).

These results are also in line with the assumption that prodrug conversion is most effective when viral gene expression (here: suicide gene expression) is at its height.
The *in vivo* data of Yamada et al confirm this: in the first experimental setting, 5-FC was added for 6 days at different time points post infection (0 days, 3 days, 6 days). Inhibition of tumor growth was most effective when 5-FC was added 3 days post infection. With this finding, the next group was treated for different time periods (6, 12 or 18 days) but from the same time point on (3 days post infection). Enhanced efficacy was achieved with a period of 12 days, whereas longer treatment did not improve the outcome anymore. Consistent with the assumption that prodrug treatment should be started when the expression of the prodrug convertase is at its peak, viral titers and DNA copy numbers were found to be highest on the third day (Yamada *et al*, 2012).
In this thesis, the most effective time point to start with prodrug treatment was found to be at 24 hpi (Yurttas *et al*, 2014), whereas Yamada et al showed that *in vivo* infection of mouse colon cancer with HSV-CD-UPRT takes 3 days for maximal gene expression; therefore, prodrug application was initiated at 3 days post virotherapy by these authors.

All these observations indicate that optimal prodrug timing depends on the individual setting of employed virus and treated tumor entity, but that prodrug application probably yields its maximal effect when administered at the peak of prodrug convertase expression (Seo *et al*, 2005; Ungerechts *et al*, 2007; Yamada *et al*, 2012).
Hence, future investigations and also clinical trials employing suicide gene encoding viruses should consider to monitor viral replication to individually determine the maximum of viral gene expression and replication and therefore the optimal time point of prodrug administration. Possible approaches could be to utilize viruses expressing soluble marker proteins (e.g. MeV-CEA) in addition to their prodrug convertase, being then used as surrogate parameters indicating the optimal time point for prodrug addition.

2. A pulsed prodrug application did not demonstrate any advantage over a continuous prodrug treatment

The idea of a pulsed 5-FC application was to let tumor cells proliferate first in the absence of 5-FU (being generated by conversion of the prodrug 5-FC), so that they could be hit in their highly vulnerable cell cycling phase by pulses of high 5-FU concentrations. A bigger percentage of tumor cells was therefore thought to be killed. Furthermore, a pulsed prodrug application was considered also to possibly enhance viral replication and thus overall effectiveness.

To investigate these hypotheses, tumor cell lines HCT116 and HCT15 were infected with MeV-SCD and 5-FC was administered at different time points (3 hpi, 24 hpi, 48 hpi or 72 hpi) and remained for a period of 6 hours in the culture medium. These prodrug pulses were either appended once or up to four times (Yurttas *et al*, 2014).

However, the results clearly showed that a pulsed application is not superior to an early and continuous prodrug treatment (Yurttas *et al*, 2014). This might be due to various reasons: prodrug convertase expressing tumor cells were shown to produce far higher 5-FU concentrations after prodrug application than achievable by systemic 5-FU therapy, thus being unable to elude the effect of 5-FU (Richard *et al*, 2007). A pulsed application to ensure proliferation of as many cancer cells as possible would therefore not be required to sufficiently kill them.

A second reason might be that the 6 hours pulses were too short to achieve the necessary effect. It could be investigated, whether pulses of 12 or 24 hours are more effective than a continuous application.

Finally, the removal of culture medium after each pulse could lead to the loss of a crucial percentage of diluted virus thus decreasing the number of progeny viruses and therefore further infectious and oncolytic particles (Yurttas *et al*, 2014).

Certainly, a continuous prodrug application enhances the oncolytic virotherapy significantly. Chalikonda et al treated human ovarian tumor cells in a xenograft model with vaccinia virus, armed with CD. A continuous application of 5-FC enhanced the efficacy at every time point, compared to the infection alone (Chalikonda *et al*, 2008).
The effectiveness of continuous prodrug availability was also observed by Yamada et al, who showed that HSV-TK persisted for at least 2 weeks intratumorally after infection with a constantly detectable expression of transgenes (Yamada *et al*, 2012). Hence, the continuous expression of prodrug convertases could efficiently convert continuously administered prodrugs. Similar conclusions were drawn by Tai et al and Lu et al. They used a replicating retrovirus (RRV) genetically modified to express CD and performed infections in a mouse model of HCC. Tumors revealed a long-term persistence and stable expression of RRV, indicating that a continuous application of a prodrug could result in a continuous conversion of the prodrug over time (Tai *et al*, 2010; Lu *et al*, 2012). Finally, also Foloppe et al were able to detect expression of CD up to 30 days in xenograft mouse models of colon cancer after systemic delivery of a vaccinia virus encoding CD and UPRT (Foloppe *et al*, 2008).

Taken together, the stable expression of suicide transgenes after infection and the ability to achieve far higher concentrations of converted prodrug within the tumor cells support the approach to continuously administer the prodrug. Application schedules to assure cellular proliferation or that the majority of cells is in a specific phase of the cell cycle are apparently not necessary to sufficiently enhance the oncolytic effect. On this basis, the design of future clinical trials is made much easier (Yurttas *et al*, 2014).

3. Prodrug addition enhances oncolysis but has an inhibitory effect on the generation of infectious progeny virus particles

The enhanced oncolytic effect by prodrug addition could be confirmed in numerous studies and also in this thesis (Yurttas *et al*, 2014). At the same time

however, some authors reported about an inhibitory effect on viral replication by prodrug conversion.

In this thesis, it was not possible to make a general statement about the inhibitory effect of converted 5-FC on viral replication and spread of MeV-SCD. In cell lines HCT116 and Hep3B, a decrease in viral titers was obvious, whereas in cell lines HCT15 and HuCCT-1 only moderate or even no effects on virus replication were visible. It was not clear, whether the virus titers measured in HCT116 and Hep3B tumor cells were lower because of (i) a direct inhibition of viral replication or because (ii) the prodrug addition enhanced oncolysis and therefore withdrew the virus' breeding grounds. In contrast to the first assumption, viral titers in HuCCT-1 tumor cells were found to be constant, although oncolysis was found to be enhanced by prodrug addition. A production of 5-FU may therefore be assumed and should have had an influence on viral replication, if 5-FU was really inhibitory. What speaks against the second hypothesis is that HCT116 tumor cells were killed only moderately after prodrug addition but showed an obvious drop of viral titers, whereas the cell mass of HCT15 tumor cells was reduced to a much larger extent, but concentrations of virus were not found to be altered (Yurttas *et al*, 2014).
To separately investigate whether converted 5-FC has an influence on MeV-SCD, it would have been necessary to enable viral infection in a host cell in the simultaneous presence of 5-FU without lysis of the host cell by neither 5-FU nor viral replication.

Seo et al, who used AdV-UPRT and 5-FU for the treatment of disseminated biliary cancer, noticed an inhibitory effect on adenoviral expression at even sub-toxic doses of 5-FU (Seo *et al*, 2005). In another study, replication of AdV-CD was suppressed in melanoma cells when 5-FC was added at 0, 24 or 48 hpi, but it could not be decided whether the effect was directly inhibitory on viral replication or indirectly by effectively killing the virus host cells (Liu & Deisseroth, 2006). The observations were not only restricted to effects of 5-FC

prodrug conversion to 5-FU, but also TK-armed AdV titers in glioma models were lowered after addition of GCV (Nanda *et al*, 2001).
Similar results were shown by Bernt et al when administering high doses of 5-FU (> 0.25 mM) to AdV-CD-UPRT infected colon and cervix carcinoma cells. In contrast, they noticed increased viral titers after administration of low 5-FU doses (0.25 - 50 µM) to *in vitro* infected tumor cells and also enhanced viral spread in liver metastases after infection and prodrug addition (Bernt *et al*, 2002).

Comparable with the results of Bernt et al, treatment of pancreatic cancer cell lines with HSV in combination with 5-FU *in vitro* increased viral progeny production in a dose dependent fashion (1 - 5 µM 5-FU). Possible explanations suppose that chemotherapeutic stress makes the host cell more susceptible to viral infection and therefore may contribute to increased viral replication (Eisenberg *et al*, 2005).
In contrast to inhibitory effects of prodrug application on AdV, investigations with HSV-CD (Nakamura *et al*, 2001) and HSV-CD-UPRT (Simpson *et al*, 2006; Simpson *et al*, 2012; Yamada *et al*, 2012) revealed that 5-FC addition directly after infection had only minimal effects on viral replication. However, if metabolites of converted prodrug 5-FC were added at the time of infection, viral replication was found to be suppressed significantly (Simpson *et al*, 2012). These data indicate that the prodrug does not inhibit early HSV replication when added from the beginning, but that its activated metabolites indeed lower viral titers.

Studies on CD-armed VV were all consistent with the finding that converted 5-FC, dependent on the dose and expression of prodrug convertase, caused a dramatic decrease in replication of vaccinia virus. Observations were made by McCart et al, when treating murine colon carcinoma *in vitro* and *in vivo* (McCart *et al*, 2000). Similar results were obtained with VV-CD and 5-FC by Chalikonda et al and Foloppe et al (Chalikonda *et al*, 2008; Foloppe *et al,* 2008). *In vitro* infection of human colon carcinoma cells with VV-CD and treatment with 5-FC

directly after infection or at 48 hpi showed that viral titers were decreased 500-fold, when 5-FC was added directly and 35-fold when administered at 48 hpi (Foloppe *et al*, 2008).

Replication of MeV-PNP was shown to be totally arrested after *in vitro* infections of Vero cells, when the prodrug was administered directly after infection and lowered 1000-fold when added at 12 or 24 hpi. These results were confirmed by *in vivo* experiments: intracranial MeV-PNP administration without prodrug addition caused lethal meningoencephalitis in mice, whereas prodrug addition from 24 hpi on avoided disease development. It was therefore concluded that early prodrug availability could control viral replication and spread (Ungerechts *et al*, 2007).

It is possible that the inhibitory effect of converted 5-FC on MeV-SCD replication is not as noticeable as reported in studies with other viruses, because MeV is an RNA virus, which could be less affected by the inhibitory effects of 5-FU metabolites than DNA viruses (AdV, HSV, VV). In fact, none of the studies published so far employed an RNA virus with CD and reported about an inhibition of virus replication after prodrug administration.

Combining all those results suggests that numbers of progeny viruses are somehow lower, when high concentrations of cyctostatics (such as 5-FU and its metabolites) are available, whereas relatively low doses could possibly even enhance viral replication and spread. This might be due to induced susceptibility of tumor cells to viral infection and replication (Eisenberg *et al*, 2005) or to a fostering of the release and dissemination of viruses from their host tumor cells (Foloppe *et al*, 2008). Nonetheless, despite the lowered number of viral particles, oncolysis is significantly enhanced by high-dose prodrug addition, if not applied too early. Ideally, administration starts, when the expression of prodrug convertases is at its peak, as any earlier addition could decrease infection, replication and suicide gene-expression, thus lowering the effectiveness of such suicide gene-armed approaches.

4. Translating the *in vitro* study results to potential clinical settings

The findings from the *in vitro* experiments clearly demand further *in vivo* investigations to determine when and how to ideally administer the prodrug after virotherapeutic infection. If there is the possibility for a continuous intravenous application of 5-FC (here simulated by our “continuous” 5-FC application regimens), our results would imply that tumor cell killing is the more efficient the longer the overall time span of the 5-FC presence is (Fig. 1); if 5-FC steady-state plasma levels can be maintained only for limited time spans (maybe due to practical reasons) it seems to be preferable to initiate the addition of 5-FC at 24 hpi and not at 3 hpi (Fig. 2); (2) when applying 5-FC orally (here simulated by our 6 hours “ON” pulses of 5-FC), addition of 5-FC at 24 hpi seems to be superior over a much earlier application at 3 hpi (Fig. 3) (Yurttas *et al*, 2014).

So far, there is no full publication on the clinical quantification of concentrations of prodrug conversion products (such as 5-FU) in plasma and/or in malignant tissues of study patients with solid tumors (Table 1). This for example is true for study NCT00415454 (“Phase I Study Combining Replication-Competent Adenovirus-Mediated Suicide Gene Therapy With Chemoradiotherapy for the Treatment of Non-Metastatic Pancreatic Adenocarcinoma”) in which a single intratumoral injection of an adenoviral vector encoding suicide genes CD and HSV-TK was followed by a 3 week period of (oral) 5-FC + valganciclovir (VGCV) prodrug therapy, which began at 72 hpi.

In the study “Phase I Trial of Replication-competent Adenovirus-mediated Suicide Gene Therapy Combined with IMRT for Prostate Cancer” the same adenoviral vector was employed via a single intraprostatic injection on day 1 (Freytag *et al*, 2007). Three days later, patients (n=9) were administered 2.6 weeks (13 days, weekdays only) of 5-FC (150 mg/kg/day; given orally in four equally divided doses every 6 hours) and VGCV (1800 mg/day; given orally in two equally divided doses every 12 hours) prodrug therapy. As some patients (cohort 3 of this study) were scheduled to receive a second adenovirus injection

on day 22, 13 days rather than 15 days of prodrug application were used in this regime to preclude the possibility that the first prodrug therapy cycle would inhibit viral replication following the second adenovirus injection (see our own discussion above).

In the subsequent phase II study (n=280) NCT00583492 (“A Randomized, Controlled Trial of Replication-Competent Adenovirus-Mediated Suicide Gene Therapy in Combination With IMRT Versus IMRT Alone for the Treatment of Newly-Diagnosed Prostate Cancer With an Intermediate Risk Profile”) the same adenoviral vector was employed again via a single intraprostatic injection; in this case, two days later (day 3), patients received a two week course (10 days, weekdays only) of (oral) 5-FC and VGCV prodrug therapy (Lu *et al*, 2011).

Unfortunately, neither in the phase I nor in the phase II study was any reporting on the determination of concentrations of prodrug conversion products (such as 5-FU) in plasma and/or in malignant tissues of the study patients exhibiting newly-diagnosed prostate cancer.

In a phase I trial on hepatocellular carcinoma (NCT00844623; “Phase I Clinical Trial Of Gene Therapy For Hepatocellular Carcinoma By Intratumoral Injection Of TK99UN (An Adenoviral Vector Containing The Thymidine Kinase (TK) Of Herpes Simplex Virus”) five consecutive cohorts of two patients received increasing doses of an HSV-TK suicide gene encoding adenoviral vector by intratumoral injection; subsequently, equal doses of either intravenous GCV or oral VGCV were applied; albeit no specific information on dosages or duration of prodrug therapies was provided.

In a further phase I study on ovarian cancer (NCT00964756; “A Phase I Study of AD5.SSTR/TK.RGD; A Tropism Modified Adenovirus Vector for Intra-peritoneal Delivery of Therapeutic Genes and Additional Capability of Non-invasive Imaging of Gene Transfer in Patients With Recurrent Ovarian and Other Selected Gynecologic Cancers (Infectivity Enhanced Adenoviral Vectors

for Ovarian CA)") patients were treated intraperitoneally for 3 days with an HSV-TK suicide gene encoding adenoviral vector followed by intravenous application of GCV (5 mg/kg BID; at a constant rate for more than 1 hour) for 14 days (Kim *et al*, 2012).

In another phase I study in patients with head and neck cancer and other malignant tumors, single intratumoral injections of an adenoviral vector encoding HSV-TK were performed on day 1 followed by systemic (intravenous) administration of GCV (50 mg/kg BID) from days 2 to 15 (Xu *et al*, 2009). Again, no data were provided on prodrug plasma levels or prodrug conversion rates.

In a most recent phase I trial, a modified vaccinia virus Ankara vector encoding the FCU-1 suicide gene (TG4023) was employed in patients exhibiting primary or metastatic unresectable liver tumors via a single intratumoral injection followed by a 2-week dosing period of the 5-FC prodrug (200 mg/kg/day; given intravenously for the first days (not specified exactly), then orally) (NCT0097810*).* Severe adverse events being related to the application of 5-FC were identified as diarrhea, hypertension, and alkaline phosphatase increase. Most interestingly, in this study, a first time comprehensive analysis on plasma and tumor 5-FC and 5-FU concentrations was performed. As a result, 5-FU concentrations were reported to be 56±30 ng/g in tumor biopsies and 1.9± 2.6 ng/mL in plasma at day 8. Thereby, a first time proof-of-concept of the *in vivo* conversion of the prodrug 5-FC into 5-FU was obtained in liver cancer patients. This hallmark clinical study paves the way for further clinical trials employing state-of-the-art virotherapeutic vectors armed with potent suicide genes, such as MeV-SCD.

In this context, our own study on “pulsed" versus "continuous" application of the prodrug 5-FC for enhancing oncolytic effectiveness of a measles vaccine virus armed with a suicide gene is of great help in designing the respective study protocols, especially when the timing and duration of the prodrug 5-FC have to be fixed (Yurttas *et al*, 2014).

5. Summary

Oncolytic virotherapy is a novel approach for the therapy of cancer that stems from the observation that tumor patients who simultaneously developed a viral infection would occasionally experience a complete remission of their disease. Meanwhile, different viruses have been preclinically and clinically investigated for the treatment of various neoplastic diseases. These viruses are characterized by their ability to selectively infect and replicate in tumor cells while sparing non-cancerous and normal cells. To further enhance the natural and innate oncolytic potency of those viruses, there are approaches to arm such viruses by incorporation of therapeutic transgenes via genetical modification.

One such concept is the complementation of the virus genome with the genetic sequence of a combinational prodrug-converting enzyme of cytosine deaminase and uracil phosphoribosyltransferase (MeV-SCD) that toxifies the prodrug 5-FC to the highly potent chemotherapeutic compound 5-FU. The fact that tumor cells are infected by virotherapeutics and that the suicide genes of the respective virotherapeutics are expressed in tumors selectively enables an additional local and highly concentrated chemotherapeutic treatment. Although this system has been shown to efficiently enhance the basic virus-mediated oncolytic effect, to date optimization of prodrug application schedules has never been undertaken.

The aim of this thesis was to utilize a suicide gene-armed measles vaccine virus for oncolytic virotherapy and to compare different application schemes ("PULSED" versus "CONTINUOUS") to identify the conditions being required for an optimal tumor treatment (Yurttas *et al*, 2014).

After having chosen suitable amounts of virus for each tumor cell line according to the results of an extensive pre-testing, the following treatment plans were investigated: 5-FC was applied at different time points after infection with armed measles vaccine virus and the prodrug remained (i) either continuously for different time periods ("continuous" application of 5-FC) or (ii)

only temporarily for defined shorter periods ("pulsed" application of 5-FC) in the tumor cell culture medium (Yurttas *et al*, 2014).

The results clearly indicate that a continuous presence of the prodrug 5-FC is most effective in terms of the anti-tumoral effect and that this regime is much superior to any pulsed application of 5-FC (Yurttas *et al*, 2014).

However, prodrug addition was found to exert an inhibitory effect on viral replication, but it is yet not clear whether viral titers were lowered by inhibition of viral replication directly or indirectly by enhancing oncolysis and therefore detracting the virus' host cells (needed for the production of progeny virus particles). Despite this inhibitory effect on generating viral progeny particles, an early and continuous addition of 5-FC was found to enhance oncolysis significantly (Yurttas *et al*, 2014).

Knowingly, our experimental *in-vitro* setting is naturally highly artificial and hence not directly convertible to clinical practice. We therefore consider our results as tentative evidence for designing clinical application regimes with a timely defined virotherapeutic treatment of MeV-SCD and the prodrug 5-FC, which we plan to hand in to the responsible German authorities (Paul-Ehrlich-Institute, Langen) for approval. Data of a corresponding clinical phase I study will then give further insight into security and antitumor efficiency of this innovative and highly promising approach.

5. Zusammenfassung

Die onkolytische Virotherapie stellt einen neuen Therapieansatz dar, der seinen Ursprung in gut dokumentierten Beobachtungen von Krebspatienten hat, die nach einer sich zufällig zeitgleich zur Tumorerkrankung ereignenden Virusinfektion eine komplette Remission ihrer Erkrankung erfuhren.

Mittlerweile wurden verschiedene Virusarten präklinisch und klinisch für die Behandlung unterschiedlicher Tumorerkrankungen untersucht. Diese Viren zeichnen sich durch ihre Eigenschaft aus, Tumorzellen selektiv zu infizieren und sich in ihnen rasant zu vermehren, was innerhalb kurzer Zeit zu deren virusvermittelter Zerstörung ("Onkolyse") führt. Gesunde Zellen bleiben dagegen unbetroffen. Um die onkolytische Wirksamkeit dieser Viren noch zusätzlich zu verbessern, gibt es Ansätze, durch Insertion von Fremdgenen in das Virus-Genom (sog. "Armierung") eine weiter verbesserte Tumorzellabtötung zu erreichen. Ein solcher Ansatz kann beispielsweise in der Armierung mit einem Fusionsgen bestehen, das für eine Enzymkombination aus Cytosindeaminase und Uracilphosphoribosyltransferase (sog. "SCD-Suizidenzym") kodiert, die das Pro-Pharmakon (sog. "Prodrug") 5-FC in das hochpotente Chemotherapeutikum 5-FU umwandelt. Die Tatsache, dass die Infektion und Suizidgen-Expression selektiv Tumorzellen trifft, ermöglicht somit eine zusätzliche lokale Hochdosis-Chemotherapie ohne begleitende sytemische Nebenwirkungen. Obwohl gezeigt wurde, dass diese Armierungs-Methode den onkolytischen Effekt signifikant verstärkt, wurde bisher kein optimales Dosierungsschema auf der Basis einer systematischen Variation der Applikationsbedingungen erarbeitet.

Das Ziel dieser Arbeit war es dementsprechend, Suizidgen-verstärkte Masernimpfviren für die onkolytische Virotherapie einzusetzen, verschiedene Regime der Pro-Pharmakongabe zu vergleichen und im Resultat ein optimales Anwendungsschema zu ermitteln (Yurttas *et al*, 2014).

Nachdem in einem umfangreichen Vorversuch für jede Tumor-Zelllinie eine für Folgeversuche jeweils am besten geeignete Virusmenge ermittelt wurde, konnten die entworfenen Behandlungspläne untersucht werden: 5-FC wurde zu verschiedenen Zeitpunkten nach der Infektion mit den Suizidgen-ver-

stärkten Masernimpfviren zugegeben und das Pro-Pharmakon blieb entweder kontinuierlich und für unterschiedliche Zeitspannen (kontinuierliche 5-FC Gabe) oder nur temporär und für definierte, kurze Zeitspannen (gepulste 5-FC Gabe) auf den Tumorzellen. Die Ergebnisse zeigen, dass eine kontinuierliche Pro-Pharmakongabe die effektivste anti-tumorale Wirkung bewirkt und einer gepulsten Applikationsweise deutlich überlegen ist (Yurttas *et al*, 2014).

Es wurde jedoch weiterhin festgestellt, dass die Pro-Pharmakonzugabe einen unterdrückenden Effekt auf die Virusvermehrung hat. Allerdings ist bislang nicht erkenntlich, ob die erniedrigten Virustiter unter Einwirkung von 5-FC durch direkte Hemmung der Virusvermehrung oder indirekt durch die verstärkte Onkolyse und damit durch den Entzug von Wirtszellmasse für das Virus zustande kommt. Interessanterweise zeigte eine frühe und kontinuierliche 5-FC Zugabe trotz des blockierenden Effekts auf die Bildung von Virusnachkommen eine signifikante Verstärkung des onkolytischen Effekts (Yurttas *et al*, 2014).

Unser experimenteller *in vitro*-Ansatz ist wohlwissend von Natur aus sehr künstlich und damit nicht direkt auf die klinische Anwendung übertragbar. Wir betrachten unsere Ergebnisse daher als vorläufige Anhaltspunkte, um nachfolgend in Kooperation mit einem Sponsor aus der biopharmazeutischen Industrie ein entsprechendes klinisches Anwendungsregime mit zeitlich optimal getakteter Behandlung mit unserem Virotherapeutikum MeV-SCD und dem Prodrug-Medikament 5-FC zu erarbeiten. Wir planen außerdem, dieses der zuständigen Bundesoberbehörde (Paul-Ehrlich-Institut) zur Genehmigung vorzulegen. Die Daten einer entsprechenden ersten klinischen Phase I-Studie werden dann weiteren Aufschluss über Sicherheit und anti-tumorale Effizienz dieses sehr vielversprechenden Ansatzes erbringen.

5. Perspectives

Oncolytic virotherapy with measles vaccine virus has already been proved to be safe and efficient. Insufficient virotherapeutic approaches can be enhanced by arming with a prodrug-converting enzyme. This thesis aimed to investigate and compare different prodrug application schemes to identify an optimal treatment plan for the novel approach of suicide gene-enhanced virotherapy.

Following infection of tumor cells with our prototypic suicide gene-enhanced vector MeV-SCD (European patent EP2605783 granted for MeV-SCD on 2015-03-25), it could be shown that a continuous and long-term availability of 5-FC yields superior oncolytic effects compared to any pulsed application of 5-FC. Further studies with cell lines of other tumor entities could be performed in the same way to investigate whether these findings are conferrable also to other solid tumors. Additionally, to also gather further data on patterns of *in vivo* bio-distribution, a suitable xenograft model should be chosen to examine and, if applicable, confirm these data.

With reference to the assumption of other authors that prodrug addition might be most effective when applied at the peak of converting-enzyme (here: SCD) expression, an appropriate method to track virus replication and spread should be utilized. One possible approach would be to use a modified MeV vector that expresses soluble marker proteins (e.g. MeV-CEA) or a radionuclide trans-porting protein (e.g. MeV-NIS) to adjust timing of prodrug addition with viral spread and suicide gene expression (termed as “peak replication guided suicide gene virotherapy”).

Although the pulsed application of 5-FC did not show any advantage over a continuous treatment (Yurttas *et al*, 2014), the application scheme could be varied and other conditions investigated. A prolonged pulse-period of 12 or 24

hours might have a beneficial effect. Quantities of multiple pulses could also be increased.

Growth curves indicated that prodrug addition and conversion had an inhibitory effect on the generation of viral progenies (Yurttas *et al*, 2014). However, it was not evident, whether this was due to a direct or indirect suppression of virus growth. It would hence be important to make these possibilities distinguishable. A possible yet difficult approach would be to infect a tumor cell line, which is resistant to both viral oncolysis and the cytostatic effects of 5-FU, but allows viral replication and gene expression. Addition of 5-FC in such a scenario then could help to read out its specific influence on viral replication.

6. Appendix

6.1. Abbreviations

5-FC	5-fluorocytosine
5-FdUDP	5-fluoro-2'-deoxyuridine-5'-diphosphate
5-FdUMP	5-fluoro-2'-deoxyuridine-5'-monophosphate
5-FdUTP	5-fluoro-2'-deoxyuridine-5'-triphosphate
5-FU	5-fluorouracil
5-FUDP	5-fluorouridine-5'-diphosphate
5-FUMP	5-fluorouridine-5'-monophosphate
5-FUTP	5-fluorouridine-5'-triphosphate
ACV	aciclovir
AdV	adenovirus
AIDS	acquired immune deficiency syndrome
CD	cytosine deaminase
CEA	carcinoembryonic antigen
CPA	cyclophosphamide
CRC	colorectal cancer
CTCL	cutaneous T-cell lymphoma
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	dimethylesulfoxide
EDTA	ethylendiamintetraacetate
F-Protein	fusion protein
FCS	fetal calf serum
FFU	fluorescence forming units
GCV	ganciclovir
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
hpi	hours post infection
H-Protein	hemagglutinin protein

HSV	herpes simplex virus
ICTV	International Committee on Taxonomy of Viruses
IFN	interferon
GM-CSF	granulocyte macrophage colony-stimulating factor
HPLC	high-performance liquid chromatography
IJA	idiopathic juvenile arthritis
ITP	idiopathic thrombocytopenic purpura
L-Protein	large protein
M-Protein	matrix protein
MeV	measles virus
MeV-CEA	measles virus expressing carcinoembryonic antigen
MeV-Edm	measles virus Edmonston strain
MeV-SCD	measles virus expressing super cytosine deaminase
MOI	multiplicity of infection
NASH	non-alcoholic steatohepatitis
NIS	sodium jodid symporter
NK cells	natural killer cells
N-Protein	nucleoprotein
OPRT	orotate phosphoribosyltransferase
P-Protein	phosphoprotein
PBS	phosphate buffered saline
PFA	paraformaldehyde
RNA	ribonucleoacid
RNP	ribonucleoparticle
rpm	rounds per minute
RPMI	Rosswell Park Memorial Institute
RRV	replicating retrovirus
SCD	super cytosine deaminase
SLAM	signaling lymphocyte activation molecule
SRB	sulforhodamine B
SSPE	subacute sclerosing panencephalitis
TBS	TRIS-buffered saline

TCA	trichloroacetate
TRIS	tris(hydroxymethyl)-aminomethane
TS	thymidylate synthase
UMP	uracil monophosphate
uPAR	urokinase-type plasminogen activator receptor
UPRT	uracil phosphoribosyltransferase
VGCV	valganciclovir
VEGF	vascular endothelial growth-factor
VSV	vesicular stomatitis virus
VV	vaccinia virus
WHO	World Health Organization

7. References

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8. Erklärungen zum Eigenanteil der Dissertationsschrift

Die Arbeit wurde in der Abteilung für Gastroenterologie, Hepatologie und Infektiologie der Universitätsklinik Tübingen unter Betreuung von Herrn Prof. Dr. med. Ulrich M. Lauer durchgeführt.

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Ich versichere, das Manuskript selbstständig verfasst und keine weiteren, als die von mir angegebenen Quellen, verwendet zu haben.

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