Novel epi-virotherapeutic treatment of pancreatic cancer combining the oral histone deacetylase inhibitor resminostat with oncolytic measles vaccine virus

TIM PATRICK ELLERHOFF1, SUSANNE BERCHTOLD1, SASCHA VENTURELLI1, MARKUS BURKARD1, IRINA SMIRNOW1, TANJA WULFF2 and ULRICH M. LAUER1

1Department of Internal Medicine I, University Hospital Tuebingen, D-72076 Tuebingen; 24SC AG, D-82152 Planegg-Martinsried, Germany

Received May 30, 2016; Accepted July 22, 2016

DOI: 10.3892/ijo.2016.3675

Abstract. Oncolytic viruses (OV) constitute highly promising innovative biological anticancer agents. However, like every other antitumoral compound, OV are also faced with both primary and secondary mechanisms of resistance. To overcome those barriers and moreover amplify the therapeutic potential of OV, we evaluated a novel combined approach composed of the oral histone deacetylase inhibitor resminostat and an oncolytic measles vaccine virus (MeV) for a future epi-virotherapy of pancreatic ductal adenocarcinoma. Cytotoxicity assays revealed that combined epi-virotherapeutic treatment of four well-characterized human pancreatic cancer cell lines resulted in a beneficial tumor cell killing as compared to either mono-therapeutic approach. Notably, epi-virotherapeutic treatment of MIA PaCa-2 and partly also of PANC-1 pancreatic cancer cells resulted in a tumor cell mass reduction being significantly more pronounced than it would be expected in case of an additive effect only, indicating a synergistic mode of action when combining resminostat with MeV. We further found that the epigenetic compound resminostat neither impaired MeV growth kinetics nor prevented the activation of the interferon signaling pathway which plays an important role in mediating primary and secondary resistances to OV. Moreover, we yielded information that the pharmacodynamic function of resminostat was presumably not altered in the course of pancreatic cancer cell infections with MeV. Taken together, these promising results favor the onset of epi-virotherapeutic clinical trials in patients suffering from advanced pancreatic ductal adenocarcinoma.

Introduction

Oncolytic viruses (OV) exhibit unique features such as: i) outstanding safety profiles (especially when vaccine-derived viral vectors are coming to application), ii) high levels of tumor selectivity, iii) an incomparable self-amplification property, iv) lack of cross-resistance with other anticancer drugs (e.g., chemotherapeutic compounds), v) superior capabilities of targeting cancer stem cells as well as, vi) distant metastases and the possibility, vii) to significantly impair the blood supply to tumor beds (1-5).

Recently, OV have made their breakthrough with respect to their implementation in daily clinical practice. Due to the favourable results of the herpes simplex virus type 1 (HSV-1)-derived virotherapeutic vector Imlygic® in a recent phase III clinical trial with patients exhibiting advanced stage melanoma (6), its approval has to be regarded as a hallmark in the clinical development of virotherapy (7). Beyond that, the unique properties of OV as self-amplifying agents that selectively infect and kill cancer cells have been successfully exploited in the treatment of patients suffering from multiple myeloma resulting in an impressive case with long-term tumor remission following a single shot, high-dose application of a marker gene-encoding recombinant measles vaccine virus (MeV-NIS) (8).

Despite these promising results, OV still have to face several limitations before taking full advantage of their great potential to kill cancer cells. On the one hand, OV like any other viruses are recognized as pathogens facing efficient elimination by the host immune system (9). On the other hand, numerous cancer cell types have been shown to be resistant toward virus-mediated oncolysis due to features such as entry receptor down-regulation as well as an insufficient extent of inactivation of anti-viral signaling pathways in the tumor cells (10-12). In this context, we have revealed that 50% of the cell lines being represented in the well-characterized NCI-60 tumor cell panel display unwanted mid and high grade resistance toward MeV-mediated oncolysis (13).

In order to address the limitations and with further respect to the advantage that there are no cross-resistances of OV with other therapeutic regimens, researchers have been prompted by...
the rationale of combining OV with other anticancer agents, including histone deacetylase inhibitors (HDACi) (recently reviewed in refs. 14,15). The impact of HDACi on cancer cells was found to be highly diversified in terms of mechanisms of action, eliciting induction of apoptosis, causing accumulation of reactive oxygen species as well as inhibiting angiogenesis and metastasis (16,17). Thereby, HDACi almost selectively affect tumor tissues, while sparing healthy cells (17). As a consequence, a reciprocal amplification of antitumor effects was hypothesized for putative HDACi plus OV combination regimens. In line with this, epi-virotherapeutic strategies already have proven to effectively boost tumor cell killing when compared with either monotherapeutic efficiencies (14,18-23), raising the novel term ‘epi-virotherapeutic approach’ (24).

Regarding the underlying molecular mechanisms of such epi-virotherapy concepts, several steps of virus-mediated oncolysis can be augmented by HDACi (Fig. 1). Among them, an HDACi-induced impairment of a proper anti-viral immune response is discussed as a potential synergistic mechanism as it is assumed to highly facilitate both virus replication and virus spreading (14). Since HDAC activity is involved in almost every step of the interferon (IFN) pathway, particularly in the transcription of IFN-β, activation of signal transducers and activators of transcription (STAT) proteins, IFN-stimulated gene factor 3 (ISGF3) formation and ultimately expression of IFN-stimulated genes (ISGs) (25-29), HDACi like VPA and TSA were shown to blunt this cellular anti-viral response (30,31). Moreover, in xenograft models it was shown that T-cell and NK-cell mediated anti-viral immune responses can be significantly impaired by concomitant treatment with entinostat (MS-275) and VPA (21,23). Since virus entry receptors are often epigenetically downregulated in different tumor cells, HDACi were shown to restore coxsackie- and adenovirus receptor (CAR) as well as the human reovirus receptor junctional adhesion molecule-1 (JAM-1) on tumor cell surfaces, thereby significantly increasing rates of primary infections with OV (14,32-34). Furthermore, several OV including vesicular stomatitis virus (VSV) and MeV have been spotted to profit from an HDACi-related enhancement of autophagy (35-37), displaying a cellular catabolic process that serves: i) for the degradation of cellular components being no longer in general use and ii) for the maintenance of energy levels in times of starvation and cellular stress (38). At last, both the translocation of OV genomes to the cell nucleus via microtubules and the expression of viral genes can be amplified by concomitant HDACi treatment (15,30,39).

OV derived from the measles virus vaccine strain Edmonston have been extensively investigated in numerous preclinical and clinical studies and have been found to constitute well suited anticancer agents (40-42). During the decades-long use as a vaccine, its safety has been comprehensively verified, whilst a reversion to wild-type MeV followed by any potential outbreak of harmful MeV infections has not been documented at any time in history (43). We studied the antitumoral potential of combining the oral HDACi resminostat with oncolytic MeV in terms of a future epi-virotherapy of advanced pancreatic adenocarcinoma, a tumor entity which is still tainted with a poor prognosis (44). Resminostat is a hydroxamic acid-based HDACi, inhibiting selectively class I, IIb and IV HDAC enzymes and has already been subject of different successful clinical trials, underlining not only its efficiency, but also its safety and tolerability (42,45,46). We further report that our novel epi-virotherapeutic combination of resminostat with oncolytic MeV resulted in an enhanced tumor cell killing in human pancreatic cancer cells. Most interestingly and in contrast to the hitherto prevailing opinion, this boosting effect was found not to be related to a resminostat-induced impairment of the anti-viral IFN response.

Materials and methods

Cell culture and non-viral compounds. Human pancreatic cancer cell lines AsPC-1, MIA PaCa-2, and PANC-1 were purchased from the American Type Culture Collection (ATCC); cell line BxPC-3 was obtained from the European Collection of Authenticated Cell Cultures (ECACC); cell line BxPC-3 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were kept in a humidified incubator at 37°C, containing 5% CO₂ and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, Munich, Germany) supplemented with 10% fetal calf serum. Resminostat was kindly provided by 4SC AG (Planegg-Martinsried, Germany).

Propagation and titration of measles vaccine virus. Construction of recombinant measles vectors MeV-GFP (measles vector encoding for green-fluorescent protein as a marker gene integrated into the viral genome) has been described (47). Virus stocks were prepared in Vero cells. For this purpose, 1x10⁷ Vero cells were seeded in 15-cm plates. The next day, cells were washed once with phosphate-buffered saline (PBS; Sigma-Aldrich) and infected for 3 h at a MOI of 0.03 in infection medium (Opti-MEM; Gibco; Grand Island, NY, USA). Subsequently, medium was replaced with DMEM containing 10% FBS. After an incubation period of 54 h, when most of the cells were infected, medium was removed and attached Vero cells were scraped into 1 ml Opti-MEM. Release of virus was achieved by one freeze-thaw cycle. After centrifugation (1,900 x g, for 15 min at 4°C), supernatants were stored at -80°C. Viral titers were determined on Vero cells according to the method of Spearman (48) and Kärber (49).

Infection of cells with measles vaccine virus. Cells were seeded in 6- or 24-well plates the day before virus infection. Then, culture medium was removed, cells were washed with PBS and subsequently virus was diluted in Opti-MEM at required multiplicities of infection (50) was added. After 3 h of incubation, the inoculum was removed and DMEM supplemented with 10% FCS and, if required additionally resminostat was added.

SRB assay. For SRB assay cells were seeded in 24-well plates with cell numbers ranging from 2x10³ for MIA PaCa-2 to 3x10⁶ for PANC-1 and 4x10⁵ per well for AsPC-1 and BxPC-3. Experiments were stopped at required time-points after treatment by removing medium, washing with PBS and subsequently fixing with trichloroacetic acid (10%, 4°C for 30 min). Afterwards, fixed cells were washed four times with tap water,
dried and then stained with SRB solution (0.4% in 1% acetic acid) for 10 min at room temperature. After washing with 1% acetic acid and drying again bound SRB was dissolved in 10 mM Tris base (pH 10.5) and the optical density was measured at a wavelength of 550 nm using a microplate reader (Tecan Genios Plus). The mean of mock-treated controls was set to 100% and treated samples were stated in percent of this control.

**Real-time cell proliferation assay.** Cells were seeded in 96-well plates (E-Plate 96, Roche Applied Science, Mannheim, Germany) in different concentrations according to their particular proliferation characteristics (AsPC-1: 1x10^4 cells/well; MIA PaCa-2: 7.5x10^3 cells/well; PANC-1: 5x10^3 cells/well). Real-time dynamic cell proliferation was monitored in 30-min intervals during a 120-h observation period using the xCELLigence RTCA SP system (Roche Applied Science). Cell index values were calculated using the RTCA Software (1.0.0.0805). At 21 h after seeding, cells were infected with MeV-GFP diluted in Opti-MEM and at 3 hpi resminostat was added in required concentrations. All values were normalized to the beginning of the treatment period (24 h after seeding) (51,52).

**Viral growth curves.** Cells were infected with MeV-GFP in 24-well plates. At 3 hpi the inoculum was removed and cells were washed three times with PBS. Then 0.5 ml medium or medium with resminostat was added. At 3, 24, 48, 72 and 96 hpi supernatants were harvested and cells were scraped off in 0.5 ml Opti-MEM. Cell lysis was performed by one freeze-thaw cycle and subsequently virus titers were determined by titrating samples on Vero cells following Spearman (48) and
Kärber (49). Therefore, Vero cells were seeded in a density of 1×10⁴ cells per well in 96-well plates in DMEM containing 5% FCS. Twenty-four hours later, cells were infected with 1:10 dilution series generated from cell lysate and supernatant samples. Tissue culture infective dose (TDC₀) was calculated by observing measles-induced cytopathic effect with a fluorescence microscope and converted into plaque forming units per ml (pfu/ml).

**Immunoblotting.** Protein samples were obtained by seeding, infecting and treating pancreatic cancer cells in 6-well plates. At required time-points, medium was removed, cells were washed with PBS and afterwards harvested in lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP40). Cell lysis was performed by three freeze-thaw cycles. Lysates were then cleared by centrifugation at 13,000 rpm for 10 min. Protein concentrations in the supernatants were determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA).

Each sample (70 µg) was mixed with 6-fold Roti Load buffer and boiled at 95°C for 5 min. Proteins were separated on a 8% polyacrylamide gel and blotted on a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond P, GE Healthcare). Membranes were blocked in 5% powdered milk in Tris-buffered saline containing 0.02% Tween-20 (TBS-T) and then incubated with primary antibodies (anti-IFIT1: GTX103452; 1:1,000; GeneTex, Irvine, CA, USA; anti-phospho-Stat1: 58D61; 1:1,000; Cell Signaling Technology, Danvers, MA, USA; anti-Stat1: sc-591; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti-β-actin: A 4700; 1:6,000; Sigma-Aldrich) overnight. After washing three times with TBS-T, membranes were exposed to the secondary antibody (goat anti-rabbit IgG; goat anti-mouse IgG; HRP-coupled; Abcam Ltd., Cambridgeshire, Uk). After washing three times with TBS-T again proteins were detected by enhanced chemiluminescence western blotting detection reagent (GE Healthcare).

**qPCR.** Cells were treated with resminostat, MeV-GFP or the combination and subsequently RNA was isolated using the NucleoSpin® RNA kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions.

Each RNA sample (500 ng) was mixed with 2 µl M-MLV RT buffer (Promega, Madison, WI, USA), 1 µl RNase-inhibitor RNasin Plus (Promega), 1 µl oligo-dT-Primer (0.5 µg/µl) (TIB MolBio, Berlin, Germany), 0.5 µl dNTP mix (Roti-Mix PCR3, Carl Roth) and added up to a total volume of 9.6 µl in RNAse-free water. Samples were then incubated at 70°C for 1 min at 95°C was followed by 1 min at 65°C and 81 cycles heating up for 15 sec at 95°C, 20 sec at 58°C, and 15 sec at 62°C. PCR was carried out with the following thermal profile: 3 min at 95°C with subsequently 40 cycles for 15 sec at 95°C, 20 sec at 58°C, and 15 sec at 62°C. Target gene expression was evaluated via the Δ∆Ct method and normalized to the housekeeping gene RPS18 and subsequently graphed relative to the respective mock sample for each time-point and expressed as ‘relative gene expression’.

**Statistical analysis.** The influence of measles and resminostat on the decadic logarithm of cell mass (in % of the mean of the cell line control) was examined by performing a two-way analysis of variance (ANOVA). Additionally, an interaction of measles and resminostat was used in the ANOVA. Calculations were done by the JMP software for windows. P-values <0.01 were considered to be statistically significant. Graphs including error bars were imaged with GraphPad Prism 4 for windows.

**Results**

**Dose- and time-dependent effects of resminostat and MeV on pancreatic cancer cells.** Following our encouraging results recently obtained in the epi-virotherapeutic treatment of human hepatoma cells (24), we now examined the antitumor potential of the epi-virotherapeutic approach consisting of the oral HDACi resminostat and MeV for the therapy of pancreatic ductal adenocarcinoma. For this purpose, we first determined the antitumor effects elicited by each agent in monotherapy on a panel of four human pancreatic cancer cell lines (AsPC-1, BxPC-3, MIA PaCa-2, and PANC-1).

First, tumor cells were treated with resminostat at different concentrations ranging from 0 to 10 µM. Experiments were stopped at different time-points and tumor cell viabilities were subsequently examined by sulforhodamine B (SRB) assays (Fig. 2).

As a result, treatment with resminostat displayed a dose- and time-dependent cytoreductive effect in all investigated human pancreatic cancer cell lines. In detail, MIA PaCa-2 cells were shown to be most sensitive exhibiting a tumor cell mass reduction of almost 100% at 5 µM resminostat at 96 h post-treatment (hpt). In contrast, PANC-1 cells were shown to be most resistant with a remaining tumor cell mass of ~70% with 5 µM resminostat at 96 h post-treatment (Fig. 2D). For further experiments, concentrations of resminostat were adjusted in a tumor cell line-specific manner ensuring remaining tumor cell masses ~75% at 96 hpt with resminostat: 1 µM resminostat for tumor cell lines MIA PaCa-2 and AsPC-1, 2.5 µM for BxPC-3 tumor cells, and 5 µM for PANC-1 tumor cells, respectively.

Next, all four human pancreatic cancer cell lines were infected with a GFP marker gene-encoding oncolytic measles vaccine virus vector (MeV-GFP) at different multiplicities of infection (50) ranging from 0.25 to 20 (i.e., using a ratio of 0.25-20 virus particles per single tumor cell to be infected) (Fig. 3). Again, tumor cell viabilities were determined by SRB assays, now at both 72 and 96 h post infection (hpi). As a result, pancreatic cancer cells displayed great differences in susceptibility towards MeV-GFP-mediated oncolysis. The tumor cell line being most sensitive to MeV-GFP-mediated
Figure 2. (A-D) Evaluation of resminostat-induced pancreatic cancer cell mass reduction. Mono-treatment with the epigenetic compound resminostat resulted in a dose- and time-dependent reduction of tumor cell masses in all tested pancreatic cancer cell lines (AsPC-1, BxPC-3, MIA PaCa-2 and PANC-1). Six concentrations of resminostat (ranging from 0 to 10 µM) were administered and tumor cell viabilities were determined at four different time-points (24, 48, 72 and 96 h after treatment) utilizing a Sulforhodamine B (SRB) viability assay. Tumor cell masses are given in % of the mean of mock-treated tumor cells (resminostat concentration of 0 µM) for each time-point. Means and SDs of three independent experiments are shown.

Figure 3. (A-D) Evaluation of MeV-GFP-induced pancreatic cancer cell mass reduction. Mono-treatment with recombined measles virotherapeutics (MeV-GFP) resulted in a dose- and time-dependent reduction of tumor cell masses in all pancreatic cancer cell lines (AsPC-1, BxPC-3, MIA PaCa-2 and PANC-1). Virotherapeutic treatments were performed at indicated multiplicities of infection (MOI), being adjusted to the oncolytic susceptibility of the respective tumor cell line. Tumor cell viabilities were determined at 72 and 96 h post-infection (hpi) using SRB viability assays. Tumor cell masses are given in % of the mean of mock-treated tumor cells for each time-point. Means and SDs of three independent experiments are shown.
Oncolysis was PANC-1 (Fig. 3D), whereas AsPC-1 tumor cells (Fig. 3A) were found to be most resistant. Considering this, >50% of AsPC-1 cells survived virus infections at a MOI of as high as 20. In contrast, a tumor cell mass reduction of 50% was obtained by infecting PANC-1 cells at a MOI of as low as 1. For further experiments, MOIs were adjusted in a tumor cell line-specific manner resulting in remaining tumor cell masses ~75% at 96 hpi: MOIs of 2.5 and 5 for MIA PaCa-2 and AsPC-1; MOIs of 0.5 and 1 for BxPC-3, and MOIs of 0.25 and 0.375 for PANC-1.

Addressing the question whether there is cross-resistance between resminostat and MeV-GFP, a remarkable trend could be observed. Tumor cell lines, which had been identified to be more resistant toward resminostat exhibited a relatively strong sensitivity toward MeV-GFP-mediated oncolysis and vice versa. The largest difference in tumor cell susceptibility was obtained in experiments with the PANC-1 tumor cell line being most resistant against resminostat treatment, but most sensitive towards MeV-GFP-mediated oncolysis (Figs. 2D and 3D).

Enhanced tumor cell-killing by epi-virotherapeutic co-treatment. To further determine whether resminostat and oncolytic MeV operate beneficially when administered in combination, pancreatic cancer cells were initially infected with MeV-GFP; then, resminostat was added following the regular change of infection culture medium at 3 hpi (Fig. 4). Tumor cell line adjusted MOIs of MeV-GFP and concentrations of resminostat were used as determined prior in the mono therapy settings.

As a result, supplementation of oncolytic MeV-GFP by resminostat resulted in beneficial effects on rates of tumor cell mass reduction in all tested pancreatic cancer cell lines. With regard to MOIs of MeV-GFP and concentrations of resminostat employed in later experiments, the reduction in tumor cell mass could be amplified from 53 to 37% for AsPC-1 (MOI 5), from 60 to 32% for BxPC-3 (MOI 0.5), from 65 to 19% for MIA PaCa-2 (MOI 2.5), and from 93 to 48% for PANC-1 (MOI 0.25) (Fig. 4). Considering that HDACi per se induce a reduction in pancreatic cancer cell masses, the most striking benefit could be obtained in the treatment of MIA PaCa-2 cells, achieving a further 45% reduction in tumor cell mass (Fig. 4C, comparison of bars 2 and 6). Whereas both agents in monotherapy reduced tumor cell viability each by 35% in comparison to the mock control, the combination led to a tumor cell mass reduction of >80% in comparison to the mock control (Fig. 4C, comparison of bars 1 and 6).

A statistical analysis was carried out to investigate whether an interaction between MeV-GFP and resminostat is verifiable that caused a more pronounced effect on tumor cell mass reduction than expected from a simple additive effect.

Figure 4. (A-D) Epi-virotherapeutic treatment is superior to any corresponding monotherapy. Tumor cells were infected with MeV-GFP (MeV) at indicated multiplicities of infection (50), being adjusted to the oncolytic susceptibility of the respective tumor cell line. At 3 h post infection (hpi), resminostat was added at the indicated concentrations. Remaining tumor cell masses were determined at 96 hpi using SRB viability assays. Means and SDs of three different experiments are shown. *P-value <0.01 of ANOVA on logarithms of tumor cell mass in % of control, comparing epi-virotherapeutic treatment with mono-treatment of resminostat (Res) and MeV. **P-value <0.01 of interaction term in ANOVA verifying a more than additive (synergistic) effect.
The interaction term in the ANOVA on the logarithms of tumor cell mass in % of control confirmed a clear significant synergistic antitumor effect for the treatment of MIA PaCa-2 cells (Fig. 4C) as compared to the cytotoxic effect that would be expected from an additive effect. With regard to the other pancreatic cancer cell lines, synergistic tumor cell killing could be significantly revealed in PANC-1 cells for only one of the two combinations (MeV MOI 0.375 and 5 µM resminostat; Fig. 4D); in contrast, no synergistic effects were found for AsPC-1 and BxPC-3 tumor cells (Fig. 4A and B), suggesting that the epi-viro-therapeutic approach does not elicit synergistic effects in all pancreatic cancer cell entities, presumably as a result of tumor cell specific features.

To confirm our results from the SRB viability assays and to gain more precise information on the entire treatment time course, real-time pancreatic cancer cell proliferation was determined using the xCELLigence system (Fig. 5). The acquired data revealed that our epi-viro-therapeutic treatment elicited beneficial effects on tumor cell viabilities in three out of the four tested pancreatic cancer cell lines (Fig. 5). Taken together, these findings underline that: i) our specific epi-viro-therapeutic treatment is much more valuable for MIA PaCa-2 and PANC-1 tumor cells than for AsPC-1 cells (BxPC-3 tumor cells were not included in this specific testing) and ii) the mode of synergistic tumor cell killing is first observed at 72 hpi in all tested pancreatic cancer cell lines (going along with MeV-mediated oncolysis phenomena taking place at this time-point).

Absence of alterations in virus growth kinetics under continuous treatment with resminostat. To examine whether the resminostat-related enhancement of MeV-GFP-mediated oncolysis is based on an accelerated virus replication and spread, virus growth kinetics were analyzed for the four tested pancreatic cancer cell lines in presence and absence of resminostat at five different time-points (at 3, 24, 48, 72, and 96 hpi). For this purpose, tumor cells were infected with indicated MOIs and treated continuously with different concentrations of resminostat (Fig. 6). Comparing the virus growth curves of MeV-GFP monotherapy with those of co-treatment with resminostat, no relevant differences were obtained.

The highest virus titers were reached in PANC-1 and MIA PaCa-2 tumor cells (Fig. 6C and D), amounting to 10^5 pfu/ml whereas in AsPC-1 and BxPC-3 titers of only 10^4 pfu/ml were detected (Fig. 6A and B). In all tumor cell lines viral titers in supernatants were almost equal to those still bound inside tumor cells. Thus, there was no clear correlation between the susceptibility of the tumor cell lines toward measles vaccine virus-mediated oncolysis and virus titers.

At later time-points (at 72 and 96 hpi) viral titers were slightly lower in supernatants as well as in tumor cell lysates in the presence of resminostat. This may be due to a greater tumor cell mass reduction induced by the combination treatment at later time-points, so that fewer tumor cells were present in the cultures at these later time-points resulting in a significantly lower cellular capacity for production of viral progeny particles.

In conclusion, enhanced oncolytic effects by the combined treatment of MeV-GFP and resminostat were not found to be caused by an enhancement of viral replication by the HDACi.

Expression of surrogate parameter zinc finger protein 64 decreased in the course of resminostat treatment of pancreatic cancer cells. Decrease in the expression of zinc finger protein 64 (zfp64) has been revealed to be a good surrogate parameter for the pharmacological activity of resminostat.
Therefore, we examined mRNA expression of zfp64 after monotreatment with either resminostat or MeV-GFP and after combination treatment (resminostat plus MeV-GFP) using the same resminostat concentrations and MOIs as in all prior experiments (Fig. 7). In the presence of resminostat, zfp64 expression was found to be downregulated in each tumor cell line as early as after five hours of treatment initiation.

Under epi-virotherapeutic co-treatment with resminostat and MeV-GFP, we still observed a lower expression of zfp64 as compared to the mock-treated control (with AsPC-1 tumor cells showing an even lower expression under co-treatment as compared to resminostat treatment alone; Fig. 7A). In contrast, different expression patterns of zfp64 were found when tumor cells had only been infected with MeV-GFP; in these cases, zfp64 was only downregulated in BxPC-3 and MIA PaCa-2 tumor cells (Fig. 7B and C), but there was no detectable regulation in AsPC-1 and PANC-1 tumor cells (Fig. 7A and D).

In conclusion, our experiments provide evidence that the pharmacodynamic function of resminostat did not seem to be impaired in MeV-GFP-infected pancreatic cancer cell lines.

Resminostat did not impair activation of IFN signaling. In most studies investigating epi-virotherapeutic approaches so far, damping of the anti-viral response by HDACi was highlighted as a potential explanation for underlying synergistic antitumoral effects of this combined treatment approach.
Accordingly, we were interested in the functionality of IFN-signaling of pancreatic cancer cells in the presence and absence of resminostat during infections with MeV-GFP.

Many tumor cells are known to exhibit defects in IFN signaling and are therefore considered to be susceptible to OV-mediated oncolysis (53). In this context, we first examined whether pancreatic cancer cells have the ability of initiating an IFN response in the course of an infection by MeV. For this purpose, pancreatic cancer cells were infected with MeV-GFP at standard MOIs (0.25 for PANC-1, 0.5 for BxPC-3, 2.5 for MIA PaCa-2, and 5 for AsPC-1, respectively). Furthermore, control samples were generated by stimulating cells with IFN-β for 24 h. Samples were taken at 24, 48, 72, and 96 hpi. In AsPC-1, BxPC-3, and PANC-1 tumor cells phosphorylation of STAT1 and expression of IFN-induced protein with tetratricopeptide repeats 1 (IFIT1) were observed at the latest at 72 hpi indicating an unaltered activation of IFN signaling (data not shown). In contrast, in MIA PaCa-2 cells neither phosphorylation of STAT1 nor expression of IFIT1 was detected after MeV-GFP infection being indicative of a severe defect in IFN signaling in this distinct tumor cell line (Fig. 8).

Figure 7. (A-D) Analysis of the resminostat pharmacodynamic function in MeV-GFP-infected pancreatic cancer cells. Unimpairment of the resminostat (Res) pharmacodynamic function in pancreatic cancer cells being infected with recombined measles virotherapeutics (MeV-GFP) was deduced from the decrease in expression of zinc-finger protein 64 (zfp64) after 5 h of epigenetic treatment. Tumor cells were infected with MeV-GFP at stated MOIs and co-treated with indicated concentrations of resminostat starting at 3 h post-infection. RNA samples were obtained after 5 h of treatment. Expression levels of zfp64, representing a well-defined surrogate parameter for the epigenetic impact of resminostat, were determined using RT-qPCR. Values were normalized to the housekeeping gene RPS 18 (ribosomal protein S18), and relative expression is displayed compared to corresponding control samples (mock; no infection with MeV-GFP and no treatment with resminostat). Data of a representative experiment are shown. MeV + Res, co-treatment with measles virus MeV-GFP and resminostat with concentrations and MOIs as used in the respective mono-treatment experiments.

Figure 8. MeV-GFP did not induce IFN signaling in MIA PaCa-2 cells. Tumor cells were either infected with MeV-GFP (MOI 2.5) or treated without infection (mock); then, samples were taken at 24, 48, 72 and 96 h post-infection (hpi) and analyzed by immunoblotting; tumor cells stimulated with interferon-β (IFN-β) were used as positive controls. Potential activation of IFN signaling by MeV-GFP was deduced from phosphorylation of STAT1 (phospho-STAT1) and expression of interferon-induced protein with tetratricopeptide repeats 1 (IFIT1). β-actin was used as a loading control.
We then investigated the impact of resminostat on MeV-GFP-induced activation of IFN signaling in AsPC-1, BxPC-3, and PANC-1 cells. As a result, resminostat monotreatment did neither result in phosphorylation of STAT1 nor in expression of IFIT1. However, both MeV-GFP infection alone as well as the epi-virotherapeutic combination resminostat plus MeV-GFP were found to activate IFN signaling at both 72 and 96 hpi, indicated by phosphorylation of STAT1 and expression of IFIT1 (Fig. 9). As MIA PaCa-2 cells did not initiate IFN signaling after MeV-GFP infection, we stimulated these tumor cells with IFN-β (please note: BxPC-3 cells were used as a control in this experiment). Some of these were additionally treated with resminostat. As a result, IFN-β treatment was found to induce IFN signaling; but similar to all prior results, resminostat was unable to inhibit phosphorylation of STAT1 and expression of IFIT1 (Fig. 10). These results clearly imply that resminostat does not impair the IFN response of pancreatic cancer cells that had been initiated by infection with MeV-GFP. Consequently, resminostat does not elicit synergistic effects due to an impairment of the anti-viral response.

**Discussion**

Oncolytic viruses have recently made a major move toward their full establishment in clinical practice by approval of Imlygic® both by the American Food and Drug Administration (FDA) and by the European Medicines Agency (EMA) (7).

In our study, an epi-virotherapeutic approach was pursued, augmenting oncolytic MeV with the oral HDACi resminostat. Both agents already have been evaluated independently as well as recently in combination for the treatment of different solid tumors with encouraging results (1,14,24,45,54-57). Here, we tested a series of four human pancreatic cancer cell lines: i) for their sensitivity to both agents in monotreatment and subsequently, ii) toward the effect of epi-virotherapeutic co-treatment.

At the outset, monotreatment experiments revealed that both agents, oncolytic MeV-GFP as well as resminostat, caused dose- and time-dependent tumor cell killing in all tested human pancreatic cancer cell lines. Strikingly, the cytotoxic effect of resminostat on a specific cancer cell line could not be predicted from the results obtained in OV cytotoxicity assays and vice versa. This is most clearly visible when comparing the virotherapeutic with the epigenetic results obtained with PANC-1 cells emphasizing that there are no cross-resistances between OV and other cytotoxic drugs such as HDACi.
Subsequently, cooperative effects were evaluated by performing SRB cell viability assays and afterwards confirmed utilizing the xCELLigence system. The results showed that the epi-virotherapeutic approach elicited beneficial cytotoxic effects in all four pancreatic cancer cell lines. Regarding MIA PaCa-2 tumor cells, considerable synergistic results were observed: virus-mediated reduction in the tumor cell masses was found to be improved in the presence of resminostat from 35 to 81% (at MOI 2.5) as well as from 55 to 92% (at MOI 5) (Fig. 4). Similarly, epi-virotherapeutic treatment of the other three cancer cell lines exhibited stronger effects than obtained in monotreatment. In further experiments we found that virus growth curves revealed no significant differences in the presence or absence of resminostat, suggesting that resminostat neither facilitated virus entry nor enhanced virus replication.

With regard to studies that have already investigated the therapeutic potential of epi-virotherapeutic treatment of different tumor entities, the most frequently examined and highlighted molecular mechanism of synergism is the ability of HDACi to impair the anti-viral immune response of host tumor cells, thereby facilitating virus replication and spread. Many underlying mechanisms have been revealed, describing involvement of HDAC activity in almost each step of IFN signaling. Virus infection leads to phosphorylation of IFN-regulatory factors (IRFs), homo- or heterodimerization and translocation into the nucleus where IFN-β expression is induced (58). Trichostatin A (TSA) was shown to prevent proper IRF-3 function, thereby hindering cells to produce IFN-β (25). Downstream signaling of the IFN-β receptor likewise requires HDAC activity, enabling proper receptor activation, STAT dimerization, and IRF-9 function as well as the formation of the IFN-stimulated gene factor-3 (ISGF3) (26-28). Also, HDAC are involved in the expression of IFN-stimulated-genes (ISGs) (29). Accordingly, HDAC inhibitors were proven to impair the expression of ISGs when tumor cells were coincidently infected with oncolytic viruses (30,36,59). Due to these findings, the enhanced oncolytic effect was retrospectively assigned to the interference with IFN signaling.

In contrast to these observations, the present epi-virotherapeutic approach did not modulate IFN signaling as indicated by an unaltered phosphorylation of STAT1 and expression of the ISG IFIT1 in any of the tested pancreatic cancer cell lines. Moreover, no obvious alteration in virus growth kinetics could be observed. For these reasons, our experiments do not support the prevailing opinion of HDACi damping the IFN-response thus enhancing OV-mediated oncolysis. In respect of implementing our epi-virotherapeutic approach into clinical practice, it is potentially not preferable to type I IFN production is impaired. Since especially IFN-α and IFN-β are essential cytokines that attract and prime cytotoxic and T helper cells by causing expression of important receptors on cancer cells (such as MHC I), type I IFN secretion from tumor sites might amplify an antitumor immune response (60,61).

Other studies having examined the potential of HDACi to enhance different virotherapeutics obtained similar findings. After having infected different infection-resistant cancer cells with vaccinia virus (VV) that had retained their B18R gene, functioning as an IFN antagonist, the HDACi TSA was still capable of amplifying OV-mediated oncolysis, suggesting that its antitumor effect was not based on an immunosuppressive function (19). In our study, MIA PaCa-2 was the only pancreatic cancer cell line which did not exhibit an activation of the IFN signaling pathway after MeV infection. Despite this lack of establishing a proper anti-viral state, it was not the most susceptible cell line to MeV-mediated oncolysis and more noteworthy, epi-virotherapeutic treatment showed the most pronounced effect in this cell line, stressing that HDACi seem to enhance virus-mediated oncolysis by eliciting other effects than damping the IFN response. This raises the question which additional mechanisms could explain the enhancement of virus-mediated cell death by epi-virotherapeutic co-treatment.

Explanations, amongst others, were provided by Liu et al (31). Using an epi-virotherapeutic approach consisting of oncolytic herpes-simplex-virus (HSV) and TSA in a panel of tumor and normal quiescent cells, they obtained beneficial cytoductive effects compared to monotreatment. These effects could be attributed neither to the dosing schedule nor to enhanced infectivity or virus replication. The authors rather ascribed the results to a decrease in expression of cyclin D1, mediating cell cycle arrest, and VEGF, reinforcing the hypothesis of vascular shutdown induced by OV (5).

Beyond the above, further replication-independent mechanisms have been illustrated, highlighting the impact of HDACi on cell signaling. Thus, HDACi cause hyperacetylation of NF-κ B, thereby increasing its nuclear retention and DNA binding capacity. Due to its promotion of HSV gene expression, this HDACi-mediated effect elicited synergistic tumor killing in oral squamous cell carcinoma (SCC) cells (62). Furthermore, combined treatment was shown to increase the expression of p21 which mediates cell cycle arrest, consequentially slowing down tumor progression and resulting in the induction of tumor cell apoptosis.

Recently, Shulak et al found a mechanism explaining NF-κ B activity accompanied by an enhanced OV-mediated oncolysis. They pointed out that hyperacetylation and nuclear retention of NF-κ B induced the expression of several autophagy-related genes. They argued that the induction of autophagy led to an impairment of IFN signaling but also to vesicular stomatitis virus (VSV)-mediated apoptosis in prostate cancer cells (36). Autophagy is a process that is per se frequently enhanced in tumor cells since it serves as a stress response to oxidative stress, lack of nutrients, and hypoxia as it is commonly present in the microenvironment of solid tumors (63). Interestingly, pancreatic cancer cells even require this catabolic process in order to prevent accumulation of ROS, thereby contributing to tumor growth as well as establishing the basis for drug resistance (64,65). Despite these pro-survival aspects, some viruses are notably capable of exploiting the autophagic machinery for the purpose of efficient replication (38). Attenuated MeV derived from the Edmonston strain actually induce and require autophagy for efficient replication (37). Since hydroxamic acid based HDACi equally increase autophagic activity (66), it is tempting to speculate that the effect elicited by resminostat in combination with oncolytic MeV is caused by an enhanced self-digestion and subsequently enhanced tumor cell death.

Physiologically, cell signaling often requires protein modifications such as phosphorylation or acetylation but beyond targeting cell proteins, even pathogenic proteins can
serve as substrates for those modifications, resulting either in enhanced or impaired activity. In this context, it was revealed that a portion of the NS-1 protein, representing the major pathogenic and most important protein for replication of the rat parvovirus H-1PV, gets acetylated during virus infection (67). Noteworthy, treatment with VPA caused hyperacetylation of NS-1 resulting in an accumulation of ROS and an enhanced transcriptional activity. Ultimately, DNA damage in cancer cells was observed consequently inducing apoptosis. Those findings were confirmed later in vivo, resulting in complete disappearance of implanted tumors in mice that had undergone co-treatment with H-1PV and VPA (18). Likewise, HDACi-related hyperacetylation of microtubules accelerated nuclear translocation of oncolytic HSV-genomes, thereby enhancing the antitumor effect in glioma stem-like cells (39).

In conclusion, our results provide evidence that the epi-virotherapeutic combination of oncolytic MeV and the HDACi resminostat constitutes a beneficial option in the treatment of advanced pancreatic ductal adenocarcinoma. We revealed an augmentation of MeV-mediated oncolysis by resminostat. Treatment of MIA PaCa-2 cells resulted even in a synergistic enhancement of the tumor-killing potential when compared to the monotherapies. Molecular mechanisms underlying the synergistic effects and the potential of our epi-virotherapeutic approach in vivo have to be elucidated in animal models in the future.

Acknowledgements

For the statistical analysis the methodological advice from the Institute of Clinical Epidemiology and Applied Biometrics of the University Hospital Tuebingen were utilized. We wish to thank Professor Martin Eichner for his excellent support, and we further wish to thank Hannes Schramm (University Hospital Tuebingen) who helped us with imaging and the augmentation of oncolytic viruses by histone deacetylase inhibitors. Dr Tanja Wulff is an employee of 4SC AG. T.P.E. was funded by the European Community’s Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 602683 (EPI-VIROTHERAPEUTIC TREATMENT OF PANCREATIC CANCER). Wulff is an employee of 4SC AG. T.P.E. was funded by the European Community’s Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 602683 (EPI-VIROTHERAPEUTIC TREATMENT OF PANCREATIC CANCER).

References


