

## ANTICANCER EFFECTS OF AN ONCOLYTIC PARVOVIRUS COMBINED WITH NON-CONVENTIONAL THERAPEUTICS ON PANCREATIC CARCINOMA CELL LINES

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**Summary.** – Standard therapies such as surgery and chemotherapy offer only minimal improvement in pancreatic cancer. However, the viruses killing cancer cells and substances like some antibiotics and phytoalexins with anticancer potential may represent a candidate non-conventional mean of cancer treatment in the future. In this study, the effect of infection with oncolytic H-1 parvovirus (H-1PV) combined with antibiotic norfloxacin (NFX) or phytoalexin resveratrol on the survival of cell lines Panc-1 and BxPC3 derived from human pancreatic carcinoma was tested. Whereas H-1PV with NFX exerted a synergistic effect, H-1PV with resveratrol resulted in an additive effect only. All the effects were partial, but they were more pronounced in Panc-1 compared to BxPC3 cells.

**Keywords:** pancreatic cancer; parvovirus; resveratrol; norfloxacin

### Introduction

Despite the undisputable progress in cancer research during the 20<sup>th</sup> century and improved prognosis for several types of cancer, pancreatic carcinoma still remains one of the malignancies with the worst survival rate (Jemal *et al.*, 2008). To downstage a tumor towards operability, neoadjuvant approaches combining radio-, chemo-, and bio-therapeutic modalities are increasingly gaining acceptance (Vulfovich and Rocha-Lima, 2008).

Treatment of cancer cells by means of viruses that specifically replicate in (oncotropism) and kill neoplastic cells (oncolysis) is a novel and promising therapeutic approach. Among oncolytic viruses, H-1PV and Minute virus of mice

belonging to the genus *Parvovirus*, the family *Parvoviridae* deserve special attention as an effective anti-cancer tool (Raykov *et al.*, 2004; Geletneky *et al.*, 2005). The major advantages of using parvoviruses in cancer therapy compared to other oncolytic viruses are: (i) parvoviruses are non-pathogenic for humans and (ii) can trigger an atypical cell death mechanism in tumor cells leading to the release of lysosomal cathepsins B and L in the cytosol (Di Piazza *et al.*, 2007). Likewise, quinolone antibiotics like ciprofloxacin and NFX beside their bactericidal effect are able to induce a lysosomal membrane permeabilization in mammalian cells (Boya *et al.*, 2003). The fact that tumor invasion and metastasis in pancreatic ductal adenocarcinoma (PDAC) are also associated with altered lysosomal trafficking and cathepsin accumulation, gives credit to the quinolones and parvoviruses treatment of this malignancy (Gocheva *et al.*, 2006).

In recent years, a phytoalexin resveratrol derived from dried grape skin, seeds or from residual winemaking products has proven to interfere with all three stages of carcinogenesis – initiation, promotion, and progression. Previous

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**Abbreviations:** cfu = center-forming units; H-1PV = H-1 parvovirus; MOI = multiplicity of infection; NFX = norfloxacin; PDAC = pancreatic ductal adenocarcinoma

reports confirm that this substance can directly inhibit the proliferation and induce apoptosis in pancreatic cancer cells (Ding and Adrian, 2002; Mouria *et al.*, 2002).

To investigate the cytotoxic effect of NFX and resveratrol combined with H-1PV infection on human pancreatic cancer cell lines, we treated *in vitro* Panc-1 and BxPC3 cultures with these agents. We estimated the killing efficiency by an MTT assay. We found that the non-conventional oncolytics have a potential to cause tumor cell death synergistically (H-1PV with NFX) or additively (H-1PV with resveratrol).

### Materials and Methods

**Virus.** Wild-type H-1PV was produced by infecting new born kidney (NBK) cells, purified by iodixanol gradient centrifugation, and dialyzed against isotonic Ringer salt solution for intravenous administration. Virus titers were determined as previously described and expressed as replication center-forming units (cfu) (Raykov *et al.*, 2004). Briefly, serially diluted purified virus was applied to NBK cells. The infected cultures were blotted onto filters 48 hrs post infection and replication centers were detected by hybridization with a virus-DNA-specific radioactive probe.

**Carcinoma cell lines.** Human pancreatic carcinoma cell lines BxPC3 and Panc-1 were obtained from ATCC and grown in medium RPMI-1640 or DMEM, respectively, supplemented with 10% fetal calf serum. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Cell survival assay.** Standard solutions of resveratrol and NFX (Sigma Aldrich) were prepared in DMSO, diluted in cultivation medium, and applied onto the cells at final concentrations from 0 to 25 µg/ml (NFX) and 0 to 100 µg/ml (resveratrol). The cell survival rates in cytotoxicity assays were done in triplicates and assessed after 3 days of drug treatment using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) colorimetric assay (Raykov *et al.*, 2004). The MTT reaction was stopped after 3 hrs and A<sub>570</sub> was measured. Arithmetic means and standard deviations (SD) were calculated using SigmaPlot 10 software.

### Results and Discussion

#### *NFX and H-1PV kill PDAC cells synergistically*

As a first step in our study we assessed the individual toxic effects of H-1PV and NFX on the Panc-1 and BxPC3 cell lines in the cytotoxic assay. H-1PV was able to kill only a minor fraction of BxPC3 cells at a low multiplicity of infection (MOI) of 0.5 and 1 cfu/cell. However, we did not detect any effect of H-1PV infection on the survival of Panc-1 cells.

Similarly, no cytotoxicity was observed with the doses of NFX ranging from 1–20 µg/ml. With the increasing concentration of NFX, the cytotoxicity remained only in the range of 10% even for the highest concentration used

(25 µg/ml) in both cell lines. In contrast, the simultaneous application of H-1PV and NFX at different concentrations of NFX and different MOIs of H-1PV led to a very efficient killing of PDAC cells exceeding the value expected from an additive effect. This was especially pronounced in Panc-1 cells, where viability was reduced to 35% after combined treatment with otherwise non-toxic doses (100% viability) of NFX (12.5 µg/ml) or H-1PV (MOI = 1 cfu/cell) (Fig. 1a). When the combined regimen was applied to BxPC3 cells, the reduction of viability by H-1PV as monotherapy (MOI = 1 cfu/cell) was enhanced only by 20% upon combination with the non-toxic NFX concentration (12.5 µg/ml) compared to 65% on Panc-1 cells at the same doses (Fig. 1b).

We could not detect any improvement in the replication efficiency of H-1PV in the presence of NFX (data not shown). Therefore, we assumed that the observed synergism between NFX and H-1PV occurs at the level of lysosome permeabilization and stimulation of cathepsin release in the cytoplasm of target cells induced by the two agents. In addition, it is interesting to note that quinolones have been shown to exert immunostimulating effects during antibacterial therapy. Oncolytic viruses can also act as adjuvants boosting anti-tumor immune responses by stimulating the release of tumor-associated antigens and danger signals. It was recently reported that both oncolytic and vaccination events participate in the antitumor effect of H-1PV infection (Raykov *et al.*, 2007). This raises the intriguing possibility that beside direct cytorreduction in tumors, the combination of H-1PV and quinolones may prime the immune system to eradicate treated tumors.

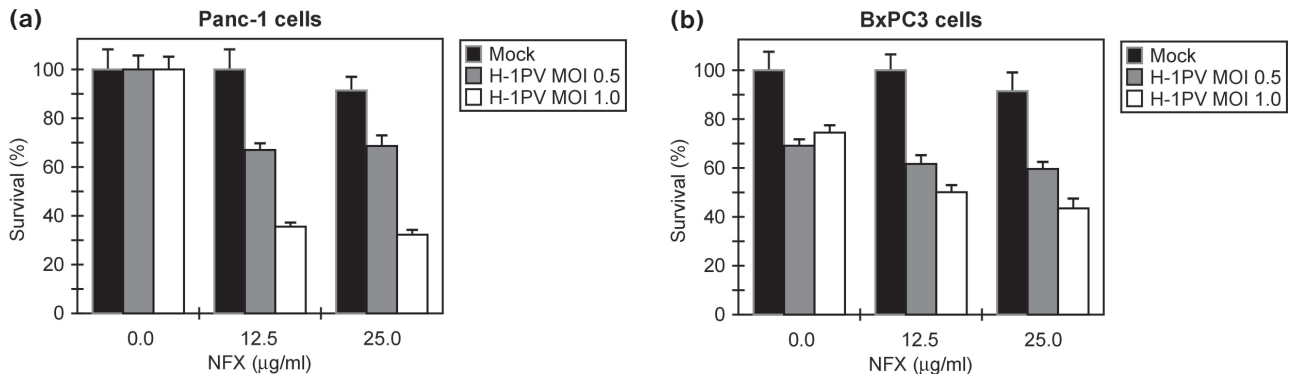
#### *Resveratrol and H-1PV kill PDAC cells additively*

The treatment of BxPC3 and Panc-1 cell lines with resveratrol in concentrations ranging from 0–100 µg/ml in short-term cytotoxic assay proved that resveratrol itself could reduce the viability of both cell lines. The viability of Panc-1 cells was dependent from the dose used. Up to 65% killing was achieved at the highest dose used (Fig. 2a). BxPC3 cells were even more sensitive to the treatment with resveratrol, reaching a killing plateau (90%) over a concentration of 50 µg/ml (Fig. 2b). Simultaneous application of resveratrol and H-1PV at different MOIs increased the fraction of dead Panc-1 cells, but the overall effect of the combination remained additive. This effect was more pronounced at resveratrol concentrations of 12.5 and 25 µg/ml and virus MOI = 0.1 and 1 cfu/cell (Fig. 2a). Viability of BxPC3 cells was already strongly reduced by resveratrol doses exceeding 12.5 µg/ml and simultaneous treatment with the virus could not enhance this effect (Fig. 2b).

Nevertheless, the combination of H-1PV and resveratrol could not reach the levels of synergism achieved with the combination of H-1PV and NFX. However, both treatments

have previously demonstrated to be nontoxic for normal human cells and even higher doses than the presently used can be applied in clinical protocols. We previously reported that oncolytic parvoviruses can be extremely effective, when

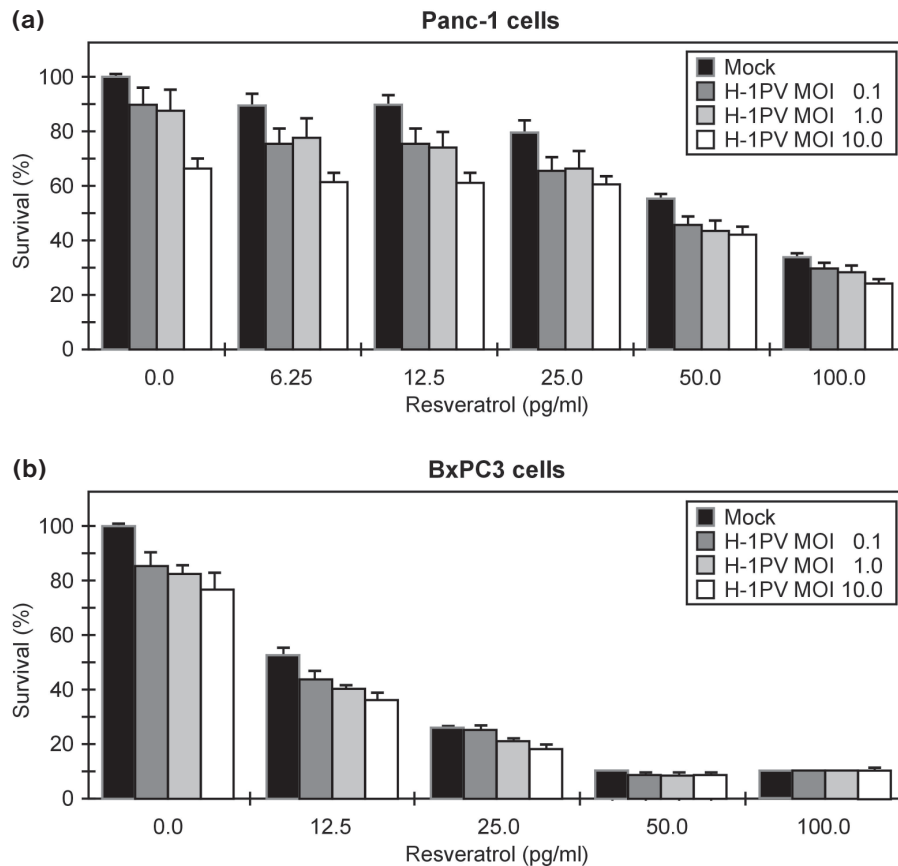
applied together with standard therapeutical methods such as radiotherapy (Angelova *et al.*, 2007). The present study extends these observations to new combinations with non-conventional treatments leading to promising results.



**Fig. 1**

**Toxicity of the combination H-1PV and NFX to PDAC cells**

Survival is expressed as % of control cells.



**Fig. 2**

**Toxicity of the combination H-1 PV and resveratrol to PDAC cells**

Survival is expressed as % of control cells.

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