

Improvement of Gemcitabine-Based Therapy of Pancreatic Carcinoma by Means of Oncolytic Parvovirus H-1PV

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Abstract Pancreatic carcinoma is a gastrointestinal malignancy with poor prognosis. Treatment with gemcitabine, the most potent chemotherapeutic against this cancer up to date, is not curative, and resistance may appear. Complementary treatment with an oncolytic virus, such as the rat parvovirus H-1PV, which is infectious but nonpathogenic in humans, emerges as an innovative option.

Purpose: To prove that combining gemcitabine and H-1PV in a model of pancreatic carcinoma may reduce the dosage of the toxic drug and/or improve the overall anticancer effect.

Experimental Design: Pancreatic tumors were implanted orthotopically in Lewis rats or subcutaneously in nude mice and treated with gemcitabine, H-1PV, or both according to different regimens. Tumor size was monitored by micro-computed tomography, whereas bone marrow, liver, and kidney functions were monitored by measuring clinically relevant markers. Human pancreatic cell lines and gemcitabine-resistant derivatives were tested *in vitro* for sensitivity to H-1PV infection with or without gemcitabine.

Results: *In vitro* studies proved that combining gemcitabine with H-1PV resulted in synergistic cytotoxic effects and achieved an up to 15-fold reduction in the 50% effective concentration of the drug, with drug-resistant cells remaining sensitive to virus killing. Toxicologic screening showed that H-1PV had an excellent safety profile when applied alone or in combination with gemcitabine. The benefits of applying H-1PV as a second-line treatment after gemcitabine included reduction of tumor growth, prolonged survival of the animals, and absence of metastases on CT-scans.

Conclusion: In addition to their potential use as monotherapy for pancreatic cancer, parvoviruses can be best combined with gemcitabine in a two-step protocol.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal gastrointestinal malignancies. It is the fourth most frequent cause of cancer-related deaths in North America, the sixth in Europe, and the fifth in the United Kingdom (1). The disease is highly resistant to current treatments. Surgical resection offers the best long-term survival, but it is feasible in only a minority of patients and is not without risk (2). In

advanced disease, the only option is chemotherapy, particularly with gemcitabine or 5-fluorouracil. However, these drugs display high general toxicity and give only modest results (3, 4). Gemcitabine has been approved by the Food and Drug Administration for first-line therapy of locally advanced or metastatic pancreatic cancer. It is a cell cycle-dependent deoxycytidine analogue of the antimetabolite class, transported into cells through human equilibrative nucleoside transporters and phosphorylated to its active triphosphate form by deoxycytidine kinase. Development of resistance to gemcitabine is a major concern. It can be due to depletion of the intracellular pool of activated gemcitabine through reduced import/phosphorylation of the drug and/or enhanced export from the cell by the ATP-binding cassette transporters and multiple drug resistance proteins (MDR and MRP1/2; ref. 5). Combining gemcitabine with other treatments is explored, with a view either to improve the anticancer effect by eradicating resistant variants or to reduce the dosage of the drug and hence its toxicity.

Cancer therapy with viruses or armed vector derivatives that specifically kill cancer cells (oncolysis) is a novel approach to the treatment of this lethal disease (6). Some autonomous parvoviruses are oncolytic (7). Parvoviruses are small (25-30 nm), nonenveloped particles containing a 5.1-kb single-stranded DNA genome from which two nonstructural proteins (NS1 and NS2) and two viral capsid proteins (VP1 and VP2) are expressed (8). Several rodent parvoviruses (H-1PV, MVM, LuIII) are under consideration for anticancer gene therapy

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Translational Relevance

Despite the undisputable progress in cancer research during the 20th century and the improvement of prognosis for several types of cancer, pancreatic carcinoma remains one of the malignancies having the worst survival rates, with standard treatments such as surgery and gemcitabine not being curative. Oncolytic viruses have been successfully combined with chemotherapeutics, accumulating already a long clinical record. We established that rat parvovirus H-1 could exert strong oncolytic activity against pancreatic cancer cells, being able to synergize with gemcitabine. H-1PV efficiently eradicated drug-resistant variants, opening the possibility to reduce the dose of the chemotherapeutic and hence its toxicity. The virus-gemcitabine combination exhibited an excellent therapeutic and safety profile *in vivo* on delayed administration of the oncolytic agent. We consider that these properties, together with recent endeavors for a phase I clinical trial for glioma treatment, make H-1PV a promising candidate for monotherapy and combined therapy of pancreatic carcinoma in patients.

applications, as they do not transform host cells, infect humans asymptotically, and propagate preferentially in (oncotropism) and kill neoplastically transformed cells (9, 10). MVMp and H-1PV have been shown to exert oncosuppressive activities *in vivo*; they can inhibit formation of spontaneous and chemically or virally induced tumors in animal models. Vectors based on a parvoviral expression cassette retain the oncotropic features of the wild-type viruses (11). Our recent success in unraveling how H-1PV exerts its antiglioma (12) effect has encouraged us to extend our studies to another human malignancy with very poor prognosis, that is PDAC.

Because clinical application of parvoviruses would not be carried out as monotherapy but concomitantly with or following currently used treatments, the present study explores the possibility of combining parvoviral therapy with gemcitabine to improve the therapeutic effect of the drug. *In vitro*, we found that cotreatment with H-1PV and gemcitabine resulted in cumulative toxicity toward PDAC-derived cell lines even when the chemotherapeutic agent was used at high doses and impaired virus replication. The combination of both agents induced an accumulation of functional cathepsin B in the cytoplasm of treated cells to a level not achievable with single treatments. Most interestingly, human PDAC cell variants selected for resistance to the drug remained sensitive to H-1PV killing. *In vivo* experiments showed that intratumoral inoculation of H-1PV 2 weeks after gemcitabine treatment, a plausible clinical scenario, offered a stronger therapeutic effect than gemcitabine alone without added toxicity. This raises hopes of improving patients' survival and quality of life by reducing the dosage of this toxic drug.

Materials and Methods

Cell culture and treatment

Human pancreatic carcinoma cell lines from primary (MiaPaCa-2, Panc-1, and BxPC-3) or metastatic (Colo357, T3M-4, and SU86.86) tumors were obtained from the American Type Culture Collection

and grown in RPMI 1640 supplemented with 10% FCS, penicillin (100 units/mL), and streptomycin (100 µg/mL).

Gemcitabine-resistant Panc-1 and BxPC-3 variants were isolated by selecting cells that survived sequential exposure to increasing doses of the drug (0.4 ng/mL for 2, 12, and 24 h followed by 4 ng/mL for the same times) with 5 rounds of selection at each treatment step (total of 30 cycles).

SV40 T-antigen-transformed 293T (American Type Culture Collection), NBK human kidney cells, and the HA-RPC cell line, derived from a chemically induced PDAC in Lewis rats (13), were grown in DMEM supplemented with 10% FCS and antibiotics. Gemcitabine (Gemzar; Lilly) was applied at the concentrations indicated in the figure legends.

For cytotoxicity assessment, cells were seeded in 96-well plates at a density of 2×10^3 per well and treated as indicated in the figure legends. Cell viability was determined by the colorimetric 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay as recommended by the manufacturer (Sigma).

Microscopy

Cultures were examined using a Leica inverted microscope at a magnification of $\times 40$. Image capture was done using a Leica DFC350 FX camera (Leica Microsystems) and the Leica FireCam software for Macintosh. EGFP fluorescence was measured using a Leica DMRBE fluorescent microscope (Leica) and the analySIS software (Olympus).

Virus production and detection

Wild-type H-1PV was produced by infecting NBK cells purified by iodixanol gradient centrifugation and dialyzed against Ringer solution. The contamination of virus stocks with endotoxins was < 2.5 EU/mL. chi-H-1/EGFP recombinant virus was produced by cotransfecting 293T cells with the corresponding recombinant vector DNA and a helper plasmid expressing the viral capsid genes *in trans* (14). Virus titers were determined as described previously (9) and expressed as replication units (RU)/mL virus suspension. To measure replication centers, serially diluted purified virus was applied to NBK cells. The infected cultures were blotted onto filters 30 h post-infection, and replication centers were detected by hybridization with a virus DNA-specific radioactive probe.

Virus transcription in the organs of treated animals and in PDAC cell cultures was measured as follows. Total RNA was extracted from samples of collected tissues or cell pellets using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA and quantified by reverse transcription-PCR as described previously (15). H-1PV transcripts were detected as 512- and 415-bp PCR fragments depending on the excision of the small intron. The primer pair used was 5'-TCAATGCGCTCACCATCTCTG-3' (forward) and 5'-TCGTAGGCTTCGTGTCT-3' (reverse). The specific primers used to detect cellular mRNAs were 5'-AAAGGAGAGAGCCAAGAGC-3' (forward) and 5'-GGCCCAACAGTCAAAGATA-3' (reverse) for human equilibrative nucleoside transporters and 5'-CCCGCATCAAGAAATCTCC-3' (forward) and 5'-TCCATCCAGTCATGCAGTC-3' (reverse) for deoxycytidine kinase. The primers used to measure expression of human β -actin, MDR, MRP1, and MRP2 genes have been described previously (16).

Animal studies

Anesthesia. All surgical and imaging procedures were done under aerosol anesthesia with 3% isoflurane (Aerrane; Baxter) in pure oxygen. A concomitant intramuscular injection of 2 to 3 mg/kg xylazine hydrochloride (Rompun; Bayer) was used for analgesia in the case of surgery.

Tumor models. Immunocompetent male Lewis rats (Janvier) weighing 180 to 200 g were used for pancreatic carcinoma implantation. A suspension of 5×10^6 cells in 200 µL PBS was prepared from subcutaneous tumors formed by implanted HA-RPC cells and injected into the pancreatic parenchyma. Tumor progression was confined to the pancreatic tail for the first 3 weeks after implantation, leading

to lymph node invasion during the fourth week. Liver metastases appeared after 5 to 6 weeks, and death from lung metastasis occurred at weeks 6 to 9 (17). Tumors in nude mice were induced through subcutaneous inoculation of 2×10^6 BxPC-3 cells. Treatment with virus or gemcitabine was initiated at a tumor size of 150 mm^3 and animals were sacrificed when the tumor masses reached $1,500 \text{ mm}^3$.

In both models, gemcitabine was administered by intraperitoneal injection (100 mg/kg), whereas H-1PV was inoculated intratumorally. In the case of orthotopic tumors, the abdominal cavity of the rats was opened under anesthesia 2 weeks after implantation, and the tumor was located in the proximity of the spleen and injected with either virus or PBS in a volume of 100 μL . The operative wound was subsequently closed with a longitudinal suture. Blood samples were taken from animal tail vein 2 weeks after the last therapeutic treatment (H-1PV or gemcitabine). Toxicologic markers were assayed at Strasbourg University Hospital using an automated multiparametric clinical laboratory analysis device (Biochime ADVIA 160; Siemens).

All animals were kept in isolators under standard conditions (temperature $22 \pm 2^\circ\text{C}$, relative humidity $55 \pm 10\%$, 12 h dark/12 h light cycle) with unrestricted access to a balanced pellet diet and water. Animal experiments were done according to the French and European Community directives for animal care (86/609/EEC, November 24, 1986).

Image acquisition and reconstruction. Images were obtained with an Imtek micro-computed tomography (micro-CT) scanner (microCAT-II; Imtek) using 80 kVp X-ray voltage and 500 μA anode current. Respiratory-gated acquisition avoided changes in abdominal organ position and consequent delineation blurring. Fenestra LC and Fenestra VC contrast agents (Alerion Biomedical) for liver and persistent vascular contrast, respectively, were concomitantly injected intraperitoneally 9 h before imaging. Image data were acquired and reconstructed using Imtek licensed software (Cobra version 4.1-4; Exxim Computing). Three-dimensional images were visualized using Amira software (Amira Advanced Visualization, Data Analysis, and Geometry Reconstruction

version 3.1). Tumors or metastases, which appeared as black defects within the contrasted liver or pancreatic lobe, were measured three-dimensionally using the Amira three-dimensional data set.

Immunohistochemistry

Paraffin-embedded tumor sections were dewaxed with xylene and rehydrated through graded alcohol solutions. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol. To block the nonspecific binding, slides were treated with nonimmune normal rabbit serum (DAKO) for 1 h. After overnight incubation (4°C) with the H-1PV NS1 protein-specific 3D9 antibody (1:50; kind gift from Dr. Nathalie Salomé, German Cancer Research Center), slides were washed and treated with rabbit anti-mouse horseradish peroxidase-labeled secondary antibody (1:200; Sigma), developed using the DAKO Envision+ System (DAKO), and counterstained with Mayer's hematoxylin.

Statistical analyses

In vitro studies. Mean \pm SD were calculated from triplicate *in vitro* experiments. To determine whether H-1PV and gemcitabine interact synergistically *in vitro*, we performed an isobolographic analysis based on the data derived from 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assays as already described for other combinations of chemotherapy and virotherapy (18). The 50% and 75% EC values (EC_{50} and EC_{75}) for gemcitabine and H-1PV were determined empirically based on a range of concentrations (0.4-4,000 ng/mL) and multiplicities of infection (1, 10, and 100 RU/cell), respectively. The isobologram was created from the data obtained for the combination of the two agents. Combination index (CI) and sensitization index (SI) were calculated using the following equations: $\text{CI} = (D_{\text{H1.c}} / D_{\text{H1.a}}) + (D_{\text{G.c}} / D_{\text{G.a}}) + (D_{\text{H1.c}} * D_{\text{G.c}} / D_{\text{H1.a}} * D_{\text{G.a}})$; $\text{SI}_{\text{H-1PV}} = D_{\text{H1.a}} / D_{\text{H1.c}}$; $\text{SI}_{\text{gemcitabine}} = D_{\text{G.a}} / D_{\text{G.c}}$, where $D_{\text{H1.c}}$, $D_{\text{H1.a}}$, $D_{\text{G.c}}$ and $D_{\text{G.a}}$ are the respective $\text{EC}_{50}/\text{EC}_{75}$ doses of H-1PV or gemcitabine, alone (H1.a, G.a), or in combination (H1.c, G.c). CI = 1 represents the conservation isobologram and indicates additive effects. CI < 1 indicates higher than the expected additive effect (synergy).

Table 1. Combined toxic effects of H-1PV and gemcitabine on human PDAC cells

Cell line	EC ₅₀ /EC ₇₅	H-1PV multiplicity of infection	Gemcitabine (ng/mL)	CI	SI _{H-1PV}	SI _{gemcitabine}
BxPC3	EC ₅₀ mono	30	25			
	EC ₅₀ comb 1	1	20	0.86	30	1.3
	EC ₅₀ comb 2	10	7.0	0.71	3	3.6
Colo357	EC ₅₀ mono	35	1.5			
	EC ₅₀ comb 1	1	1.3	0.92	35	1.2
	EC ₅₀ comb 2	10	0.6	0.80	3.5	2.5
MiaPaCa-2*	EC ₇₅ mono	20 (4)	30 (9)			
	EC ₇₅ comb 1	1	18	0.68	20	1.7
	EC ₇₅ comb 2	10	2.0	0.60	2	15.0
Panc-1	EC ₅₀ mono	35	400			
	EC ₅₀ comb 1	1	200	0.54	35	2.0
	EC ₅₀ comb 2	10	60	0.48	3.5	6.7
SU86.86*	EC ₇₅ mono	30 (4)	17 (2.5)			
	EC ₇₅ comb 1	1	8.0	0.52	30	2.1
	EC ₇₅ comb 2	10	1.3	0.44	3	13.1
T3M-4*	EC ₇₅ mono	45 (10)	2.5 (1.2)			
	EC ₇₅ comb 1	1	2.4	1.00	45	1.0
	EC ₇₅ comb 2	10	1.5	0.96	4.5	1.7

NOTE: Cultures were seeded in 96-well plates at 2×10^3 per well, treated with gemcitabine (0.4-4,000 ng/mL), and infected 24 h later with H-1PV at different multiplicities of infection (1, 10, and 100 RU/cell). Cytotoxicity was assessed using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assays 72 h after infection. EC_{50} values for single (mono) and combined (comb) treatments were calculated from isobolograms that were created using MTT measurements obtained at different multiplicities of infection (H-1PV) and concentrations (gemcitabine). CI and SI were determined as described in Materials and Methods.

*Due to the hypersensitivity of indicated cell lines to H-1PV killing, EC_{75} values were used for statistical analyses. The EC_{50} values for H-1PV and gemcitabine monotherapy are given in parentheses.

In vivo studies. Tumor volumes, determined *in vivo* by micro-CT scanning, were tested for statistical differences between animal groups using one-way ANOVA followed by a parametric Student's unpaired *t* test. Rat survival curves were generated according to the Kaplan-Meier method, and differences between animal groups were put to the log-rank test. The InStat 2.00 Macintosh software (GraphPad Software) was used for these analyses. *P* values < 0.05 were considered statistically significant.

Results

H-1PV, both alone and in cooperation with gemcitabine, kills cultured human pancreatic cancer cells, causing a release of active cathepsin B in the cytoplasm. Six human PDAC cell lines, Colo357, T3M-4, SU86.86, MiaPaCa-2, Panc-1, and BxPC-3, were tested for their sensitivity to H-1PV and gemcitabine toxicity (Table 1; Supplementary Fig. S1). MiaPaCa-2, SU86.86, and T3M-4 were found to be hypersensitive to virus-induced killing, whereas Colo357, SU86.86, and T3M-4 were most susceptible to gemcitabine. Importantly, the cell lines that showed stronger resistance to gemcitabine treatment (Panc-1, BxPC-3, and MiaPaCa-2) were sensitive to H-1PV. As indicated in Table 1, the cytotoxic effect of the combined therapy was in most cases synergistic (*CI* < 1), particularly in MiaPaCa-2, Panc-1 and SU86.86 cells. The effective virus dose could be reduced by a factor (*SI*_{H-1PV}) of up to 35-fold in the presence of gemcitabine doses ranging from 1.3 ng/mL (Colo357, combination 1) to 200 ng/mL (Panc-1, combination 1). Conversely, the effective drug concentration required to inhibit cell proliferation was reduced by a factor (*SI*_{gemcitabine}) of up to 15-fold when the cells were infected with H-1PV (MiaPaCa-2,

combination 2). The ability of all cell lines to form colonies over a 2-week period was also efficiently inhibited by both H-1PV and gemcitabine (data not shown).

We reported previously that the toxic effects of H-1PV on human glioma cells (12) were mainly due to the cytosolic relocation of lysosomal proteases, particularly cathepsin B. These data prompted us to determine whether the subcellular distribution of cathepsin B activity in PDAC-derived lines was also altered after the above treatments. This was tested using three human PDAC cell lines. As shown in Supplementary Fig. S2, the cytosolic accumulation of functional cathepsin B was significantly enhanced by the H-1PV/gemcitabine combination, strongly suggesting that this mechanism might participate in the observed cumulative toxicity.

H-1PV can kill both gemcitabine-sensitive and gemcitabine-resistant cells with similar efficiency. Because the development of resistance to gemcitabine is a major drawback of long-term treatment of PDAC patients with this drug, we tested the cytopathic effects of H-1PV on gemcitabine-resistant cell variants derived from two of the above-mentioned lines (Panc-1 and BxPC-3) that differed in their natural sensitivity to gemcitabine (Table 1). Resistant populations were isolated by sequentially treating cells with increasing doses of the drug. Resistant variants were distinguishable from the respective parental cell lines by their slower growth and stable phenotypic changes, such as enhanced expression of the MDR and MRP1/2 drug-export markers (Fig. 1A). As the levels of import (human equilibrative nucleoside transporters) and activation (deoxycytidine kinase) markers remained unchanged or slightly reduced (Fig. 1A), it seems that the resistant phenotype was mostly due to more intensive gemcitabine export. The

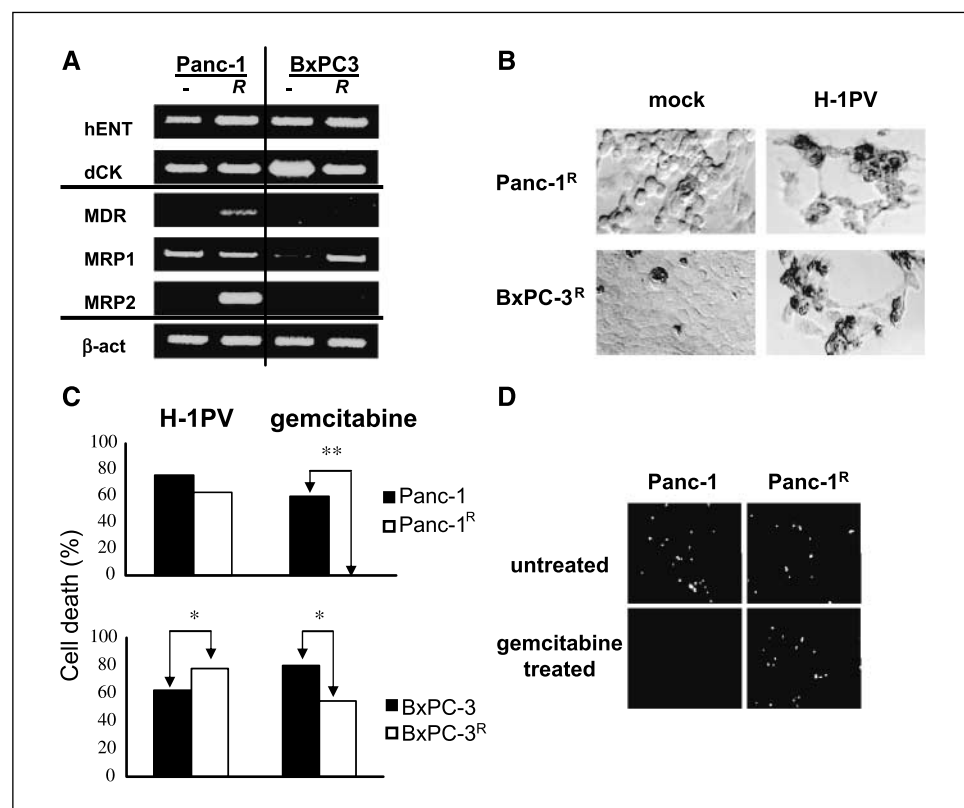


Fig. 1. Characterization of gemcitabine-resistant cell lines and their sensitivity to H-1PV. **A**, expression of drug resistance markers in parental (-) and Panc-1^R and BxPC-3^R cells as measured by reverse transcription-PCR. β -Actin transcript levels served as a reference. **B**, micrographs of Panc-1^R or BxPC-3^R cells infected (H-1PV) or not (mock) with H-1PV at multiplicity of infection = 10 RU/cell. Magnification, $\times 40$. **C**, sensitivity of Panc-1^R and parental Panc-1 (*top*) and BxPC-3^R (*bottom*) cells to H-1PV versus gemcitabine treatment. Cells were seeded at a density of 2×10^3 per well in 96-well plates and treated either with H-1PV (10 RU/cell, H-1PV columns) or gemcitabine (40 ng/mL, gemcitabine columns), 3-(4,5-Dimethylthiazolyl)-2,5-diphenyltetrazolium bromide cytotoxicity assays were done 144 h post-treatment. Results of three independent experiments. *, *P* < 0.05; **, *P* < 0.005, statistically significant differences. **D**, EGFP transduction by a recombinant H-1PV into Panc-1 and Panc-1^R cells. Cultures were treated (*bottom*) or not (*top*) with gemcitabine (40 ng/mL) and concomitantly infected with the viral vector (5 RU/cell). Cells expressing the transduced gene were detected by fluorescence microscopy at 48 h post-infection. Representative fields.

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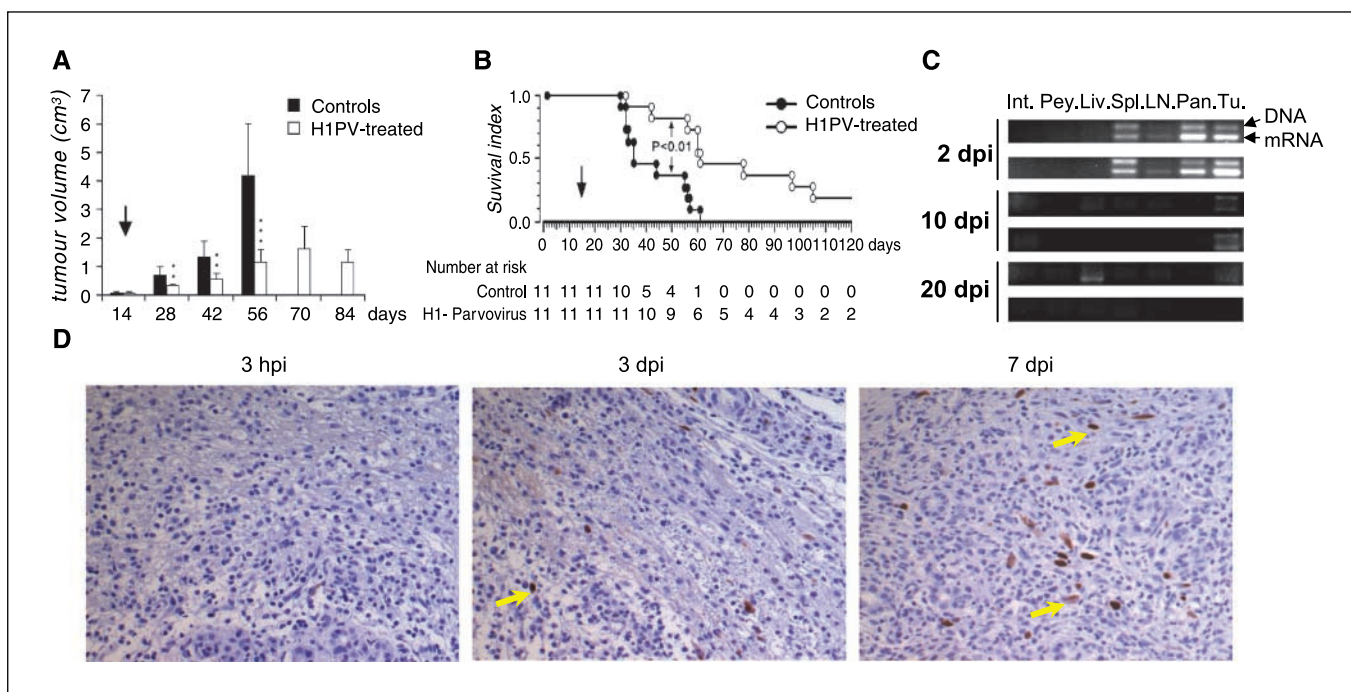


Fig. 2. Effect of H-1PV on a rat model of PDAC formation. Rats ($n = 26$) received an intrapancreatic injection of a cell suspension deriving from subcutaneous HA-RPC tumors, and the developing neoplasias were treated 2 wk later by intratumoral inoculation of 1×10^9 RU H-1PV ($n = 16$) or mock-treated ($n = 10$). **A**, growth of H-1PV versus mock-treated tumors. Tumor volumes were measured as a function of time by micro-CT scanning. Mean \pm SD. **B**, survival of H-1PV versus mock-treated animals. Ten rats of each group were monitored over 120 d, at which time the experiment was terminated. **C**, distribution of H-1PV expression in tumor-bearing rats. Two rats in the H-1PV-treated group were sacrificed on days 2, 10, and 20 after infection, and their organs were processed for reverse transcription-PCR detection of viral transcripts. PCR products corresponding to viral DNA and precursor RNA (DNA/RNA) and mRNA are shown for the intestine (*Int*), Peyer's patches (*Pey*), liver (*Liv*), spleen (*Spl*), lymph nodes (*LN*), pancreas (*Pan*), and tumor (*Tu*). **D**, immunohistochemical detection of H-1PV NS1 protein (arrows) in infected pancreatic tumors. Three PDAC-bearing rats were sacrificed at 3 h, 3 d, and 7 d after intratumoral inoculation. Immunohistochemistry was done on paraffin-embedded tumor sections Magnification, $\times 20$.

drug-resistant variants remained sensitive to H-1PV infection (Fig. 1B and C, H-1PV column), whereas they resisted an extended incubation with gemcitabine (40 ng/mL for 144 h) that was toxic for the original lines (Fig. 1C, gemcitabine column). A slight but significant increase in H-1PV-induced killing was even observed in BxPC-3^R versus BxPC-3 cells. This suggests that H-1PV might be used as a second-line treatment of PDAC to circumvent acquired resistance to gemcitabine. Furthermore, the gemcitabine-resistant phenotype was found to correlate with reduced interference of toxic drug doses with progression of the parvoviral lifecycle. Indeed, marker-protein EGFP expression, driven by a recombinant parvoviral vector, persisted in Panc-1^R cells even after they were exposed to a gemcitabine dose abolishing EGFP transduction in parental cells (Fig. 1D). We conclude that chemoresistant tumor cell variants remain targets of H-1PV even under conditions in which gemcitabine therapy is continued. Efficient virus killing was similarly observed after infection of gemcitabine-resistant Colo357 and T3M-4 cells selected using short-term treatment with high gemcitabine doses (Supplementary Fig. S3). In agreement with these data, the resistant and parental Colo357 and T3M-4 lines had a similar capacity for virus replication (data not shown).

Altogether, *in vitro* experiments suggest that H-1PV may improve the therapeutic effect of gemcitabine both by reinforcing the overall killing of drug-sensitive cells and by eradicating chemoresistant variants emerging at late stages of drug treatment. This prompted us to evaluate the anti-PDAC potential of H-1PV *in vivo*.

H-1PV induces partial to full suppression of orthotopic pancreatic tumors, prolonging animal survival. To mimic the clinical situation more closely, a syngeneic rat model of orthotopically implanted PDAC was used to evaluate the anticancer activity of H-1PV. Because the rat is the natural host of H-1PV, the system is also suitable for toxicologic assessment of this oncolytic agent, another prerequisite to its clinical application. The rat PDAC cells used in the model (HA-RPC) were first tested *in vitro* for their susceptibility to H-1PV infection and were proven to have the same range of sensitivity to virus and gemcitabine toxicity as the above-mentioned human cells (Supplementary Fig. S4A).

H-1PV was then administered *in vivo* through a single intratumoral injection 2 weeks after implantation of HA-RPC cells into the pancreas. Tumor size (measured by micro-CT scanning and macroscopic inspection after death), animal survival, and virus distribution were determined. Virotherapy caused a delay in tumor growth (Fig. 2A), and as illustrated in Fig. 2B, rats in the virus-treated group survived significantly longer than the mock-treated controls, with 20% remaining disease-free for 16 weeks (until the end of the experiment). Importantly, H-1PV was expressed selectively in tumors as opposed to normal tissues. To confirm this, we tested visceral organs for the presence of virus transcripts (by reverse transcription-PCR). As shown in Fig. 2C, we observed an initial burst of virus expression shortly after infection in the tumor and the surrounding pancreatic tissue. In agreement with our previous observations in other models, H-1PV was also distributed to lymphoid organs (15). From day 10 on,

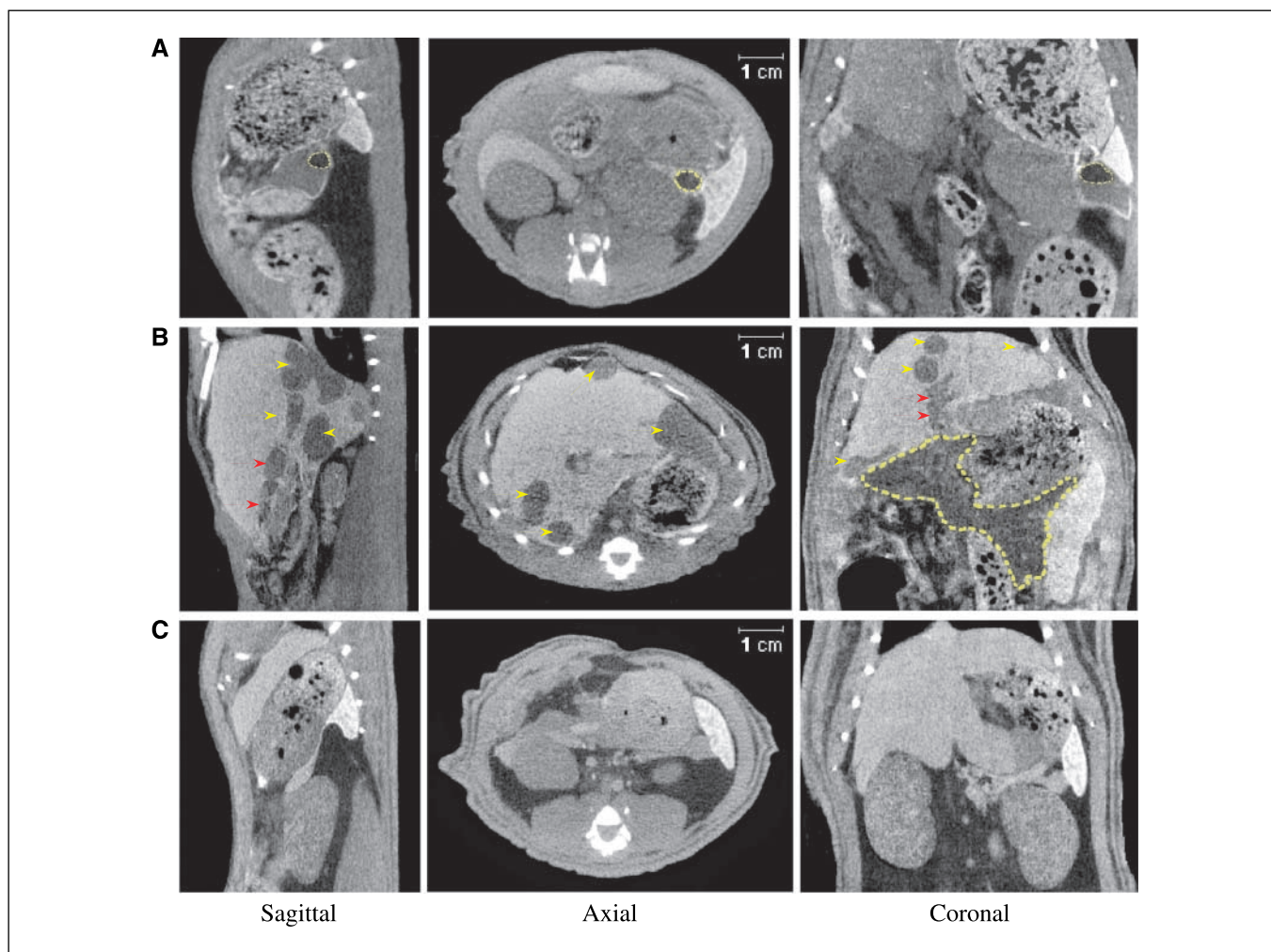


Fig. 3. micro-CT imaging of orthotopic pancreatic tumors. *A*, abdominal CTscans of a rat at an early stage (2 wk) after tumor initiation, showing a tumor ~5 mm in diameter (dotted lines) in the tail of the pancreas. *B*, evolution of PDAC in the absence of virotherapy, with a large primary tumor mass (dotted lines) and metastases in the lymph nodes and liver (arrows) 8 wk post-initiation. *C*, regression of primary tumor and absence of metastases in a rat treated with H-1PV and examined 2 and 8 wk post-initiation. Sagittal, axial, and coronal micro-CTscan images were selected to illustrate the localization of primary tumors and metastases.

virus expression faded most likely due to the appearance of virus-neutralizing antibodies reducing virus spread (19). However, it persisted in the tumor for up to 20 days post-inoculation. In addition, the intratumoral dissemination of the virus could be confirmed by immunohistochemical analysis (Fig. 2D).

It has to be noted that, in some cases, complete disappearance of preexisting tumors was observed on micro-CT scans (compare Fig. 3A and C). Besides local expansion of the primary tumor, lymphogenous and hematogenous metastases affecting the visceral lymph nodes of the upper abdominal cavity and liver, respectively, play a major role in PDAC mortality. As expected, micro-CT monitoring of uninfected rats revealed metastatic invasion of local pancreatic, pyloric, and hepatic lymph nodes and of the liver (Fig. 3B, arrows). Inoculation of the primary tumor with H-1PV at an early stage (corresponding to the Fig. 3A images) resulted in 45% suppression of distant metastases as well as of the primary tumor at a later time (Fig. 3C). Interestingly, the spreading of metastatic disease to the liver in this model correlated with late

virus expression in the organ (Fig. 2C), suggesting that H-1PV can actively control tumor invasion.

H-1PV suppresses PDAC tumors escaping gemcitabine treatment. We then investigated whether H-1PV can enhance the therapeutic effect of gemcitabine *in vivo* as observed *in vitro* (Table 1; Supplementary Fig. S1). To mimic a clinically plausible scenario, PDAC-bearing rats were first treated with gemcitabine, and 2 weeks later, the tumor was inoculated intraoperatively with H-1PV. As depicted in Fig. 4A (gemcitabine pre-H-1PV), this led to significantly prolonged survival of the animals compared with mock treatment (control) or monotherapy with the chemotherapeutic (gemcitabine). When both agents were applied simultaneously (H-1PV and gemcitabine), H-1PV failed to improve the therapeutic effect of gemcitabine. This is probably due to negative interference of the genotoxic drug with the parvoviral lifecycle (see also Fig. 1D).

To test the effectiveness of gemcitabine and H-1PV in a different *in vivo* model using human cells, BxPC-3 tumors were induced in nude mice and treated with a similar regimen as the

one used in rats (Fig. 4B). All animals receiving therapy had a prolonged survival compared with controls. Combined treatment (gemcitabine pre-H-1PV, $n = 5$) and H-1PV monotherapy ($n = 5$) achieved the highest level of antitumor protection, with one animal from each of these groups remaining tumor-free up to 70 days after the beginning of the experiment. At this time, 40% of the animals from the combined treatment group were still alive, whereas all control mice had to be sacrificed by 40 days post-implantation.

Altogether, these data confirm that gemcitabine provides transient protection against PDAC and that subsequent administration of H-1PV can extend the animals' survival. Furthermore, toxicologic assessment of the therapeutic regimens was carried out in the rat model, indicating that the blood-borne markers of bone marrow activity were largely unaffected (Fig. 5A), apart from a drop in reticulocyte and monocyte levels due to gemcitabine treatment. Clinical reports prompted us to monitor the liver and kidney functions as well (Fig. 5B). The bilirubin, aspartate aminotransferase, and alanine aminotransferase levels were elevated in the untreated and gemcitabine-treated groups, revealing a low-grade lytic process in the livers of PDAC-bearing rats. Additional parvovirus therapy restored these markers to levels within the physiologic range. Creatinine levels remained stable, showing unaffected

kidney clearance. In conclusion, the detected blood variable anomalies were fully attributable to gemcitabine treatment and were not aggravated by subsequent H-1PV administration.

Discussion

We have shown that parvovirus H-1PV monotherapy has anti-PDAC potential, because the virus can kill a panel of PDAC cell lines *in vitro* and suppress primary and metastatic pancreatic cancer in animal models. Furthermore, H-1PV can synergize with gemcitabine, a standard anti-PDAC chemotherapeutic agent, and boost its effect when administered after the drug. This regimen has two major advantages: the virus shows no added general toxicity, improving in this way the therapeutic index, and suppresses PDAC cells escaping gemcitabine treatment.

Gemcitabine is a known inducer of apoptosis, and drug-resistant cells are suggested to have altered expression of antiapoptotic genes (20). Some oncolytic viruses (e.g., herpes simplex or adenovirus) and vector derivatives armed with suicide or tumor suppressor genes have been combined with chemotherapy and radiotherapy in attempts to enhance apoptosis in pancreatic cancer cells (21–23). Notably, parvovirus H-1PV has the unique advantage of triggering, at least in brain and some other tumors, a distinct death process: cytosolic relocation and activation of lysosomal proteases (cathepsins; ref. 12). This mechanism, which does not depend on caspase-3 activation, should circumvent any antiapoptotic features acquired during PDAC progression. Similarly, a cytosolic relocation of cathepsins was detected in the PDAC cell lines tested after H-1PV infection. Interestingly, this change was significantly enhanced in cells cotreated with gemcitabine, also under conditions in which the drug alone had little effect. These observations are in keeping with the lack of cross-resistance to gemcitabine and H-1PV and the enhanced efficacy of dual therapy. Furthermore, because gemcitabine-resistant cells show enhanced gemcitabine export, they may constitute a refuge for H-1PV from the negative influence of the drug. Subsequent oncolysis of these cells should result in release of a burst of infectious progeny particles that can further spread to neighboring cells from the residual or relapsing tumor.

H-1PV administration as a second-line treatment after gemcitabine seems compatible with the clinical situation where the drug is used as a standard chemotherapeutic for PDAC patients. The frequent relapse observed after chemotherapy, resulting in tumors resisting all conventional treatments, makes it imperative to devise innovative therapies. In this regard, oncolytic H-1PV stands as a potential candidate. A promising clinical scenario might be to inoculate patients with this virus perioperatively or during fibroscopic examination, a few days after the course of gemcitabine infusions. Clinical trials involving the endoscopically controlled delivery of ONYX-15 adenovirus in combination with gemcitabine failed to reveal a major therapeutic improvement but showed the safety of the combined treatment (6). This makes a similar approach with parvoviruses seem feasible.

An advantage of the rat PDAC model used here is that tumors are initiated using a suspension of cells that derive from a subcutaneously developed neoplasia containing both malignant and stromal cells. Furthermore, the neoplastic process takes place in its natural environment (in the pancreas) and progresses to the formation of metastases in clinically relevant

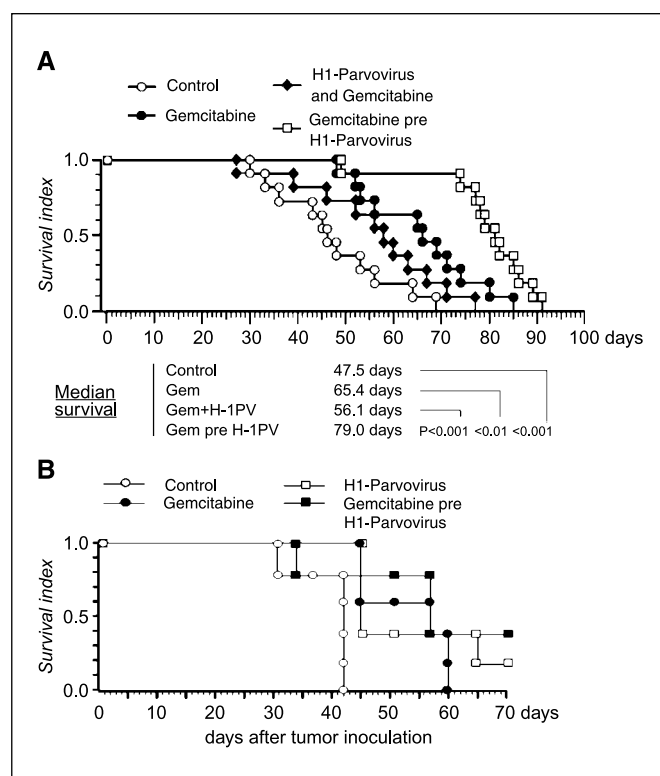


Fig. 4. Survival of tumor-bearing animals after combined treatment with gemcitabine and parvovirus. *A*, rats bearing intrapancreatic tumors were divided into four groups ($n = 11$) and treated with PBS (control), gemcitabine alone (gemcitabine), gemcitabine and virus simultaneously (H-1PV and gemcitabine), or gemcitabine first and H-1PV 14 d later (gemcitabine pre-H-1PV). Animal survival was monitored over a period of 100 d and is presented as Kaplan-Meier curves, with the median survival and P values indicated below. *B*, nude mice bearing subcutaneous BxPC-3 tumors were divided into four groups ($n = 5$) and treated 21 d after tumor initiation with PBS (control), gemcitabine alone (gemcitabine), virus (3×10^8 RU) alone (H-1PV), or gemcitabine first and H-1PV 7 d later (gemcitabine pre-H-1PV).

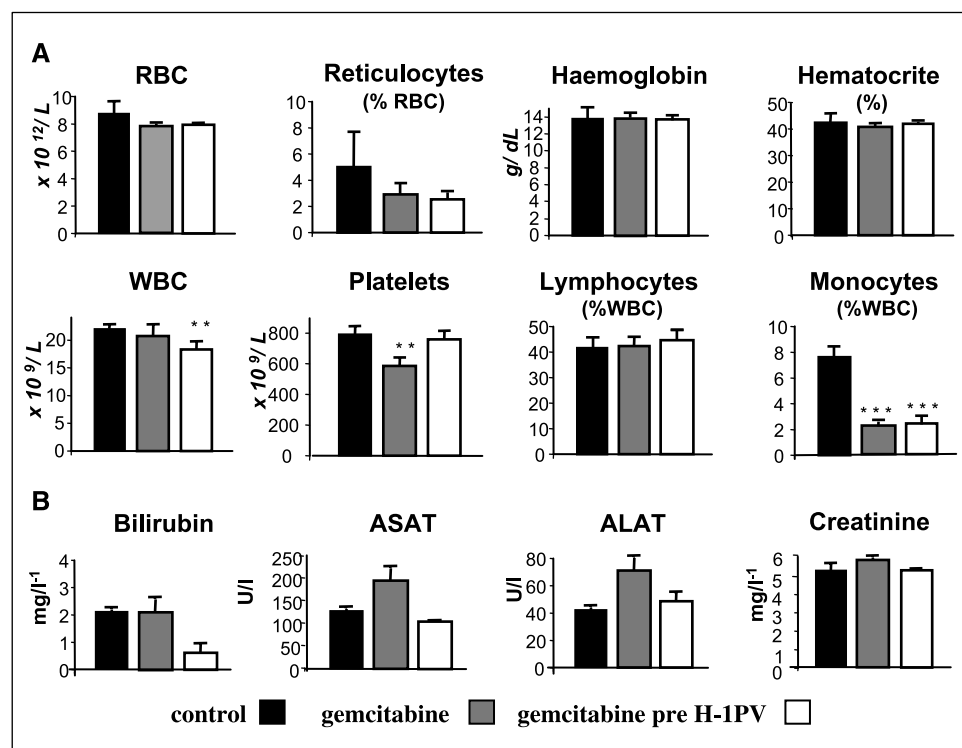


Fig. 5. Toxicologic assessment of the gemcitabine and H-1PV combination. Blood was collected from three PDAC-bearing rats each of the control, gemcitabine, and gemcitabine pre-H-1PV groups (see Fig. 4A) 2 wk after the last therapeutic treatment. Blood samples were analyzed for (A) RBC, platelet, and WBC counts and related variables and (B) liver (aspartate aminotransferase and alanine aminotransferase) and kidney (creatinine) markers. Mean \pm SD.

organs, particularly the liver. This progression correlates with the tissue distribution of H-1PV gene expression, which is mainly in the pancreas a few days post-infection and does not become detectable in the liver until 10 to 20 days later. This is in keeping with the known oncotropism of rodent parvoviruses (7, 15). A recognized drawback of virotherapy lies in the limitations to virus spreading through the stromal matrix of tumors, which applies in particular to collagen-rich pancreatic lesions. The small size of parvoviruses (20-30 nm) should give them an advantage in this respect over most of the viruses presently used for cancer therapy (60 to >300 nm). This was actually supported by evidence of time-dependent propagation of H-1PV infection through rat PDAC lesions, as observed by immunohistochemical analysis (Fig. 2D), in the presence of detectable stromal reaction (Supplementary Fig. S4B). The latter was additionally confirmed by Aniline blue staining (data not shown).

Furthermore, the rat model is suitable for determining the safety profile of H-1PV in its natural host species. Whether administered as monotherapy or in combination with gemcitabine, H-1PV failed to induce any pathologic signs, the toxic effects seen after the combined treatment being fully attributable to gemcitabine. Because both H-1PV and gemcitabine kill PDAC cells, and H-1PV shows no pathogenicity, combining both agents should improve the therapeutic index by reducing drug dosage and drug-related side effects.

The immune response of the host contributes significantly to the outcome of oncolytic virotherapy (24). Tumor cells infected with an oncolytic agent are also targeted by the immune system, thereby constituting an autologous vaccine with the virus acting as an adjuvant. This may account, at least in part, for the lower protective effect achieved in the present study with a single H-1PV application in the nude mouse model relative to the immunocompetent rat system, although no direct compar-

ison can be made due to the use of different tumor cells. Immunostimulation by H-1PV has recently been shown, enhancing the therapeutic vaccination effect of lysed tumor cells (25). Chemotherapy is generally toxic and unfavorable to immunotherapy, but gemcitabine, unlike most nucleoside analogues, lacks any straightforward immunosuppressive properties (26). Instead, this drug exhibits immunomodulating features, causing reduction of B-cell and antibody reactions with a shift toward a Th1 antitumor response (27). Thus, the influence of gemcitabine on the cellular and humoral immune components may actually be quite beneficial to the outcome of oncolytic virus infection through reduced production of virus-neutralizing antibodies and enhanced CTL-mediated antitumor effects. Therefore, combining gemcitabine with oncolytic viruses for the treatment of pancreatic cancer seems even more promising. We are currently investigating the plausibility of this exciting scenario.

In conclusion, the oncolytic properties and safety profile of H-1PV make this virus a promising candidate for monotherapy of pancreatic carcinoma. Furthermore, delayed administration of the oncolytic agent after gemcitabine significantly improves the overall anticancer effect, implying that drug-resistant cells are targeted by the parvovirus.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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