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PNC-28, a p53-derived peptide that is cytotoxic to cancer cells, blocks pancreatic cancer cell growth *in vivo*

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PNC-28 is a p53 peptide from its mdm-2-binding domain (residues 17–26), which contains the penetratin sequence enabling cell penetration on its carboxyl terminal end. We have found that this peptide induces necrosis, but not apoptosis, of a variety of human tumor cell lines, including several with homozygous deletion of p53, and a ras-transformed rat acinar pancreatic carcinoma cell line, BMRPA1. Tuc3. On the other hand, PNC-28 has no effect on untransformed cells, such as rat pancreatic acinar cells, BMRPA1, and human breast epithelial cells and no effect on the differentiation of human stem cells. In this study, we now test PNC-28 *in vivo* for its ability to block the growth of BMRPA1. Tuc3 cells. When administered over a 2-week period in the peritoneal cavities of nude mice containing simultaneously transplanted tumors, PNC-28 causes complete destruction of these tumors. When delivered concurrently with tumor explantation at a remote site, PNC-28 causes a complete blockade of any tumor growth during its 2-week period of administration and 2 weeks posttreatment, followed by weak tumor growth that plateaus at low tumor sizes compared with tumor growth in the presence of a control peptide. When administered after tumor growth has occurred at a site remote from the tumor, PNC-28 causes a decrease in tumor size followed by a slow increase in tumor growth that is significantly slower than growth in the presence of control peptide. These results suggest that PNC-28 may be effective in treating cancers especially if delivered directly to the tumor.

Key words: PNC-28 peptide; p53; pancreatic cancer; nude mice

Activation of latent wild-type p53 in cancer cells is an emerging strategy in the treatment of human cancers.[1] p53 is known to bind to the mdm-2 (hdm-2 in humans) protein, that targets p53 for ubiquitination and ultimate proteosomal degradation,[1] using residues 12–27 in its trans-activating amino terminal regulatory domain.[2] The X-ray crystal structure of the p53 12–27 fragment bound to the p53-binding domain of hdm-2 has been determined[3,4] and shows that the p53 peptide forms an amphipathic alpha-helix and binds with residues Phe 19, Trp 23 and Leu 26 to a deep hydrophobic pocket of hdm-2.[3,4] Since the hdm-2 binding domain of p53 contains many conserved residues and since the 12–27 p53 peptide forms a stable, structurally well-defined complex with hdm-2, several groups have proposed to utilize this p53 segment or analogues of it to block the p53–hdm-2 interaction[5,6] thereby prolonging the half-life of wild-type p53. A study utilizing *in vitro* binding[5] or phage-display peptide libraries[6] has found that the p53 12–26 peptide blocks the p53–hdm-2 interaction and that other homologous peptides are even more effective than the parent p53 peptide in blocking this interaction.[6]

Modified peptides and small molecules that block p53–hdm-2 interactions, with affinities for hdm-2 several thousand-fold greater than that of the parent p53 sequence, have been introduced into human cancer cells containing wild-type p53, many of which overexpress hdm-2, and have been found to induce p53-dependent apoptosis.[7–11] In several of these studies (*e.g.*, Ref. 7), use of peptides that block the p53–hdm-2 interaction were found not to induce cell death in p53-null cells, such as the SAOS2 osteogenic sarcoma cell line, consistent with their predicted mechanism of action, prolongation of the half-life of wild-type p53. Recently, using a combined chemical library in a rapid throughput assay for agents that block p53–hdm-2 interactions, a class of imidazoline compounds (nutlins) was found to block p53–hdm-2 interaction with IC50's in the 100 nM range, the most powerful of which was nutlin-3. This compound induced apoptosis of cells containing wild-type p53 (like HCT116) but had no effect on the proliferation of cell lines that were p53-null or that contained mutant p53.[11]

High affinity peptides and small molecules, like nutlin-3, have further been tested on wild-type p53-containing tumor xenografts in animal models. Nutlin-3 was found to reduce by 90% the growth of tumors containing wild-type p53 explanted into nude mice. In another study, a peptide was synthesized containing 12 residues (the exact sequence was not specified) from the mdm-2 binding domain of p53 and an amino terminal membrane-penetrating sequence from the tat protein enabling cell penetration and was found to induce apoptosis of uveal melanoma and retinoblastoma cell lines explanted into the anterior chambers of rabbit eyes but had only minimal effects on their normal cell counterparts.[10] All of these cell lines tested contained wild-type p53, which presumably activates apoptosis of the cancer cells; while the active peptide was not tested against p53-null cells, it was found to induce apoptosis in the cervical cell line, C33A, that contains mutant p53.[10]

In a series of studies,[12–15] we have found that peptides corresponding to residues 12–26, 12–18 and 17–26 of the hdm-2-binding domain of p53 attached to the membrane-penetrating antenna-

This paper is dedicated to the memories of Paula and Richard Schroeder, who both devoted their lives to the development of new methods for the early detection and effective treatment of pancreatic cancer and greatly aided our efforts in both endeavors.

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pedia penetratin sequence are cytotoxic to a variety of human cancer cells. We assumed that these peptides would bind to hdm-2 and so placed the highly positively charged penetratin sequence on their carboxyl terminal ends where they would stabilize alpha-helical formation[12] as observed in the X-ray structure of p53 12–27 peptide bound to the hdm-2 fragment.[3,4] Each of these p53 peptides, 12–26, 12–18 and 17–26, attached to penetratin is termed PNC-27, PNC-21 and PNC-28, respectively. PNC-28 (p53 residues 17–26-penetratin) is cytotoxic to a rat pancreatic cancer cell line, called BMRPA1.Tuc3,[12,16,17] that we have developed by transfecting the Ki-ras oncogene into a normal pancreatic acinar cell line, established in our laboratory and called BMRPA1.[12,16] Treatment of BMRPA1.Tuc3 cells for 3 days with PNC-28 results in 90–100% cell killing while treatment of BMRPA1 cells over the same time period results in no cell death and no inhibition of the growth of these cells into contact-inhibited monolayers.[12] These peptides have no effect on the viability or on the growth of normal cells, including human hematopoietic stem cells, which form colonies in response to growth factors whether or not these peptides are present.[12] In contrast, but as expected from our structural considerations,[12] the p53 17–26 peptide linked to penetratin on its amino terminal end, was only about 5% as effective in inducing cell killing as PNC-28 with penetratin on its carboxyl terminal end (Refs. 12,13; Kanovsky M., Michl J. and Pincus M.R., unpublished observations).

Surprisingly, in contrast to the results of other studies using p53 peptides and peptidomimetics,[5,7–11] BMRPA1.Tuc3 cells treated with PNC-28 do not express high levels of any of the protein markers for p53-induced apoptosis and cell cycle arrest, including BAX, waf-p21 and caspases.[12,13] The DNA from these cells is fragmented and does not form the ''ladder pattern'' typical of DNA from cells undergoing apoptosis.[12] In contrast to the findings in the other studies discussed above,[5,7–11] PNC-27 and PNC-28 were cytotoxic to several different known human p53-null cancer cell lines including MDA-MB-453 human breast cancer, H1299 human colon cancer, SAOS2 human osteosarcoma and SW1417 human colon cancer cell lines.[12] (The latter were obtained from the American Type Culture Collection as p53-null cells were found to express no p53 in blots using polyclonal anti-p53 antibody[12]). These findings suggested that PNC-28 induces cancer cell cytotoxicity by mechanisms that are at least partially p53-independent.

These results were further corroborated in studies on a series of breast cancer cell lines using PNC-27 and PNC-28.[13] Both peptides induced rapid total cell necrosis (within 1 hr) of a number of breast cancer cell lines but did not affect the untransformed MCF-10-2A breast epithelial cell line.[13] In the breast cancer cell lines treated with these peptides, there was no elevation of caspases, as would be expected for cells undergoing apoptosis, consistent with our findings in the BMRPA1.tuc3 cell line, but there was a marked elevation of LDH in the cell media, suggesting necrosis as the mechanism of action of these peptides. Time-lapse electron micrographic studies of MDA-MB-468 breast cancer cells treated with PNC-27 revealed the development of pores in both cell and nuclear membranes[13] suggesting lysis of the cell membrane as an important component in the mechanism of action of these peptides. That membrane damage may be an important component of the cytotoxic effect of these peptides is further suggested by the 3D structure of PNC-27. Using 2D NMR, we have found that this peptide forms an amphipathic structure consisting of 2 alpha-helices in the p53 peptide segment and the penetratin segment, separated by a loop, a motif that has been described for several membrane-lytic peptides such as melittin and maganin.[14,15] These results all suggest that our peptides, in contrast to the agents that block p53–hdm-2 interactions,[5,7–11] induce necrosis of cancer cells selectively using mechanisms that are largely independent of classical apoptotic pathways induced by activated p53 protein and its ''surrogates'' such as p63 and p73 proteins[1] and that involve, at least in part, damage to cancer cell membranes.

Since our peptides are cytotoxic to cancer cells but not to normal cells, we have elected to test one of these peptides, PNC-28, the shorter of the 2 effective p53–penetratin peptides, for its ability to block tumor cell growth in a nude mouse animal model. In these studies, we have xenotransplanted BMRPA1.TUC3 pancreatic cancer cells into nude mice and have infused PNC-28 or a control peptide into these mice to determine if PNC-28 specifically blocks tumor growth *in vivo*.

Material and methods

Peptides

PNC-28 peptide-containing residues 17–26 of human p53 (bold) attached to penetratin (italics), whose sequence is **ETFSDLWKL-L***KKWKMRRNQFWVKVQRG* and a negative control peptide, called PNC-29, which consists of a peptide from cytochrome P450 (also called X13) (bold) attached to penetratin (italics), whose sequence is **MPFSTGKRIMLGE***KKWKMRRNQFWVKVQRG*, were synthesized using solid phase methods by the Biopeptide Company (La Jolla, CA). On the day of the implantation of the mini-osmotic pumps, which are described later, the peptides were dissolved in sterile PBS at the concentrations defined later for the experiments. Once the peptides had apparently dissolved, the peptide solutions were sonicated twice for 30 sec in a sonicator bath followed by sterilization by passing the solution through a 0.22-μm syringe filter (Millipore, Billerica, MA). The sterile peptide solution was then dispensed under sterile conditions into each pump (described later) (0.095 ml/14 days pump) using the specially designed needle provided by the company.

Pancreatic carcinoma cells

BMRPA1.Tuc3 cells, rodent pancreatic carcinoma cells, were derived from BMRPA1 cells by transfection with a plasmid containing an activated human K-ras oncogene (single base mutation at codon 12, valine substitution for the wild-type glycine in the ras protein [K-rasval12, a kind gift of Dr. M. Perucho (IBR, La Jolla, CA)]).[12,17] In contrast to BMRPA1 cells, which are untransformed, rat pancreatic acinar cells expressing a normal epithelial phenotype and differentiated acinar cell functions including a normal response to secretagogue,[12,16] BRMPA1.Tuc3 cells display a transformed cellular phenotype, grow significantly faster than their parent cells, form colonies *in vitro* and tumors *in vivo* in Nu/Nu mice (Refs. 12,17; Bradu S., Huynh R., Akman H.O., Zheng T., Bao L-Y., Pincus M.R. and Michl J. manuscript in preparation). BMRPA1.Tuc3 cells were maintained in culture in cRPMI.[12,16–18] Cells were passaged by release from their culture dish surface by trypsin-EDTA (GIBCO), and their cell number adjusted to 10^7–10^8 live cells/ml for xenotransplantation by injection of 0.1 ml of the respective cell concentration into Nu/Nu mice (see below).

In some experiments, cells were retrieved from sterile collected ascites and phosphate buffered saline (PBS) washes, respectively, of the peritoneal cavities of the mice.[19] The cells present in the ascites and the PBS washes were pelletized by centrifugation, washed with sterile PBS, and 2×10^6 nucleated cells were dispersed into sterile tissue culture dishes (100 mm diameter). Within 24 hr after seeding, nonadherent cells were washed off and the adherent cells continued in culture for up to 1 week. Cells were observed for growth and morphology.

Mice

Hsd:Athymic Nu/Nu mice of 7–8 wks (22–24 g) were obtained from Harlan-Sprague Dawley (Indianapolis, IN). The mice were held in an aseptic room inside BioIncubator units.

Tumor growth inhibition studies – in vivo tumor model

All experiments were performed according to NIH guidelines for animal research with animal research protocols reviewed annually and in force by the Animal Experiment Review Committee at the SUNY Downstate Medical Center Institutional IACUC Committee. An *in vivo* tumor model was established according to the procedure described previously.[17] Briefly, Nu/Nu mice were xeno-

TABLE I – EFFECTS OF PNC-28 AND CONTROL PNC-29 PEPTIDES ON TUC-3 CELLS (1×10^6/MOUSE) IMPLANTED IN THE PERITONEAL CAVITY

Numbers of Nu/Nu mice	Pump location	PNC-28 (10 mg/mouse)	PNC-29 (10 mg/mouse)	Results
4	s.c.	+	−	Day 21: no ascites; no liver metastasis
4	s.c.	−	+	Day 21: ascites, liver metastasis; carcinomatosis
5	i.p.	+	−	Day 21: no ascites; no liver metastasis
5	i.p.	−	+	Day 22: ascites; carcinomatosis; liver metastasis

s.c., subcutaneousi.p., intraperitoneal

transplanted by subcutaneous (s.c.) injection with a 28 gauge needle on the right side and above the hind leg with 10^6–10^7 BMRPA1.Tuc3 cells. Under drug-free conditions and over a period of 6–14 days the cells grew into tumors with a range from 20–240 mg/mouse. Tumor sizes were measured in mm in 2 dimensions (length = l, width = w) using an electronic digital caliper (Control Company, Friendswood, TX), and the tumor masses calculated according to the equation *tumor mass* (mg) = $l \times (w)^2/2$.[20] The tumor masses of multiple tumors in the same mouse were added together as indicative of the (visible) tumor load of the animal. Immediately prior to pancreatic carcinoma cell transplantation and at the time of each tumor measurement the weight of each mouse was obtained as well using an electronic balance (Ohaus, Denver, CO).

Implantation of osmotic pumps

Alzet mini-osmotic pumps were purchased from the Durect Corp.(Cupertino, CA). A mini-osmotic pump infuses its contents at a defined constant rate of 0.10–0.25 μl/hr for periods from 14 to 28 days. Mice were surgically implanted s.c. in the region above the left front leg with a single Alzet mini-osmotic pump per mouse. Surgery was performed under strictly sterile conditions. The animals were anesthetized by intraperitoneal (i.p.) injection of pentobarbital at 65 mg/kg body weight.[21] The anesthetized animal was placed in a biological hood under laminar flow conditions on its belly on a sterile surgical drape, the skin was cleaned with ethanol, and, *via* a 0.5 cm cut in the skin on the back and above the left front leg, a small subcutaneous pocket was made, and the pump was moved gently into this pocket with the nozzle in the upward (head) direction. The cut was closed with nonabsorbable nurolon (3-0, Ethicon) thread, which was removed 7–10 days later when the wound had completely closed. The animals were then placed, 1 per cage, under a warming lamp, and closely watched until they had completely recovered from anesthesia. During the period of anesthesia, the animals in each group were marked using the standard ear-marking system. The mice were kept in their aseptic BioIncubators, 1 animal per cage, to avoid the biting off the sutures and, consequently, to keep the wounds closed and healing. Upon having been returned to individual cages, the mice were inspected daily for 1 week to assure their recovery. Once the wound had closed and the sutures were removed, the mice were moved together up to 4 animals/cage. In addition to observing their recovery, the mice were examined following the implantation of the minipumps to evaluate the effects of treatment on tumor growth. Tumor size was measured and tumor weight was calculated as described previously. An identical group of mice underwent the same procedure of mini-osmotic pump implantation that infused, in several experiments, PNC-29 control peptide or, as described later, in some experiments, sterile physiological saline.

Microscopy and photography

Cells maintained in culture in cRPMI medium were fixed overnight in 3% buffered (phosphate-buffered saline pH 7.2) and photographed in a Zeiss inverted microscope using TMX-100 film with 10× and 40× objectives, respectively.

Statistical analyses

All experiments were conducted at least 2 times with each experimental group consisting of 4 or 5 mice each from the same littermate with the exception of the i.p. pump implantation, which was done once. Each longitudinal dataset was analyzed by applying a general linear mixed model. Tumor mass was analyzed as a function of time, and its interaction with the treatment groups was assessed. Therefore, to test this treatment-based hypothesis a type III repeated measures ANCOVA test was employed. Estimation of regression parameters was performed, and these parameters were contrasted to estimate the treatment effect. We found that ARIMA[22] covariate structure best fit the repeated measures data; however, other structures such as compound symmetry and unstructured fits were also analyzed. To assess the experimental effects, log tumor volume was analyzed as a function of time (days), and its interaction with the treatment groups (PNC-28 and PNC-29) and phase of treatment (treatment and posttreatment). Therefore, a 3-way repeated measures ANCOVA test was applied to perform repeated measures regression using days as a continuous variable. Estimation of regression parameters was performed, and these parameters were contrasted to estimate the treatment effect.

Results

The effect of PNC-28 on the establishment and growth of xenotransplanted pancreatic carcinoma cells was examined in homozygous Nu/Nu mice in 2 experimental conditions: infusion of PNC-28 at virtually the same time as the s.c. implantation (simultaneous, Condition I), and after the s.c. xenotransplantation of the pancreatic carcinoma cells (posttreatment, Condition II). While the effect of PNC-28 released by the mini-osmotic pumps implanted s.c. was, in most experiments, examined on pancreatic carcinoma cells implanted s.c., in one separate experimental design, pancreatic carcinoma cells were injected i.p. and the pumps implanted in the peritoneal cavity and s.c., respectively, as described later.

Condition I – Effect of simultaneous treatment with PNC-28: Intraperitoneal tumor implantation

In the first experiment, a group ($n = 4$) of Nu/Nu mice was implanted s.c. with 14 days mini-osmotic pumps releasing PNC-28 (2 mg/mouse) at a rate of 0.25 μl/hr. While the mice were still under anesthesia, the animals were injected i.p. with 10^6 BMRPA1.Tuc3 cells/mouse. A control group ($n = 4$) of mice was implanted with mini-osmotic pumps that released PNC-29 and several hours later injected i.p. with 10^6 BMRPA1.Tuc3 cells. The animals were observed every other day for up to 21 days during which time the mice in the control group developed ascites so that they had to be sacrificed by day 21 (Table I, row 2). In contrast, no ascites was seen in the PNC-28-treated animals (Table I, Row 1). Cells obtained by peritoneal lavage with sterile saline of the mice treated with PNC-28 were seeded into tissue culture plates, which were then incubated for 8 days; microscopic inspection of this fluid revealed the presence of no tumor cells, but abundant macrophages and some lymphocytes which, as expected, did not grow in culture (Fig. 1, left). In contrast, the ascites from the mice that had received PNC-29 for the first 14 days of simultane-

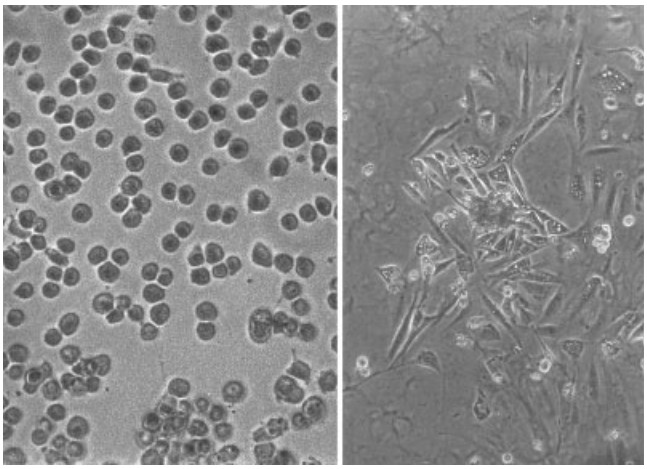

FIGURE 1 – Left: Peritoneal aspirate from Nu/Nu mice injected with BMRPA1.Tuc3 cells and treated with PNC-28 for 2 weeks. The microphotograph shows the characteristic morphology of adherent macrophages and no tumor cells were present. Right: Peritoneal aspirate from Nu/Nu mice containing untreated BMRPA1.Tuc3 cells allowed to grow in the peritoneum for 3 days showing foci formation and tumor cell (spindle-shaped) growth. Both pictures were taken 3 days into the cell culture.

ous treatment and tumor implantation contained a large number of cells that rapidly divided in *in vitro* culture and that microscopically displayed a spindly phenotype and formed numerous foci (Fig. 1, right). We note here that tumor size in mice treated with PNC-29 was statistically the same as that in mice treated with sterile saline (see below) indicating that PNC-29 has no inhibitory effect on tumor growth (results not shown).

All of these findings are characteristic of a tumor cell phenotype and, in this case, of the previously injected pancreatic carcinoma BMRPA1.Tuc3 (Hannan *et al*. [2006] manuscript submitted). The presence of tumor cells in the ascites and their absence in the peritoneal cavity of the PNC-28- treated mice strongly suggest that even when infused simultaneously and at a location distant from the site of tumor growth, PNC-28 can be strongly effective in inhibiting this particular tumor. The absence of tumor cells in the PNC-28-treated animals further suggests that part of the peptide's effect is cytotoxicity against tumor cells and, thus, similar to the *in vitro* effect.[12,13]

In a similar experimental design, mini-osmotic pumps delivering PNC-28 (2 mg/mouse; rate = 0.25 μl/hr) over a 14 days period were implanted i.p. into 4 Nu/Nu mice. Approximately 12 hr later, the same mice were injected i.p. with 10^6 BMRPA1.Tuc3 cells/mouse. Correspondingly, a control group of 5 Nu/Nu mice was first implanted with mini-osmotic pumps delivering sterile physiological saline followed 12 hr later by injection i.p. with 10^6 BMRPA1.Tuc3 cells/mouse. After their return to their aseptic cages, the nude mice in each group were examined for 1 week daily and then every other day for signs of tumor growth. Within 12 days, the control group of mice had developed clearly visible ascites, begun to show signs of distress and all of the animals died before the 22nd day with large distended abdomens and metastatic cancer of the peritoneum and abdominal viscera including liver (Table I, Row 4). In contrast, after 18 days, neither any signs of distress were observed nor was ascites present in the mice that had been treated i.p. with PNC-28. The mice were sacrificed by day 21 and inspection of the peritoneal cavity showed no sign of tumor growth, and there were no signs of peritoneal cancer as in the mice treated with PNC-28 s.c. described previously (Fig. 1). These findings suggest that, similar to the observations in *in vitro* tumor killing by PNC-28,[12] the delivery of the peptide directly to the site of a tumor results in total cell killing and may be an effective method of delivery of this peptide into tumors.

Our results for the effects of PNC-28 administered either at the same peritoneal site or at a site remote from the peritoneal seeding site of the BMRPA1.Tuc3 cells are summarized in Table I. Clearly PNC-28 is cytotoxic to pancreatic cancer cells in the peritoneal cavities of these mice, implying the possibility that it may be effective in blocking the growth of pancreatic cancer metastatic to the peritoneum.

Condition I – Simultaneous subcutaneous implantation of osmotic pumps and tumor cells

We performed experiments on 2 sets of 5 nude mice in which we implanted tumor cells and pumps delivering peptide (PNC-28, $n = 5$, PNC-29, $n = 5$). In each set of experiments, a group ($n = 5$) of Nu/Nu mice was implanted s.c. with mini-osmotic pumps releasing PNC-28 (2 mg/mouse) at a rate of 0.25 μl/hr for 14 days. While the mice were still under anesthesia, the animals were xenotransplanted s.c. at the right hind leg region with BMRPA1.Tuc3 cells (10^6 BMRPA1.Tuc3 cells/ mouse). Nu/Nu mice of a control group ($n = 5$) were implanted with mini-osmotic pumps that were loaded with PNC-29 (2 mg/mouse) followed immediately by the xenotransplantation s.c. of 10^6 BMRPA1.Tuc3 cells. Mice were observed daily for 10 days, then every other day for up to 4 weeks. As shown in Figure 2*a*, which presents the results of each individual mouse in 1 of the 2 groups of 5, simultaneous treatment of Nu/Nu mice with PNC-28 strongly delayed the growth of pancreatic carcinoma cells in their s.c. inoculation site. By the 13th day after the virtually simultaneous implantation of the mini-osmotic pumps releasing PNC-28 and the xenotransplantation s.c. of BMRPA.-Tuc3 pancreatic carcinoma cells, tumor growth was nearly completely inhibited (Fig. 2*b*, filled circles; $p = 0.001$, day 13). In contrast, tumor growth continued uninhibited in the control peptide PNC-29-treated group (Fig. 2*b*, open squares). Figure 2*c* shows the statistical significance of the mean values plotted in Figure 2*b* computed from ANCOVA analysis, based on repeated measures regression in order to predict log tumor size using days as a continuous variable, treatment condition (2 levels: control and PNC-28), and treatment phase (2 levels: during and posttreatment) of the log-transformed data in Figure 1*b* on the set of 5 mice. Almost identical results were obtained on the second group of 5 Nu/Nu mice (data not shown). This figure clearly shows a rapidly expanding statistical significance in the small tumor masses seen in the PNC-28-treated animals of the experimental group versus the large tumor masses developing in the group of animals infused with the control peptide. In fact, both Figures 2*b* and 2*c* show that, even after day 15, when the pumps ceased delivery of peptide, until around day 30, *i.e*., until close to the end of the observation period, there was no significant tumor growth in the PNC-28-treated animals while there was strong tumor growth in the PNC-29 control peptide-treated group.

Beginning at day 30, there is an increase in tumor size in the PNC-28-treated mice (Figs. 2*b* and 2*c*). The actual increase in tumor size, however, over the period from day 30–35 can be seen in Figure 2*b* to be much greater in the control group, PNC-29-treated than in the PNC-28-treated mice.

We have further analyzed the effects on the rates of tumor growth during treatment and posttreatment for PNC-28- and PNC-29-treated mice. The results of ANCOVA-based analysis of the slopes that give a measure of the rate of tumor growth, of the log-transformed data shown in Figure 2*c* are shown in Figure 2*d*. Table II summarizes the statistical significance of the slope analysis from Figure 2*d*. In these analyses, the slopes were calculated and contrasted within the regression model, in order to assess differences in growth rate between the control and PNC-28 groups during the different treatment phases.

Clearly, tumor growth rate is strongly inhibited in the PNC-28-treated group as compared with the control group ($p = 0.00012$ in Table II). This difference becomes less significant in the posttreatment phase, barely achieving statistical significance ($p = 0.049$), although, as noted above, the absolute increase in tumor size is

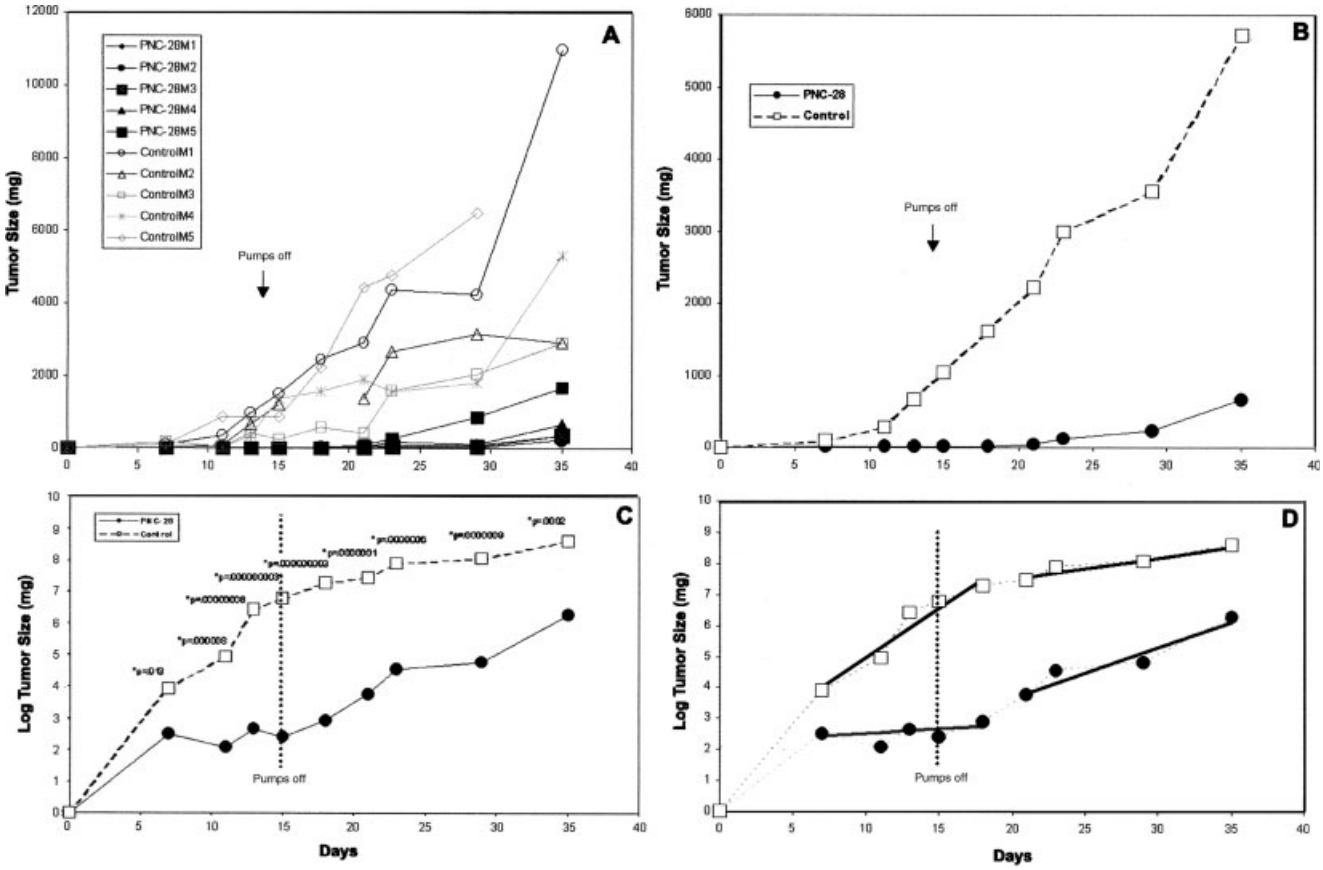

FIGURE 2 – *a*: Tumor cell growth in individual Nu/Nu mice (labeled as ''M1, M2,'' *etc*.) either treated with control PNC-29 peptide or PNC-28 therapeutic peptide delivered simultaneously with s.c. tumor implantation in Nu/Nu mice. Inset shows scheme for symbols. *b*. Comparison of means from Fig. 1*a* (see legend for Figure 1) for PNC-28-treated mice (filled circles) versus control peptide, PNC-29 (open squares). *c*. Plot of the log-transformed data in Figure 1*b*. *d*. Analysis of the slopes for tumor cell growth during treatment and posttreatment (data to the right of the ''pumps off'' vertical line). Slope comparisons are shown in Table II. Symbols are the same as in Figures 1*b* and 1*c*.

TABLE II – COMPARISONS OF SLOPES IN FIGURE 2*D*

Condition	*p* Value for slope comparison[1]
PNC-28- and 29-treated during treatment phase	0.00012
PNC-28 and 29 during posttreatment phase	0.043
PNC-28 during and posttreatment	0.034
PNC-29 during and posttreatment	0.0003

[1] p values ≤ 0.05 are considered to denote statistically significant differences.

much lower in the PNC-28-treated mice in the posttreatment observation period (Fig. 2*b*). Also, this loss of statistical significance in slopes between treated and control groups may be attributed to a leveling in the growth of tumors in the control group due to such effects as the extreme size of the tumors, possibly outgrowing their blood supply. On the other hand, the increase in tumor size in the posttreatment period for the PNC-28-treated group may be caused by proliferation of tumor cells that were not accessible to the peptide. This issue is discussed further in the Discussion section. The increase in growth of tumors after 30 days in the PNC-28-treated mice results in a marginally statistically significant increase in slope in the posttreatment period ($p = 0.034$, Fig. 2*d* and Table II), and the decrease of tumor growth in the control group in the posttreatment phase causes a significant decrease in slope for the posttreatment control group ($p = 0.0003$, Fig. 2*d* and Table II).

These effects have been further ANCOVA-analyzed as summarized in Table III. For single parameter tests, *i.e*., treatment group, treatment phase (treatment or posttreatment) and days, statistically significant main effects were found for treatment group and days but not for treatment phase, the latter resulting from omission of treatment group. All of the 2-way interactions were significant except for treatment phase by days, again resulting from omission of treatment group. In the critical analysis for this experiment, the 3-way interaction of group by treatment phase by days was found to be highly significant (Table III). This implies that the differences in the slopes of tumor growth between both treatment conditions depends on phase of the treatment as concluded from the results in Figure 2*d*.

Although it is evident that PNC-28 did not result in the elimination of all implanted tumor cells, significant tumor growth in the experimental group of Nu/Nu mice was absent for at least another 2 weeks after the end of peptide infusion (Fig. 2*b*). This suggests an effect of this peptide on the tumor cells *in vivo* that extends beyond the limited treatment period as discussed further in the Discussion Section.

Condition II – Tumor implantation followed by PNC-28 treatment

Since virtually all human tumors are discovered after tumor establishment and growth have occurred, we explored the effect of the administration of PNC-28 subsequent to tumor cell implantation. To this end Nu/Nu mice were xenotransplanted s.c. with pancreatic carcinoma BMRPA1.Tuc3 cells (5×10^6–10^7 cells/mouse). When the tumors reached sizes from 40 to 260 mg/mouse,

TABLE III – TYPE III ANCOVA TESTS FOR STATISTICAL SIGNIFICANCE FOR SIMULTANEOUS TUMOR CELL AND PEPTIDE DELIVERY PUMP IMPLANTATION

Parameter tested	Significance	*F* (difference)	Statistical significance (*p* value)[1]
Group	Comparison of the mean of tumor size between treated and control groups without regard to phase	11.24	0.0023
Treatment phase	Comparison of difference in means between treatment phase *vs*. posttreatment phase, ignoring treatment group	2.44	0.1289
Days	Test of whether slope of least squares best fit line through all tumor size points differs from zero	64.33	0.00000
Group treatment phase	Comparison of the difference in means of tumor size between control and treated groups during treatment with the difference posttreatment	14.07	0.00083
Group days	Comparison of slopes of tumor growth for treated and control groups, independent of treatment phase	7.08	0.01286
Treatment phase days	Comparison of slopes of best fit line of tumor size *vs*. days, irrespective of treatment group	1.96	0.172217
Group treatment phase days	Comparison of difference in slopes for tumor growth of treated *vs*. control groups during treatment with that posttreatment	20.24	0.000113

[1] *p* values that are $\leq$ to 0.05 indicate significant differences.

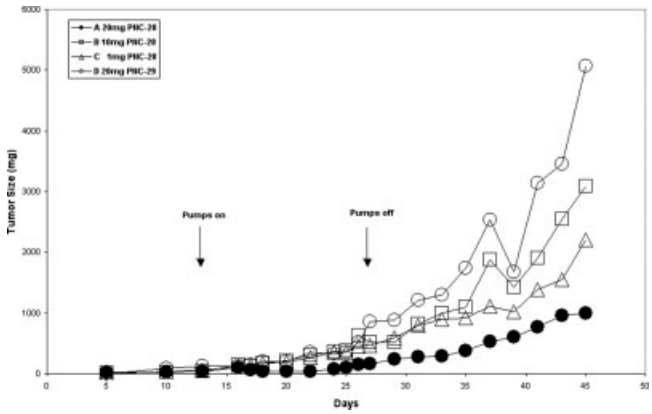

FIGURE 3 – Dose-response curves as a function of time for treatment after tumors were explanted and grew to a minimum mass of 40 mg. Peptide was then delivered at different concentrations for a period of 2 weeks, after which observations were continued for up to 45 days. Filled circles (*a*), 20 mg/mouse PNC-28; open triangles (*b*), 10 mg PNC-28/mouse; open squares (*c*), 1 mg/mouse PNC-28; open circles (*d*) 20 mg/mouse, PNC-29.

mini-osmotic pumps were implanted s.c. that released, over a period of 14 days, 1 mg, 10 mg and 20 mg of PNC-28 dissolved in sterile PBS ($n = 5$ for each treatment group). To each experiment was added a fourth group of mice ($n = 5$) carrying BMRPA1.Tuc3 pancreatic carcinomas of sizes similar to that in the experimental groups, and that were implanted with mini-osmotic pumps releasing 20 mg of PNC-29 in sterile PBS over the same time period as the experimental groups. Mice were observed daily and tumors measured in intervals of 2–5 days.

As shown in Figure 3, when BMRPA1.Tuc3 pancreatic carcinoma-bearing mice are infused over a 2-week period at a constant dose of 59.5 μg/hr for a total of 20 mg of PNC-28/animal, continued growth of the carcinoma is strongly inhibited when compared with tumor growth in animals treated with control peptide PNC-29. In fact, within 6 days after initiation of PNC-28 treatment, a reduction in tumor size was observed from an average of 96 mg to <30 mg that lasted for about 1 week. Even after the pumps had exhausted their release of PNC-28, BMRPA1.Tuc3 tumor cells demonstrated a significantly reduced ability to recover their growth rate throughout the rest of the observation period over 18 days and, at the 20 mg/mouse dose, reached a plateau after which no further growth occurred (Fig. 3, curve A, filled circles), as we observed for tumors treated from the time of implantation (Fig. 2*b*).

TABLE IV – STATISTICAL SIGNIFICANCE OF TUMOR SIZE IN PNC-28 (20 MG/MOUSE)-TREATED NUDE MICE VS TUMOR SIZE IN PNC-29 (20 MG/MOUSE)-TREATED NUDE MICE (GROUP A *VS*. GROUP D, FIG. 3).

Day	*p* Value[1]	Condition
10	0.196	No pumps
13	0.165	Pumps implanted
16	0.827	
17	0.226	
18	0.039	
20	0.016	
22	0.001	
24	0.016	
27	0.008	Pumps cease delivery
29	0.030	
31	0.015	
33	0.033	
35	0.008	
37	0.006	
39	0.036	
41	0.012	
43	0.030	
45	0.003	

[1] *p* values $\leq$ 0.05 indicate statistical significance

Table IV summarizes the statistical significance of the results for the difference in tumor size at each time point for tumors treated with PNC-28 20 mg/mouse in 14 days *vs*. those treated with PNC-29 (20 mg/mouse in 14 days) using the same method discussed previously for the analysis shown in Figure 2*b*. As can be seen in this table, 4 days after pump implantation, tumor sizes in the PNC-28-treated group were significantly smaller than those treated with PNC-29 including all points after pumps ceased to deliver peptide. Tumor sizes in the PNC-28 (20 mg/mouse)-treated animals during the 14 day posttreatment period remained at 840 ± 235 mg/mouse, well below the tumor sizes measured (5200 + 1640 mg) in the animals previously treated with PNC-29 peptide. The body weights of the PNC-28-treated mice were, on average, 26.5 g/mouse (day 27) and not different from the weights measured in control mice (average 27.4 g/mouse) that had the same age but had neither been implanted with pancreatic carcinoma cells nor had been treated with PNC-28. This strongly suggests that PNC-28 itself had no adverse effects on the mice.

Treatment of explanted tumors with the lower doses of PNC-28 (1 and 10 mg) per mouse over the same time period resulted in less inhibition of tumor growth during the 14 day post-pump implantation period in a dose-related manner, as shown in Figure 3. Nevertheless, both lower doses, especially the 10 mg/mouse dose, continued to inhibit tumor growth after discontinuation of the pumps.

TABLE V – ANCOVA STATISTICAL ANALYSIS OF PAIRWISE COMPARISONS OF THE RATE OF BMRPA.TUC3 TUMOR CELL GROWTH IN NUDE MICE UNDER DIFFERENT TREATMENT CONDITIONS

Groups compared with respect to slope of tumor growth without regard to treatment phase	Statistical significance (p value)[1]
20 mg/mouse PNC-28 *vs*. 0 mg/mouse PNC-29 (A *vs*. D , Fig. 3)	0.0002
20 mg/mouse PNC-28 *vs*. 1 mg/mouse PNC-28 (A *vs*. C, Fig. 3)	0.0062
20 mg/mouse PNC-28 *vs*. 10 mg mouse PNC-28 (A *vs*. B, Fig. 3)	0.0180
10 mg/mouse PNC-28 *vs*. 20 mg/mouse PNC-29 (B *vs*. D, Fig. 3)	0.1375
10 mg/mouse PNC-28 *vs*. 1 mg/mouse PNC-28 (B *vs*. C, Fig. 3)	0.6970
1 mg/mouse PNC-28 *vs*. 20 mg/mouse PNC-29 (C *vs*. D, Fig. 3)	0.2712

[1] p values ≤ 0.05 indicate statistical significance.

Statistical analysis of tumor growth rates

We have analyzed the data in Figure 3 using repeated measures regression based on a general linear mixed model. We found that the main effect for slope tumor growth by day was significant ($p = 0.002$) for group days for all treatment groups compared with the control group, as performed for the condition of simultaneous treatment and tumor implantation (Condition 2), as explained in Table II. Moreover, the analysis that tests the hypothesis that the growth of tumor by days depends on the group examined (group by slope of days interaction) was highly significant ($p = 0.001$). On the other hand, we analyzed the different growth rates among the 4 treatment conditions, in a pairwise manner, by computing the slopes of tumor growth by day, using a test for equality of slopes for group pairs. The results are summarized in Table V. This analysis demonstrated that there was a statistically significant difference in tumor growth rates between the 20 mg/mouse dose of PNC-28-treated mice and those in each other treatment condition. On the other hand, the rates of tumor growth for the other 2 doses of PNC-28, 1 and 10 mg/mouse, were not significantly different either from one another or from the control (Table V). We conclude from these results that a dose of 20 mg PNC-28 per mouse appears to be the minimal effective dose in inhibiting tumor cell growth *in vivo* under the stringent conditions employed in this study.

Discussion

Our results with PNC-28 in treating a pancreatic cancer cell line *in vivo* show that this peptide is effective in blocking tumor growth *in vivo* when administered concurrently with tumor implantation and after tumor growth has occurred. This effect is specific because administration of the negative control peptide PNC-29 has no effect on tumor cell growth in either set of circumstances. Because Nu/Nu mice were used in these experiments, our findings further suggest that the antitumor effect of PNC-28 is initiated by PNC-28 itself rather than by cytotoxicity due to an immune response. The reduced tumor growth in the treatment and posttreatment period in the experimental group of mice versus the rapid tumor growth seen in the control animals strongly suggests a reduced ability for the tumor cells to grow and/or an actual reduction in the number of tumor cells in the PNC-28-infused animals.

Administration of PNC-28 to Nu/Nu mice concurrently with tumor cell implantation, at a site removed from the location of tumor cell implantation, results in complete blockade of tumor cell growth over the 2-week treatment period. This blockade lasts for 2 weeks after cessation of PNC-28 administration, suggesting the possibility that it is cytotoxic to many of the cells exposed to it and that remaining viable tumors cells may have been injured by peptide treatment. Our observation in Figures 2 and 3 that there is some growth 1 week after cessation of PNC-28 treatment implies that not all cells may have been killed by this peptide.

On the other hand, in the experiments in which PNC-28 is administered to the pancreatic cancer cells in the peritoneum, *i.e*., is delivered directly to the same site as tumor implantation, it appears that total cell killing results. Importantly, even when PNC-28 is delivered at a site remote from the peritoneal implantation site, there appears to be total cell killing although the observation period for this condition was less extended. This difference may be due to the increased vascular supply of the peritoneum compared with a subcutaneous implantation site, resulting in the delivery of more of the peptide to the tumor site. Our results on the contiguous implantation of PNC-28-delivering pumps and tumor cells in the peritoneal cavity are parallel to those we obtained with this and other tumor cell lines in cell culture in which we found that PNC-28 induces total cell killing over periods ranging from 1 hr to 3 days.[12,13] Taken together, these findings suggest that direct injection of PNC-28 into tumors or administration of PNC-28 into the vicinity of these cancers may result in total cell killing.

Even when administered 13 days after tumor cell implantation and when tumor growth has occurred, PNC-28 also blocks tumor cell growth in a similar manner as shown in Figure 3. There is minimal cell growth during the treatment period; after cessation of treatment, there is some growth of the tumors. However, this growth is significantly less even 18 days after cessation of treatment than tumor growth in the presence of PNC-29 control peptide (curve A *vs*. curve D in Fig. 3). Furthermore, this growth appears to plateau at day 40 while it increases rapidly in tumors treated with PNC-29 (Fig. 3). These results suggest that PNC-28 is lethal to most BMRPA1.Tuc3 cells *in vivo* and that there may be peptide-induced injury to remaining viable tumor cells.

In these experiments, it is clear that there is a pronounced dose-dependence of PNC-28 in inhibition of tumor growth. Doses of 1 and 10 mg administered either for 2 weeks do not a cause statistically significant difference in the rate of tumor growth when compared with the rate in the presence of PNC-29 (20 mg/mouse). On the other hand, 20 mg of PNC-28 administered over the same time period results in a significant level of tumor growth inhibition. Thus, this dose appears to be approximately the minimal effective dose *in vivo*.

The relatively low level of tumor growth observed after cessation of treatment with PNC-28 after tumor implantation (Condition II) may be attributed either to the lack of exposure of some of the BMRPA1.Tuc3 cells to PNC-28 or to the resistance of populations of these cells to this peptide. In this low level of growth, plateaus (Figs. 2 and 3) occur ultimately whether or not treatment is concurrent with (Fig. 2) or subsequent (Fig. 3) to PNC-28 administration. In view of our findings that this peptide kills 100% of BMRPA1.Tuc3 cells *in vitro* by inducing necrosis and that PNC-28 also kills 100% of these cells when administered to the tumor in the peritoneal cavity *in vivo* (experimental Condition I), irrespective of whether it is contiguously or noncontiguously administered, we conclude that the observed growth occurs due to absence of exposure of cancer cells to the peptide, *i.e*., the nonoptimal alignment of the pumps with respect to the tumor cells. Not only were both located remotely from one another but also the pumps were delivering peptide into tissues and not even directly into the circulation. It would therefore be desirable to increase the dose of PNC-28 and the time of treatment in this Nu/Nu mice model system and to inject the peptide at sites more proximate to the tumor. We are currently undertaking these studies.

Based on our findings that PNC-28 and PNC-27 both induce tumor cell necrosis in 11 different tumor cell lines,[12,13] several of which were p53-homozygously deleted, we assume that the tumor

eradication that we observed in tumors implanted in the peritoneal cavities of the nude mice (Table I, Fig. 1) also occurred by this mechanism. Since PNC-28 contains a segment of the mdm-2-binding domain (residues 17–26) of p53, it is possible that its mechanism of action changes *in vivo* to block the p53–mdm-2 interaction to induce apoptosis. We consider this possibility to be remote in view of the facts that PNC-28 contains 10 of the 15 residues involved in mdm-2 binding, lowering its affinity for this target; that this peptide induces tumor cell necrosis, not apoptosis, in a wide variety of tumor cell lines, containing highly varied genetic status (some containing p53, others containing no p53, some containing oncogenic ras, others containing progesterone and estrogen receptors, *etc*), making a switch in mechanism all the less likely; and that PNC-28 induces only necrosis of TUC-3 cells, used in the present *in vivo* experiments, in culture, irrespective of the concentration of peptide used (10, 50 and 100 μg/ml) and the time of incubation (from 3–7 days).

An important aspect of this study is the effect of PNC-28 on the general ability of the mice to function. None of the Nu/Nu mice treated with this peptide, whether infused concurrently with tumor cells or infused posttumor cell implantation, and even at the highest dose of 20 mg administered over the 2 week period, were found to undergo any loss of weight and continued to function normally. This observation suggests that PNC-28 is nontoxic to these mice and is consistent with our prior observations that this peptide has no effect on the viability or growth of normal cells in culture, including stem cells derived from cord blood.[12]

We note here that maintenance of weight and normal responses are considered the most commonly made observations for ruling out drug toxicity, including recent studies on the effects of naturally occurring peptides on tumor growth in nude mice.[23] However, in a few studies (12 out of 11,225 nude mouse studies listed in PUBMED), almost entirely those using known marrow-suppressing agents, hematological parameters, and less commonly, other parameters such as liver function tests, have also been measured to survey for possible adverse drug effects. We have not performed these analyses in part because we have already found that PNC-28 has no effect on the ability of stem cells from cord blood to differentiate into hematopoietic lines.[12] In addition, effects of marrow-suppressive drugs require approximately 3–6 weeks to be observed,[24,25] at the limit of, or exceeding, our observation periods. Finally, it is difficult to determine whether decreases in cell counts should be attributed to the drug or to the tumor.

Other studies have been performed in which p53 peptides, and small molecules, that block p53–hdm-2 interactions have been administered to animals in which tumors have been xenografted (*e.g.*, Refs. 10 and 11). Perhaps most closely related to our present study is the one utilizing the hdm-2-binding p53 peptide attached to the membrane-penetrating tat sequence on its amino terminus[10]; this peptide induces apoptosis in uveal melanoma and retinoblastoma cell lines explanted into the eyes of rabbits but has little effect on untransformed counterpart cell lines and causes minimal damage to surrounding ocular tissue.[10] Similarly, PNC-28 induces tumor cell death but does not affect normal cells in culture.[12,13] Since PNC-28 does not affect the viability of nude mice treated with it and does not cause damage to the peritoneal cavities of these mice (Fig. 1), we infer that PNC-28 likewise does not destroy normal tissue in nude mice. In addition, when we treat tumors with PNC-28 in the confined space of the peritoneal cavity, we obtain tumor eradication (Table I).

However, a major difference between the 2 studies involves the peptides themselves. The prior study[10] utilized a tat sequence on the amino terminal end of the p53 effector peptide while we employed a membrane-penetrating sequence, penetratin, from antennapedia protein, (both sequences contain a high percentage of positively charged residues) on the carboxyl terminus of our p53 effector peptide. As we noted in the Introduction, placement of the penetratin sequence on the amino terminal end of the p53 peptide results in significant loss of tumoricidal activity. In addition, these differences in membrane-penetrating sequences and in their placement on the amino or carboxyl terminal end of the p53 peptide results in induction of either apoptosis or necrosis, respectively. Our peptide induces necrosis such that the presence of p53 or its p73 surrogate, is not required for its effect as it apparently is for the tat-p53 peptide.[10]

Our present study also differs from the prior one[10] in that the prior study was confined to ocular tumors, and the peptide could be directly administered to tumor cells in an exposed and enclosed domain. In our protocols, while we tested PNC-28 on tumors in the confined space of the peritoneal cavity, we also administered it from a site that is remote from the tumor implanted internally as would be necessary in the treatment of most cancers. We find that even when administered at distant sites and not even directly into the circulation, we obtain major inhibition of tumor growth (Fig. 3). In addition, unlike in the prior study,[10] in our experimental protocols, we further followed tumor growth for several weeks after discontinuation of peptide infusion and found that tumor growth is sluggish (Figs. 2*b* and 3), suggesting that damage to remaining tumor cells may have occurred.

Compared with p53 peptides that block p53–hdm-2 interactions, peptidomimetics such as modified peptides[8,9] and small molecules, such as nutlin-3,[11] that likewise efficiently block p53–hdm-2 interactions, have the advantage of longer half-lives due to resistance to proteases and the ability to be administered orally rather than internally, *e.g.*, intravenously or *via* implanted pumps. In this regard, nutlin-3 has been found to block tumor cell growth of SJSA-1 cells in nude mice when administered orally for several weeks.[11] However, unlike PNC-28, the anti-tumor activity of nutlin-3 is confined to tumor cells expressing wild-type p53[11] and has no effect on the growth of tumor cells containing mutant p53 and, presumably, on cells that are p53-null, although these cell lines were not tested. In addition, although it does not affect ultimate cell viability, this agent, unlike PNC-28, has been found to block the growth of untransformed cells.[11]Furthermore, the fate of residual tumor cells after withdrawal of the drug was not observed as we have performed in this study (Figs. 2*b* and 3).

Based on our current findings that PNC-28 blocks tumor cell growth *in vivo* and appears not to induce toxic side effects, we conclude that this peptide may be an effective agent in the treatment of cancers.

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References

1. Fleischer A, Ghadiri A, Dessauge F, Duhamel M, Rebollo MP, Alvarez-Franco F, Rebollo A. Modulating apoptosis as a target for effective therapy. Mol Immunol 2006;43:1065–79.
2. Haffner R, Oren M. Biochemical properties and biological effects of p53. Curr Opin Genet Dev 1995;5:84–94.
3. Kussie PH, Gorina S, Marechal V, Elenbaas B, Moreau J, Levine AJ, Pavletich NP. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science 1996;274:921–22.
4. Chi SW, Lee SH, Kim DH, Ahn MJ, Kim JS, Woo JY, Torizawa T, Kainosho M, Han KH. Structural details on mdm2-p53 interaction. J Biol Chem 2005;280:38795–802.
5. Picksley SM, Spicer JF, Barnes DM, Lane DP. The p53-MDM2 interaction in a cancer-prone family, and the identification of a novel therapeutic target. Acta Oncol 1996;35:429–34.
6. Bottger V, Bottger A, Howard SF, Picksley SM, Chene P, Garcia-Echeverria C, Hochkeppel HK, Lane DP. Identification of novel mdm2 binding peptides by phage display. Oncogene 1996;13:2141–7.

7. Wasylyk C, Salvi R, Argentini M, Dureuil C, Delumeau I, Abecassis J, Debussche L, Wasylyk B. p53 mediated death of cells overexpressing MDM2 by an inhibitor of MDM2 interaction with p53. Oncogene 1999;18:1921–34.
8. Chene P, Fuchs J, Carena I, Furet P, Garcia-Echeverria C. Study of the cytotoxic effect of a peptidic inhibitor of the p53-hdm2 interaction in tumor cells. FEBS Lett 2002;529:293–7.
9. Garcia-Echeverria C, Furet P, Chene P. Coupling of the antennapedia third helix to a potent antagonist of the p53/hdm2 protein-protein interaction. Bioorg Med Chem Lett 2001;11:2161–4.
10. Harbour JW, Worley L, Ma D, Cohen M. Transducible peptide therapy for uveal melanoma and retinoblastoma. Arch Ophthalmol 2002; 120:1341–6.
11. Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N, Liu EA. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science 2004;303:844–8.
12. Kanovsky M, Raffo A, Drew L, Rosal R, Do T, Friedman FK, Rubinstein P, Visser I, Robinson P, Brandt-Rauf PW, Michl J, Fine RL et al. Peptides from the amino terminal mdm-2 binding domain of p53, designed from conformational analysis, are selectively cytotoxic to transformed cells. Proc Natl Acad Sci USA 2001;98:12438–43.
13. Do TN, Rosal RV, Drew L, Raffo AJ, Michl J, Pincus MR, Friedman FK, Petrylak DP, Cassai N, Szmulewicz J, Sidhu G, Fine RL et al. Preferential induction of necrosis in human breast cancer cells by a p53 peptide derived from the mdm-2 binding site. Oncogene 2003;22: 1431–44.
14. Rosal R, Pincus MR, Brandt-Rauf PW, Fine RL, Michl J, Wang H. NMR solution structure of a peptide from the mdm-2 binding domain of the p53 protein that is selectively cytotoxic to cancer cells. Biochemistry 2004;43:1754–861.
15. Rosal R, Brandt-Rauf PW, Pincus MR, Wang H, Mao Y, Fine RL. The role of alpha-helical structure in p53 peptides as a determinant for their mechanism of cell death: necrosis versus apoptosis. Adv Drug Deliv Rev 2005;57:653–60.
16. Bao LY, Thelmo WL, Somnay S, Madahar C, Michl J. Characterization of an acinar cell line, BMRPA. 430, derived from adult rat pancreas. FASEB J 1994;8:64A.
17. Bradu S. An in vitro model of pancreatic carcinogenesis: characterization of transformation-associated biological and molecular alterations induced by the tobacco smoke carcinogen NNK in rat pancreatic BMRPA1 cells. Doctoral dissertation, SUNY Downstate Medical Center. 1999.
18. Nam J, Raffaniello RD, Lin J, Bao LY, Michl J, Raufman J-P. Protein kinase C isoform expression and function in transformed and nontransformed pancreatic acinar cell lines. Biochem Biophys Res Commun 1998;246:166–71.
19. Michl J, Ohlbaum D, Silverstein SC. 2-Deoxyglucose selectively inhibits Fc and complement receptor-mediated phagocytosis in mouse peritoneal macrophages. II. Dissociation of the inhibitory effects of 2-deoxyglucose on phagocytosis and ATP generation. J Exp Med 1976;144:1484–93.
20. Corbett T, Polin L, Roberts BJ, Lawson AJ, Leopold WR, White K, Kushner J, Paluch J, Hazeldine S, Moore R, Rake J, Horwitz JP. Transplantable syngeneic rodent tumors. In: Teicher BA, ed. Tumor models in cancer research. Totowa, NJ: Humana Press, 2002.41–71.
21. Lovell DP. Variation in pentobarbital sleeping time in mice. 2. Variables affecting test results. Lab Anim 1986;20:91–6.
22. Munro BH. Statistical methods for health care research. Philadelphia, PA: J.B. Lippincott, 1986.
23. Vesely DL, Clark LC, Garces AH, McAfee QW, Soto J, Gower ,WR, Jr. Novel therapeutic approach for cancer using four cardiovascular hormones. Eur J Clin Invest 2004;34:674–82.
24. Kikuchi Y, Kita T, Hirata J, Sasa H, Nagata I, Fukushima M. Adjuvant effects of antineplastic prostoglandins to cisplatin in nude mice bearing human ovarian cancer cells. J Cancer Res Clin Oncol 1992; 118:453–7.
25. Roodman GD, Johnson RA, Clibon U. Tumor necorsis factor alpha and the anemia of chronic disease: effects of chronic exposure to TNF on erythropoiesis in vivo. Adv Exp Med Biol 1989;271:185–96.