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Poptosis: A Novel Mechanism for the Selective Killing of Cancer Cells

Pincus MR[1*], Bowne W[2] and Sarafraz-Yazdi E[3]

[1]Department of Pathology, SUNY Downstate Medical Center, 450 Clarkson Avenue Brooklyn, USA

[2]Department of Surgery, Jefferson Medical Center,1100 Walnut Street, Philadelphia, USA

[3]Nomocan 310 East 67th Street, New York, USA

[*]Corresponding author:

Matthew R. Pincus,
Department of Pathology, SUNY Downstate Medical Center, 450 Clarkson Avenue Brooklyn, NY, 11203, USA

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1. Introduction

A new class of peptides has recently been discovered that kill cancer cells but have no effect on untransformed cells. The prototype of these peptides is PNC-27 [1]. As shown in Figure 1, this peptide (peptide 1) contains amino acid residues 12-26 of p53, shown in red, from its domain that binds to HDM-2, attached to a transmembrane-penetrating sequence, also called penetratin, shown in yellow. Other peptides from this same domain of p53 are also shown in this figure. Peptide 2 and another similar peptide PNC-26 are shorter peptides and have similar specificities of PNC-27 but significantly lower activities. Peptide 3, PNC-28, also a shorter version of PNC-27 has residues 17-26 containing all of the important residues necessary for binding to HDM-2 and has anti-cancer activity similar to that of PNC-27 [2].

In the nucleus, binding of p53 to HDM-2 in its amino terminal binding domain (amino acids 1-109) [3] results in ubiquitination of p53 and its hydroylsis in the proteosome limiting its ability to block proliferation of cancer cells by inducing apoptosis. PNC-27 was designed by our research group to block the binding of p53 to HDM-2 in the nuclei of cancer cells as a decoy peptide, i.e., the peptide would cross the cancer cell and nuclear membranes using its penetratin sequence and bind in HDM-2 in place of p53 in the nucleus allowing p53 to induce apoptosis of the cancer cell.

In a number of initial studies, we found that PNC-27 and PNC-28 induced rapid cancer cell necrosis but did not affect untransformed cells [1]. An example of this finding is shown in Figures 2 in which rat pancreatic acinar cancer cells called TUC-3 (spindle shaped cancer cells in Figure 1A) and their normal counterpart pancreatic acinar cells called BMRPA1 (Figure 1B) were incubated with PNC-27 for 24h. As shown I Figure 1C, all of the TUC-3 cells were killed by PNC-27 while, as shown in Figure 2D, the BMRPA1 remained viable [1].

The dose response curves are shown in Figures 3 for PNC-27 in killing a number of different cancer cell lines including TUC-3 rat pancreatic, MIA-PaCa-2 human pancreatic, A2058 human melanoma cell lines and normal human fibroblasts (AG13145I in serum-supplemented culture media (left panels) and in phosphate-buffered saline (PBS, right panels) [4].The red dots shown in the different dose response curves in Figures 3 show that the negative control peptide, PNC-29, that contains an unrelated sequence from human cytochrome P450 attached to the penetratin sequence (peptide 4 in Figure 1) has no effect on any of the cells tested. Importantly, PNC-27 is seen to have no effect on the growth of the normal AG13145 human fibroblasts indicating that the cytotoxic effect of PNC-27 is specific for cancer cells.

As can also be seen in Figures 3, PNC-27 kills primary human ovarian cancer cells [5,6]. An example is shown for OVCA4 primary ovarian cancer cells in the lower rightmost curve in the left panel set. These are cells that have been removed from patients who have undergone excision of these tumors under IRB protocol and have been incubated in culture with PNC-27. As can be seen from the figure, PNC-27 kills all of the cells while the negative control peptide, PNC-29, has no effect on these cells.

We have tested many different cancer and normal cell lines using both PNC-27 and PNC-29 and have found that PNC-27 effectively kills all of these cells while the negative control PNC-29 peptide

has no effect on cell growth. Examples of the range of cancer and normal cells tested are summarized in Figures 4 and 5, respectively. In addition to the cell lines shown, numerous other cell lines have been tested using either peptide that include four primary ovarian cell cancers (OVCA1 is listed in Figure 4 and OVCA3 is shown in Figure 3), OVCAR-3 and SKOV3 ovarian cancer cells [6], human colon cancers (HCT-116, CTA, CTP, CTR and SW1222) [7] and a variety of hematopoietic (non-solid tissue) tumors acute myelogenous leukemia (AML) cell lines including U-937, OCI-AML3 and HL-60, and chronic myelogenous leukemia [8]. As is discussed below, PNC-27 has been effective in eradicating human AML in vivo [9].

It should be noted that, among the cancer cell lines that are killed by PNC-27 and PNC-28 listed, several, such as OVCAR-3 and SKOV3 ovarian cancers, are known to be chemotherapy resistant (6). Also, a number of colon cancer cell lines such as CTA, CTR and SW1222, are enriched in CD44-positive tumor stem cells. Thus, these peptides are cytotoxic to tumor stem cells. As discussed below, PNC-27 is cytotoxic to human acute myelogenous tumor stem cells [9] without affecting normal hematopoietic stem cells [1] as discussed above.

In Figure 5, it may be seen that normal human hematopoietic stem cells were unaffected by PNC-28 (Figure 1) [1]. In these experiments, the hematopoietic stem cells were isolated from the cord blood of five different patients and were incubated either with PNC-28 or PNC-29 in the presence of growth factors inducing colony formation of differentiated cell lines. As shown in Figure 6, the growth factor-induced colony counts for each of the patient's stem cells were the same irrespective of the presence of either peptide or in the absence of either peptide. This result strongly implies that PNC-27 and PNC-28 do not affect bone marrow and would not induce off-target effects involving bone marrow suppression in patient treated with these peptides. As we discuss below, these peptides kill cancer cells in vivo with no observable off-target effects.

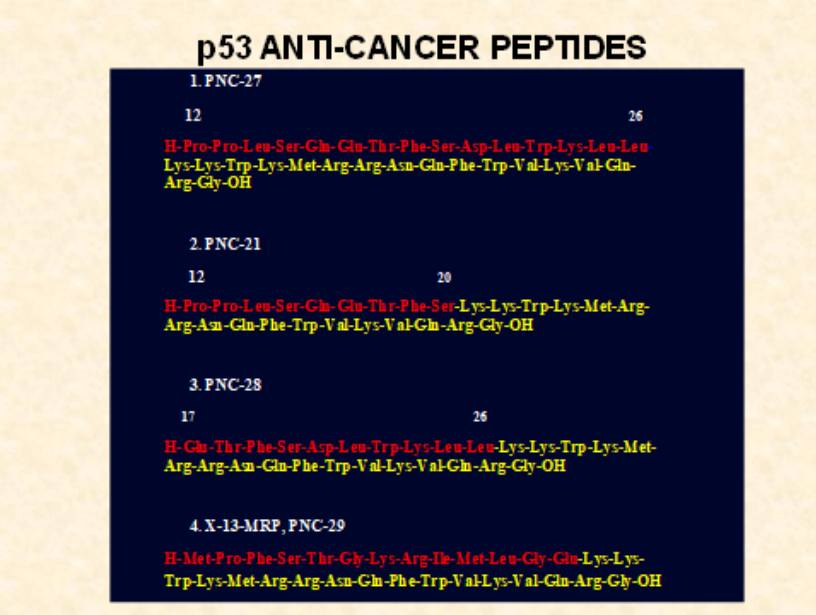

Figure 1: Anti-cancer peptides with the p53 sequences that are involved in the binding of p53 to HDM-2.

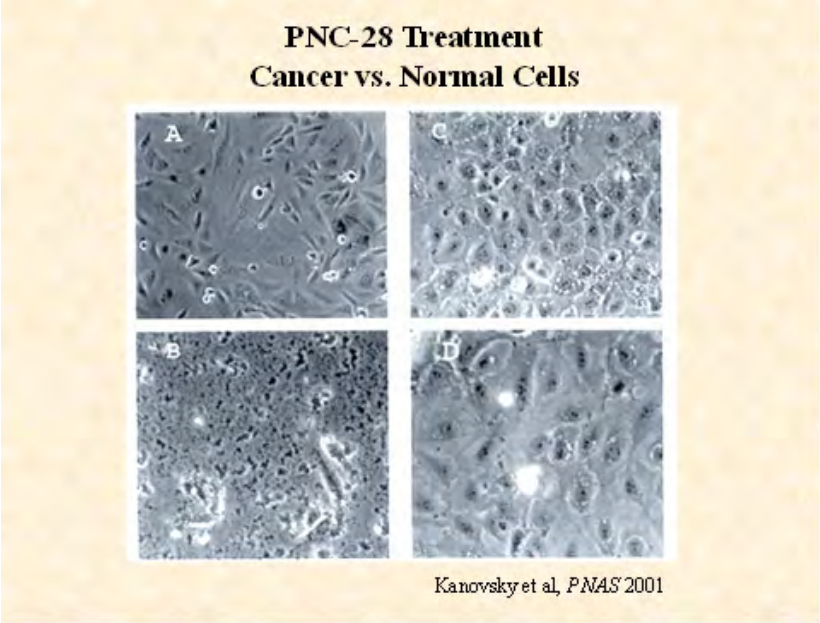

Figure 2: A.TUC-3 rat acinar pancreatic cancer cells prior to treatment with PNC-27 showing malignant spindle-shaped cells.
B. Normal counterpart rat pancreatic BMRPA1 cells showing well differntaited round acinar cells.
C. TUC-3 cells after treatment with PNC-27 showing no viable cells and only cellular debris.
D.BMRPA1 cells after treatment with PNC-27 showing normal, rounded acinar cells. There was no cell death.

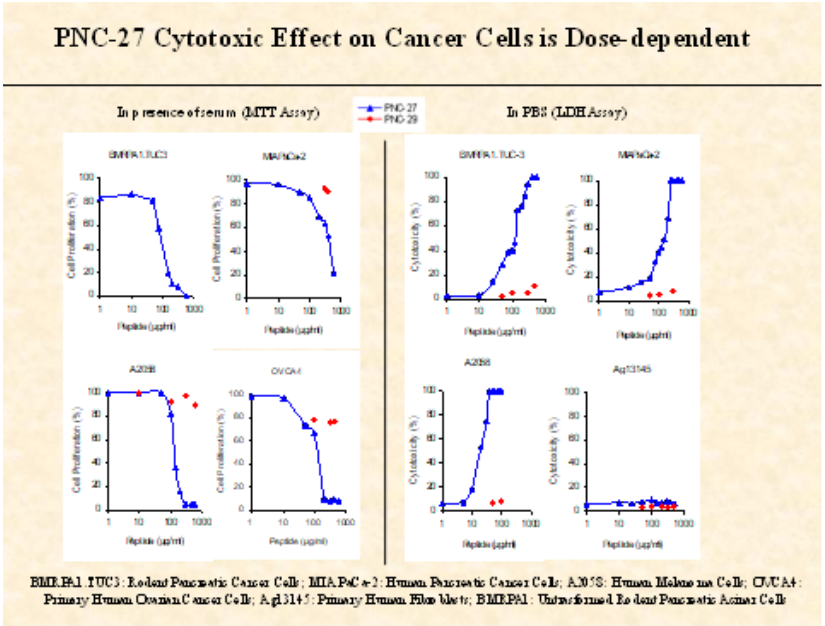

Figure 3: PNC-27 cytotoxic effect on cancer cells is dose-dependent. Dose response curves for different cancer cell lines as labeled in the figure.
The dose response curves on the left panel are for cancer cells in culture media as assayed by the MTT cell viability assay. The dose response curves on the right panel are for cancer cells in phosphate buffered saline (PBS) as assayed by lactate dehydrogenase (LDH) release.

PNC-27/-28 Effect on Cancer Cell Lines

CELL LINE	DOSE & PEPTIDE	CELL TYPE	TIME TO TOTAL CELL DEATH ($1x10^6$)	REFERENCE
BMRPA1.TUC-3[1]	30mM PNC-28 20mM PNC-27	Rat Pancreatic Cancer hu oncogenic k-ras^{val12}	72 h 72 h	1 This study
E-49	30mM PNC-28	Rat Brain Angiosarcoma	72 h	1
B16	60mM PNC-27	Mouse Melanoma	24 h	This study
HeLa	30mM PNC-28	Hu[1]) Cervical Squamous Cell Cancer	72 h	1
A549	30mM PNC-28	Hu Non-small Cell Lung Cancer	72 h	1
H1299[1])	30mM PNC-28	Hu Non-small Cell Lung Cancer p53 null	1 h	1
MDA-MB-468[1])	30mM PNC-27	Hu Breast Cancer p53 mut.	30 min	2
MDA-MB-157[1])	30mM PNC-27	Hu Breast Cancer; p53 null	30 min	2
MDA-MB-453[1])	30mM PNC-27	Hu Breast Cancer; p53 null	1 h	2
MCF-7[1])	30mM PNC-27	Hu Breast Cancer	48 h	2
SW-1417[1])	30mM PNC-28	Hu Colon Cancer p53 null	48 h	1
SAOS2[1])	30mM PNC-28	Hu Osteosarcoma p53 null	1 h	3
A2058	20mM PNC-27	Hu Melanoma	24 h	This study
MIA-PaCa-2	75mM PNC-28 60mM PNC-27	Hu Pancreatic Cancer	72 h 48 h	4 This study
OVCA1	60 µM PNC27	Primary Hu Ovarian cancer	2h	Dr.C.Gorelick & this study

Figure 4: Partial list of cancer cells that are killed by PNC-27 or PNC-28.

Normal cells are unaffected by PNC-27 treatment

Cell lines	Dose & peptide	Cell type	Peptide Incubation time	Reference
BMRPA-1	30 µM PNC27	Normal Rat pancreatic acinar	3 days	1
MCF-10	30 µM PNC27	Human breast	24h	1
Primary Human cells				
Ag 13145	30 µM PNC27	Primary Human Fibroblasts	48h	This study
Keratinocytes	30 µM PNC27	Primary Human Keratinocytes	24h	1
Hematopoetic stem cells	30 µM PNC27	Umbilical cord Stem cells from 5 donors	14 days	1

Figure 5: Partial list of normal cells that have been tested and which are not affected by PNC-27 or PNC-28.

	Absolute colony counts		
Sample	Untreated	X13-Leader*	PNC-28*
1	2,000	3,000	2,500
2	22,667	17,500	17,667
3	11,167	11,000	14,000
4	6,667	7,000	6,333
5	5,333	8,000	5,333

Growth factors consisted of 1.5 units/ml erythropoietin (Epo), 2×10^{-10} M granulocyte colony-stimulating factor (GCSF), 100 ng/ml stem cell factor (SCF), 4.5×10^{-10} M granulocyte-macrophage colony-stimulating factor (GM-CSF), and 2×10^{-10} M interleukin-3 (IL-3).
*All peptides were present at a concentration of 100 µg/ml.

Figure 6: PNC-28 does not affect hematopoietic stem cells isolated from the cord blood of five patients in culture. Cell counts after addition of appropriate growth factors listed below the table and incubation for 24h are given for each of three conditions: no peptide, PNC-29 negative control peptide (125 ug/mL): PNC-28 (125 ug/mL). As can be seen the cell counts are similar for the stem cells from each of the five patients.

2. PNC-28 Kills TUC-3 Cells in Vivo

As discussed above, PNC-27 and PNC-28 kill TUC-3 cells that are murine pancreatic acinar carcinoma in culture. These cells form highly metastatic tumors in nude mice within two weeks after transplantation. We have treated these cancers with PNC-28 administered to the mice via Alcet minipumps that delivered PNC-28 over a two-week period [2]. The results are shown in Figure 6. As can be seen in this figure, PNC-28 effectively eradicated these cancer cells while the negative control peptide, PNC-29, has no effect on tumor growth [2]. The effect of PNC-28 lasted for two weeks after the pumps ceased after which there was a small increase in size. Biopsy of this small mass showed that it was accumulated necrotic tissue with abundant edema and macrophages but no tumor cells while the expanding mass obtained in the mice treated with PNC-29 were found to contain massive numbers of spindle shaped tumor cells such as those shown in Figure 1A. In the mice treated with PNC-28, there were no off-target effects observed and the mice gained weight that was statistically insignificantly different from a control group of untreated mice.

3. PNC-27 and PNC-28 Kill Cancer Cells by a p53-Independent Mechanism

An unexpected finding obtained for the cell lines studied was that PNC-27 and 28 both induced rapid cell death, that occurred in minutes after incubation of cell lines with either peptide. In none of these studies were the usual markers for apoptosis expressed such as DNA laddering [1], annexin V labeling of membrane phospholipid, and caspase expression [6-8]. On the other hand release of LDH occurred almost immediately after incubation of cell lines with either peptide indicative of tumor cell death. An example is shown in Figure 8 in which MIA-PaCA-2 cells were incubated with PNC-28, and both LDH and caspase were assayed at the end of a 24h incubation [10]. There are high levels of LDH but only baseline levels of caspase in cells treated with this peptide. There was no release of LDH or caspase in cells treated with negative control peptide PNC-29. Thus the cells were undergoing tumor cell necrosis and not apoptosis as was expected. In addition, a number of cell lines killed by PNC-27 shown in Figure 4 such as MDA-MB-453 breast cancer cells and SAOS2 osteogenic sarcoma cells are known to be p53 gene homozygously deleted. Thus both peptides must be inducing cancer cell death by a non-p53-dependent mechanism explaining the absence of expected apoptosis.

4. PNC-27 Causes Transmembrane Pore Formation in Cancer Cells Resulting in Extrusion of Intracellular Contents, Called Poptosis

To further investigate how PNC-27 kills cancer cells, we subjected tumor cells including breast cancer cells (MDA-MB-468) [11], pancreatic cancer cells (MIA-PaCa-2) and melanoma cells (A2058) to transmission electron microscopy after incubating them for 15 minutes with PNC-27 [10,12]. In each case, there were marked pores formed across the cell membranes of these cells and selective damage to mitochondria (suggesting that PNC-27 does enter cells), not seen when normal cells like human fibroblasts were incubated over the same time period and using the same dose of PNC-27. The structure of these pores is discussed further below. Time lapse photographs of cancer cells treated with PNC-27 show that this process is explosive, hence the term “poptosis.” The question arose as to how PNC-27 induced such pores.

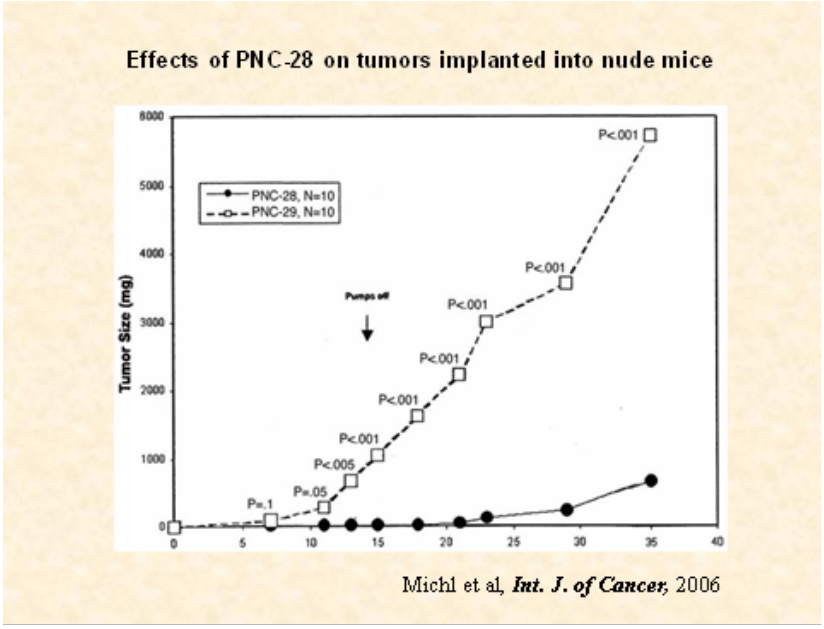

Figure 7: Effects of PNC-28 on BMRPA1-TUC-3 cells implanted subdermally in nude mice. Open squares, negative control, PNC-29; filled circles, PNC-28. p values for each time point are for tumor size difference for tumors treated by each peptide. P values ≤0.05 are considered to be statistically significant.

5. PNC-27 Adopts a Membrane Active Conformation

A clue to answer this question was provided in the three-dimensional structure of PNC-27 that was determine by two-dimensional NMR as shown in Figure 9, a space-filling model of PNC-27 [13]. The structure is an amphipathic alpha-helix-turn-alpha helix structure. The two helices are formed with the amino terminal residues corresponding to p53 residues 12-26 followed by a bend at the first two residues of the penetratin sequence followed by another alpha helix for the remainder of this domain. In the color scheme of Figure 9, all green-colored residues are hydrophobic amino acid residues whe the blue and red residues are charged hydrophilic residues, red for negative charged residues, blue for positively charged residues. The hydrophobic residues line one face of the molecule while the hydrophilic residues line the opposite face resulting in a so-called amphipathic conformation. This conformation has been observed in a number of so-called membrane active peptides which induce lysis of cell membranes presumably by forming transmembrane pores. These include melittin [14], an active component of bee venom which induces lysis of erythrocytes and magainin, an anti-microbial peptide that induces lysis of bacterial cell walls or membranes [15]. Both of these bioactive peptides adopt conformations that are similar to our structure for PNC-27.

Importantly, we have found that our PNC-27 structure is superimposable on the x-ray crystal structure of a p53 peptide corresponding to residues 17-29 bound to residues 1-109 of HDM-2, the binding site of HDM-2 for p53 [12,16]. PNC-27 has p53 residues 12-26, and we found that residues 17-26 of PNC-27 superimposed on the same residues of the x-ray structure as shown in Figure 10. This finding raised the question as to whether PNC-27 was binding to HDM-2 in the cancer cell membrane.

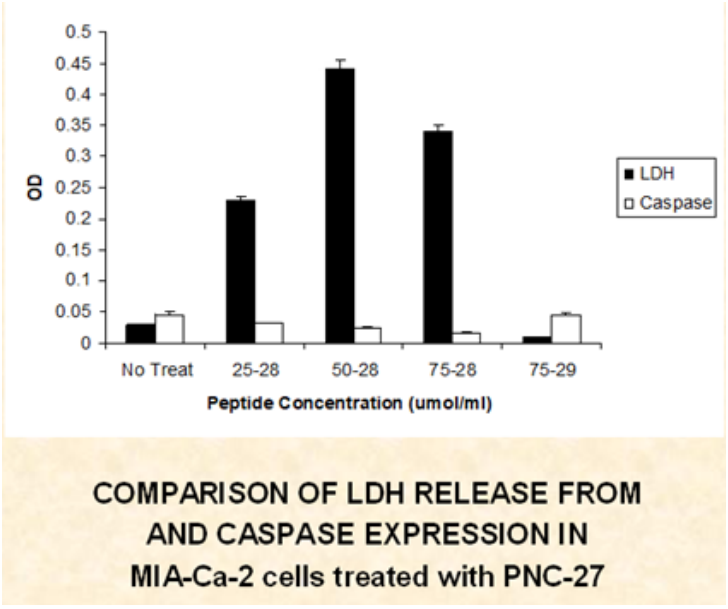

Figure 8: Comparison of LDH release from (filled bar graphs) and caspase expression (open bar graphs) in MIA-PaCa-2 human pancreatic cancer cells treated with PNC-28. The numbers on the X-axis are dose of peptide in ug/mL-peptide number (PNC-28 or PNC-29).

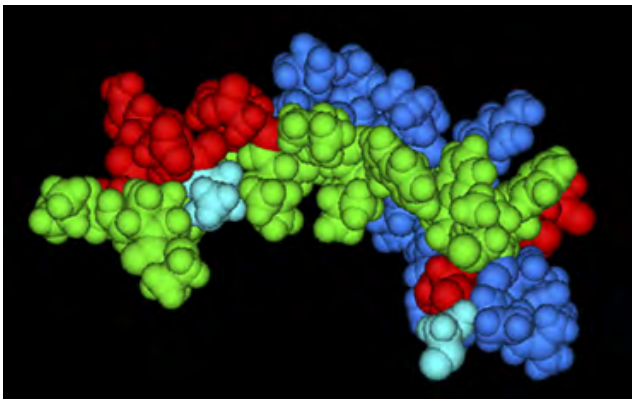

Figure 9: Space filling model of PNC-27 as determined by 2D-NMR. The amino terminus is at the left, the carboxyl terminus is at the right. Color scheme: green, hydrophobic residues; dark blue, positively charged amino acid residues (Lys and Arg); red negatively charged amino acid residues (Asp and Glu);cyan, uncharged, neutral residues.

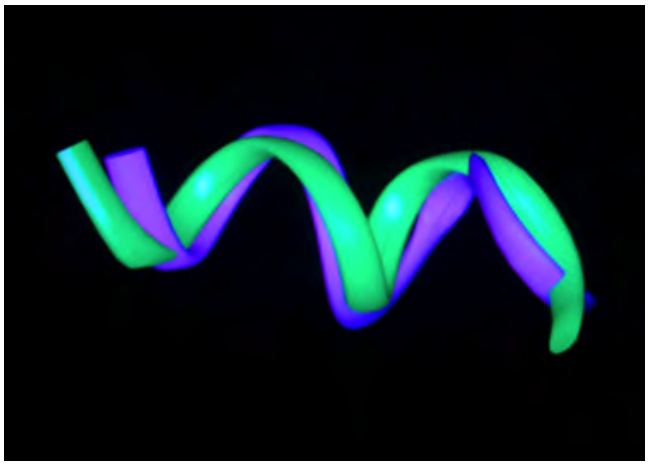

Figure 10: Ribbon representation of the superposition of residues 17-26 of PNC-27 (green0 on the same residues in the x-ray crystal structure of the p53 peptide 17-29 (blue) bound to the p53 binding domain of HDM-2 (residues 1-109).

6. PNC-27 Kills Cancer Cells by a Novel, Cancer Cell-Specific Mechanism

If PNC-27 binds to HDM-2 in cancer cell membranes, it would be expected that this protein would be detectable in cancer cell membranes. Since PNC-27 does not affect normal cells, this protein should not be detectable in the membranes of these normal cells. We obtained the cell membrane and nuclear fractions of a series of untransformed cells and another series of cancer cells to detect whether the above findings actually held. As can be seen in Figure 11 [16], Western blots were performed on the membranes and nuclear fractions of three normal cell lines (MCF-10-2A human breast epithelial cells), BMRPA1 rat pancreatic acinar cell line and AG13145 human fibroblast cell line (top lanes 1-3) and also on four different cancer cell lines, TUC-3 rat pancreatic acinar cell cancer, MIA-PaCa-2 human pancreatic cancer, MCF-7 (human breast cancer) and A20548 human melanoma cancer cell lines (top lanes 4-7) [16]. The figure shows only low levels of membrane-bound HDM-2 in lanes 1-3 for the normal cells although there were significant levels in their nuclear fractions (lower lanes 1-30) [16]. On the other hand, top lanes 4-7 show high levels of HDM-2 in the membranes of the four cancer cell lines and also high levels in their nuclear fractions. Quantitation of the levels of membrane-bound HDM-2 in all seven cell lines is shown in the bar graph at the bottom of the figure showing that there is a markedly higher expression of HDM-2 in the cancer cell lines compared with that in the three normal cell lines [16].

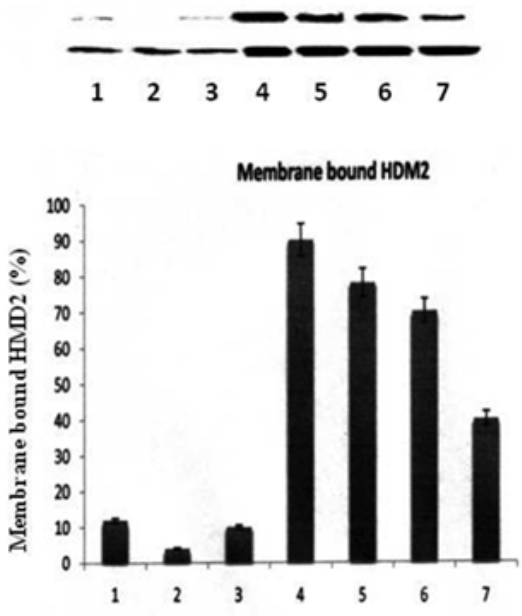

Figure 11: Top: Western blots of membrane fractions of seven different cell lines for membrane-bound HDM-2: lane: 1, MCF-102A; 2, BMRPA1; 3, AG13145; 4, BMRPA1-TUC3; 5, MIAPaCa-2;MCF-7;A2058.Middle: Western blots of nuclear fractions of these cell lines; bottom , plot of percent of total HDM-2 for each cell line that is membrane bound.

7. PNC-27 Colocalizes with HDM-2 in the Cancer Cell Membrane [16]

Figure 12 shows the immunofluorescence pattern when cancer cells are incubated for short periods with PNC-27 and then incubated with two antibody systems one a green fluorescent-labeled anti-PNC-27 and a red fluorescent labeled anti-HDM-2. In this figure A2058 human melanoma cells were incubated in the above-described manner with PNC-27 and the two labeled antibody systems. The top panels show low power cells in which PNC-27 labeled green (leftmost panel) and HDM-2 labeled red (middle panel) fluoresce, but show yellow fluorescence as merged images indicating that both fluorescent probes must be proximate to one another. The two lower sets of panels are high powered views of two such cells showing that all of the fluorescence is on the cell mem-

brane. The combined green and red fluorescence is seen to give a yellow-colored fluorescence due to the proximity of the probes in the cell membrane to one another indicating colocalization of PNC-27 with HDM-2. Identical results have been obtained on numerous other cancer cell lines including TUC-3, MIA-PaCa-2, MCF-7, SKOV-3, OVCAR-3, HCT-116, CT-26, CTA, CTP, CTR, SW1222, U937, OCI-AML3, HL60 but not on any normal cells including MCF-10-2A, BMRPA1, AG13145, HUVEC, CCD-18Co and rat mononuclear cells [6-8,16].

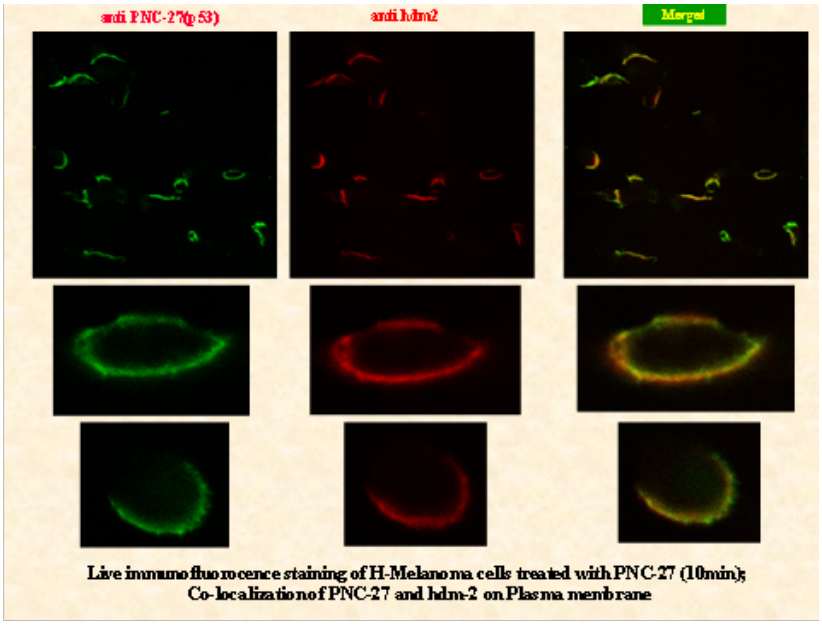

Figure 12: Confocal microscopic images showing colocalization of PNC-27 and membrane-bound HDM-2 in A2058 human melanoma cells. Cells were incubated with PNC-27, washed and incubated sequentially with anti-PNC-27 (green fluorophor) and anti-HDM-2 (red fluorophore) antibody systems.for each set of panels, leftmost image shows green fluorescence, middle image, red fluorescence, rightmost image shows merged fluorescence, yellow color (combined red and green) indicating colocalization. Uppermost panel shows cells labeled with each of these systems and appreciable merged yellow surface fluorescence in the third figure of this panel. Middle and bottom panels show two different cells showing that the green and red fluorescence is on the cell surface and that there is a strong yellow signal on the surfaces of each of the cells indicating colocalization.

8. Expression of PNC-27 on the Cancer Cell Surface is Necessary for PNC-27-Induced Tumor Cell Necrosis [16]

To determine if HDM-2 expression in the cell membrane is required for PNC-27-induced tumor cell necrosis, we have taken untransformed cells, i.e., MCF-10-2A human breast epithelial cells which are not affected by PNC-27 (see above), and transfected them with plasmids expressing full length HDM-2 with a nuclear localization peptide sequence on their carboxyl terminal ends. These sequences target proteins to the cell membrane. The plasmid used was designed by Origene called the Precision Shuttle Destination Vector. We prepared three plasmids that encoded full length HDM-2, full length HDM-2 with the membrane localization peptide and HDM-2 lacking its amino terminal 1-109 p53 binding domain also with the carboxyl terminal membrane localization peptide. Figure 13 shows empty vector and each construct transfected into these cells. As can be seen in Figure 14, Western blots of the membrane fractions of the two cell lines expressing the HDM-2 proteins with the membrane localization peptide showed high levels of expression in the cell membranes in contrast to those found in cells treated with empty vector or transfected with full length HDM-2 without the membrane localization peptide. In addition, PNC-27 was found to colocalize with the membrane-expressed HDM-2 with the membrane localization peptide on cells that had been transfected with the plasmid expressing this form of HM-2.

As shown in Figure 15 and summarized in Figure 13, each of these transfected cell lines was incubated with PNC-27 to determine if they were killed by this peptide. As shown in Figure 15, only the cells expressing full length HDM-2 with the membrane localization peptide were killed by PNC-27. These results suggest that, to induce tumor cell necrosis in cancer cells, PNC-27 binds to membrane-expressed HDM-2 resulting in transmembrane pore formation and that PNC-27 binds to HDM-2 in its p53 binding domain involving residues 1-109 since it colocalizes only with full length membrane-expressed HDM-2 and kills only these cells.

HDM-2 CONSTRUCTS AND ACTIVITIES

CONSTRUCT	PRESENT IN MEMBRANE	RESPONSE OF MCF-10-2A CELLS TO PNC-27
EMPTY VECTOR	-	-
FULL HDM-2	-	-
FULL HDM2-CAAX	+	+
DEL 1-109-HDM-2-CAAX	+	-

Figure 13: Summary of transfected Precision Shuttle vector constructs expressing different forms of HDM-2, into MCF-10-2A cells, their cellular locations (membrane-bound or not) and whether the cells expressing the different HDM-2 forms were killed by PNC-27.

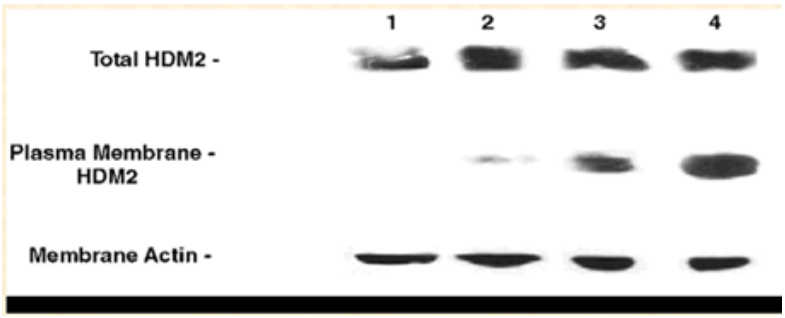

Figure 14: Western blots for HDM-2 in membrane and nuclear fractions in cells transfected with Precision Shuttle vectors expressing: no HDM-2, empty vector (lane 1); full length HDM-2 (lane 2); HDM-2 with carboxyl terminal membrane localization peptide lacking residues 1-109 (lane 3) and full length HDM-2 with the membrane localization peptide (lane 4). Membrane actin loading controls are shown at the bottom of each lane indicating same amounts of protein in each lane.

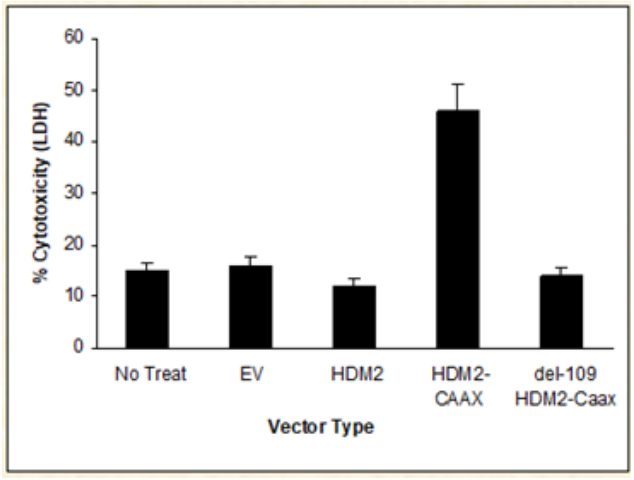

Figure 15: Bar graphs showing responses of transfected MCF-10-2A cells to PNC-27 as measured by release of LDH into the medium. Negative controls were untreated MCF-10-2A cells (first graph); MCF-10-2A cells transfected with empty vector (labeled as EV in the figure) (second graph); MCF-10-2A cells transfected with plasmid expressing full length HDM-2 with no membrane localization peptide (third graph); MCF-10-2A cells transfected with plasmid expressing full length HDM-2 with the membrane localization peptide (labeled as CAAX in the figure) (fourth graph); MCF-10-2A cells transfected with plasmid expressing HDM-2 with deleted residues 1-109 (the p53 binding domain) with the membrane localization peptide.

9. Structure of PNC-27-Induced Transmembrane Pores [12]

We have performed high resolution immune scanning electron microscopy (SEM) with high back-scatter on cancer cells treated with PNC-27 to visualize the overall features of the pores induced by this peptide. In these studies, we incubated the cancer cells treated for short periods with PNC-27 and then with gold-labeled secondary antibodies in the anti-PNC-27 and anti-HDM-2 antibody system described above in the colocalization section. Uniform gold particles of 6μ were used in the anti-PNC-27 antibody system and 15μ for the anti-HDM-2 antibody system.

Figure 16 shows several magnifications of SEMs of untreated MIA-PaCa-2 cells. The membranes are regular although ruffled, an effect of the presence of the oncogenic k-ras-p21 protein. Figure 17 are SEMs of the same cells incubated for 15 min with PNC-27 [12]. In contrast to the cell surfaces seen in Figure 16, the treated cells in Figure 17 contain discrete transmembrane pores best seen in the lower leftmost panel where multiple spherical particles sur-

round holes in the membrane. Figure 18 shows human AG13145 untransformed fibroblasts that were subjected to the same treatment as the MIA-PaCa-2 cells. The upper two panels show the results for untreated cells use membranes appear smooth while the lower two panels show the results for treated cells. As can be seen in this figure, there are no differences in the appearance of the membranes, and no pore formation is evident.

Figure 19 [12] shows the structure of the transmembrane pores induced in the MIA-PaCa-2 cells shown in Figure 17. The yellow arrows point to 15 μ gold particles identifying HDM2 while the red arrow point to the 6 μ gold particles identifying PNC-27. There appear to be doublets of PNC-27-HDM-2 complexes surrounding the pores estimated from sizing to be about 37 μ in diameter. Thus, pore formation occurs directly involving PNC-27 complexed with HDM-2.

Figure 20 is a model for the action of PNC-27 (and PNC-28) on cancer cells [12]. At the top of the figure, PNC-27 is represented by a blue (p53 residues 12-26) and a red (penetratin) alpha helix separated by a hairpin turn. HDM-2 is represented as a transmembrane red structure to which PNC-27 binds. Upon binding to HDM-2 the PNC-27 and HDM-2 form doublets, i.e., two sets of PNC-27-HDM-2 complexes based on the results of the gold-labeling-immune-SEM. These doublets can polymerize to quartets and high order complexes. PNC-27 are also shown to enter the cancer cell either through newly formed transmembrane pores or via a pathway in which the leader sequence allows for passage through the cell membrane. In either event intracellular PNC-27 can induce mitochondrial damage.

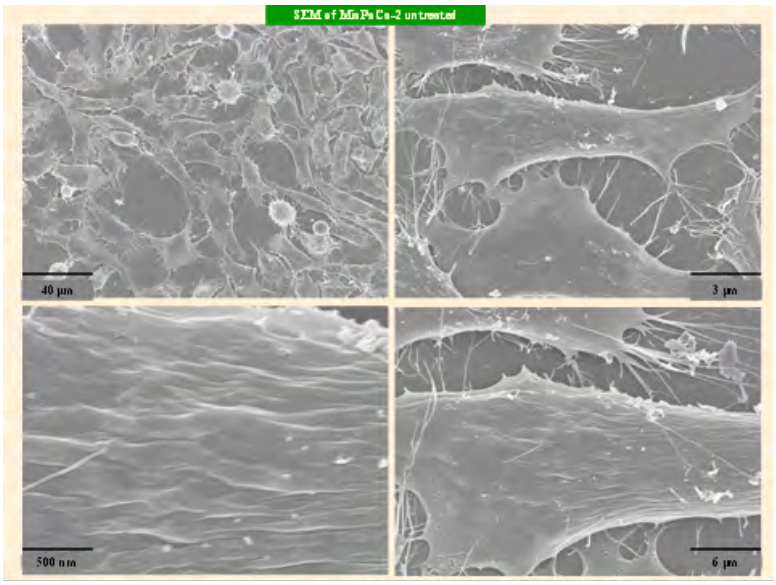

Figure 16: Scanning electron microscopic view of untreated MIA-PaCa-2 human pancreatic cancer cells at different magnifications as labeled on the four figures. At the highest magnification (lower left figure) the cell membrane appears regular and ruffled.

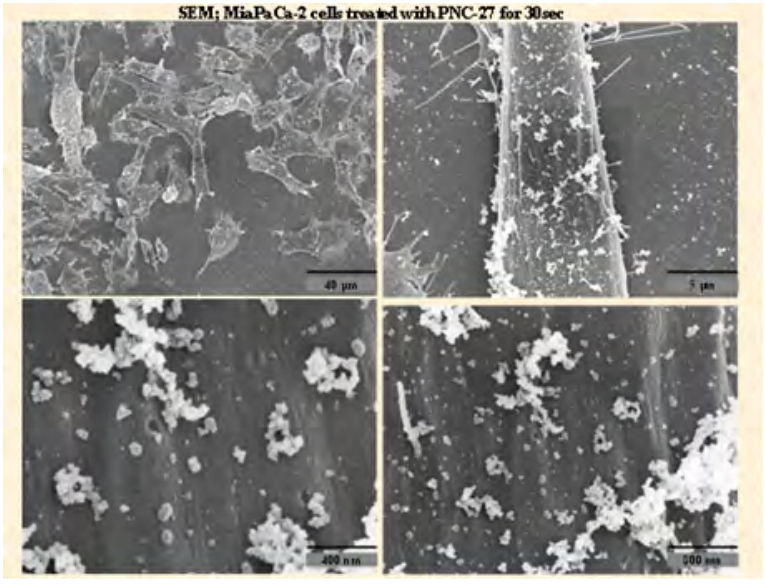

Figure 17: Scanning electron microscope view of the same cells treated for 15 min with PNC-27. At the two highest magnifications (bottom two views) numerous holes are seen in the cell membrane that are pores many of which surrounded by spherical particles.

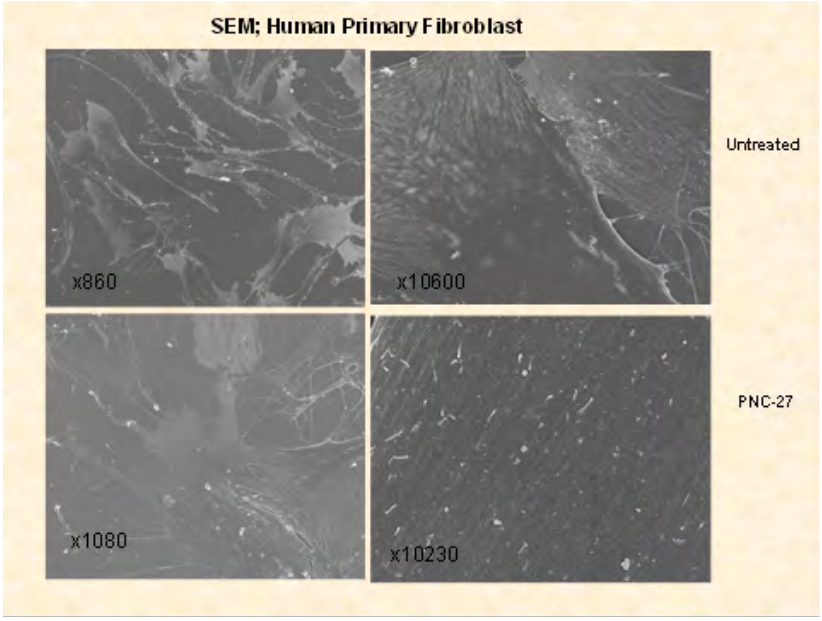

Figure 18: Scanning electron micrographs of normal AG13145 human fibroblast membranes in cells: untreated (upper two views with different magnifications) and treated with PNC-27 (lower two views). At both magnifications the membranes appear the same showing no pores or clusters of spherical particles.

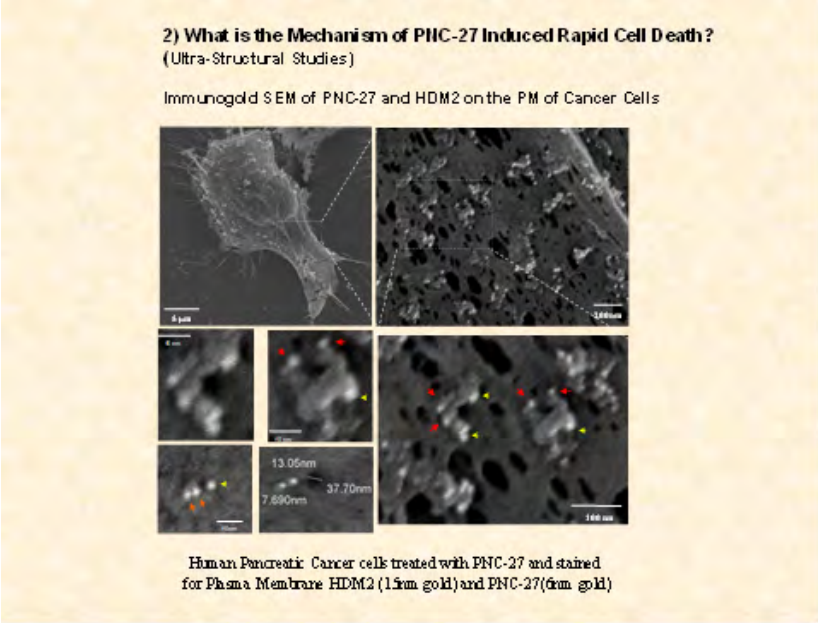

Figure 19: Scanning electron micrographs of MIA-PaCa-2 cells treated for 15 min with PNC-27 and then incubated with 6μ gold particle-labeled anti-PNC-27 antibody system and 15μ gold particle-labeled anti-HDM-2 antibody system. The upper two figures are lower power views of the cells that are similar in pattern to that seen in Figure 17. The bottom two views are at high magnification showing that there are multiple transmembrane pores many of which are surround with pairs of 6μ (red arrow heads) and 15 μ (yellow arrow heads) gold particles. The lower left figure is split into four sections. The upper left part of this figure is an overall view of the membrane while the upper right is at higher magnification with labeled gold particle sizes (red and yellow arrow heads). Particle sizing is shown in the lower left panel of this figure while the lower right panel gives the diameters of the transmembrane pores formed (37 μ) together with the measured diameters of the gold particles for the PNC-27 and HDM-2 systems.

Figure 20: Model for the actions of PNC-27 on cancer cells. The red structures depict HDM-2 molecules as inserting into the lipid bilayer of the cell membrane. PNC-27 is represented as having a red p53 12-26 sequence domain and a blue penetratin domain. These latter molecules bind to HDM-2 such that they form double dyads or two PNC-27-HDM-2 complexes together to initiate formation of transmembrane pores. Some PNC-27 molecules can enter the cell from pores formed by other PNC-27 molecules or by their penetratin sequence. PNC-27 that enters the cell causes damage to intracellular organelles like mitochondria contributing to cell death. See text for further explanation.

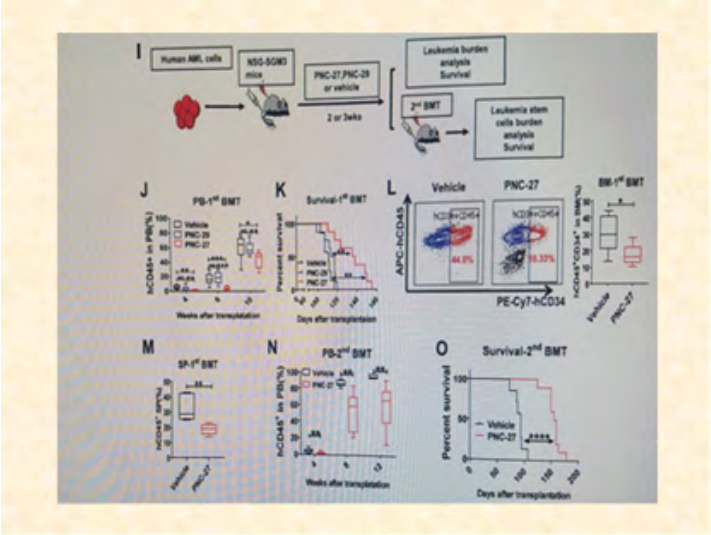

Figure 21: Pincus et al.

10. Large in vivo Study of PNC-27 in Nude Mice on Stem-Cell-Enriched Human Acute Myelogenous Leukemia Cells [9]

The ultimate test of a potential anti-cancer drug is its ability to treat cancers in vivo. As we noted above, PNC-28 was found to eradicate a highly metastatic rat pancreatic cancer (TUC-3) in nude mice. More recently, a large in vivo study was performed at the City of Hope Medical Center in Los Angeles on stem-cell enriched acute myelogenous leukemia (AML) cells harvested from nine different patients. These cells were implanted into the bone marrows of nude mice which developed AML and high white cell counts. These mice were then treated with daily intraperitoneal injections of PNC-27 over a three-week period. At the end of this period, the white cell counts normalized. Bone marrow cells were then explanted from these mice into the bone marrows of innocent mice whose white cell counts were followed and found to be normal. Both cohorts of mice showed markedly prolonged Kaplan-Meier survival curves compared with those of treated mice with negative control peptide, PNC-27.

Simultaneously, in these studies, the stem cell-enriched AML cells from each patient were placed into culture and dose-response curves for cell killing by PNC-27 were obtained. At the same time, quantitation of HDM-2 in the cell membranes of these cells was performed using flow cytometry. These studies showed a linear correlation of HDM-2 expression with the IC50 of PNC-27 in cancer cell killing. Importantly, a potential target of HDM-2 in the cancer cell membrane was identified in these studies as being cadherin -3, a protein involved in cell-cell adhesions that result in in-

hibition of cell proliferation and that was identified in prior studies on metastatic breast cancers.

These results of studies strongly support that PNC-27 has great potential in treating human cancers.

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