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ORIGINAL ARTICLE

The anti-cancer peptide, PNC-27, induces tumor cell lysis as the intact peptide

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Abstract
Purpose PNC-27, a peptide that contains an HDM-2-binding domain from p53 attached to a membrane-penetrating peptide on its carboxyl terminal end, is cytotoxic to cancer, but not normal, cells. It forms transmembrane pores in the cancer cell membrane. Our purpose is to determine if the whole peptide or critical fragments induce pore formation in cancer cells.
Methods We have prepared PNC-27 with a green fluorescent label on its amino terminus and a red fluorescent label on its carboxyl terminus and treated MCF-7 breast cancer cells and untransformed MCF-10-2A breast epithelial cells with this double-labeled peptide to determine if combined yellow fluorescence occurs in the membrane of the cancer cells during cancer cell killing.
Results At 30 min, there is significant combined punctate yellow fluorescence, indicative of intact peptide, in the cell membrane of cancer cells that increases during cancer cell lysis. MCF-10-2A cells show initial (30 min) uniform combined yellow membrane fluorescence that subsequently disappears. Unlike the cancer cells, these untransformed cells remain viable.
Conclusions PNC-27 induces cancer cell membrane lysis by acting as the whole peptide, not fragments. The punctate yellow fluorescence is due to interaction of PNC-27 with intramembrane targets of MCF-7 cells that do not exist in the membrane of the untransformed cell line. This interaction increases the lifetime of PNC-27. Absence of these targets in the membranes of the untransformed MCF-10-2A cells results in initial uniform fluorescence of the double-labeled peptide in their membranes after which the peptide is degraded.

Keywords PNC-27 · Anti-cancer peptide · Tumor cell necrosis · Pore formation · Double fluorescent-labeled peptide · Confocal microscopy · Intramembrane target

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Introduction

PNC-27 is a peptide that contains an HDM-2-binding sequence derived from p53 attached to a transmembrane penetrating sequence. This peptide is cytotoxic to cancer cells by inducing transmembrane pores but has no effect on

normal cells [1–5]. We have determined the three-dimensional structure of PNC-27 using two-dimensional NMR and found that it adopts a well-defined strongly amphipathic alpha-helix-loop-alpha-helix structure [6]. The amino terminal alpha-helix involves residues 17–26 that are involved in interactions with HDM-2. The carboxyl terminal helix involves most of the residues of the transmembrane-penetrating sequence [6]. This structural motif has been described for a number of membrane-active peptides and proteins [7].

In a recent study, we explored the role of the transmembrane-penetrating sequence in inducing cancer cell death [4]. We transfected a plasmid that encoded the 17–26 p53 peptide from its HDM-2-binding domain without the transmembrane-penetrating sequence into MIA-PaCa-2 pancreatic cancer cells to determine if the peptide induced cell death by necrosis due to membrane lysis or by p53-dependent apoptosis [4]. We found that cell death occurred via p53-dependent apoptosis as revealed by increased levels of expression of p53, annexin V and waf^{p21} and caspase [4]. These findings were in contrast to results obtained with the same cells treated with PNC-27 [4]. These cells did not express any of these markers for apoptosis above background levels but underwent membranolysis as revealed by rapid release of lactate dehydrogenase (LDH) into the medium [4]. Transmission electron microscopy on these PNC-27-treated cells showed extensive trans-membrane pore formation and cell membrane destruction explaining the release of LDH [4].

These results suggested that the transmembrane-penetrating sequence is pivotal in changing the mechanism of action of the HDM-2-binding domain peptide from inducing cytotoxicity by apoptosis to inducing cytotoxicity by membrane pore-dependent tumor cell necrosis. In control experiments, we found that the isolated p53 peptide 12–26, the isolated trans-membrane-penetrating sequence and combined p53 12-26 and transmembrane-penetrating peptide have no effect on the growth of tumor cells [1, 2]. These results suggested that, to be active, PNC-27 must remain intact and not undergo cleavage into its two component domains.

Treatment of different cancer cells with PNC-27 results in total cell death over time periods that vary from minutes to several hours in PBS media-free solution to 24–48 h in supplemented media, suggesting that elements of the media such as proteins may either block pore formation or seal formed pores [1]. In the latter circumstance, the fact that PNC-27 induces total tumor cell necrosis suggests that it maintains its structural integrity during tumor cell lysis. However, this inference, and the results of the control experiments discussed above in which we found that the component domains of PNC-27 do not induce tumor cell necrosis, do not preclude the possibility of cleavage of this peptide into different fragments that may themselves be membrane-active.

Therefore, to determine in this study if PNC-27 maintains its structural integrity during tumor cell lysis, we have prepared a double fluorescent probe-labeled form of PNC-27 with a green fluorescent probe on the amino terminal and a red-fluorescent probe on the carboxyl terminal end of the peptide. We have treated a cancer cell line with this peptide and observed the fluorescence pattern over the time course of PNC-27-induced tumor cell necrosis. If the peptide remains whole, simultaneous fluorescence of both fluorophores should occur showing a yellow fluorescence. In contrast, if the peptide is cleaved on the cell membrane, two fragments should result showing non-combined fluorescence, i.e., discrete green and red fluorescence.

Materials and methods

Materials

Peptides

PNC-27, **H-Pro-Pro-Leu-Ser-Gln-Glu-Thr-Phe-Ser-Asp-Leu-Trp-Lys-Leu-Leu-***Lys-Lys-Trp-Lys-Met-Arg-Arg-Asn-Gln-Phe-Trp-Val-Lys-Val-Gln-Arg-Gly-OH* (1 g), was synthesized using solid phase methods (Shaanxi Zhongbang Pharma-Tech Corp., NanguanZhengjie, Xi'an, China) and was >95% pure by HPLC and mass spectrographic analysis. The bold sequence corresponds to amino acid residues 12–26 of the hdm-2-binding domain of human p53 while the italicized sequence corresponds to the membrane residency peptide (MRP) segment that allows entry of the whole peptide into cells. PNC-28, which contains p53 residues 17–26 linked to the MRP, was likewise synthesized by solid phase methods (Biopeptide Corp., La Jolla, CA). A double fluorescent probe-labeled form of PNC-27 was prepared containing an amino terminal fluorescein isothiocyanate (FITC) fluorophore and a carboxyl terminal rhodamine fluorophore (Biopeptide Corp.). The negative control peptide, PNC-29 [1–5], containing the X13 peptide from cytochrome P450 (bold) attached to the MRP (italics), **H-Met-Pro-Phe-Ser-Thr-Gly-Lys-Arg-Ile-Met-Leu-Gly-Glu-** *Lys-Lys-Trp-Lys-Met-Arg-Arg-Asn-Gln-Phe-Trp-Val-Lys-Val-Gln-Arg-Gly-OH*, was likewise synthesized by solid phase methods and was likewise >95% pure.

FITC- and TAMRA-labeled PNC-27 peptide

This peptide was synthesized with two fluorescent labels: amino terminal 5,6-carboxy-fluorescein (green fluorescence) and carboxyl terminal 5-tetramethyl Rhodamine (TAMRA) (red fluorescence). The double-labeled peptide

was prepared automatically using an ACT-396 synthesizer starting with 0.6 g of the Lys(5-tetramethyl Rhodamine [TAMRA])-NovaSyn TGR Resin (0.18 meq/g). All acylation reactions were carried out using a fivefold excess of Fmoc-amino acid activated with HBTU(1 eq.) in presence of HOBt(1 eq.) and NMM(2 eq.). A coupling time of 60 min was used throughout. DMF was used as a solvent and 20% piperidine/DMF treatment for 5 and 15 min was used for Fmoc removal. The final coupling of 5,6-carboxyfluorescein was done manually, using DIC/HOBt for 2 h. Cleavage of the peptide from the resin and side-chain deprotection was carried out by treatment of the peptidyl resin with TFA/TIS/water (95:2.5:2.5) for 3 h. The crude peptide (0.6 g) was purified by RP chromatography on Polymerex C18 10 × 250 mm column by elution at a flow rate 6 ml/min, using the gradient 0.1%TFA/water to 40%ACN/0.1%TFA/water in 2 h. The fractions containing the product with purity >95% by analytical HPLC were pooled and lyophilized to yield 10 mg of the final product.

Cell lines

MCF-7 and MCF-10-2A cells were obtained from the American Type Culture Collection (Manassas, VA) and were incubated in the appropriate media as described previously [1–3].

Methods

Time course for cell killing by PNC-27

To test the efficacy of PNC-27 over time, we treated MCF-7 breast epithelial cancer cells, as well as their untransformed counterpart, MCF-102A, with PNC-27, and the effects were observed and recorded over a 48-h period.

Approximately 2×10^4 cells were seeded into each well of 3 sterile 24-well tissue culture dishes and incubated overnight. The following day the cells were treated, in triplicate, with PNC-27 peptide at a standard concentration of 50 μg/ml. Prior to treatment, the peptide was briefly sonicated and then added to the media. After the initiation of peptide treatment samples of media and cells were taken at different time points (0, 2, 4, 12, 24 and 48 h), and the following three assays were performed at each time point:

MTT assay For each time point as stated above, after peptide treatment was started, 10 μl of MTT substrate (Promega, WI) was added to each specific well and incubated for 4 h. After 4 h, 100 μl of MTT stop solution was added and the absorbance was read in a SpectraMax 90 at 595 nm.

LDH assay At each time point, a volume of 100 μl of supernatant from each well was taken and stored at −80 degrees. After completion of the time course experiment, a volume of 50 μl of each sample, along with total lysate, was added to a 96-well plate. To these samples, we added 50 μl of LDH substrate (Promega) and incubated the samples at room temperature for 30 min. After incubation, 50 μl of stop solution was added to each well and the absorbance was read at 492 nm in the spectrophotometer microplate reader.

Capsase assay To determine if apoptosis occurred in PNC-27-treated cells, for each time point, cells from each well were washed with PBS, harvested, and lysed with 50 μl of cell lysis buffer (Biovision Inc, Mountainview, CA) for 10 min in ice with intermittent gentle vortexing. For the positive control experiment, a total of 50 μl of total lysate along with 50 μl of staurosporine (Sigma, St. Louis, MO, USA) at a concentration of 45 μl/ml was added to a 96 well plate. 2X Reaction buffer (50 μl) and 5 μl of caspase 3,7 substrate (Biovision, Inc) were then added. The plate was incubated at 37°C, according to manual protocol and then read at 405 nm. The above procedure was repeated in parallel for the untransformed counterpart, MCF-10-2A, cell line.

Confocal microscopy

MCF-7 and MCF-10 cells were grown to 60% confluence on glass coverslips and were then incubated for different times (30 min, 4, 24 and 48 h) with 2.5 μM (approx 10 μg/ml) FITC-TRITC-labeled PNC-27 peptide +50 μg/ml of "cold" PNC-27. The coverslips were washed with PBS, fixed in 70% methanol for different time point and used for confocal microscopy. After extensive washing, the cover slips were mounted over antifade (Molecular Probes, Invitrogen, Carlsbad, CA) on glass slides and examined with a laser-equipped Olympus Confocal microscope 1X76. Results were digitally recorded. Green, red and combined green and red (yellow) fluorescence were determined.

Results and discussion

Time course for PNC-27-induced tumor cell necrosis

Figure 1 summarizes the time course for MCF-7 cell-killing induced by PNC-27 in media. Previously, we performed the same study for cells in PBS over 1 h [2] over which time 100 percent cell killing occurred. Release of LDH occurs rapidly and appears to reach a maximum at about 24 h although there is a mild increase over the remainder of the time course. Tumor cell death also increases over the same time course although more slowly than LDH release but reaches 80 percent in 24 h, after which time LDH and cell death correlate with one another (Fig. 1). At 48 h, virtually 100 percent cell death and maximal LDH release occur. In

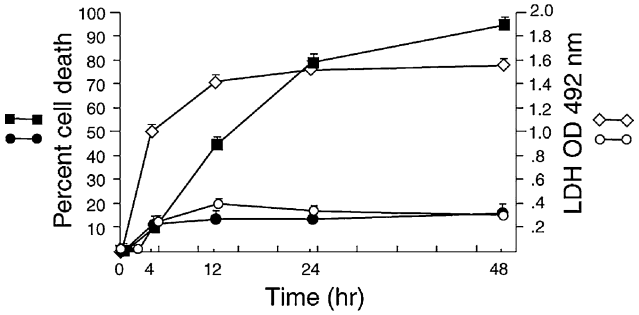

Fig. 1 PNC-27-induced cell death (left *Y*-axis) (MTT assay) and LDH release (right *Y*-axis) of MCF-7 cells (*closed squares* and *open diamonds*, respectively) and of untransformed MCF-10-2A cells (*closed* and *open circles*, respectively) over a 48-h time course

contrast, the negative control PNC-29 peptide had no effect on the rate of cell growth (not shown). As also shown in Fig. 1, PNC-27 had virtually no effect on LDH release and caused no increase in the rate of cell death in the control untransformed MCF-10-2A cell line over the background rate for negative control PNC-29-treated cells (not shown), as we found previously [2].

Cell killing occurs by tumor cell necrosis, not apoptosis

We previously found that PNC-27 does not induce caspase activity in MCF-7 cells in PBS, indicating that PNC-27 does not kill tumor cells using p53-dependent or independent pathways but rather induces tumor cell necrosis as evidenced by early LDH release [2]. As shown in Fig. 2, we likewise find that caspase activity in MCF-7 tumor and untransformed MCF-10-2A cells in media does not

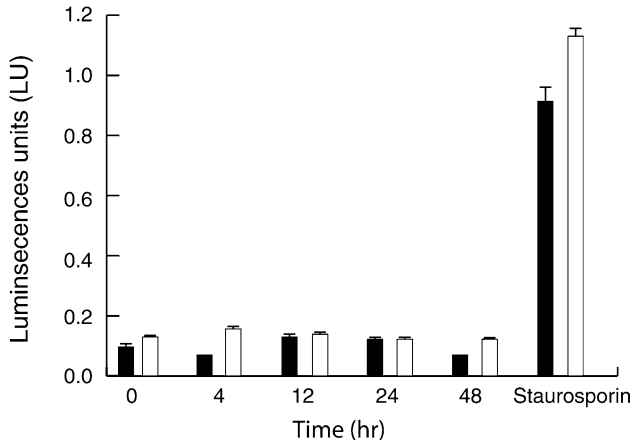

Fig. 2 Results of caspase assays on MCF-7 (*dark bar graphs*) and MCF-10-2A (*white bar graphs*) on cells treated with 50 μg/ml PNC-27 over a 48-h time period. The results for the positive staurosporine (labeled "staurosporin" on the *X*-axis) control for both cell lines is shown after the 48-h results on the *right* of the figure

increase above the baseline (0 time) value over the entire 48-h period while the control agent, staurosporine, which is known to induce apoptosis in cancer cells [8], is seen to induce high levels of caspase activity in both cell lines.

Confocal microscopy shows whole PNC-27 peptide binds to the cancer cell membrane where it induces pore formation

We then treated MCF-7 breast cancer cells with the double-fluorophore-labeled PNC-27 and observed the fluorescence pattern over the 48-h period. The results are shown for representative cells in Fig. 3. In this figure, for each time point (0.5, 4, 24 and 48 h), red (carboxyl terminal), green (amino terminal) and combined red and green (yellow-orange) fluorescence are shown, respectively, in the frames from left to right.

Beginning at the 30-min (0.5 h) time point, each of the representative cells shows punctate red and green fluorescence over the cell surface. For the 0.5 and 4 h time points, the yellow fluorescence pattern (combined red and green indicative of the presence of the whole peptide) corresponds to these individual red and green fluorescence patterns suggesting that the whole peptide is bound to the cell membrane. The punctate pattern suggests that PNC-27 binds to discrete sites in the cell membrane rather than non-specifically intercalating into it. For the 4-h time point, a small level of green fluorescence occurred both on the nuclear membrane and in the nucleus, suggesting that a small amount of peptide hydrolysis occurred wherein only (an) amino terminal fragment(s) entered into the cell.

The yellow fluorescence pattern on the cell membrane increased in intensity from 0.5 to 4 h (compare the rightmost frames for the 0.5 and 4 h time points in Fig. 3). At 4 h, about 60 percent release of LDH occurred as shown in Fig. 1. Since we have previously found that PNC-27 and its shorter homologue, PNC-28, induce pore formation in the membranes of cancer cells [1–5], inducing LDH release, it appears that PNC-27 induces pore formation as the whole peptide.

Surprisingly, the yellow fluorescence on the cell surface continued to increase in intensity and extent for the remaining viable cells at 24 h, when over 80 percent cell death occurred (Fig. 1). In other studies, we have obtained evidence that peptide degradation occurs extensively over a 24-h period [1]. However, these present results suggest that peptide half-life is significantly prolonged if the peptide binds to the cancer cell membrane. Nonetheless, that some peptide degradation occurred can be seen in Fig. 3 for the 24-h time point by significant green fluorescence in the nuclei of the remaining viable cells. Interestingly, there was no red or yellow fluorescence in the nuclei of these cells, suggesting that only (an) amino terminal fragment(s)

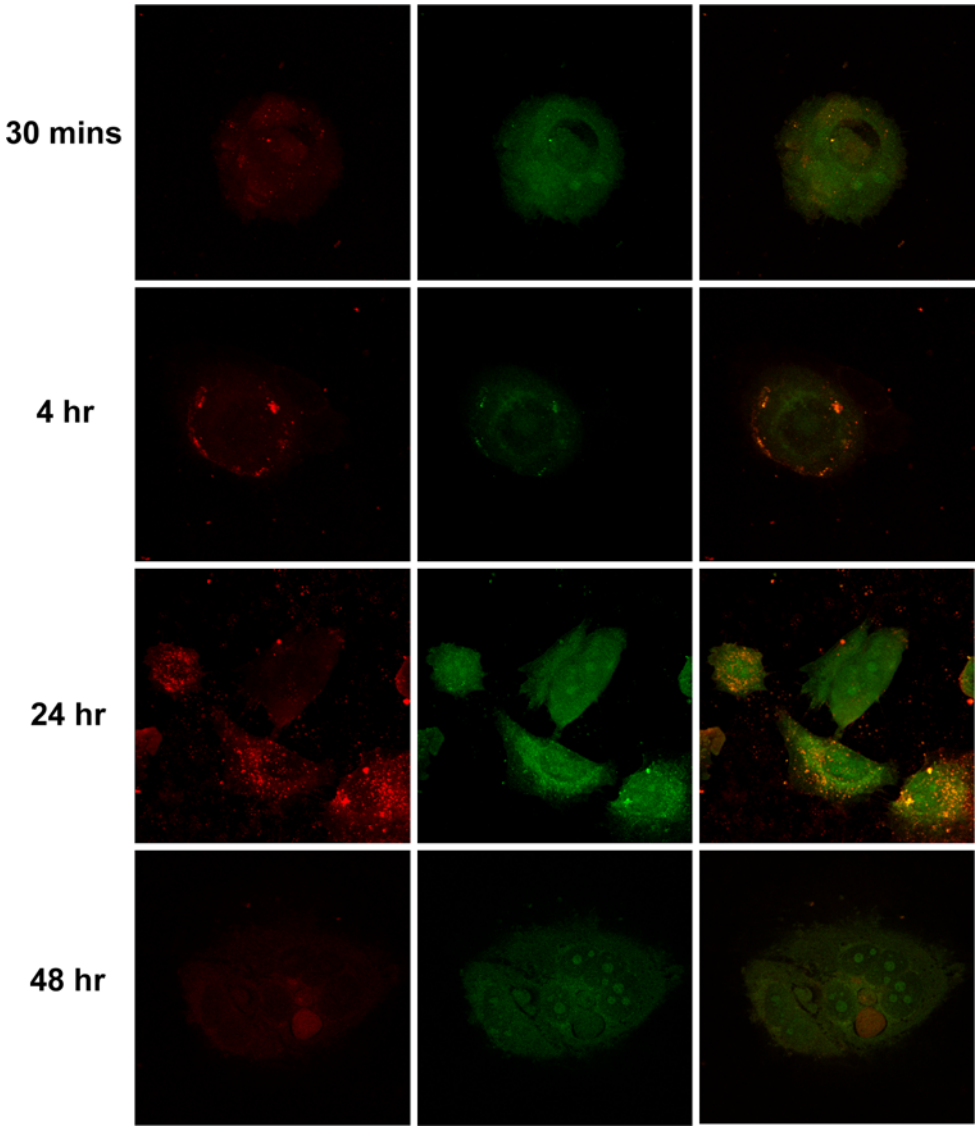

Fig. 3 Confocal microscopy results for MCF-7 cells treated with double-fluorescent-labeled PNC-27, with *green* fluorescent tag on the amino terminus and *red* fluorescent tag on the carboxyl terminus. Time frames proceed from *top* to *bottom* as labeled on the *left* of the figure. For each time frame, the three fluorescent results are shown from *left* to *right*: *red* fluorescence, *left*; *green* fluorescence, *middle*; and *combined red* and *green* (*yellow*) fluorescence, *right*

entered the nuclei. As can be seen in Fig. 3, at 48 h, there were no viable cells remaining but only enlarged cell fragments with inclusions of amino terminal fragments and of whole peptide (orange-colored) inclusions.

Overall, the above results suggest that significant amounts of whole peptide bind uniquely to the cancer cell membrane in increasing amounts over time. This increase in whole peptide binding to the cancer cells' membranes appears to correlate with increased release of LDH and cancer cell death over the time course of this study. Since, at 24 h, increased presence of whole peptide on the membranes of the cells was accompanied by increased presence of amino terminal peptides in the nucleus, there is a possibility that these peptide fragments induce membrane pore formation and tumor cell lysis. This possibility is unlikely in view of the absence of these fragments in the cell membrane where pore formation occurs and in view of our finding (Fig. 1) that LDH release was elevated at 4 h when almost all of the whole peptide was present in the cell membrane and only a small fraction of amino terminal fragment was observed in the nucleus. Since PNC-27 induces rapid tumor cell lysis by the same mechanism, i.e., induction of membrane pore formation, in a wide variety of tumor cells [1–3], including a variety of breast cancer cells [2], such as MCF-7 (this study), MDA-MB-468 and MDA-MB-157, we conclude that PNC-27 produces these effects as the intact peptide.

Confocal microscopy studies on untransformed MCF-10-2A cells

We performed the same studies on untransformed human breast epithelial MCF-10-2A cells. As shown in Fig. 4, at 0.5 h, there is intense red and green fluorescence on the surfaces of the representative cells that were incubated with double-fluorophore-labeled PNC-27. As can be seen in the

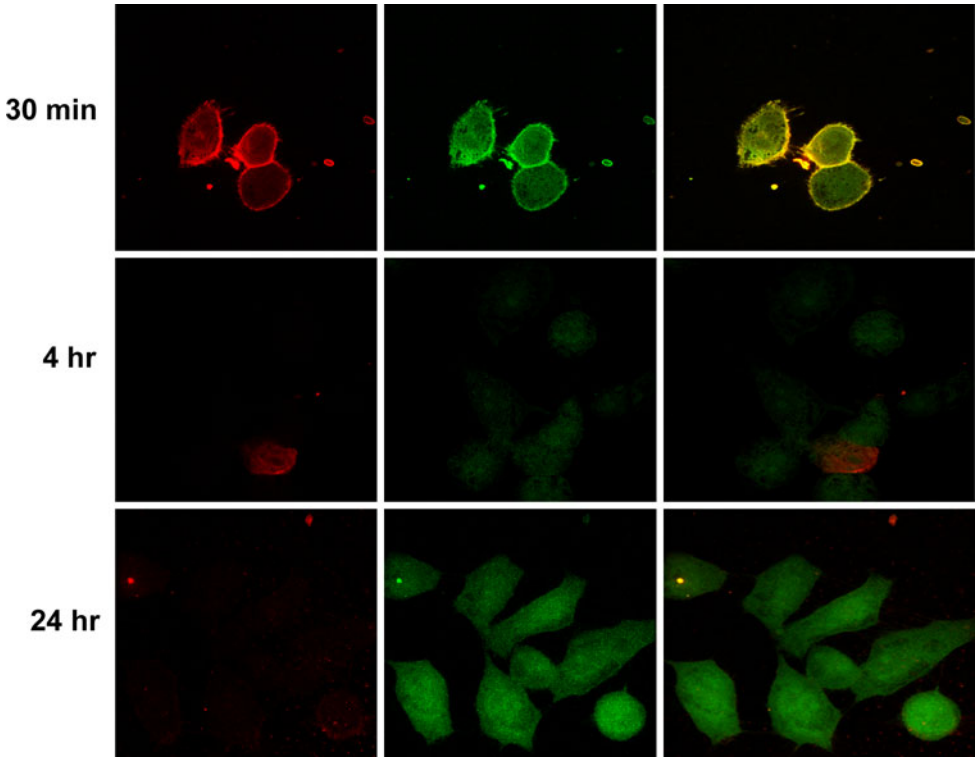

Fig. 4 Confocal microscopy results for MCF-10-2A cells treated with double-fluorescent-labeled PNC-27 as described in the legend to Fig. 3. As in Fig. 3, the time frames proceed from *top* to *bottom*, the 30-min time frame occurring at the *top* of the figure and the 48 time frame at the *bottom*, as labeled on the *left* of the figure. For each time frame, *red*, *green* and combined (*yellow*) fluorescence is shown from *left* to *right*, respectively

merged fluorescence (rightmost frame), virtually all the peptide in the cell membrane is whole peptide as evidenced by intense yellow membrane fluorescence. However, in contrast to the results for the breast cancer cells, that showed punctate yellow fluorescence (Fig. 3), the yellow fluorescence for the normal cells was distributed uniformly over the cell surface. This finding suggests that PNC-27 inserts into the cell membrane but does not bind to specific sites in the membrane while it binds to discrete targets in the membranes of the cancer cells giving rise to the punctate pattern.

In striking contrast to the MCF-7 breast cancer cells treated with PNC-27, the cells at 4 h have virtually no red or yellow fluorescence in any cell fractions but contain only green fluorescence, indicative of amino terminal fragments that are distributed throughout the cell. This pattern is enhanced at 24 h. Also in contrast to the MCF-7 cells, cell viability was maintained in all cells at 24 and 48 h (Fig. 1) compatible with the results in Fig. 1 showing no increased release of LDH and no significant cell death over the 48-h time period. These results suggest that PNC-27 is hydrolyzed rapidly in the cell membrane; the membrane penetrating sequence (red fluorescence) is either degraded or diffuses out of the cell while the amino terminal fragments distribute throughout the cell fractions but cause no membrane lysis or cell death. This result is compatible with our prior observation that expression of the amino terminal amino acid residues 17–26 from the peptide PNC-28 in the untransformed pancreatic acinar BMRPA1 cell line does not induce cell death [4]. Since accumulation of these fragments do not induce LDH release or cell death, this result further supports our conclusion, discussed above, that accumulation of amino terminal fragments in the MCF-7 cells likewise are not likely to cause LDH release and cell death in these cells. They further imply that when PNC-27 interacts with specific membrane targets, it may be protected from hydrolysis by cellular proteases; these interactions do not occur in the untransformed cell line since these targets may not be present in their cell membranes.

Conclusions

Our results suggest that PNC-27 causes cancer cell death as the intact peptide. This is based on our finding that the occurrence of combined amino and carboxyl terminal fluorescence occurs concurrently in the membranes of the cancer cells and increases with time while both LDH release and cell death simultaneously increase. They further point to the possibility that PNC-27 interacts with specific targets in the membranes of cancer cells that do not occur in the membranes of untransformed cells. This conclusion is based on our observation that the combined yellow amino and carboxyl terminal concurrent fluorescence occurs in a punctate pattern for cancer cells but in a homogeneous pattern for untransformed cells. The latter finding suggests that PNC-27 intercalates into the cell membrane of the untransformed cells without binding to specific targets. Interaction

of PNC-27 with specific membrane targets increases its lifetime allowing for prolonged action on the cell membrane. Absence of these targets in normal or untransformed cells results in hydrolysis of the peptide into amino and carboxyl terminal fragments allowing for diffusion of the former throughout the cell and expulsion and/or degradation of the latter.

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