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Molecular Targeting of H/MDM-2 Oncoprotein in Human Colon Cancer Cells and Stem-like Colonic Epithelial-derived Progenitor Cells

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Abstract. *Background/Aim: We have tested whether the anti-cancer peptide, PNC-27, that kills cancer cells but not normal cells by binding to cancer cell membrane HDM-2 forming pores, kills CD44+ colon cancer stem cells. Materials and Methods: Flow cytometry determined the CD44 and HDM-2 expression on six-colon cancer cell lines and one normal cell line (CCD-18Co). MTT, LDH release, annexin V binding and caspase 3 assays were used to assess PNC-27-induced cell death. Bioluminescence imaging measured PNC-27 effects on in vivo tumor growth. Results: High percentages of cells in all six tumor lines expressed CD44. PNC-27 co-localized with membrane HDM-2 only in the cancer cells and caused total cell death (tumor cell necrosis, high LDH release, negative annexin V and caspase 3). In vivo, PNC-27 caused necrosis of tumor nodules but not of normal tissue. Conclusion: PNC-27 selectively kills colon cancer stem cells by binding of this peptide to membrane H/MDM-2.*

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Key Words: Cancer stem cells, PNC-27, H/MDM-2, tumor cell necrosis, peritoneal carcinomatosis.

Colorectal cancer is the third most common cause of cancer and cancer-related deaths worldwide. In patients with metastatic, unresectable disease, systemic therapy is the treatment of choice (1, 2). However, the majority of patients with advanced disease will become refractory to treatment and develop disease progression despite first-line therapy, becoming potential candidates for second-line systemic therapy (3). Mutations in cancer stem cells (CSCs) and their self-renewal properties make them a root cause of systemic disease through metastasis-initiating cells (MICs) and disease relapse (4). Therefore, identification of therapeutic agents against CSC targets has become critical in developing new cancer treatment strategies.

One candidate agent that has been found to be effective against CSCs is the anti-cancer peptide, PNC-27 and its shorter homologue, PNC-28. PNC-27 contains an HDM-2 binding segment from human p53 (corresponding to residues 12-26) attached on its carboxyl terminal end to a leader peptide, called the membrane residency peptide (MRP), that inserts into cell membranes (5). Both peptides have been found to induce tumor cell necrosis in a wide variety of solid tissue and hematopoietic cancer cells both *in vivo* and *in vitro* but have no effect on the viability or growth of normal cells including, importantly, human hematopoietic stem cells (5, 6). A number of studies have suggested that specificity of these

peptides is caused by their binding to the double minute-binding protein of human or mouse origin (H/MDM-2) that is uniquely expressed in the cell membranes of cancer cells at significantly higher levels than that expressed on normal or untransformed cells (7). Formation of this peptide-protein complex induces transmembrane pore formation resulting in rapid tumor cell necrosis (6-8). Subsequent studies have further confirmed that PNC-27 selectively induces tumor cell necrosis by co-localizing with H/MDM-2 resulting in rapid tumor cell necrosis (9-11).

In a major *in vivo* and *in vitro* study of the effects of PNC-27 on stem cell-enriched human acute myelogenous leukemia (AML) cells from multiple patients with this disease, identified by their expression with the stem cell marker protein, CD-34, PNC-27 was found to be strongly cytotoxic to each AML tumor, with IC_{50} values that correlated with the extent of expression of HDM-2 on the tumor cell surfaces (12). In addition, AML-cancer stem cell (CD-34-expressing)-enriched tumor cells were implanted in the bone marrows of nude mice that were treated with PNC-27 or the negative control peptide, PNC-29. After treatment, bone marrow explants from these mice were transplanted into the bone marrows of nude mice. White cell counts in the PNC-27-treated groups were found to be reduced to normal levels and their survival times were significantly longer than those for the negative control peptide-treated group whose white cell counts remained elevated and exhibited no off-target effects (12). These results suggested that PNC-27 is a potent agent that kills hematopoietic cancer stem cells, but does not affect normal cells, including normal (non-malignant) hematopoietic stem cells as found in our prior studies (5).

In this article, we have extended our studies on the effects of PNC-27 on cancer stem cells to human solid tissue tumors, in particular human colon cancers. Like hematopoietic cancers, colon cancers have been found to contain tumor stem cells (13-16) as indicated by expression of surface markers identifying these cell types, most notably the glycoprotein, CD-44. This protein is expressed on colon cancer stem cell surfaces in different splice variant forms, all of which bind to hyaluronan to activate mitogenic signal transduction pathways. Solid tissue tumor cells expressing this protein, but not those that do not express this surface protein, have been found to regenerate themselves and to divide into a variety of more differentiated forms of tumor cells when transplanted into nude mice (13).

We, herein, show that PNC-27 is effective in killing a variety of colon cancer cells, each of which contains significant CD-44 surface protein expression and colocalizes with surface membrane expression of H/MDM-2 on each cancer cell line but does not affect normal colonic fibroblast negative control cells, which do not express significant levels of membrane HDM-2.

Materials and Methods

Peptides and chemicals. The test peptide, PNC-27, whose sequence is **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*, contains the MDM-2 binding domain from human p53 and is comprised of p53 aa 12-26 (bold) linked to the membrane penetrating sequence or membrane residency peptide (MRP) (italics). It was synthesized using a solid phase method with HPLC purity >95% (Biopeptides Corp, USA). The negative control peptide, PNC-29, whose sequence is **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* (aa sequence from human cytochrome p450 in bold, MRP sequence in italics) was also synthesized by solid phase method with >95% HPLC purity (Biopeptides Corp, USA). TritonX-100 and Bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin and Pen Strep were obtained from Life Technologies (Grand Island, NY, USA). All other chemicals were purchased from Sigma Aldrich.

Cell culture. Modified human colonic epithelial cells (HCEC) 1CTA, 1CTP and 1CTR were a generous gift from Dr. Jerry W. Shay, University of Texas Southwestern, Dallas, TX, USA. Stem cells were cultured in 4:1 DMEM: Media 199 + 2% Cosmic calf serum + 10 μg/ ml insulin + 20 μg/ ml human recombinant epidermal growth factor + 1 μg/ml hydrocortisone + 2 μg/ml apo-transferin + 0.05pM sodium selenite. The human stem-like colonic progenitor cells, 1CTA and 1CTP, were derived from HCECs by shRNA knockdown of the APC (adenomatous polyposis coli) gene and p53 respectively whereas, 1CTR are HCECs expressing $KRAS^{V12}$. HCECs have been reported to express significant levels of stem cell markers, CD29, CD44 and CD166 (17-19). The human male colon cancer cell line, SW-1222, was a generous gift from Meenhard Herlyn, Wistar Institute, Philadelphia, PA, USA, and these cells were cultured in DMEM + 10% FBS + 1% pen/strep.

The human male colon cancer cell line, HCT-116, the murine colon cancer cell line, CT-26, and the normal CCD-18Co human female colonic fibroblast cell line were acquired from the American type culture collection, Manassas, VA, USA. HCT-116 cells [containing $CD24^{+}CD44^{+}$ CSC subpopulations, that cannot differentiate but result in more aggressive and metastatic tumors (14)] were cultured in McCoy's 5A + 10% FBS + 1% pen/strep, and CCD-18Co cells were cultured in EMEM + 10% FBS + 1% pen/strep. All cells were maintained in a humidified incubator with 5% CO_2 at 37˚C.

CT-26 cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep). DMEM, RPMI, Media 199, FBS and cosmic calf serum were acquired from Thermo Fischer Scientific, Waltham, MA, USA.

Flow cytometry. Cells were harvested and washed in chilled Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (DPBS) and diluted to have 100,000 cells/ 100 μl/ assay in chilled FACS buffer. Ten percent fetal bovine serum (FBS) + 2 mM ethylenediaminetetraacetic acid (EDTA) was added to DPBS to prepare FACS buffer. Cells were incubated with polyclonal rabbit anti-HDM-2 (1:40) or mouse anti-CD44 (1:100) and isotype controls for 1 h at 4˚C after blocking them in FACS buffer for 30 min on ice. At the end of incubation, cells were washed 3X with chilled FACS buffer and incubated with DyLight® 650 labeled goat

anti-rabbit (1:250) and Alexa Fluor 488 labeled anti-mouse (1:500) antibodies at 4˚C, 1 h. Cells were then washed 3X with chilled FACS buffer. Fluorescent signals from anti-CD44 and anti-MDM2 stained cells were measured using a BD Accuri™ C6 cytometer system. Polyclonal rabbit anti-HDM-2 (catalog# AF1244) and rabbit isotype control (catalog# AB-105-C) were obtained from Novus Biologicals, Centennial, CO, USA. Mouse isotype control (catalog# sc3878) were obtained from Santa Cruz Biotechnology, CA, USA. Monoclonal mouse anti-CD44 (catalog# 156-3C11) was purchased from Thermo Fischer Scientific, MA, USA. Alexa Fluor 488 labeled anti-mouse antibody (catalog# ab96879) and DyLight® 650 labeled goat anti-rabbit (catalog# ab96902) was obtained from Abcam, Cambridge, MA, USA.

Confocal microscopy. Colocalization experiments using confocal microscopy were performed on all cell lines in a manner identical to the procedure used in a prior publication (10). Briefly, cells grown in confocal dishes overnight to 50-60% density were treated for 1h and maintained in 5% CO_2 humidified air at 37˚C. 1CTA, 1CTP, 1CTR and SW-1222 were treated with 60 μM of PNC-27 while HCT-116 and CT-26 were treated with 25 μM of PNC-27. After 3X washes with 1X DPBS (containing Ca^{2+} and Mg^{2+}), cells were immediately fixed in 4% formaldehyde. Fixation was performed for 10 min at 37˚C. Fixation was followed by blocking in 2% BSA in 1X DPBS (containing Ca^{2+} and Mg^{2+}) for 1 h at 25˚C. Cells were then incubated in polyclonal rabbit anti-MDM-2 (catalog# AF1244) at a dilution of 1:10 and polyclonal mouse anti-p53 (DO-1) at a dilution of 1:40 with shaking overnight at 4˚C. Cells were washed 3X with 1XDPBS (containing Ca^{2+} and Mg^{2+}) and incubated with polyclonal anti-rabbit red fluorophore (DyLight® 650) and polyclonal anti-mouse green fluorophore (DyLight® 488) at a dilution of 1:200 in the dark for 1 h at 25˚C. After 3X washes with 1X DPBS (containing Ca^{2+} and Mg^{2+}) and nuclei staining with DAPI, cell were visualized in confocal dishes under the Olympus Fluoview FV3000 confocal microscope (Olympus America INC, Center Valley, PA, USA) at 60X resolution. Image analysis was performed using cellSens software. Polyclonal mouse anti-p53 (DO-1) was obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA and polyclonal anti-mouse green fluorophore (DyLight® 488) was obtained from Abcam Inc., Cambridge, MA, USA.

MTT cell proliferation assay, LDH cytotoxicity assay. Cells were seeded in 24 well culture plate and grown to 70-80% confluence. Cells were then treated with increasing concentrations of PNC-27 and PNC-29 for 4 h. At the end of the treatment, MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) assay (Promega, Madison, WI) was carried out to determine the effect of PNC27/29 on cell proliferation. LDH (lactate dehydrogenase) release from cells was determined using CytoTox96 assay (Promega) with lysis buffer as a positive control. Manufacturer's instructions were followed for both assays.

Annexin V/PI apoptosis assay. Cells were seeded at a density of 100,000 cells/well in 6-well plates and grown to 60-70% confluence. Cells were then treated either with PNC-27 or an apoptosis inducer, staurosporine. Incubation times and agent concentrations were optimized to obtain the maximal level of apoptosis with the positive control agent, staurosporine, for each cell line as follows, respectively: CTA, 16 h, 80 μM, 1 μM; CTP, 6 h, 80 μM, 1 μM; CTR, 6 h, 80 μM, 2 μM; SW122, 6h, 80 μM , 1 μM; HCT116, 16 h, 50 μM, 0.5 μM; CT-26, 6 h, 50 μM, 5 μM. Following treatment, cells were harvested and washed in chilled DPBS. After centrifugation, cells were diluted to 1×10^6 cells/ml in chilled annexin V buffer to have 100,000 cells/100 μl/assay. Subsequently, cells were incubated with 5 μl FITC annexin V and 5 μl of 10 μg/ml propidium iodide (PI) for 15 min at room temperature. Stained cells were then analyzed using BD Accuri™ C6 cytometer system. Annexin V buffer, FITC annexin V reagent and propidium iodide were provided in the apoptosis detection kit from BD Biosciences (BD Biosciences, CA, USA).

Caspase activity assay. All cells were cultured at a concentration of $3\text{-}5\times10^6$ cells in a T150 flask and grown to confluence. For each cell line, untreated cells (negative control), cells treated with staurosporine that is known to induce apoptosis (positive control) and cells treated with PNC-27 were assayed for caspase- 3 activity using Caspase-3 Colorimetric Assay Kit (BioVision, Inc., Milpitas, CA, USA) as per the manufacturer's instructions. Staurosporine was present at 1 μM for CTA, CTP and SW-1222 cells, 2 μM for CTR cells, 0.5 μM for HCT cells, and 5 μM for CT-26 cells. PNC-27 was present at a concentration of 80 μM and an incubation time of 24 h for 1CTA, 1CTP, 1CTR and SW-1222 cells; a concentration of 50 μM and incubation time of 24h for HCT116 cells and at a concentration of 50 μM and an incubation time of 10h for CT-26 cells.

In vivo studies. To test whether PNC-27 induces tumor cell necrosis *in vivo* without affecting normal cells in tissues, we treated a murine colon cancer cell line (CT-26, see above in this section) that was transplanted intraperitoneally into nude mice. Our animal protocol, approved by the Drexel University Institutional Animal Care and Use Committee (IACUC), was adhered to throughout. Five to six-week-old female Nu/Nu nude mice were acclimatized for three to five days in the barrier housing where they were provided with food, bedding and water for the duration of the experiment. Peritoneal carcinomatosis was induced by intraperitoneal (IP) injection of 2.5×10^6 luciferase-transfected CT-26 (CT-26-Luc) cells in 500 μl of Dulbecco's phosphate-buffered saline (DPBS). Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Rockford, IL, USA) was used to transfect CT-26 cells with a firefly luciferase gene (luc2) containing plasmid (manufacturer protocol was followed). Stable transfection was confirmed *in vitro via* bioluminescent imaging. *In vivo*, established tumor in our murine colon cancer peritoneal carcinomatosis model was monitored using the In Vivo Imaging System (IVIS) Lumina XR series III [Perkin Elmer (Caliper), Waltham, MA]. The mice were monitored *via* IVIS on days 1, 3, 5, 7 and 9. After establishment of tumor on day 9 as evidenced by IVIS, mice were divided into two groups and treated as follows: Intraperitoneal (IP) injection, once per 24 h, with 1. PNC-29 (100 μg/10 μl sterile water/ g mouse, negative control) and 2. PNC-27 (100 μg/10 μl sterile water/g mouse, treatment cohort) for 14 days. On day 24, mice were sacrificed *via* CO_2 inhalation and tumors were excised. Tumor size and extent of hemorrhage were quantified on necroscopy. Marginal coagulation necrosis was assessed using histological sections prepared from fixed tumor nodules from each of the treatment groups. A treatment-blinded rater was used to examine necropsy specimens. An average of twenty-two tumor nodules were examined per animal. Necrosis was quantified histologically using Aperio eSlide Manager (Leica Biosystems, Buffalo Grove, IL, USA).

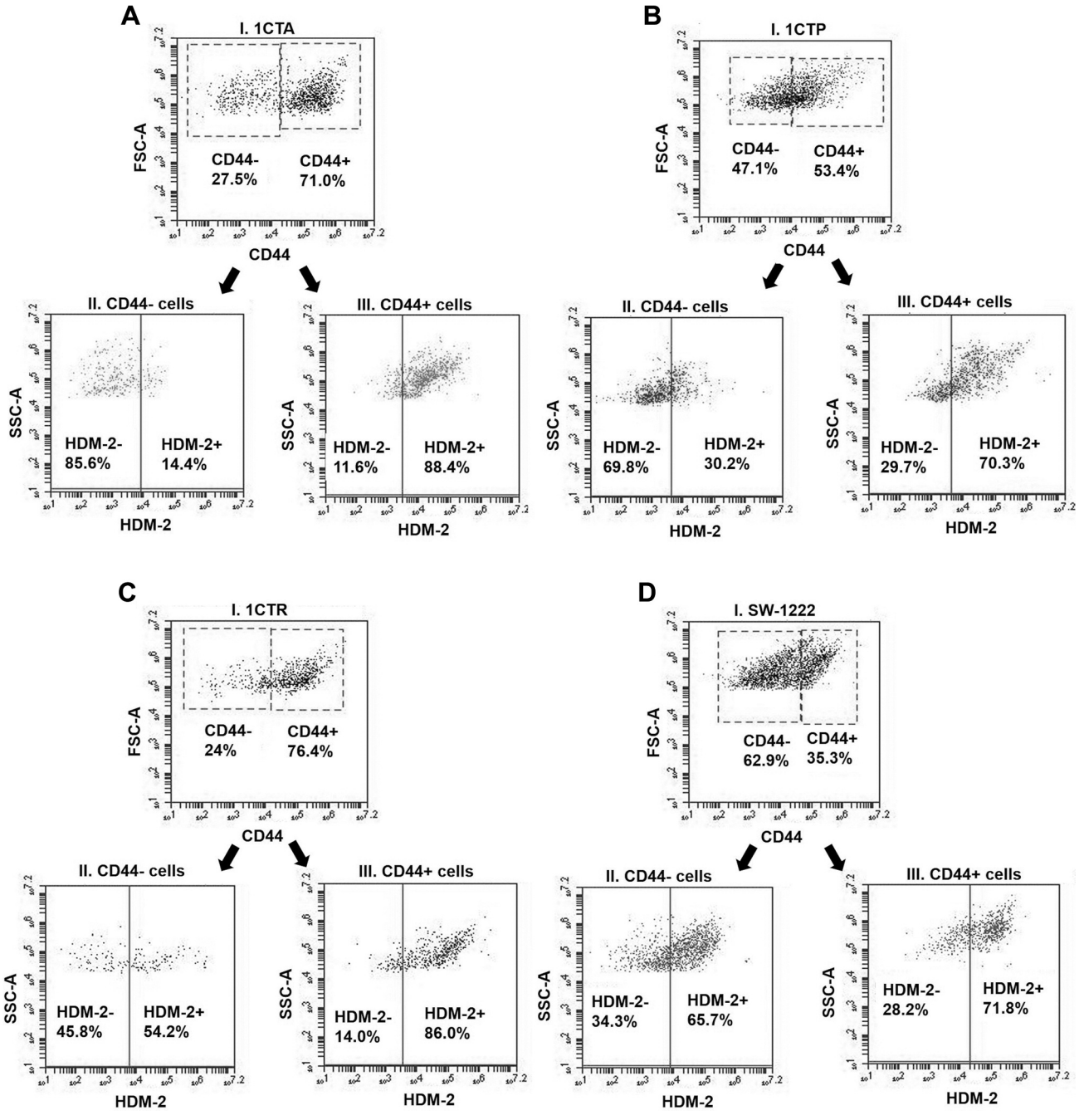

Figure 1. *Continued*

Computational and statistical methods. GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA) was used to analyze all quantitative assay results from flow cytometry, confocal microscopy, MTT assays and LDH assays. Results are shown as means±1 SEM. Statistical significance between assay values was determined using the two-tailed Student's *t*-test. *p*-Values of 0.05 were considered to indicate statistically significant difference.

Results and Discussion

Expression of CD44 and HDM-2 in colon cancer cell membranes. Human and murine colon cancer cells were validated for their heterogeneity and presence of colon cancer stem cell marker, CD44. CD44 has been widely

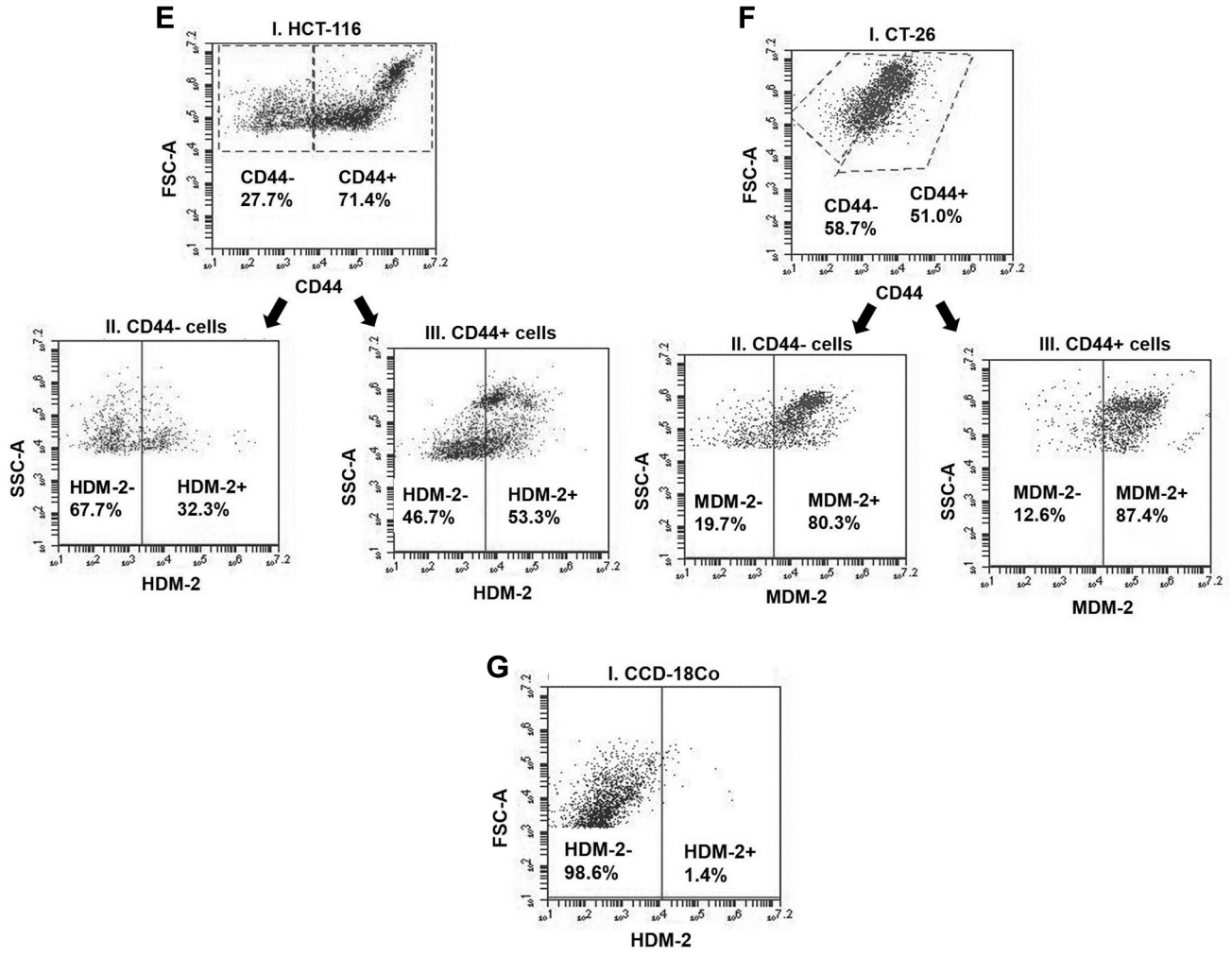

Figure 1. *Membrane expression of H/MDM-2 in CD44+ and CD44-subpopulations of colon cancer cells. Flow cytometry was utilized to identify the expression of stem cell-associated marker, CD44 in colon cancer cells. In (A) I. 1CTA, (B) I. 1CTP, (C) I. 1CTR, (D) I. SW-1222, (E) I. HCT-116 and (F) I. CT-26, cells were gated based on isotype controls and sorted into CD44+ and CD44– subpopulations. To evaluate levels of H/MDM-2 in CD44+ and CD44– subpopulations of each cell, all cells were co-stained with H/MDM-2 as shown in panels II and III. CD44+ and CD44– cells stained positively for membrane H/MDM-2 are shown in quadrant labeled as H/MDM-2+. Membrane H/MDM-2 was detected in all 6 colon cancer cell lines and a significantly high percentage of CD44+ cells expressed membrane H/MDM-2. (G) I shows percentage of non-malignant/ normal CCD-18Co cells stained with membrane H/MDM-2 were negligible compared to colon cancer cells. Very few (about 1.4%) of these cells were positive for HDM-2 which indicates that H/MDM-2 is specifically upregulated in malignant cells. Dot plots shown for each cell line are representative plots; data are from 3 independent experiments.*

studied as a CSC surface marker and utilized to segregate CSCs in many cancers (20). In our study, we found a significantly high fraction of CD44+ CSCs in various human and murine colon cancer cells (~35-76%) as shown in Figure 1. Furthermore, high percentages of cells in all six-colon cancer cell lines expressed H/MDM-2 in their cell membranes (lowest, 47% in HCT 116 cells, highest 92% in CT 26 cells). Moreover, a high percentage of the CD44+ CSCs were positively stained for the oncoprotein, membrane H/MDM-2 (Figure 1A-F). In contrast, membrane H/MDM-2 was negligible in normal CCD-18Co cells (which contained no CD44 positive cells) (Figure 1G). This finding suggests that membrane H/MDM-2 can be a potential therapeutic target in CD44+ CSCs, which can ameliorate aggressive tumors that develop resistance to the conventional chemotherapies without affecting normal cells.

PNC-27 interacts with HDM-2 on the membrane of colon cancer cells. PNC-27 was designed to target overexpressed membrane HDM-2 and promote cell death. Our aim was to

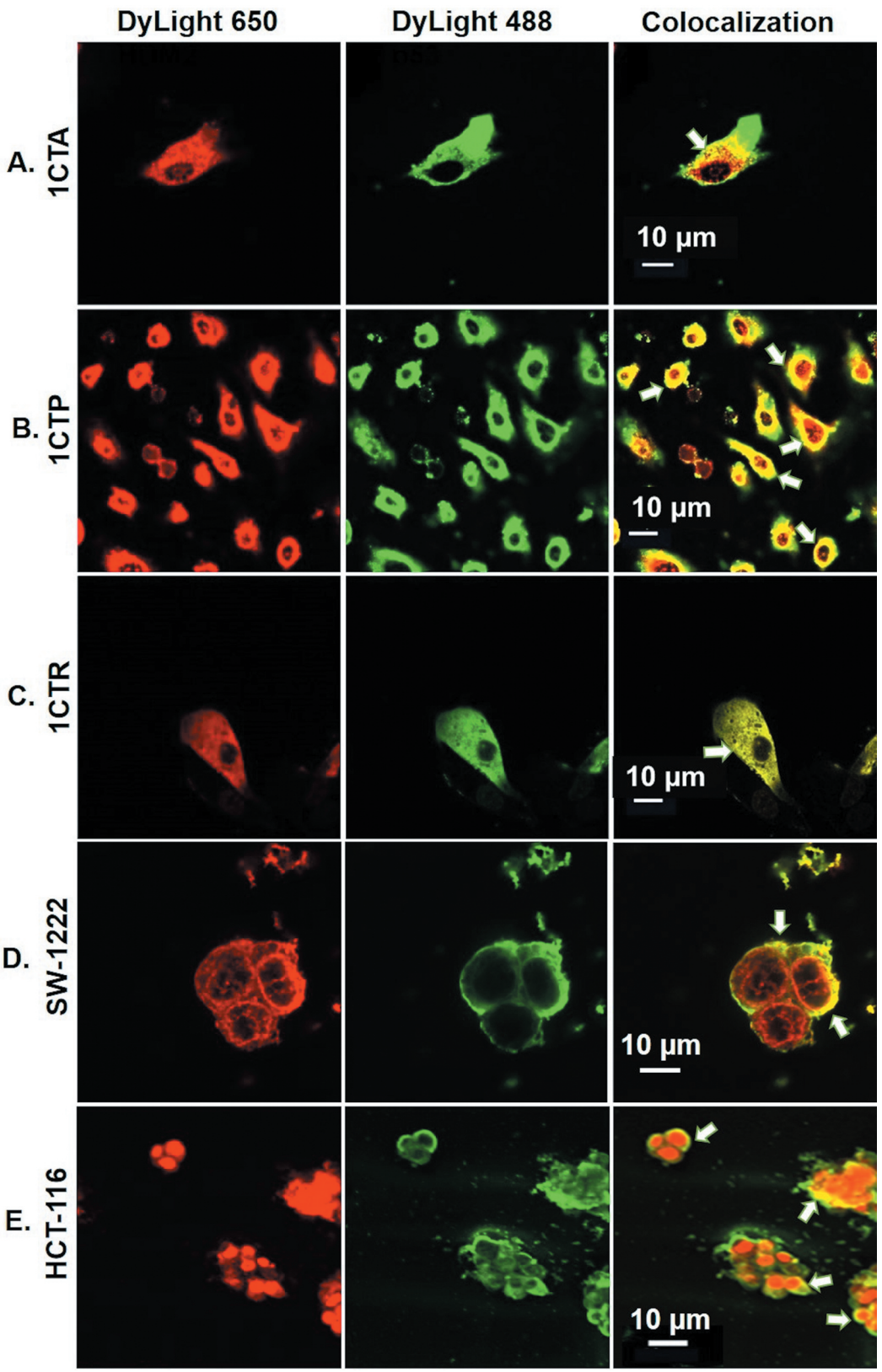

Figure 2. *Continued*

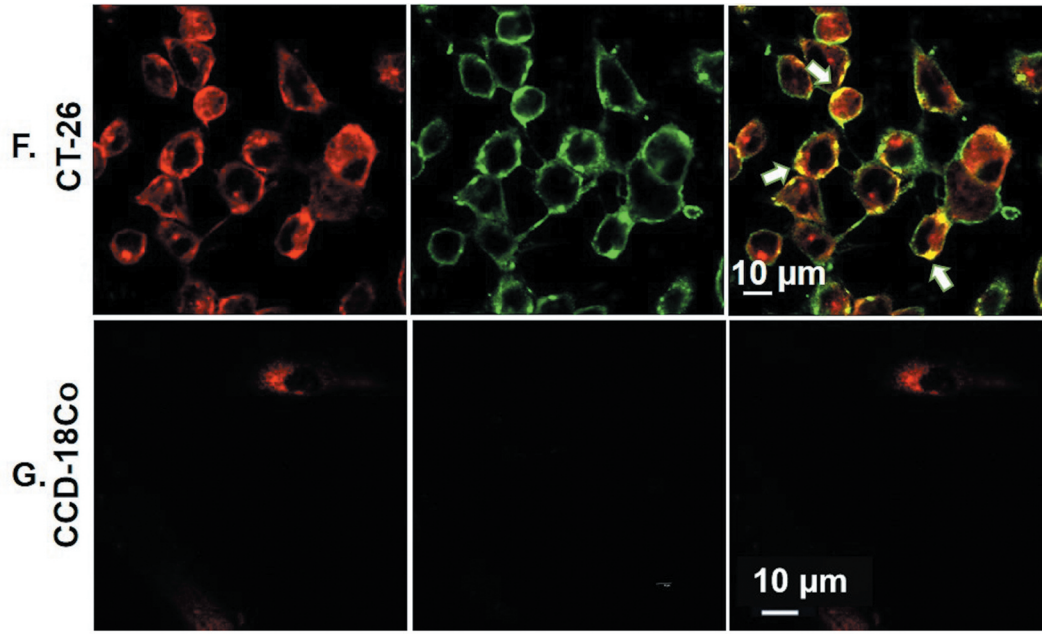

Figure 2. *Colocalization of PNC-27 with HDM-2 on the colon cancer cell plasma membrane. (A) 1CTA, (B) 1CTP, (C) 1CTR, (D) SW-1222, (G) CCD-18Co were treated with 60 µM whereas (E) HCT-116 and (F) CT-26 cells were treated with 25 µM of PNC-27 for 1 h followed by imaging under Olympus Fluoview FV3000 confocal microscope at 60× magnification. Green and red fluorescence correspond to DyLight® 488 anti-hu-p53/PNC-27 and DyLight® 650 anti-H/MDM2, respectively. Yellow fluorescence was produced by the overlap of green and red emission signals at the membrane of colon cancer cells. (G) Normal CCD-18Co cells did not emit any yellow fluorescence.*

determine if membrane HDM-2 expressed on colon cancer cells containing significant populations of cancer stem cells can be a targeted by PNC-27. We performed co-localization studies using confocal microscopy to determine if PNC-27 can interact with HDM-2 on the plasma membrane of cancer stem cells. As shown in Figure 2, PNC-27, indicated by green fluorescence and HDM-2, indicated by red fluorescence are both localized to the cell surface. Prominent yellow fluorescence on the cell surface, resulting from direct overlap of green and red emission, clearly demonstrates co-localization of PNC-27 and HDM-2 on the membrane of colon cancer cells.

Binding of PNC-27 to HDM-2 results in cell death by necrosis and shows no sign of apoptotic activity. Further studies were done to determine if the PNC-27-HDM-2 complex on the cell surface of cancer cells will result in their cell death. The MTT cell viability assay was performed on these cells at different concentrations of PNC-27 and negative control peptide PNC-29. As summarized in Figure 3A-F (black bar graphs), we observed a dose-dependent decrease in cell viability of colon cancer cells treated with PNC-27 for an incubation time of 4 h. This effect was not seen with our control peptide, PNC-29 (Figure 3A-F, gray bar graphs). Additionally, we found that PNC-27 is not toxic to normal CCD-18Co cells (Figure 3G).

In order to understand the mechanism of cell death, we measured the levels of necrotic, early apoptotic, and late apoptotic markers in these cells after treatment with PNC-27. As shown in Figure 4A-F, 1CTA, 1CTP, 1CTR, SW-1222, HCT-116 and CT-26 cells treated with increasing doses of PNC-27 for a 4 h incubation time resulted in LDH leakage, indicative of loss of membrane integrity, in a dose-dependent manner. Treatment with increasing doses of our control peptide, PNC-29, did not yield an increase in LDH. CCD-18Co cells did not undergo necrosis when treated with PNC-27 or PNC-29.

Importantly, for all six cancer cell lines, at specific concentrations of PNC-27, the level of LDH release was found to be the same as that for the lysis controls, *i.e.*, the positive controls in which each cell line was lysed with lysis buffer to determine the level of LDH representing total cell lysis or total cell death. Thus, PNC-27 killed all tumor cells in each cell line, a finding that indicates that this peptide killed all tumor (CD44-expressing) stem cells.

In contrast, results from the annexin V assay for apoptosis demonstrated no significant difference in detection of annexin V-positive cells in the PNC-27 treated group of cancer cells compared to stained untreated cells (Figure 5). Likewise, similar absence of caspase-3 activity in PNC-27-treated cells compared to untreated cells further confirmed

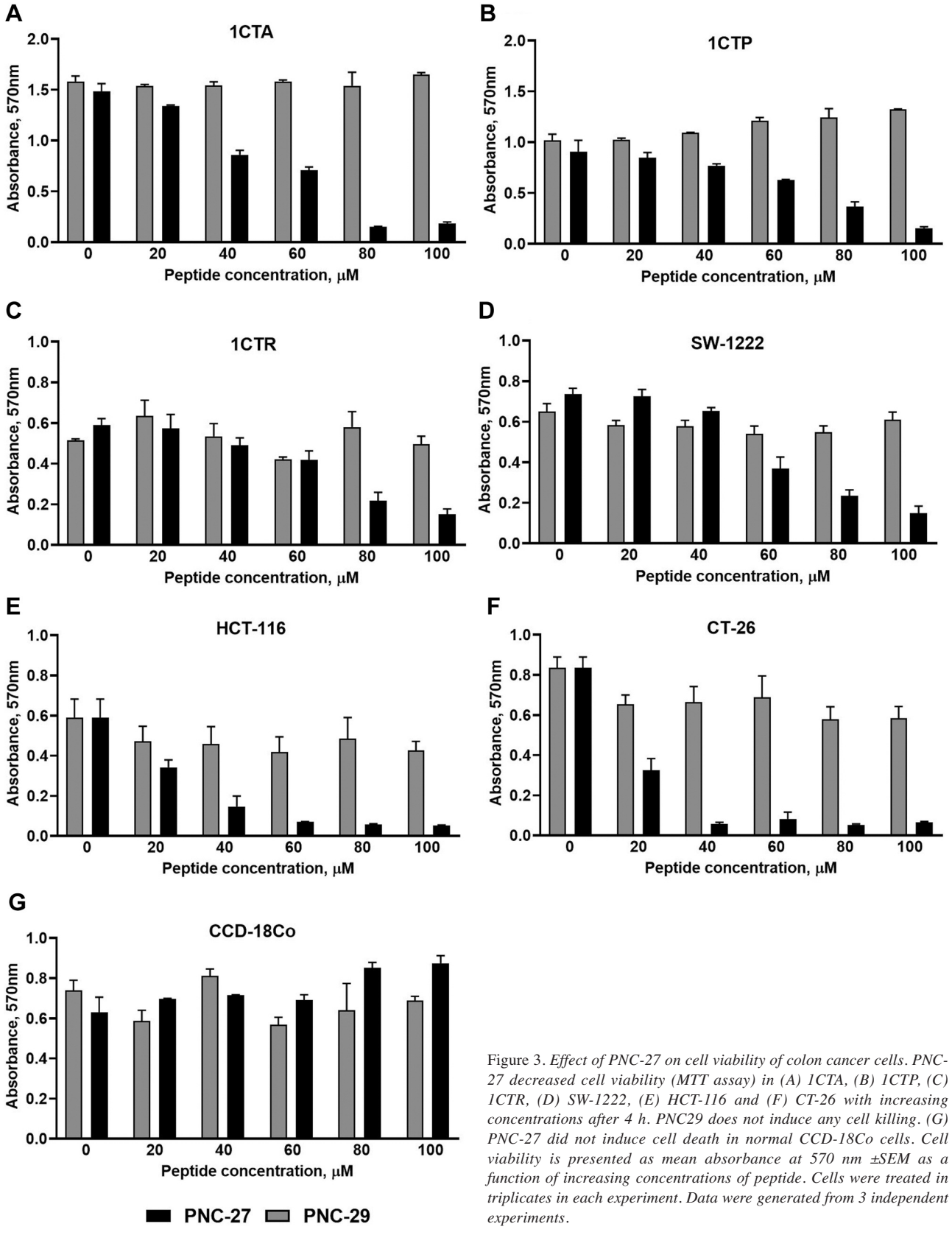

Figure 3. *Effect of PNC-27 on cell viability of colon cancer cells. PNC-27 decreased cell viability (MTT assay) in (A) 1CTA, (B) 1CTP, (C) 1CTR, (D) SW-1222, (E) HCT-116 and (F) CT-26 with increasing concentrations after 4 h. PNC29 does not induce any cell killing. (G) PNC-27 did not induce cell death in normal CCD-18Co cells. Cell viability is presented as mean absorbance at 570 nm ±SEM as a function of increasing concentrations of peptide. Cells were treated in triplicates in each experiment. Data were generated from 3 independent experiments.*

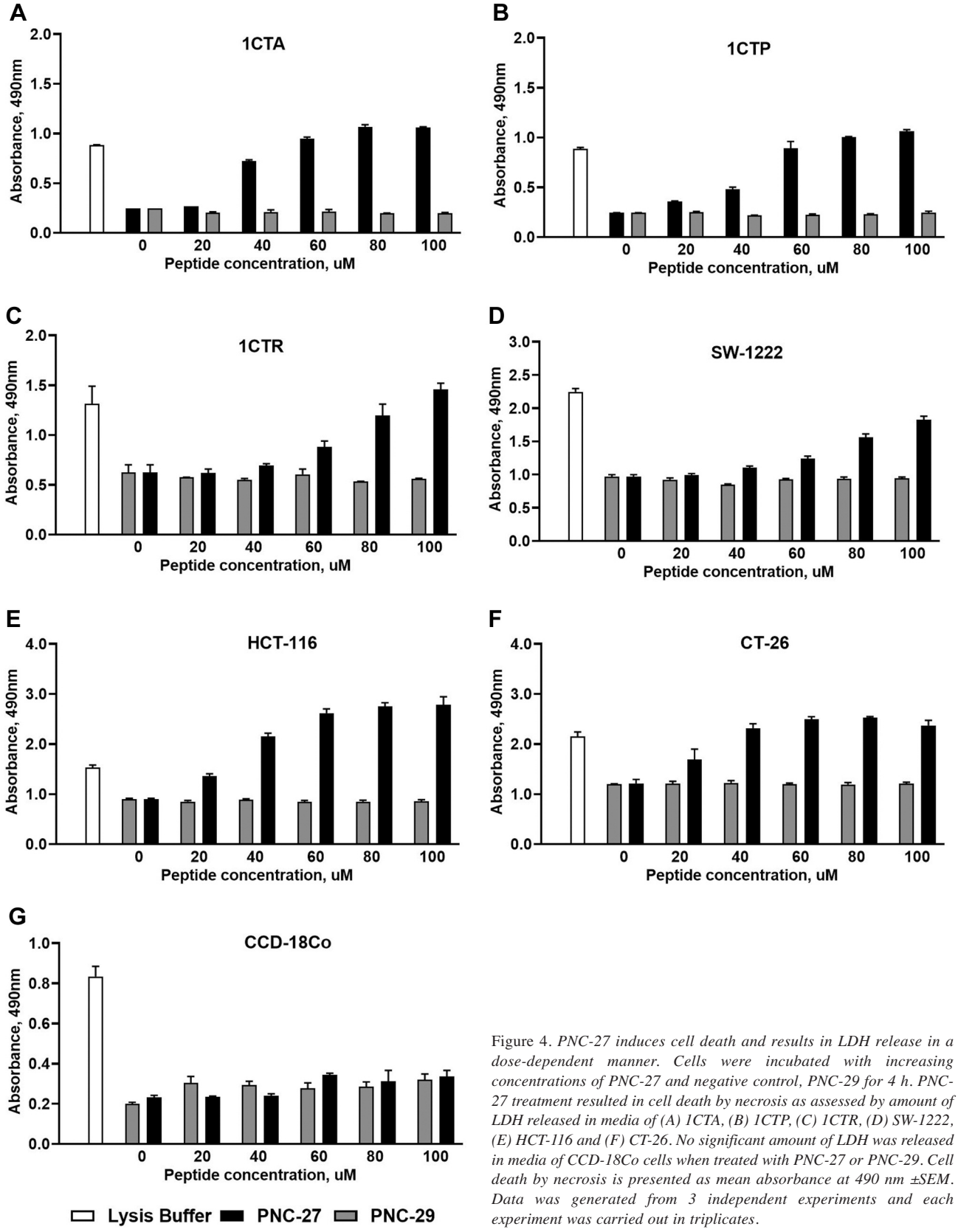

Figure 4. *PNC-27 induces cell death and results in LDH release in a dose-dependent manner. Cells were incubated with increasing concentrations of PNC-27 and negative control, PNC-29 for 4 h. PNC-27 treatment resulted in cell death by necrosis as assessed by amount of LDH released in media of (A) 1CTA, (B) 1CTP, (C) 1CTR, (D) SW-1222, (E) HCT-116 and (F) CT-26. No significant amount of LDH was released in media of CCD-18Co cells when treated with PNC-27 or PNC-29. Cell death by necrosis is presented as mean absorbance at 490 nm ±SEM. Data was generated from 3 independent experiments and each experiment was carried out in triplicates.*

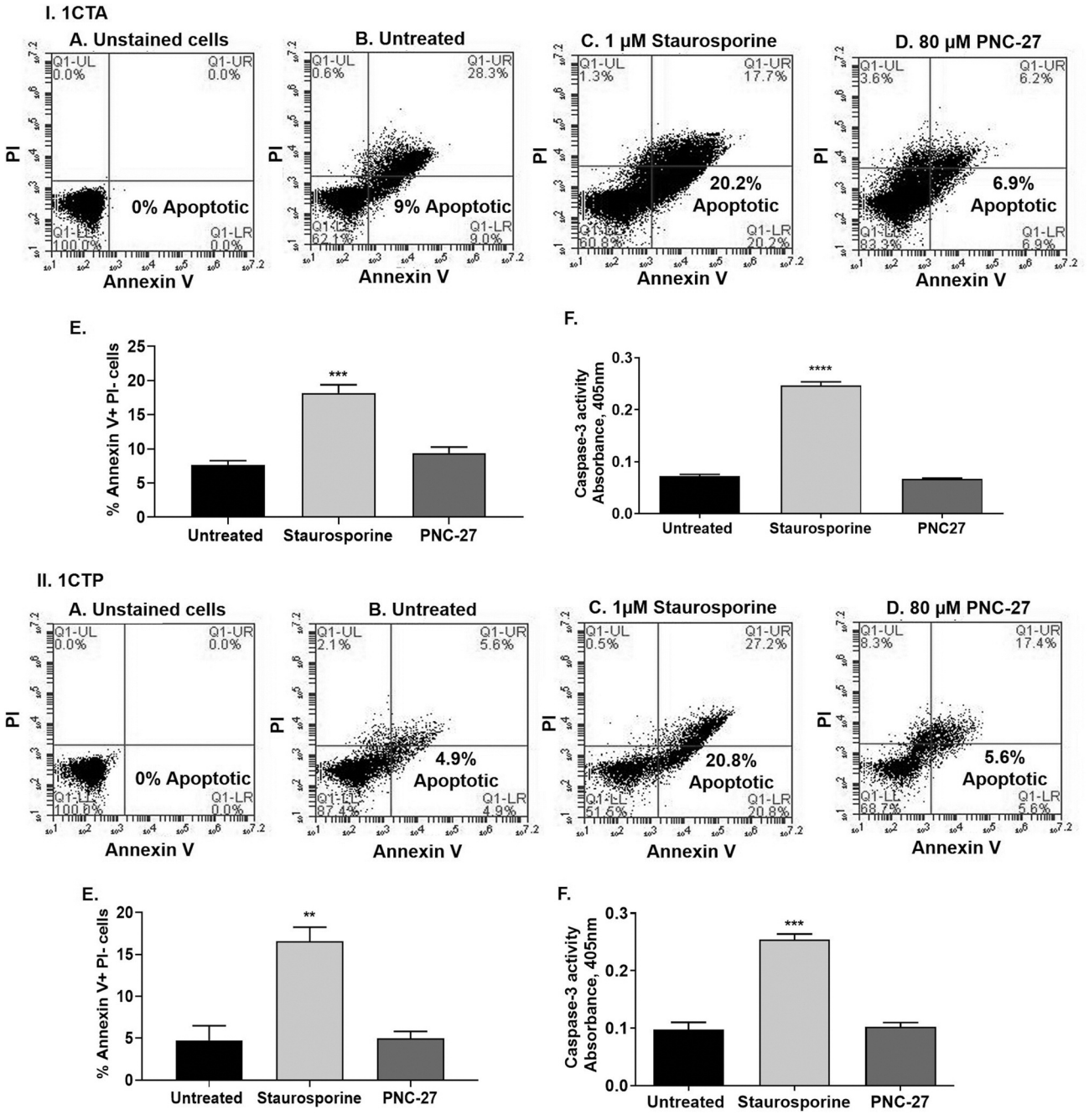

Figure 5. *Continued*

the cell death mechanism to be independent of apoptosis. On the other hand, our positive control, staurosporine, a known activator of apoptosis, resulted in significant annexin V-positive cell and caspase-3 activity (Figure 5). Therefore, PNC-27 induces its cytotoxic effects *via* necrosis independent of apoptosis, as we found for all of the other cell lines that we have studied previously (5-11, 21).

PNC-27 induces significant tumor-specific hemorrhagic necrosis in vivo. To test whether PNC-27 induces tumor cell necrosis without affecting normal cells *in vivo*, we established a murine colon cancer peritoneal carcinomatosis model by IP injection of CT-26-Luc in athymic Nu/Nu mice. Tumor development was monitored *via* IVIS bioluminescence imaging and relative luminescence units

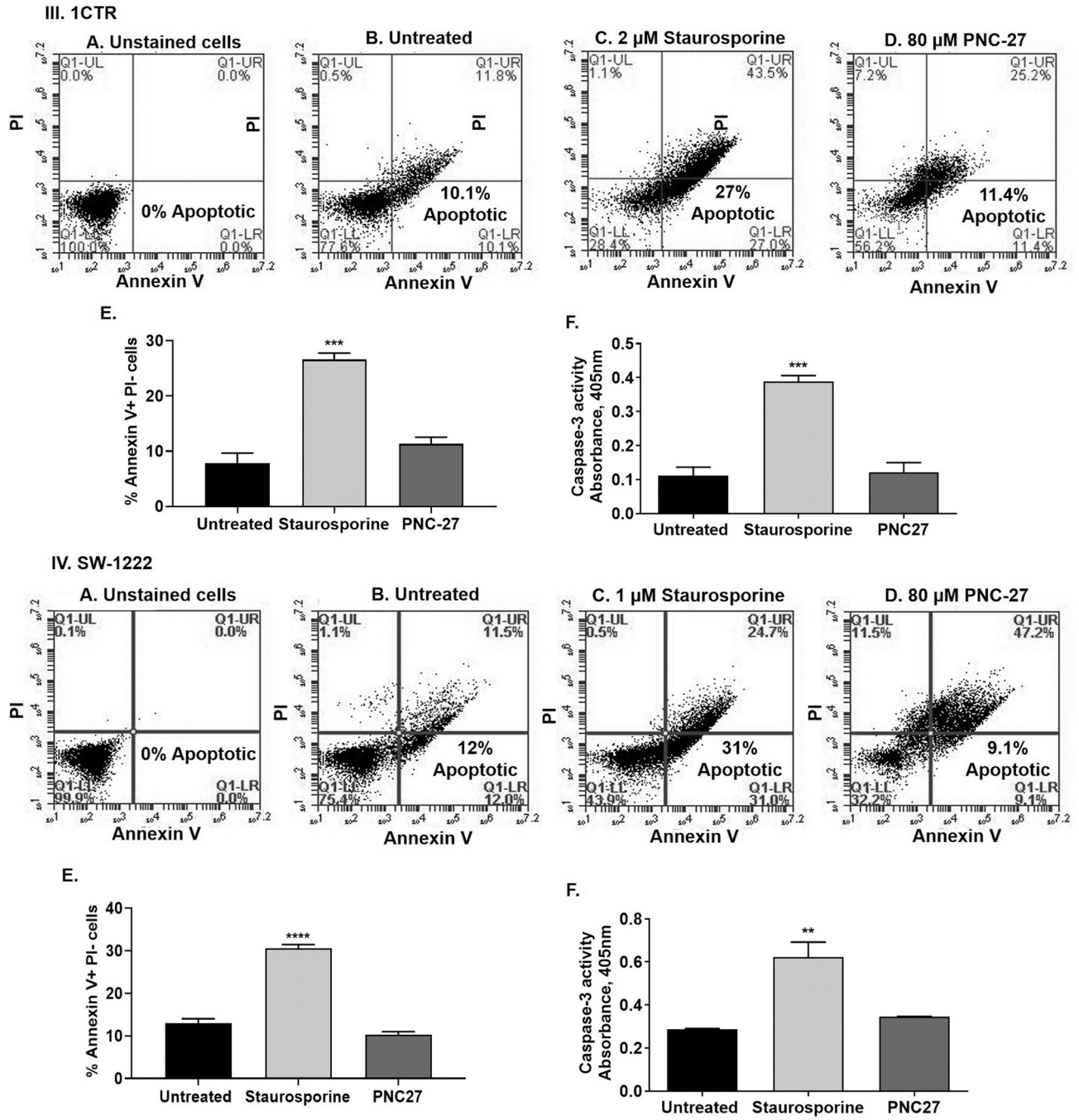

Figure 5. *Continued*

(RLU) were recorded as shown in Figure 6A and B. Once tumors were established, PNC-27 was tested for its efficacy for inducing tumor-specific necrosis without damaging normal cells. Treatment was initiated when the presence of tumor was confirmed *via* IVIS. Upon necropsy on day 15 post treatment, tumors of mice treated with PNC-27 demonstrated multiple peritoneal areas of gross hemorrhage (Figure 6C, left) while tumors of mice treated with the negative control PNC-29 peptide showed significantly fewer regions of hemorrhage that were also significantly smaller than found in mice treated with PNC-27 (Figure 6C, right). These results are quantitated in Figure 6D showing that hemorrhagic necrosis was dramatically higher in the PNC-27-treated tumors.

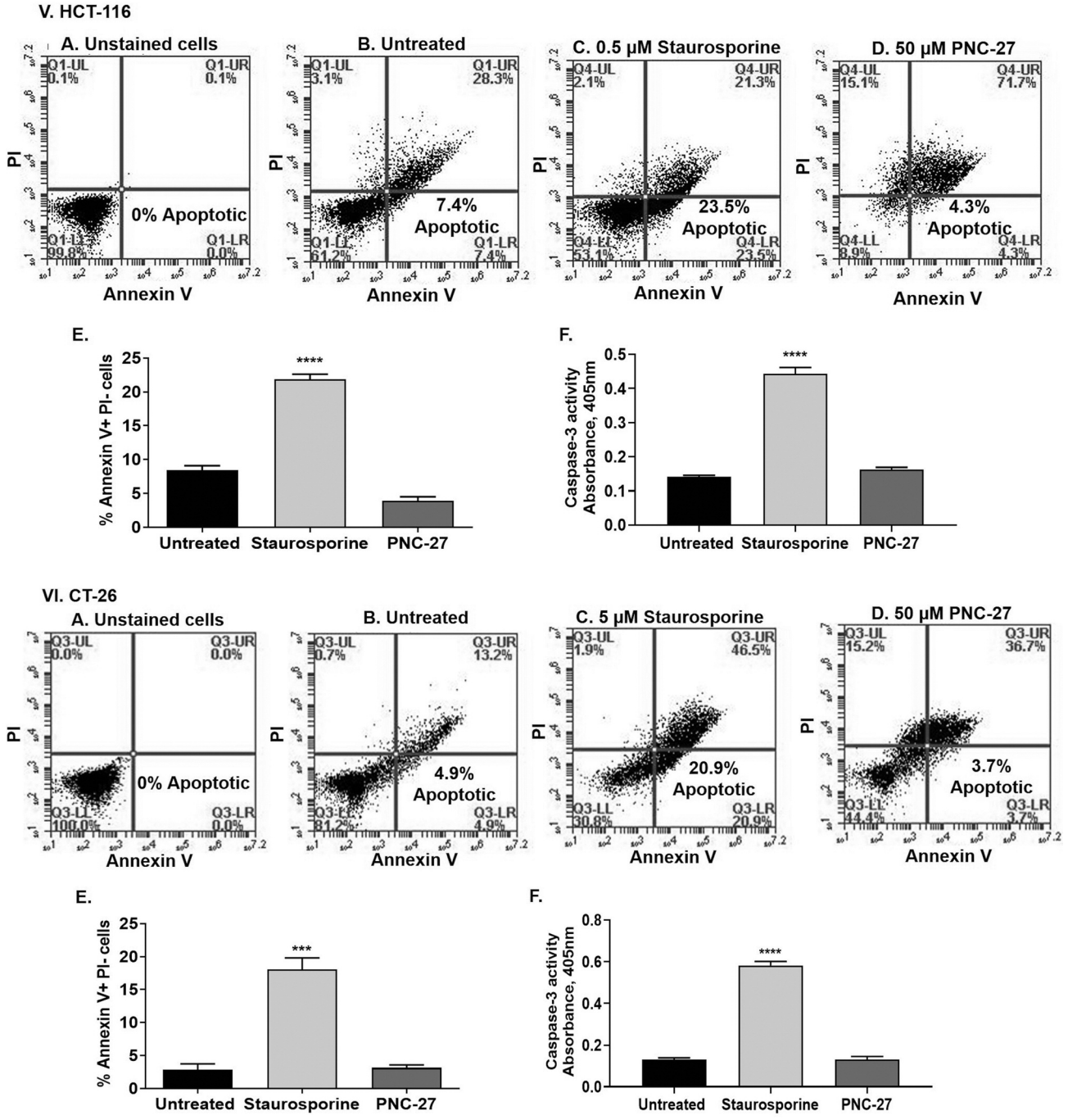

Figure 5. *Anti-tumor effect of PNC-27 does not induce apoptotic markers in the six colon cancer cell lines. Percent apoptosis induced in response to PNC-27 treatment was determined by staining cells with FITC-annexin V to detect early apoptotic marker, phosphatidylserine using flow cytometry. The X-axis is the staining intensity for annexin V, and the Y-axis is the intensity of staining for propidium iodide (PI) that stains the cell nuclei. (A-D): For each of the six cell lines, four flow cytometry patterns are shown, labeled sequentially from left to right as (A-D). (A) is the pattern for untreated cells, not stained with either agent (annexin V or PI); (B) is the pattern for untreated cells that have been stained with both agents; (C) is the pattern for cells treated with staurosporine; and (D) is the pattern for cells treated with PNC-27. Percentages of staining patterns of cells are given for each cell line for each quadrant in the flow cytometric pattern. Percent cells positive for apoptosis, i.e., positive for annexin V and negative for PI, were detected in the lower right quadrant (Q-LR) for each cell line. (E) is a bar graph of % apoptotic cells (annexin V+ PI– cells) in the three groups: no treatment [corresponding to graph (B)], treated with staurosporine [corresponding to graph (C)] and treated with PNC-27 [corresponding to graph (D)]. Results are presented as mean of % annexin V+PI– cells ±SEM and were generated from 3 independent experiments. (F) is a bar graph summarizing caspase levels that were measured after treatment with PNC-27 in the same three groups, respectively, as described in (E) above. Caspase levels are represented as mean of caspase-3 activity at absorbance 405 nm±SEM. Data was generated from 3 independent experiments and ****p≤0.0001, ***p≤0.001 and **p≤0.01 in comparison to untreated cells.*

Further demonstration of PNC-27-induced hemorrhagic necrosis of the peritoneal tumor is shown in Figure 6E showing the histopathology of hemotoxylin-eosin stains of sections taken from the tumor. In this section, the eosin-staining sections (red-colored areas enclosed by the blue boundary curve) show hemorrhagic necrosis while the hemoxylin-staining (blue-colored) sections are tumor cells. Section 6E inset shows the same area at a higher magnification. Figure 6F shows that in tumors treated with the negative control peptide, PNC-29, the histopathology on similar sections through the tumor shows absence of any hemorrhagic necrosis and only tumor cells. The results of histopathologic quantitation of the percentage of tumor nodules showing hemorrhagic necrosis in mice treated with PNC-27 or with PNC-29 (negative control peptide) are shown in Figure 6G in which it is clear that hemorrhagic necrosis is significantly more prominent in the PNC-27-treated mice.

Since PNC-27 was administered intraperitoneally, it was possible that it exerted a general cytotoxic effect that affected tumor and non-tumor cells *i.e.*, off target effects. As shown in Figure 6H, histopathological sections taken from other major tissues, *i.e.*, liver, bowel and spleen, revealed no cytotoxic effects on these tissues. Thus, the cell-killing effects of PNC-27 in this *in vivo* model are specific for the tumor cells and not normal cells showing no gross toxicity or apparent cell death in healthy GI organs of PNC-27 treated mice.

Conclusion

PNC-27 is cytotoxic to all six colon cancer cell lines used in this study. This effect is specific to colon cancer cells since this peptide had no effect on the viability of control normal colonic fibroblasts (CCD-18Co cells). Specificity of PNC-27 for killing tumor cells exclusively was further supported by our findings in the *in vivo* peritoneal carcinomatosis model in nude mice in which this peptide induced widespread hemorrhagic tumor cell necrosis of an intraperitoneally implanted syngeneic CT-26 colon cancer with no evidence of cell damage to normal cells in a variety of tissues in these mice.

Furthermore, in our *in vitro* studies, all six-colon cancer cell lines expressed significant levels of H/MDM-2 in their membranes (Figure 1) while the normal control cells expressed barely detectable membrane levels of this protein suggesting that cancer cell membrane expression of this protein is important for the cytotoxic effects of PNC-27 on these cancer cells. As found in a number of previous studies, PNC-27 colocalized with H/MDM-2 in the membranes of the colon cancer cells (Figure 2). As shown in these previous studies, binding of PNC-27 to HDM-2 in cancer cell membranes induces the formation of transmembrane pores resulting in cell death (6, 8, 12). This mechanism of PNC-27-induced cancer cell death for the cells tested in this study was very likely due to pore formation given that the rise in cytosolic LDH was rapid and, at higher concentrations of peptide, reached maximal levels within four hours as observed in prior studies (9-11). Our findings that PNC-27 induced rapid cancer cell death without expression of early (annexin V staining of exposed phospholipid) or late (caspase 3) markers for apoptosis in any of the cell lines tested in this study (Figures 5) strongly support tumor cell necrosis and not apoptosis as the mechanism of cell death. Further evidence that apoptosis was not the cause of PNC-27-induced cancer cell death is that staurosporine, that is known to be a strong inducer of apoptosis, required from 10-24 h to induce total cancer cell death in each of the cell lines that we tested while PNC-27 induced total cancer cell death in 4 h for each cell line tested and induced maximal release of LDH over this time course. In prior studies, we have found that PNC-27 actually induces immediate release of LDH into the medium suggesting early cancer cell membrane damage (5-11, 21). Electron microscopic analysis of these cancer cells treated with PNC-27 after several minutes of incubation shows transmembrane pore formation in these cells (6, 8). Although the initial events in apoptosis leading to cell death can occur early after an initiating event, cell death with membrane damage resulting in release of LDH requires hours to days (22).

An important finding in this study was that a significant fraction of each of the cell lines expressed the CD44 protein marker for colonic stem cells. Since, as suggested by the cell viability and LDH release data shown in Figures 3 and 4, respectively, at higher concentrations of PNC-27 for each cell line, there was 100 percent cell death, we conclude that PNC-27 is cytotoxic to colon cancer stem cells. Since it is also toxic to leukemia stem cells (12), it may be an effective agent against tumor stem cells in general.

It is also of interest that PNC-27 was cytotoxic to six different cell lines that differed significantly phenotypically and genotypically from one another. For example, as noted in the Materials and Methods section, HCT 116 cells express, in addition to CD 44, CD 24 and, unlike the other cell lines, cannot differentiate but result in more aggressive and metastatic tumors. Human stem-like colonic progenitor cells, 1CTA and 1CTP, were derived from HCECs by shRNA knockdown of APC and p53 respectively whereas, 1CTR are HCECs expressing $KRAS^{V12}$. Thus, the cytotoxic effects of PNC-27 on these cells is independent of the pathways involved in cell transformation and very likely result from the observation that all cell lines express H/MDM-2 in their membranes enabling PNC-27 binding to HDM-2 with subsequent transmembrane pore formation.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

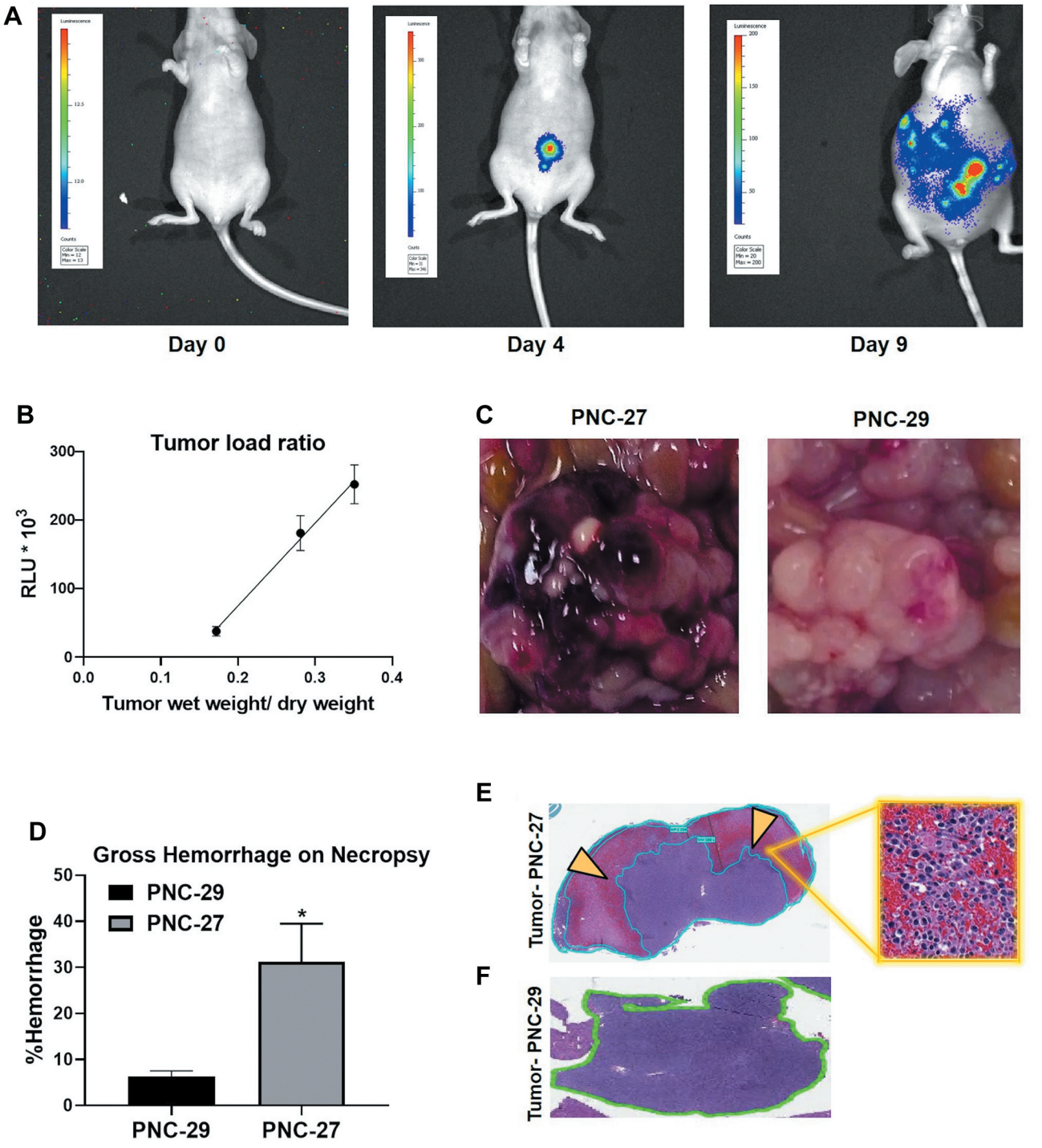

Figure 6. *Continued*

Authors' Contributions

AT designed and conducted to experiments, acquired and analyzed the data, wrote the article. WFM, MK, BDB, EG, MFS, YP, and EMG conducted experiments, acquired, and analyzed the data. DSF, LS and PMC analyzed the data and reviewed the manuscript. WBB, BL and MRP designed experiments, analyzed the data, wrote the article and provided administrative support.

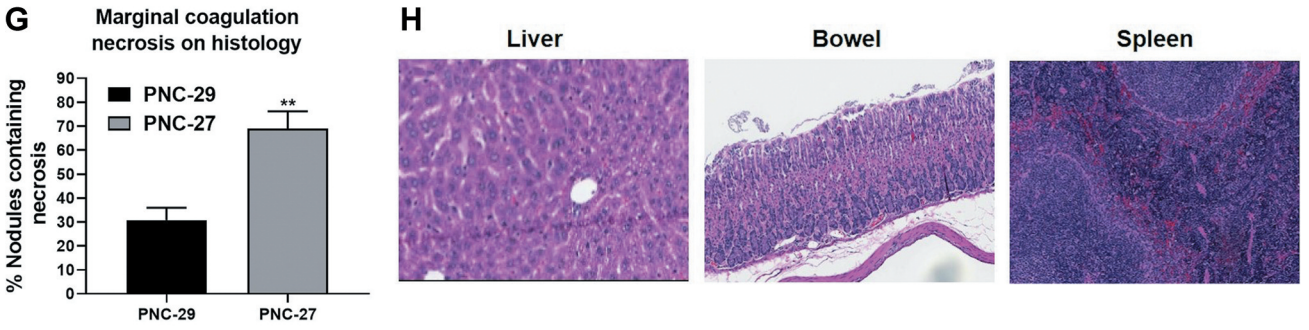

Figure 6. *Effect of PNC-27 in vivo on CT-26 peritoneal carcinomatosis (CT-26 inoculated mice). Nu/Nu mice were inoculated with CT-26-Luc cells and IVIS was used to detect the bioluminescent signal from tumors (See Materials and Methods Section). (A) Shows tumor development in nude mice monitored using IVIS on day 0, 4 and 9. (B) Shows tumor development in Nu/Nu mice represented by RLU measured by IVIS as a function of tumor wet weight/ dry weight. (C) Left panel shows necrotic hemorrhage of tumors from mice treated with PNC-27; Right panel: there was no hemorrhage seen in tumors from PNC-29 treated mice. (D) The bar graph shows the histomorphology analysis of tumors with significantly higher percent gross hemorrhage of tumors treated with PNC-27 as compared with tumors treated with PNC-29. Percent gross hemorrhage was calculated as % area of necrosis relative to area of full specimen. (E) Histopathology of a peritoneal tumor nodule in PNC-27-treated mouse showing marginal coagulation necrosis (eosinophilic area) surrounding area of tumor (hematoxylin or blue stain). E-inset is a higher power view of (E) showing hemorrhagic necrosis. (F) section of a typical tumor nodule taken from the peritoneum of a PNC-29 treated mouse showing only tumor cells and no necrosis. (G) Bar graph represents relatively higher percentage of nodules containing necrosis from PNC-27-treated mice as compared with PNC-29-treated mice. Results are represented as mean±SEM with **$p \leq 0.01$ and *$p < 0.05$ compared with the PNC-29 treated group (n=5). (H) Tissues bearing no tumors isolated from PNC-27-treated animals showed no signs of necrosis or cell damage.*

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