

## Original Article

# Polyisoprenylated cysteinyl amide inhibitors induce caspase 3/7- and 8-mediated apoptosis and inhibit migration and invasion of metastatic prostate cancer cells

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**Abstract:** Metastatic castration-resistant prostate cancer (mCRPC) is the most aggressive and deadly form of prostate cancer. It is characterized by the overexpression of epidermal growth factor receptors whose signals are mediated by small monomeric G proteins of the Ras superfamily. These require polyisoprenylation for functional activity. Polyisoprenylated cysteinyl amide inhibitors (PCAls) of polyisoprenylated methylated protein methyl esterase (PMP-MEase) were developed as potential targeted therapies to mitigate excessive growth signaling in mCRPC either by inhibiting PMPMEase and/or perturbing the polyisoprenylation-dependent functional interactions. We investigated the effects of PCAls on the viability of prostate cancer PC 3, DU 145, MDA PCa 2b, LNCaP and 22Rv1 cells, determined the effect of the PCAls on PC 3 cell proliferation, survival and caspase-mediated apoptotic cell death. Metastatic PC 3 and DU 145 cell migration and invasion in the presence of NSL-BA-040 were determined using the scratch and matrigel invasion assays. We further investigated the effect of NSL-BA-040 on F-actin organization in TagRFP F-actin marker-transfected metastatic PC 3 cells. The PCAls suppress mCRPC cell viability with  $EC_{50}$  values ranging from 1.3 to 4.0  $\mu$ M for the most potent of the PCAls against PC 3, DU 145, MDA PCa 2b, LNCaP and 22Rv cells. PCAls induced apoptotic cell death in PC 3 and DU 145 cells as determined by annexin V/propidium iodide flow cytometry analysis through the activation of caspases 3 and 8 while also inhibiting migration and invasion through the disruption of F-actin organization. Taken together, our studies show the anti-cancer effects on mCRPC cells through induction of caspase-mediated apoptosis and F-actin-mediated inhibition of cell motility and invasion thereby indicating the anti-tumor and anti-metastatic potential of the PCAls.

**Keywords:** F-actin, cell motility, polyisoprenylated protein, prostate cancer, G proteins

## Introduction

Metastatic castration-resistant prostate cancer (mCRPC) remains an incurable disease that poses a major challenge to therapy. Consequently, the vast majority of the 26,120 expected deaths in 2016 [1-3] are likely to be from mCRPC given that this form of the disease is resistant to the androgen-deprivation therapy (ADT) [4]. Lack of understanding of the molecular phenomena driving PCa metastasis implies that therapies targeting the 'drivers' are limited. In the absence of new effective therapies, the survival rates are expected to remain low. Current chemotherapies such as Docetaxel, mitoxantrone and cabazitaxel only extend the

lives of men with mCRPC by 2 to 4 months [5, 6] while also causing severe adverse effects [7]. Abiraterone, enzalutamide or sipuleucel-T are also used without much success [8]. Therefore, identifying the molecular mechanisms involved in the promotion of mCRPC is imperative for the development of targeted and more effective therapies.

The epidermal growth factor receptor (EGFR) and its ligands are overexpressed and assume the autocrine control in mCRPC tumor growth [9, 10], suggesting that EGFR signaling plays an important role in PCa progression to the metastatic disease. It has also been shown that cancer cells overexpress EGFR as a resistance

mechanism to monoclonal antibodies and tyrosine kinase inhibitors therapies that block EGFR [11]. Downstream mediators of EGFR signaling such as Ras have been shown to be hyperactive and appear to drive PCa metastasis [12]. Furthermore, cancers with activated Ras do not respond to anti-EGFR drugs. This, in addition to the EGFR overexpression, makes Ras a very important target in the search for more effective mCRPC therapies. The Ras superfamily of GTPases consists of Ras and Rho subfamilies that regulate cell proliferation, differentiation, apoptosis and cytoskeletal organization involved in cell migration and invasion [13]. Oncogenic Ras, together with c-myc, has been reported to confer metastatic capability on cells in culture while Rho subfamily members are implicated in cancer metastasis [14]. The Rho subfamily includes Rho, Rac and Cdc42 that regulate assembly and disassembly of actin cytoskeleton required for cell migration and invasion [14]. Cell movement is regulated through cell-substrate adhesion, cell-cell adhesion, vesicular trafficking and transcription [14]. Rho A regulates stress fibers and the formation of focal adhesions, Rac 1 stimulates actin polymerization and formation of lamellipodia which then initiate cell movement [15] while Cdc42 regulates filopodia formation and directs cell movement [14].

A major post-translational pathway that is vital for the functions of Ras proteins is polyisoprenylation [16]. The modifications facilitate Ras proteins' association with the inner surfaces of the plasma membranes [17]. The polyisoprenylation pathway terminates in the only reversible step regulated by polyisoprenylated protein methyl transferase (PPMTase) [18] and polyisoprenylated methylated protein methyl esterase (PMPMEase) [19]. Work in our laboratory [20, 21] showed that inhibition of PMPMEase with synthetic and natural small molecules results in cancer cell death, inhibition of cancer cell migration as well as significant changes in the expression of cancer-related genes [22, 23]. Cushman *et al* (2013) showed that knockdown of PMPMEase (CES1) diminished Rho A demethylation, inhibited migration and altered the morphology of breast cancer MDA-MB-231 cells [24]. Our previous studies indicate that castration-resistant PCa cell lines have high levels of PMPMEase expression and activity [21] that when inhibited results in diminished cell viability and migration [21]. In this study, we investi-

gate the effects of PCAIs against the molecular processes that drive mCRPC.

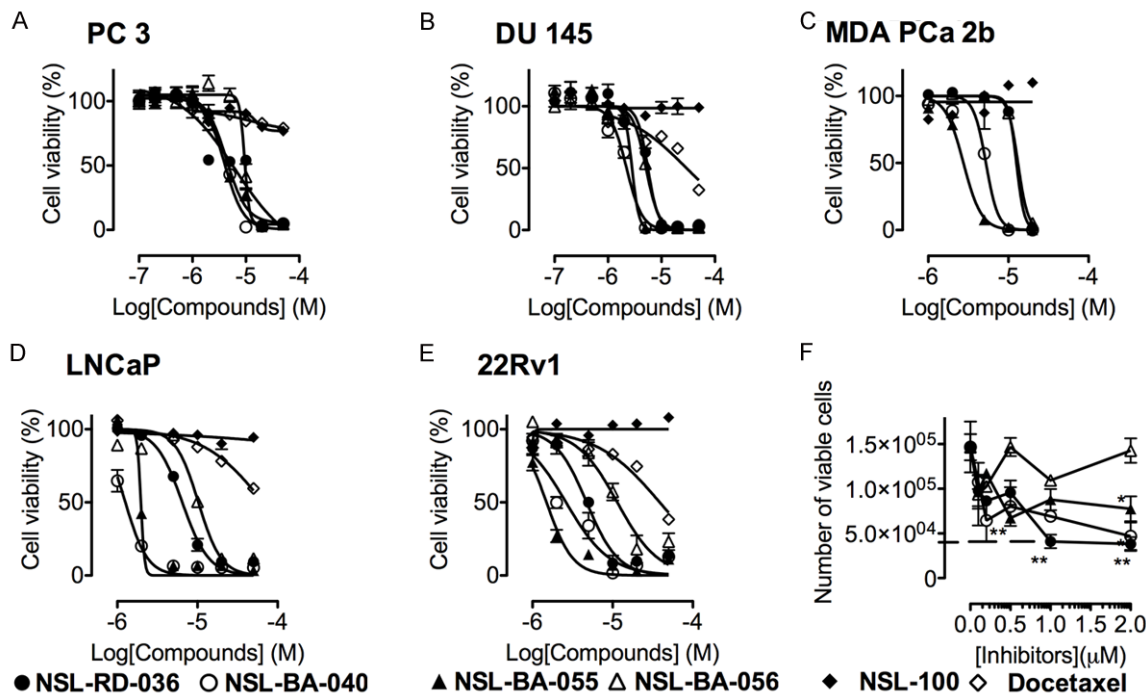
### Materials and methods

#### *Cell lines and cell culture*

Human androgen-sensitive PCa, LNCaP and 22Rv1 cells, and castration-resistant PC 3, DU 145 and the African American androgen-independent MDA PCa 2b cell lines were obtained from American Type Culture Collection (Manassas, VA). LNCaP and 22Rv1 were maintained in RPMI 1640, PC 3 were cultured in Ham's F12K while DU145 were maintained in MEM. All media for these cell lines, except MDA PCa 2b, were supplemented with 10% fetal bovine serum (FBS) and PSN (100 U/mL penicillin, 50 µg/mL streptomycin and 50 µg/mL neomycin) from Life Technologies (Grand Island, NY). MDA PCa 2b cells were cultured in Ham's F12K supplemented with 20% FBS, 25 ng/mL cholera toxin, 10 ng/mL mouse epidermal growth factor, 0.005 mM phosphoethanolamine, 100 pg/mL hydrocortisone and 45 nM selenious acid and 0.005 mg/mL bovine insulin. All cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. pCMVLifeAct-TagRFP plasmid expressing F-actin marker was obtained from ibidi (Madison, WI), Lipofectamine 3000 was obtained from Life Technologies Corporation (Grand Island, NY) and used to transfect plasmid into PCa cells. G 418 disulfate salt from Sigma (St Louis, MO) was used to select for transfected cells since pCMVLifeAct-TagRFP plasmid has neo<sup>r</sup> gene. BD matrigel-coated 24 well inserts were obtained from BD Biosciences. The polyisoprenylated cysteinyl amide inhibitors (PCAIs), NSL-RD-036 (NSL-BA-036), NSL-BA-040, NSL-BA-055 NSL-BA-056 as well as the non-farnesylated analog, NSL-100 were synthesized in our lab as previously described [20, 25].

#### *Effect of the PCAIs on the viability and proliferation of PCa cells*

The human PCa cell lines were seeded at a density of  $2 \times 10^4$ /well in 96 well plates and allowed to attach over 24 h in media containing 5% FBS except for the MDA PCa 2b cells where 10% FBS was used. These were then treated for 48 h with 1 to 20 µM of the PCAIs dissolved in 1 µL of acetone. Control cells were treated with an equal volume of acetone. Cell Titre-Blue solution (20 µL) was then added to each well



**Figure 1.** PCAIs-induced-PCa cell death is polyisoprenylation-dependent. (A) PC 3, (B), DU145 (C) African American MDA PCa 2b (D) LNCaP and (E) 22Rv1 were seeded in 96-wells and treated with 0 to 50 µM of the respective PCAIs, docetaxel or with 0-100 µM of the non-farnesylated analogs of the PCAIs as described in the methods. After 48 h, the cell viabilities were determined using Cell Titre Blue and the number of viable cells counted for the effect on cell proliferation (F). Each point represents the mean ± SEM of 4 determinations. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001 compared to the untreated cells using Student's t-test.

and incubated for 1-2 h after which the cell viability was determined as previously described [23]. To determine the effect of PCAIs on PC 3 cell proliferation, 2 × 10<sup>4</sup> cells were plated into a 24-well plate and allowed to attach overnight. These were then treated with 0.1 to 2.0 µM of the PCAIs for 48 h. Viable cells were counted using a Countess II Automated Cell Counter from Life Technologies (Grand Island, NY).

*Effect of PCAIs on PC 3 colony formation*

Metastatic PC 3 cells were plated at a density of 1.5 × 10<sup>5</sup>/mL in T-25 flasks and allowed to attach overnight. The cells were treated with NSL-BA-040 (1.0-4.0 µM) for 48 h after which they were trypsinized, counted and re-plated in 6-well plates at a density of 500-1000 cells per well and maintained in complete growth media for 14 days. They were then fixed with a solution of acetic acid in methanol (1:7) and stained with 1% crystal violet solution in methanol. Colonies consisting of 50 or more cells were counted with a stereomicroscope. The results were expressed as a percentage of the controls.

*Effect of PCAIs on PC 3 spheroid formation*

Metastatic PC 3 cells were seeded in round bottom 96 well Nunclon Sphera microplates at 5000 cells per well. They were then treated with 1-5 µM of NSL-BA-040 or NSL-BA-055 for 48 h after which 10 µL of acridine orange/ethidium bromide (AO/EB) (100 µg/mL) was added followed by incubation at 37°C for 15 min. Images were taken with Nikon Eclipse Ti 100 inverted fluorescent microscope (Nikon Instruments Inc., Melville, NY).

*Apoptotic-effect of PCAIs on metastatic PCa cell lines*

The mechanism by which the PCAIs induce cell death was determined using the AO/EB staining, annexin V/Propidium iodide flow cytometry and caspase 3/7 activation assays. PC 3 cells were plated in 24 wells at 5 × 10<sup>4</sup> per well and allowed to attach overnight. They were treated with 0.5 to 5.0 µM NSL-BA-040 for 48 h after which 10 µL AO/EB solution (100 µg/mL) was added to each treatment and allowed to incubate for 15 min. Changes in the nuclei after treatment were visualized and pictures taken

## Apoptotic and antimetastatic effects of PCAIs

**Table 1.** EC<sub>50</sub> values for the PCAIs inhibition of PCa cell viabilities as determined from the data in **Figure 1**

COMPOUND	EC <sub>50</sub> (μM)				
	PC 3	DU 145	MDA PCa 2b	LNCaP	22Rv1
NSL-RD-036	6.3	5.6	12.5	5.4	4.7
NSL-BA-040	4.0	2.2	5.2	1.3	2.4
NSL-BA-055	4.3	2.8	2.7	2.0	1.5
NSL-BA-056	9.4	5.1	13.1	10.3	14.8
NSL-100	>50.0	>50.0	>50.0	>50.0	>50.0
Docetaxel	>50.0	>50.0	>50.0	>50.0	>50.0

with Nikon Eclipse Ti 100 inverted fluorescent microscope (Nikon Instruments Inc., Melville, NY). For the flow cytometry analysis, 1 mL of the metastatic PC 3 and DU 145 cells were seeded at  $1 \times 10^5$  cell per mL in six well plates and left overnight to attach. These were then treated with 1 to 8 μM NSL-BA-040 in media supplemented with 5% FBS for 48 h. Apoptosis was determined using ApopNexin FITC Apoptosis Detection Kit (EMD Millipore, Temecula CA) according to the manufacturer's protocol. Briefly, cells were trypsinized and centrifuged at  $500 \times g$  for 5 min. Cell pellets were washed twice with  $1 \times$  PBS and re-suspended in binding buffer. Annexin FITC and propidium iodide were added to the cell suspensions and incubated for 10 min at room temperature in the dark. Analysis was done using the FAS Calibur with Cell Quest Pro software (BD Biosciences NJ).

Caspase activation was determined using the CaspaTagCaspase-3/7 *in situ* Assay Kit, Fluorescein (EMD Millipore, Temecula CA) according to the manufacturer's instructions. PC 3 cells were treated with PCAIs (1-10 μM) or 200 nM staurosporin (positive control) for 48 h. The cells were incubated with  $30 \times$  FLICA reagent in treatment media for 1 h at 37°C under 5% CO<sub>2</sub>. FLICA-containing media was removed and Hoechst solution (0.5% v/v in  $1 \times$  PBS) added to the CaspaTag-labeled cells to stain the nuclei. Cells were washed with  $1 \times$  wash buffer and fixative added. Fluorescent images were obtained with the Nikon Eclipse Ti 100 inverted microscope (Nikon Instruments Inc., Melville, NY) equipped with a Nikon DS Qi2 digital camera and S Plan Fluor ELWD 20×Ph1 ADM objective with a numerical aperture of 0.45. The effect of PCAIs treatment on caspase activation was further investigated by Western blotting. Cells were plated in 60 mm dishes at  $3.5 \times 10^5$  per mL and treated with NSL-BA-040 (5 μM) for 16 h after which they were lysed on ice with RIPA

lysis buffer supplemented with  $1 \times$  protease inhibitors cocktail (Sigma, St. Louis, MO) and the protein concentrations determined using the Bradford assay. Aliquots of each sample containing 50 μg of protein were resolved on 10-20% gradient SDS-PAGE (Bio-Rad) and transferred onto PVDF membranes. After blocking in 3% non-fat dried milk (Sigma, St Louis MO), membranes were incubated at 4°C overnight with caspase 3, 7 (Cell Signaling Technology, Danvers, MA) and cleaved caspase 8 (Sigma, St Louis MO) primary antibodies.

After incubating at room temperature for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG from Santa Cruz Biotech (Santa Cruz, CA), Clarity Western ECL Substrate was added and the chemiluminescence images obtained using the ChemiDoc Imaging Systems (Bio-Rad, CA).

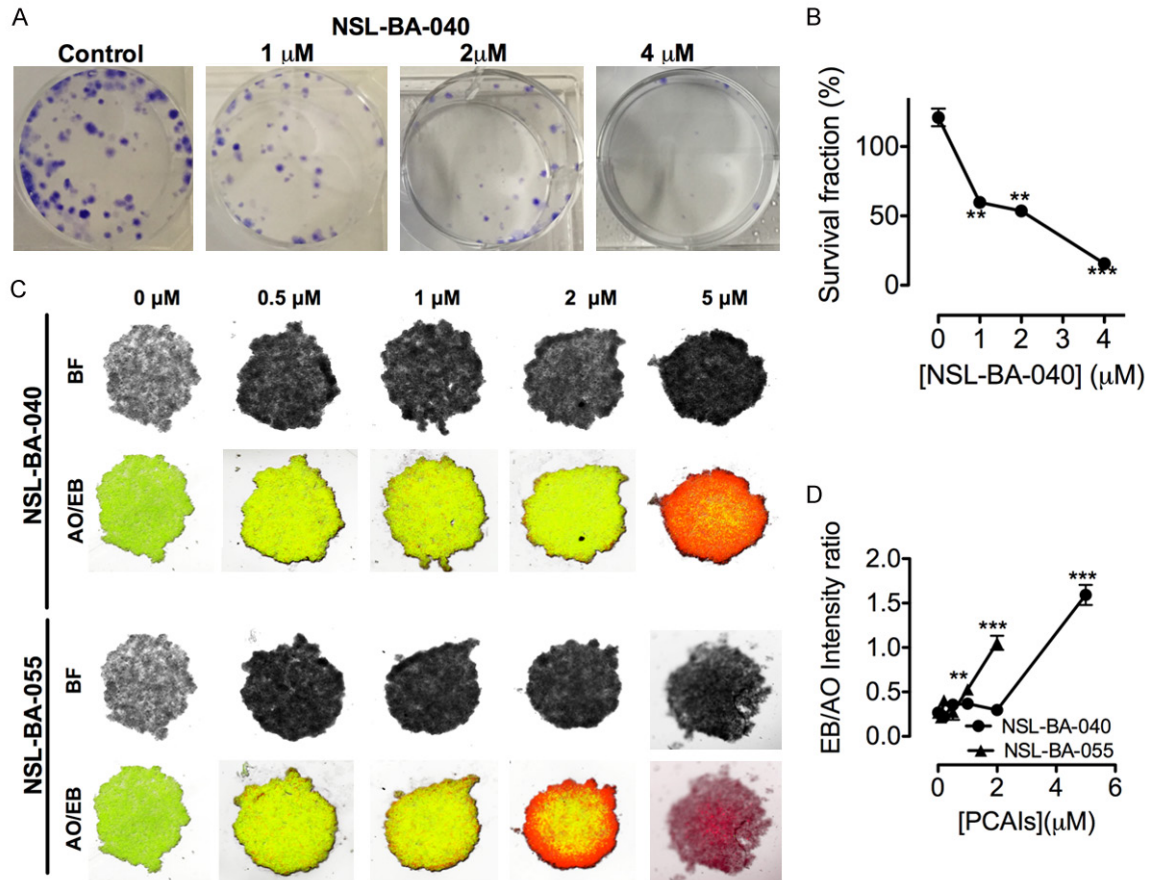
### *Effect of PCAIs on PCa cell migration and invasion*

The ability of the PCAIs to inhibit PCa cell migration and invasion were determined using the wound-healing and matrigel invasion methods. Cells were seeded in ibidi cell culture inserts, allowed to grow to confluence and serum-starved for 24 h. After treatment with 0.5 to 2 μM NSL-BA-040, wound closure was monitored by capturing images with the Nikon Eclipse Ti 100 inverted microscope (Nikon Instruments Inc., Melville, NY) at 0, and 24 h post-treatment and the images analyzed as previously described [21]. In order to determine the effects on cell invasion, matrigel-coated 24-well inserts (Corning) with 8 μm pores were used. PC 3 or DU 145 cells ( $1 \times 10^5$ ) were seeded into the inserts containing 500 μL of media supplemented with 1% FBS while 750 μL of media supplemented with 10% FBS was added to the lower chamber for chemo-attraction. After 24 h, cells that had migrated through the membrane lining of the insert were fixed with 3.7% formaldehyde in  $1 \times$  PBS and stained with 1% crystal violet in methanol. Microscope images of the stained cells were obtained with an Olympus DP70 microscope using 4× objective. The captured images invaded cells counted and expressed as percentages of the control.

### *Effect of PCAIs on F-actin organization in metastatic PC 3 cells*

PC 3 cells were seeded in 6-well plates in media containing 10% FBS for 24 h. These were trans-

## Apoptotic and antimetastatic effects of PCAls



**Figure 2.** PCAls inhibit colony formation and induce apoptosis in spheroids. Treatment with NSL-BA-040 for 48 h inhibited PC 3 colony formation (A) as shown in the concentration-dependent reduction in survival fraction (B). Images of PC 3 spheroids treated with the indicated concentrations of the PCAls for 48 h were captured following staining with acridine orange and ethidium bromide (AO/EB), the former turning live cells green and the latter turning apoptotic cells red (C). The graph shows the ratio of TRITC to FITC intensity (D). The ratio could not be obtained for spheroids treated with 5 mM NSL-BA-055 due to the absence of live cells. Each point represents the mean  $\pm$  SEM of 4 determinations. \* $P < 0.05$ . \*\* $P < 0.01$  \*\*\* $P < 0.001$  compared to the controls using Student's t-test.

ected with 1  $\mu$ g of pCMVLifeAct-TagRFP F-actin marker plasmid using 4  $\mu$ L of lipofectamine 3000 (Life Technologies Corporation Grand Island, NY) per well Transfected cells were selected with G 418 (300  $\mu$ g/mL) for a month. PC 3 cells expressing the LifeAct -TagRFP F-actin marker were plated in 24-well plates at  $5 \times 10^4$ /well and allowed to attach. After cell had attached, they were treated with 1.0-10  $\mu$ M of NSL-BA-040 and imaged for its effects on F-actin organization using Nikon Eclipse Ti 100 inverted microscope (Nikon Instruments Inc., Melville, NY) for 48 h.

### Statistical analysis

All results were expressed as the means  $\pm$  SEM. Statistical analysis was performed with Graph Pad Prism software (San Diego, CA).

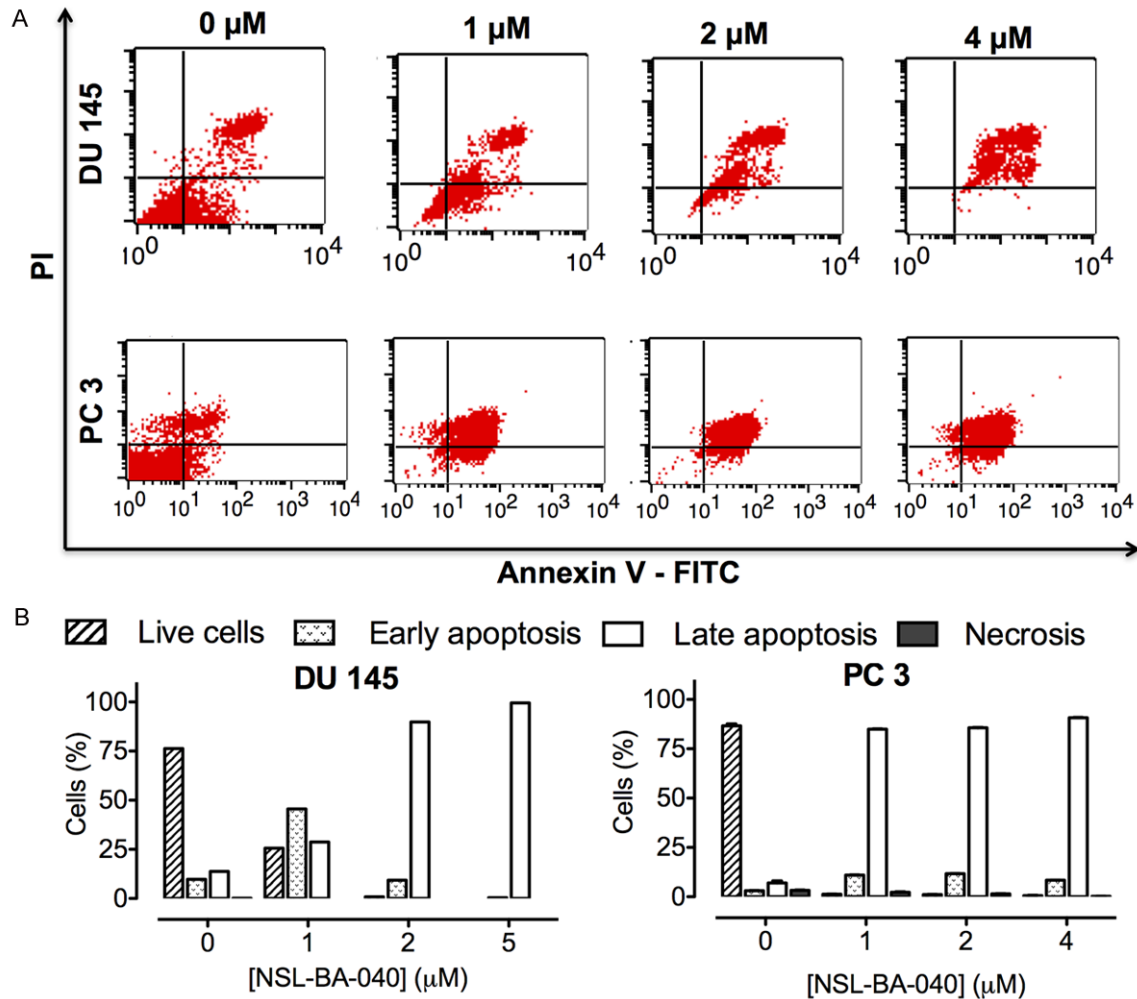
One-way ANOVA analyses with Bonferroni's post hoc test were used for comparisons between multiple groups. The Student's t-test was used for statistical comparison between two groups.  $p$  values  $\leq 0.05$  was considered significant.

### Results

#### *PCAls inhibit the viability and proliferation of PCa cells*

All four PCAls (NSL-RD-036, NSL-BA-040, NSL-BA-055 and NSL-BA-056) used in this study were effective at inducing cell death in virtually the PCa cell lines at concentrations of about 10 to 20  $\mu$ M. The most potent of the PCAls were NSL-BA-040 and NSL-BA-055 with  $EC_{50}$  values ranging from 2.0-to 5.0  $\mu$ M and 1.5 to 4.3  $\mu$ M,

## Apoptotic and antimetastatic effects of PCAIs



**Figure 3.** NSL-BA-040 induces apoptosis in metastatic PCa cells. (A) DU 145 and PC 3 cells were treated with the indicated concentrations of NSL-BA-040 for 48 h, stained with annexin V and propidium iodide and analyzed using flow cytometry as described in the methods (B) Bar graphs showing the distribution of cells as determined by flow cytometry analysis.

respectively for the different cell lines. NSL-RD-036 ( $\text{EC}_{50}$  values of 4.7 to 13  $\mu\text{M}$ ) and NSL-BA-056 ( $\text{EC}_{50}$  values 5.1 to 14.8  $\mu\text{M}$ ) were also very effective against the PCa cell lines (Figure 1 and Table 1). The non-farnesylated analog of the PCAIs, NSL-100 was not effective at inhibiting the viabilities of the cells (Figure 1). As shown in Figure 1, docetaxel, currently used clinically to manage metastatic prostate, was much less effective against the PCa cell lines when compared to the PCAIs with  $\text{EC}_{50}$  in excess of 50  $\mu\text{M}$  (Table 1). This implies that docetaxel is at least 11- to 33-fold and 3- to 10-fold less effective than the most and least potent of the tested PCAIs. PC 3 cell growth and proliferation was significantly inhibited ( $P < 0.01$ ) in a concentration-dependent manner by all the PCAIs tested. As shown in Figure 1F, 2  $\mu\text{M}$  of NSL-RD-036

and NSL-BA-040 completely blocked PC 3 cell proliferation. When we conducted the colony-forming assay to determine the ability of NSL-BA-040 to attenuate PC 3 cell growth relapse, the compounds inhibited colony formation in a concentration dependent manner ( $P < 0.001$ ) (Figure 2A, 2B).

### PCAIs induce cell death in PC 3 spheroids

The ability of PCAIs to potentially induce apoptosis in tumor cells was assessed *in vitro* by treating PC 3 spheroids with NSL-BA-040 and NSL-BA-055. The PCAIs induced concentration-dependent cell death in the PC 3 spheroids as revealed by the progressive increase in ethidium bromide staining. By comparison, the untreated control spheroids were predominant-

ly green due to the exclusion of ethidium bromide by the intact live cells (**Figure 2C**). The increasing intensity ratios of EB over AO with increasing PCAIs concentrations (**Figure 2D**) is an indication of the increasing proportion of dead over live cells in the spheroids since AO stains live cell nuclei green and EB stain the nuclei of dead cells red.

### *NSL-BA-040 induces apoptotic cell death in metastatic PC 3 and DU 145 cells*

Apoptotic cell death is associated with changes in cell morphology and significant changes in DNA. Therefore, we determined the mechanism of the PCAIs-induced cell death by treating PC 3 cells with NSL-BA-040 and stained with acridine orange and ethidium bromide (AO/EB) dye. AO stains live cell nuclei green while EB stains the nuclei of dead cells red. Changes in nuclei morphology and color have been used to demonstrate apoptotic cell death [26]. Treatment with increasing concentrations of NSL-BA-040 (1.0-2.0  $\mu\text{M}$ ) revealed progressive decline in the number of live cells as shown by decrease in AO-labeled nuclei and an increase in the number of EB-labeled nuclei.

The apoptotic effect of NSL-BA-040 was further studied using flow cytometry analysis. Propidium iodide and FITC annexin V staining of DU 145 and PC 3 cells treated with NSL-BA-040 were analyzed with a flow cytometer. A concentration-dependent induction of apoptosis was observed as shown in **Figure 3A** and **3B**. The populations of early (20%) and late apoptotic (8%) cells were significantly increased (20%) with the treatment compared to the control. Activation of executioner caspases 3/7 in apoptotic cell death has been shown in several studies [27]. We thus investigated the involvement of caspase 3/7 in the apoptotic effect of NSL-BA-040 in PC 3 cells using the CaspaTag™Caspase-3/7 *In Situ* Assay (EMD Millipore). PC 3 cells treated with NSL-BA-040 (5  $\mu\text{M}$ ), showed prominent activation of Caspase-3/7, as indicated by intense green fluorescence in the cells compared to the control cells. The other PCAIs at similar concentrations were less effective at activating the caspases 3/7 as shown in **Figure 4A**. This indicates that NSL-BA-040 induces apoptotic cell death in PC 3 cells through activation of Caspase-3/7. Western blot analysis revealed that treatment with NSL-BA-040 (5  $\mu\text{M}$ ) resulted in a one-fold decrease in un-cleaved caspase 3 and 7 in

both cell lines (**Figure 4B** and **4C**). Cleaved caspase 8 showed a two-fold increase with treatment in DU 145 cells and a slight increase in PC 3 cells (**Figure 4D** and **4E**).

### *NSL-BA-040 inhibits migration and invasion of metastatic PC 3 and DU 145*

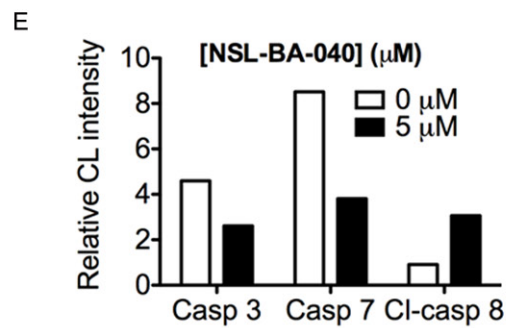
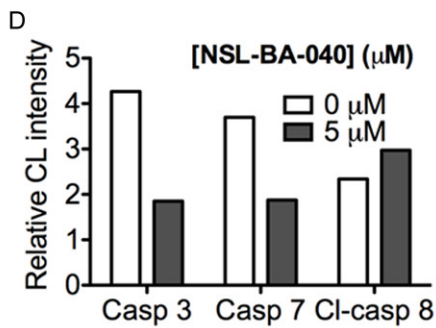
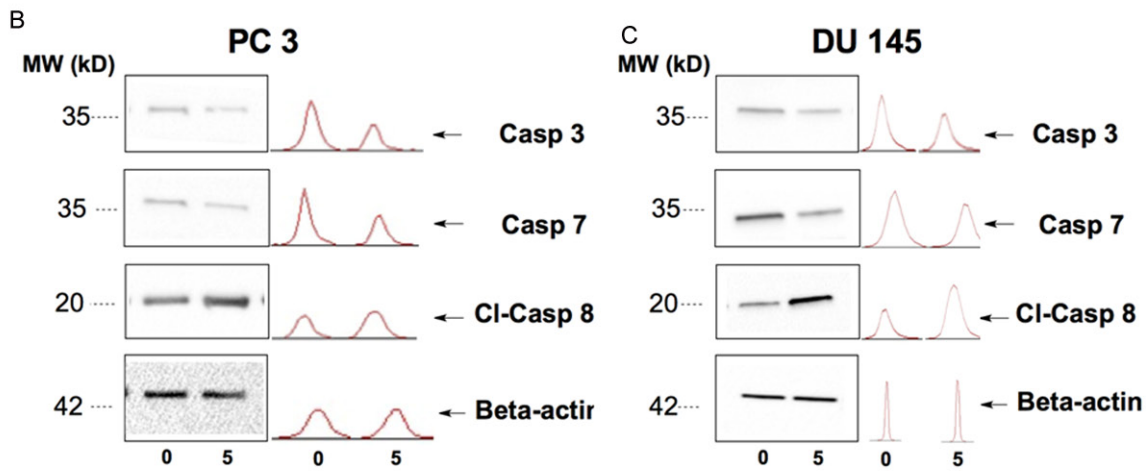
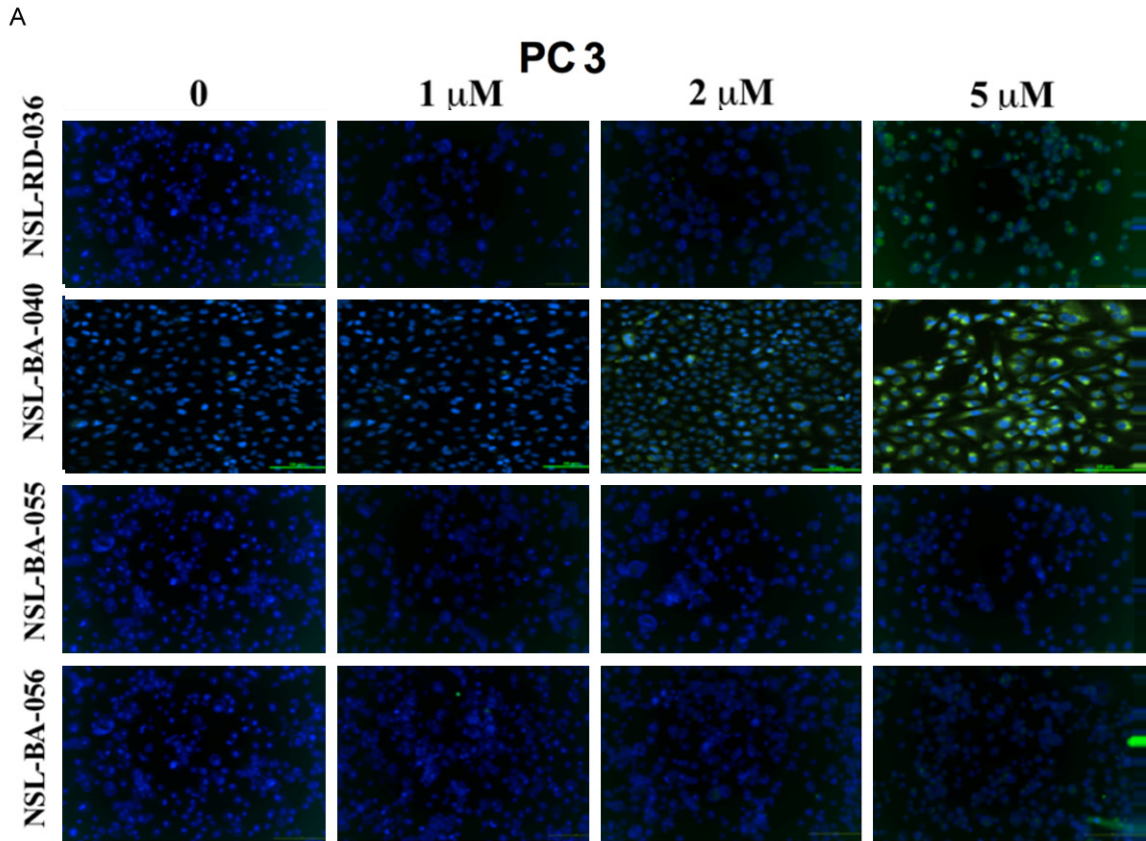
Malignancy in PCa is dependent on the migration and invasion of nearby and distant tissues. The wound-healing assay was used to investigate the ability the PCAIs to limit the motility of PC 3 cells. As shown in **Figure 5A** and **5B**, the number of cells that migrated into the wound area following treatment with NSL-BA-040 decreased significantly in a concentration dependent manner when compared to the control. NSL-BA-040 at 0.5, 1.0 and 2.0  $\mu\text{M}$ , inhibited PC 3 migration by 23%, 35% and 40%, respectively compared to the control (**Figure 5A** and **5B**). Using the matrigel invasion assay, it was determined that NSL-BA-040 significantly inhibited PC 3 and DU 145 cell invasion at sub-micro-molar concentrations. NSL-BA-040 at 0.1  $\mu\text{M}$  reduced number of PC 3 and DU 145 cells that invaded through the matrigel by 27% and 23%, respectively. At the higher concentration of 2.0  $\mu\text{M}$ , PC 3 and DU 145 cell invasion was reduced by 91% and 94%, respectively when compared to the controls (**Figure 5C** and **5D**). These results indicate that at non-cytotoxic concentrations, NSL-BA-040 inhibits migration and invasion of highly metastatic PCa cells in a concentration-dependent manner.

### *NSL-BA-040 disrupts F-actin organization and morphology of PC 3 cells*

PC 3 cells expressing the TagRFP-actin marker protein showed significant alterations in F-actin organization upon exposure to NSL-BA-040. As shown in **Figure 6**, the control cells showed intact well-organized F-actin and normal cell shape. However, after treatment with 1.0-5.0  $\mu\text{M}$  NSL-BA-040, the cells rounded up and had no visible or collapsed F- actin filaments.

## Discussion

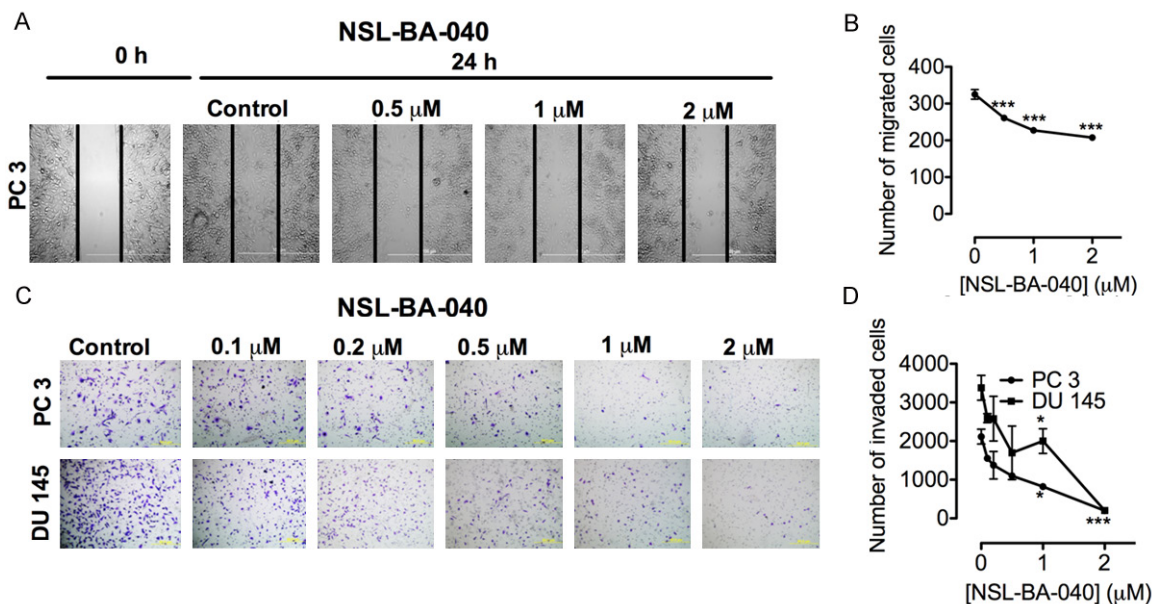
Several studies to elucidate the molecular mechanisms that underlie PCa relapse and metastasis have revealed a strong correlation with amplification-driven activation of the EGFR signaling pathway [28]. However, mCRPC is resistant to the tyrosine kinase inhibitors that target EGFR [29]. Polyisoprenylated proteins





## Apoptotic and antimetastatic effects of PCAIs

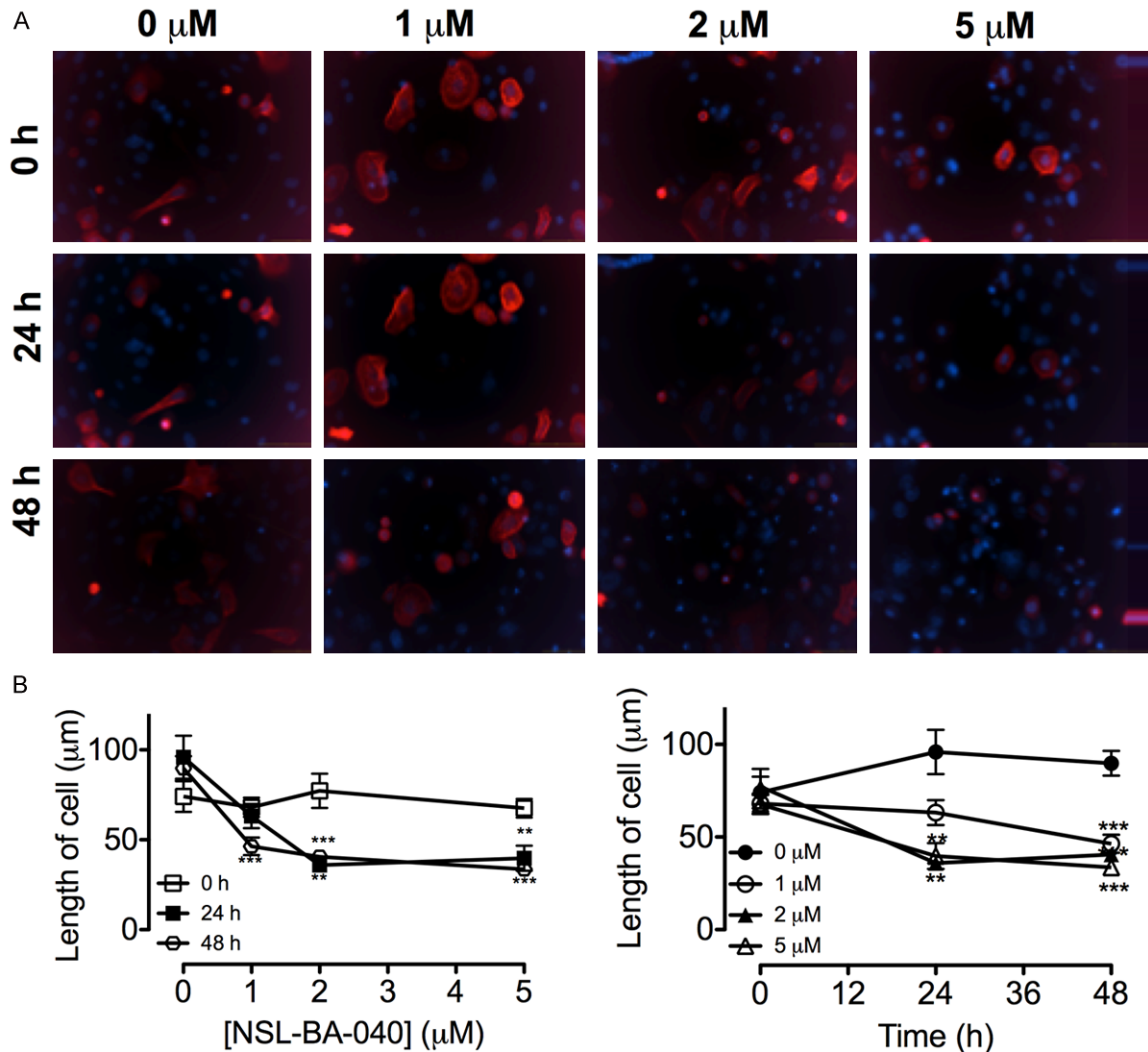
**Figure 4.** PCAIs-induced apoptosis in PCa cells lines is associated with caspase 3, 7 and 8 activation. A: Caspase 3/7 activation was observed after treating PC 3 cells with 1 to 5  $\mu\text{M}$  PCAIs for 48 h and then reacting with the fluorescent caspase 3/7 irreversible inhibitor; green FLICA. Images were taken with Nikon DS Qi2 digital camera linked to a Nikon Eclipse Ti 100 inverted microscope. B, D: Western blot analysis showing the expression of caspase 3, 7 and cleaved caspase 8 in DU 145 and PC 3 cells after treatment with 5  $\mu\text{M}$  NSL-BA-040. C, E: Bar graph showing the chemiluminescence intensity (CL) of the protein bands relative to control.



**Figure 5.** NSL-BA-040 inhibits migration and invasion of PC 3 and DU 145 cells. A: Wounds were created in monolayers of cells and treated with the indicated concentrations of NSL-BA-040 for 24 h as described in the methods. Images were then taken showing the inhibition PC 3 cell migration. B: NSL-BA-040 significantly decreased the number of cells that migrated into the wound compared to the control in a concentration dependent manner. C: PC 3 and Du145 cells were seeded in matrigel-coated inserts and treated with or without NSL-BA-040. After 24 h invaded cells were fixed, stained and counted. D: The number of cells that invaded through matrigel decreased significantly with the treatment compared to the respective untreated controls. Each point represents the mean  $\pm$  SEM of 3 determinations. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus untreated control cells compared by Student's t-test.

such as Ras, Rho and related monomeric G proteins mediate EGFR signaling pathways. Activation of Ras GTPases has been reported to be essential for PCa metastasis as it drives the over expression of EGFR ligands that promote the tumor growth [10]. Consequently, polyisoprenylated small molecules such as the PCAIs have the potential to mitigate the hyperactivity of monomeric G-proteins by disrupting the metabolism and/or the polyisoprenylation-dependent protein-protein interactions of polyisoprenylated proteins [20]. The potential effectiveness of the PCAIs is clearly demonstrated by the fact that the least potent of the PCAIs, NSL-BA-056, was 3-10 fold more effective against the viability of the PCa cell lines than docetaxel, the treatment of choice for mCRPC [30]. Previous studies from our laboratory revealed that the PCAIs are 10-fold more potent

than farnesyl thiosalicylic (FTS), a small polyisoprenylated molecules that has been demonstrated to dislodge K-Ras from cell membranes leading to apoptosis, [20, 31]. In light of these, the PCAIs could be acting in similar fashion by disrupting the interactions between Ras and Rho family of monomeric G-proteins and their effector. This is because Ras and Rho are known to promote cell growth, proliferation, survival, apoptosis and metastasis [32, 33]. These effects on the PCa cells are consistent with an earlier study by Aguilar et al (2014) showing the effects of the PCAIs on pancreatic cancer cell viability and cell death [20]. The polyisoprenyl moiety is essential for the observed effects since the non-farnesylated cysteinyl amide analogs of the PCAIs were not effective against the PCa cells. This further demonstrates the possible interference with



**Figure 6.** NSL-BA-040 disrupts F-actin organization in TagRFP F-actin marker transfected PC 3 cells. A: PC 3 cells expressing the LifeAct-TagRFP F-actin marker was treated with 1 to 5  $\mu\text{M}$  of NSL-BA-040 as described in the methods. The fluorescent images were captured using Nikon DS Qi2 digital camera connected to Nikon Eclipse Ti 100 inverted microscope. B: The length of cells decreased significantly with the treatment compared to the control. Each point represents the mean  $\pm$  SEM ( $n = 25$ ). \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

polyisoprenylated protein-mediated processes.

Further investigations to determine the mechanisms of cell death indicate that the PCAIs induce apoptotic cell death, triggered either through the intrinsic or extrinsic pathway. The extrinsic pathway involves the activation of the death receptors and subsequent activation of caspase 8 while the intrinsic pathway involves the mitochondria and activation of caspase 9 [34]. Activated caspases 8 and 9 from their respective pathways activate caspases 3 and 7, resulting in apoptosis. The observed activation of caspase 8 following treatment with the PCAIs is therefore consistent with the observed

activation of caspase 3/7. This indicates that the apoptotic effect of the PCAIs on metastatic PC 3 and DU 145 prostate cells involve the extrinsic pathway. The apoptotic cell death induced by the PCAIs in PCa cells is potentially of immense therapeutic significance given that the transitions from androgen-dependence, through androgen-independence to mCRPC transition is associated with profound resistance to current therapies [35, 36]. Androgen-independent PCa cell lines have also been reported to evade apoptosis after ADT which drives their continuous survival and proliferation [37]. The ability of PCAIs to induce significant cell death in androgen-dependent and

metastatic androgen-independent as well as African American prostate cancer cell lines makes them a potential single therapeutic agent that can target and eliminate incurable metastatic forms of prostate cancer. This is significant because African American men have higher risks of being diagnosed and dying from metastatic advanced PCa than other ethnic groups [38].

The aberrant activities of the polyisoprenylated Rho A, Rho C, Rac 1 and Cdc42 proteins have also been implicated in invasion and metastasis in prostate and other cancers [39]. Reorganization of actin cytoskeleton, focal adhesion formation and cell migration, which constitute the hallmarks of metastasis, depend mostly on Rho signaling [39]. However, polyisoprenylation is necessary for Rho and related proteins to be functional [40]. In the presence of the PCAIs, the invasive and migratory ability of metastatic PCa cell lines, PC 3 and DU 145, were significantly inhibited. This suggests that PCAIs probably interfere with Rho interactions, resulting in the disruption of Rho-mediated migratory and invasive properties in these metastatic cell lines. The inhibitory effect of the PCAIs on invasion and migration is further supported in the TagRFP F-actin marker transfected metastatic PC 3 cells, which revealed complete disruption of F-actin organization with corresponding morphological changes on treatment with the PCAIs. Similar morphological changes were reported by Cushman *et al* in the triple negative breast cancer cell line (MDA-MB 231), following knockdown of CES1/PMPMEase, indicating the importance of PMPMEase in Rho methylation and F-actin cytoskeletal function [24].

### Conclusions

Taken together, our data show that the PCAIs have the potential to limit PCa tumor growth by inducing apoptotic cell death through the activation of caspases 8 and then 3/7 as well as inhibit metastasis by disrupting F-actin-mediated migration. The effects of the PCAIs are most likely through the disruption of polyisoprenylated protein signaling. This is significant since EGFR is frequently overexpressed in mCRPC and the Ras proteins require polyisoprenylation to mediate the EGFR signaling.

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### Disclosure of conflict of interest

None.

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