Effects of 3-butenyl isothiocyanate on phenotypically different prostate cancer cells

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Abstract. Isothiocyanates (ITCs) have gained increasing attention since they have been attributed the merits for the potential beneficial effects of cruciferous vegetable dietary consumption on cancer. The aim of the present study was to determine the cytotoxic effects of 3-butenyl ITC (3-BI) on prostate cancer (PC) cells under *in vitro* conditions. Two androgen-insensitive human PC cell lines, PC-3 and DU145, were assayed. Cells were cultured in the presence of increasing concentrations of 3-BI (5, 10, 30 and 50 μM) in the absence or presence of the chemotherapeutic drug docetaxel (DOCE) (1 and 2 nM). The cytotoxic effects of these compounds were analyzed using the trypan blue exclusion assay at 24, 48 and 72 h. Apoptosis and migration assays were also performed. The results showed that 3-BI induced a dose-dependent cytotoxic effect on PC-3 cells at 24, 48 and 72 h. These effects were significantly higher than those found with DOCE at 72 h of culture. Moreover, 3-BI also potentiated the effects of DOCE in a dose-dependent manner. Additionally, 3-BI showed inhibition of the migration of PC-3 cells. Nevertheless, 3-BI was not effective in the DU145 PC cell line. These results show a promising role for the 3-BI compound as a co-adjuvant agent in DOCE-based therapy in certain types of PC.

Introduction

Prostate cancer (PC) is a major health problem causing high morbidity and mortality worldwide (1). PC is a heterogeneous cancer phenotypically and biologically, particularly with regard to its hormone sensitivity. In order to reproduce this context *in vitro*, DU145 and PC-3 androgen-insensitive PC cells (AIPC) may be used. These cells differ in terms of tumor suppressor proteins, cell adhesion molecules and aggressiveness (2-5).

Although the synthesis of androgens and androgen receptors (AR) constitutes a therapeutic target (6), there is currently no definitive treatment for PC beyond surgery or radiotherapy in the early stages of the disease. When recurrence occurs, androgen ablation therapy (ADT) is a standard systemic therapy. Although the initial response to treatment is high, after ~18 months there is a state of resistance to androgens, termed castration-resistant PC (CRPC) (7), which is associated in up to 40% of cases with metastasis and heterogeneity in the intra patient intermetastasis therapeutic response. The expected survival rate for patients with PC is ~18%, but when recurrences appear it decreases to 5-10% (8). Treatment options for CRPC and metastatic CRPC (mCRPC) include docetaxel (DOCE)-based regimens (9) alone or in association with, for example, ADT (10-13) or anti-angiogenic (bevacizumab), anti-proliferative and anti-migratory (tyrosine kinase inhibitors) drugs (14,15). DOCE has been extensively used and is likely to continue to be used in PC, improving tumor response rate, time to progression and overall survival time (7). Although DOCE is a first-line reference drug in CRPC, side effects (15) and DOCE resistance (16) limit its clinical efficacy. Additionally, the widespread use of novel drugs is limited by the high cost (17). Therefore, there is a requirement to develop novel therapeutic strategies to improve efficacy and reduce adverse effects.

Phytochemicals are a heterogeneous set of bioactive compounds that are found naturally in vegetables, fruits, grains and other plant products. The compounds are often responsible for certain unique plant characteristics, including smell and pigmentation, and a number are vital for the protection of the host against parasites, viruses and other externally damaging agents. Moreover, phytochemicals could be compounds of interest in cancer chemoprevention due to their lower toxic effects compared with conventional chemotherapeutic drugs, their action on molecular targets involved in carcinogenesis (18) or their resensitizing effects on anti-androgen-resistant cells (19). An inverse correlation between consumption of cruciferous vegetables and cancer risk has been observed in PC and breast, colon, lung and gastric

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cancer (20-22). This effect has been attributed to a group of plant compounds, the isothiocyanates (ITCs), which are present in substantial concentrations in all brassica vegetables. ITCs are formed by the hydrolysis of their precursor parent compounds, the glucosinolates. The glucosinolates are a large group of sulphur-containing compounds that are present in all Brassica crops; a β-D-thioglucose group, a sulphonated oxime moiety and a variable side-chain derived from methionine, tryptophan or phenylalanine forms their common structure. Upon damage to the plant tissues during consumption, the endogenous enzyme myrosinase (thioglucoside glycohydrolase EC 3:2:3:1) and other gut enzymes hydrolize the glucosinolates to release a range of breakdown products, including the biologically active ITCs (23) (Fig. 1). ITCs have inhibitory effects on the metabolic activation of carcinogenic dietary or tobacco components (24), along with the inhibition of mutagenesis (25) and anti-carcinogenic effects *in vitro* (26-30) and *in vivo* (31,32). In PC, ITCs modulate epigenetic changes, induce the arrest of the cell cycle, activate apoptosis pathways and increase chemotherapeutic agent sensitivity; leading to the inhibition of cell proliferation, progression and invasion-metastasis (33).

ITCs are compounds with the structure R-N=C=S, where R is an alkyl or aryl group. One such ITC is 3-Butenyl ITC (3-BI), found particularly in brassica crops from *Brassica rapa* species, including pak choi, Chinese cabbage, turnips, turnip greens and turnip tops (34), where it is present as the glucosinolate gluconapin (Fig. 2). 3-BI is known to have multiple effects, including anti-proliferative and anti-apoptotic effects, on colon (26,35), prostate, bone, cervical, liver, breast and neuroblastoma tumor cells (30). To date, there are no data on the effects of combination therapy of 3-BI and DOCE in PC. In order to elucidate this interaction, the present study examined the *in vitro* anticancer effects of 3-BI, with and without DOCE, using primary cultures of healthy human prostate epithelial cells (PECs) and the AIPC PC-3 and DU145 cell lines. Comparing the effects of 3-BI with or without DOCE on normal versus cancer cell lines will provide insight into their role in the treatment of PC.

Materials and methods

Cell culture. Tumorigenic cell lines, DU145 (HTB-81) and PC-3 (cat. no. CRL-1435), and primary PECs (PCS-440-010; used to determine non-toxic nature of 3-BI only) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured according to company protocols with F-12K Medium (cat. no. 30-2004) and a Prostate Epithelial Cell Growth kit (cat. no. PCS-440-040) (both from ATCC), supplemented with fetal bovine serum (FBS; cat. no. 10270; Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 U/ml penicillin and 100 mg/ml streptomycin (Penicillin-Streptomycin Solution 30-2300; LGC Standards SL, Barcelona, Spain). Stock solutions of 3-BI (cat. no. I0443; TCI, Paris, France) and DOCE (cat. no. 01885) were prepared in dimethyl sulfoxide (DMSO; cat. no. D2650) (both from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and diluted with complete medium (medium supplemented with FBS, penicillin and streptomycin). An equal volume of DMSO (final concentration <0.05%) was added to the controls.

Cell viability assay. The effect of 3-BI and/or DOCE treatments on cell viability was determined by trypan blue dye exclusion assay as described previously (36). Briefly, $5x10^3$ cells in 1 ml complete medium were plated in 6-well plates and allowed to attach overnight. The medium was replaced with fresh complete medium containing different concentrations of 3-BI (0, 5, 10, 30 and 50 μM) and/or DOCE (0, 1 and 2 nM), and the plates were incubated for 24, 48 or 72 h at 37˚C. At the end of the incubation period, floating and adherent cells were collected and suspended in 25 ml phosphate-buffered saline (PBS; cat. no. D8537; Sigma-Aldrich; Merck KGaA). The cells were then mixed with 5 ml 0.4% trypan blue solution (cat. no. T6146; Sigma-Aldrich; Merck KGaA), and live and dead cells were counted under an Olympus inverted microscope (Olympus, Tokyo, Japan).

Determination of apoptosis. Apoptosis induction by 3-BI and/or DOCE treatments, at the same concentrations that were used to evaluate cellular viability, was assessed by fluorescence microscopy following staining with 4',6-diamidino-2-phenylindole (DAPI), caspase-3 activation assay and cleaved poly(ADP-ribose) polymerase (PARP) assay.

Fluorescence microscopy. Cells ($2x10^4$) were plated on coverslips, allowed to attach overnight, and exposed to DMSO or 3-BI and/or DOCE at the same concentrations that were used to evaluate cellular viability, for 72 h. The cells were washed with PBS and fixed with 3% paraformaldehyde for 1 h at room temperature. The cells were washed three times with PBS, permeabilized with 1% Triton X-100 (cat. no. 142314.1611142314.1611; Panreac Quimica SLU, Barcelona, Spain) for 4 min, washed again with PBS and stained with 1 μg/ml DAPI (cat. no. D9542; Sigma-Aldrich; Merck KGaA) for 5 min. The cells with condensed and fragmented DNA (apoptotic cells) were counted under a fluorescence microscope at x40 magnification (37).

Caspase-3 activity and cleaved PARP assay. The effect of 3-BI and/or DOCE treatments on caspase-3 activity and cleaved PARP level in PC-3 cells was determined using commercially available ELISA kits. Active caspase-3 and cleaved PARP levels (ng/mg total protein of cell lysate) were determined, and the results were expressed relative to the control. Briefly, $1x10^4$ cells were plated in 24-well plates and allowed to attach overnight. Next, the cells were treated with DMSO, 50 μM 3-BI, 2 nM DOCE or 50 μM 3-BI + 2 nM DOCE for 72 h at 37˚C. The cells were collected and lysed in cell extraction buffer (cat. no. FNN0011; Thermo Fisher Scientific, Inc.) mixed with protease inhibitor cocktail (cat. no. P-2714) and 1 mM phenyl methane sulfonyl fluoride (cat. no. 10837091001) (both from Sigma-Aldrich; Merck KGaA). ELISA assays for caspase-3 activity (cat. no. KHO1091) and cleaved PARP level (cat. no. 10650704) (both from Thermo Fisher Scientific, Inc.) were performed according to the manufacturer's protocols.

In vitro migration assay. The effect of 3-BI (0, 5, 10, 30 and 50 μM) on PC-3 and DU145 cell migration was assessed using 24-well Transwell cell culture chambers (6.5-mm diameter, 8.0-μm pore size, polycarbonate membrane) (cat. no. C6932; Sigma-Aldrich; Merck KGaA) with a Millicell Cell Culture

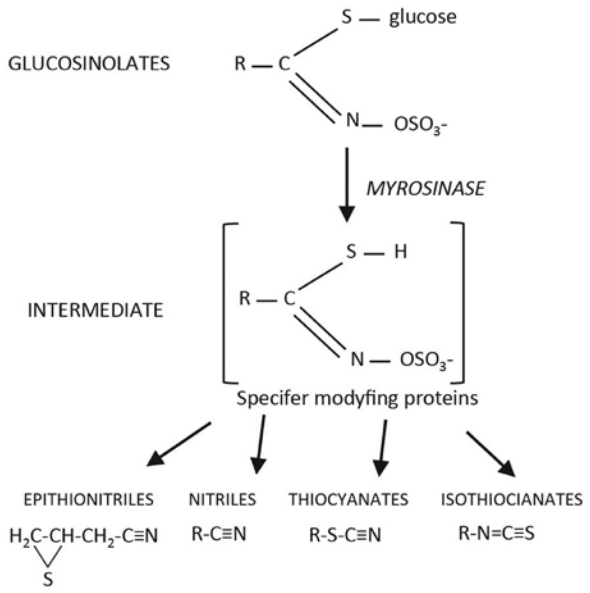

Figure 1. General structure of glucosinolates and their enzymatic degradation products.

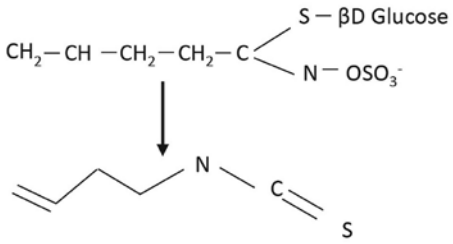

Figure 2. Structure of glucosinolate gluconapin and its enzymatic degradation product, 3-butenyl isothiocyanate.

Insert (cat. no. PI8P01250; Merck KGaA) (38). Briefly, $1x10^5$ cells were added into the upper chamber with 100 μl serum-free medium. The lower chamber was filled with 600 μl complete medium containing 10% fetal bovine serum (Life Technologies; Thermo Fisher Scientific, Inc.). After 24 h of incubation at 37˚C in a 5% CO_2 incubator, the cells in the upper chamber were carefully removed with a cotton swab and the cells that had migrated through the membrane and had stuck to the lower surface of the membrane were fixed with 4% paraformaldehyde (20 min at room temperature) and stained with crystal violet (cat. no. C6158; Sigma-Aldrich; Merck KGaA) (15 min at room temperature). For quantification, the stained cells were counted by capturing images of the membrane under a microscope in five randomly selected fields using an Olympus microscope with a digital camera (Olympus, Tokyo, Japan). At least three chambers from three different experiments were analyzed.

Confocal microscopy. As 3-BI exhibited noticeable effects on PC-3 cells but not in DU 145 cells (see Results section), PC-3 cells were seeded in glass coverslips into incubation chambers for 24 h at 37˚C and with 5% CO_2 for full adherence. Next, the medium was replaced with fresh complete medium without adding any treatment (control) or containing 3-BI 50 μM and/or DOCE (1 and 2 nM), and the cells were incubated for 72 h at 37˚C. Subsequently, the PC-3 cells were fluorescently stained for F-actin (Phalloidin-ATTO 647N; 1:500; cat. no. 65906; Sigma-Aldrich; Merck KGaA), nuclei (DAPI) and cytoplasm [Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE); cat. no. 1351201EDU; Bio-Rad Laboratories, Inc., Hercules, CA, USA] according to the following procedure. The culture medium was replaced by PBS solution containing fluorescent dye CFDA-SE at 1 mM and the cells were incubated for 15 min at room temperature. Following incubation, CFDA-SE solution was removed and the cells were washed with PBS, fixed with 70% ethanol for 5 min, washed again with PBS and stained for with Phalloidin-ATTO for 1 h at room temperature. Next, the cells were washed with 0.9% sodium chloride (cat. no. S7653; Sigma-Aldrich; Merck KGaA) and stained with DAPI at 1 μg/ml for 10 min at room temperature. Thereafter, slides were embedded in Vecta Shield antifade mounting medium (cat. no. H-1000; Vector Laboratories, Inc., Burlingame, CA, USA). A Leica confocal microscope (Leica TCS SP5 X microscope; Leica Microsystems, Inc., Buffalo Grove, IL, USA) was used for the analysis of the cells (39).

Statistical analysis. The results represent the mean of at least three independent experiments (mean ± standard deviation). The significance of difference in measured variables between control and treated groups was studied by two-way analysis of variance. Significance between multiple experimental groups was determined using Tukey's post hoc analysis. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Inhibition of PC cell proliferation by 3-BI and/or DOCE. The effect of 3-BI treatment on the survival of PC-3 and DU145 cells was assessed by trypan blue dye exclusion assay (Fig. 3). The viability of the DU145 cells was not affected by 3-BI (Fig. 3B), whereas the compound significantly inhibited the survival of PC-3 cells in a concentration- and time-dependent manner (Fig. 3A). The inhibitory effect of 3-BI was most pronounced at doses of 50 μM (30% at 24 h). Otherwise, survival of PECs was minimally affected ($P>0.05$) by 3-BI. Thus, ~96% of PECs were viable following a 72-h exposure to 50 μM 3-BI (Table I), whereas only ~30% of PC-3 survived under similar conditions to 3-BI treatment.

3-BI was more effective in PC-3 cells than DOCE only at 72 h; 50 μM 3-BI reduced PC-3 cell viability by ~70%, whereas 2 nM DOCE alone inhibited the growth of these cells by ~35%.

Sensitization of PC cells to growth suppression by DOCE. Growth inhibition of PC-3 cells (but not of DU145 cells) by DOCE was increased by 3-BI. Fig. 3 shows the viability of PC cells following 24-72 h of exposure to 3-BI and/or DOCE, as assessed by trypan blue dye exclusion assay.

The 3-BI and DOCE combination was significantly more efficacious against the viability of PC-3 cells compared with 3-BI or DOCE treatment alone. This effect was not observed for DU145 cells, so the 3-BI-mediated sensitization to growth suppression by DOCE was a cell line-specific response. Analyzing the results using a previously described method (40), it was observed that 3-BI synergistically enhanced the anti-proliferative effects of DOCE on PC-3 cells at 24 and 48 h when the lowest dose of the chemotherapy drug

was administered (Table II). For example, the viability of the PC-3 cells was slightly affected in the presence of 1 nM DOCE alone for 48 h (95%), while exposure to 50 μM 3-BI resulted in a survival rate of 73% compared with that in the DMSO-treated control group. The viability of PC-3 cells was reduced by ~53% by a 48-h co-treatment with 50 μM 3-BI and 1 nM DOCE in comparison with that of the vehicle-treated control cells, with an observed combination index of 1.47, indicating synergy between 3-BI and DOCE (Table II).

Apoptosis. Apoptotic induction by 3-BI was assessed by fluorescence microscopy subsequent to staining with DAPI (Fig. 4). Furthermore, taking into account that characteristic events of apoptosis include proteolytic activation of caspase-3, and proteolytic cleavage of PARP-1, PARP and caspase-3 cleavage measurements were performed. It was concluded that the 3-BI-mediated death of androgen-independent PC cells induced by 3-BI cannot be explained by apoptotic mechanisms, as apoptotic cells were rarely observed in the cultures of PC-3 cells following a 72-h treatment with 3-BI (Fig. 5).

Migration. Transwell chamber assays were used to assess the effects of 3-BI on PC-3 and DU145 cell migration. In contrast to that in DU145 cells, following 3-BI treatment for 72 h, the migration ability of the PC-3 cells was decreased, with its maximum effect at 50 μM (Fig. 6).

Confocal microscopy. To investigate the role of 3-BI in cell damage in PC-3, cytopathic changes were examined (Fig. 7). As the maximum effect had previously been observed at 50 μM 3-BI, this dose was used as the treatment concentration in this experiment. In the control group, PC-3 cells showed elongated morphology, cell appendages (arrows), ellipsoid nuclei with euchromatin and heterochromatin, actin filaments localized mainly beneath the plasma membrane and the cytoplasm stained homogenously with CFDA-SE. In the treatment groups (50 μM 3-BI, 50 μM 3-BI and 1 nM DOCE, and 50 μM 3-BI and 2 nM DOCE) the cells exhibited a rounded shape as the predominant phenotype and actin skeleton reorganization with actin dislocation into the perinuclear zone.

Table I. Effects of the treatment with 3-BI (72 h) on proliferation of PEC cells, as determined by trypan blue dye exclusion assay.

		Cell proliferation	
DOCE, nM	3-BI, μM	Mean (%)	SD
0	0	98.04	0.52
0	5	97.55	1.37
0	10	98.52	1.26
0	30	96.52	0.57
0	50	95.64	1.32
1	0	93.43	0.97
1	5	96.75	1.01
1	10	96.40	1.34
1	30	95.32	0.98
1	50	95.35	1.19
2	0	96.48	1.08
2	5	96.37	1.33
2	10	95.51	0.95
2	30	96.48	0.77
2	50	96.04	0.90

Results are expressed relative to the control. No significant differences were found compared with the control treatment. 3-BI, 3-butenyl isothiocyanate; DOCE, docetaxel; SD, standard deviation.

Discussion

The present study found that the PC-3 cell line, but not the DU145 cell line, was sensitive to survival inhibition induced by 3-BI. This was a time- and dose-dependent effect. At the highest doses and more prolonged times of exposure, PC-3 cell survival was ~30%. By contrast, survival of PECs was ~96%. These findings may be noteworthy since the ability of a chemopreventive agent to cause specific cytotoxicity in cancer cells and not in normal cells is an important determinant of its clinical relevance and safety.

There are a few studies on the effects of 3-BI on cancer cells under *in vitro* conditions (26,30,35). Other 3-butenyl ITCs, including 4-methylthio-3-BI-ITC (raphasatin) or 4-methylsulfinyl-3-BI-ITC (sulforaphene), have been found not to cause toxicity in T lymphocytes, despite being cytotoxic in Jurkat T-leukemia (41), colon cancer (LoVo, CaCo-2, HCT-116 and HT-29) (42,43) and breast cancer (MDA-MB-231 and MCF-7) (43) cells.

Unlike other ITCs, the molecular mechanisms by which 3-BI produces an inhibitory effect on the survival and proliferation of cancer cells are not fully known. The anti-proliferative effect of ITCs is usually associated with cell cycle arrest and the activation of apoptosis (33,44,45). Regarding cell cycle arrest, previous studies have found that sulforaphene inhibits proliferative activity by increasing the expression of p21 (cyclin-dependent kinase inhibitor), which in turn promotes G1 cell cycle phase arrest (esophageal carcinogenesis *in vivo*). By contrast, sulforaphene induces apoptosis through increased expression of p21 (32), downregulation of the B-cell lymphoma-2 (Bcl-2)/apoptosis regulator BAX (Bax) ratio (*in vivo*) and activation of caspase-3 and -9 (*in vitro*, A549 lung cancer cells) (46). In the present study, few apoptotic cells were observed following 3-BI treatment of PC-3 cells, suggesting that the effect of 3-BI could be mediated through other pathways, which will be investigated in future studies. These controversial results may be due to the different cell and ITC characteristics and/or ITC treatment concentrations.

In an attempt to overcome DOCE resistance, the effective cytotoxic, apoptotic and anti-migratory concentrations of the microtubule-targeting drug DOCE and 3-BI, and the synergistic effects in DU145 and PC-3 CRPC lines were studied. DU145 cells are p53-mutant, Bax-negative, signal transducer and activator of transcription 3 (STAT3)-positive and E-cadherin-positive, while PC-3 cells are p53-null, STAT3-negative, and phosphatase and tensin homologue (PTEN)-negative, with negative or low E-cadherin expression and high matrix metalloproteinase (MMP)1 and 3 expression; therefore making PC-3 cells more aggressive (2-5,47). Furthermore, PC-3 cells show features that are characteristic of prostatic small cell neuroendocrine PC (48).

Table II. Analysis of synergy between 3-BI and DOCE calculated by survival rate for PC-3 cells.

	DOCE			3-BI			Combination treatment			
Time, h	Dose, nM	MSR[a]	P-value[b]	Dose, nM	MSR	P-value	Expected[c]	Observed[d]	P-value	Index[e]
24	1	0.99	>0.05	5	0.97	>0.05	0.96	0.58	<0.05	1.66
24	1	0.99	>0.05	10	0.72	<0.05	0.71	0.36	<0.05	1.97
24	1	0.99	>0.05	30	0.69	<0.05	0.68	0.36	<0.05	1.89
24	1	0.99	>0.05	50	0.71	<0.05	0.70	0.33	<0.05	2.12
24	2	0.37	<0.05	5	0.97	>0.05	0.36	0.48	<0.05	0.75
24	2	0.37	<0.05	10	0.72	<0.05	0.27	0.37	<0.05	0.73
24	2	0.37	<0.05	30	0.69	<0.05	0.26	0.36	<0.05	0.72
24	2	0.37	<0.05	50	0.71	<0.05	0.26	0.34	<0.05	0.76
48	1	0.95	>0.05	5	0.75	<0.05	0.71	0.55	<0.05	1.29
48	1	0.95	>0.05	10	0.75	<0.05	0.71	0.46	<0.05	1.54
48	1	0.95	>0.05	30	0.76	<0.05	0.72	0.47	<0.05	1.53
48	1	0.95	>0.05	50	0.73	<0.05	0.69	0.47	<0.05	1.47
48	2	0.38	<0.05	5	0.75	<0.05	0.29	0.55	<0.05	0.53
48	2	0.38	<0.05	10	0.75	<0.05	0.29	0.41	<0.05	0.71
48	2	0.38	<0.05	30	0.76	<0.05	0.29	0.39	<0.05	0.74
48	2	0.38	<0.05	50	0.73	<0.05	0.28	0.39	<0.05	0.72
72	1	0.95	>0.05	5	0.56	<0.05	0.53	0.54	>0 .05	0.98
72	1	0.95	>0.05	10	0.45	<0.05	0.43	0.46	>0 .05	0.93
72	1	0.95	>0.05	30	0.32	<0.05	0.30	0.48	>0 .05	0.63
72	1	0.95	>0.05	50	0.30	<0.05	0.29	0.47	<0.05	0.62
72	2	0.33	<0.05	5	0.56	<0.05	0.18	0.46	<0.05	0.39
72	2	0.33	<0.05	10	0.45	<0.05	0.15	0.42	<0.05	0.36
72	2	0.33	<0.05	30	0.32	<0.05	0.11	0.30	<0.05	0.37
72	2	0.33	<0.05	50	0.30	<0.05	0.10	0.30	<0.05	0.33

3-BI, 3-butenyl isothiocyanate; DOCE, docetaxel; MSR, mean survival rate. [a]MSR of treated group compared with dimethyl sulfoxide-treated control group. [b]P-value was determined by one-way analysis of variance followed by Tukey's test. [c]Survival rate of DOCE group multiplied by survival rate of the 3-BI group. [d]Survival rate of combination treatment group (DOCE + 3-BI). [e]Index was calculated by dividing the expected survival rate by the observed survival rate. An index of >1 indicates a synergistic effect and an index of <1 indicates less than additive effect.

The present results demonstrated that 3-BI synergizes with DOCE against cultured human PC-3 AIPC cells. To the best of our knowledge, the present study is the first published report to document this synergistic anti-proliferative effect. This effect could be explained by interaction with a DOCE molecular mechanism (some of which are already under investigation by our group): i) DOCE is a well-known microtubule-target agent (49). Certain ITCs, including allyl-ITC (45), benzyl-ITC, phenethyl-ITC and 4-methyl-sulphynilbutyl-ITC (SFN) (50-52), interact with microtubules causing inhibition of tubulin polymerization, tubulin depolymerization, histone deacetylase inhibition or tubulin acetylation (Fig. 8). ii) DOCE induces Bcl-2 phosphorylation, which in turn causes cellular apoptosis through different routes (53). Bcl-2 is associated with the development of AIPC, being overexpressed in advanced stages of the disease. Bcl-2 proteins, independent of their known anti-apoptotic effects, are also implicated in cancer cell migration and invasion. Certain ITCs can upregulate Bcl-2 (33). iii) DOCE inhibits androgen-dependent and -independent activation of AR. The association between AR and tubulin, and the preferential binding of microtubules to AR can recruit AR to determine apoptotic-signaling promotion and tumor growth inhibition (54). SFN can decrease the expression of AR in a dose- and time-dependent manner in PC cells (55). The chemical structure of this ITC determines this effect. Thus, while propyl-, butyl- and pentyl-thio analogues have similar effectiveness, sulfonyl derivatives of SFN are either inactive or less effective than the thio- or sulfinyl-derivatives (56).

On the other hand, the present study observed that 3-BI-mediated DOCE sensitization to growth suppression was significantly higher in the PC-3 cell line as opposed to that in the DU145 cell line. Although the precise mechanism underlying this divergence is not known, it could likely be attributed to differential cells characteristics, particularly with regard to p53, STAT3 and PTEN. The tumor suppressor p53 is expressed in response to different stresses (57), orchestrating biological outputs such as cell proliferation, apoptosis, cell-cycle arrest or autophagy (58). In advanced PC, a complete loss of p53 is found (59) and a lack or mutation of p53 is associated with chemotherapeutic resistance (60-62). As the PC-3 and DU145 cell lines lack functional p53, similarities could be expected; however, according to Muller and Vousden (63), mutant p53

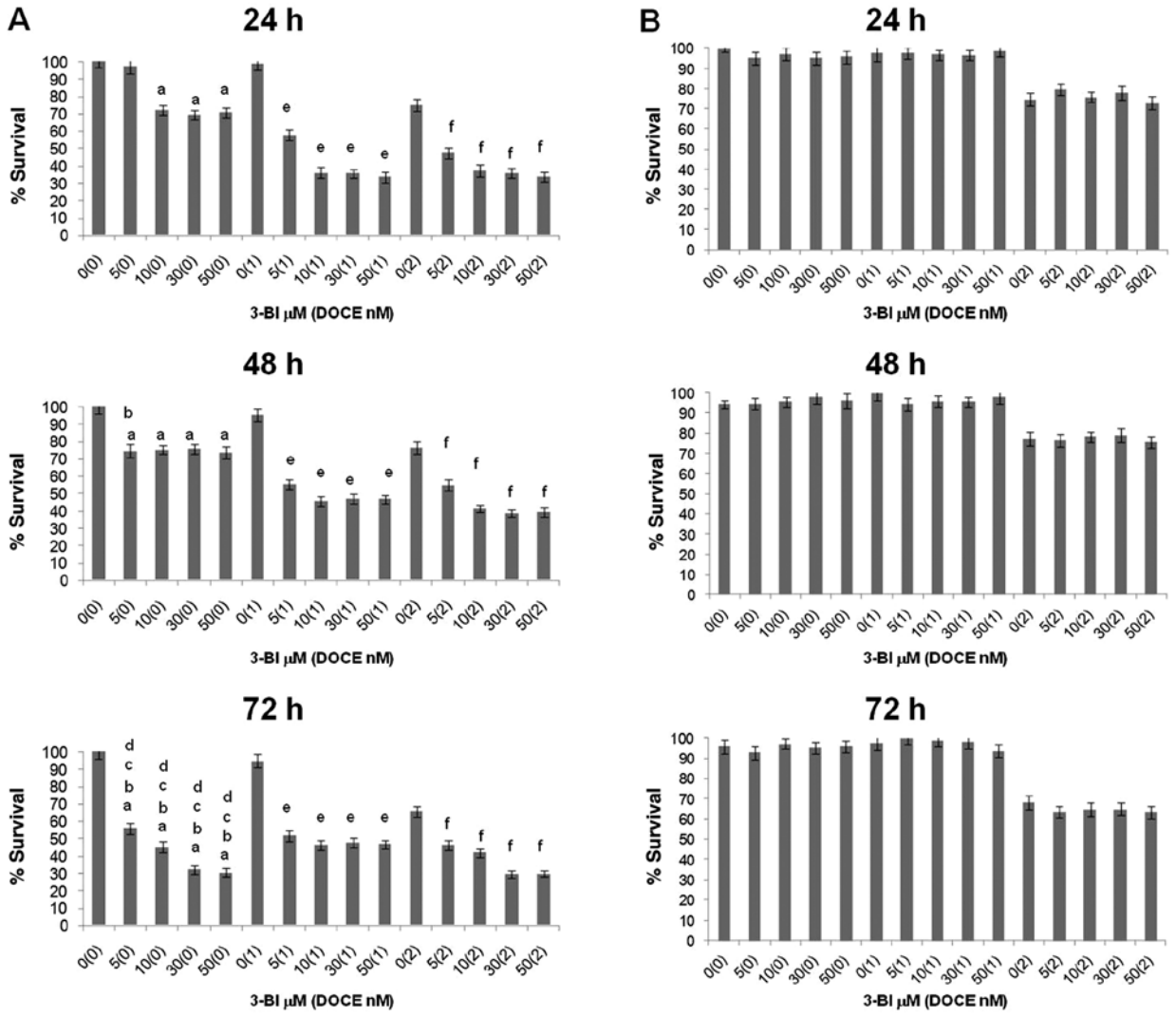

Figure 3. Time-course and dose-response of 3-BI treatment effects on proliferation of (A) PC-3 and (B) DU145 cells, as determined by trypan blue dye exclusion assay. Columns, mean; bars, standard deviation (n=3). [a]P<0.05, 3-BI treatment alone compared with control treatment; [b]P<0.05, 3-BI treatment alone for 48 or 72 h compared with 3-BI treatment alone for 24 h; [c]P<0.05, 3-BI treatment alone for 72 h compared with 3-BI treatment alone for 48 h; [d]3-BI treatment alone compared with 2 nM DOCE alone; [e]combination treatment (3-BI + 1 nM DOCE) compared with 1 nM DOCE alone; [f]combination treatment (3-BI + 2 nM DOCE) compared with 2 nM DOCE alone (one-way analysis of variance and Tukey's post hoc). 3-BI, 3-butenyl isothiocyanate; DOCE, docetaxel.

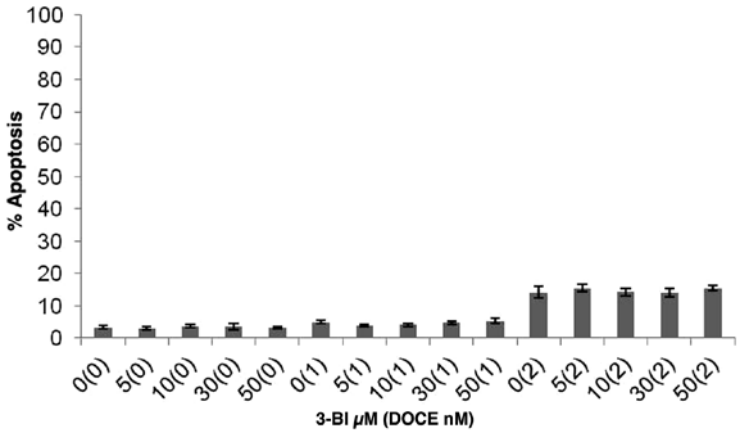

Figure 4. Quantitation of PC-3 apoptotic cells following 72 h of treatment with 3-BI and/or DOCE. No significant differences were found compared with the control treatment. 3-BI, 3-butenyl isothiocyanate; DOCE, docetaxel.

proteins could acquire oncogenic properties that are different to those resulting from loss of wild-type tumor-suppressing p53 function and that could promote proliferation, survival, migration, invasion and metastasis. STAT3 is an oncogenic

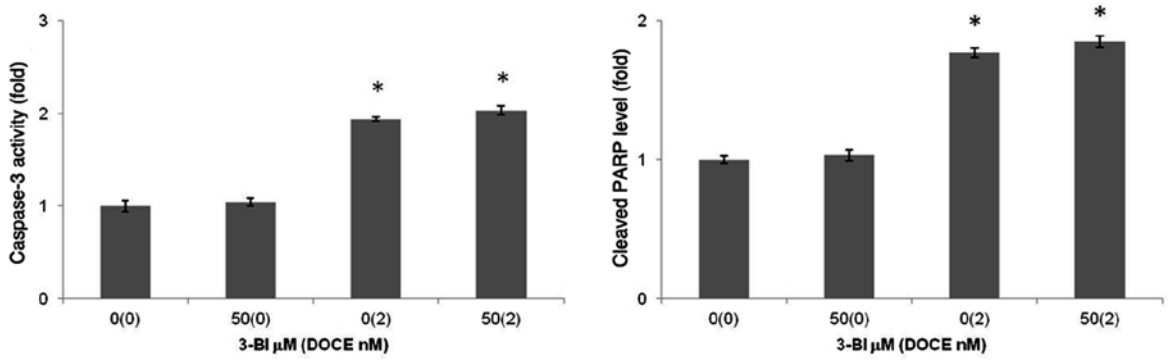

Figure 5. Active caspase-3 activity and cleaved PARP level in PC-3 cells following 72 h of treatment with dimethyl sulfoxide, 3-BI and/or DOCE. *P<0.05 compared with control treatment. 3-BI, 3-butenyl isothiocyanate; DOCE, docetaxel; PARP, poly(ADP-ribose) polymerase.

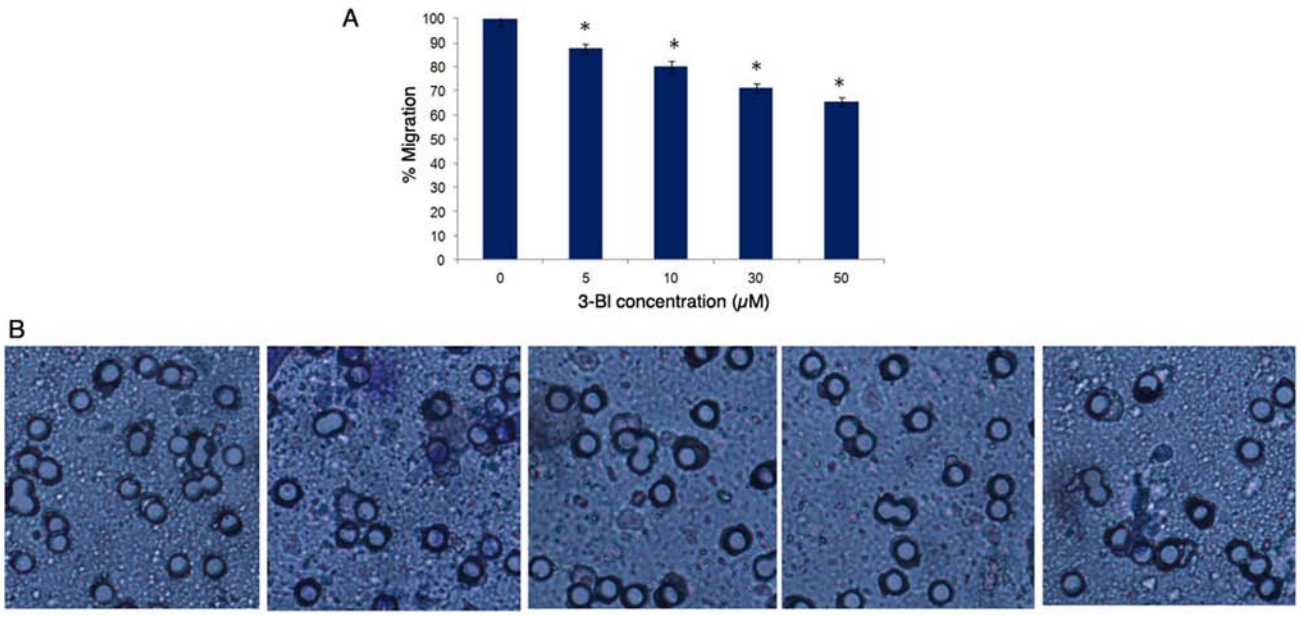

Figure 6. Effects of 3-BI on PC-3 cell migration. (A) 3-BI inhibited PC-3 cell migration. Experiments were repeated 3 times. *P<0.05 compared with control treatment. (B) Representative microphotographs of PC-3 cells (x40 magnification) that migrated through the chamber membranes following treatment with dimethyl sulfoxide (control) or 5, 10, 30 and 50 μM 3-BI (panels from left to right). 3-BI, 3-butenyl isothiocyanate.

transcription factor implicated not only in development, but also in PC progression, and is constitutively active in the DU145 cell line, but not in the PC-3 cell line. PTEN, a tumor suppressor gene, is mutated or lost in 50-80% of primary PC cases, with the complete loss associated with aggressiveness and metastasis. Likewise, Akt is constitutively active in the PC-3 cell line, but not in the DU145 cell line, due to the lack and the robust expression of PTEN, respectively (5). Nevertheless, the PTEN loss-induced Akt pathway promotes cell survival, proliferation and metastasis in DU145 and PC-3 cells (64).

With regard to the ability of PC to metastasize, the present study showed that the migration of PC-3 cells is reduced significantly by 3-BI treatment. Migration involves changes in the motility and adhesion of PC cells that are associated with metastasis (65). Inhibition of E-cadherin expression is considered as one of the main indicators of the epithelial/mesenchymal phenotype transition of PC, diminishing cell-cell adhesion and facilitating metastasis (66). Certain ITCs can modulate the expression of a variety of metastasis target genes. The regulation of migration is quite complex and is mediated by Bcl-2, p53, Akt, PTEN, E-cadherin and MMP, among others (33). Further studies are required to solve the differential 3-BI-induced response in PC-3 and DU145 cells. This could be of note, as anti-migratory drugs such as tyrosine kinase inhibitors are commonly used in CRPC (14,15).

In addition, visualization of cytopathic changes by confocal microscopy could be a sensitive indicator for malignant progression and for the anti-invasive and cytotoxic drug effects. In particular, the reduction of cell size and the loss of cell appendages are considered as indicators of growth inhibitory and anti-invasive effects of anticancer compounds in PC-3 cells (67). In the present study, it was found that 3-BI with or without DOCE exhibits cytopathic effects in PC-3 cells, manifested by cell morphology changes and actin skeleton reorganization. Härmä *et al* (67) showed that cell migration can be more rapid in elongated bipolar PC-3 cells in comparison to that in rounded cells. The changes in cell morphology and motility could be driven by organization of the actin cytoskeleton, likely

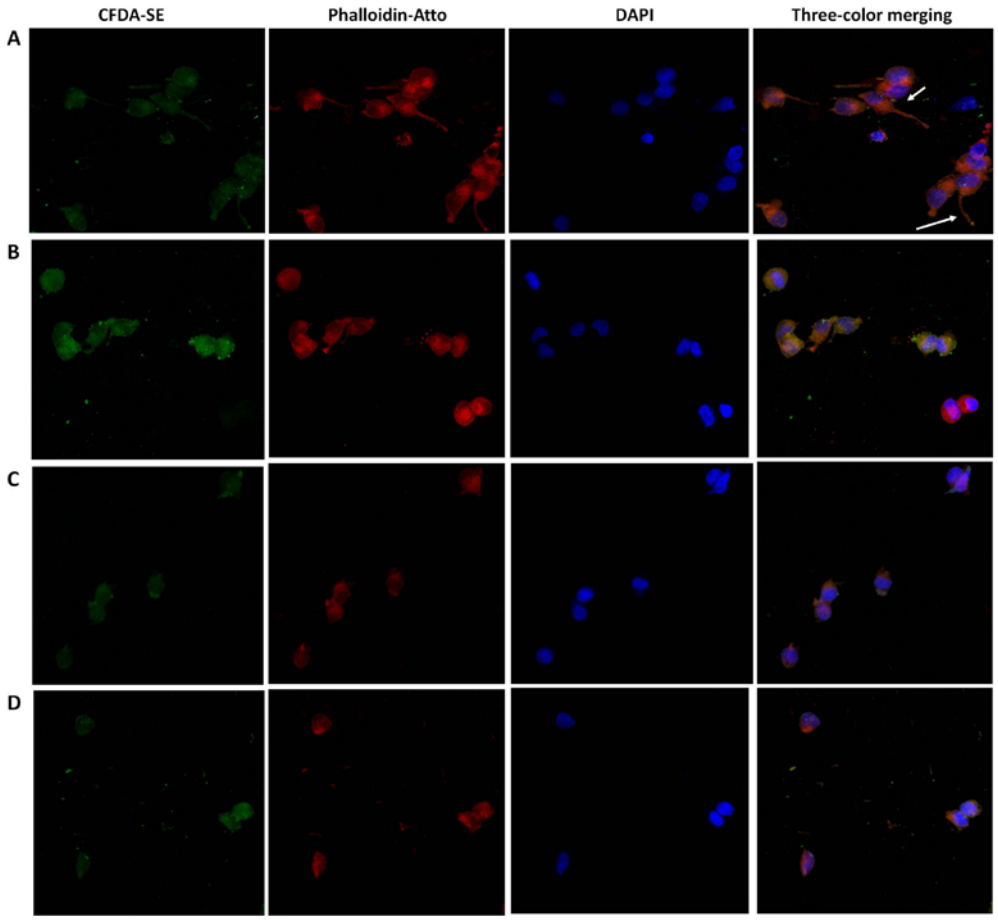

Figure 7. Scanning confocal microscopy of PC-3 cells. The images depict green (CFDA-SE), red (Phalloidin-Atto 647N) and blue (DAPI) stained cells, and a merger of the three channels. (A) Untreated PC-3 cells showed a more elongated phenotype with cytoplasmic projections (arrows) as compared with treated PC-3 cells. The cells treated with (B) 3-BI or co-treated with 3-BI and (C) 1 nM or (D) 2 nM showed predominantly a rounded shape phenotype with DNA condensation. DAPI, 4',6-diamidino-2-phenylindole; CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; 3-BI, 3-butenyl isothiocyanate.

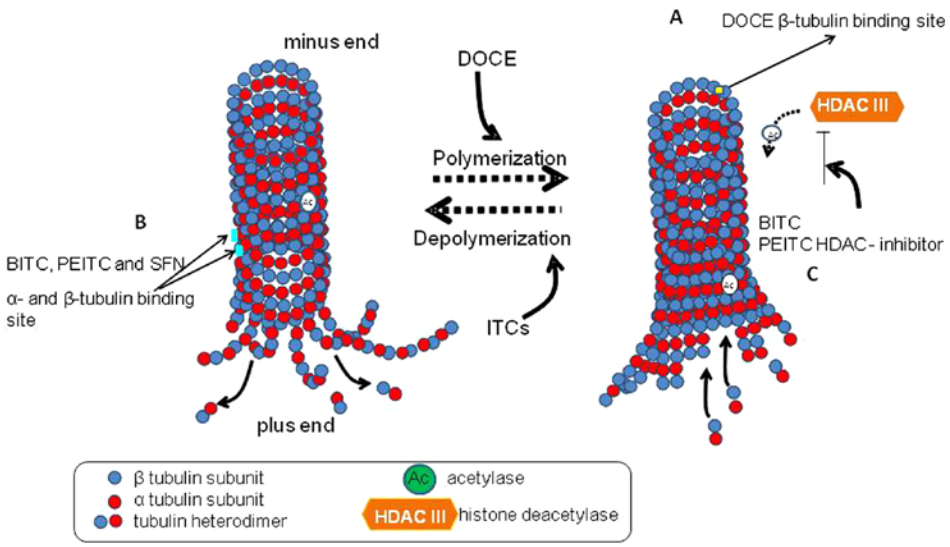

Figure 8. Possible mechanisms of 3-butenyl isothiocyanate microtubule-targeting effects. Tubulin-containing microtubules serve a pivotal role in different cellular processes, including cell division, motility and intracellular trafficking. Microtubules are dynamic polymers, formed by α and β tubulin heterodimers, which undergo periods of polymerization and depolymerization. The dynamic process of polymerization and depolymerization is required to form mitotic spindles that are necessary to segregate the replicated chromosomes of the daughter cells. (A) DOCE binds to β-tubulin and stabilizes it. Subsequent to the union, the microtubules cannot be disassembled, so the static polymerization arrests the normal mitotic process in the G2M phase and finally induces cell death (49). (B) ITCs can bind to α and β tubulin, inhibiting microtubule polymerization *in vitro* (human lung cancer A549 cells, human cervix cancer HeLa cells and mouse mammary carcinoma F3II cells) (50,51,67), causing depolymerization (51,67) or (C) inhibiting HDAC with increasing microtubule acetylation (51). The degree of binding affinity and microtubule polymerization inhibition is conditioned by the ITC structure (51,52). DOCE, docetaxel; HDAC, histone deacetylase; ITC, isothiocyanate; BITC, benzyl ITC; PEITC, phenethyl ITC; SFN, sulphynilbutyl-ITC.

mediated by putative motility modifiers (68,69). A detailed study of these mechanisms requires further study and future research.

In conclusion, DOCE-based combination therapy was shown to increase the number and/or the duration of the therapeutic response in the present study. 3-BI demonstrated anti-proliferative and anti-migratory effects. Furthermore, 3-BI showed a synergic effect with DOCE without toxic effects on prostatic normal cells. According to these findings, 3-BI could be clinically attractive as an adjuvant to conventional chemotherapy with DOCE. The present results are promising, but further studies are required to determine the molecular mechanisms involved in 3-BI-induced cytotoxicity DOCE synergism.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CG contributed substantially to the conception, design, drafting, critical revision and supervision of the submitted manuscript. PS contributed substantially to the analysis and interpretation of data, drafting, and technical and administrative support for the submitted manuscript. MEC and PV contributed substantially to the acquisition of data, drafting and administrative support for the submitted manuscript. SN, MJNI and MFG contributed substantially to the analysis and interpretation of data, design, drafting, critical revision and supervision of the submitted manuscript. All authors approved the final version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

Authors declare that they have no competing interests.

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