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Relative Cytotoxic Potencies and Cell Death Mechanisms of α_1-Adrenoceptor Antagonists in Prostate Cancer Cell Lines

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BACKGROUND. Some α_1-adrenoceptor antagonists possess anti-cancer actions that are independent of α_1-adrenoceptors and the aim of these studies was to assess the relative cytotoxic potencies of α_1-adrenoceptor antagonists and the mechanisms involved in these actions.
METHODS. PC-3 and LNCap human prostate cancer cells were exposed to α_1-adrenoceptor antagonists (0.01–100 μM) and cell survival assessed after 24–72 hr. The levels of apoptosis, autophagy and stress related proteins were also determined.
RESULTS. The relative cytotoxic potency order was prazosin = doxazosin > terazosin = silodosin = alfuzosin > tamsulosin on both cell types, but LNCaP cells were significantly more sensitive to these effects than PC-3 cells. Prazosin and doxazosin increased levels of apoptotsis and autophagy in both cell lines, and activated EphA2 receptors in PC-3 cells. Autophagy contributed to survival of LNCaP, but promoted cell death in PC-3 cells. Treatment with prazosin (30 μM) altered the expression of several cell stress-related proteins: elevating phospho-p38α and reducing S6 kinase in both cell lines. Surprisingly some proteins were differentially affected in the two prostate cancer cell lines: Akt and p27 increasing and HIF-1α decreasing in LNCap cells but not PC-3, while ADAMTS1 was increased in PC-3 cells only.
CONCLUSIONS. Prazosin and doxazosin demonstrated cytotoxic actions on both castration-resistant PC-3 and androgen-sensitive LNCap prostate cancer cells. The mechanisms involved included changes in a number of proliferation and apoptosis regulatory proteins. The role of autophagy depended on the cell type, but contributed to cell death in PC3 cells. *Prostate 76:757–766, 2016.* © 2016 Wiley Periodicals, Inc.

KEY WORDS: **α_1-adrenoceptor antagonists; PC-3; LNCaP; autophagy; apoptosis**

INTRODUCTION

Alpha$_1$-adrenoceptor antagonists are the first line treatment for the lower urinary tract symptoms associated with benign prostatic hyperplasia, where they reduce the dynamic component of bladder outlet obstruction [1]. However in vitro, some of these antagonists, at concentrations above those required for α_1-adrenoceptor antagonism, have been shown to exert cytotoxic effects on prostate cancer cell lines. Thus in vitro, doxazosin has been shown to induce apoptosis and cell death in LNCap, PC3 and DU-147 prostate cancer cell [2–6] via a mechanism that is independent of the α_1-adrenoceptor [7,8]. These potential anti-tumor

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actions have been demonstrated for doxazosin and terazosin, two quinazoline based antagonists, but they have not been observed with tamsulosin, which has a phenylethanolamine structure [7,8]. This led to the suggestion that it is the quinazoline structure that is responsible for the cytotoxic actions of these agents [9], a conclusion supported by the demonstration that prazosin, another quinazoline based antagonist, also has cytotoxic effects on PC3 prostate cancer cells [4]. Since a number of tyrosine kinase inhibitors developed as anti-tumor agents (e.g., Iressa) are also based on the quinazoline structure, it has been suggested that the mechanism involved in the cytotoxic actions of α_1-adrenoceptor antagonists involves an inhibition of tyrosine kinase receptors and doxazosin has been shown to inhibit phosphorylation of the EGF receptor in one breast cancer cell line [3]. An alternative mechanism proposed to explain the cytotoxic actions of these drugs involves an activation of erythropoietin-producing hepatocellular (Eph) receptors, a family of tyrosine kinases critically involved in the control of cell-cell interactions and tissue growth. Petty et al. [10] have reported that doxazosin is a potent agonist at this receptor, and activates apoptosis via EphA2-receptors. This action did not involve the quinazoline component of doxazosin.

In vivo studies in mice suggest that the cytotoxic effects observed with high concentrations of α_1-adrenoceptor antagonists in vitro, are observed at therapeutic doses in vivo. Treatment of mice with doxazosin increases apoptosis in the prostate [11] and a number of studies have shown that in patients with benign prostatic hyperplasia treatment with α_1-adrenoceptor antagonists results in elevated levels of apoptosis and reduced vascularity in prostatic tissues [8,12–14]. The clinical benefits of these effects have also been shown in retrospective studies where treatment is associated with a significantly reduced risk of developing prostate cancer [15] compared to unexposed men.

The anticancer effects of these antagonists are known to occur independent of α_1-adrenoceptors and are mediated primarily via apoptotic cell death mechanisms without effects on cell proliferation [7,16]. However, the full spectrum of cell death mechanisms remains to be fully elucidated. In addition to apoptosis, autophagy may contribute to α_1-adrenoceptor antagonist-mediated programmed cell death. Autophagy is a catabolic process by which a cell recycles damaged or unneeded cytosolic components, and is well known to play differential roles to promote prostate cancer survival or death in a cell type- and stimuli-dependent manner [17–19]. Interestingly, one study demonstrated the onset of autophagic cell death following prazosin exposure in rodent myocytes [20], suggesting a potential role for autophagy in α_1-adrenoceptor antagonist toxicity.

The aim of the current study was to investigate the relative cytotoxic potencies of a range of α_1-adrenoceptor antagonists currently in clinical use. These included the quinazolines prazosin, doxazosin, terazosin and the non-quinazolines tamsulosin, silodosin and alfuzosin. The relative cytotoxic potencies of each antagonist were determined on androgen-sensitive LNCaP and the castration-resistant PC-3 prostate cancer cell lines. In addition, cell stress-related proteins were assessed to investigate the possible intracellular pathways involved in cytotoxicity in the two prostate cancer cell lines. In particular the role of autophagy and EphA2 receptors was also investigated.

MATERIALS AND METHODS

Cell Culture

Human prostate cancer PC-3 and LnCAP cell lines were purchased from ATCC (Manassas). PC-3 cells were cultured in Ham's F-12K medium (Life Technologies, Mulgrave, Australia) containing L-glutamine and supplemented with 10% fetal bovine serum (FBS) and 50 mg gentamicin. LnCAP cells were cultured in RMPI 1640 (Life Technologies) containing L-glutamine and supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 10% FBS, and 50 mg gentamicin. Cell were grown and maintained in a humidified 37°C incubator with 5% CO_2/95% air. LnCAP cell culture flasks and plates were pre-coated with sterile-filtered 0.01% poly-L-lysine solution (Sigma–Aldrich, St. Louis). Unless otherwise stated, all experiments were conducted in complete culture medium.

Cell Viability

PC-3 and LnCap cells were seeded in 96-well plates at a density of 3×10^{-4} and 5×10^{-4} cells/ml, respectively, in 100 μl of culture medium. PC-3 and LNCaP cells were incubated for either 24 hr (PC-3) or 48 hr (LNCap), to allow for attachment. Cells were treated with a range of concentrations (0.01–100 μM) of α_1-drenoceptor antagonists (prazosin from Sigma–Aldrich; doxazosin, tamsulosin and alfuzosin from Selleck Chemicals, Huston; silodosin from LKT Laboratories, St. Paul) or vehicle for 24, 48, or 72 hr. Cell proliferation was determined by cell-mediated reduction of non-fluorescent resazurin to the non-toxic, highly fluorescent by-product, resorufin, as previously described [21,22]. Briefly, culture medium above the cells was replaced with fresh complete culture medium containing 44 μM resazurin and incubated at 37°C for 1.5–3 hr depending on cell type and density. Resorufin fluorescence (ex: 530 nm, em: 590 nm) was read using a

Modulus Microplate fluorescent plate reader (Promega, Madison). The concentration of drug that resulted in a 50% reduction in cell survival (IC_{50} with 95% confidence limits) was determined for all drugs, time points and cell lines. Preliminary experiments showed that resazurin data corresponded well to results obtained using the CyQuant® NF cell proliferation assay kit (Life Technologies, data not shown).

To investigate the contribution of autophagy to antagonist-mediated toxicity, cells were pre-treated with the autophagy inhibitor 3-methyladenine (3-MA, 5 mM) for 30 min [23,24], prior to co-treatment with prazosin or doxazosin (10–30 μM) for 24 hr.

Caspase-3 Activity Assay

Caspase-3 activity was used as an index of apoptosis. PC-3 and LnCAP cells were treated with doxazosin, prazosin (10–30 μM), terazosin, tamsulosin, silodosin, alfuzosin (30 μM) or vehicle for 24 hr. To evaluated the effects of the autophagy inhibitor 3-MA on caspase-3 activity, cells were pretreated with 3-MA (5 mM) for 30 min prior to co-treatment with either doxazosin or prazosin (10–30 μM) for 24 hr. Caspase-3 activity was determined using a caspase-3 fluorescence assay kit (Cat #: 10009135, Cayman Chemicals, Ann Arbor). Caspase-3 activity was normalized for cell density (resazurin data) and expressed as a fraction of untreated vehicle control.

Autophagy Vesicle Detection

The specific autophagy-related vesicle fluorescent probe, CytoID® (Enzo Life Sciences, Farmingdale) was used to quantify autophagic vesicles after treating cells with antagonists (10 or 30 μM) or vehicle for 24 hr. Cyto-ID® (ex: 480 nm, em: 530 nm) and Hoechst 33342 (ex: 340 nm, em: 480 nm) fluorescence was read using a Modulus microplate fluorescent plate reader (Promega). Cyto-ID® fluorescence values were normalized to Hoechst 33342 fluorescence and expressed as a fraction of untreated vehicle control. Fluorscent microscopy was conducted using Evos® FL Cell Imaging System (Life Technologies). The effects of the autophagy inhibitor 3-methyladenine (3-MA 5 mM) [23] for 30 min, prior to co-treatment with prazosin or doxazosin (10–30 μM) for 24 hr was also examined.

Human Cell Stress Array

Protein expression following 24 hr treatment of PC-3 and LNCaP cells with 30 μM tamsulosin, doxazosin, or prazosin was determined using a Human Cell Stress Array Kit (R&D Systems, Minneapolis). The relative expression of 26 human cell stress proteins were assessed: ADAMTS1, Bcl-2, carbonic anhydrase IX, cited-2, COX-2, cytochrome c, DKK-4, FABP1/L-FABP, HIF-1apha, HIF-2alpha, phospho-HSP27, HSp60, HSP70, IDO, phosphor-JNK Pan, NFkappaB1, p21/CIP1, p27/Kip1, phosphor-p38alpha (T180/Y182), phosphor-p53 (S46), PON1, PON2, PON3, thioredoxin-1, SIRT2, and SOD2. Following treatment, cells were lysed in buffer supplemented with 1X protease inhibitor cocktail and 1 mM PMSF (Sigma–Aldrich). Protein content was quantified using Bradford's reagent (Sigma–Aldrich). Membranes were blocked and incubated with 350 μg of cell lysate overnight (16 hr) on a platform rocker at 4°C. Relative membrane-bound protein was determined by chemiluminecence and pixel density quantified using ImageJ (version 1.48 for Mac OS X) software. Protein signals were normalized to the positive control and data expressed as the mean (±sem).

Human Phospho-EphA2 Detection

Changes in phosphorylation (activation) of EphA2 were measured using a Human Phospho-EphA2 DuoSet IC ELISA kit (R&D Systems). Cells were lysed using kit recommended lysis buffer supplemented with protease inhibitor cocktail and 1 mM PMSF (Sigma–Aldrich). A protein concentration of 2 mg/ml was used of each sample in duplicate. Following plate preparation, samples were incubated for 2 hr at room temperature in capture antibody coated high bind 96-well plate. Then the wells were washed, and HRP-linked detection antibody was added to wells and incubated for an additional 2 hr at room temperature. Wells were washed and substrate solution (Cat. No. DY999, R&D Systems) was added to wells and incubated at room temperature. The reaction was stopped using 2N H_2SO_4 (Cat No. DY994; R&D Systems), and absorbance (450 nm) was read using a Modulus Microplate multimode plate reader (Promega).

Statistical Analysis

Data were analyzed and presented using Graphpad Prism version 6. One-way ANOVA with Dunnett's post hoc test was used for comparisons between control and treated values. For comparisons of multiple drugs or time points, a two-way ANOVA with Tukey-Kramer post hoc test was used. Statistical significance was defined as $P < 0.05$.

RESULTS

Relative Cytotoxic Potency

In both LNCap and PC-3 cells, prazosin and doxazosin exhibited the greatest cytotoxic potency

and this was observed at all three time-points (Fig. 1, Table I). On PC-3 cells doxazosin and prazosin were equipotent at all time points, while on LNCap cells prazosin was more potent than doxazosin at all time points but the difference was only statistically significant at 48 hr ($P < 0.01$). Maximum effects on cell survival were also similar, with both drugs completely abolishing survival at 100 μM after 72 hr. The main difference between the actions of prazosin and doxazosin appeared to be the time course for their effects on cell viability (Fig. 1B). In particular, the time for a 50% reduction in viable LNCap cells with prazosin (30 μM) was half that observed for doxazosin (30 μM) (Fig. 1B). Also, at all time points and for both drugs. LNCap were more sensitive to the cytotoxic actions than PC-3 and this different was statistically significant for prazosin at all time-points and for doxazosin at 48 hr (Table I).

The other α_1-adrenoceptor antagonists were less toxic to the prostate cancer cells and even the highest concentration (100 μM) of terazosin, silodosin and alfuzosin, resulted in only modest reductions of cell survival. These effects were greatest at 72 hr, where the three antagonists reduced survival by $42.2 \pm 2.2\%$ ($P < 0.001$), $25.5 \pm 1.4\%$ ($P < 0.001$), and $38.7 \pm 2.7\%$ ($P < 0.001$), respectively, in PC-3 cells and by $30.0 \pm 2.6\%$ ($P < 0.001$), $26.6 \pm 4.6\%$ ($P < 0.01$), and $14.0 \pm 2.8\%$ ($P > 0.05$), respectively, in LNCap cells. In contrast, tamsulosin and silodosin at concentrations up to 100 μM failed to have any significant effect on cell viability even after 72 hr of continuous treatment. For all time points and in both cell lines, the relative potency order for these antagonists was found to be: prazosin/doxazosin > terazosin = alfuzosin > solifenacin = tamsulosin (see Table I).

Apoptosis

Basal levels of caspase-3 activity was greater ($P < 0.001$) in PC-3 than LNCaP cells after normalizing for cell number (data not shown). Doxazosin and prazosin treatment resulted in concentration-dependent increases in caspase-3 activity in both cell lines (Fig. 2). In PC-3 cells the activation of caspase-3 was similar for prazosin and doxazosin. However, in LNCap cells, caspase-3 activation was greater than in PC-3 cells and this was particularly obvious for prazosin (30 μM) where activation was increased sixfold in LNCap, but only twofold in PC-3 cells (Fig. 2C and D, $P < 0.05$). In contrast, no significant changes were observed in caspase-3 activation after treatment with the other antagonists at a concentration of 30 μM (data not shown). In both cell lines, proteins that regulate mitochondrial-mediated apoptosis (cytochrome c and Bcl-2) remained unchanged following treatment (Supplementary Data).

Autophagy

Basal autophagic vesicle formation was greater in LNCaP cells than PC-3 cells (Fig. 2). In both cell lines, doxazosin treatment (10–30 μM) resulted in significant

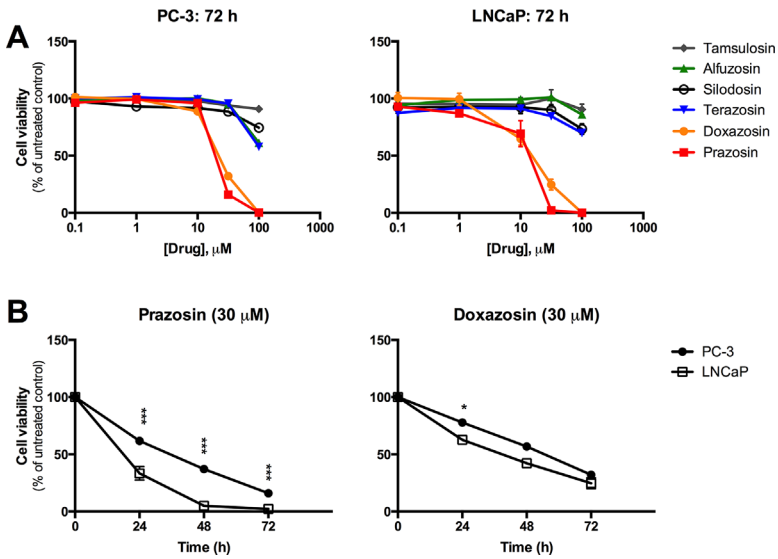

Fig. 1. PC-3 and LNCaP cell viability following treatment with α_1-adrenoceptor antagonists. (**A**) Cells were treated with drug (0.01–100 μM) for 72 hr and cell viability determined using the resazurin reduction assay. (**B**) Effect of treatment duration on PC-3 and LNCap cell survival following treatment with doxazosin (30 μM) or prazosin (30 μM). Survival is expressed as the percentage values of untreated vehicle control and values are the mean (±sem) of six independent determinations each conducted in triplicate. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to corresponding values for LNCap cells.

TABLE I. Cytotoxic Potencies (IC50) for Prazosin and Doxazosin on PC-3 and LNCap Cells

	IC_{50} (μM)		
	24 hr	48 hr	72 hr
Prazosin			
PC-3	55.3 (47.8–64.1)	26.8 (25.5–28.2)	21.3 (19.5–23.3)
LNCaP	22.0 (18.8–25.8)***	14.3 (11.6–17.5)***	13.0 (8.6–19.6)**
Doxazosin			
PC-3	46.8 (42.5–51.5)	34.1 (32.0–36.4)	23.3 (21.0–25.7)
LNCaP	35.4 (30.6–41.0)	23.4 (20.3–27.0)* ##	17.2 (10.9–27.1)

Mean values ($n \leq 5$) with 95% confidence limits are shown for cells treated with drug for 24, 48, or 72 hr. Potency increased along with treatment duration $^{*}P < 0.05$ compared to 24 hr treatment. Prazosin was more potent than doxazosin ($^{\#}p < 0{,}05$ compared to corresponding times for PC-3 cells). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for comparisons between PC-3 and LNCaP cells for indicated drug and treatment time. $^{\#\#}P < 0.01$ for comparisons between prazosin and doxazosin for indicated cell line and treatment time.

increases in autophagy as did prazosin treatment in PC-3 cells. No significant change in the presence of autophagic vesicles were observed following treatment with the other α_1-adrenoceptor antagonists (30 μM, 24 hr) in either cell line (tamsulosin data shown in Fig. 2). To investigate whether doxazosin and prazosin-induced autophagy may be contributing to cell survival or to cell death, cells were pretreated for 30 min with the autophagy inhibitor 3-MA (5 mM) prior to concurrent treatment with doxazosin or prazosin (10–30 μM) for 24 hr. Inhibition of autophagy with 3-MA without antagonists present, significantly reduced LNCaP cell survival by $41.1 \pm 1.1\%$ ($P < 0.001$), but it had no effect on PC-3 survival (Fig. 3). In LNCap cells, the effects of the autophagy inhibitor significantly enhanced the cytotoxic effects of both α_1-adrenoceptor antagonists. Treatment with 3-MA was also accompanied by an increase in caspase-3 activity, although this was only statistically significant for the higher concentration of prazosin.

In PC-3 cells inhibition of autophagic processes with 3-MA had only a minor effect. Neither concentration of doxazosin (10 and 30 μM), nor the lower concentration of prazosin (10 μM) had any effect on caspase-3 activity or cell viability (Fig. 3). However, 3-MA had some effect on cellular responses to the higher concentration of prazosin (30 μM), with caspase-3 activity being reduced (20.7%, $P < 0.001$) and cell survival slightly increased (12.0%, $P < 0.01$) following inhibition of autophagy.

Expression of Cell Stress-Related Proteins

In both cell lines, treatment of the cells with α_1-adrenoceptor antagonists (30 μM) resulted in a number of changes to stress proteins (Fig. 4). In both cell lines S6 MAP kinase was significantly reduced and phopho-p38 was elevated after treatment (Fig. 4), while some other proteins involved in cell growth and proliferation (EGFR, ERK1/2) were not altered in either cell line following treatment with the α_1-adrenoceptor antagonists. One interesting feature was the finding that some proteins were differentially affected in the two prostate cancer cell lines: Akt and p27 increasing and HIF-1α decreasing in LNCap cells only while ADAMTS1 was increased in PC-3 cells only.

Activation of EphA2

In PC-3 cells, basal EphA2 phosphorylation (activation) was low, but 1 hr after treatment with doxazosin (30 μM) or prazosin (30 μM) in PC-3 cells, levels of phosphorylated EphA2 were greatly increased by 7,150-fold and 575-fold, respectively. However, following 2 hr treatment with doxazosin, EphA2 activation had dropped back by 87.7% ($P < 0.001$), whereas EphA2 activation remained unchanged between 1 and 2 hr following prazosin treatment of cells ($P > 0.05$). In contrast, basal levels of EphA2 in LNCaP cells were lower than PC-3 cells (fivefold lower) and treatment with either doxazosin or prazosin did not affect EphA2 phosphorylation status (Supplementary Data).

DISCUSSION

For decades the α_1-adrenoceptor antagonists have been used to treat the lower urinary tract symptoms associated with benign prostatic hyperplasia. A significant number of these drugs are now globally available for clinical use where they act by reducing prostatic contraction and relieving bladder outlet obstruction. However, some of these antagonists also exert cytotoxic effects at higher concentrations and an increase in apoptosis has been observed in prostatic tissues from

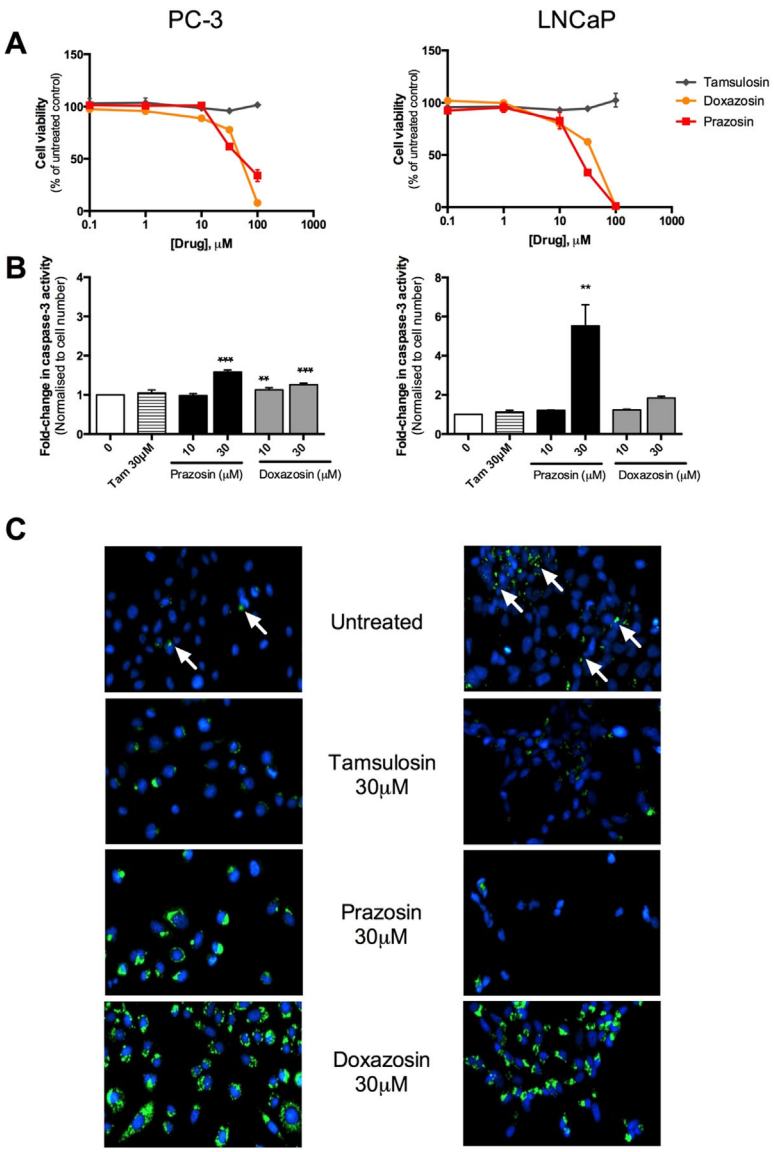

Fig. 2. Cytotoxicity, caspase-3 activity and autophagy induced by treatment of PC-3 and LNCap cells with prazosin or doxazosin for 24 hr. (**A**) Cell viability versus drug concentration. Cell viability is expressed as the percentage of untreated vehicle control and values are the mean (±sem) of six independent determinations each conducted in triplicate. (**B**) Changes in caspase-3 activity were quantified by fluorescence (ex: 480 nm, em: 530 nm) and normalized to cell number. Mean (±sem) values are expressed as a fraction of untreated control from three independent determinations each conducted in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control untreated cells. (**C**) The presence of autophagosomes was visualized by fluorescent microscopy.

patients treated with doxazosin for benign prostatic disease [25]. The current study is the first to compare the cytotoxic efficacies of the antagonists currently in clinical use for benign prostatic disease. The cytotoxic efficacy ranged from prazosin and doxazosin which induced total cell death, to silodosin, terazosin, and alfuzosin with some weak cytotoxic actions, and tamsulosin which was devoid of these actions. The relative potencies of these drugs was similar on both cell lines: prazosin = doxazosin > terazosin = alfuzosin = silodosin > tamsulosin. The antagonists were effective on both the androgen-sensitive LNCaP cells and the castration-resistant PC-3 cells, but overall LNCap cells were slightly more sensitive to the cytotoxic effects of these antagonists than PC-3 cells. Thus, after 72 hr of treatment prazosin and doxazosin abolished cell survival in both cell lines, but the effects were observed at a twofold lower concentration on LNCap cells than PC-3 cells. This small difference is unlikely to be clinically relevant and suggest that the drugs will be equally effective on androgen-sensitive and castration-resistant prostate cancer cells.

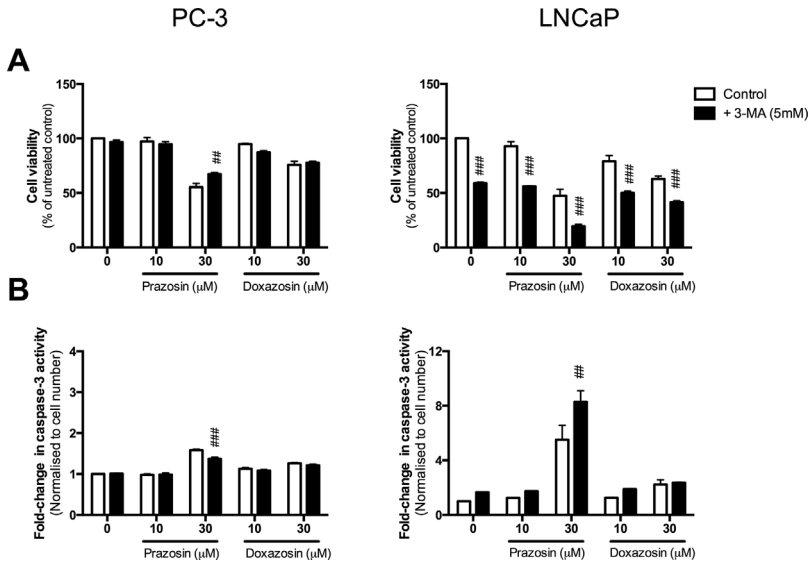

Fig. 3. Effect of the autophagy inhibitor 3-MA on cell survival and caspase-3 activity in (**A**) PC-3 and (**B**) LNCaP cells. Cells were treated with 3-MA (5 mM) prior to treatment with doxazosin, prazosin (10–30 μM) or vehicle for 24 hr. Resazurin reduction assay was used as measurement of cell viability and caspase-3 activity used as an index of apoptosis (measured using a commercially available kit, Cayman Chemicals). Mean values (±sem) are represented as the percentage (resazurin data) or fold-change (caspase-3 data, normalized for cell number) of untreated vehicle controls obtained from three independent determinations each conducted in triplicate. $^{\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to corresponding values in the absence of 3-MA.

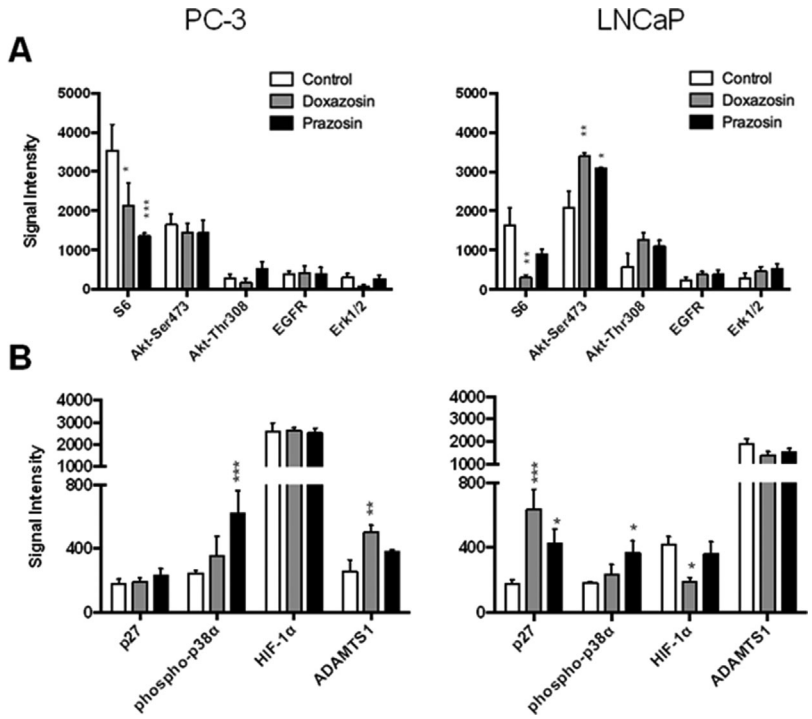

Fig. 4. Expression of receptor tyrosine kinases (RTK) (**A**) and cell stress-related proteins (**B**) in PC-3 and LNCaP cells following 24 hr treatment with antagonists (30 μM). The expression of cell stress-related proteins was determined using the human cell stress array kit (R&D Systems). The activation of receptor tyrosine kinases was determined using RTK signaling antibody array kit (Cell Signaling Technologies). Proteins were semi-quantified using pixel densitrometry (ImageJ software). Values are represented as the signal intensity. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control untreated cells.

The anti-cancer activity of doxazosin has largely been attributed to apoptotic mechanisms [7–9,11,16,25]. This was supported by the current data where cell death was associated with a concentration-related increase in caspase-3 activity for the two potent drugs prazosin and doxazosin, but no significant changes in apoptosis could be detected for the other antagonists. Furthermore, these drugs increased caspase-3 activity more in LNCap cells than PC-3 cells, again in line with a greater cytotoxic potency. It has previously been reported that treatment of benign and malignant prostate cells with doxazosin [2] and mesothelioma with prazosin [26] results in activation of caspase-8 which is indicative of a death receptor-mediated apoptotic pathway. Furthermore, proteins that regulate mitochondrial-mediated apoptosis, cytochrome c and Bcl-2, remained unchanged following treatment with doxazosin and prazosin in the present study, giving further evidence that these drugs induce apoptosis via a death receptor-mediated pathway.

Autophagy, a cellular process that can result in type II programmed cell death was also examined in our studies. Autophagy is a cellular process that operates to maintain cellular homeostasis through protein and organelle degradation. It has been implicated in a number of physiological and pathological conditions, including cancer [27] and drug resistance to chemotherapy where it has been shown to protect cells from the actions of cytotoxic agents [28]. Only two previous studies have examined the role of autophagy in the cytotoxic actions of α_1-adrenoceptor antagonists. In rodent myocytes, Yang et al. [20] reported autophagic cell death in response to prazosin exposure, but in PC-3 prostate cancer cells no autophagic activity was identified following acute doxazosin or prazosin treatment. Prazosin and doxazosin increased autophagy in PC-3 and LNCaP cells, however with paradoxical outcomes; contributing to antagonist cytotoxicity or survival mechanisms, respectively. In support of the current findings, previous separate studies demonstrated a similar cell-line specific role for autophagy in promoting prostate cancer cell survival or death [24], as well as autophagic mechanisms underlying prazosin-mediated cytotoxicity of rodent cardiac myoblast cells [20].

Interestingly, Akt, a signaling kinase with a wide array of substrates, including the indirect activation of mammalian target of rapamicin complex I (mTORC1) and subsequent inhibition of autophagy, was partially activated (phosphorylation at Ser473, but not Thr307) by doxazosin and prazosin in LNCaP cells. The conflicting findings in LNCaP cells between Akt activation and downstream autophagy suggest that mTORC1 inhibition, and subsequently autophagy, occurs downstream of Akt-mediated regulation of mTORC1. Likewise, in PC-3 cells, Akt phosphorylation remained unchanged, suggesting prazosin- and doxazosin-induced autophagy also occurs independently of Akt in these cells. The autophagic response of both cell lines following treatment with prazosin and doxazosin was further confirmed by the significant inhibition of S6 ribosomal kinase, a downstream target of mTORC1. However, the precise molecular mechanism of mTORC1 inhibition by prazosin and doxazosin, and thus induction of autophagy, remains unclear. A previous study demonstrating prazosin-induced autophagic cytotoxicity in rodent cardiac cells, reported increased activation of AMPK which correlated with decreased mTORC1 and S6 activation [20]. The AMPK pathway is known to crosstalk with the mTORC1 signaling pathway at several points [29], including the phosphorylation of raptor [30,31], thereby contributing to mTORC1 inhibition and the induction of autophagy. Together with the present findings, this suggests doxazosin and prazosin-mediated AMPK activation may contribute to antagonist-induced autophagy in prostate cancer cells and specifically contribute to PC-3 cytotoxicity.

The cell cycle inhibitor, p27(KIP1) was also found to be activated in response to prazosin and doxazosin, a finding previously reported for several cell lines [32,33]. Interestingly, AMPK activation and subsequent autophagy may also be associated with significant increases in p27 expression and the AMPK pathway has been reported to regulate p27-mediated inhibition of cell cycle progression and autophagy [34]. With increased p27 phosphorylation and previous reports of doxazosin and prazosin-mediated inhibition of Rb [35], it is possible the cytotoxic actions of these drugs occurs via the induction of autophagy and subsequent p27-mediated inhibition of the cell cycle. However, antagonist-induced autophagy has a paradoxical role by contributing to LNCaP survival and PC-3 cytotoxicity. Indeed, autophagy has been previously implicated as a downstream androgen-dependent survival mechanism, this mechanism protecting PC-3 cells against the cytotoxic actions of docetaxel and Src kinase inhibitors [18]. Unlike that observed with LNCap cells, the only significant effect observed with the autophagy inhibitor 3-MA on PC-3 cells was a small (15%) increase in caspase-3 activity and reduction in PC-3 cell survival at the highest prazosin concentration, but not at the lower concentration or when using doxazosin. Our data suggest that this mechanism also operates in LNCap cells to protect against the toxicity of α_1-adrenocptor antagonists. Furthermore the lack of this protective mechanism against α_1-adrenoceptor antagonist toxicity, despite previous reports of protection against

docetaxel and Src inhibitors, suggests a selective role for autophagy against specific toxic agents.

Several studies have suggested that these drugs are able to induce cell cycle arrest as a mechanism of cytotoxicity [4,32,36]. In separate studies, terazosin [31] and KMUP-1 [36], a piperazine-based α_1-adrenoceptor antagonist, up-regulated p27 following treatment in prostate cancer cells. Likewise, prazosin was previously found to halt cell cycle progression through the inactivation of the cell cycle promoter Cdk1 [4]. Since Cdk1 is negatively regulated by p27, these findings may in fact be due to the up-regulation of p27 following prazosin treatment. Together, these findings support the present study, suggesting that p27 is an important regulator in α_1-adrenoceptor antagonist-mediated toxicity of LNCaP cells.

Another mechanism suggested for α_1-adrenoceptor antagonist activation of apoptotic pathways has been EphA2 receptor activation [10]. These receptors are involved in cell-cell interactions and can inhibit tumor growth and angiogenesis [37] and it has been reported that doxazosin mediates its cytotoxic effects by acting as an agonist of the EphA2 receptor [10]. In the present study ADAMTS, a metallinoproteinase responsible for shedding extracellular components of transmembrane proteins and playing diverse roles in cell-cell interactions, was increased in PC-3 cells. This is particularly interesting because an increase in the level of ADAMTS has previously been shown to activate EphA2 receptors [37]. The present results suggest that the effects reported previously may be mediated indirectly via an activation of ADAMTS. Thus prazosin and doxazosin may induce apoptosis via different mechanisms; cleaving of EphA receptors (ADAMTS) in PC-3 cells and inhibition of CdK (p27) in LNCaps.

In the present study, prazosin treatment also increased phosphorylation of p38α in LNCaP cells. p38α (MAPK14) is a member of the mitogen-activated kinase superfamily and its activation promotes cell proliferation, differentiation, migration and death [38], suggesting this kinase may be partially responsible for prazosin-induced cytotoxicity in these cells. These findings are supported by previous work in cardiomyocytes, which demonstrated p38-dependent apoptosis following treatment with doxazosin [20].

CONCLUSION

In conclusion, α_1-adrenoceptor antagonists have cytotoxic actions on prostate cancer cells, with doxazosin and prazosin being the most potent. Consistent with previous findings, doxazosin and prazosin induce prostate cancer apoptosis in both AR-positive LNCaP and AR-negative PC-3 prostate cancer cell lines. A number of cellular pathways were affected by these drugs and their actions may include EPhA2 as an initial step. Furthermore this is also the first report that autophagy may contribute to antagonist-mediated cytotoxicity in PC-3 cells.

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