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Growth Inhibitory Effect of Doxazosin on Prostate and Bladder Cancer Cells. Is the Serotonin Receptor Pathway Involved?

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Abstract. *Doxazosin, an alpha1-adrenoceptor antagonist, is used for the treatment of benign prostatic hyperplasia (BPH) and hypertension. Alpha-adrenoceptor antagonists also inhibit growth and induce apoptosis in malignant prostatic cells. The apoptotic activity is independent of their capacity to antagonize alpha-adrenoceptors. The effect of doxazosin on the growth of prostate and bladder cancer cell lines was assessed and whether the growth inhibitory effect of doxazosin on prostate cancer cells is serotonin (5-hydroxtryptamine; 5HT)-dependent was investigated. Materials and Methods: PC3 (androgen-independent prostate cancer) and HT1376 (grade III transitional cell carcinoma) cells were plated. The cells were incubated with doxazosin. After 72 h, cell viability was assessed (crystal violet assay). Studies were also performed after incubating the PC3 cells with 5HT or $5HT_{1B}$ agonists for a short duration, followed by the addition of doxazosin. Cell viability was assessed at 72 h. Results: Doxazosin caused a dose-dependent inhibition of PC3 and HT1376 cell growth with a maximum inhibition of 80% ($n=12$, $p<0.0001$) and 91% ($n=12$, $p<0.0001$), respectively, at a concentration of 10^{-4}M, at 72 h. Incubation of PC3 cells with 5HT or $5HT_{1B}$ agonist, followed by addition of doxazosin, increased the percent of viable cells as compared to when the cells were treated with doxazosin alone. Conclusion: Doxazosin significantly inhibited prostate (PC3) and bladder cancer (HT1376) cell growth. Furthermore, prior incubation of PC3 cells with 5HT or $5HT_{1B}$ agonist increased cell viability as compared to treatment with doxazosin alone. These findings may be related to the similarity between subtype 1 serotonin and adrenergic receptors. The effect of alpha1-adrenoceptor antagonists on tumour cell growth merits further investigation.*

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Prostate cancer is the second most common malignancy affecting men in Europe and the USA (1). One in twelve men over the age of 60 develops prostate cancer and this figure is expected to rise to three in twelve in the next 20 years (1). At the age of 50 about 15% of prostates contain islands of cancer and by 80 this figure rises to nearly 100% (2). For patients who have organ-confined disease, effective treatment options are available (surgery or radiotherapy). However, about 20% of the patients have evidence of metastases on presentation (3). The mainstay of treatment for these patients is androgen ablation therapy, however patients on this regime eventually relapse and develop an androgen-independent tumour (4-6). This aggressive stage of the disease carries a high morbidity and mortality.

Bladder cancer constitutes a significant proportion of the workload in urology, due to its high prevalence and recurrent nature (7). It is the fifth and fourth most common malignancy in Europe and the United States, respectively (7). It affects about 1 in 4000 people and accounts for 5% of all diagnosed cancers. The disease has a spectrum of clinical severity varying from superficial bladder cancer to muscle invasive or metastatic disease, which carries a poor prognosis (7). Because of this, one of the main thrusts of research is to prevent progression from superficial disease to muscle invasive and metastatic bladder cancer.

Alpha1-adrenoceptor antagonists may be quinazoline-based (*e.g.* doxazosin and terazosin) or sulfonamide-based (*e.g.* tamsulosin). Doxazosin is used for the treatment of benign prostatic hyperplasia (BPH), as it has an action in relaxing the musculature within the prostate gland and around the bladder neck (8, 9). It is also widely used for the treatment of hypertension (8).

Alpha-adrenoceptor antagonists, have been documented to inhibit growth and induce apoptosis in malignant

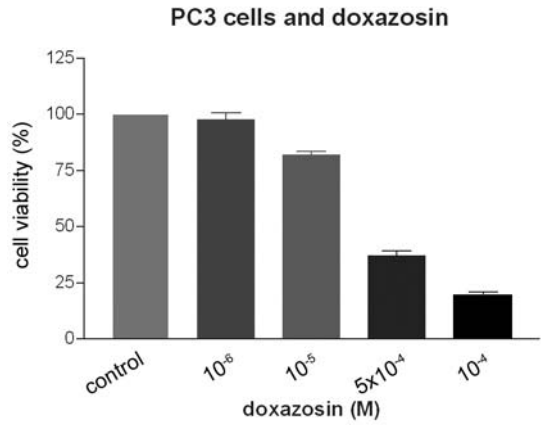

Figure 1. *Dose-dependent inhibition of PC3 (androgen-independent prostate cancer) cell growth by doxazosin with a maximum effect seen at a concentration of 10^{-4} M. The bars represent the standard error of the mean.*

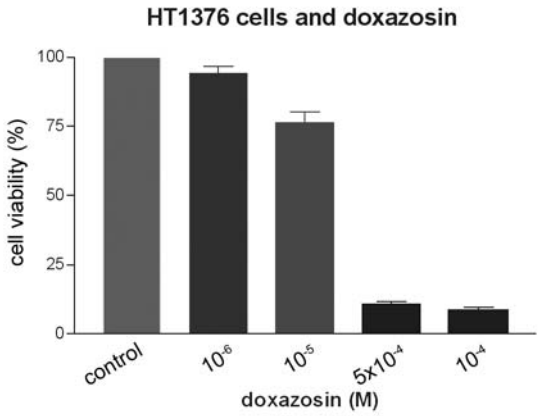

Figure 2. *Dose-dependent inhibition of HT1376 (grade III transitional cell carcinoma bladder) cell growth by doxazosin with a maximum effect seen at a concentration of 10^{-4} M. The bars represent the standard error of the mean.*

prostatic cells (10-26). The apoptotic activity of alpha1-adrenoceptor antagonists (doxazosin and terazosin) against prostate cancer cells is independent of: (a) their capacity to antagonize alpha-adrenoceptors, and (b) the hormone sensitivity status of the cells (10, 11, 17-20, 22). The search continues for the exact mechanism(s) involved.

Serotonin (5-hydroxytryptamine; 5HT) increases the proliferation of PC3 cells (27-29), and 5HT antagonists have a significant growth inhibitory effect on these cells (27,29). Doxazosin probably inhibits 5HT-induced platelet shape change *via* the $5HT_2$ receptor (8). Doxazosin also significantly inhibits 5HT-mediated contractions in the rabbit detrusor (9). This effect appears to be mainly mediated *via* $5HT_3$ receptor inhibition. Autoradiographic evidence suggests that doxazosin reduces 5HT binding in the rabbit detrusor (9). It is possible that the growth inhibitory effect of doxazosin on prostate and bladder cancer cells is mediated *via* the 5HT receptors.

The effect of doxazosin on PC3 (androgen-independent prostate cancer) and HT1376 (grade III transitional cell carcinoma) cell growth was assessed *in vitro*. The effect of 5HT on this process was also evaluated.

Materials and Methods

Two malignant cell lines were used: PC3 (passage 7), an androgen-independent prostate cancer cell line and HT1376 (passage 9), a grade III transitional cell carcinoma of the bladder cell line. Both were obtained from the (ATCC) American Type Culture Collection (Teddington, Middlesex, UK).

The PC3 cells were maintained in nutrient mixture F-12 Ham medium supplemented with 8% foetal bovine serum (FBS) and 1% antibiotic antimycotic solution. HT1376 cells were maintained in minimum essential medium Eagle supplemented with 8% FBS and 1% antibiotic antimycotic solution.

Both PC3 and HT1376 cells were incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Reagents. Doxazosin (alpha1-adrenoceptor antagonist) was obtained from Pfizer Ltd. (Tadworth, Surrey, UK). 5HT (serotonin creatinine sulphate complex) was purchased from Sigma-Aldrich Company Ltd. (Dorset, UK), whereas the $5HT_{1B}$ agonist (CP93129 dihydrochloride) was bought from Tocris Laboratories (Bristol, UK). The nutrient mixture F-12 Ham medium (for PC3 cells), minimum essential medium Eagle (for HT1376 cells), FBS, dimethyl sulfoxide (DMSO), MEM non essential amino acid solution and 1% antibiotic antimycotic solution were purchased from Sigma-Aldrich Company Ltd. Dulbecco's phosphate-buffered saline (PBS) was used for washing the cells and distilled water was used as a control.

In vitro proliferation assay. Cells were seeded in a 96-well plate, 5,000 cells per well in 100 μl serum-containing medium and were incubated at 37˚C. After 24 h, 10 μl of the serum-containing medium was removed and replaced with 10 μl of doxazosin, at different concentrations, dissolved in distilled water.

In the combination experiments, cells were incubated with 5HT (10^{-8} and 10^{-4} M) or $5HT_{1B}$ (10^{-5} and 10^{-4} M) agonist. After 45 min, 10 μl of the medium was removed and replaced with 10 μl of doxazosin, at different concentrations (10^{-6}, 10^{-5}, $5x10^{-4}$ and 10^{-4} M), dissolved in distilled water. A cell proliferation study was carried out, and changes in cell number were quantified using a crystal violet colorimetric assay, 72 h after addition of the drugs.

For the colorimetric assay, a solution of 0.5 g of crystal violet, 0.85 g of NaCl, 5 ml of 10% formal saline, 50 ml of absolute ethanol and 45 ml of distilled water was used. The medium was gently aspirated from the wells of a 96-well plate, and 100 μl of colorimetric assay mixture was added to each well and incubated at room temperature for 10 min. This mixture allowed simultaneous fixation of cells and penetration of crystal violet dye into the living cells. After washing three times in PBS, 33% acetic acid was used to elute color from the cells, and optical density was read at 570 nm using the spectrophotometric plate reader.

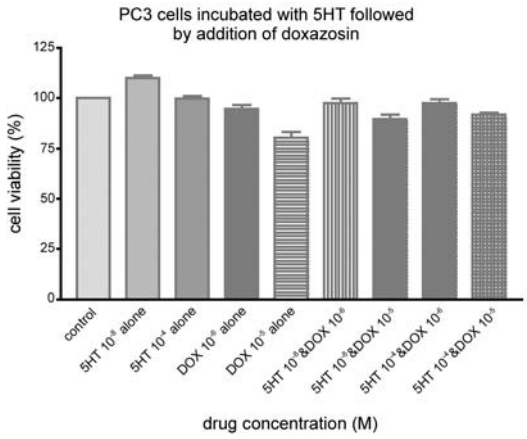

Figure 3. *The growth proliferatory effect of 5HT (10^{-8} and 10^{-4} M) and the growth inhibitory effect of doxazosin (10^{-6} and 10^{-5} M) on PC3 (androgen-independent prostate cancer) cell growth. The effect of incubation of PC3 cells with 5HT (10^{-8} and 10^{-4} M) for 45 min followed by the addition of doxazosin (10^{-6} and 10^{-5} M) is also shown. There was a rise in percent cell viability as compared to when PC3 cells are treated with doxazosin in the absence of 5HT. The bars represent the standard error of the mean.*

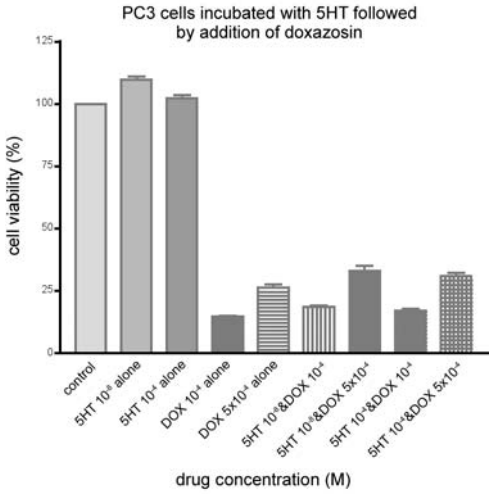

Figure 4. *The growth proliferatory effect of 5HT (10^{-8} and 10^{-4} M) and the growth inhibitory effect of doxazosin (10^{-4} and $5x10^{-4}$ M) on PC3 (androgen-independent prostate cancer) cell growth. The effect of incubation of PC3 cells with 5HT (10^{-8} and 10^{-4} M) for 45 min followed by the addition of doxazosin ($5x10^{-4}$ and 10^{-4} M) is also shown. There was a rise in percent cell viability as compared to when PC3 cells are treated with doxazosin in the absence of 5HT. The bars represent the standard error of the mean.*

Values are expressed as the percent of cell viability relative to control cultures.

Statistical analysis. Each proliferation assay was repeated on three separate occasions, each time with quadruple samples. Data analysis was performed using Microsoft Excel XP and Graphpad Prism 3.0 software. One way analysis of variance (ANOVA) and paired *t*-test were carried out between groups.

Results

A) Effect of doxazosin on PC3 and HT1376 cell lines.

(i) PC3 cells: Doxazosin caused a dose-dependent inhibition of PC3 cell growth with a maximum inhibition of 80% [*i.e.* percentage cell viability = 20%] (n=12, $p<0.0001$) at a concentration of 10^{-4} M at 72 h. Doxazosin at concentrations of 10^{-6} M and 10^{-5} M resulted in a percentage cell viability of 95% ($p=0.0150$) and 80% ($p<0.0001$), respectively, at 72 h (Figure 1).

(ii) HT1376 cells: Doxazosin caused a dose-dependent inhibition of HT1376 cell growth with a maximum inhibition of 91% [*i.e.* percentage cell viability = 9%] (n=12, $p<0.0001$) at a concentration of 10^{-4} M at 72 h. Doxazosin at concentrations of 10^{-6} M and 10^{-5} M resulted in a percentage cell viability of 94% ($p=0.039$) and 76% ($p=0.0001$), respectively, at 72 h (Figure 2).

B) Incubation of PC3 cells with 5HT followed by the addition of doxazosin.

(i) Effect of 5HT alone on PC3 cell growth: 5HT caused a 10.2% ($p<0.0001$) and 0.3% ($p=0.81$) increase in cell growth at concentrations of 10^{-8} M and 10^{-4} M, respectively, as compared to controls at 72 h. A 6% ($p=0.04$) and 4% ($p=0.24$) increase in cell proliferation was observed at concentrations of 10^{-7} and 10^{-6} M, whereas a 2% ($p=0.287$)

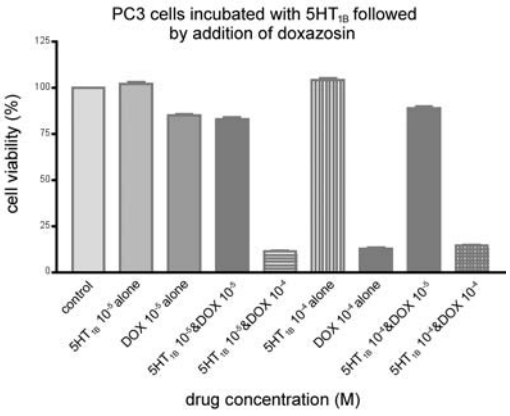

Figure 5. *The growth proliferatory effect of $5HT_{1B}$ agonist (CP93129 dihydrochloride) (10^{-5} and 10^{-4} M) and the growth inhibitory effect of doxazosin (10^{-5} and 10^{-4} M) on PC3 (androgen-independent prostate cancer) cell growth was assessed. The effect of incubation of PC3 cells with $5HT_{1B}$ agonist (10^{-5} and 10^{-4} M) for 45 min followed by the addition of doxazosin (10^{-5} and 10^{-4} M) is also shown. There was a rise in percent cell viability as compared to when PC3 cells are treated with doxazosin in the absence of 5HT. The bars represent the standard error of the mean.*

decrease in cell growth took place at 10^{-5} M as compared to control. In the combination studies (see below), the highest and lowest concentrations of 5HT (*i.e.* 10^{-4} and 10^{-8} M) we evaluated were used.

(ii) Effect of doxazosin alone on PC3 cell growth: Doxazosin at concentrations of 10^{-6} and 10^{-5} M resulted in a percentage cell viability of 94.7% ($p=0.015$) and 80.4% ($p<0.0001$), respectively, at 72 h. Doxazosin at concentrations of $5x10^{-4}$ and 10^{-4} M led to a percentage cell viability of 26.2% ($p<0.0001$) and 14.6% ($p<0.0001$), respectively, at 72 h.

(iii) Incubation of PC3 cells with 5HT followed by the addition of doxazosin: Incubation of PC3 cells with 5HT at a concentration of 10^{-8} M for 45 min, followed by the addition of doxazosin at concentrations of 10^{-6}, 10^{-5}, $5x10^{-4}$ and 10^{-4} M, demonstrated a cell viability of 97.5%, 89.2%, 33.1% and 18.7% respectively, at 72 h. Therefore, there was a 2.9% ($p=0.32$), 8.8% ($p=0.0015$), 6.8% ($p=0.03$) and 4.0% ($p<0.0001$) rise in cell viability at 72 h compared to doxazosin alone at the same concentrations (Figures 3 and 4).

Incubation with 5HT at a concentration of 10^{-4} M for 45 min followed by addition of doxazosin at concentrations of 10^{-6}, 10^{-5}, $5x10^{-4}$ and 10^{-4}M resulted in a cell viability of 97.6%, 91.7%, 30.9% and 17.1%, respectively, at 72 h. Thus, there was a 2.9% ($p=0.21$), 11.3% ($p=0.002$), 4.6% ($p=0.013$) and 2.5% ($p=0.002$) increase in cell viability at 72 h as compared to doxazosin alone at the same concentrations (Figures 3 and 4).

C) Incubation of PC3 cells with $5HT_{1B}$ agonist followed by the addition of doxazosin.

(i) Effect of $5HT_{1B}$ agonist alone on PC3 cell growth: The $5HT_{1B}$ agonist (CP93129 dihydrochloride) (n=12) caused a 2.1% ($p=0.0735$) and 5% ($p=0.0004$) increase in cell growth at concentrations of 10^{-5} and 10^{-4} M, respectively, as compared to controls at 72 h. A 2% ($p=0.35$) increase in cell proliferation was seen at both $5HT_{1B}$ agonist concentrations of 10^{-7} and 10^{-6} M.

(ii) Effect of doxazosin alone on PC3 cell growth: Treatment with doxazosin at concentrations of 10^{-5} and 10^{-4} M resulted in a percentage cell viability of 85.0% ($p<0.0001$) and 12.3% ($p<0.0001$), respectively, at 72 h.

(iii) Incubation of PC3 cells with $5HT_{1B}$ agonist followed by the addition of doxazosin: Incubating the PC3 cells for 45 min with the $5HT_{1B}$ agonist (CP93129 dihydrochloride) at a concentration of 10^{-5} M, followed by doxazosin at concentrations of 10^{-5} and 10^{-4} M, demonstrated a cell viability of 88.2% and 13.1%, respectively, at 72 h. These results indicate a 3.1% ($p=0.0001$) and 1.3% ($p=0.0007$) increase in cell viability at 72 h as compared to doxazosin alone at the same concentrations (Figure 5).

Incubation with $5HT_{1B}$ agonist at a concentration of 10^{-4} M for 45 min followed by doxazosin at concentrations of 10^{-5} and 10^{-4} M resulted in a cell viability of 89.9% and 14.8%, respectively, at 72 h. Thus, there was a 4.9% ($p<0.0001$) and 3.0% ($p<0.0001$) increase in cell viability at 72 h as compared to doxazosin alone at the same concentrations (Figure 5).

Discussion

Alpha-adrenoceptor antagonists can inhibit growth and induce apoptosis in prostatic cancer cells (10-20, 22-26). The growth inhibitory effect of doxazosin may be mediated *via* the up-regulation of transforming growth factor (TGF-β_1). TGF-β_1 is a major regulator of prostate growth by inhibiting cell proliferation, inducing apoptosis and regulating cell migration (24).

Xu *et al.* proposed that the sensitivity of prostate cancer cells (PC3 and DU145) to terazosin was not affected by the presence of either functional p53 or Rb. However, terazosin-induced cell death was associated with G1-phase cell cycle arrest and up-regulation of p27KIP1. In addition, up-regulation of Bax and down-regulation of Bcl-2 was observed, indicating that these two apoptotic regulators play a role in terazosin-mediated cell death (26).

Keledjian *et al.* reported that Bcl-2 overexpression in prostate cancer cells exerts an antagonistic effect against the quinazoline-mediated apoptotic effect by suppressing cell attachment to the gelatine matrix without affecting cell invasion (15). Growth factors such as TGF-β, basic fibroblast growth factor and vascular endothelial growth factor contribute to the angiogenic response of tumours *via* the modulation of integrin expression (15).

Alpha-adrenoceptor antagonists enhance the apoptotic effect of ionizing radiation against human prostate cancer cells (12). They also decrease the vascularity of prostate tumours (14, 15). Studies continue to investigate the exact mechanism by which alpha-adrenoceptor antagonists inhibit prostate cancer cell growth.

5HT, a monoamine neurotransmitter, mediates a wide range of activities (27-29) including acting as a growth factor (30) on several non-tumoral cells (*e.g.* vascular smooth muscle, lung fibroblasts and renal mesangial cells) (31, 32). 5HT also has a growth stimulatory activity on carcinoid valve disease (33), pancreatic carcinoid cells (34), small cell lung carcinoma cells (35-37) and colonic carcinoma (38-41) in rats. Depending on the tumour type, either $5HT_2$ or $5HT_1$ receptor antagonists can inhibit the 5HT-induced increase in tumour growth. The $5HT_{1A}$ receptor ($5HTR_{1A}$) has been associated with prostate cancer growth (27-29).

$5HT_2$ receptors have also been identified in platelets (8), $5HT_4$ receptors are present in the human bladder and $5HT_3$ receptors are involved in the 5HT-mediated contraction of the rabbit detrusor (9). It has been suggested that penile erections in rats are modulated by $5HT_{1B}$ receptors (42).

5HT has a growth effect on prostate cancer cells and $5HT_{1A}$ antagonist and 5HT uptake inhibitors caused growth inhibition in prostate cancer cell lines PC3, DU145 and LNCaP *in vitro* (27-29).

Doxazosin inhibits 5HT-induced platelet shape change *via* the $5HT_2$ receptor (8) and also significantly inhibits 5HT-mediated contractions in the rabbit detrusor (9). The latter effect appears to be mediated *via* $5HT_3$ receptor inhibition, since autoradiographic evidence suggested that doxazosin reduced 5HT binding (9). This finding may be attributed to the similarity between 5HT receptors alpha adrenergic receptors (27). Therefore, it is possible that, in the present study, the growth inhibitory effect of doxazosin on prostate and bladder cancer cells was mediated *via* 5HT receptors. Furthermore, the beneficial effects of doxazosin in bladder outflow obstruction may be partly attributed to 5HT antagonism (9).

We found that incubating PC3 cells for a short duration with 5HT, followed by exposure to doxazosin, led to a greater percent of viable cells at 72 h, as compared to when PC3 cells are treated with doxazosin in the absence of 5HT. Similarly, incubating PC3 cells with a $5HT_{1B}$ agonist for a short duration, followed by exposure to doxazosin, led to an increased percentage of cell viability at 72 h as compared to treatment with doxazosin alone. A possible explanation is that early binding to the 5HT receptors decreased any 5HT receptor-mediated growth inhibition of doxazosin. However, it is also possible that doxazosin and 5HT act through different pathways.

Our results indicate that doxazosin causes a dose-dependent growth inhibitory effect on both prostate and bladder cancer cells. The inhibition of HT1376 cells is a novel and potentially important finding, suggesting that alpha adrenoceptor antagonists may have a role in the treatment of transitional cell carcinoma of the bladder. An interesting aspect in future studies will be to determine the effect of lower concentrations of doxazosin (10^{-7}, 10^{-8} M) on the growth of PC3 and HT1376 cells after longer incubation periods (5-7 days).

Autoradiographic studies in prostate and bladder cancer cells may identify the ability of doxazosin to displace 5HT from 5HT receptors. Such a finding would support the concept that doxazosin exerts at least some of its growth inhibitory effect at the level of these receptors.

Conclusion

The alpha1-adrenoceptor antagonist, doxazosin, significantly inhibited prostate (PC3) and bladder cancer (HT1376) cell growth. Incubation of PC3 cells with 5HT or $5HT_{1B}$ agonist partially reversed the growth inhibitory effect of doxazosin. Doxazosin may modulate the action of 5HT at the receptor level. Autoradiographic studies are required to clarify this issue.

Further research is essential to obtain a better understanding of the anti-proliferative effect of doxazosin on prostate and bladder cancer cells.

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