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THE ROLE OF SEROTONIN (5HT) AND 5HT ANTAGONISTS IN UROLOGICAL CANCERS

A thesis presented for the degree of Doctor of Medicine to the Faculty of Medicine of the University of London.

Submitted

By

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ABSTRACT

Neuroendocrine (NE) differentiation takes place in both prostate and bladder cancer tissue. These NE cells release growth factors including neuropeptides and biogenic amines which correlate with tumour progression, differentiation, androgen independence, angiogenesis and poor prognosis.

Serotonin (5-hydroxytryptamine; 5HT), a monoamine neurotransmitter, plays a role in regulation of growth, gene expression and may function as a proto-oncogene. 5HT acts as a growth factor in tumours such as small cell carcinoma of the lung and colonic carcinoma.

The *in vitro* effect of 5HT and 5HT antagonists on prostate and bladder cancer cell growth was evaluated. Cell culture and proliferation assay studies established a significant increase in growth proliferation by 5HT. Furthermore, there was a significant growth inhibition by $5HT_{1A}$ and $5HT_{1B}$ antagonists on prostate cancer (PC3) cells and bladder cancer (HT1376) cells. FACS analysis in PC3 cells demonstrated cell cycle arrest and apoptosis when treated with $5HT_{1A}$ and $5HT_{1B}$ antagonists. Immuno-staining for $5HT_{1A}$ and $5HT_{1B}$ receptors was seen in PC3 cells, HT1376 cells, as well as in human prostate and bladder cancer tissue. Western blot analysis demonstrated $5HT_{1A}$ and $5HT_{1B}$ receptor proteins in both prostate and bladder cancer cells and tissue. Ethical approval was obtained and the Helsinki Declaration of Human Rights was strictly adhered to throughout this study. These results imply that a 5HT system exists in both prostate and bladder cancer tissue. $5HT_{1A}$, and in particular $5HT_{1B}$ receptor antagonists, warrant further investigation as potential anti-neoplastic agents.

A growth inhibitory effect of an alpha adrenoceptor antagonist (doxazosin) on PC3 and HT1376 cells was also demonstrated. This growth inhibition is independent of the antagonist action on $alpha_1$-adrenoceptors. 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, possibly at the receptor level. Doxazosin (or more effective analogues) commonly used in the treatment of benign prostatic hyperplasia, may be potential chemotherapeutic agents.

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ABBREVIATIONS

ABC	Avidin Biotin Complex
ACTH	Adrenocorticotrophic Hormone
APUD	Amine Precursor Uptake and Decarboxylation
ATCC	American Type Culture Collection
BFGF	Basic Fibroblast Growth Factor
BPH	Benign Prostatic Hyperplasia
cAMP	Cyclic AMP
CEA	Carcino-embryonic Antigen
CNS	Central Nervous System
COX	Cyclooxygenase
CT	Computerised Tomography
DAB	3,3'-diaminobenzidin tetrahydrochloride
DMSO	Dimethyl Sulfoxide
DRE	Digital Rectal Examination
dsDNA	Double-stand Deoxyribonucleic Acid
dsRNA	Double-stand Ribonucleic Acid
FAA	Flavone-8-acetic acid
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GCC	Giant Cell Carcinoma
GIST	Gastrointestinal Stromal Tumour
H&E	Haematoxylin and Eosin
5HIAA	5-hydroxyindoleacetic acid

5HT	5-hydroxytryptamine
IHC	Immunohistochemistry
IV	Intravenous
LELC	Lymphoepithelioma-like Carcinoma
LHRH	Luteinising Hormone Releasing Hormone
LOX	Lipoxygenase
LUTS	Lower Urinary Tract Symptoms
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
NE	Neuroendocrine
NIH	National Institute of Health
NSAIDs	Non-steroidal Anti-inflammatory Drugs
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PI	Propidium Iodide
PSA	Prostate Specific Antigen
PTH	Parathyroid Hormone
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SC	Subcutaneous
SCID	Severe Combined Immunodeficient
SCLC	Small Cell Lung Carcinoma
SEM	Standard Error of the Mean

TGF	Transforming Growth Factor
TGF-β1	Transforming Growth Factor Beta-1
TRUS	Transrectal Ultrasound Guided Prostate Biopsy
TURBT	Transurethral Resection of Bladder Tumour
TURP	Transurethral Resection of the Prostate
UTI	Urinary Tract Infection
VEGF	Vascular Endothelial Growth Factor

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PUBLICATIONS RELATED TO THIS THESIS

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6. The role of serotonin in tumour growth. Oncol Reports, 2005. Emad J Siddiqui, Cecil S Thompson, Dimitri P Mikhailidis, Faiz H Mumtaz *(In press).*

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CHAPTER 1

INTRODUCTION

1.1. Prostate cancer

1.1.1 Introduction

Prostate cancer is the second most common cancer affecting men in Europe and the USA. One in twelve men over the age of 60 develop prostate cancer and this figure is expected to rise to three in twelve in the next 20 years (Fournier et al., 2004; Parchment Smith C, 2002). At the age of 50 about 15% of prostates contain islands of cancer and by 80 this figure rises to nearly 100% (Amling, 2004).

For patients who have apparent organ confined disease, effective treatment options are available in the form of surgery or radiotherapy. However, about 20% of patients have evidence of metastases on presentation (Parchment Smith C, 2002). The mainstay of treatment for these patients is androgen ablation therapy. Unfortunately, these patients eventually relapse and develop an androgen independent tumour (Ahmad, 2004; Amling, 2004; Ansari et al., 2002). This aggressive stage carries a high morbidity and mortality. At present the treatment for hormone refractory prostate cancer is unsatisfactory and a search for alternative forms of therapy continues.

1.1.2 Aetiology

It is well established that the prevalence of prostate cancer varies in different races. It is relatively uncommon in Japanese and Asian men, whereas, it is more common in Africans (including Black Americans)(Fournier et al., 2004; Spangler et al., 2005).

Migration studies reveal that when men relocate from a low- to a high-risk region and abandon their traditional dietary patterns in favour of their hosts

common diet, their incidence of prostate cancer rises to that of the local population. This observation, thus suggests a role of western dietary factors in prostate cancer (Steinberg et al., 1998; Villers et al., 1997).

Numerous studies implicate dietary and nutritional factors in the onset and progression of prostate cancer. Hence, it is possible that bioactive compounds (anti-oxidants) like vitamins A, C, D and E, minerals like selenium and carotenoids like lycopene can be a part of chemopreventive strategies (Ansari et al., 2002; Harris et al., 2004; Oakley-Girvan et al., 2004). Ongoing studies on nutrition and prostate cancer may provide the required evidence to support what is still only a hypothesis.

A family history (first degree relative) is associated with a 2 to 3 fold higher risk of prostatic cancer. This familial aggregation can be used to define a high-risk group suitable for screening (Bostwick et al., 2004; Hemminki et al., 2005; Spangler et al., 2005; Villers et al., 1997).

There has been a misconception with regards to whether vasectomy can increase the risk of developing prostate cancer. However, an extensive review by the United States National Institutes of Health (NIH) concluded that there was insufficient evidence of a real association between vasectomy and prostate cancer. The recommendation was that vasectomy should continue to be provided (Pollack, 1993).

1.1.3 Pathology and Clinical Features

Prostate cancer is an adenocarcinoma that arises from the peripheral zone of the gland. However, in 10-15% of cases, for example when benign prostatic hyperplasia (BPH) is present, the cancer may develop in the transitional zone

of the prostate. This type of cancer is usually less aggressive and spreads at a slower rate (Ansari et al., 2002; Spangler et al., 2005; Sternberg, 2003).

Early prostate cancer often does not cause symptoms. However, patients may present with lower urinary tract symptoms (LUTS) complaining of increased urinary frequency, nocturia, urgency, hesitancy, weak or interrupted stream and post-micturition dribbling. Haematuria, hematospermia, anorexia and weight loss, are other possible symptoms. Pain or stiffness in the lower back, hips or upper thighs may indicate bony metastasis. Some of the above symptoms may be caused by prostate cancer or by BPH. Therefore, patients with such symptoms should be investigated.

1.1.4 Investigations and Evaluation

It is important to perform a digital rectal examination (DRE). A hard, nodular, craggy and fixed prostate strongly suggests a diagnosis of prostate cancer.

Additionally serum PSA (Prostate Specific Antigen) is measured. PSA is a proteolytic enzyme that is secreted by the prostate gland and has a main role in liquefaction of the ejaculate. As PSA can be used to detect prostate cancer, it is often referred as a biological or tumour marker. PSA >4.0 ng/ml has a sensitivity of 40-90%, specificity of 60-90% and a positive predictive value of 33% for prostate cancer (Ansari et al., 2002; Sternberg, 2003). The greater the PSA level, the greater the likelihood of diagnosing prostate cancer on biopsy (Cooperberg et al., 2004; Ross et al., 2003). In general, patients with a PSA >4 ng/ml should have a transrectal ultrasound guided prostate biopsy (TRUS). However, one should also be aware of variability of PSA value with

age. The norm ranging from 2.5 ng/ml in a 40-49 year old, to 6.5 ng/ml in a 70-79 year old (Cooperberg et al., 2004; Parchment Smith C, 2002)

Furthermore, before subjecting patients to PSA testing, one should consider other conditions which may lead to its elevation. For example, urinary tract infection (UTI), acute retention of urine, BPH, prostatitis, post-ejaculation and post-TURP (transurethral resection of the prostate) (Balmer & Greco, 2004; Parchment Smith C, 2002). DRE, catheterization, cystoscopy or TRUS biopsy do not lead to an elevation of PSA (Parchment Smith C, 2002; Ross et al., 2003).

To date, there has been no prostate cancer screening program in the U.K. Experts disagree on the usefulness of PSA measurement in the screening of prostate cancer, as it is not yet known whether such testing will reduce mortality rates. In the future, as more evidence evolves, PSA might be adopted as a screening tool for prostate cancer.

PSA velocity is one parameter of PSA that can be used to further assess an elevated PSA. A rise of >0.75 ng/ml per year strongly suggests the presence of prostate cancer (Balmer & Greco, 2004; Sternberg, 2003)}.

TRUS biopsy is the gold standard for diagnosing prostate cancer (Ansari et al., 2002; Fournier et al., 2004). Biopsies are performed under ultrasound guidance, allowing visualization of suspicious areas of the gland and directing biopsy. The procedure can be performed with a periprostatic local anaesthetic injection (Ansari et al., 2002).

Histological examination of the biopsy cores serves a dual purpose; it diagnoses and grades the tumour according to the Gleason System (Calabrese, 2004; Fournier et al., 2004). This system is based on the degree

of differentiation and tumours can vary from well differentiated to poorly differentiated tumours. Generally, the Gleason system correlates well with the clinical behaviour of the tumour and its response to treatment (Calabrese, 2004; Fournier et al., 2004; Ross et al., 2003).

Currently, the majority of patients with prostate cancer are diagnosed on TRUS biopsy, <5% are diagnosed following TURP and a few from secondary spread (e.g. pathological bone fracture).

Further staging includes a CT scan or MRI of the pelvis. This determines any extra capsular involvement of the pelvic organs (e.g. seminal vesicles, rectum or pelvic wall). These imaging studies also identify pelvic lymph node involvement (Amling, 2004).

A Tc^{99M} radio-isotope bone scan is necessary to identify any bony metastasis. The tumour usually spreads to the spine, pelvis, long bones, ribs, scapula and skull in order of decreasing frequency (Iwamoto & Maher, 2001; Peneau et al., 1998). Once staging of the disease is complete, a decision is made for the most appropriate treatment.

The TNM staging system is commonly used in which 'T' stands for Tumour, 'N' for nodal involvement and 'M' for metastasis (Peneau et al., 1998; Sternberg, 2003). 'T' is further subdivided into Tis, T0, T1, T2, T3 and T4 representing carcinoma in-situ, no tumour, clinically unapparent tumour diagnosed histologically on needle biopsy or incidentally on TURP, tumour confined to but deforming gland, extra capsular tumour involving seminal vesicles and fixed tumour invading neighbouring structures, respectively (Peneau et al., 1998).

1.1.5 Management

Various options are available for the management of patients with prostate cancer depending on the age, general health, grade and stage of the disease. When considering localised disease, watchful waiting is probably the best treatment option for older men with well and perhaps moderately differentiated, low volume prostate cancer who have a life expectancy of less than 10 years (Steinberg et al., 1998). This is because many elderly men have a small cancer in their prostate, but less than 0.5% die of it: 99.5% do not (Ross et al., 2003; Steinberg et al., 1998).

However, this conclusion derived from watchful waiting studies of older men cannot and should not be applied to younger, healthier men or to those with more advanced or aggressive disease (Steinberg et al., 1998; Villers et al., 1997). If treated ineffectively, many of these men will die of prostate cancer. Most men with prostate cancer who have a life expectancy greater than 10 to 15 years should be treated with curative intent (Steinberg et al., 1998).

Good results are obtained in patients with moderately and poorly differentiated prostate cancer ranging from stage T0-T2, after radical prostatectomy or radiotherapy (Amling, 2004). Apart from being used to cure early stage prostate cancer, radiotherapy is also used to control locally advanced disease and effectively palliate symptoms of metastases (Peneau et al., 1998).

Surgery or radiotherapy alone cannot cure clinical stage T3 prostatic cancer (Amling, 2004; Iwamoto et al., 2001; Peneau et al., 1998). The choice of treatment for stage T3, N0, M0 depends on the patient's quality of life and life expectancy. If the option of a curative treatment in a young subject can be

reasonably considered, combination therapy in the form of hormonal treatment followed by radiotherapy should be utilised. This approach has been proved to be superior to radiotherapy alone in several randomized trials (Iwamoto et al., 2001; Peneau et al., 1998).

For non-localized or metastatic disease the mainstay of treatment is hormone ablation therapy aimed at decreasing the level of circulating testosterone. This is achieved by medical or surgical castration (Ahmad, 2004). Medical castration includes the use of luteinising hormone releasing hormone (LHRH) agonists (Ahmad, 2004). Initially these agents can cause tumour flare that is covered with short term simultaneous administration of anti-androgens (Ahmad, 2004; Calabrese, 2004). The advantage surgical castration has over medical castration is that in surgically castrated patients, drug compliance is not an issue.

In the long term, LHRH agonists act by reducing circulating testosterone levels thus controlling disease progression. Steroidal and non-steroidal anti-androgens may be another medical treatment option. Surgical castration is carried out by bilateral orchidectomy (Parchment Smith C, 2002).

Most (80%) of these patients will initially show an adequate response to such therapy, the mean period of response being approximately 18 months (Calabrese, 2004). However, eventually these patients transit into a "hormone refractory" or "androgen independent" phase. Once at this stage the patient has a poor prognosis with a median survival time of 9-12 months (Calabrese, 2004). At this stage oestrogens and progesterone may be used as a second line with some benefit.

1.1.6 Comments

The incidence of prostate cancer has risen by 60-75% in the western world in the last 15 years. This number is increasing due to the demographic shift to longevity, increased awareness and PSA testing with consequently more biopsies.

Potentially effective treatment in the form of surgery and radiotherapy is available for individuals with localised disease, and the effectiveness of different combination therapy is being assessed to improve the outcome further.

For hormone refractory disease, management is as yet unsatisfactory. The search for new agents for the treatment of hormone refractory prostate cancer continues.

1.2 Bladder cancer

1.2.1 Introduction

Bladder cancer is a significant proportion of the general workload in Urology due to its high prevalence and recurrent nature. It is the fifth most common malignancy in Europe and the fourth most common malignancy in the United States (Jensen et al., 1990). It affects 1 in 4000 people and accounts for 5% of all diagnosed cancers (Jensen et al., 1990). The peak incidence is in the fifth and seventh decade. About 75 % of patients with bladder cancers are men. 90% of all bladder cancers are transitional cell carcinomas (Clavel et al., 1989).

1.2.2 Aetiology

Around 50% of all transitional cell carcinomas of the bladder are associated with cigarette smoking. Smokers have a fourfold higher incidence of bladder cancer than the general population (Clavel et al., 1989). This risk correlates with the number of cigarettes smoked and the duration of smoking (Augustine et al., 1988). Discontinuing smoking is beneficial, even after many years, as ex-smokers have a reduced incidence of bladder cancer compared with current smokers (Chen & Ahsan, 2004; Karagas et al., 2004).

The association between the exposure to benzidene, aromatic amines and β-naphtylamine, typically in the dye and rubber industries (Veys, 2004), is associated with a statistically significant increased risk of developing bladder cancer when compared with the general population. Data collected from 11 case-control studies (3346 cases and 6840 controls, aged 30-79 years) conducted between 1976-1996 in six European countries revealed that metal workers, machinists, transport equipment operators and miners are among the major occupations contributing to occupational bladder cancer in men in Western Europe. In this population one in 10 to one in 20 cancers of the bladder can be attributed to occupation (Colt et al., 2004; Kogevinas et al., 2003). Exposure to carbon black experienced by dockyard workers was associated with a two-fold increased risk of bladder cancer (Puntoni et al., 2004).

Bilharzia is associated with the development of squamous cell carcinoma of the bladder and occurs commonly in areas where schistosomiasis is endemic (Shokeir, 2004).

1.2.3 Pathology and Clinical features

Histologically, bladder cancer may be transitional cell carcinoma, squamous cell carcinoma or adenocarcinoma (Dundr et al., 2003). Transitional cell carcinoma is the most commonly seen (Dundr et al., 2003). Bladder cancers may have elements of NE differentiation; these tumours are usually aggressive and have a poor prognosis (Lertprasertsuke et al., 1991; Mills et al., 1987).

Haematuria is the cardinal symptom of bladder cancer (Augustine et al., 1988; Mikhailidis et al., 1998). Therefore, frank or microscopic haematuria requires investigation, especially in those aged over forty (Augustine et al., 1988; Mikhailidis et al., 1998).

Around 15 % of patients will present with lower urinary tract symptoms (LUTS) of frequency, dysuria and urgency. This may be associated with or without haematuria.

1.2.4 Investigations and Evaluation

Ultrasonography of the bladder may identify a tumour. However, cystoscopy is the gold standard investigation for the diagnosis of bladder cancer. The other investigations for haematuria include a urine culture, urine cytology and intravenous urography which can demonstrate transitional cell carcinoma in the kidney and ureter. Several urinary markers and tests such as BTA Stat, BTA TRAK, NMP22, telomerase, HA and HAse tests, Immunocyt, Quanticyt, FDP, BLCA-4, FISH, CYFRA-21-1 have enough potential for future clinical use (Dey, 2004). BTA stat, NMP22 (bladder check) and AccuDX (FDP) tests are presently point of care tests. The other tests are laboratory-based and

may need trained technicians. To date, there is no urinary marker or test that can replace the need of cystoscopy. However, some markers have the potential for future clinical use (Dey, 2004).

The TNM system is the universally accepted method for staging bladder tumours (Sobin L.H & Wittekind C, 1997). This is based on the depth of invasion through the bladder wall and the presence or absence of pelvic lymph node involvement and distant metastasis. A tumour that is limited to the mucosa and lies flat is Tis (carcinoma in situ), a tumour that is papillary and limited to the mucosa is Ta and a tumour that penetrates the lamina propria but not the muscularis is T1. Stage T2 tumours invade the muscularis layer and are highly malignant with a strong potential to metastasize preferentially to regional lymph nodes, lungs, liver and bone. A tumour extending into the perivesical fat is T3 and tumours invading the surrounding organs, pelvic or abdominal wall are T4. Muscle-invasive tumours are staged clinically with a combination of bimanual examination under anaesthetic and imaging with computed tomography or MRI, chest radiograph and isotope bone scans (Sobin L.H & Wittekind C, 1997).

1.2.5 Management

(i) Treatment of superficial tumours

Superficial bladder cancers can be treated completely via endoscopic transurethral resection. However, 50-70% of patients have a recurrence within two years. Patients are therefore given adjuvant intra-vesical instillation of mitomycin C and bacillus Calmette-Guerin (BCG) (Pawinsky A et al., 1996). Evidence suggests that BCG is the most effective intravesical therapy for the

treatment of superficial bladder cancer and that maintenance therapy is superior to an induction course alone (Borden et al., 2004). Endoscopic surveillance is performed by flexible cystoscopy at three-month intervals for the first year, six-monthly intervals for the second year and then annually (Donat, 2003).

(ii) Treatment of muscle invasive tumours

At the time of diagnosis, 20% of bladder cancers have muscle invasion, which cannot be eradicated by transurethral resection or intravesical therapy. These have a 3-year survival of less than 5% in untreated cases. Definitive treatment is either by radical cystectomy or by radical radiotherapy. The nodal status and extent of lymphadenectomy have been shown to correlate with survival after radical cystectomy (Borden et al., 2004). Despite these treatments being available for the past 50 years, the survival of patients has not improved (Gospodarowicz et al., 1995; Whitmore, Jr., 1983).

Radiotherapy has the advantage of bladder preservation, but may be associated with frequency and urgency along with bladder contracture. About 50% of patients will have a complete response to radiotherapy. Following irradiation, regular cystoscopic surveillance is resumed.

Radical cystectomy with the formation of an ileal conduit has become the treatment of choice stage T2 and above tumours. Pre-operative assessment of general fitness and cardiorespiratory status is an important consideration before surgery. Improvements in surgical techniques and post-operative care have taken place and it is possible to create an orthoptic neo bladder utilising intestine (Hautmann et al., 1993).

The role of chemotherapy in the treatment of bladder cancer continues to evolve (Borden et al., 2004; van der Meijden, 1998). Neoadjuvant chemotherapy has recently demonstrated a survival benefit, and trials are ongoing to define the optimal regimen of chemotherapy for urothelial carcinoma (Borden et al., 2004).

The prognosis in muscle-invasive bladder cancer is dependent on disease stage. The 5-year survival rates are 85% for T2 cancers, 55% for T3 and less than 25% for T4 cancers. The mean survival of patients with metastatic bladder cancer is 6-12 months. Treatment is targeted at palliation of symptoms, which may involve local tumour resection or palliative radiotherapy.

1.2.6 Comments

Bladder cancer remains a major health problem. Due to the tendency of bladder cancer to recur repeated cystoscopies and resections are often required. Because of this, one of the main thrusts of research is to find a way of preventing the progression from superficial disease to muscle invasive and metastatic bladder cancer. Furthermore, new treatments need to be developed to improve the relatively poor prognosis of invasive disease. Among preventive measures, smoking cessation is paramount (Mikhailidis et al., 1998).

1.3 Serotonin (5-hydroxytryptamine; 5HT)

Serotonin (5-hydroxytryptamine; 5HT) a monoamine neurotransmitter mediates a wide range of physiological activities by binding to receptor

subtypes (Abdul et al., 1994; Abdul et al., 1995; Dizeyi et al., 2004). It is produced and stored in a number of tissues throughout the body in particular the central nervous system (CNS) (Shields & Eccleston, 1973), and platelets (Cortellazzo et al., 1985; Marmaras & Mimikos, 1971; Yazaki et al., 1987). It has also been found in gastrointestinal enterochromaffin cells (Dizeyi et al., 2004) and prostatic NE cells (Abdul et al., 1994; Abdul et al., 1995; Abrahamsson et al., 1986; Dizeyi et al., 2004).

5HT has been implicated in the regulation of circadian rhythmicity, sleep-wakefulness, sexual behaviour, mood changes, appetite and nociception. 5HT-analogs are currently used in the treatment of disorders including depression, anorexia, anxiety, migraine and chemotherapy-induced emesis. Deficient serotonin neurotransmission has been implicated in panic attacks (Maron et al., 2005; Saudou et al., 1994). 5HT plays a fundamental role in tumour growth, differentiation and gene expression. The activation of a brain specific $5HT_{1C}$ receptor, in the unnatural environment of a fibroblast (NIH 3T3) resulted in the generation of transformed foci (Julius et al., 1989). Cells derived from these transformed foci when injected into nude mice resulted in the generation of tumours (Julius et al., 1989). Therefore, $5HT_{1C}$ receptors may function as a proto-oncogene when expressed in fibroblasts (Julius et al., 1989).

1.3.1 Synthesis of 5HT

With the exception of platelets, 5HT is synthesized in most tissues in which it is stored. The biosynthesis of 5HT, starts with the hydroxylation of the essential amino acid L-tryptophan; this first, rate limiting, step is catalyzed by

the enzyme trytophan hydroxylase and results in the formation of 5-hydroxytrytophan which is transformed to 5HT in a reaction catalyzed by the non-specific enzyme aromatic L-amino acid decarboxylase. In man, about 2% of the ingested tryptophan is utilised for the daily synthesis of approximately 10 mg of 5HT (Saxena et al., 1990).

Tryptophan

↓

5-hydroxytryptophan

↓

5-hydroxytryptamine

1.3.2 Metabolism of 5HT

The principal metabolic pathway of 5HT is the oxidative deamination by monoamine oxidase which results in the production of 5-hydroxyindoleacetic acid (5-HIAA). Besides 5HT, monoamine oxidase catalyzes the deamination of several other biogenic amines such as dopamine, noradrenaline, tyramine and tryptamine. In the first step, the deamination of 5HT leads to the formation of 5-hydroxyindoleacetaldehyde which, in most cases, is oxidised to 5-HIAA by the enzyme aldehyde dehydrogenase. 5-hydroxyindoleacetaldehyde can also be reduced to 5-hydroxtrytophol; this metabolite has been detected in several peripheral tissues such as the liver (Saxena et al., 1990).

1.4 5HT receptors

There are a number of 5HT receptors (5HTR). Seven have been identified to date ($5HT_1$-$5HT_7$) on the basis of their structural, functional and pharmacological characteristics (Alhaider & Wilcox, 1993; Anzini et al., 1989; Boess et al., 1992; Dickenson & Hill, 1998; Goadsby, 1998; Peroutka, 1988). Each receptor is further subdivided into different sub-types. $5HTR_1$ alone comprises of five sub-types ($5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, $5HT_{1E}$ and $5HT_{1F}$) (Goadsby, 1998). Apart from $5HT_3$ receptor which has a ligand gated ion channel, all the other 5HT receptors are G-protein coupled (Peroutka, 1988). Among all the 5HT sub-type receptors, the $5HT_{1A}$ receptor is the most widely studied, mainly due to early availability of potent and selective agonist, 8-OH-DPAT (Dizeyi et al., 2004).

5HT receptors have been implicated in psychiatric and neurological disorders (Stahl, 1992). For example, depressed subjects treated with antidepressants downregulate markers of both $5HT_2$ and $5HT_{1A}$ receptors in a time course consistent with their recovery from depression (Stahl, 1992). Evidence from animal studies and human genetics suggested that $5HT_{1B}$ receptors may be involved in the mechanism of action of antidepressants and may become important targets of drug therapy (Massot et al., 1996). Experiments conducted in male mice indicated that $5HT_{1B}$ receptor agonists have anti-aggressive effects in mice that show moderate as well as high levels of aggression (de Almeida & Miczek, 2002; Saudou et al., 1994). Further studies on mice revealed that post synaptic $5HT_{1B}$ receptors are required for the antidepressant activity of selective 5HT receptor inhibitors (SSRIs) (Trillat et al., 1998). Neuroendocrine techniques described to study this include

fenfluramine-induced changes in ACTH, cortisol and prolactin secretion (for $5HT_2$ receptors) and ipsapirone-induced changes in ACTH and cortisol (for $5HT_{1A}$ receptors) (Stahl, 1992). The 5HT receptor implicated in migraine is $5HT_1$. Currently, effective and relatively specific anti-migraine compounds, as a group, are potent $5HT_{1B/1D}$ agonists (Goadsby, 1998). Their possible mechanisms of action include carotid territory vasoconstrictor effects and inhibitory effects on both the peripheral and central terminals of the trigeminal innervation of the pain-producing intracranial structures (Goadsby, 1998). $5HT_{1A}$ and $5HT_{1B}$ receptors have been identified in post-mortem in the hippocampus, ventral and prefrontal cortex of human brain tissue (Marazziti et al., 1994; Varnas et al., 2005). $5HT_{1A}$ and $5HT_{1B}$ receptors have also been localised in human cholangiocytes (Marzioni et al., 2005). $5HT_2$ receptors have been identified in platelets (Jagroop & Mikhailidis, 2001). $5HT_4$ receptors stimulate aldesterone secretion in aldesterone-producing adrenocortical adenomas (Lefebvre et al., 2002). Immunohistochemistry showed expression of $5HT_{1B}$ receptors in the medial layer, but did not reveal any obvious difference in $5HT_{1B}$ receptor expression between normal and atherosclerotic coronary arteries in humans (Edvinsson et al., 2005). The results of the study support the notion that triptans are selective vasoconstrictors of cerebral over coronary arteries (Edvinsson et al., 2005). Contractions of the fundus of the stomach are mediated via 5HT receptors (Cohen & Fludzinski, 1987; Cohen & Wittenauer, 1986). 5HT receptors are present in rat spinal cord (Marazziti et al., 1994; Matsumoto et al., 1992) and brain tissue where they mediate neurotransmission (Wirtshafter & Cook, 1998). The stimulation of the postsynaptic $5HT_{1B}$ receptors inhibits electroshock-induced seizures in rats

(Stean et al., 2005). The hypophagic effect induced by 5HT requires activation of $5HT_{1A}$ and $5HT_{1B}$ receptors, the $5HT_{1A}$ receptors showing higher behavioural sensitivity (Mancilla-Diaz et al., 2005; Vickers & Dourish, 2004). These results suggest that $5HT_{1A}$ and $5HT_{1B}$ receptors may have a significant role in the treatment of diet control and obesity in humans in the future. The coronary arteries (Feletou et al., 1994) and saphenous vein in rabitts also contains 5HT receptors (Wurch et al., 1997). Valvular subendocardial cells contain $5HT_{1B}$ and $5HT_{2B}$ receptors (Elangbam et al., 2005; Rajamannan et al., 2001).

$5HT_4$ receptors are present in the human bladder (Khan et al., 2000). The rabbit detrusor contains $5HT_3$ receptors where they are involved in the 5HT-mediated contraction of the bladder (Khan et al., 2000). It has been suggested that penile erections in rats are mediated via $5HT_{1B}$ receptors (Berendsen & Broekkamp, 1987). The penile erections were induced by the 5HT uptake inhibitors (zimelidine, fluoxetine, citalopram, Org 6997), by the 5HT-releasing agent fenfluramine and by the putative $5HT_{1B}$ receptor agonist (1-3'-chlorophenyl-piperazine). The $5HT_{1A}$ agonist (8-OH-DPAT) did not induce penile erections (Berendsen & Broekkamp, 1987). In rats, the central effect of 5HT on ejaculation is relatively established; 5HT also results in concentration dependent inhibition of hypogastric nerve stimulation induced seminal vesicle pressure change (Kim & Paick, 2004). RT-PCR demonstrated mRNA of $5HT_{1A}$, $5HT_{1B}$ and $5HT_{2C}$ receptors (Kim et al., 2004).

Immunocytochemical analysis performed on prostate cancer cell lines (PC3, HT1376 and LNCaP), demonstrated stronger staining for 5HT receptors in PC3 cells (androgen-independent prostate cancer) as compared to DU145

(androgen-independent prostate cancer) and LNCaP cells (androgen-sensitive prostate cancer) (Abdul et al., 1994).

5HT receptor sub-type 1A and 1B ($5HTR_{1A}$ and $5HTR_{1B}$) immunoreactivity was demonstrated in benign and malignant epithelial cells (Dizeyi et al., 2004). In prostate tumour areas, staining intensity was related to Gleason grade, with a strongest signal detected in the more advanced grade 4 and 5 tumours (Dizeyi et al., 2004). The staining intensity of 5HT receptor sub-type 1A and 1B in tumours with Gleason grade 3 and lower was relatively weak (Dizeyi et al., 2004). These results indicate that aggressive prostate cancers with higher Gleason grades have greater 5HT receptor expression. $5HT_{1A}$ and $5HT_{1B}$ receptors were also identified in BPH tissue (Dizeyi et al., 2004). $5HT_{1D}$ receptors were localised in vascular endothelial cells of both benign and malignant tissue (Dizeyi et al., 2004). Western blot analysis demonstrated 5HT receptor 1A, 1B and 1D proteins in benign and malignant prostate tissue (Dizeyi et al., 2004). Among the cell lines, $5HTR_{1A}$ protein was the only protein that could be demonstrated, and only in the PC3 cell line. RT-PCR demonstrated $5HTR_{1A}$ mRNA expression in all three cell lines and in hPCPs cells (Dizeyi et al., 2004).

Sub-type 1 5HT receptors exhibit a substantial similarity to adrenergic receptors (alpha 1,2 and beta 1,2) and this is why a number of adrenergic agents, including propranolol and pindolol bind to the $5HT_{1A}$ receptor (Abdul et al., 1994).

Furthermore, a $5HT_{1A}$ antagonist, Pindobind, was shown to be a potent antagonist of both beta-$_2$ adrenergic receptor and $5HT_{1A}$ receptor activity (Fenrick et al., 1996).

1.5 5HT; as a growth factor in non-tumoural and tumoural cells

5HT acts as a growth factor (Seuwen & Pouyssegur, 1990) on several types of non-tumoural and tumoural cells. 5HT is a mitogen for fibroblasts, vascular smooth muscle cells, renal mesangial cells, hepatocytes, jejunal crypt cells (Ishizuka et al., 1992; Nemecek et al., 1986; Takuwa et al., 1989; Vicaut et al., 2000) and normal as well as transformed intestinal epithelial cells (Abdul et al., 1994). Its effect may be either direct or through synergy with other growth factors (Seuwen et al., 1990) and involves inhibition of adenyl cyclase or activation of pathways involving phospholipase C or a pertussis toxin sensitive G protein (Vicaut et al., 2000). 5HT stimulated smooth muscle cell mitogenesis to the same extent as human platelet-derived growth factor (PDGF) (Nemecek et al., 1986). When smooth muscle cells were incubated with sub-maximal concentrations of 5HT and PDGF, synergistic rather than additive mitogenic responses were observed. Thus, 5HT has a significant mitogenic effect on smooth muscle cells in vitro, which appears to be mediated by specific plasma membrane receptors (Nemecek et al., 1986).

Several contradictory effects of 5HT on tumour growth have been reported (Seuwen et al., 1990). On one hand, 5HT is regarded as a growth factor for several types of non-tumoural and tumoural cells. In this context, it has been proposed to take part in the autocrine loops of growth factors contributing to cell proliferation in aggressive tumours such as small cell lung carcinoma and colonic carcinoma (Cattaneo et al., 1993; Cattaneo et al., 1995). It has been reported that either $5HT_1$ or $5HT_2$ receptor antagonists inhibit tumour growth (Vicaut et al., 2000). In contrast, others report that 5HT and $5HT_2$ agonist also inhibit tumour growth (Rudzit et al., 1970; Vicaut et al., 2000). Most often this

effect of 5HT, $5HT_2$ and to lesser extent $5HT_1$ agonists has been considered to be related to the specific vasoconstrictor action on the vessels irrigating the tumour, as shown by intravital microscopy (Rudzit et al., 1970; Vicaut et al., 2000). In addition, 5HT has been shown to be involved in the effects of anticancer treatments associated with the reduction of tumour flow (Vicaut et al., 2000). Several other anticancer agents with experimental or clinical activity are associated with the reduction in tumour blood flow such as flavone-8-acetic acid (FAA), vincristine, vinblastine and colchicine (Vicaut et al., 2000).

5HT has growth stimulatory activity in carcinoid valve disease (Rajamannan et al., 2001), pancreatic carcinoid cells (Ishizuka et al., 1992), small cell lung carcinoma cells (Cattaneo et al., 1993; Cattaneo et al., 1995; Codignola et al., 1993), and colonic carcinoma (Dolezel et al., 1969; Tutton & Barkla, 1978; Tutton & Barkla, 1982; Tutton & Steel, 1979). Elevated serum 5HT is associated with carcinoid heart disease, the hallmark of which is valvular thickening. 5HT is a powerful mitogen for valvular subendocardial cells. The mitogenic effect is at least partly mediated via $5HT_{1B}$ receptors. Subendothelial cell proliferation is significantly elevated in human carcinoid valves in vivo. This data suggest a mechanism whereby 5HT may contribute to valvular proliferation in carcinoid heart disease. It is postulated that 5HT has a direct mitogenic effect on cardiac valvular subendocardial cells, and that this effect is mediated by 5HT receptors (Rajamannan et al., 2001).

5HT stimulated growth of human pancreatic carcinoid cells (BON cells). It stimulated phosphatidylinositol (PI) hydrolysis and inhibited cyclic AMP production in a dose-dependent fashion. The $5HT_{1A/1B}$ receptor antagonist,

SDZ 21-009, prevented the reduction of cyclic AMP production evoked by 5HT and inhibited the mitogenic action of 5HT. The $5HT_{1C/2}$ receptor antagonist, mesulergine, competitively inhibited PI hydrolysis, but did not affect the mitogenic action of 5HT. These results suggest that 5HT is an autocrine growth factor for BON cells and that the mitogenic mechanism of 5HT involves receptor-mediated inhibition of the production of cyclic AMP (cAMP) (Ishizuka et al., 1992).

The mitogenic effect of 5HT on human small cell lung carcinoma (SCLC) is at least partly due to stimulation of a $5HT_{1D}$ receptor type. The $5HT_{1A}$ receptor agonist (8-OH-DPAT) is also capable of stimulating [3H]thymidine incorporation into the SCLC cell line GLC-8, although with lower efficacy than 5HT. The simultaneous administration of maximal doses of $5HT_{1A}$ and the $5HT_{1D}$ receptor agonist sumatriptan reproduced the maximal [3H]thymidine incorporation observed with 5HT alone. The $5HT_{1A}$ receptor antagonists piperone and SDZ 216-525 completely abolished the effect of 8-OH-DPAT. Accordingly, the two drugs partially inhibited the mitogenic effect of 5HT. Therefore, the mitogenic effect of 5HT in SCLC cells involves both $5HT_{1A}$ and $5HT_{1D}$ receptor types (Cattaneo et al., 1995). Hyper-serotoninaemia has also been observed very early in some patients suffering from small cell lung carcinoma (Vicaut et al., 2000).

Interesting observations were made by Pratesi et al who reported that a high dose of 5HT (200 μgm/ daily) increased the growth of SCLC cells grafted in athymic nude mice, whereas a low dose (20 μgm/ daily) inhibited tumour growth (Vicaut et al., 2000). This observation led to the hypothesis that high doses of 5HT exert a direct mitogenic effect on tumour cells whereas low

doses of 5HT reduce tumour growth by decreasing the oxygen tension and blood supply to the tumours.

Two specific inhibitors of 5HT uptake, citalopram and fluoxetine, were examined for their effects on cell proliferation and tumour growth. Each of the agents was found to suppress cell division in dimethylhydrazine-induced colonic tumours in rats, and to retard the growth of 2 out of 3 lines of human colonic tumours propagated as xenografts in immune-deprived mice (Tutton et al., 1982). In another study the anti-serotoninergic drug BW 501C caused short-term suppression of tumour growth in xenografts in immune-deprived mice (Tutton et al., 1979).

5HT stimulates aldosterone secretion in aldosterone-producing adrenocortical adenomas through activation of $5HT_4$ receptors (Lefebvre et al., 2002). Eight patients with an aldosteronoma received a single oral dose of placebo or $5HT_4$ receptor agonist (cisapride 10 mg). Cisapride administration significantly increased plasma aldosterone within 120 min without any significant change in renin, cortisol or potassium levels. The effects of 5HT and selective $5HT_4$ ligands on aldosterone production from aldosteronoma tissues were studied in vitro. 5HT alone and the $5HT_4$ receptor agonist cisapride both stimulated aldosterone secretion from aldosteronoma slices. 5HT and cisapride-evoked aldosterone responses were inhibited by concomitant administration of the specific $5HT_4$ receptor antagonist GR 113808. PCR amplification revealed the expression of $5HT_4$ receptor mRNA in 13 of 14 aldosteronomas studied. Taken together, this data shows that most aldosteronomas express functional $5HT_4$ receptors. Results also suggest that 5HT, which can be locally released

by intra-tumoural mast cells, plays a role in the pathophysiology of these tumours (Lefebvre et al., 2002).

5HT causes parathyroid hormone (PTH) hyper-secretion in parathyroid adenomas (Zimmerman et al., 1980). Incubation with 5HT elicited a marked increase of 90-150% in cAMP content in slices of parathyroid adenoma tissue. Stimulation by 5HT was dose-dependent, with the highest stimulation being achieved at 10^{-4} M. The 5HT antagonists, methylsergide and cinanserin, blocked the stimulatory effect of 5HT on cAMP increase. These observations demonstrate that parathyroid adenoma tissue has a high content of 5HT, which stimulates cAMP accumulation in this tissue. Since cAMP acts as a mediator of PTH release, 5HT could be one of the factors regulating PTH hyper-secretion in parathyroid adenomas.

Platelets appear to be involved in tumour cell lodgement, since thrombocytopenia significantly reduces the number of lodged tumour cells. Platelets activated by tumour cells are known to release 5HT which in turn plays a role in tumour cell lodgement and metastasis (Skolnik et al., 1985).

In vitro proliferative effects of 5HT and anti-proliferative effect of $5HT_{1A}$ antagonists on the growth of PC3 and DU145 cells (androgen-independent prostate cancer) as well as LNCaP cells (androgen-sensitive prostate cancer) have been described (Abdul et al., 1994; Dizeyi et al., 2004). $5HT_{1A/1B}$ receptor antagonist (SDZ 21-009) and a $5HT_{1A}$ receptor antagonist (Pindobind) inhibited PC3 cell growth by 40% and 90%, respectively at a concentration of 10^{-5}M at 5 days (Abdul et al., 1994). In vivo growth inhibition of PC3 cells has also been shown in subcutaneous xenografts in athymic nude mice (Abdul et al., 1994). Radioligand binding studies indicated the

presence of 5HT binding sites on all three cell lines (PC3, DU145 and LNCaP) (Abdul et al., 1994; Dizeyi et al., 2004). The effect of three 5HT uptake inhibitors (6-nitroquipazine, zimelidine and fluoxetine) on cell growth was also studied (Abdul et al., 1995). All three drugs caused inhibition of PC3, DU145 and LNCaP in a dose-dependent manner. The growth of subcutaneous PC3 xenografts in athymic nude mice was also significantly inhibited by fluoxetine (Abdul et al., 1995). In a recent study performed by Dizeyi et al, NAN-190 hydrobromide, a $5HT_{1A}$ antagonist significantly inhibited the proliferation of PC3 and DU145 cells, but inhibited LNCaP and hPCPs less significantly, thus indicating that $5HT_{1A}$ antagonists have a more pronounced growth inhibitory effect on androgen- independent prostate cancer cells. A maximum effect was seen at a concentration of 10^{-6}M. 5HT significantly stimulated growth of PC3, DU145, LNCaP and hPCPs cells in a dose-dependent manner with a maximum effect at 10^{-8}M (Dizeyi et al., 2004).

1.6 Neuroendocrine differentiation

Until recently the available publications on neuroendocrine differentiation in the prostate were few compared with the voluminous literature related to neuroendocrine cells in other organs (e.g. the gastrointestinal tract and lung). This situation has begun to change, with a marked increase in the number of publications related to NE differentiation in prostate carcinoma.

Endocrine-paracrine cells of the prostate, also known as APUD (amine precursor uptake and decarboxlation) or neuroendocrine cells (NE), constitute in addition to the basal and exocrine secretory cells, a third population of highly specialized epithelial cells in the prostate gland (di Sant'Agnese, 1992a;

di Sant'Agnese, 1992b; di Sant'Agnese, 1998a; di Sant'Agnese, 1998b; di Sant'Agnese & Cockett, 1994; di Sant'Agnese & Cockett, 1996; di Sant'Agnese & Mesy Jensen, 1987). These endocrine-paracrine cells are located in all regions of the human prostate at birth, but rapidly decrease in the peripheral prostate after birth and then reappear at puberty (di Sant'Agnese et al., 1987). After puberty, the number of NE cells seems to increase until an apparently optimum level is reached, which persists from 25 to 54 years old (Battaglia et al., 1994).

There are markedly fewer NE cells and NE secretory products in mature nodules of BPH. However, it was also noted that small, proliferating hyperplastic nodules, and what appear to be growth foci in somewhat larger nodules, contained abundant NE cells (Cockett et al., 1993). NE differentiation occurs in virtually all common prostatic adenocarcinomas (Abrahamsson, 1996; Bonkhoff & Fixemer, 2004).

NE cells are further characterised by the presence of cytoplasmic dense core granules, and ultra-structural studies have shown these constitutive NE cell organelles to be markedly heterogeneous in size and form, suggesting the existence of several cell variants (Abrahamsson, 1996; Abrahamsson et al., 1986).

NE cells contain and most likely secrete, 5HT and calcitonin, as well as variety of other peptides (di Sant'Agnese et al., 1994). Little is known of the functional role of these cells, but they probably have a paracrine or local regulatory role. These endocrine-paracrine cells most likely regulate growth and differentiation, as well as the secretory functions of the mature prostate gland (di Sant'Agnese et al., 1994). NE cells release growth factors including

neuropeptides (somatostatin and bombesin) and biogenic amines (5HT), which act on the adjacent (exocrine) tumour cells and thus are related to tumour growth, differentiation, androgen independence, angiogenesis and poor prognosis (Abrahamsson, 1996; Bonkhoff et al., 2004; di Sant'Agnese, 1992b; di Sant'Agnese, 1998b; Hansson & Abrahamsson, 2001). Another important product of prostatic NE cells is chromogranin A. Increased serum levels of chromogranin A were found in patients with androgen-insensitive prostate tumours (Cussenot et al., 1996).

NE differentiation in prostate carcinoma is a frequent occurrence and manifests itself in several forms, including (1) small cell carcinoma, (2) carcinoid and carcinoid-like tumours, and, (3) conventional adenocarcinoma with focal NE differentiation. This latter pattern is the most common, and there is evidence that all or nearly all prostatic adenocarcinomas show at least some focal NE differentiation (di Sant'Agnese, 1992b; di Sant'Agnese, 1998b). NE differentiation leads to tumour progression and poor prognosis (Dizeyi et al., 2004). Data indicates an increase in NE cells in malignant prostate tissue (Dizeyi et al., 2004). Results also indicate that a substantial proportion of prostate cancer metastases contain a subpopulation of cells expressing a NE phenotype similar to primary tumours (Aprikian et al., 1993; Aprikian et al., 1994).

NE differentiation has also been reported in bladder cancer (Acs et al., 2000; Amichetti et al., 1992; Blomjous et al., 1988; Blomjous et al., 1989; Chin et al., 1992; Dundr et al., 2003; Grignon et al., 1992; Helpap & Kloppel, 2002; Lertprasertsuke & Tsutsumi, 1991; Martignoni & Eble, 2003; Mazzucchelli et al., 1992; Mills et al., 1987; Swanson et al., 1988; van Hoeven & Artymyshyn,

1996; Vincendeau et al., 2003), with positive immunohistochemistry for 5HT (Lertprasertsuke et al., 1991; Mills et al., 1987). The carcinomas of the urinary bladder in which NE differentiation has been found include small cell undifferentiated carcinoma (SCUC), giant cell carcinoma (GCC), lymphoepithelioma-like carcinoma (LELC), and large cell NE carcinoma (LCNEC) (Dundr et al., 2003). These tumours are either pure or can be associated with other components, such as transitional cell carcinoma, squamous cell carcinoma and adenocarcinoma (Dundr et al., 2003). Bladder cancers with NE differentiation are usually aggressive tumours with poor prognosis. It is essential that we obtain a better understanding of NE differentiation in both prostate and bladder cancer in order to treat them effectively.

1.7 Alpha-adrenoreceptor antagonists

Alpha$_1$-adrenoceptor antagonists possess an affinity for and thus bind to the alpha$_1$ receptors. They also bind to alpha$_2$, beta$_1$ or beta$_2$ receptors, but with a lower affinity. These antagonists maybe quinazoline based (doxazosin and terazosin) or sulphonamide-based (tamsulosin). Alpha$_1$-adrenoceptor antagonists, have been documented to inhibit growth and induce apoptosis in malignant prostate cells (Anglin et al., 2002; Benning & Kyprianou, 2002; Cuellar et al., 2002; Glassman et al., 2001; Keledjian et al., 2001; Keledjian & Kyprianou, 2003; Kyprianou, 2000; Kyprianou, 2003; Kyprianou & Benning, 2000a; Kyprianou et al., 2000b; Kyprianou & Jacobs, 2000c; Liu et al., 2004; Partin et al., 2003; Tahmatzopoulos & Kyprianou, 2004a; Tahmatzopoulos et al., 2004b; Xu, Wang et al., 2003a; Xu et al., 2003b). Recent evidence

suggests that the quinazoline-based alpha$_1$-adrenoceptor antagonists, doxazosin and terazosin, exhibit a potent apoptotic effect against prostate tumour epithelial cells, whereas tamsulosin, a sulphonamide-based alpha$_1$-adrenoceptor antagonist, was ineffective in inducing a similar apoptotic effect against prostate cells (Anglin et al., 2002; Benning et al., 2002; Kyprianou, 2000; Kyprianou, 2003; Kyprianou et al., 2000a; Kyprianou et al., 2000b; Kyprianou et al., 2000c; Partin et al., 2003). Furthermore, an in vivo efficacy trial demonstrated that doxazosin administration (at tolerated pharmacologically relevant doses) in SCID mice bearing PC3 prostate cancer xenografts resulted in a significant inhibition of tumour growth (Kyprianou et al., 2000a). Doxazosin and terazosin affect prostate growth via an alpha$_1$-adrenoceptor-independent action. The apoptotic activity alpha$_1$-adrenoceptor antagonists (doxazosin and terazosin) against prostate cancer cells is independent of: (a) their capacity to antagonise alpha$_1$-adrenoceptors; and (b) the hormone sensitivity status of the cells (Anglin et al., 2002; Benning et al., 2002; Kyprianou, 2000; Kyprianou, 2003; Kyprianou et al., 2000a; Kyprianou et al., 2000c; Partin et al., 2003). It has been suggested that the growth inhibitory effect is possibly mediated via the upregulation of TGF-β_1 (Anglin et al., 2002; Glassman et al., 2001; Kyprianou, 2003; Partin et al., 2003). Transforming growth factor-β (TGF-β) is a major regulator of the dynamics of prostate growth by inhibiting cell proliferation, inducing apoptosis and regulating cell migration. (Tahmatzopoulos et al., 2004a). TGF-β_1 exerts anti-apoptotic effects by binding to its high affinity TβRII. TβRII then interacts with TβRI to initiate a signalling cascade, via which it exerts its mechanism of

action. TGF-β_1 inhibits target cells from entering the S phase of the cell cycle via induction of key cell cycle regulators (Glassman et al., 2001).

It was 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, suggesting that the terazosin-induced cell death was independent of p53 and Rb (Xu et al., 2003b). However, the 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 also observed indicating that these two apoptotic regulators may play important roles in terazosin-mediated cell death pathway (Xu et al., 2003b).

Another study reported that Bcl-2 overexpression in prostate cancer cells exerts an antagonistic effect against the quinazoline - mediated apoptotic effect by suppressing cell attachment to gelatine matrix without effecting cell invasion (Keledjian et al., 2003). Growth factors such as transforming growth factor (TGF), basic fibroblast growth factor (BFGF) and VEGF, contribute to the angiogenic resonse of tumours via the modulation of integrin expression (Keledjian et al., 2003).

Alpha$_1$-adrenoceptor antagonists enhance the apoptotic effect of ionizing radiation against human prostate cancer cells (Cuellar et al., 2002). It has also been proven that they decrease the vascularity of prostate tumours (Keledjian et al., 2001; Keledjian et al., 2003).

Studies continue to investigate the exact mechanism by which alpha-adrenoceptor antagonists inhibit prostate cancer cell growth.

5HT receptors of subtype 1 exhibit a substantial similarity to adrenergic receptors (alpha$_1$, alpha$_2$, beta$_1$ and beta$_2$) and this is why a number of

adrenergic agents, including propranolol and pindolol bind to the $5HTR_{1A}$ receptor with a high affinity (Abdul et al., 1994). Furthermore, a $5HT_{1A}$ antagonist, Pindobind, was shown to be an antagonist of both $beta_2$ adrenergic receptor and $5HT_{1A}$ receptor activity (Fenrick et al., 1996).

1.8 Other agents inhibiting prostate and bladder cancer cell growth

Researchers have been looking into the effect of various agents on prostate and bladder cancer cell growth in an attempt to find an agent which can be used as an effective anti-neoplastic drug. The apoptotic effect of alpha-adrenoceptor antagonists (Anglin et al., 2002; Benning et al., 2002; Cuellar et al., 2002; Glassman et al., 2001; Keledjian et al., 2001; Keledjian et al., 2003; Kyprianou, 2000; Kyprianou, 2003; Kyprianou et al., 2000a; Kyprianou et al., 2000b; Kyprianou et al., 2000c; Liu et al., 2004; Partin et al., 2003) has already been discussed. A number of other agents have been studied.

Fluroquinolone antibiotics such as ciprofloxacin and ofloxacin cause a dose- and time-dependent inhibition of transitional cell carcinoma of the bladder and inhibit DNA synthesis at concentrations that are easily attainable in the urine of patients(Aranha et al., 2000; Seay et al., 1996; Zehavi-Willner & Shalit, 1992). The mechanism of this effect is not clear but research continues. Nitrofurantoin is another antibiotic found to inhibit bladder cancer cell growth(Bulbul et al., 1985). A selective 5HT reuptake inhibitor (Fluoxetine) inhibits the growth of bladder cancer cells(Bendele et al., 1992; Tang et al., 2001).

Non steroidal anti-inflammatory agents have been shown to inhibit the growth of both prostate and bladder cancer cell growth. Ibuprofen inhibits bladder

cancer cell growth in vitro (Khwaja et al., 2004), however more selective cyclooxygenase (Cox-1 and Cox-2) inhibitors have shown to inhibit the proliferation of both prostate and bladder cancer cells (Farivar-Mohseni et al., 2004; Grubbs et al., 2000; Mohseni et al., 2004; Pommery et al., 2004) in vitro and in experimental animals (Grubbs et al., 2000). Lipoxygenase (LOX) inhibitors have shown to have a similar response in bladder cancer cells (Ikemoto et al., 2004; Matsuyama et al., 2004). Several molecular mechanisms have been postulated to explain the anticancer effects of NSAIDs. These drugs do not only involve the inhibition of cyclooxygenase-2, and a more proximate initiator molecule may be regulated by NSAIDs to inhibit growth (Grubbs et al., 2000). Retinoic acid antagonists inhibit the growth of prostate (Keedwell et al., 2004) and bladder cancer cells (Wang et al., 2000; Zou et al., 2005).

Other agents that inhibit the proliferation of prostate cancer cells are imatinib (Kubler et al., 2004), resveratrol (Kim et al., 2003), liver X receptor agonist (Fukuchi et al., 2004), endothelin receptor antagonist (Lassiter & Carducci, 2003), avocado extract(Lu et al., 2005), epiguard (Lu et al., 2004), hypericum perforatum methanolic (Martarelli et al., 2004) and pygeum africanum extracts (Santa Maria et al., 2003), anandamide (Mimeault et al., 2003), oligonucleotides (Rubenstein et al., 2003), organoselenium compounds (Shi et al., 2003), inositol hexaphosphate (Singh et al., 2004), acacetin (Singh et al., 2005) and branched chain fatty acids (Yang et al., 2003).

Other agents documented to inhibit the proliferation of bladder cancer cells are epigallocatechin gallate (found in green tea) (Chen et al., 2004; Kemberling et al., 2003; Yu et al., 2004), flavopiridol (Chien et al., 1999),

phenylretinamide (Clifford et al., 2001), p21 adenovirus (Hall et al., 2000), ganoderma lucidum extract (Lu et al., 2004), platinum analogs (Niell et al., 1985), N-trifluroacetyladriamycin-14 valerate (Niell et al., 1987), linoleic acid (Oh, Lee, Cho, Lee, Jung & Park, 2003), essiac (Ottenweller et al., 2004), carboxyamido-triazole (Perabo et al., 2004), OK-432 (an immunomodulating agent like BCG) (Sakano et al., 1997), suramin (Sandgren & Belting, 2003; Walther et al., 1994), N-butyl-N-(4-hydroxybutyl) nitrosamine (found in green tea) (Sato, 1999), tyrosine kinase inhibitors (Sion-Vardy et al., 1995), arsenic trioxide (Tong et al., 2001) and honey (Swellam et al., 2003).

1.9 Hypothesis

Neuroendocrine differentiation takes place in prostate cancer tissue, especially after long-term anti-androgen therapy and thus the treatment options for androgen independent prostate cancer are limited (di Sant'Agnese, 1992a; di Sant'Agnese, 1992b; di Sant'Agnese, 1998a; di Sant'Agnese, 1998b; di Sant'Agnese & Cockett, 1994; di Sant'Agnese & Cockett, 1996; di Sant'Agnese & Mesy Jensen, 1987). NE differentiation has also been reported in bladder cancer (Acs et al., 2000; Amichetti et al., 1992; Blomjous et al., 1988; Blomjous et al., 1989; Chin et al., 1992; Dundr et al., 2003; Grignon et al., 1992; Helpap et al., 2002; Lertprasertsuke et al., 1991; Martignoni et al., 2003; Mazzucchelli et al., 1992; Mills et al., 1987; Swanson et al., 1988; van Hoeven et al., 1996; Vincendeau et al., 2003). NE cells release growth factors including neuropeptides (somatostatin and bombesin) and biogenic amines (5HT) which act on the adjacent (exocrine) tumour cells and thus correlate to tumour growth, differentiation, androgen independence, angiogenesis and

poor prognosis (Abrahamsson, 1996; Bonkhoff et al., 2004; di Sant'Agnese, 1992b; di Sant'Agnese, 1998b; Hansson et al., 2001). Positive immuno-staining for 5HT has been demonstrated in both prostate and bladder cancer tissue (Dizeyi et al., 2004; Lertprasertsuke et al., 1991; Mills et al., 1987).

Based on the fact that 5HT acts as a growth factor for several types of non-tumoural and tumoural cells, we investigate the effect of 5HT on the growth of PC3 cells (androgen independent prostate cancer) and HT1376 cells (grade III transitional cell carcinoma of the bladder) using crystal violet proliferation studies. Using the same technique, we also determine the effect of various 5HT receptor sub type antagonists on PC3 and HT1376 cell growth. We seek to prove the hypothesis that 5HT shall cause proliferation of prostate and bladder cancer cell growth and 5HT antagonists shall inhibit the growth.

Using techniques of immunohistochemistry and western blotting, we examine the 5HT receptor profile in both human prostate and bladder cancer cells and tissue. Apoptosis and cell cycle arrest in prostate cancer cells treated with 5HT antagonists are examined using FACS analysis.

Doxazosin, an alpha$_1$-adrenoceptor antagonist, commonly used for the treatment of benign prostatic hyperplasia (BPH) and hypertension, has recently been shown to inhibit prostate cancer cell growth (Anglin et al., 2002; Benning et al., 2002; Cuellar et al., 2002; Glassman et al., 2001; Keledjian et al., 2001; Keledjian et al., 2003; Kyprianou, 2000; Kyprianou, 2003; Kyprianou et al., 2000a; Kyprianou et al., 2000b; Kyprianou et al., 2000c; Liu et al., 2004; Partin et al., 2003). This inhibitory effect is independent of the direct action of alpha-receptor blockade by the drug (Anglin et al., 2002; Benning et al., 2002; Kyprianou, 2000; Kyprianou, 2003; Kyprianou et al., 2000a; Kyprianou et al.,

2000c; Partin et al., 2003). Using the crystal violet proliferation assay technique, we aim to confirm the inhibitory effect of doxazosin on prostate cancer cell growth and extend this study to examine the effect of this drug on bladder cancer cell growth.

Doxazosin inhibits 5HT-induced shape change in platelets, via the $5HT_2$ receptor (Jagroop et al., 2001). It significantly inhibits 5HT-mediated contractions in the rabbit detrusor (Khan et al., 2000; Khan et al., 2001) through $5HT_3$ receptor inhibition. Autoradiographic evidence suggests that doxazosin reduces 5HT binding in the rabbit detrusor (Khan et al., 2000). The beneficial effects of doxazosin in bladder outflow obstruction may be due, at least in part, to 5HT antagonism (Khan et al., 2000).

Based on these observations, it is possible that the growth inhibitory effect of doxazosin on prostate and bladder cancer cells is mediated via 5HT receptors. Using crystal violet proliferation studies, we aim to prove this hypothesis.

CHAPTER 2

IN VITRO PROLIFERATION STUDIES OF PROSTATE CANCER (PC3) AND BLADDER CANCER (HT1376) CELL LINES WITH 5HT AND 5HT ANTAGONISTS

2.1 Introduction

Cell proliferation assays are essential to develop an understanding of the molecular mechanisms that modulate cell growth and differentiation.

From the very beginning, scientists tried to develop tests to assess the efficacy of anticancer treatment. One of the greatest achievements in cancer research was the brilliant work of R. Schrek in the 1960s. In his published work, there are obvious clues to a practicable testing method, to improve clinical research and drug selection in cancer treatment. But timing is everything, the drugs available in the 1960s were not very effective, and no one appreciated the importance of *apoptosis* (programmed cell death) (www.weisenthal.org).

In the early 80s, cancer was considered to be a disease in which the primary problem was disordered cell division and proliferation (www.weisenthal.org). There are numerous techniques available today to determine cell proliferation and death; for example the MTT assay, thymidine assay, alamar Blue assay and crystal violet assay. We used the crystal violet assay for our experiments which is based on the principle that crystal violet is only incorporated into living cells. The optical density of the crystal violet eluted by the cells after treatment with acetic acid is read using a spectrophotometric plate reader, providing an assessment of cell viability when compared with control.

In this chapter, we investigate the effect of 5HT on the growth of PC3 cells (androgen independent prostate cancer) and HT1376 cells (grade III transitional cell carcinoma of the bladder) using crystal violet studies. Using the same technique, we also look into the effect of various 5HT receptor sub-type antagonists on PC3 and HT1376 cell growth. We intend to determine

whether 5HT causes proliferation of prostate and bladder cancer cells and if 5HT antagonists inhibit their growth.

2.2 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% Fetal Bovine Serum (FBS) and 1% Antibiotic Antimycotic Solution.

The HT1376 cells were maintained in Minimum Essential Medium Eagle supplemented with 8% Fetal Bovine Serum (FBS), 1% Antibiotic Antimycotic Solution and 1% MEM Non Essential Amino Acid Solution .

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

2.3 Reagents

5HT (serotonin creatinine sulphate complex) was purchased from Sigma-Aldrich Company Ltd (Dorset, UK). 5HT antagonists; (NAN-190 hydrobromide; $5HT_{1A}$), (SB224289 hydrochloride; $5HT_{1B}$), (Ketansarin; $5HT_{1D}$ & $5HT_2$), (Y25130 hydrochloride; $5HT_3$) and (RS 23597-190 hydrochloride; $5HT_4$) were purchased from Tocris Laboratories (Bristol, UK).

The Nutrient Mixture F-12 Ham Medium (for PC3 cells), Minimum Essential Medium Eagle (for HT1376 cells), Fetal Bovine Serum (FBS), Dimethyl

Sulfoxide (DMSO), MEM Non Essential Amino Acid Solution and Antibiotic Antimycotic Solution were all purchased from Sigma-Aldrich Company Ltd (Dorset, UK). Dulbecco's Phosphate buffered saline (PBS) was used for washing the cells and distilled water was used as a control for the experiments.

2.4 In Vitro Proliferation Assay

Prostate cancer cells were seeded in a 96 well plate, at 5000 cells per well in 100 μl serum containing medium and incubated at 37°C. After 24 h, 10 μl of the serum containing medium was removed and replaced with 10 μl of 5HT or 5HT antagonist ($5HT_{1A}$, $5HT_{1B}$, $5HT_{2/1D}$, $5HT_3$, $5HT_4$) solutions at varying concentrations prepared in distilled water. A cell proliferation study was carried out and changes in cell number quantified using a crystal violet colorimetric assay, 72 h after adding the drugs.

Proliferation studies were carried out at 24, 48 and 72 h, after addition of 5HT. In brief, a solution containing 0.5 g of crystal violet, 0.85 g of NaCl, 5 ml of 10% formal saline (0.5 ml of formaldehyde and 4.5 ml of normal saline), 50 ml of absolute ethanol, 45 ml of distilled water was prepared. 100 μl of medium was gently aspirated from each well and replaced by an equal volume of the colorimetric assay mixture 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 colour from cells and changes in optical density read at 570 nm using a spectrophotometric plate reader. Cell viability was expressed

as a percentage of the control OD value, where control OD values were considered 100 %.

2.5 Statistical analysis

Each proliferation assay experiment was repeated an average of three times, each with quadruple samples. Data analysis was performed using Microsoft Excel XP and SPSS Graphpad software. Repeated measure analysis of variance test was carried out to assess the effect of drug concentrations within the groups. When an overall difference among the drug concentrations was identified, Dunnett's Post Hoc comparison tests were applied in order to assess the difference between various concentrations of a drug and the control. Statistical significance was accepted at $P<0.05$

2.6 Results

5HT caused proliferation of PC3 cells at 24, 48 and 72 h. A maximum proliferation of 15% ($n = 12$, $P < 0.0001$) was seen at a concentration of 10^{-8} M at 72 h, as compared to control (Figure 1, Tables 1,2 & 3).

$5HT_{1A}$ (NAN-190 hydrobromide) and $5HT_{1B}$ (SB224289 hydrochloride) antagonists had a dose-dependent inhibitory effect on the growth of PC3 cells at 72 h. At a concentration of 10^{-4} M, $5HT_{1A}$ and $5HT_{1B}$ antagonists had a 20% ($n = 12$, $P < 0.0001$) and 78% ($n = 12$, $P < 0.0001$) inhibitory effect on PC3 cell growth, respectively as compared to control (Figures 2 & 3, Tables 4 & 5). Thus, SB224289 hydrochloride, a selective $5HT_{1B}$ receptor antagonist, had a greater inhibitory effect on PC3 cell growth as compared to a $5HT_{1A}$

antagonist. The IC_{50} value for the $5HT_{1B}$ antagonist (SB224289 hydrochloride) is 3.23×10^{-6} (Figure 4).

The remaining 5HT receptor subtype antagonists ($5HT_{1D/2}$ (Ketansarin), $5HT_3$ (Y25130 hydrochloride) and $5HT_4$ (RS 23597-190 hydrochloride) had no significant inhibitory effect on cell growth ($5HT1_{D/2}$ $P = 0.439$, $5HT_3$ $P = 0.168$ and $5HT_4$ $P = 0.106$)(Figures 5,6 & 7, Tables 6,7 & 8).

All the above experiments were performed in quadruples and were repeated at least three times.

The effect of 5HT and various 5HT receptor antagonists on HT1376 (grade III transitional cell carcinoma bladder) cell growth was investigated. 5HT caused proliferation of HT1376 cell growth at 24, 48 and 72 h. A maximum proliferation of 12% ($n = 12$, $P < 0.0001$) was seen at a concentration of 10^{-8} M at 72 h, as compared to controls (Figure 8, Tables 9,10 & 11).

$5HT_{1A}$ (NAN-190 hydrobromide) and $5HT_{1B}$ (SB224289 hydrochloride) antagonists had a dose-dependent inhibitory effect on the growth of HT1376 cells at 72 h, as compared to controls. At a concentration of 10^{-4} M, $5HT_{1A}$ and $5HT_{1B}$ antagonists had a 10% ($n = 12$, $P < 0.0001$) and 93% ($n = 12$, $P < 0.0001$) inhibitory effect, respectively, on HT1376 cell growth (Figures 9 & 10, Tables 12 & 13). The IC_{50} value for the $5HT_{1B}$ antagonist (SB224289 hydrochloride) is calculated at 1.85×10^{-6} (Figure 11).The other 5HT-receptor subtype antagonists ($5HT_{1D/2}$ (Ketansarin), $5HT_3$ (Y25130 hydrochloride) and $5HT_4$ (RS 23597-190 hydrochloride) had no significant inhibitory effect on cell growth ($5HT_{1D/2}$ $P = 0.219$, $5HT_3$ $P = 0.805$ and $5HT_4$ $P = 0.677$) (Figures 12, 13 &14, Tables 14, 15 & 16).

Experiments were performed in quadruples and were repeated at least three times.

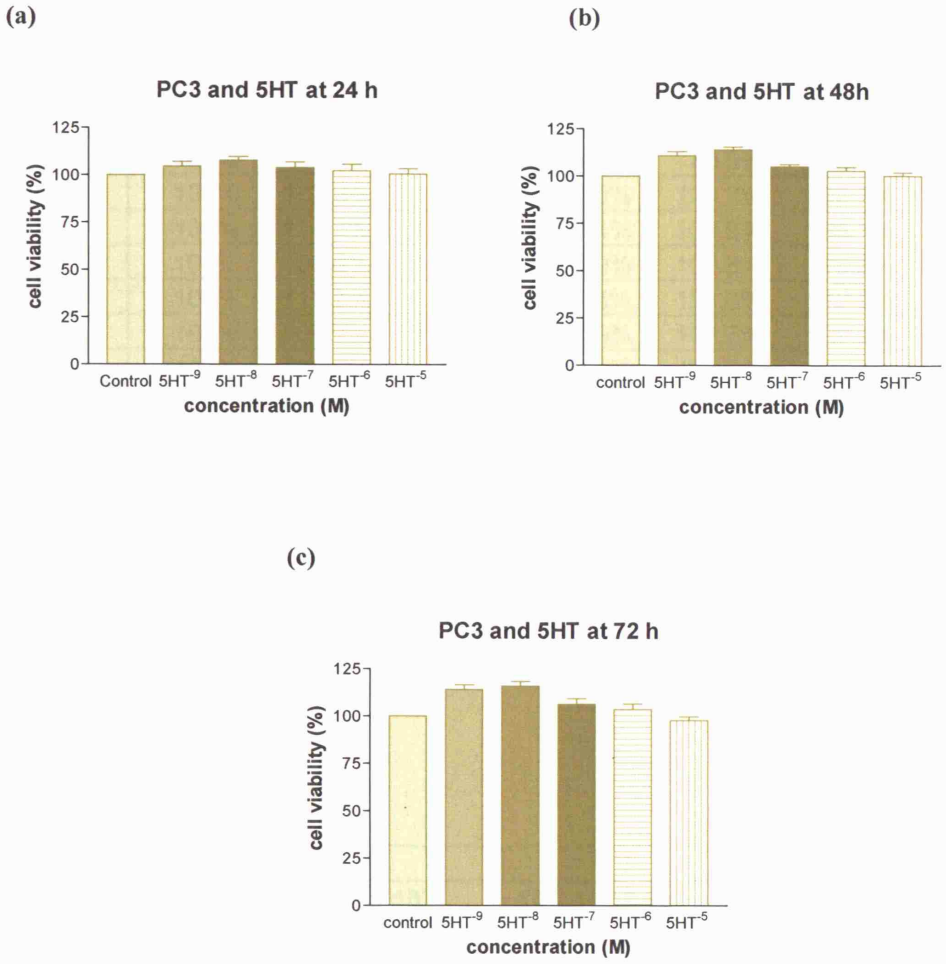


Figure 1: An increase in growth proliferation of PC3 cells by 5HT, at (a) 24 h, (b) 48 h and (c) 72 h, respectively. A maximum effect is seen at a concentration of 10^{-8} M at 72 h. The bars represent the standard error of the mean (SEM).

	24h					
	Control	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}
1	100%	101.0%	105.1%	109.2%	110.2%	106.1%
2	100%	90.9%	95.0%	109.9%	90.9%	90.1%
3	100%	96.9%	98.4%	84.4%	80.5%	85.9%
4	100%	112.6%	114.6%	101.0%	125.2%	108.7%
5	100%	114.7%	106.3%	94.4%	104.9%	95.1%
6	100%	109.6%	117.1%	95.9%	104.8%	96.6%
7	100%	113.7%	114.4%	111.5%	103.6%	112.9%
8	100%	107.7%	110.8%	114.6%	107.7%	109.2%
9	100%	105.5%	109.1%	118.2%	110.9%	106.4%
10	100%	112.1%	113.6%	112.1%	95.5%	115.2%
11	100%	93.5%	105.1%	97.1%	84.8%	93.5%
12	100%	98.6%	103.6%	100.7%	108.6%	87.9%
Mean	100%	104.7%	107.8%	104.1%	102.3%	100.6%
SEM	0	2.4	2.0	2.9	3.6	3.0

Table 1: Percentage cell viability of PC3 cells at 24 h, after treatment with 5HT.

	48h					
	Control	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}
1	100%	124.5%	110.0%	105.5%	100.0%	107.5%
2	100%	100.0%	99.6%	99.1%	93.0%	92.1%
3	100%	104.5%	116.9%	101.0%	105.0%	100.0%
4	100%	109.2%	115.6%	104.6%	113.8%	93.1%
5	100%	106.9%	115.4%	104.3%	97.3%	105.3%
6	100%	106.4%	117.2%	99.5%	96.1%	95.1%
7	100%	110.6%	114.3%	105.3%	107.4%	110.1%
8	100%	107.4%	111.2%	109.8%	98.1%	97.2%
9	100%	117.6%	118.8%	105.3%	110.0%	94.7%
10	100%	120.4%	119.1%	107.6%	108.9%	98.7%
11	100%	109.4%	113.2%	106.9%	100.6%	105.0%
12	100%	115.3%	117.2%	112.7%	105.7%	104.5%
Mean	100%	111.0%	114.0%	105.1%	103.0%	100.3%
SEM	0	2.0	1.5	1.2	1.8	1.7

Table 2: Percentage cell viability of PC3 cells at 48 h, after treatment with 5HT.

	72h					
	Control	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}
1	100%	107.8%	113.5%	93.4%	93.4%	89.2%
2	100%	111.9%	96.7%	95.8%	97.3%	87.8%
3	100%	118.4%	125.1%	112.4%	101.0%	99.7%
4	100%	102.0%	123.5%	108.5%	110.5%	105.8%
5	100%	103.4%	117.7%	113.4%	99.6%	89.2%
6	100%	118.8%	118.8%	120.0%	97.1%	108.3%
7	100%	115.9%	116.8%	90.1%	103.9%	97.0%
8	100%	130.1%	117.5%	106.1%	93.4%	99.1%
9	100%	112.9%	109.6%	117.2%	108.1%	95.2%
10	100%	117.8%	127.2%	103.9%	129.4%	106.1%
11	100%	122.8%	119.2%	113.5%	109.3%	92.7%
12	100%	107.7%	104.8%	102.9%	100.0%	102.4%
Mean	100%	114.1%	115.4%	106.4%	103.6%	97.7%
SEM	0	2.4	2.5	2.8	2.9	2.0

Table 3: Percentage cell viability of PC3 cells at 72 h, after treatment with 5HT.

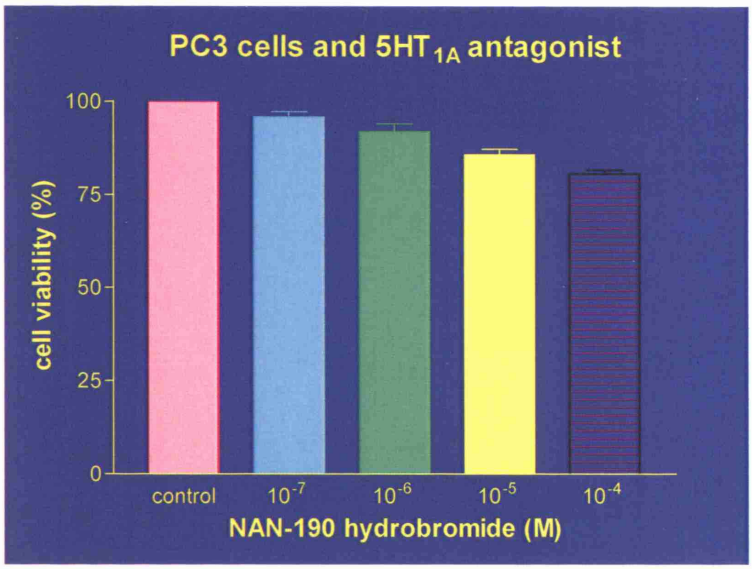


Figure 2: A dose-dependent growth inhibition of PC3 cells by a $5HT_{1A}$ antagonist (NAN-190 hydrobromide) with a maximum effect seen at a concentration of 10^{-4} M at 72 h. The bars represent the standard error of the mean (SEM).

	Control	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
1	100%	96.8%	84.1%	81.8%	79.1%	
2	100%	93.2%	91.2%	83.9%	76.6%	
3	100%	86.7%	88.8%	81.6%	79.6%	
4	100%	103.4%	105.6%	93.3%	79.8%	
5	100%	89.8%	82.9%	77.6%	76.4%	
6	100%	97.7%	95.9%	93.6%	86.8%	
7	100%	91.9%	89.8%	81.3%	85.4%	
8	100%	92.0%	84.3%	84.7%	77.9%	
9	100%	102.7%	96.9%	87.3%	82.7%	
10	100%	100.9%	87.7%	88.6%	81.1%	
11	100%	98.8%	102.1%	90.1%	82.3%	
12	100%	96.0%	94.2%	85.0%	81.0%	
Mean	100%	95.8%	92.0%	85.8%	80.4%	
SEM	0	1.5	2.1	1.4	0.9	

Table 4: Percentage cell viability of PC3 cells at 72 h, after treatment with a $5HT_{1A}$ antagonist (NAN-190 hydrobromide).

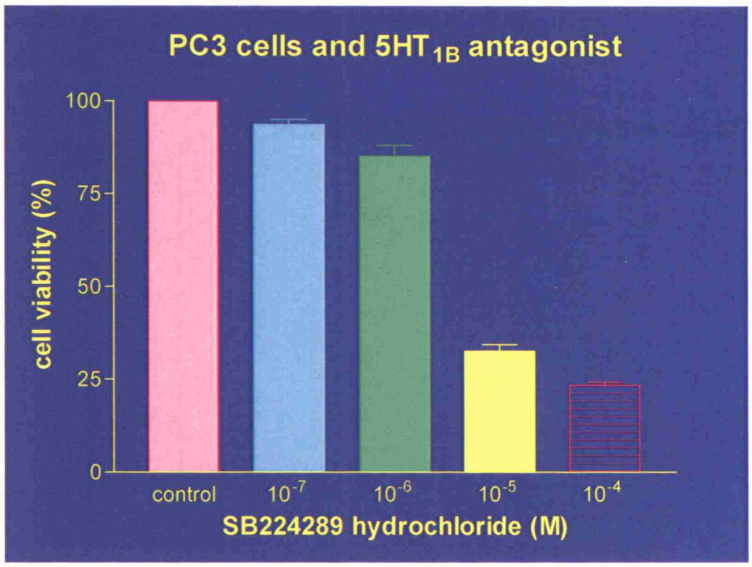


Figure 3: A dose-dependent growth inhibition of PC3 cells by a $5HT_{1B}$ antagonist (SB224289 hydrochloride) with a maximum effect seen at a concentration of 10^{-4} M at 72 h. The bars represent the standard error of the mean (SEM).

	control	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
1	100%	85.0%	70.5%	32.7%	21.8%	
2	100%	99.5%	89.6%	42.7%	18.8%	
3	100%	90.3%	90.8%	31.1%	24.5%	
4	100%	95.5%	89.9%	38.8%	23.6%	
5	100%	89.9%	64.7%	25.9%	21.1%	
6	100%	89.1%	71.7%	27.8%	20.00%	
7	100%	97.4%	88.2%	37.4%	23.6%	
8	100%	100.0%	95.4%	40.6%	25.4%	
9	100%	93.6%	87.8%	27.5%	24.0%	
10	100%	95.2%	85.0%	34.3%	24.2%	
11	100%	93.0%	95.4%	22.8%	21.9%	
12	100%	95.9%	92.4%	29.1%	29.6%	
Mean	100%	93.7%	85.1%	32.6%	22.4%	
SEM	0	1.3	3.0	1.8	0.8	

Table 5: Percentage cell viability of PC3 cells at 72 h, after treatment with a $5HT_{1B}$ antagonist (SB224289 hydrochloride).

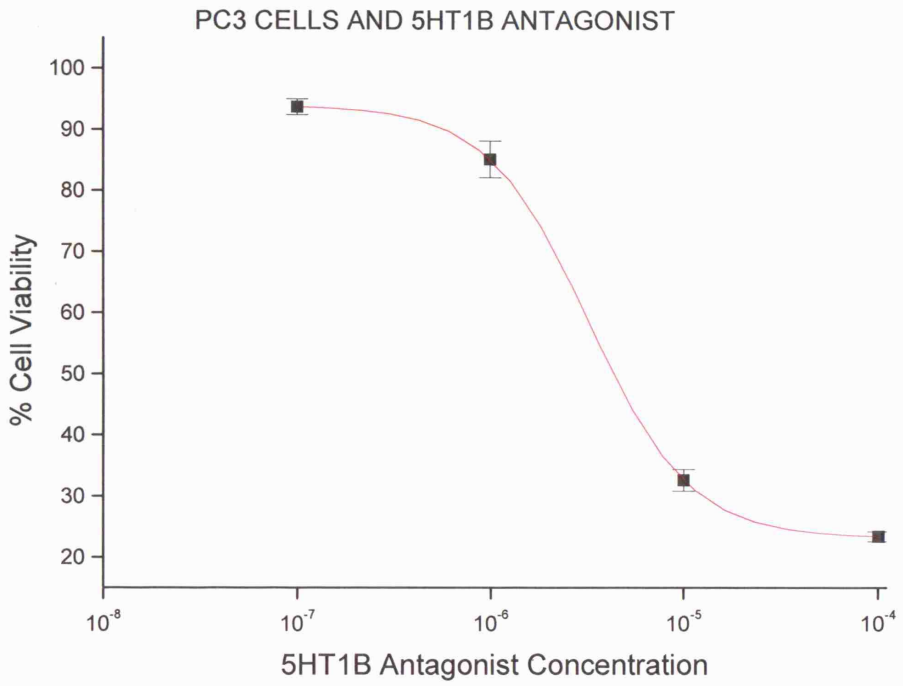


Figure 4: A dose response curve of PC3 cells by a $5HT_{1B}$ antagonist (SB224289 hydrochloride), indicating a maximum effect seen at a concentration of 10^{-4} M at 72 h. The IC_{50} value is calculated at 3.23 X 10^{-6} M

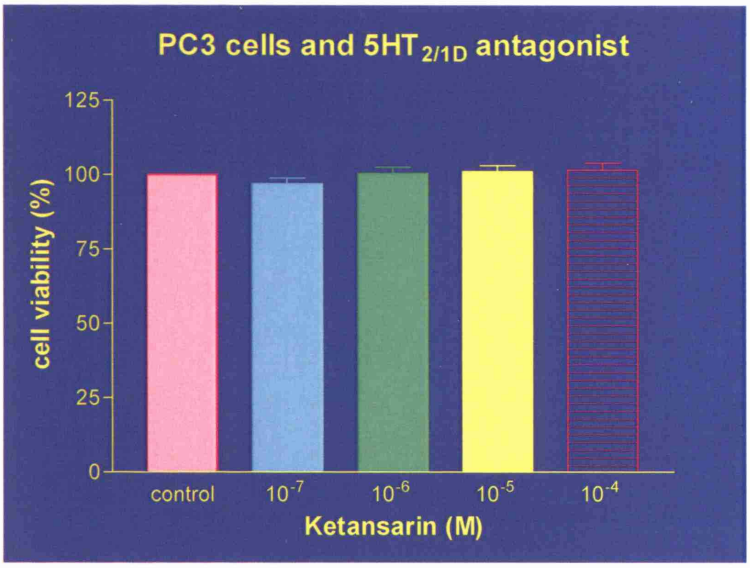


Figure 5: No significant effect seen on the growth of PC3 cells with a $5HT_{2/1D}$ antagonist (ketansarin) at 72 h. The bars represent the standard error of the mean (SEM).

	control	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
1	100%	100.0%	93.6%	98.7%	97.5%	
2	100%	98.1%	94.7%	95.0%	101.9%	
3	100%	106.7%	94.6%	107.1%	97.3%	
4	100%	108.1%	101.9%	96.5%	107.8%	
5	100%	95.9%	107.1%	111.9%	108.6%	
6	100%	99.3%	106.1%	104.8%	100.7%	
7	100%	89.9%	108.4%	110.4%	89.9%	
8	100%	95.1%	103.3%	104.0%	102.6%	
9	100%	98.4%	91.4%	88.6%	100.0%	
10	100%	94.9%	111.9%	93.6%	102.1%	
11	100%	85.7%	92.3%	103.1%	91.1%	
12	100%	92.3%	100.5%	98.6%	119.1%	
Mean	100%	97.0%	100.4%	101.0%	101.6%	
SEM	0	1.8	2.0	2.0	2.3	

Table 6: Percentage cell viability of PC3 cells at 72 h, after treatment with a $5HT_{2/1D}$ antagonist (ketansarin).

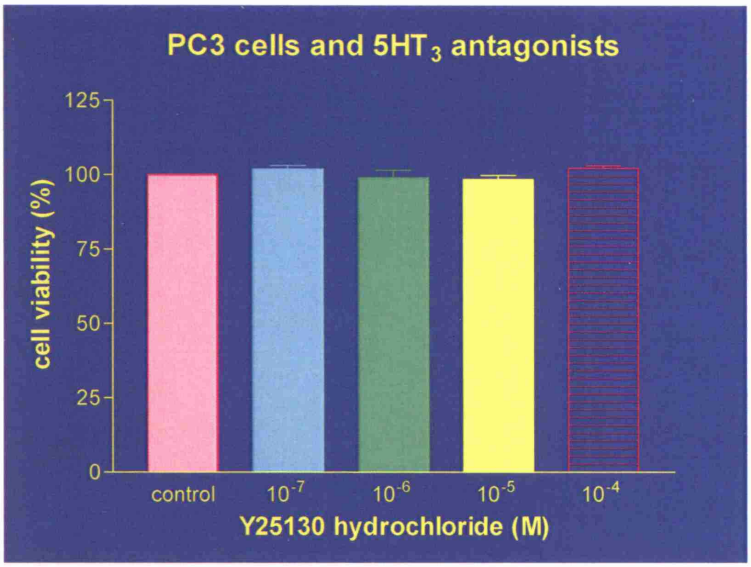


Figure 6: No significant effect seen on the growth of PC3 cells with a $5HT_3$ antagonist (Y25130 hydrochloride) at 72 h. The bars represent the standard error of the mean (SEM).

	control	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
1	100%	101.9%	81.5%	101.2%	102.3%	
2	100%	98.8%	107.3%	104.4%	100.4%	
3	100%	100.8%	100.8%	90.5%	106.2%	
4	100%	101.3%	102.6%	98.2%	102.6%	
5	100%	102.7%	107.7%	99.6%	96.5%	
6	100%	106.6%	102.2%	100.9%	104.4%	
7	100%	105.1%	100.0%	100.0%	100.4%	
8	100%	108.4%	108.4%	97.9%	104.2%	
9	100%	102.0%	101.2%	106.1%	102.9%	
10	100%	95.7%	86.7%	95.2%	104.3%	
11	100%	95.6%	89.1%	89.1%	97.2%	
12	100%	105.4%	101.5%	97.6%	104.9%	
Mean	100%	102.0%	99.1%	98.4%	102.2%	
SEM	0	1.2	2.5	1.4	0.9	

Table 7: Percentage cell viability of PC3 cells at 72 h, after treatment with a $5HT_3$ antagonist (Y25130 hydrochloride).

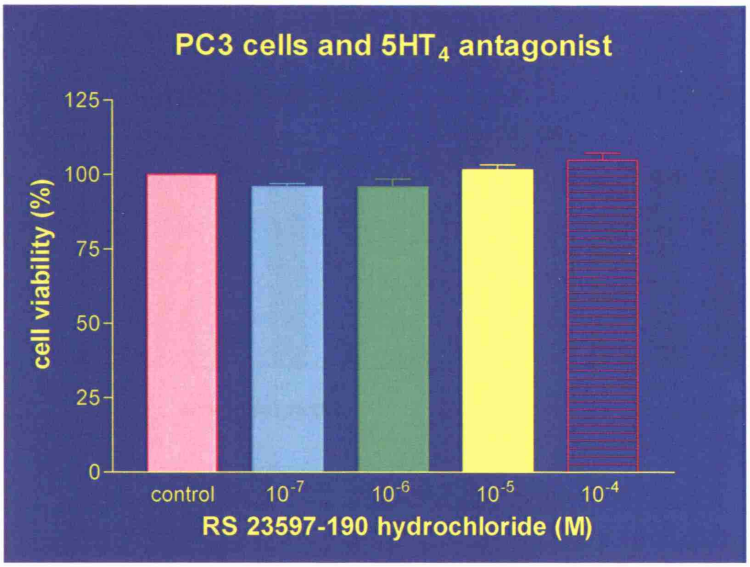


Figure 7: No significant effect seen on the growth of PC3 cells with a $5HT_4$ antagonist (RS 23597-190 hydrochloride) at 72 h. The bars represent the standard error of the mean (SEM).

	control	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
1	100%	95.2%	100.4%	103.0%	101.7%	
2	100%	98.3%	94.7%	95.1%	98.3%	
3	100%	97.5%	101.4%	105.3%	105.6%	
4	100%	102.7%	100.5%	98.7%	98.7%	
5	100%	99.4%	94.2%	99.0%	91.6%	
6	100%	94.6%	92.5%	97.5%	100.4%	
7	100%	101.2%	103.5%	105.5%	99.2%	
8	100%	100.0%	94.0%	99.7%	99.7%	
9	100%	100.8%	99.6%	101.6%	107.1%	
10	100%	96.8%	96.4%	104.0%	103.6%	
11	100%	101.3%	103.0%	100.9%	105.6%	
12	100%	96.8%	102.3%	103.7%	104.6%	
Mean	100%	98.7	98.5	101.2	101.3	
SEM	0	0.7	1.1	0.9	1.2	

Table 8: Percentage cell viability of PC3 cells at 72 h, after treatment with a $5HT_4$ antagonist (RS 23597-190 hydrochloride).

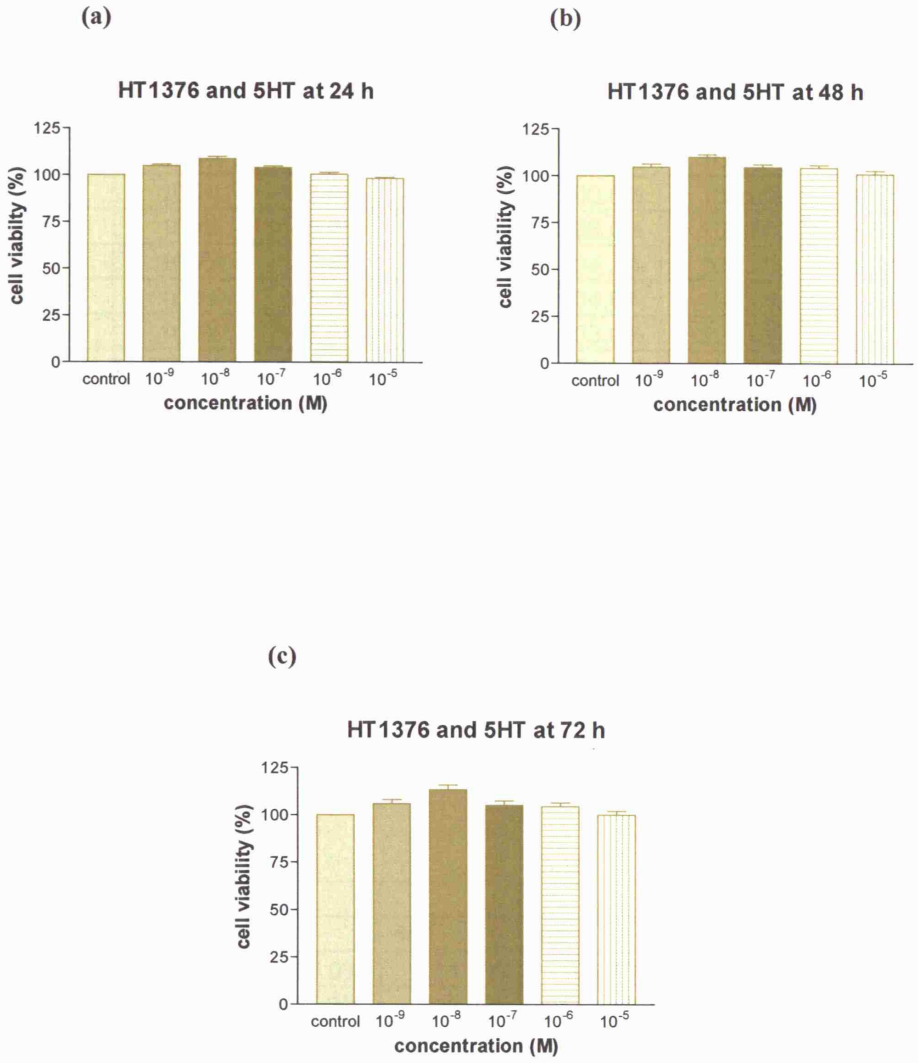


Figure 8: An increase in growth proliferation of HT1376 cells by 5HT, at (a) 24 h, (b) 48 h and (c) 72 h, respectively. A maximum effect is seen at a concentration of 10^{-8} M at 72 h. The bars represent the standard error of the mean (SEM).

	24h						
	control	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	
1	100%	100.8%	106.5%	103.0%	97.3%	96.7%	
2	100%	99.7%	103.2%	98.4%	97.0%	98.4%	
3	100%	104.5%	109.5%	105.0%	102.5%	100.6%	
4	100%	106.6%	106.6%	100.6%	100.6%	101.9%	
5	100%	108.2%	105.2%	103.9%	100.9%	95.7%	
6	100%	106.4%	112.8%	104.3%	101.7%	95.3%	
7	100%	108.3%	112.2%	104.8%	95.7%	95.7%	
8	100%	106.6%	118.0%	103.9%	100.0%	100.9%	
9	100%	106.4%	109.3%	106.4%	105.1%	95.8%	
10	100%	101.9%	106.4%	104.8%	98.7%	101.3%	
11	100%	104.4%	105.3%	105.6%	100.3%	99.1%	
12	100%	105.8%	110.3%	107.7%	105.8%	98.4%	
Mean	100%	105.0%	109.0%	104.0%	101.0%	98.0%	
SEM	0	0.8	1.2	0.7	0.9	0.7	

Table 9: Percentage cell viability of HT1376 cells at 24 h, after treatment with 5HT.

	48h						
	control	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	
1	100%	112.0%	111.4%	106.7%	104.8%	105.7%	
2	100%	111.2%	115.4%	112.4%	108.2%	101.1%	
3	100%	105.1%	117.1%	110.6%	105.7%	107.7%	
4	100%	99.4%	115.6%	105.6%	110.9%	97.2%	
5	100%	100.5%	104.1%	104.3%	105.2%	91.4%	
6	100%	107.5%	105.0%	109.3%	106.8%	107.8%	
7	100%	99.8%	107.5%	99.8%	100.2%	93.1%	
8	100%	105.7%	108.8%	103.3%	110.3%	105.0%	
9	100%	102.0%	106.1%	96.5%	99.1%	94.3%	
10	100%	109.5%	111.8%	104.7%	104.3%	107.1%	
11	100%	110.2%	111.9%	104.2%	102.3%	104.4%	
12	100%	95.2%	106.3%	98.7%	93.7%	96.1%	
Mean	100%	105.0%	110.0%	105.0%	104.0%	101.0%	
SEM	0	1.6	1.3	1.4	1.4	1.8	

Table 10: Percentage cell viability of HT1376 cells at 48 h, after treatment with 5HT.

	72h						
	control	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	
1	100%	106.3%	116.2%	102.1%	103.6%	96.4%	
2	100%	113.3%	119.6%	106.6%	118.4%	87.6%	
3	100%	103.4%	122.1%	91.1%	106.7%	103.1%	
4	100%	114.8%	122.9%	121.7%	100.1%	115.5%	
5	100%	108.0%	113.7%	105.7%	97.7%	103.8%	
6	100%	112.7%	117.4%	108.4%	110.2%	106.4%	
7	100%	88.9%	94.4%	95.1%	88.7%	90.7%	
8	100%	109.3%	116.7%	108.7%	107.6%	94.9%	
9	100%	106.4%	111.3%	103.6%	103.9%	100.7%	
10	100%	105.1%	109.9%	104.4%	102.8%	99.4%	
11	100%	100.3%	107.6%	105.0%	106.7%	99.6%	
12	100%	105.7%	103.6%	110.5%	106.0%	102.8%	
Mean	100%	106.2%	112.3%	105.2%	104.4%	100.1%	
SEM	0	2.0	2.5	2.2	2.0	2.1	

Table 11: Percentage cell viability of HT1376 cells at 72 h, after treatment with 5HT.

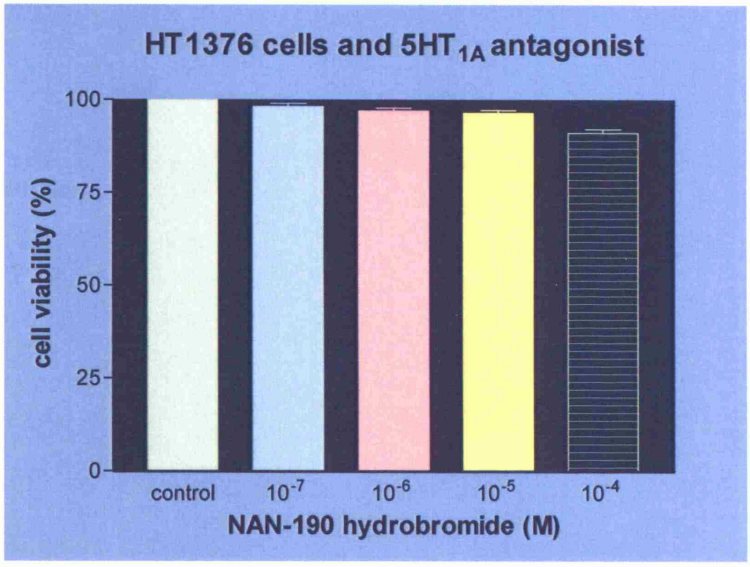


Figure 9: A dose-dependent growth inhibition of HT1376 cells by a $5HT_{1A}$ antagonist (NAN-190 hydrobromide) with a maximum effect seen at a concentration of 10^{-4} M at 72 h. The bars represent the standard error of the mean (SEM).

	control	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
1	100%	100.1%	101.1%	99.7%	93.8%	
2	100%	96.2%	96.5%	94.8%	91.0%	
3	100%	97.4%	96.0%	91.1%	89.8%	
4	100%	97.4%	95.5%	91.3%	87.2%	
5	100%	100.8%	101.4%	97.7%	94.7%	
6	100%	97.1%	93.4%	98.6%	88.9%	
7	100%	91.5%	95.1%	97.3%	90.7%	
8	100%	94.9%	94.3%	95.5%	84.3%	
9	100%	98.7%	99.3%	98.2%	94.1%	
10	100%	99.4%	97.0%	97.0%	93.7%	
11	100%	101.1%	98.6%	98.6%	88.8%	
12	100%	103.0%	95.8%	96.7%	93.5%	
Mean	100%	98.1%	97.0%	96.4%	90.1%	
SEM	0	0.9	0.7	0.8	1.0	

Table 12: Percentage cell viability of HT1376 cells at 72 h, after treatment with $5HT_{1A}$ antagonist (NAN-190 hydrobromide).

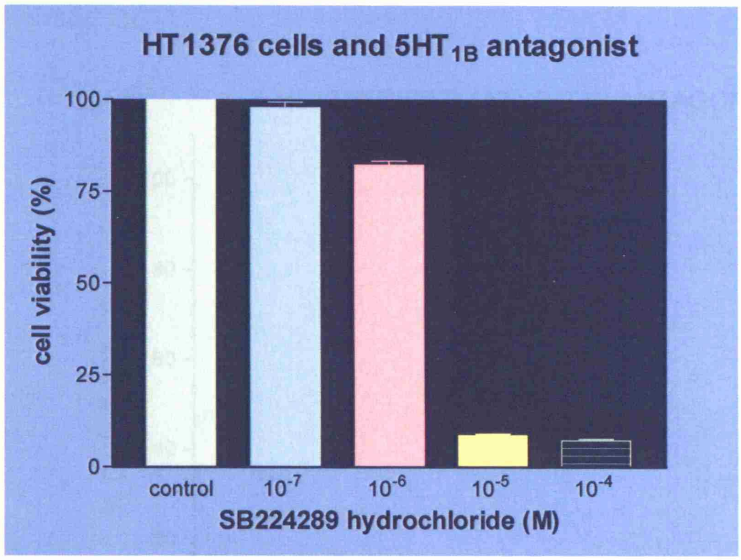


Figure 10: A dose-dependent growth inhibition of HT1376 cells by a $5HT_{1B}$ antagonist (SB224289 hydrochloride) with a maximum effect seen at a concentration of 10^{-4} M at 72 h. The bars represent the standard error of the mean (SEM).

	control	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
1	100%	100.1%	84.4%	6.2%	5.4%	
2	100%	98.0%	84.4%	6.5%	5.7%	
3	100%	95.1%	80.9%	6.9%	6.5%	
4	100%	91.0%	82.5%	7.7%	6.3%	
5	100%	99.1%	83.5%	9.3%	7.8%	
6	100%	95.4%	85.6%	8.1%	7.7%	
7	100%	107.6%	79.7%	8.7%	8.5%	
8	100%	100.6%	81.8%	10.0%	7.9%	
9	100%	95.0%	84.5%	9.8%	6.3%	
10	100%	98.3%	78.4%	11.0%	8.7%	
11	100%	104.1%	73.0%	9.1%	8.7%	
12	100%	89.5%	86.9%	9.2%	7.2%	
Mean	100%	97.8%	82.1%	8.5%	7.2%	
SEM	0	1.5	1.1	0.4	0.3	

Table 13: Percentage cell viability of HT1376 cells at 72 h, after treatment with a $5HT_{1B}$ antagonist (SB224289 hydrochloride).

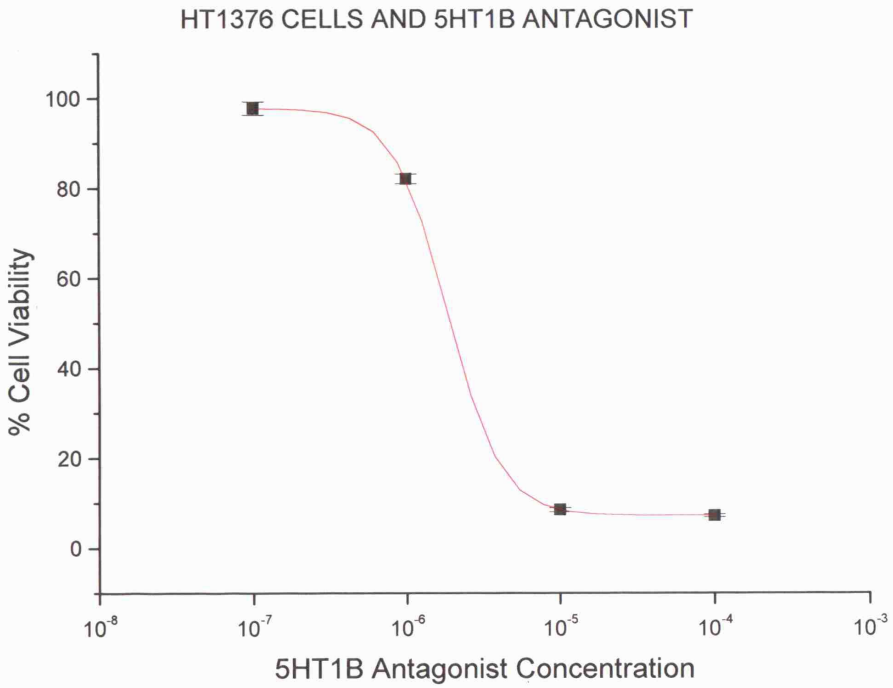


Figure 11: A dose response curve of HT1376 cells by a $5HT_{1B}$ antagonist (SB224289 hydrochloride), indicating a maximum effect seen at a concentration of 10^{-4} M at 72 h. The IC_{50} value is calculated at 1.85 X 10^{-6} M.

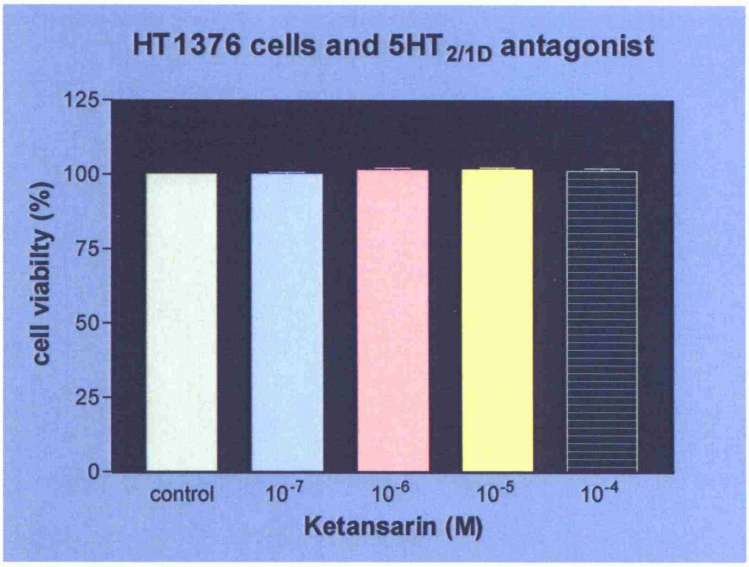


Figure 12: No significant effect seen on the growth of HT1376 cells with a $5HT_{2/1D}$ antagonist (ketansarin) at 72 h. The bars represent the standard error of the mean (SEM).

	control	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
1	100%	101.2%	103.0%	100.0%	98.6%	
2	100%	100.0%	103.6%	100.9%	97.7%	
3	100%	101.4%	103.4%	107.1%	105.3%	
4	100%	100.5%	103.4%	102.9%	104.7%	
5	100%	100.6%	101.3%	103.0%	103.4%	
6	100%	97.5%	96.0%	102.3%	102.1%	
7	100%	99.1%	104.3%	100.9%	99.1%	
8	100%	103.0%	98.3%	98.9%	99.4%	
9	100%	96.1%	97.8%	103.9%	104.7%	
10	100%	96.7%	102.5%	100.8%	101.3%	
11	100%	102.3%	103.3%	99.2%	99.2%	
12	100%	101.5%	99.0%	99.5%	99.3%	
Mean	100%	99.9%	101.3%	101.6%	101.2%	
SEM	0	0.6	0.8	0.7	0.8	

Table 14: Percentage cell viability of HT1376 cells at 72 h, after treatment with $5HT_{2/1D}$ antagonist (ketansarin).

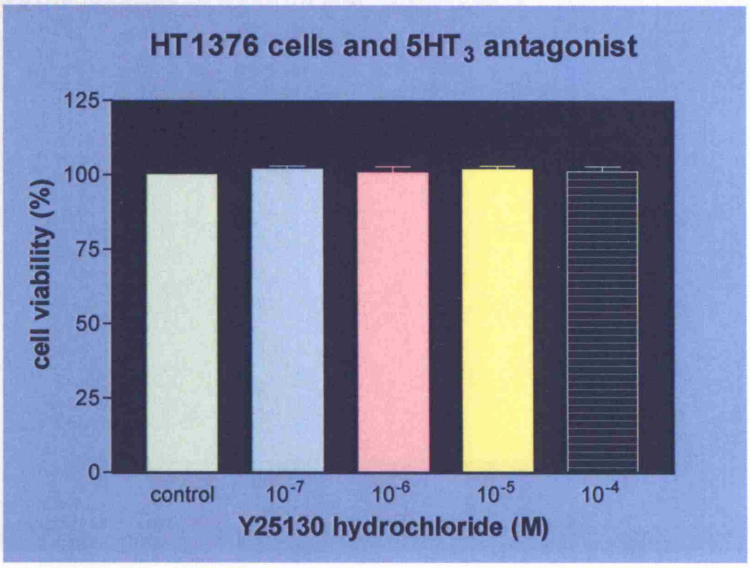


Figure 13: No significant effect seen on the growth of HT1376 cells with $5HT_3$ antagonist (Y25130 hydrochloride) at 72 h. The bars represent the standard error of the mean (SEM).

	control	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
1	100%	100.6%	102.5%	95.9%	99.4%	
2	100%	95.1%	96.9%	100.6%	100.3%	
3	100%	104.9%	102.6%	100.0%	101.7%	
4	100%	107.9%	105.9%	107.1%	102.6%	
5	100%	97.7%	104.2%	107.6%	108.6%	
6	100%	104.3%	92.9%	106.1%	92.3%	
7	100%	101.9%	111.0%	96.3%	103.3%	
8	100%	104.4%	101.6%	105.2%	95.8%	
9	100%	104.4%	105.8%	100.2%	107.9%	
10	100%	97.4%	85.2%	102.2%	100.4%	
11	100%	103.3%	105.3%	103.1%	109.6%	
12	100%	101.3%	95.0%	97.6%	93.4%	
Mean	100%	101.9%	100.7%	101.8%	101.3%	
SEM	0	1.0	2.0	1.2	1.6	

Table 15: Percentage cell viability of HT1376 cells at 72 h, after treatment with a $5HT_3$ antagonist (Y25130 hydrochloride).

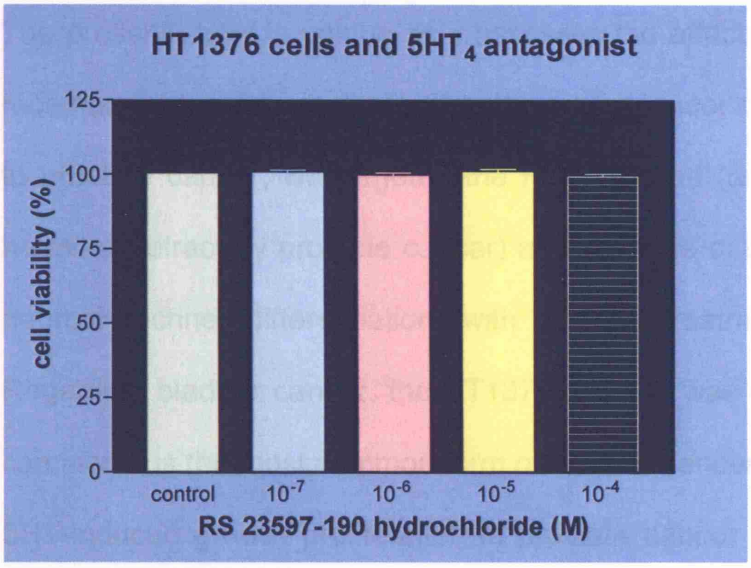


Figure 14: No significant effect seen on the growth of HT1376 cells with a $5HT_4$ antagonist (RS 23597-190 hydrochloride) at 72 h. The bars represent the standard error of the mean (SEM).

	control	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
1	100%	95.2%	101.7%	100.0%	99.3%	
2	100%	101.7%	97.4%	97.8%	98.7%	
3	100%	103.3%	100.6%	104.1%	102.6%	
4	100%	98.1%	98.4%	99.0%	95.6%	
5	100%	103.0%	95.1%	100.7%	97.9%	
6	100%	97.9%	102.0%	95.3%	99.5%	
7	100%	100.0%	107.6%	99.1%	99.5%	
8	100%	98.4%	93.0%	97.4%	93.0%	
9	100%	98.7%	95.3%	97.8%	101.1%	
10	100%	107.5%	101.2%	102.7%	104.6%	
11	100%	103.3%	103.1%	109.9%	99.8%	
12	100%	97.6%	96.3%	102.8%	99.1%	
Mean	100.00%	100.4%	99.3%	100.5%	99.2%	
SEM	0	1.0	1.2	1.1	0.9	

Table 16: Percentage cell viability of HT1376 cells at 72 h, after treatment with a $5HT_4$ antagonist (RS 23597-190 hydrochloride).

2.7 Discussion

The present study is unique as it assesses the effect of a wide range of 5HT receptor antagonists on prostate and bladder cancer cell growth. With regards to prostate cancer, we targeted the PC3 cell line (androgen-independent or hormone refractory prostate cancer) as this stage of the disease has marked neuroendocrine differentiation with limited treatment options available. Regarding bladder cancer, the HT1376 cell line was used as transitional cell carcinoma is the most common form of bladder cancer encountered.

5HT-induced growth proliferation in prostate cancer cells (PC3, DU145 and LNCaP) has been reported (Abdul et al., 1994; Dizeyi et al., 2004), with a maximum proliferation seen at a concentration of 10^{-8}M (Dizeyi et al., 2004). Our results indicate, a 5HT-induced growth proliferation of 15% in PC3 cells at the same concentration of 10^{-8}M, at 72 h. Pindobind and NAN-190 hydrobromide; $5HT_{1A}$ antagonists have a significant growth inhibitory effect on prostate cancer cells (PC3, DU145 and LNCaP) (Abdul et al., 1994; Dizeyi et al., 2004). In addition, 5HT uptake inhibitors (6-nitroquipazine, zimelidine and fluoxetine) also caused inhibition of PC3, DU145 and LNCaP cell growth in a dose-dependent manner (Abdul et al., 1995). We found that a $5HT_{1A}$ antagonist; NAN-190 hydrobromide, caused a 20% inhibition of PC3 cell growth at a concentration of 10^{-4}M at 72 h. More importantly, SB224289 hydrochloride; a $5HT_{1B}$ antagonist caused a 78% growth inhibition of PC3 cells at a concentration of 10^{-4}M at 72 h. Other 5HT receptor subtype antagonists ($5HT_{1D/2}$, $5HT_3$ and $5HT_4$) had no significant inhibitory effect on PC3 cell growth.

We are in agreement with Abdul et al and Dizeyi et al that $5HT_{1A}$ antagonists cause a dose-dependent inhibition of PC3 cell growth. We, however, differ with both Abdul et al and Dizeyi et al who proposed that in PC3 cells a maximum inhibition with a $5HT_{1A}$ antagonist takes place at 10^{-5} M and 10^{-6}M, respectively. Our results indicate that the $5HT_{1A}$ antagonist (NAN-190 hydrobromide) causes a maximum growth inhibition in PC3 cells, at 10^{-4}M. Furthermore, Abdul et al demonstrated a maximum growth inhibition of 90% by a $5HT_{1A}$ antagonist, as compared to the 20% found by ourselves. Dizeyi et al did not express their results as a percentage growth inhibition and therefore a direct comparison could not be made. A possible explanation for the above discrepancies may be the longer incubation period of 120 h and 96 h, used by Abdul et al and Dizeyi et al, respectively. Moreover, Abdul et al used the $5HT_{1A}$ antagonist (Pindobind) as compared to NAN-190 hydrobromide used by Dizeyi et al and ourselves. Thus, the variation in the magnitude of the effect of both $5HT_{1A}$ antagonists might be attributed to differences in receptor selectivity (Fenrick et al., 1996). Our argument is further strengthened by the observations of Dizeyi et al that out of the three $5HT_{1A}$ antagonists (NAN-190, WAY100135 and BMY7378) used; only NAN-190 significantly inhibited prostate cancer cell growth (Dizeyi et al., 2004).

A novel finding from our study is that SB224289 hydrochloride; a selective $5HT_{1B}$ receptor antagonist causes a 78% growth inhibition in PC3 cells at a concentration of 10^{-4}M, at 72 h. The growth inhibition by $5HT_{1B}$ antagonist on PC3 cells was far greater and more significant than the 20% growth inhibitory effect demonstrated by $5HT_{1A}$ antagonist; NAN-190 hydrobromide. The IC_{50} value was 3.23×10^{-6}M.

NE cells release growth factors including 5HT, which are related to tumour growth, differentiation and progression. NE differentiation takes place in both prostate and bladder cancer (di Sant'Agnese, 1998b; di Sant'Agnese & Cockett, 1996; di Sant'Agnese & Mesy Jensen, 1987; Acs et al., 2000; Dundr et al., 2003; Helpap et al., 2002; Martignoni et al., 2003; Vincendeau et al., 2003). Hence, we also investigated the effect of 5HT and 5HT receptor antagonists on bladder cancer cell growth (HT1376).

Our findings indicated that 5HT caused a growth proliferation of HT1376 cells with a maximum proliferation of 12% seen at a concentration of 10^{-8} M, at 72 h (Siddiqui et al., 2005b). The concentration (10^{-8}M) at which 5HT caused maximum proliferation in HT1376 cells was similar to that established in experiments performed on PC3 cells by both Dizeyi et al and us. Furthermore, $5HT_{1A}$ and $5HT_{1B}$ antagonists had a dose-dependent growth inhibitory effect on HT1376 cell growth with a maximum inhibition of 10% and 93%, respectively, at a concentration of 10^{-4} M, at 72 h (Siddiqui et al., 2005b). Similar, to PC3 cells, $5HT_{1A}$ and $5HT_{1B}$ antagonists caused a maximum growth inhibition in HT1376 cells at 10^{-4}M. Other 5HT receptor subtype antagonists ($5HT_{1D/2}$, $5HT_3$ and $5HT_4$) had no significant inhibitory effect on HT1376 cell growth.

We believe that $5HT_{1B}$ antagonist is the more significant of the two receptor antagonists and requires further investigation as a potential anti-neoplastic agent for the treatment of both androgen independent prostate cancer and transitional cell carcinoma of the bladder.

CHAPTER 3

IN VITRO PROLIFERATION STUDIES OF PROSTATE CANCER (PC3) AND BLADDER CANCER (HT1376) CELL LINES WITH AN ALPHA$_1$-ADRENOCEPTOR ANTAGONIST (DOXAZOSIN), AND IN COMBINATION WITH 5HT AND A 5HT$_{1B}$ AGONIST

3.1 Introduction

In this chapter, using the crystal violet proliferation assay, the effect of an alpha$_1$-adrenoceptor antagonist, doxazosin on the growth of PC3 cells (androgen independent prostate cancer) and HT1376 cells (grade III transitional cell carcinoma of the bladder) was investigated.

Doxazosin inhibits 5HT-induced shape change in platelets, via the $5HT_2$ receptor (Jagroop et al., 2001). Autoradiographic evidence suggests that doxazosin reduces 5HT binding in the rabbit detrusor (Khan et al., 2000). It is possible that the growth inhibitory effect of doxazosin on prostate and bladder cancer cells is mediated via 5HT receptors. Again, using the crystal violet proliferation assay we have tried to establish this link.

3.2 Materials and Methods

The malignant cell lines used were: 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 American Type Culture Collection (ATCC).

The PC3 cells were maintained in Nutrient Mixture F-12 Ham Medium supplemented with 8% Fetal Bovine Serum (FBS) and 1% Antibiotic Antimycotic Solution.

The HT1376 cells were maintained in Minimum Essential Medium Eagle supplemented with 8% Fetal Bovine Serum (FBS), 1% Antibiotic Antimycotic Solution and 1% MEM Non Essential Amino Acid Solution.

Both PC3 and HT1376 cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2.

3.3 Reagents

Doxazosin (alpha$_1$-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), Fetal Bovine Serum (FBS), Dimethyl Sulfoxide (DMSO), MEM Non Essential Amino Acid Solution and Antibiotic Antimycotic Solution were all purchased from Sigma Laboratories Ltd. Dulbecco's Phosphate buffered saline (PBS) was used for washing the cells and distilled water was used as a control for the experiments.

3.4 In vitro proliferation assay

PC3 and HT1376 cells were seeded in a 96 well plate, 5000 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 (10^{-6}, 10^{-5}, $5x10^{-4}$, 10^{-4}), dissolved in distilled water.

In the combination experiments on the PC3 cell line, 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 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, 45 ml of distilled water was used. Medium was gently aspirated from 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 colour from cells and optical density was read at 570 nm using the spectrophotometric plate reader. Cell viability was expressed as a percentage of the control OD value, where control OD values were considered 100 %.

3.5 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.

3.6 Results

3.6.1 [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. 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 cell viability of 95% ($P = 0.0150$) and 80% ($P < 0.0001$), respectively, at 72 h (Figure 15, Table 19).

(ii) HT1376 cells

Doxazosin caused a dose-dependent inhibition of HT1376 cell growth with a maximum inhibition of 91% [i.e. 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 cell viability of 94% (P = 0.039) and 76% (P = 0.0001), respectively, at 72 h (Figure 16, Table 22).

3.6.2 [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 (Figure 18, Table 24). 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) decrease in cell growth took place at 10^{-5} M as compared to control (Figure 1, Table 3). In the combination studies, we used the highest and lowest concentrations of 5HT (i.e. 10^{-4} and 10^{-8} M) that we evaluated. At higher concentrations the proliferatory effect of 5HT on prostate cancer cell growth decreases. This is most likely due to toxicity developing at high concentrations of 5HT.

(ii) Effect of doxazosin alone on PC3 cell growth:

Doxazosin at concentrations of 10^{-6} and 10^{-5} M resulted in a cell viability of 94.7% (P = 0.015) and 80.4% (P < 0.0001), respectively, at 72 h (Figure 18, Table 24). Doxazosin at concentrations of $5x10^{-4}$ and 10^{-4} M led to a cell

viability of 26.2% ($P < 0.0001$) and 14.6% ($P < 0.0001$), respectively, at 72 h (Figure19, Table 25).

(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 (Figure 18 & 19, Table 24 & 25). 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.

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 (Figure 18 & 19, Table 24 & 25). 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.

3.6.3 [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

$5HT_{1B}$ 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 (Figure 20, Table 26). A 2%

(P = 0.35) increase in cell proliferation was seen at both $5HT_{1B}$ agonist concentrations of 10^{-7} and 10^{-6} M (Figure 17, Table 23).

(ii) Effect of doxazosin alone on PC3 cell growth:

Treatment with doxazosin at concentrations of 10^{-5} and 10^{-4} M resulted in a cell viability of 85.0% ($P < 0.0001$) and 12.0% ($P < 0.0001$), respectively, at 72 h (Figure 20, Table 26).

(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 (Figure 20, Table 26). 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.

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 (Figure 20, Table 26). 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.

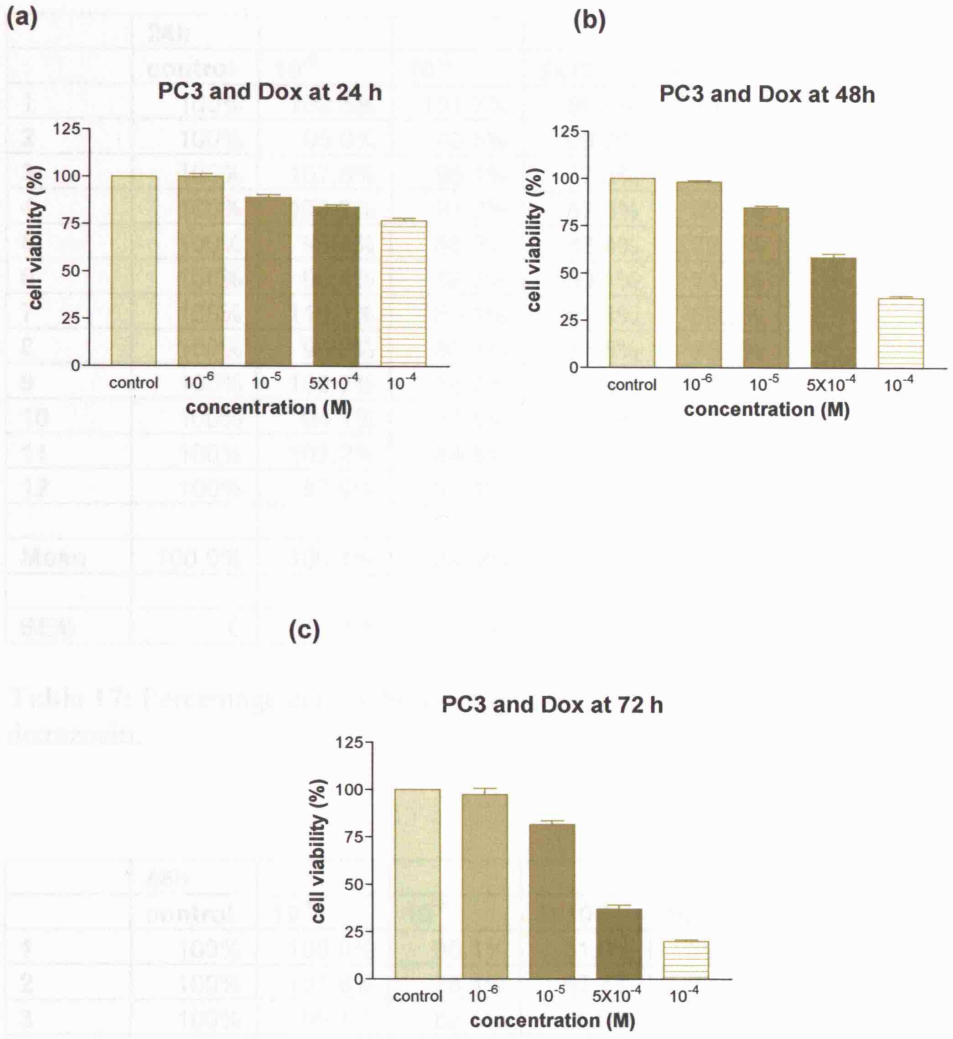


Figure 15: Dose-dependent inhibition of PC3 (androgen-independent prostate cancer) cell growth by doxazosin at (a) 24 h, (b) 48 h and (c) 72 h, respectively. A maximum inhibition is seen at a concentration of 10^{-4} M at 72 h. The bars represent the standard error of the mean (SEM).

	24h				
	control	10^{-6}	10^{-5}	$5x10^{-4}$	10^{-4}
1	100%	102.5%	101.7%	89.8%	84.7%
2	100%	95.0%	83.5%	89.3%	83.5%
3	100%	107.8%	96.1%	77.3%	78.1%
4	100%	103.2%	91.9%	89.5%	83.1%
5	100%	95.4%	88.2%	82.4%	72.5%
6	100%	96.4%	82.5%	80.1%	71.1%
7	100%	110.1%	88.1%	81.8%	69.2%
8	100%	96.3%	88.1%	87.5%	75.6%
9	100%	100.7%	88.7%	80.9%	74.5%
10	100%	94.1%	87.5%	78.9%	77.0%
11	100%	102.2%	84.8%	86.2%	75.4%
12	100%	97.9%	87.1%	85.7%	80.0%
Mean	100.0%	100.1%	89.0%	84.1%	77.1%
SEM	0	1.5	1.6	1.3	1.4

Table 17: Percentage cell viability of PC3 cells at 24 h, after treatment with doxazosin.

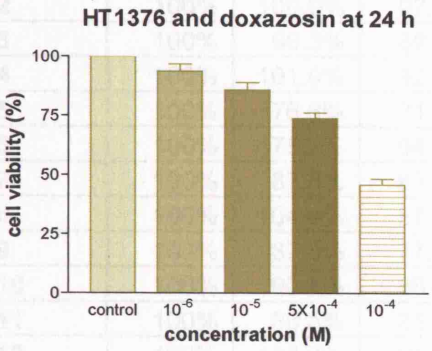
	48h				
	control	10^{-6}	10^{-5}	$5x10^{-4}$	10^{-4}
1	100%	100.9%	86.1%	51.9%	39.4%
2	100%	101.8%	78.4%	53.7%	29.1%
3	100%	96.1%	82.5%	55.9%	38.9%
4	100%	94.5%	89.4%	65.1%	39.9%
5	100%	95.0%	83.2%	70.2%	42.9%
6	100%	100.4%	79.5%	56.7%	35.0%
7	100%	96.7%	85.4%	63.6%	36.0%
8	100%	97.4%	89.8%	66.4%	38.9%
9	100%	100.8%	86.0%	51.6%	32.0%
10	100%	98.3%	87.0%	50.4%	37.4%
11	100%	98.7%	82.2%	51.7%	35.7%
12	100%	98.2%	85.9%	63.6%	37.3%
Mean	100.0%	98.2%	84.6%	58.4%	36.9%
SEM	0	0.7	1.0	2.0	1.0

Table 18: Percentage cell viability of PC3 cells at 48 h, after treatment with doxazosin.

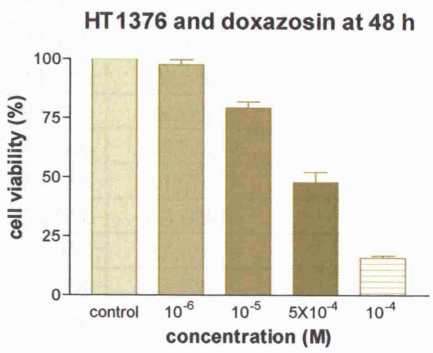
	72h				
	control	10^{-6}	10^{-5}	$5x10^{-4}$	10^{-4}
1	100%	89.9%	68.8%	29.7%	17.0%
2	100%	90.9%	80.0%	34.5%	17.1%
3	100%	95.4%	88.2%	37.4%	20.6%
4	100%	80.8%	82.6%	31.7%	19.6%
5	100%	87.7%	77.5%	51.8%	25.1%
6	100%	112.2%	79.8%	36.9%	16.7%
7	100%	116.1%	79.9%	46.2%	21.4%
8	100%	115.0%	80.6%	49.7%	18.4%
9	100%	93.4%	79.7%	25.3%	17.1%
10	100%	95.6%	86.7%	33.0%	20.7%
11	100%	98.1%	80.4%	30.4%	20.7%
12	100%	94.7%	96.5%	36.0%	24.1%
Mean	100.0%	97.5%	81.7%	36.9%	19.9%
SEM	0	3.2	1.9	2.4	0.8

Table 19: Percentage cell viability of PC3 cells at 72 h, after treatment with doxazosin.

(a)



(b)



(c)

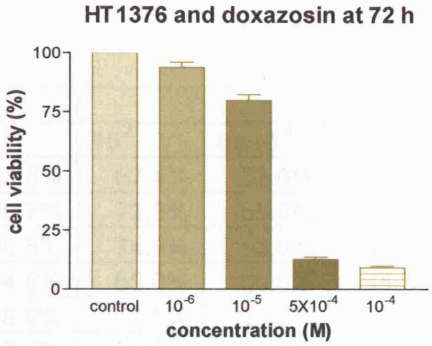


Figure 16: Dose-dependent inhibition of HT1376 (Grade III transitional carcinoma of the bladder) cell growth by doxazosin at (a) 24 h, (b) 48 h and (c) 72 h, respectively. A maximum inhibition is seen at a concentration of 10^{-4} M at 72 h. The bars represent the standard error of the mean (SEM).

	24 h					
	control	10^{-6}	10^{-5}	$5X10^{-4}$	10^{-4}	
1	100%	109.0%	97.0%	71.4%	51.5%	
2	100%	100.0%	97.6%	71.1%	58.1%	
3	100%	98.3%	89.6%	71.4%	62.7%	
4	100%	101.9%	92.8%	71.9%	49.3%	
5	100%	76.6%	71.4%	84.4%	39.0%	
6	100%	78.7%	64.7%	71.5%	34.5%	
7	100%	87.0%	83.5%	79.1%	41.7%	
8	100%	104.4%	87.7%	93.0%	43.0%	
9	100%	87.5%	77.3%	59.4%	37.4%	
10	100%	90.4%	86.6%	70.7%	47.5%	
11	100%	88.5%	85.7%	72.6%	36.4%	
12	100%	103.2%	95.5%	71.8%	49.0%	
Mean	100%	94.0%	86.0%	74.0%	46.0%	
SEM	0	3.0	3.0	2.4	2.6	

Table 20: Percentage cell viability of HT1376 cells at 24 h, after treatment with doxazosin.

	48 h					
	control	10^{-6}	10^{-5}	$5X10^{-4}$	10^{-4}	
1	100%	103.6%	67.4%	52.6%	13.5%	
2	100%	106.7%	72.8%	55.6%	12.0%	
3	100%	100.6%	74.9%	53.0%	16.7%	
4	100%	104.8%	65.0%	50.6%	11.3%	
5	100%	86.9%	77.0%	24.8%	16.9%	
6	100%	97.3%	82.3%	31.8%	19.5%	
7	100%	83.4%	73.5%	27.8%	19.6%	
8	100%	91.4%	91.2%	32.5%	18.6%	
9	100%	101.1%	85.1%	52.5%	15.3%	
10	100%	99.5%	97.4%	59.2%	13.5%	
11	100%	95.3%	83.3%	65.1%	15.6%	
12	100%	99.1%	83.3%	67.1%	15.2%	
Mean	100%	98.0%	79.0%	48.0%	16.0%	
SEM	0	2.1	2.7	4.2	0.8	

Table 21: Percentage cell viability of HT1376 cells at 48 h, after treatment with doxazosin.

	72 h					
	control	10^{-6}	10^{-5}	$5X10^{-4}$	10^{-4}	
1	100%	79.7%	71.4%	11.0%	5.4%	
2	100%	90.1%	74.2%	8.0%	5.3%	
3	100%	86.2%	68.4%	11.6%	7.9%	
4	100%	86.1%	76.7%	11.3%	7.5%	
5	100%	102.5%	81.4%	17.6%	11.4%	
6	100%	100.0%	78.1%	18.9%	12.7%	
7	100%	91.7%	70.4%	12.0%	11.3%	
8	100%	106.1%	86.2%	16.7%	12.1%	
9	100%	99.3%	89.8%	10.7%	8.7%	
10	100%	92.2%	82.9%	10.3%	9.4%	
11	100%	92.7%	88.4%	11.2%	8.5%	
12	100%	99.3%	93.1%	12.1%	10.2%	
Mean	100%	94.0%	80.0%	13.0%	9.0%	
SEM	0	2.5	4.1	0.9	0.9	

Table 22: Percentage cell viability of HT1376 cells at 72 h, after treatment with doxazosin.

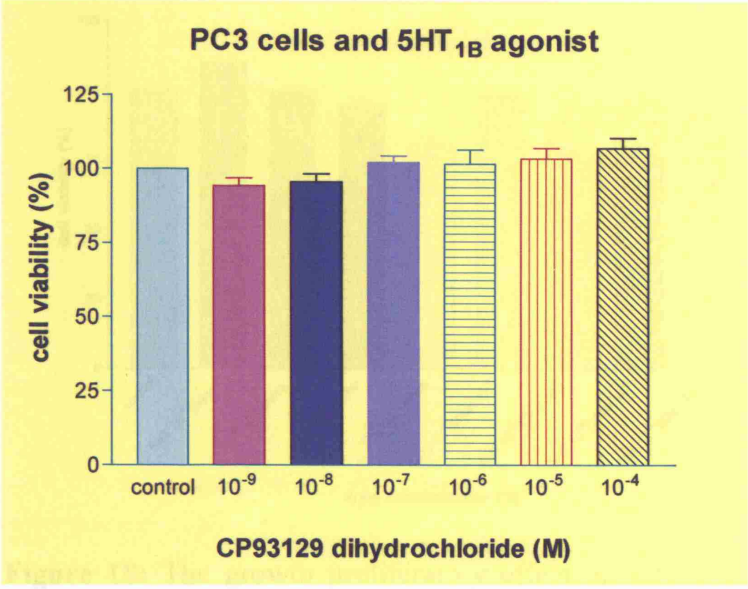


Figure 17: A dose-dependent growth proliferation of PC3 cells by $5HT_{1B}$ agonist (CP93129 dihydrochloride) with a maximum effect seen at a concentration of 10^{-4} M at 72 h. The bars represent the standard error of the mean (SEM).

	control	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
1	100%	87.0%	100.0%	113.0%	81.7%	94.7%	98.2%	
2	100%	87.7%	88.8%	97.8%	77.1%	88.3%	100.6%	
3	100%	97.4%	87.6%	93.5%	88.9%	92.2%	97.4%	
4	100%	88.9%	90.9%	103.3%	98.0%	98.7%	95.4%	
5	100%	99.5%	88.6%	101.0%	100.0%	114.4%	98.5%	
6	100%	85.8%	105.9%	98.8%	127.8%	120.1%	117.2%	
7	100%	105.5%	101.8%	92.6%	117.8%	109.2%	122.1%	
8	100%	111.0%	114.3%	113.0%	125.3%	126.0%	127.3%	
9	100%	89.7%	86.0%	94.9%	92.8%	94.5%	89.4%	
10	100%	104.8%	96.5%	110.5%	118.8%	103.9%	114.9%	
11	100%	88.8%	102.2%	110.9%	95.7%	91.3%	108.0%	
12	100%	86.7%	85.6%	97.0%	95.9%	110.0%	116.7%	
Mean	100%	94.4%	95.7%	102.2%	101.7%	103.6%	107.1%	
SEM	0	2.6	2.7	2.2	4.9	3.6	3.5	

Table 23: Percentage cell viability of PC3 cells at 72 h, after treatment with $5HT_{1B}$ agonist (CP93129 dihydrochloride).

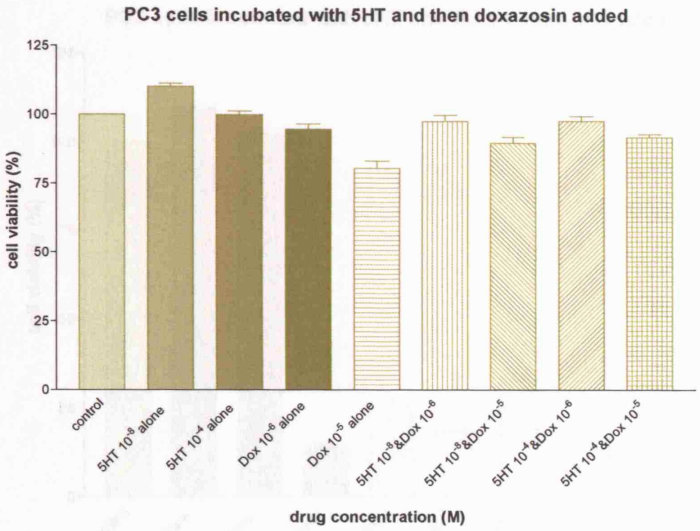


Figure 18: The growth proliferatory effect of 5HT (10^{-8} and 10^{-4} M) and growth inhibitory effect of doxazosin (10^{-6} and 10^{-5} M), on PC3 (androgen-independent prostate cancer) cell growth. The effect of incubation of PC3 cells with 5HT (10^{-8} and 10^{-4} M) for 45 min followed by the addition 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 (SEM).

	control	(5HT)10^{-8}	(5HT)10^{-4}	(Dox)10^{-6}	(Dox)10^{-5}	(5HT)10^{-8} & (Dox)10^{-6}	(5HT)10^{-8} & (Dox)10^{-5}	(5HT)10^{-4} & (Dox)10^{-6}	(5HT)10^{-4} & (Dox)10^{-5}
1	100%	108.1%	100.8%	91.4%	76.6%	93.0%	87.7%	88.9%	95.7%
2	100%	113.8%	101.3%	92.3%	87.2%	106.1%	85.4%	97.1%	90.4%
3	100%	108.6%	100.8%	86.5%	69.2%	86.5%	87.6%	94.3%	83.7%
4	100%	110.0%	95.3%	105.3%	67.4%	97.6%	84.0%	98.2%	94.2%
5	100%	109.8%	110.6%	97.5%	90.5%	101.3%	108.7%	99.8%	90.9%
6	100%	108.4%	98.4%	90.8%	85.2%	95.3%	91.4%	90.6%	95.7%
7	100%	103.6%	100.2%	98.8%	90.8%	86.4%	91.6%	100.2%	87.2%
8	100%	108.2%	99.6%	98.9%	93.8%	108.8%	98.1%	98.9%	94.0%
9	100%	119.3%	103.6%	96.4%	71.4%	103.0%	85.4%	96.7%	90.9%
10	100%	112.6%	102.2%	90.6%	85.5%	107.0%	84.7%	113.4%	95.4%
11	100%	110.9%	97.7%	84.2%	70.0%	94.6%	87.6%	92.5%	86.6%
12	100%	108.8%	93.4%	103.2%	77.4%	91.0%	84.3%	100.5%	96.0%
Mean	100%	110.2%	100.3%	94.7%	80.4%	97.5%	89.7%	97.6%	91.7%
SEM	0	1.1	1.3	1.9	2.7	2.2	2.1	1.8	1.7

Table 24: Percentage cell viability of PC3 cells at 72 h, after incubation with 5HT (10^{-8} and 10^{-4} M) for 45 min, followed by addition of doxazosin (10^{-6} and 10^{-5} M).

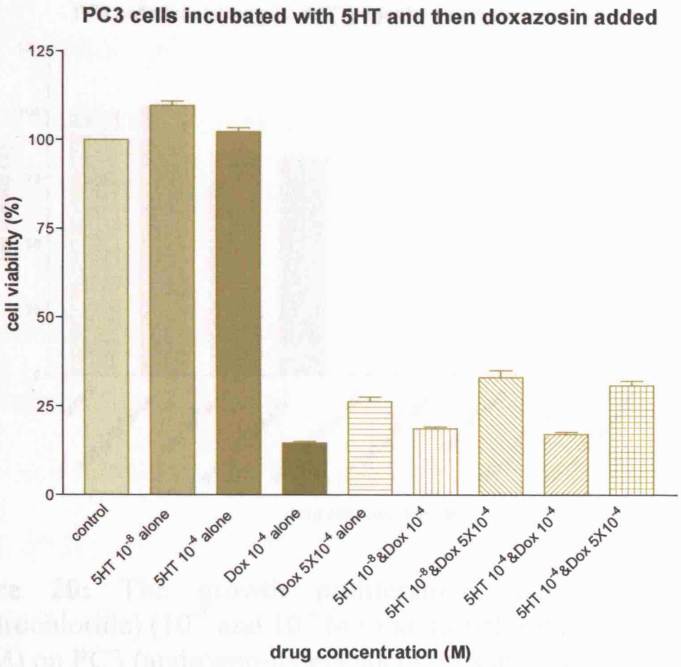


Figure 19: The growth proliferatory effect of 5HT (10^{-8} and 10^{-4} M) and growth inhibitory effect of doxazosin (10^{-4} and 5 x 10^{-4} M), on PC3 (androgen-independent prostate cancer) cell growth. The effect of incubation of PC3 cells with 5HT (10^{-8} and 10^{-4} M) for 45 min followed by the addition of doxazosin (5 x 10^{-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 (SEM).

	Control	(5HT)10^{-8}	(5HT)10^{-4}	(Dox)10^{-4}	(Dox)5X10^{-4}	(5HT)10^{-8} & (Dox)10^{-4}	(5HT)10^{-8} & (Dox)5X10^{-4}	(5HT)10^{-4} & (Dox)10^{-4}	(5HT)10^{-4} & (Dox)5X10^{-4}
1	100%	103.1%	99.5%	12.9%	33.3%	19.9%	33.0%	18.3%	32.7%
2	100%	107.9%	101.6%	15.0%	26.8%	18.2%	41.1%	19.5%	40.0%
3	100%	109.4%	94.5%	13.7%	21.4%	17.6%	34.1%	14.7%	30.1%
4	100%	105.4%	102.9%	14.5%	25.3%	16.4%	34.8%	15.7%	32.1%
5	100%	106.4%	103.8%	12.2%	21.4%	16.0%	48.6%	15.5%	29.8%
6	100%	113.1%	107.9%	14.7%	23.0%	20.2%	38.0%	13.6%	29.8%
7	100%	105.8%	96.2%	14.9%	22.4%	17.6%	26.2%	18.0%	21.2%
8	100%	114.2%	103.8%	16.9%	22.6%	19.9%	28.9%	21.0%	32.7%
9	100%	115.8%	105.3%	15.2%	29.1%	21.9%	32.7%	16.3%	30.5%
10	100%	112.6%	107.0%	17.4%	33.2%	19.6%	26.8%	16.9%	33.8%
11	100%	112.9%	104.0%	14.0%	25.8%	19.8%	26.9%	19.2%	31.8%
12	100%	110.0%	101.6%	13.9%	30.6%	17.0%	25.9%	16.8%	25.9%
Mean	100%	109.7%	102.3%	14.6%	26.2%	18.7%	33.1%	17.1%	30.9%
SEM	0	1.2	1.2	0.4	1.3	0.5	2.0	0.6	1.3

Table 25: Percentage cell viability of PC3 cells at 72 h, after incubation with 5HT (10^{-8} and 10^{-4} M) for 45 min, followed by addition of doxazosin (10^{-4} and 5 x 10^{-4} M).

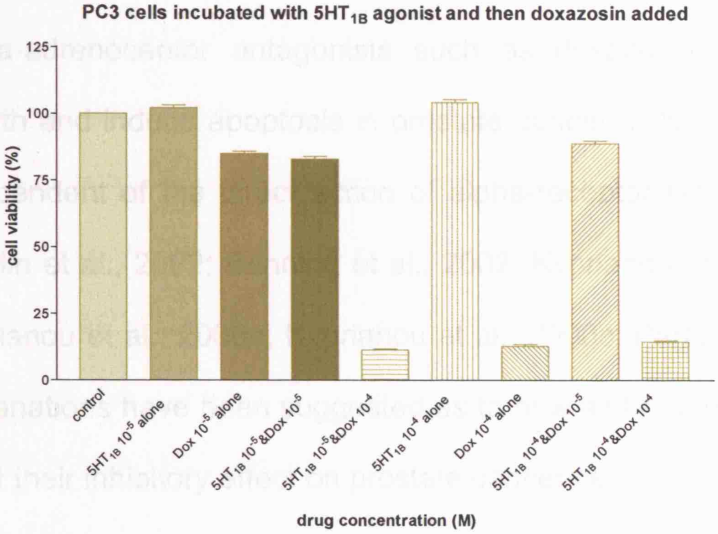


Figure 20: The growth proliferatory effect of $5HT_{1B}$ agonist (CP93129 dihydrochloride) (10^{-5} and 10^{-4} M) and growth inhibitory effect of doxazosin (10^{-5} and 10^{-4} M) on PC3 (androgen-independent prostate cancer) cell growth was assessed. The effect of incubation of PC3 cells with $5HT_{1B}$ 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 (SEM).

	Control	($5HT_{1B}$Ag) 10^{-5}	(Dox)10^{-5}	($5HT_{1B}$Ag) 10^{-5} & (Dox)10^{-5}	($5HT_{1B}$Ag) 10^{-5} & (Dox)10^{-4}	($5HT_{1B}$Ag) 10^{-4}	(Dox)10^{-4}	($5HT_{1B}$Ag) 10^{-4} & (Dox)10^{-5}	($5HT_{1B}$Ag) 10^{-4} & (Dox)10^{-4}
1	100%	102.3%	86.1%	89.6%	13.9%	104.6%	11.6%	90.3%	13.9%
2	100%	106.0%	78.3%	85.0%	12.5%	100.0%	12.0%	85.7%	14.1%
3	100%	99.8%	873%	88.6%	14.1%	105.7%	12.7%	90.9%	14.1%
4	100%	106.7%	83.9%	86.2%	12.4%	104.6%	11.3%	91.2%	12.8%
5	100%	96.0%	84.9%	87.9%	12.3%	103.4%	11.5%	86.4%	15.7%
6	100%	102.0%	86.7%	89.1%	13.8%	100.4%	11.4%	89.3%	15.9%
7	100%	96.5%	81.3%	87.3%	13.1%	102.5%	11.5%	86.2%	14.4%
8	100%	102.7%	87.3%	88.2%	13.8%	112.7%	12.5%	89.3%	17.6%
9	100%	102.9%	85.0%	88.5%	13.9%	107.5%	11.1%	91.6%	16.2%
10	100%	106.1%	83.2%	87.8%	12.2%	106.1%	12.0%	93.3%	13.6%
11	100%	98.6%	88.4%	90.7%	13.2%	105.0%	11.4%	91.8%	15.5%
12	100%	106.1%	88.0%	89.1%	12.5%	107.5%	13.0%	93.0%	14.3%
Mean	100%	102.1%	85.0%	88.2%	13.1%	105.0%	11.8%	89.9%	14.8%
SEM	0	1.1	0.9	0.4	0.2	1.0	0.2	0.8	0.4

Table 26: Percentage cell viability of PC3 cells at 72 h, after incubation with $5HT_{1B}$ agonist (CP93129 dihydrochloride) (10^{-5} and 10^{-4} M) for 45 min, followed by addition of doxazosin (10^{-5} and 10^{-4} M).

3. 7 Discussion

Alpha-adrenoceptor antagonists such as doxazosin and terazosin inhibit growth and induce apoptosis in prostate cancer cells. This inhibitory effect is independent of the direct action of alpha-receptor blockade by these drugs (Anglin et al., 2002; Benning et al., 2002; Kyprianou, 2000; Kyprianou, 2003; Kyprianou et al., 2000a; Kyprianou et al., 2000c; Partin et al., 2003). Various explanations have been suggested as to how alpha-adrenoceptor antagonists exert their inhibitory effect on prostate cancer cell growth.

For example, the growth inhibitory effect of doxazosin may be mediated via the upregulation of transforming growth factor (TGF-β_1). TGF-β_1 is a major regulator of prostate growth that acts by inhibiting cell proliferation, inducing apoptosis and regulating cell migration (Tahmatzopoulos et al., 2004a).

Xu et al proposed that terazosin-induced prostate cancer cell death was associated with G_1 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 (Xu et al., 2003b). Keledjian et al reported that Bcl-2 over-expression in prostate cancer cells exerts an antagonistic effect against the quinazoline-mediated apoptotic effect by suppressing cell attachment to gelatine matrix without affecting cell invasion (Keledjian et al., 2003).

As previously mentioned, 5HT acts as a growth factor on several non-tumoural cells (e.g. vascular smooth muscle, lung fibroblasts and renal mesangial cells) (Nemecek et al., 1986; Seuwen et al., 1990; Takuwa et al., 1989). 5HT also has a growth stimulatory effect on prostate cancer cells (Abdul et al., 1994; Dizeyi et al., 2004), small cell lung carcinoma cells

(Cattaneo et al., 1993; Cattaneo et al., 1995; Codignola et al., 1993) and colonic carcinoma (Dolezel et al., 1969; Tutton et al., 1978; Tutton et al., 1982; Tutton et al., 1979). $5HT_1$ or $5HT_2$ receptor antagonists can inhibit tumour growth. In prostate cancer cell lines PC3, DU145 and LNCaP, $5HT_{1A}$ antagonists and 5HT uptake inhibitors cause growth inhibition *in vitro* (Abdul et al., 1994; Abdul et al., 1995; Dizeyi et al., 2004). $5HT_4$ receptors are present in the human bladder and $5HT_3$ receptors are implicated in the 5HT-mediated contraction of the rabbit detrusor, following partial bladder outlet obstruction (Khan et al., 2000). Autoradiographic evidence suggests that doxazosin reduces 5HT binding in the rabitt bladder, and the beneficial effects of doxazosin in bladder outflow obstruction may be partly attributed to 5HT antagonism (Khan et al., 2000). Previous studies have also established that, doxazosin inhibits 5HT-induced platelet shape change via the $5HT_2$ receptor (Jagroop et al., 2001). The fact that doxazosin, an alpha-adrenoceptor antagonist exerts such profound 5HT blockade, would give credence to the reported similarity between 5HT receptors and alpha-adrenergic receptors (Abdul et al., 1994).

In our study, we established that doxazosin causes a dose-dependent growth inhibitory effect on both prostate and bladder cancer cells (Siddiqui et al., 2005a). The growth inhibition of HT1376 cells is a novel and potentially important finding, since it suggests that $alpha_1$-adrenoceptor antagonists may have a role in the treatment of transitional cell carcinoma of the bladder. Furthermore, we demonstrated that incubating PC3 cells for a short duration with 5HT or a $5HT_{1B}$ agonist, followed by exposure to doxazosin, led to a greater percent of viable cells being present at 72 h, as compared to when

PC3 cells are treated with doxazosin in the absence of 5HT or a $5HT_{1B}$ agonist (Siddiqui et al., 2005a). A possible explanation is that the early binding of 5HT or the $5HT_{1B}$ agonist to the 5HT receptors decreases any 5HT receptor-mediated growth inhibition of doxazosin. However, it is also possible that doxazosin and 5HT act through independent pathways.

An interesting approach for future studies will be that autoradiographic studies using 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. Moreover, it will be interesting to determine the effect lower concentrations of doxazosin (10^{-7}, 10^{-8} M) have on the growth of PC3 and HT1376 cells, after longer incubation periods (5-7 days).

CHAPTER 4

IMMUNOHISTOCHEMISTRY STUDIES ON PC3 AND HT1376 CELL LINES AND HUMAN PROSTATE AND BLADDER CANCER TISSUE

4.1 Introduction

Immunohistochemistry is the process of detection of cellular or tissue constituents (antigens) using (antibodies). The basic principle behind immunohistochemistry is the antigen-antibody interaction, the site of antibody binding being identified either by direct labelling of the antibody, or by use of a secondary labelling method .

The antibodies can be polyclonal or monoclonal in origin, the monoclonal ones being more specific in nature. Immunohistochemistry is widely used for diagnosis of cancers. Specific markers are known for various cancers, for example Carcino-embryonic antigen (CEA): used for colon cancer, CD15 and CD30: used for Hodgkin's disease, Alpha fetoprotein for yolk sac tumors and hepatocellular carcinoma and CD117 for gastrointestinal stromal tumours (www.myWiseOwl.com).

As $5HT_{1A}$ and $5HT_{1B}$ antagonists have a significant growth inhibitory effect on both PC3 and HT1376 cells *in vitro*, the purpose of the experiments in this chapter were to identify these two receptors ($5HT_{1A}$ and $5HT_{1B}$) in PC3 and HT1376 cells, as well as, human metastatic hormone refractory prostate cancer tissue and bladder cancer tissue.

4.2 Materials and Methods

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, were the two cell lines used in this experiment. Both were obtained from the American Type Culture Collection (ATCC).

Human hormone refractory prostate cancer tissue was obtained during surgery from three patients known to be suffering from prostate cancer, undergoing channel transurethral resection of the prostate (TURP) for bladder outlet obstruction. Human bladder cancer tissue was obtained from three patients undergoing transurethral resection of bladder tumour (TURBT) for transitional cell carcinoma of the bladder. Ethical approval was obtained and the Helsinki Declaration of Human Rights was strictly adhered to throughout this study.

4.3 Reagents

5HT receptor antibodies were purchased from Santa Cruz (St. Louis, MO). The 5HTR1A antibody used was SR-1A (H-119): sc-10801 and the 5HTR1B antibody used was SR-1B (C-19): sc-1460. For 5HTR1A the secondary antibody used was a goat anti-rabbit antibody whereas in the case of 5HTR1B it was a rabbit anti-goat antibody. Both of these secondary antibodies were purchased from Vector Laboratories Ltd. In the case of the ABC (Avidin Biotin Complex) staining in tissue the ABC was purchased from Vector Laboratories Ltd. Normal donkey serum and DAB reagent was used in the processes which were obtained from Sigma Laboratories Ltd.

Fitc, Cy3 and Dapi secondary antibodies were used in immunoflorescence which were purchased from Upstate Laboratories. Vector shield used for mounting purposes was obtained from Vector Laboratories Ltd.

4.4 Immunohistochemistry

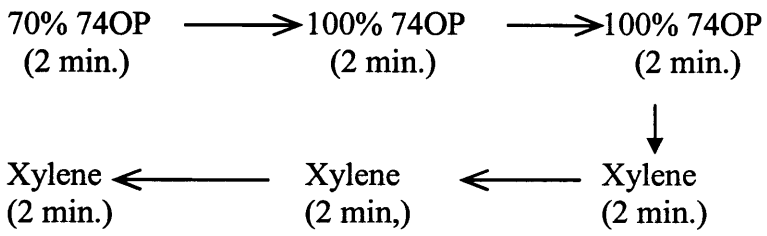
The PC3 and HT1376 cells were fixed with 4% paraformaldehyde. This was followed by the addition of 0.5% Tween in PBS for a few seconds to increase the permeability of the cell membrane. After this the cells were given several washes with PBS. The cells were then incubated with normal donkey serum for 1 h at a dilution of 1:30 at room temperature with the intention to block non-specific binding. The primary antibodies were then added at a dilution of 1:2000 and were kept at 4°C for 24 h. After washing with PBS the Fitc or Cy3 secondary antibodies (for identification of $5HT_{1A}$ and $5HT_{1B}$ receptors, respectively); raised in donkey, were added and the cells were left in a dark room for 45 min. The cells were then mounted in Vector shield with Dapi (for identification of the cell nucleus) and were examined under a flourescence microscope.

For tissue immunohistochemistry (IHC), the prostate and bladder cancer tissue specimens were fixed in 4% neutral buffered formaldehyde for 24 h and then embedded in paraffin. After hematoxylin and eosin (H & E) staining the specimens were examined to confirm cancer and grade it (Figures 30 & 41). Paraffin sections (3 μm) of prostate and bladder cancer tissue were then deparaffinized and rehydrated, and for antigen retrieval, they were incubated with citrate buffer (10 mM, pH 6.0) and heated twice in a microwave oven at 750 watts for 5 min. The sections were incubated with primary polyclonal antibodies; rabbit ($5HTR_{1A}$) and goat ($5HTR_{1B}$). The $5HTR_{1A}$ antibody was raised against a recombinant protein corresponding to amino acids 218-336 mapping near the carboxy terminus of 5HT receptor 1A of human origin

whereas the $5HT_{1B}$ antibody was raised against a peptide mapping at the carboxy terminus of 5HT receptor 1B of human origin.

Both antibodies were titrated prior to staining to obtain an optimal dilution producing crisp staining with minimum background. The cells were incubated with the primary antibodies at a dilution of 1:1000 for 24 h at 4°C. After washing three times with PBS, the sections were incubated with biotinylated secondary antibodies against rabbit or goat IgG. (Rabbit anti goat immunoglobulin – biotinylated and Goat anti-rabbit immunoglobulin – biotinylated) The tissue was left for 45 min at room temperature and then washed three times with PBS. This was followed by the addition of the Avidin Biotin Complex for 45 min followed by a repeat series of washes with PBS.

DAB (3,3'-diaminobenzidin tetrahydrochloride reagent as included in the DAKO ChemMate™ kit) was then added to the tissue for approximately 10 min. The tissue sections were counter stained with haematoxylin, dehydrated, cleared and mounted in Pertex according to the protocol below.



4.5 Results

$5HT_{1A}$ and $5HT_{1B}$ immunoreactivity was seen in PC3 (n=3) and HT1376 cells (n=3) (Figures 21, 23, 33 & 35). $5HT_{1A}$ immunoreactivity was demonstrated in the cell membrane and cytoplasm by green immunofluorescent staining using

Fitc secondary antibody. $5HT_{1B}$ immunoreactivity was demonstrated in the cell membrane and cytoplasm by red immunofluorescent staining using Cy3 secondary antibody. The Dapi antibody was used to identify the cell nucleus (blue immunofluorescent stain).

Immuno-staining for $5HT_{1A}$ and $5HT_{1B}$ receptors was also demonstrated in paraffin sections of malignant metastatic hormone refractory prostate cancer tissue (n=3) and bladder cancer tissue (n=3), using the Avidin Biotin Complex (ABC) stain, thus confirming the presence of the two receptor subtypes (Figures 25, 27, 37 & 39). The brown staining in the cross sections of the prostate and bladder cancer tissue highlights the $5HT_{1A}$ and $5HT_{1B}$ receptors. Immunohistochemistry is a technique used to identify receptors and not quantify them, however, the staining of the majority of the prostate and bladder cancer cells and tissue, provides an indication of the abundant distribution of $5HT_{1A}$ and $5HT_{1B}$ receptors.

No immunoreactivity was demonstrated in negative controls for PC3 and HT1376 cells, in which the primary antibody was exluded (Figures 22, 24, 34, 36). This was evident by the absence of the green (Fitc) and red (Cy3) immuno-staining in the cell membrane and cytoplasm of PC3 and HT1376 cells. In malignant hormone refractory prostate cancer tissue and bladder cancer tissue, no immunoreactivity was demonstrated in negative controls. This was evident by the absence of brown staining in the prostate and bladder cancer tissue in which the primary antibody was excluded (Figures 26, 28, 38 & 40).

Immunohistochemistry for $5HT_{1A}$ and $5HT_{1B}$ was also performed in PC3 cells using the Avidin Biotin Complex (ABC) stain (Figures 31 & 32). Receptors

were highlighted in the cell membrane and cytoplasm of the PC3 cells by brown staining.

H & E stained sections of the prostate and bladder tissue were examined by a Consultant Histopathologist who confirmed the presence of cancer (Figures 30 & 41). H & E staining was also performed in PC3 cells (Figure 29).

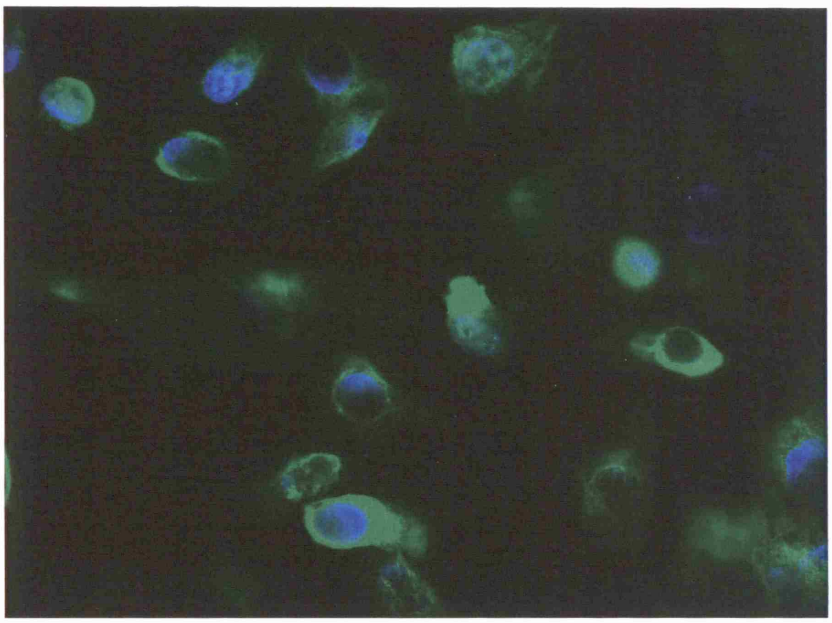


Figure 21: Immunohistochemical localisation of $5HT_{1A}$ receptors in the cell membrane and cytoplasm of PC3 cells is shown by green fluorescent staining using Fitc secondary antibody. The cell nuclei are stained blue with the Dapi antibody.

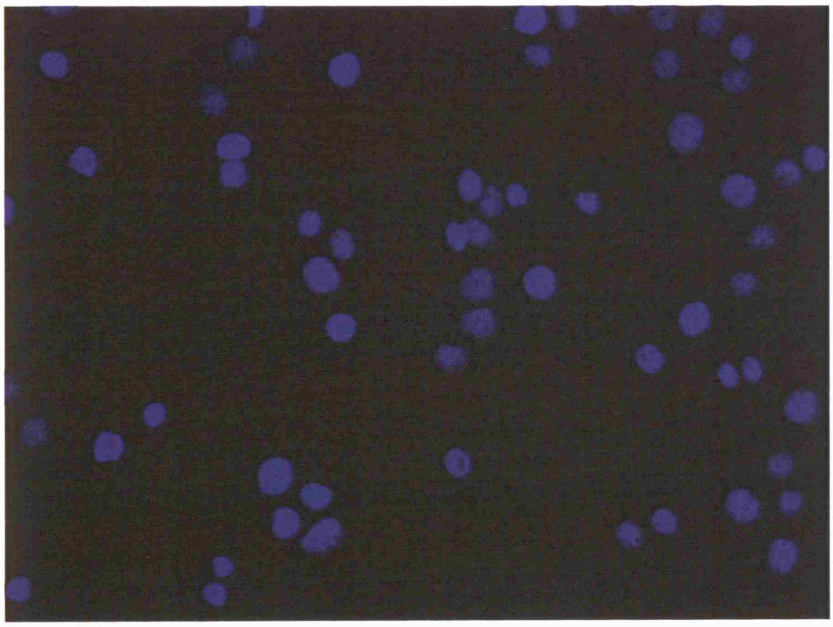


Figure 22: *Negative control for $5HTR_{1A}$ in PC3 cells;* No green immunofluorescent staining (Fitc) for $5HT_{1A}$ is seen in the cell membrane and cytoplasm of PC3 cells, when the primary antibody is excluded. The cell nuclei are stained blue with the Dapi antibody.

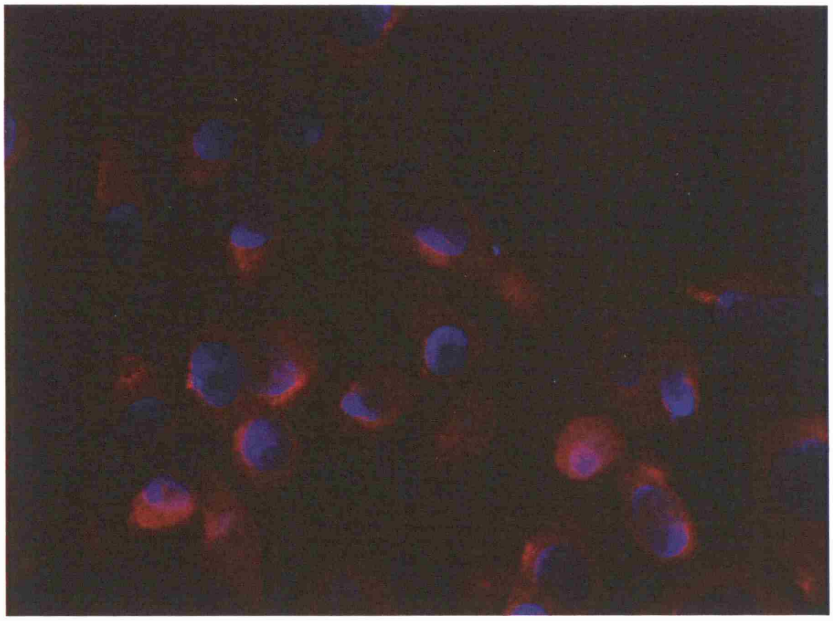


Figure 23: Immunohistochemical localisation of $5HT_{1B}$ receptors in the cell membrane and cytoplasm of PC3 cells is shown by red fluorescent staining using Cy secondary antibody. The cell nuclei are stained blue with the Dapi antibody.

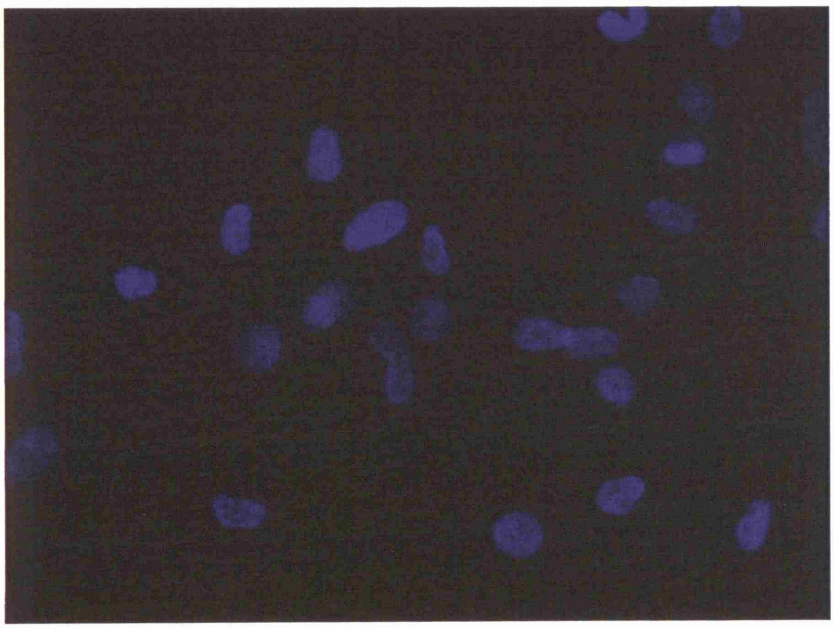


Figure 24: *Negative control for $5HTR_{1B}$ in PC3 cells;* No red immunofluorescent staining (Cy) for $5HT_{1B}$ is seen in the cell membrane and cytoplasm of PC3 cells, when the primary antibody is excluded. The cell nuclei are stained blue with the Dapi antibody.

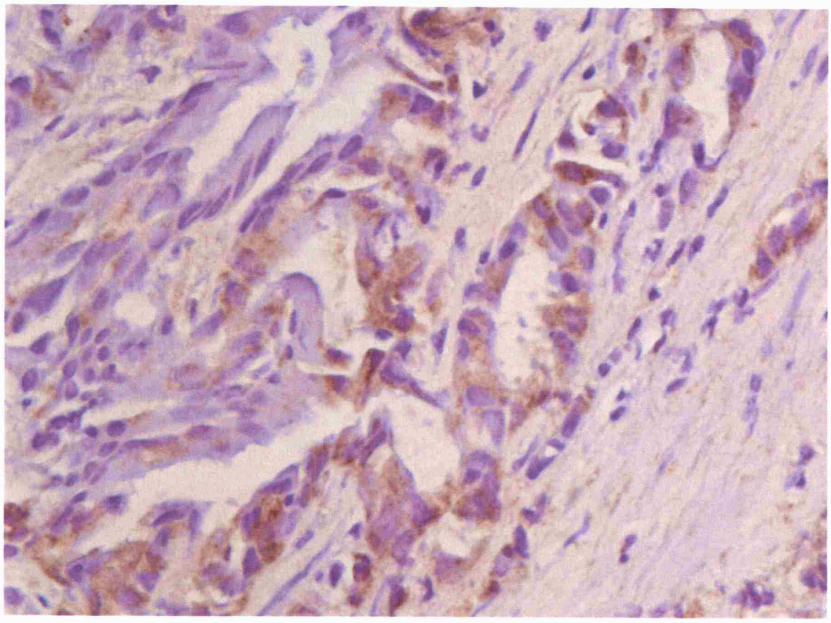


Figure 25: Immunostaining for $5HT_{1A}$ receptors in human prostate cancer tissue is shown by the brown colouration using the ABC (Avidin Biotin Complex).

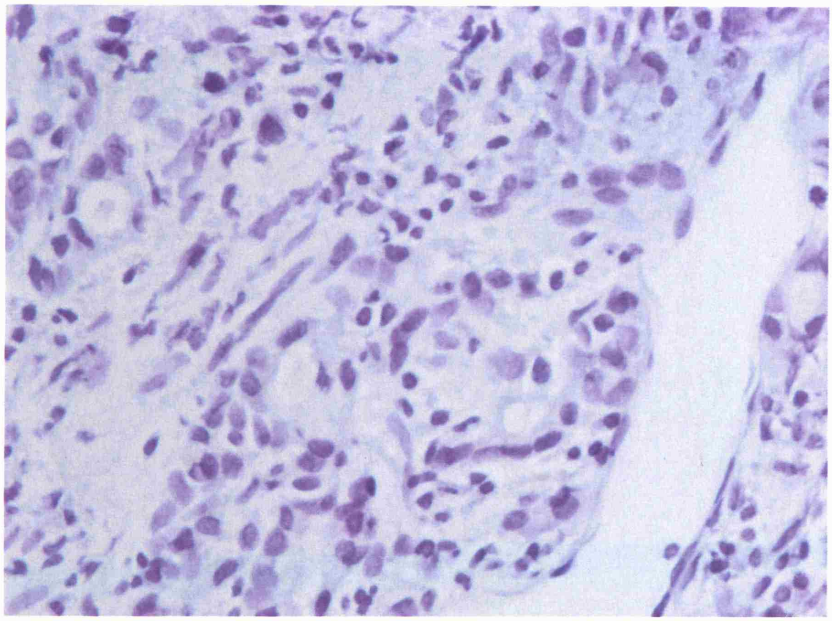


Figure 26: *Negative control for $5HTR_{1A}$ in human prostate cancer tissue;* No brown immunostaining for $5HT_{1A}$ is seen in a 3 μm paraffin section of human prostate cancer tissue using the ABC (Avidin Biotin Complex) stain, when the primary antibody is excluded.

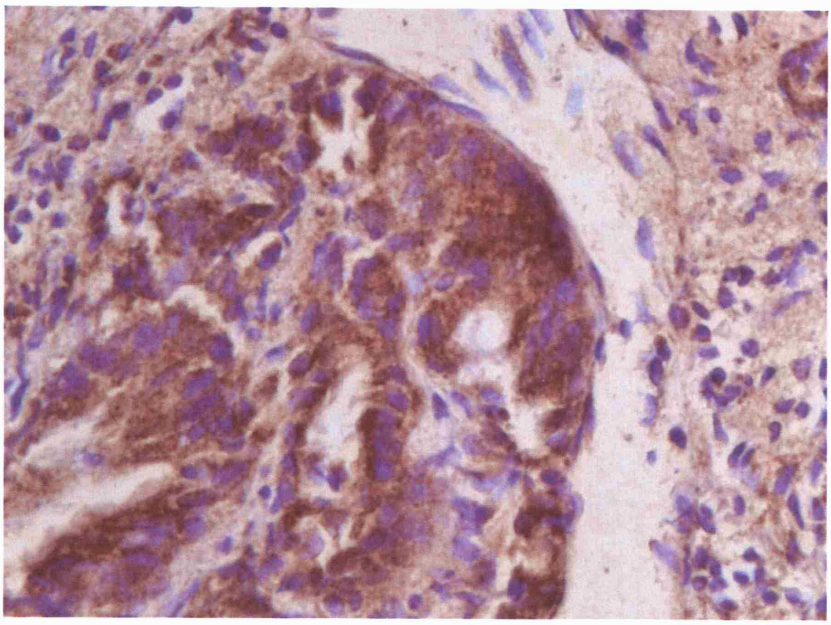


Figure 27: Immunostaining for $5HT_{1B}$ receptors in human prostate cancer tissue is shown by the brown colouration using the ABC (Avidin Biotin Complex).

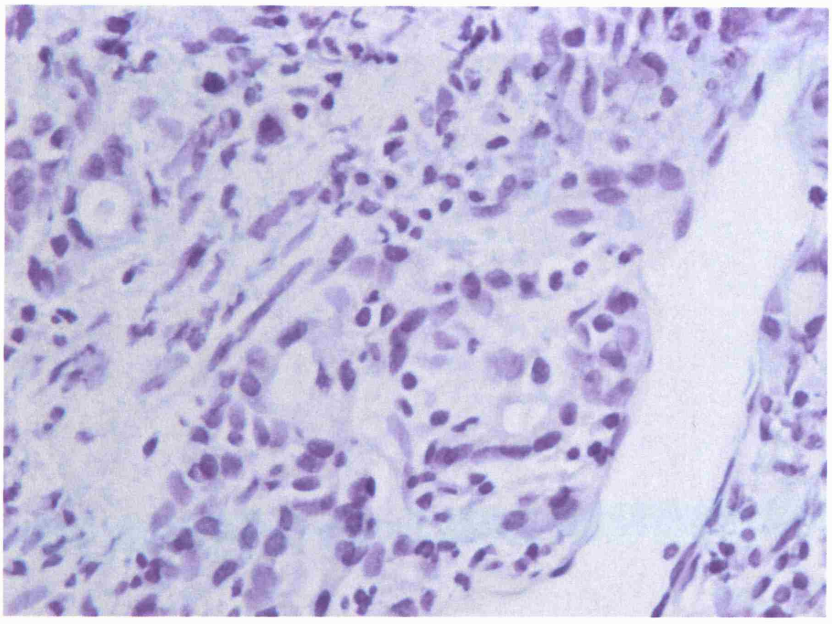


Figure 28: *Negative control for $5HTR_{1B}$ in human prostate cancer tissue;* No brown immunostaining for $5HT_{1B}$ is seen in a 3 μm paraffin section of human prostate cancer tissue using the ABC (Avidin Biotin Complex) stain, when the primary antibody is excluded.

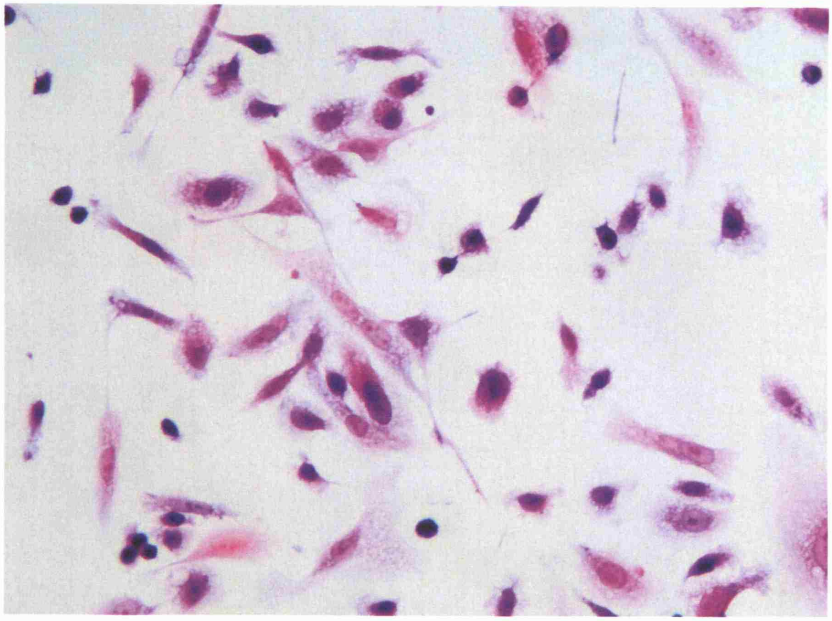


Figure 29: H & E staining X 10 of PC3 cells.

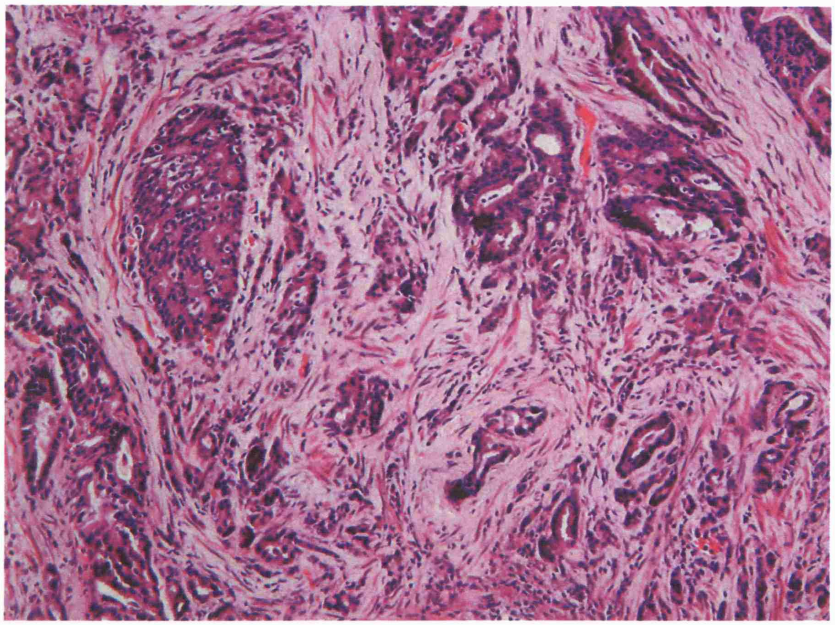


Figure 30: H & E staining X 10 of human prostate cancer tissue.

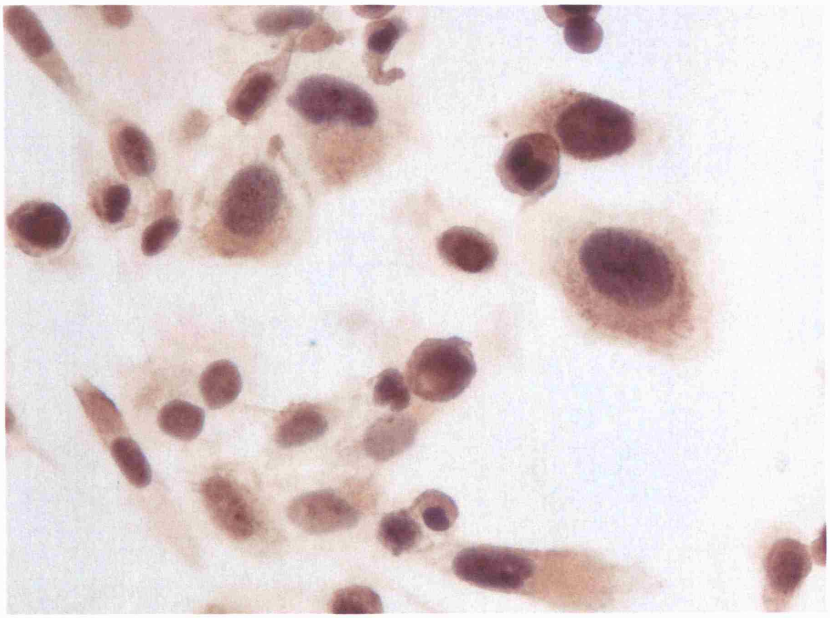


Figure 31: An immunoreaction of $5HT_{1A}$ is seen in PC3 cells using the ABC (Avidin Biotin Complex) stain X 40.

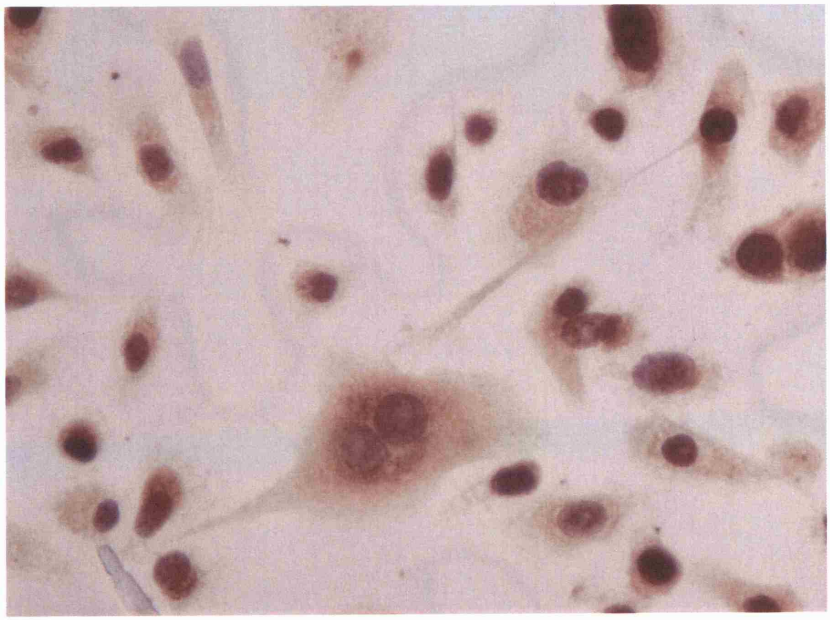


Figure 32: An immunoreaction of $5HT_{1B}$ is seen in PC3 cells using the ABC (Avidin Biotin Complex) stain X 40.

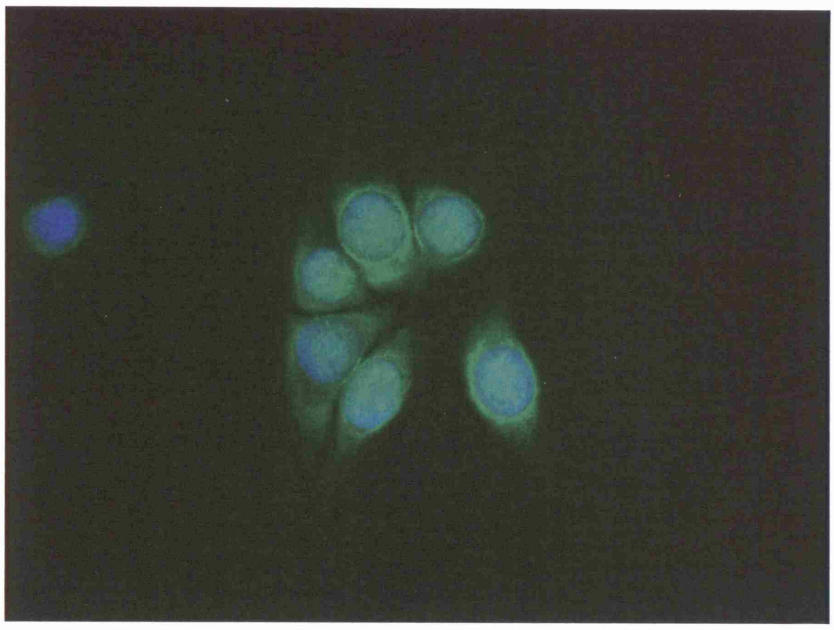


Figure 33: Immunohistochemical localisation of $5HT_{1A}$ receptors in the cell membrane and cytoplasm of HT1376 cells is shown by green fluorescent staining using Fitc secondary antibody. The cell nuclei are stained blue with the Dapi antibody.

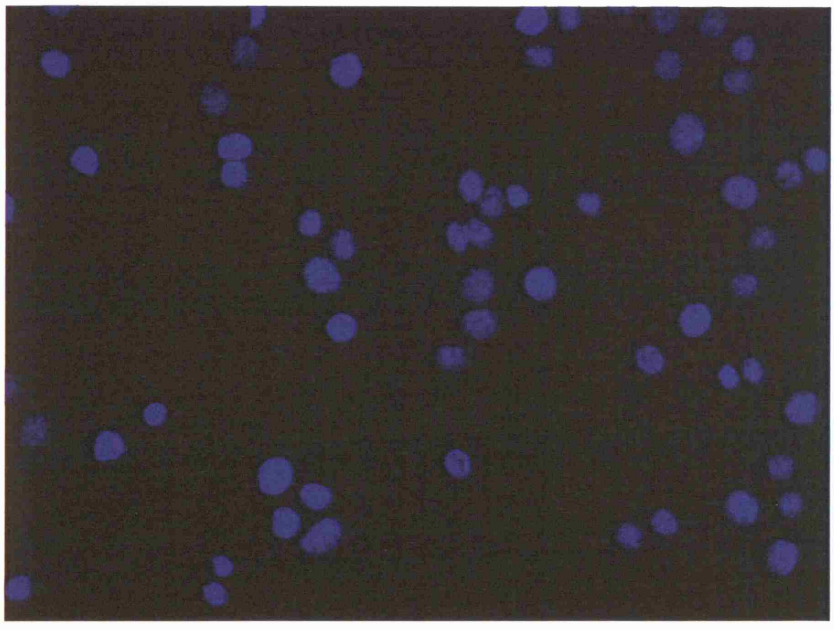


Figure 34: *Negative control for $5HTR_{1A}$ in HT1376 cells;* No green immunofluorescent staining (Fitc) for $5HT_{1A}$ is seen in the cell membrane and cytoplasm of HT1376 cells, when the primary antibody is excluded. The cell nuclei are stained blue with the Dapi antibody.

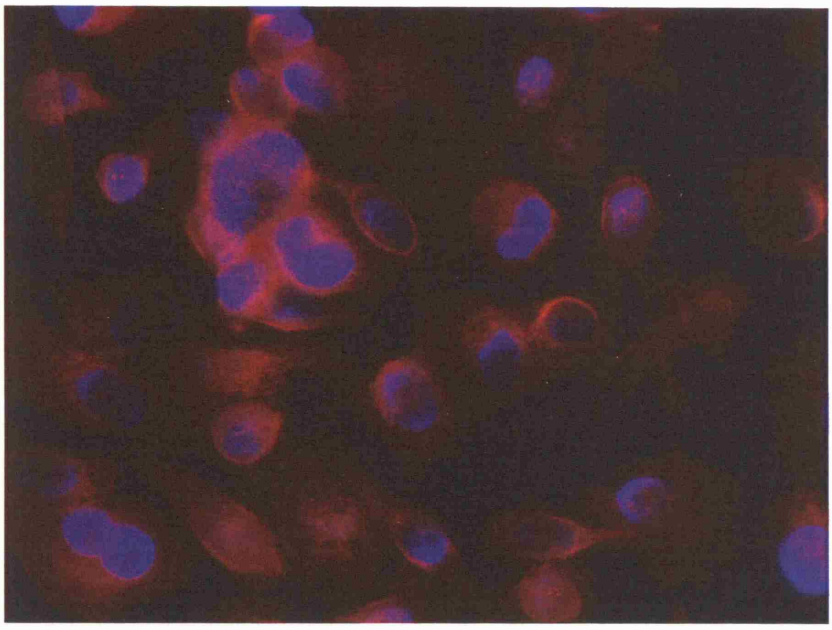


Figure 35: Immunohistochemical localisation of $5HT_{1B}$ receptors in the cell membrane and cytoplasm of HT1376 cells is shown by red fluorescent staining using Cy secondary antibody. The cell nuclei are stained blue with the Dapi antibody.

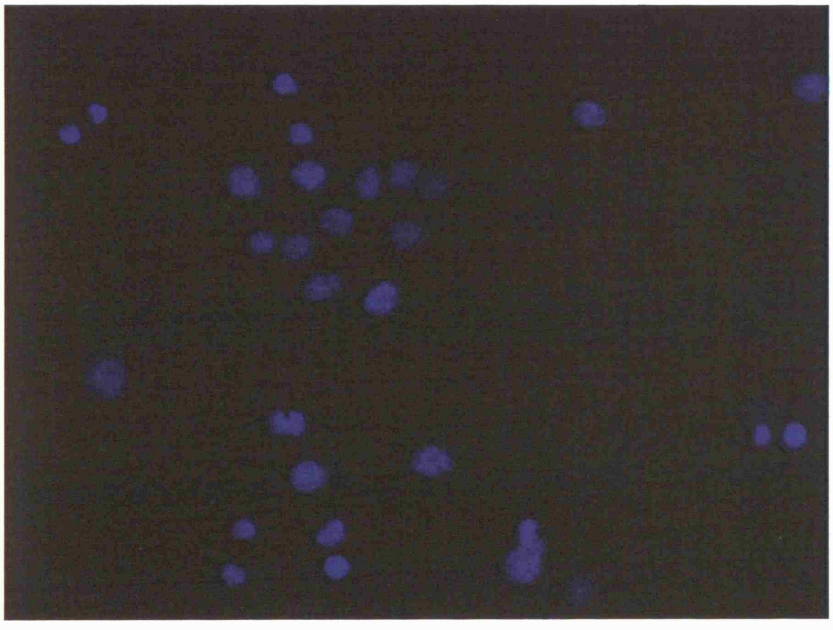


Figure 36: *Negative control for $5HTR_{1B}$ in HT1376 cells;* No red immunofluorescent staining (Cy) for $5HT_{1B}$ is seen in the cell membrane and cytoplasm of HT1376 cells, when the primary antibody is excluded. The cell nuclei are stained blue with the Dapi antibody.

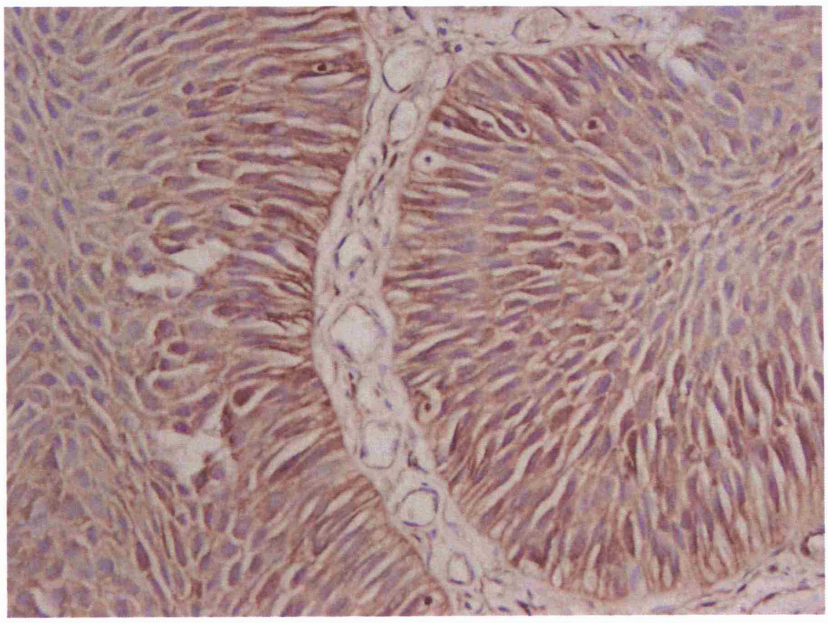


Figure 37: Immunostaining for $5HT_{1A}$ receptors in human bladder cancer tissue is shown by the brown colouration using the ABC (Avidin Biotin Complex).

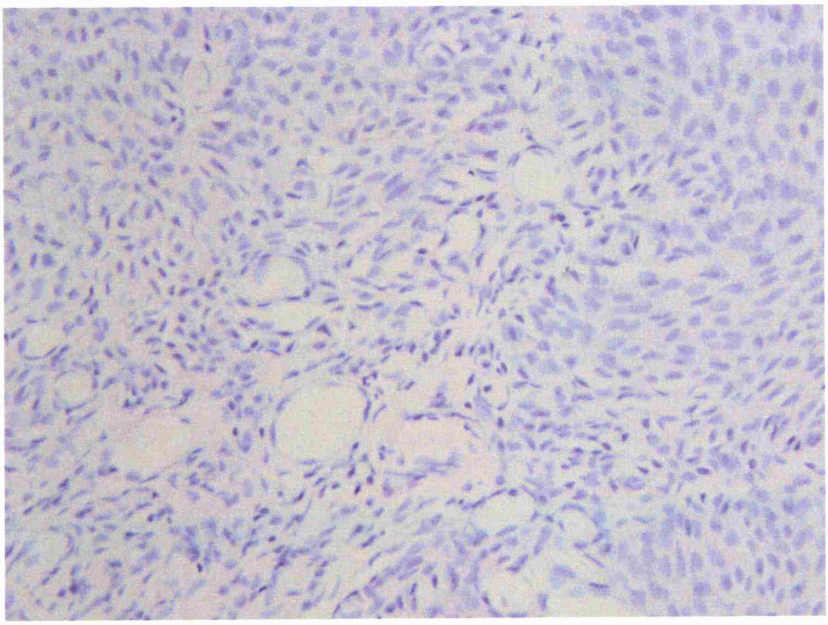


Figure 38: *Negative control for $5HTR_{1A}$ in human bladder cancer tissue;* No brown immunostaining for $5HT_{1A}$ is seen in a 3 μm paraffin section of human bladder cancer tissue using the ABC (Avidin Biotin Complex) stain, when the primary antibody is excluded.

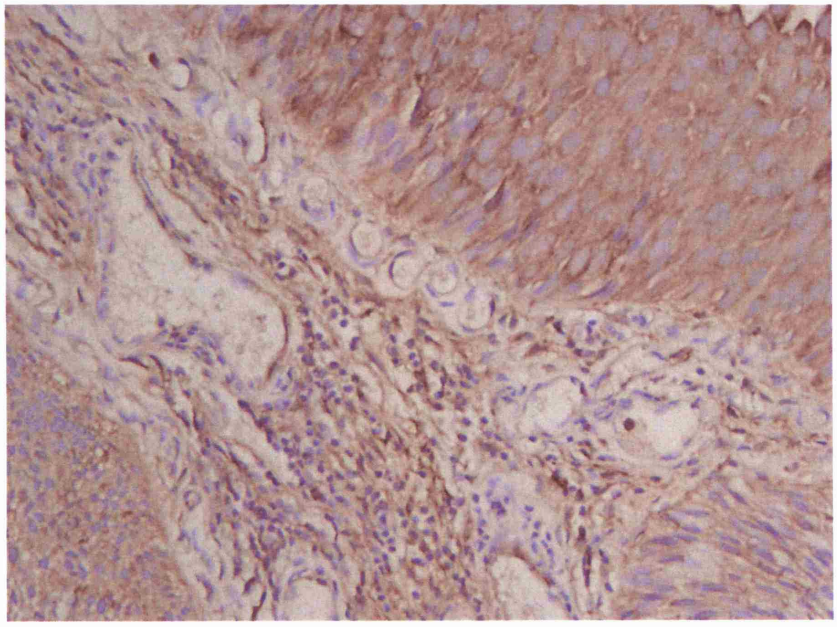


Figure 39: Immunostaining for $5HT_{1B}$ receptors in human bladder cancer tissue is shown by the brown colouration using the ABC (Avidin Biotin Complex).

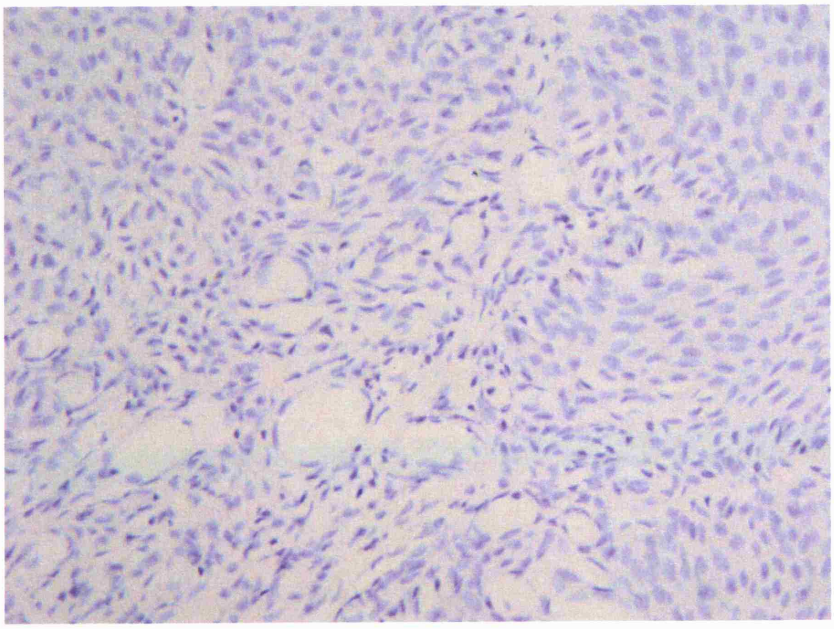


Figure 40: *Negative control for $5HTR_{1B}$ in human bladder cancer tissue;* No brown immunostaining for $5HT_{1B}$ is seen in a 3 μm paraffin section of human bladder cancer tissue using the ABC (Avidin Biotin Complex) stain, when the primary antibody is excluded.

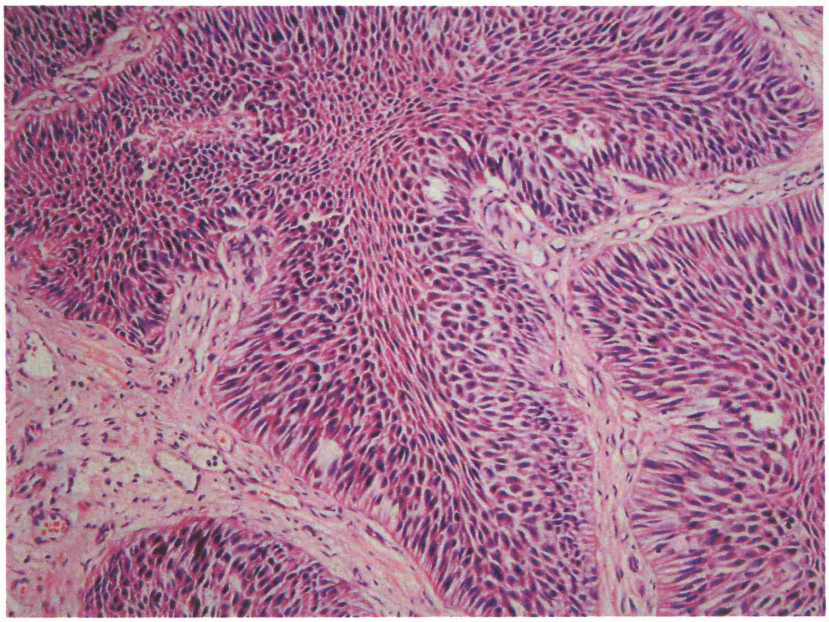


Figure 41: H & E staining X 10 of human bladder cancer tissue.

4.6 Discussion

Our cell proliferation studies determined that only two antagonists ($5HT_{1A}$ and $5HT_{1B}$) were functionally active in causing growth inhibition in PC3 and HT1376 cells. Therefore, our immunohistochemical studies concentrated on the localisation of these receptors ($5HTR_{1A}$ and $5HTR_{1B}$) in PC3 cells, HT1376 cells, hormone refractory prostate cancer tissue and bladder cancer tissue. Dizeyi et al also localised $5HT_{1A}$ and $5HT_{1B}$ receptors using immunohistochemistry in PC3 cells and prostate cancer tissue. Immunohistochemistry on malignant and benign epithelial cells demonstrated $5HTR_{1A}$ and $5HTR_{1B}$ immunoreactivity in the cytoplasm (Dizeyi et al., 2004). Immunohistochemistry detected small foci of $5HTR_{1A}$ and $5HTR_{1B}$ in BPH tissue but only on intense immunostaining. In prostate tumour, the strongest staining for $5HT_{1A}$ and $5HT_{1B}$ receptors was seen in Gleason grade 4 and 5 tumours. Grades below this had weak staining (Dizeyi et al., 2004). Therefore, it was concluded that the higher the grade of prostate cancer, greater is the $5HTR_{1A}$ and $5HTR_{1B}$ expression. $5HTR_{1A}$ immunoreactivity was also demonstrated in the cytoplasm of PC3 cells xenografted subcutaneously in athymic nude mice, lymph node and bone metastases as well as NE cells (Dizeyi et al., 2004).

Abdul et al demonstrated stronger staining for 5HT in the PC3 cell line as compared to DU145 and LNCaP cells.

It is evident that our results concur with those of Dizeyi et al that $5HT_{1A}$ and $5HT_{1B}$ receptors exist in PC3 cells and human hormone refractory prostate cancer tissue. In addition, we have localised $5HT_{1A}$ and $5HT_{1B}$ receptors in

HT1376 cells and bladder cancer tissue, thus establishing a 5HT receptor profile for bladder cancer.

Based on the immunohistochemical localisation of $5HT_{1A}$ and $5HT_{1B}$ receptors in human prostate and bladder cancer tissue, we infer that administration of $5HT_{1A}$ and $5HT_{1B}$ antagonists in prostate and bladder cancer patients is likely to inhibit the cancer growth.

CHAPTER 5

WESTERN BLOT ANALYSIS ON PC3 AND HT1376 CELL LINES AND HUMAN PROSTATE AND BLADDER CANCER TISSUE

5.1 Introduction

Western blot analysis is a procedure in which proteins separated by electrophoresis in polyacrylamide gels are transferred (blotted) onto nitrocellulose or nylon membranes and identified by specific complexing with antibodies that are either pre-or post-tagged with a labelled secondary protein (www.biologyonline.org).

Western blot analysis can detect one protein in a mixture of any number of proteins while giving information about the size of the protein. It does not matter whether the protein has been synthesised *in vivo* or *in vitro*. This method is, however, dependent on the use of a high-quality antibody directed against a desired protein. Therefore, it is necessary that at least a small portion of the protein is obtained from a cloned DNA fragment. In this technique the antibody is used as a probe to detect the protein of interest (www.lsvl.la.asu.edu.com).

In this chapter, using western blotting, we examine the 5HT receptor profile in both human prostate and bladder cancer cells and tissue. Our intention was primarily to identify the $5HT_{1A}$ and $5HT_{1B}$ receptor proteins, and to also determine the other 5HT receptor proteins present in PC3 and HT1376 cells as well as, in human metastatic hormone refractory prostate cancer tissue and bladder cancer tissue.

5.2 Materials and Methods

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 were used in this experiment. Both cell lines were obtained from the American Type Culture Collection (ATCC).

Human prostate cancer tissue was obtained during surgery from three patients with metastatic hormone refractory prostate cancer, undergoing channel transurethral resection of the prostate (TURP) for bladder outlet obstruction. Human bladder cancer tissue was obtained from three patients undergoing transurethral resection of transitional bladder tumour (TURBT) for transitional cell carcinoma of the bladder. Ethical approval was obtained and the Helsinki Declaration of Human Rights was strictly adhered to throughout this study.

5.3 Reagents

5HT receptor antibodies were purchased from Santa Cruz (St. Louis, MO). The 5HTR antibodies used were SR-1A (H-119): sc-10801, SR-1B (C-19): sc-1460, SR-1D (H-70): sc-25644, SR-2A (H-18): sc-15074, SR-2B (I-20): sc-15076, SR-2C (N-19): sc-15081, SR-3 (C-20): sc-19152 and SR-4 (N-16): sc-19154.

Lysis buffer was used for homogenising the tissue and cells.

The contents of this lysis buffer were as follows:

Lysis Buffer

Final concentration	Stock	To prepare 20 ml
Tris 50 mM pH 7.4	1 M	1 ml
NaCl 50 mM	1 M	1 ml
SDS 1%	10%	2 ml
Leupeptin 1 μg/ml	5 mg/ml	4 μl
Chymostatin 10 μg/ml	5 mg/ml	40 μl
Pepstatin 1 μg/ml	1 mg/ml	20 μl
Bestatin 40 μg/ml	5 mg/ml	160 μl
TLCK 50 μg/ml	5 mg/ml	200 μl
EDTA 2 mM	0.5 M pH 8	80 μl
PMSF 1 mM		6.97 μl
Water		11.49 ml

The BioRad protein assay kit was used to determine protein concentrations. SDS gel 4-12% used for loading samples was purchased from Invitogen Laboratories. 3% non-fat milk in Tris-buffered saline containing 0.2% Tween 20 (TBST) used to block non-specific binding of proteins was purchased from Avesham International.

5.4 Western Blot Analysis

Frozen samples of human prostate and bladder cancer tissue were homogenised at 0°C in lysis buffer (contents of which are mentioned in the box above).

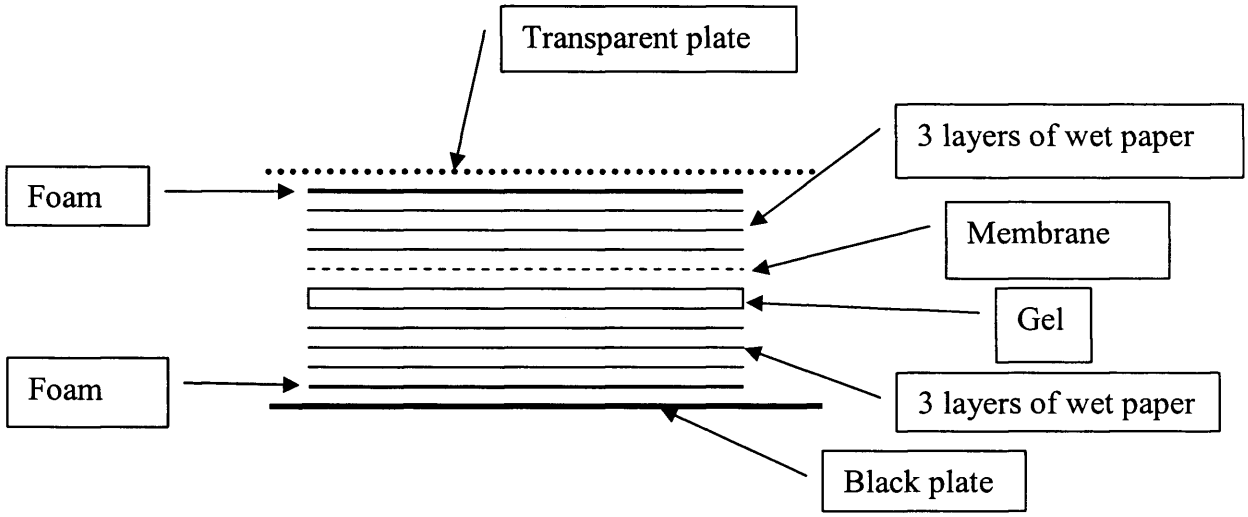
Protein extractions from PC3 and HT1376 cell lines were prepared as follows; cells were lysed in 1ml lysis buffer, (contents of which are mentioned in the box above) containing complete protease inhibitor (Boehringer-Mannheim). After centrifugation at 15,000 g for 45min, the supernatant containing the proteins was kept. Protein concentrations were determined using the BioRad protein assay kit. Aliquots of extractions containing 5-10 mg/ml of protein were prepared. Samples were loaded into pre-cast SDS gel 4-12%. After electrophoresis at 200 constant volts, the proteins were transferred onto a nitrocellulose membrane. The process for this transfer was as follows:

Transfer buffer	
Tris base	5.81 g
Glycine	2.93 g
SDS	0.37 g
Methanol	200 ml
The final volume of the buffer is made up to 1 litre.	

- Cut out 6 cm x 9 cm rectangles of blotting paper. Six pieces of blotting paper are required for each gel.

  Also cut out 6 cm x 9cm rectangles of the Hybond nitrocellulose filter paper, one for each gel that is being transferred.

- Pre-soak six of the pieces of blotting paper and the pieces of foam in transfer buffer.



- First place one of the pre-soaked pieces of foam onto the black plate of the transfer cassette.

- Place three of the pre-soaked pieces of blotting paper on top of the foam.

- Carefully remove the gel from the running apparatus and place on top of the three layers of blotting paper. Keep the gel wet with transfer buffer at all times to prevent the gel tearing.

- Place a pre-soaked piece of the nitrocellulose filter on top of the gel.
- Place three pre-soaked pieces of blotting paper on top of the nitrocellulose filter.

- Place a pre-soaked piece of foam on top of the layers of blotting paper.

- The cassette can now be closed and placed into the transfer tank making sure that the black surface is aligned with the black surface of the transfer tank.

- Place the ice block into the transfer tank and top up with more transfer buffer put a magnetic flea into the tank.

- Place lid on tank making sure that the black pin plugs into the black socket and that the red pin plugs into the red socket.

- Plug the apparatus into the power supply (IN COLD ROOM) again making sure that black goes to black and red goes to red.

- Place transfer tank onto magnetic stirrer and turn on power supply.

- Set voltage to 250 mA and transfer time for 2 h. Press RUN.

Non-specific binding of proteins was blocked with 3% non-fat milk in Tris-buffered saline containing 0.2% Tween 20 (TBST) for 30 min at RT. The membranes were subsequently incubated for 1 h with anti- 5HT1A, 1B, 1D, 2A, 2B, 2C, 3 and 4 IgGs at a dilution of 1:5000 in TBS. The cells and tissue were then washed three times with TBST at intervals of 15 min. This was followed by incubation with alkaline- phosphatase linked secondary antibody at a dilution of 1:10,000 in TBST for 45 min. Washing with TBST was repeated. Immunoreactive components were now visualised by enhanced chemiluminescent ECL Plus detection.

5.5 Results

Western blot analysis demonstrated 5HTR1A, 1B, 1D, 2A, 2B and 2C proteins in PC3 cells (n = 3) and human metastatic hormone refractory prostate cancer tissue (n = 3).

5HTR1A, 1B, 1D, 2A, 2B, and 2C proteins were also demonstrated in HT1376 cells (n= 3) and human bladder cancer tissue (n = 3).

$5HTR_3$ and $5HTR_4$ proteins could not be identified in PC3 cells, HT1376 cells or human prostate and bladder cancer tissue.

Polyclonal anti-$5HTR_{1A}$ and anti-$5HTR_{1B}$ IgGs recognised single bands of 46 kDa and 43 kDa respectively, in PC3 and HT1376 cells as well as, in human hormone refractory prostate cancer tissue and bladder cancer tissue (Figures 42 & 43).

Polyclonal anti-5-HTR_{1D} IgGs showed a single band of 41 kDa in PC3 cells, HT1376 cells, human prostate and bladder cancer tissue (Figure 44).

Bands of 52kDa, 54kDa and 51kDa were demonstrated with Polyclonal anti-5-HTR_{2A}, anti-5-HTR_{2B} and anti-5-HTR_{2C} IgGs respectively, in PC3 and HT1376 cells as well as prostate and bladder cancer tissue (Figures 45, 46 and 47).

The positive control used was rat brain tissue which demonstrated 5HT receptor proteins.

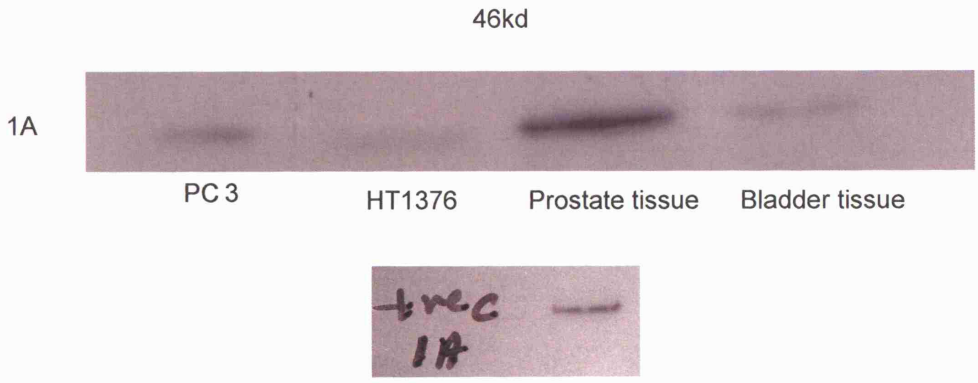


Figure 42: $5HT_{1A}$ receptor protein is demonstrated in PC3 and HT1376 cells as well as human prostate and bladder cancer tissue. The positive control for $5HT_{1A}$ receptor protein is also shown in rat brain.

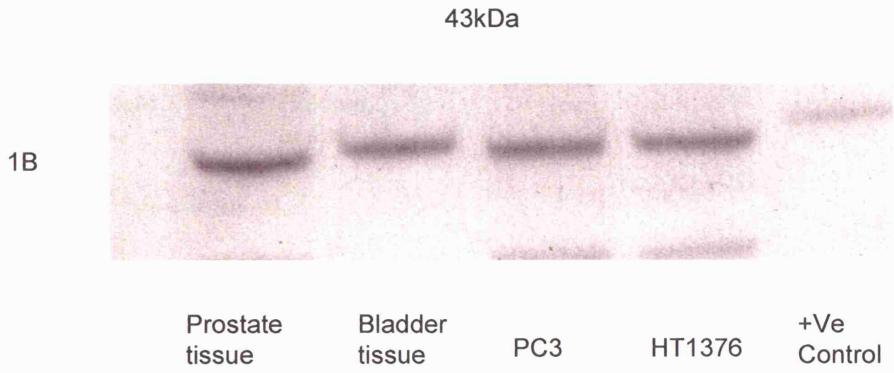


Figure 43: $5HT_{1B}$ receptor protein is demonstrated in PC3 and HT1376 cells as well as human prostate and bladder cancer tissue.

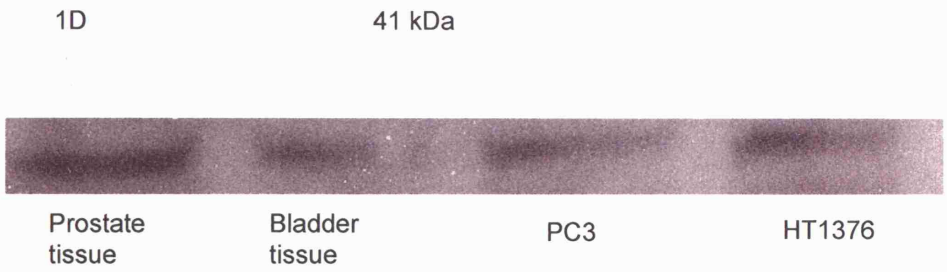


Figure 44: $5HT_{1D}$ receptor protein is demonstrated in PC3 and HT1376 cells as well as human prostate and bladder cancer tissue.

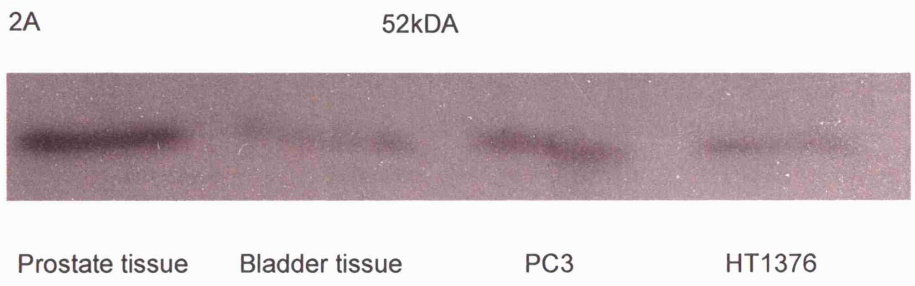


Figure 45: $5HT_{2A}$ receptor protein is demonstrated in PC3 and HT1376 cells as well as human prostate and bladder cancer tissue.

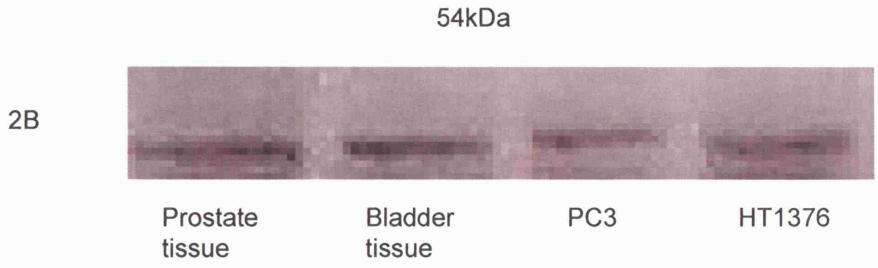


Figure 46: $5HT_{2B}$ receptor protein is demonstrated in PC3 and HT1376 cells as well as human prostate and bladder cancer tissue.

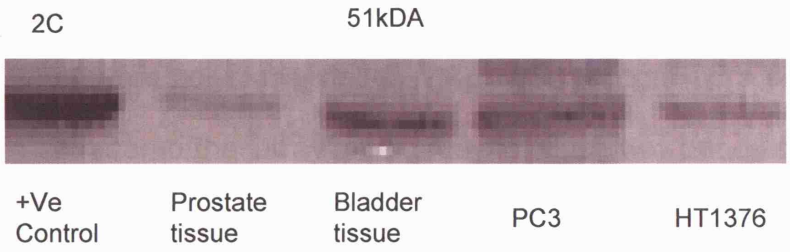


Figure 47: $5HT_{2C}$ receptor protein is demonstrated in PC3 and HT1376 cells as well as human prostate and bladder cancer tissue.

5.5 Discussion

Western blot analysis was conducted to identify a 5HT receptor profile in both PC3 cells and human metastatic hormone refractory prostate cancer tissue. We identified receptor proteins for 5HTR1A, 1B, 1D, 2A, 2B and 2C in PC3 cells and human hormone refractory prostate cancer tissue. Hence, we have been able to reinforce the immunohistochemical localisation of $5HT_{1A}$ and $5HT_{1B}$ receptors in prostate cancer cells and tissue.

We have found that polyclonal anti-$5HTR_{1A}$ IgGs recognised a single band of 46 kDa, in contrast to the Dizeyi et al study in which two bands of 60kDa and 65 kDa were recognised. Polyclonal anti-$5HTR_{1B}$ and anti-$5HTR_{1D}$ IgGs showed single bands of 43 kDa and 41 kDa, respectively, approximately similar to 45 kDa demonstrated by Dizeyi et al. Furthermore, we demonstrated bands of 52 kDa, 54 kDa and 51 kDa with polyclonal anti-$5HTR_{2A}$, anti-$5HTR_{2B}$ and anti-$5HTR_{2C}$ IgGs, respectively.

We have carried out a detailed analysis and identified $5HTR_{1A}$, $5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{2A}$, $5HTR_{2B}$ and $5HTR_{2C}$ proteins in PC3 cells and human prostate cancer tissue. This suggests that the study of Dizeyi et al is not thorough since they demonstrated only the $5HTR_{1A}$ protein in PC3 cells and $5HTR_{1A}$, $5HTR_{1B}$ and $5HTR_{1D}$ proteins in benign and malignant prostate tissue.

Western blot analysis performed in HT1376 cells and human bladder cancer tissue identified a similar 5HT receptor profile (5HTR1A, 1B, 1D, 2A, 2B and 2C) to that seen in PC3 cells and prostate cancer tissue.

The establishment of a 5HT receptor profile in human prostate and bladder cancer tissue has strengthened the suggestion that $5HT_{1A}$ and $5HT_{1B}$

antagonists may play a role in treating patients suffering from prostate and bladder cancer. Furthermore, the determination of an elaborate 5HT receptor profile in prostate and bladder cancer will surely instigate further research into the effects of 5HT and 5HT antagonists in other urological conditions such as BPH, over active bladder and erectile dysfunction.

CHAPTER 6

FLOW CYTOMETRY ON THE PC3 CELL LINE

(Cell cycle and apoptosis analysis)

6.1 Introduction

Flow cytometry is a means of measuring certain physical and chemical characteristics of cells or particles as they travel in suspension one by one past a sensing point. Flow cytometers can measure physical characteristics such as cell size, shape and internal complexity and, of course, any cell component or function that can be detected by a fluorescent compound (http://science.cancerresearchuk.org/sci/facs/fac_labinfo/flow_cytometry/?version=1).

The applications of flow cytometry are numerous, and this has lead to the widespread use of this instrument in the biological and medical fields.

A number of fluorescent probes have been developed for the flow cytometric analysis of cycling cells. The prototype for single-colour flow cytometric analysis of cycling cells uses propidium iodide (PI) staining of the total cellular DNA content expressed by the individual cells within activated cell populations. PI is the most widely-used fluorescent dye for staining DNA in whole cells (or isolated nuclei). PI intercalates into the DNA helix of fixed and permeabilised cells. Because PI can stain both double-stranded RNA (dsRNA) and DNA (dsDNA), cells must be treated with RNase to ensure that PI staining is DNA specific. PI cannot cross the intact plasma membrane of viable cells. However, PI can readily enter dead cells and late apoptotic (which have damaged plasma membrane) and can stain their dsRNA and dsDNA. For this reason, PI is also widely used as a discriminator of live and dead cells in experiments using immunofluorescent staining of unfixed cells with flow cytometric analysis (BrdU Staining and Cell Cycle Analysis, www.bdbiosciences.com).

The eukaryotic cell cycle consists of a series of events that are involved in the growth, replication and division of cells. The cell cycle can be subdivided into two major stages, interphase (a phase between mitotic events) and mitosis. There are three distinct, successive stages within interphase, called G1, S and G2 phases. During G1 (first gap), cells "monitor" their environment and upon receipt of requisite signals, they induce growth (synthesis RNA and proteins). If conditions are right, cells "commit" to DNA synthesis (S phase) and replicate their chromosomal DNA. A G2 phase (second gap) follows in which cells continue to direct and prepare for mitosis. The G2 gap allows time for the cell to ensure DNA replication is complete before initiating mitosis. In mitosis (division), there are four successive phases called prophase, metaphase, anaphase and telophase that are accompanied by cytoplasmic division (cytokinesis) giving rise to two daughter cells (BrdU Staining and Cell Cycle Analysis, www.bdbiosciences.com).

In this chapter, the purpose of FACS analysis in PC3 cells treated with $5HT_{1A}$ antagonist (NAN-190 hydrobromide) or $5HT_{1B}$ antagonist (SB224289 hydrochloride) was to demonstrate apoptosis and cell cycle arrest in these cells. This would implicate the mechanism leading to growth inhibition in PC3 cells treated with $5HT_{1A}$ and $5HT_{1B}$ antagonists.

6.2 Materials and Methods

Cell line

The malignant cell line used was PC3 (passage 7), an androgen independent prostate cancer cell line, obtained from the American Type Culture Collection (ATCC). The PC3 cells were maintained in Nutrient Mixture F-12 Ham

Medium supplemented with 8% Fetal Bovine Serum (FBS) and 1% Antibiotic Antimycotic Solution. PC3 cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Flow cytometry could not be performed on HT1376 cells due to technical limitations. HT1376 cells are large, and have a property of clumping together. Thus, HT1376 cells clog up the channels in the FACS analysis machine and therefore an apoptotic and cell cycle analysis could not be performed.

6.3 Reagents

5HT antagonists were purchased from Tocris Laboratories (Bristol, UK). The 5HT antagonists used were $5HT_{1A}$ (NAN-190 Hydrobromide) and $5HT_{1B}$ (SB224289 hydrochloride).

The Nutrient Mixture F-12 Ham Medium, Fetal Bovine Serum (FBS), Dimethyl Sulfoxide (DMSO), Antibiotic Antimycotic Solution were all purchased from Sigma-Aldrich Company Ltd (Dorset, UK). Dulbecco's Phosphate buffered saline (PBS), used for washing the cells was purchased from Sigma-Aldrich Company Ltd (Dorset, UK).

Propidium Iodide (PI) Staining Solution, Annexin V-FITC and Annexin V Binding Buffer used in apoptosis and cell cycle analysis were purchased from BD Biosciences Pharmingen (Cowley, Oxford, UK).

6.4 Cell cycle analysis

The PC3 cells were treated with $5HT_{1A}$ and $5HT_{1B}$ antagonists at a concentration of 10^{-4} M for 24 h. Control samples were kept in media without addition of $5HT_{1A}$ and $5HT_{1B}$ antagonists. At 24 h, the cells were harvested by

trypsinization and centrifuged at 2000 rpm for 5 min. They were then resuspended in 500 µl of cold PBS and fixed with 70% ethanol for 30 min at 4°C. The cells were washed with PBS and centrifuged at 2000 rpm. To ensure that only DNA is stained the cells were treated with 20 µl of 10 mg/ml RNase and were incubated at 37°C for 15-30 min. Propidium iodide (25-50 µg/ml) was then added, followed by incubation for at least 20 min at RT. Finally, the cells were analysed by flow cytometry within 1 h.

6.5 Apoptosis analysis

The PC3 cells were treated with $5HT_{1A}$ and $5HT_{1B}$ antagonists at a concentration of 10^{-4} M for 24 and 48 h. Control samples were kept in media without addition of $5HT_{1A}$ and $5HT_{1B}$ antagonists. At 24 and 48 h, respectively, the cells were harvested by trypsinization and centrifuged at 2000 rpm for 5 min. They were washed twice with cold PBS. They were then resuspended in 1 X binding buffer. 100 µl of the solution was transferred to a 5 ml culture tube. 5 µl of Annexin V-FITC and 5 µl of PI were then added. The cells were then gently vortexed and incubated for 15 min at RT in the dark. Finally, 400 µl of 1 X binding buffer was added to each tube and a flow cytometry analysis was performed within 1 h.

6.6 Results

6.6.1 $5HT_{1A}$ and $5HT_{1B}$ antagonists induce G_0-G_1 Cell Cycle Arrest in PC3 cells

In controls, 52.68% of the PC3 cells were in the G_0-G_1 phase, 17.26% in the S phase, 22.75% in the G_2-M phase and 1.32% of the cells had undergone apoptosis at 24 h (Figure 48).

In $5HT_{1A}$ antagonist treated cells, the number of PC3 cells in the G_0-G_1 phase increased to 64.48% whereas the number of cells in the S phase and G_2-M phase decreased to 14.85% and 9.09%, respectively, at 24 h. 1.49% of the cells had undergone apoptosis at 24 h (Figure 49) .

Furthermore, in $5HT_{1B}$ antagonist treated cells, the number of cells in the G_0-G_1 phase increased to 64.36% whereas the number of cells in the S phase and G_2-M phase decreased to 16.53% and 14.03%, respectively, at 24 h. 1.74% of the cells had undergone apoptosis at 24 h (Figure 50).

This data, indicating an increase in the number of cells in the G_0-G_1 phase, provides evidence that $5HT_{1A}$ and $5HT_{1B}$ antagonists, cause cell cycle arrest and in turn growth inhibition of PC3 cells, in the G_0-G_1 phase of the cell cycle. The growth inhibition could be attributed to apoptotic cell death. Hence, we investigated whether $5HT_{1A}$ and $5HT_{1B}$ antagonists could induce apoptotic cell death in PC3 cells.

6.6.2 $5HT_{1A}$ and $5HT_{1B}$ antagonists induce apoptosis in PC3 cells

In PC3 cells, the controls (untreated cells) were primarily Annexin V-FITC and PI negative, indicating that they were viable and not undergoing apoptosis. These living cells are located in the lower left quadrant (Figure 51).

After 24 h and 48 h treatment with a $5HT_{1A}$ antagonist (NAN-190 hydrobromide), there were primarily two population of cells. Cells that were viable and not undergoing apoptosis located in the lower left quadrant

(Annexin V-FITC and PI negative) and cells undergoing apoptosis seen in the upper left quadrant (Annexin V-FITC positive and PI negative) (Figures 52).

After 24 h and 48 h treatment with a $5HT_{1B}$ antagonist (SB224289 hydrochloride), there were once again two population of cells. Cells that were viable and not undergoing apoptosis located in the lower left quadrant (Annexin V-FITC and PI negative) and cells undergoing apoptosis seen in the upper left quadrant (Annexin V-FITC positive and PI negative) (Figures 52). In both $5HT_{1A}$ and $5HT_{1B}$ antagonist treated PC3 cells, a minor population of cells were observed to be Annexin V-FITC and PI positive, indicating that they were in the end stage of apoptosis or early death (Figures 52 & 53).

The number of cells undergoing apoptosis was greater in $5HT_{1B}$ antagonist-treated cells as compared to those treated with $5HT_{1A}$ antagonist.

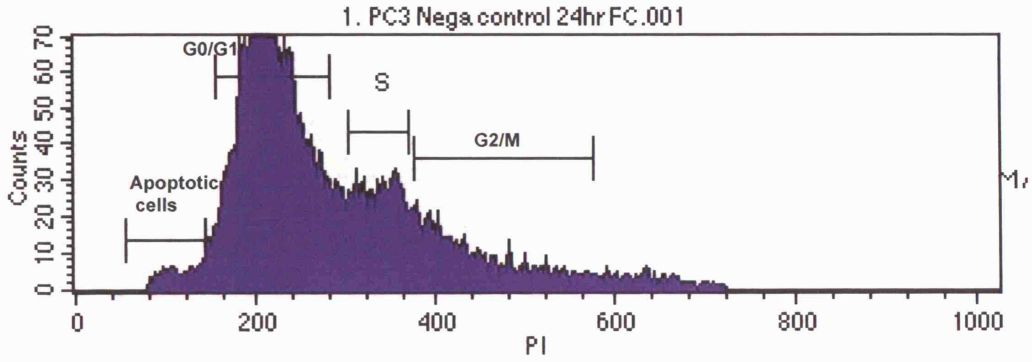


Figure 48: Cell cycle analysis at 24 h, in control PC3 cells. $5HT_{1A}$ or $5HT_{1B}$ antagonists were not added into this sample.

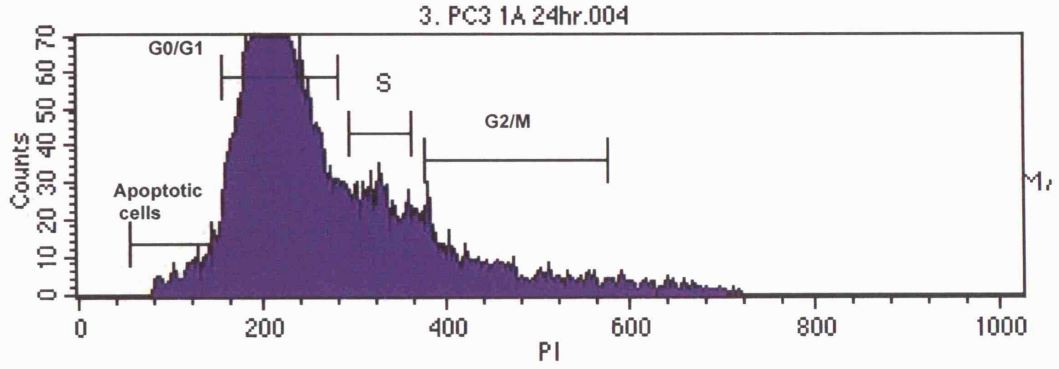


Figure 49: Cell cycle analysis at 24 h, in PC3 cells treated with $5HT_{1A}$ antagonist (NAN-190 hydrobromide). There was an increase in the percentage of cells in the G_0-G_1 phase of the cell cycle, as compared to control.

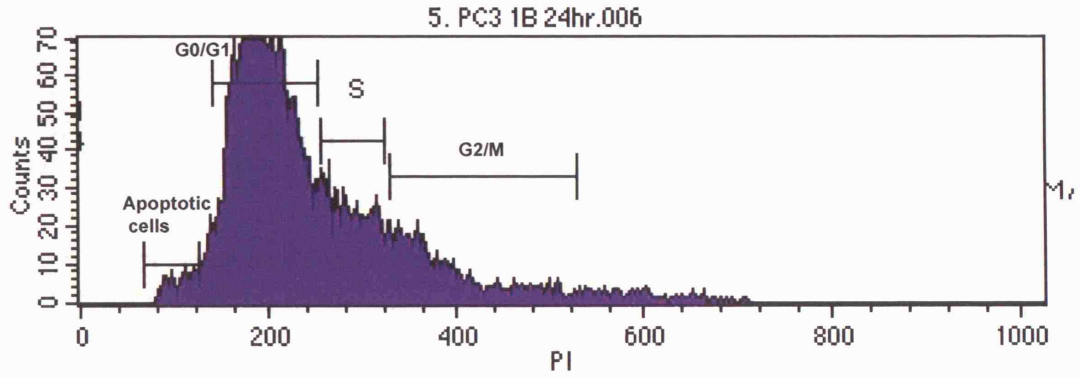


Figure 50: Cell cycle analysis at 24 h, in PC3 cells treated with $5HT_{1B}$ antagonist (SB224289 hydrochloride). There was an increase in the percentage of cells in the G_0-G_1 phase of the cell cycle, as compared to control.

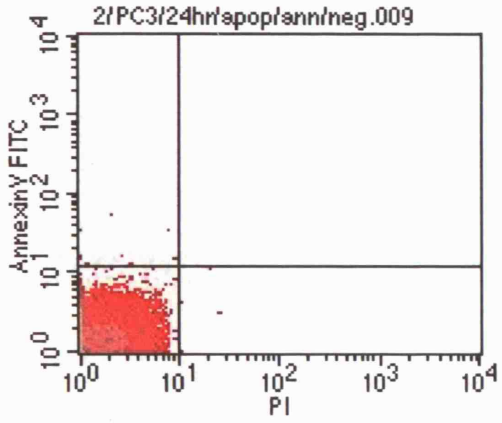


Figure 51: Apoptosis analysis in untreated PC3 cells (control) at 24 h, with almost all the cells alive (lower left quadrant).

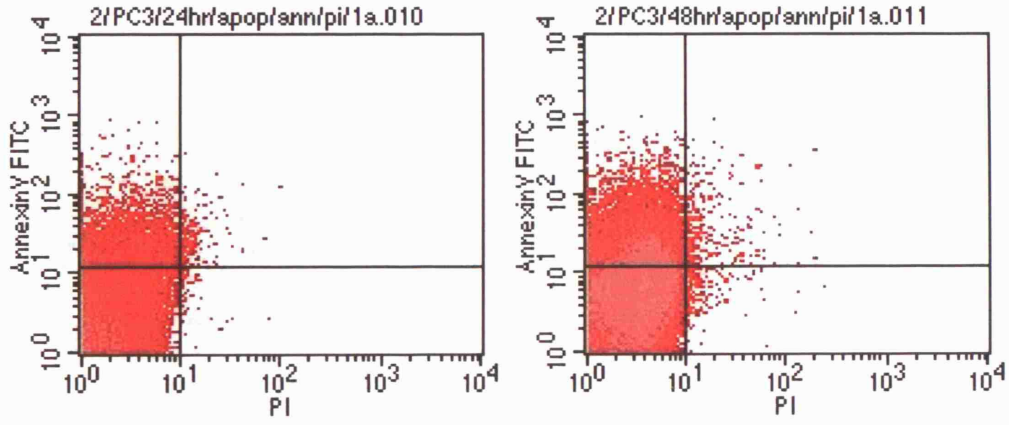


Figure 52: Apoptosis analysis in $5HT_{1A}$ (NAN-190 hydrobromide) antagonist treated PC3 cells at 24 h and 48 h, showing living cells (lower left quadrant) and apoptotic cells (upper left quadrant).

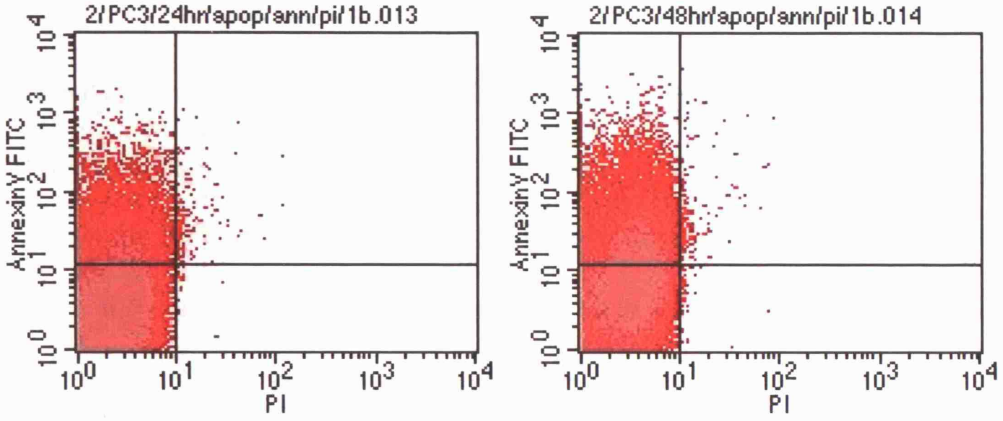


Figure 53: Apoptosis analysis in $5HT_{1B}$ (SB224289 hydrochloride) antagonist treated PC3 cells at 24 h and 48 h, showing living cells (lower left quadrant) and apoptotic cells (upper left quadrant).

6.7. Discussion

In order to investigate the possible mechanism by which $5HT_{1A}$ and $5HT_{1B}$ antagonists mediate growth inhibition in PC3 cells, apoptosis analysis and cell cycle analysis was carried out.

Our results indicate that $5HT_{1A}$ and $5HT_{1B}$ antagonists, cause cell cycle arrest and in turn growth inhibition of the PC3 cells in the G_0-G_1 phase of the cell cycle, as an increase in the number of cells in the G_0-G_1 phase was evident, 24 h after the exposure to 5HT antagonists.

Cell death was confirmed in PC3 cells, as apoptosis was demonstrated at 24 h and 48 h, following the exposure to either a $5HT_{1A}$ or $5HT_{1B}$ antagonist. There were a greater number of apoptotic cells following treatment with the $5HT_{1B}$ antagonist, than those found following $5HT_{1A}$ antagonist exposure. This was consistent with the findings from our cell proliferation studies.

Previous studies have shown that $5HT_{1A}$ antagonists cause a dose-dependent growth inhibition in prostate cancer cells (Abdul et al., 1994; Dizeyi et al., 2004). These studies, however, did not establish whether cell cycle arrest or apoptosis were implicated in the growth inhibition in prostate cancer cells. This omission was rectified in the present study.

When the results of functional studies, immunohistochemistry, western blot analysis, cell cycle analysis and apoptosis analysis are considered collectively, it is apparent that a functional 5HT system exists in prostate cancer cells after androgen ablation therapy. This functional 5HT system merits further investigation *in vivo*.

CHAPTER 7

GENERAL DISCUSSION

Several opposing effects of 5HT on tumour growth have been reported (Seuwen et al., 1990). 5HT is regarded as a growth factor for several types of non-tumoural and tumoural cells. In this context, it has been proposed to take part in the autocrine loops of growth factors contributing to cell proliferation in aggressive tumours such as small cell lung carcinoma and colonic carcinoma (Cattaneo et al., 1993; Cattaneo et al., 1995). It has been reported that $5HT_1$ and $5HT_2$ receptor antagonists inhibit tumour growth (Vicaut et al., 2000). In contrast, others report that 5HT and $5HT_2$ agonist also inhibit tumour growth (Rudzit et al., 1970; Vicaut et al., 2000). Often the effect of 5HT, $5HT_2$ and to lesser extent $5HT_1$ agonists has been considered to be related to the specific vasoconstrictor action on the vessels supplying the tumour (Rudzit et al., 1970; Vicaut et al., 2000).

5HT-induced growth proliferation in prostate cancer cells (PC3, DU145 and LNCaP) has been reported (Abdul et al., 1994; Dizeyi et al., 2004), with maximum proliferation seen at a concentration of 10^{-8}M (Dizeyi et al., 2004). In our study, a maximum proliferation of 15% was demonstrated at the same concentration of 10^{-8}M, in PC3 cells at 72 h. Pindobind and NAN-190 hydrobromide; $5HT_{1A}$ antagonists have a significant growth inhibitory effect on prostate cancer cells (PC3, DU145 and LNCaP) (Abdul et al., 1994; Dizeyi et al., 2004). In addition, 5HT uptake inhibitors (6-nitroquipazine, zimelidine and fluoxetine) also cause inhibition of PC3, DU145 and LNCaP cell growth in a dose-dependent manner (Abdul et al., 1995). We found that the $5HT_{1A}$ antagonist (NAN-190 hydrobromide) caused a 20% inhibition of PC3 cell growth at a concentration of 10^{-4}M at 72 h. More importantly, SB224289 hydrochloride; a $5HT_{1B}$ antagonist caused a 78% growth inhibition of PC3

cells at a concentration of 10^{-4}M at 72 h. Other 5HT receptor subtype antagonists ($5HT_{1D/2}$, $5HT_3$ and $5HT_4$) had no significant inhibitory effect on PC3 cell growth.

Our findings demonstrate a discrepancy in the magnitude of inhibition caused by $5HT_{1A}$ antagonists on prostate cancer cell growth, as well as a difference in the concentrations at which a maximum effect is established. We concur with the conclusion of Abdul et al and Dizeyi et al that $5HT_{1A}$ antagonists cause a dose-dependent inhibition of PC3 cell growth. We, however, differ with both Abdul et al and Dizeyi et al who proposed that in PC3 cells a maximum inhibition with a $5HT_{1A}$ antagonist takes place at 10^{-5}M and 10^{-6}M, respectively. Our results indicate that the $5HT_{1A}$ antagonist (NAN-190 hydrobromide) causes a maximum growth inhibition in PC3 cells, at 10^{-4}M. Furthermore, Abdul et al demonstrated a maximum growth inhibition of 90% by a $5HT_{1A}$ antagonist, as compared to the 20% found by ourselves. As Dizeyi et al did not express their results as a percentage growth inhibition; therefore a direct comparison could not be made. A possible explanation for the above discrepancies might be due to the longer incubation period of 120 h and 96 h, used by Abdul et al and Dizeyi et al, respectively. The different $5HT_{1A}$ antagonists used in the experiments must also be considered; Abdul et al used Pindobind; a $5HT_{1A}$ antagonist whilst NAN-190 hydrobromide was used by Dizeyi et al and ourselves. Thus, the variation in the magnitude of the effect of both $5HT_{1A}$ antagonists on PC3 cell growth might be attributed to differences in receptor selectivity (Fenrick et al., 1996). This may be further supported by the observations of Dizeyi et al that out of the three $5HT_{1A}$

antagonists (NAN-190, WAY100135 and BMY7378) they used; only NAN-190 significantly inhibited prostate cancer cell growth (Dizeyi et al., 2004).

A novel finding from our study is that SB224289 hydrochloride; a selective $5HT_{1B}$ receptor antagonist causes a 78% growth inhibition in PC3 cells at a concentration of 10^{-4}M, at 72 h. The growth inhibition by $5HT_{1B}$ antagonist on PC3 cells was far greater and more significant than the 20% growth inhibitory effect demonstrated by $5HT_{1A}$ antagonist; NAN-190 hydrobromide. The IC_{50} value was 3.23×10^{-6}M. Naturally, at such a low concentration, the use of this drug or similar analogues *in vivo* would cause minimal concerns of drug toxicity.

Our cell proliferation studies have indicated that only $5HT_{1A}$ and $5HT_{1B}$ receptors are functionally active in causing growth inhibition in PC3 cells. Therefore, our immunohistochemical studies concentrated on the localisation of $5HT_{1A}$ and $5HT_{1B}$ receptors in PC3 cells and hormone refractory prostate cancer tissue. Dizeyi et al also localised $5HT_{1A}$ and $5HT_{1B}$ receptors using immunohistochemistry in PC3 cells and prostate cancer tissue. In prostate tumour, they found the strongest staining for $5HT_{1A}$ and $5HT_{1B}$ receptors was seen in Gleason grade 4 and 5 tumours. Grades below this had weak staining (Dizeyi et al., 2004). It was concluded, therefore, that the higher the grade of prostate cancer, greater is the $5HTR_{1A}$ and $5HTR_{1B}$ expression. It is evident that our results concur with those of Dizeyi et al in that $5HT_{1A}$ and $5HT_{1B}$ receptors exist in PC3 cells and human hormone refractory prostate cancer tissue.

Western blot analysis identified receptor proteins for 5HTR1A, 1B, 1D, 2A, 2B and 2C in PC3 cells and human hormone refractory prostate cancer tissue.

Thus, we were able to reinforce our immunohistochemical findings on localisation of $5HT_{1A}$ and $5HT_{1B}$ receptors. Additionally, we identified receptor proteins for the 5HT sub type receptors (5HTR1D, 2A, 2B and 2C) in PC3 cells and human prostate tissue. These receptors, however, were found not to be functionally active in the regulation of tumour growth.

We have carried out detailed western blot analysis and identified $5HTR_{1A}$, $5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{2A}$, $5HTR_{2B}$ and $5HTR_{2C}$ proteins in PC3 cells and human prostate cancer tissue. This suggests that the study of Dizeyi et al is limited since they demonstrated only the $5HTR_{1A}$ protein in PC3 cells and $5HTR_{1A}$, $5HTR_{1B}$ and $5HTR_{1D}$ proteins in benign and malignant prostate tissue.

We further investigated the possible mechanism by which $5HT_{1A}$ and $5HT_{1B}$ antagonists mediate growth inhibition in PC3 cells. Our results indicate that $5HT_{1A}$ and $5HT_{1B}$ antagonists, cause cell cycle arrest and in turn growth inhibition of the PC3 cells in the G_0-G_1 phase of the cell cycle, as an increase in the number of cells in the G_0-G_1 phase was evident, 24 h after the exposure to $5HT_{1A}$ and $5HT_{1B}$ antagonists.

Apoptosis in PC3 cells was demonstrated at 24 h and 48 h, following the addition of either a $5HT_{1A}$ or $5HT_{1B}$ antagonist, thus confirming cell death in these cells. There were a greater number of apoptotic cells following treatment with the $5HT_{1B}$ antagonist, than those found following $5HT_{1A}$ antagonist exposure. These findings complement our cell proliferation assay results which demonstrate that in PC3 cells, $5HT_{1B}$ antagonists have a greater growth inhibitory effect than $5HT_{1A}$ antagonists.

NE differentiation is a feature of bladder cancer (Acs et al., 2000; Amichetti et al., 1992; Chin et al., 1992; Dundr et al., 2003; Grignon et al., 1992; Helpap et al., 2002; Lertprasertsuke et al., 1991; Martignoni et al., 2003; Mazzucchelli et al., 1992; van Hoeven et al., 1996; Vincendeau et al., 2003). Hence, we also investigated the effect of 5HT and 5HT receptor antagonists on bladder cancer cell growth (HT1376).

Our findings indicate that 5HT caused growth proliferation in HT1376 cells with a maximum effect of 12% seen at a concentration of 10^{-8} M, at 72 h (Siddiqui et al., 2005b). The concentration (10^{-8}M) at which 5HT caused maximum proliferation in HT1376 cells was similar to that established in experiments performed on PC3 cells by both Dizeyi et al and us. Furthermore, $5HT_{1A}$ and $5HT_{1B}$ antagonists had a dose-dependent growth inhibitory effect on HT1376 cell growth. The $5HT_{1A}$ antagonist; NAN-190 hydrobromide caused a maximum growth inhibition of 10% in HT1376 cells at 72 h, whereas, the $5HT_{1B}$ antagonist, SB224289 hydrochloride demonstrated a far greater growth inhibitory effect of 93% in the same cell line at 72 h (Siddiqui et al., 2005b). Similar, to PC3 cells, the concentration at which $5HT_{1A}$ and $5HT_{1B}$ antagonists cause a maximum growth inhibition in HT1376 cells is 10^{-4}M. Other 5HT receptor subtype antagonists ($5HT_{1D/2}$, $5HT_3$ and $5HT_4$) had no significant inhibitory effect on HT1376 cell growth.

To complement the functional work carried out in HT1376 cells, immunohistochemical studies on HT1376 cells and human bladder cancer tissue concentrated on the localisation of the $5HT_{1A}$ and $5HT_{1B}$ receptors.

Western blot analysis identified a similar 5HT receptor profile (5HTR1A, 1B, 1D, 2A, 2B and 2C) to that seen in PC3 cells and prostate cancer tissue (Siddiqui et al., 2005b).

We believe that $5HT_{1B}$ antagonist is the more significant of the two receptor antagonists, and therefore, it requires further investigation *in vivo* as a potential anti-neoplastic agent for the treatment of both androgen independent prostate cancer and transitional cell carcinoma of the bladder.

The effect of 5HT, 5HT antagonists and 5HT uptake inhibitors on the growth of subcutaneous cancer xenografts in athymic nude mice has been studied. Pratesi et al reported that a high dose of 5HT (200 μgm/ daily) increased the growth of SCLC cells grafted in athymic nude mice (Vicaut et al., 2000). The growth of subcutaneous PC3 xenografts in athymic nude mice was significantly inhibited by $5HT_{1A}$ antagonist (Pindobind) and a 5HT uptake inhibitor (fluoxetine) (Abdul et al., 1994; Abdul et al., 1995). Two specific inhibitors of 5HT uptake, citalopram and fluoxetine, were found to retard the growth of human colonic tumours propagated as xenografts in immune-deprived mice (Tutton et al., 1982).

Since the effect of 5HT, 5HT antagonists and 5HT uptake inhibitors on the growth of subcutaneous cancer xenografts have been documented, future *in vivo* experiments could follow a similar approach. We suggest that an animal model of prostate and bladder cancer using SCID (severe combined immunodeficient) mice should be established, in order to assess the *in vivo* effect of 5HT receptor antagonists ($5HT_{1A}$ and $5HT_{1B}$) on tumour growth.

In the years to come, if $5HT_{1A}$ and $5HT_{1B}$ antagonists are to be used as chemotherapeutic agents, we need to determine the distribution of functional

$5HT_{1A}$ and $5HT_{1B}$ receptors in the human body. In animals $5HT_{1A}$ and $5HT_{1B}$ receptors have been localised in the brain, penis, seminal vesicles, spinal cord and heart valves (Elangbam et al., 2005; Berendsen & Broekkamp, 1987; Stean et al., 2005). In humans the localisation of the $5HT_{1A}$ and $5HT_{1B}$ receptors is limited to the brain, cholangiocytes, lung cancer cells as well as coronary and cerebral arteries (Edvinsson et al., 2005; Varnas et al., 2005; Cattaneo et al., 1995; Marzioni et al., 2005). Therefore, the selection of a 5HT antagonist, for prostate and bladder cancer therapy, must take into account the impact the drug has on other systems of the body. Equally, the most suitable route of administration and therapeutic dose causing minimal side effects will have to be determined. With regards to the treatment of bladder cancer, the advantage of the intra-vesical administration of drugs, limits drug toxicity and side effects. However, in prostate cancer, careful consideration needs to be given to this issue.

Alpha-adrenoceptor antagonists, have been documented to inhibit growth and induce apoptosis in malignant prostate cells (Anglin et al., 2002; Benning et al., 2002; Cuellar et al., 2002; Glassman et al., 2001; Keledjian et al., 2001; Keledjian et al., 2003; Kyprianou, 2000; Kyprianou, 2003; Kyprianou et al., 2000a; Kyprianou et al., 2000b; Kyprianou et al., 2000c; Liu et al., 2004; Partin et al., 2003; Tahmatzopoulos et al., 2004a; Tahmatzopoulos et al., 2004b; Xu et al., 2003a; Xu et al., 2003b). The apoptotic activity of doxazosin against prostate cancer cells is independent of: (a) their capacity to antagonise alpha-adrenoceptors, and, (b) the hormone sensitivity status of the cells (Anglin et al., 2002; Benning et al., 2002; Kyprianou, 2000; Kyprianou, 2003; Kyprianou et al., 2000a; Kyprianou et al., 2000c; Partin et al., 2003).

An important component of our study was the demonstration of the growth inhibition of PC3 and HT1376 cells, by the alpha$_1$-adrenoceptor antagonist (doxazosin). The growth inhibition of HT1376 cells, by doxazosin is novel (Siddiqui et al., 2005a). Efforts were made to establish whether the growth inhibition of PC3 cells by doxazosin is mediated via 5HT receptors.

Our results demonstrate that pre-incubating PC3 cells with 5HT or 5HT$_{1B}$ agonists, followed by the addition of doxazosin led to an increase in percent viable cells as compared to when PC3 cells were treated with doxazosin alone (Siddiqui et al., 2005a). 5HT or 5HT$_{1B}$ agonists were unable to however, completely abolish the effect of doxazosin. This suggests that it possible that doxazosin has some 5HT receptor-mediated mechanism for growth inhibition but acts predominantly through some other pathway. However, it might well be that the increase in percent viable cells was due to the growth proliferative effect of 5HT or 5HT$_{1B}$ agonist and not due to the inability of doxazosin to cause its 5HT-receptor mediated growth inhibition. Autoradiographic studies might clarify this issue by demonstrating whether doxazosin displaces 5HT from the 5HT receptors.

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

LIMITATIONS OF THE STUDY

Flow cytometry could not be performed on HT1376 cells due to technical limitations. HT1376 cells are large, and have a property of clumping together. Thus, HT1376 cells clog up the channels in the FACS analysis machine and therefore an apoptotic and cell cycle analysis could not be performed.

A further limitation to our study was lack of funding to extend the study in vivo and perform animal studies. Efforts are however, underway to obtain funding to extend the above study in vivo.

CONCLUSIONS

$5HT_{1A}$ and $5HT_{1B}$ antagonists have a significant inhibitory effect on prostate cancer (PC3) and bladder cancer (HT1376) cell growth *in vitro*. This effect is most likely mediated via $5HT_{1A}$ and $5HT_{1B}$ receptors which we have localised in PC3 cells, HT1376 cells, human metastatic hormone refractory prostate cancer tissue and transitional cell bladder carcinoma tissue. The $5HT_{1A}$ and $5HT_{1B}$ antagonists induce apoptosis and cell cycle arrest in PC3 cells. Our results, therefore, imply that $5HT_{1A}$ and in particular $5HT_{1B}$ receptor antagonists warrant further *in vivo* investigation to determine their role as potential anti-neoplastic agents.

The $alpha_1$-adrenoceptor antagonist, doxazosin, significantly inhibits 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. Thus, 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.

SUGGESTIONS FOR FUTURE WORK

In the future, our research group intends to extend our studies using *in vivo* techniques. We propose to develop an animal model of prostate and bladder cancer (with ethical and Home Office approval) using SCID (severe combined immunodeficient) mice, in which the cancer cells have been inoculated either subcutaneously or intra-prostatically. Using this model the *in vivo* effect of 5HT receptor antagonists ($5HT_{1A}$ and $5HT_{1B}$) can be assessed. We feel that these in vivo studies are vital to determine the role of $5HT_{1A}$ and more importantly $5HT_{1B}$ antagonists as potential anti-neoplastic agents. Other members in our research group are also examining the effect of $5HT_{1A}$ and $5HT_{1B}$ antagonists in renal cancer.

Initial studies regarding *in vivo* inhibition by the alpha-adrenoceptor antagonist (doxazosin) on prostate and bladder cancer growth, in SCID mice have already been initiated with encouraging results.

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