

MODULATION OF THE ANDROGEN RECEPTOR BY
HYPERTHERMIA IN HUMAN PROSTATE
CANCER CELL LINES

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY IN THE FACULTY OF SCIENCE

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This thesis is dedicated to my husband Ricky Austin

Whose interest and encouragement

I will always be grateful for

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DMP Dimethyl pimelimidate dihydrochloride

ABBREVIATIONS

AAH	atypical adenomatous hyperplasia
Ab	Antibody
ACTH	adrenocorticotrophic hormone
ANOVA	One-way analysis of variance
AR	androgen receptor
ARE	androgen response element
BPH	benign prostatic hyperplasia
BpM	bound picomolar
BSA	Bovine Serum Albumin
cDNA	complementary DNA
CKII	casein kinase II
cpm	counts per minute
DES	Diethylstilboestrol
DHT	5 α -dihydrotestosterone
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
eIF-2 α	eukaryotic peptide initiation factor 2 α
ER	estrogen receptor
ERE	estrogen response element
FGF	fibroblast growth factor
GAM	goat anti-mouse
GAM-Sep	GAM linked CNBr-sepharose
Gly	Glycine
Glu	Glutamic acid
GR	glucocorticoid receptor

GRE	glucocorticoid response element
HRE	hormone response element
HSE	heat shock element
HSF	heat shock factor
hsp	heat shock protein
kD	kilodaltons
Kd	dissociation constant
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
MR	mineralocorticoid receptor
NLS	nuclear localisation signal
OH-Flut	hydroxyflutamide
PBS	Phosphate Buffered Saline
PIN	Prostatic intraepithelial neoplasia
POMC	pro-opiomelanocortin
PSA	prostate specific antigen
RAR	retinoic acid receptor
RPM	rotations per minute
S. D.	Standard deviation
SDGA	sucrose density gradient analysis
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrilimide gel electrophoresis
Ser	Serine
SHBG	sex hormone-binding globulin
SR	steroid receptor
T	Testosterone
TAF	transcriptional activation function
TBST	Tris buffered saline with tween

TEMED	N,N,N',N', tetramethylethylenediamine
TFIIB	transcription factor IIB
TFIID	transcription factor IID
TFIIE	transcription factor IIE
TFIIF	transcription factor IIF
TFIIH	transcription factor IIH
TFIIJ	transcription factor IIJ
TGF- α	transforming growth factor- α
TGF- β	transforming growth factor - β
T ₃ R	thyroid hormone receptor
Val	Valine
VitDR	vitamin D receptor

Summary

Many types of tumour cells are more sensitive to elevated temperatures than normal cells. This observation and the accessibility of the prostate gland to an external heat source has led to the development of thermotherapy as a treatment for prostate disease. One possible problem with this type of therapy is the development of thermotolerance. Thermotolerance is the acquisition of a resistance to a thermokilling temperature by pre-exposure of the cell to a sublethal dose of heat and is due to an increased level of a group of proteins known as heat shock proteins (hsp). One of the members of the hsp family is hsp90. Hsp90 is associated with steroid receptors and forms part of the unactivated 8S receptor.

Work described in this thesis has shown that thermotolerance in three prostate cancer cell lines (LNCaP, DU145 and PC3) is a transient property which can be established by pre-exposing the cells to 39°C for 2 hours. This temperature has been shown to induce hsp90 synthesis in prostate cell lines, shown using immunoprecipitation of hsp90 after incorporation of S^{35} methionine and Western blotting.

The androgen sensitive prostate cell line LNCaP is not normally growth inhibited by the antiandrogen HO-Flut.. However if the cells are treated with hyperthermia therapy (2 hours at 39°C and 42°C) before exposure to HO-Flut the cells exhibit an increased cell kill. This increased sensitivity to cell kill was shown to be maximised 12 hours after heat shock and had fallen by 72 hours to control levels. Re-establishment of both thermotolerance and the increased cell kill in conjunction with antiandrogens can be achieved by a further heat shock administration. Thus, induction of thermotolerance in the prostate cancer cells may not be too serious for patients or clinicians because these cells may now be more sensitive to cell kill by antiandrogens.

Androgen receptor ligand binding assays were used to study the effect of heat shock on the binding affinity of androgen receptors in LNCaP and DU145 cells. A gradual increase (0.232 ± 0.050 - 0.513 ± 0.019) in the disassociation constant (K_d) of the type I binding site was seen during the first 12 hours following heat shock. To answer the question of whether this change in K_d reflects some underlying change in the composition of the androgen receptor following heat shock sucrose density gradient analysis was used to investigate the possible changes in the androgen receptor complex composition and the ratio of 8S:4S receptor types. After heat shock a temporal shift in favour of the 8S receptor complex was seen, the time course for this mimics that of the acquisition and loss of thermotolerance as shown by cell survival to exposure to normally lethal temperatures. An additional peak around 11S can be seen between 10-12 hours after heat shock. A model is presented to provide a molecular basis for these observations.

CHAPTER 1

INTRODUCTION

1:1 Introduction

In 1992 prostate cancer killed 693 men in Scotland alone, its incidence is second only to lung cancer (Scottish Health Statistics 1993). It is becoming the most commonly diagnosed cancer in men in the western world [Silverberg *et. al.*, 1990]. It is therefore easy to see why it is a major medical problem and why research new methods of diagnosis and treatment is important.

The highest incidences appear to be in the black population of the USA, which is more than double that of the white population [WHO, 1992]. Oriental males have the lowest mortality rate [Griffiths *et. al.*, 1993]. 67% of all prostate tumors originate in the peripheral zone (see figure 1). Only 15% of patient with stage A prostatic cancer (where the tumour is confined to the prostate and hasn't penetrated the capsule surrounding the prostate) go on to develop progressive disease [Zang, 1992].

1:2 The prostate gland

The prostate gland surrounds the neck of the bladder and the proximal end of the urethra in males and is attached to the rectum by dense fibrous tissue. It is to produce and secrete a lubricating fluid which helps nourish the spermatozoa. The prostate can be divided into 4 zones; the anterior, the central, the transitional and the peripheral lobes [McNeal, 1981], see figure 1 for details.

Androgens are essential for normal prostate development and maintenance [Davies & Eaton, 1991]. However androgens are not the sole factors involved. Several growth factors have been shown to be important including epidermal growth factor (EGF), fibroblast growth factor (FGF) and transforming growth

factor (TGF)- β . EGF and FGF have mitogenic effects on prostate cell growth which are balanced by TGF- β 's inhibitory effect [Davies & Eaton, 1991].

1:3 Prostatic diseases

Benign prostatic hyperplasia

Major diseases of the prostate include benign prostatic hyperplasia (BPH) and prostate cancer. At the present time more than half the male population over sixty five suffers from some degree of BPH [Carter & Coffey, 1990]. BPH is characterised by nonmalignant nodular transformation of the transition zone of the prostate (see figure 1) [Algaba, 1992]. Although it is not normally a fatal condition, it causes obstruction of the urethra which often requires surgery and creates a great deal of distress to the patient. BPH was originally believed to be a premalignant condition but it is now believed that BPH and prostate cancer have separate etiologies and originate in different regions of the prostate [Griffiths *et al.*, 1991]. It is important to remember that 20% of prostate cancers do occur in the same zone as BPH, the transitional zone.

Prostatic intraepithelial neoplasia and atypical adenomatous hyperplasia

Prostatic intraepithelial neoplasia (PIN) is a putative precancerous state which occurs most commonly in the peripheral zone of the prostate. Increasing severity of PIN is associated with progressive disruption of the basal cell layer and basement membrane [Bostwick, 1992]. PIN is found in 82% of patients with prostate cancer. It has been suggested that high-grade PIN and invasive carcinoma represent different stages of the same process [Nagle *et al.*, 1991]. Another premalignant lesion atypical adenomatous hyperplasia (AAH) which is found in the central transition zones of the prostate is believed to be a precursor of central carcinomas [Kovi, 1988].

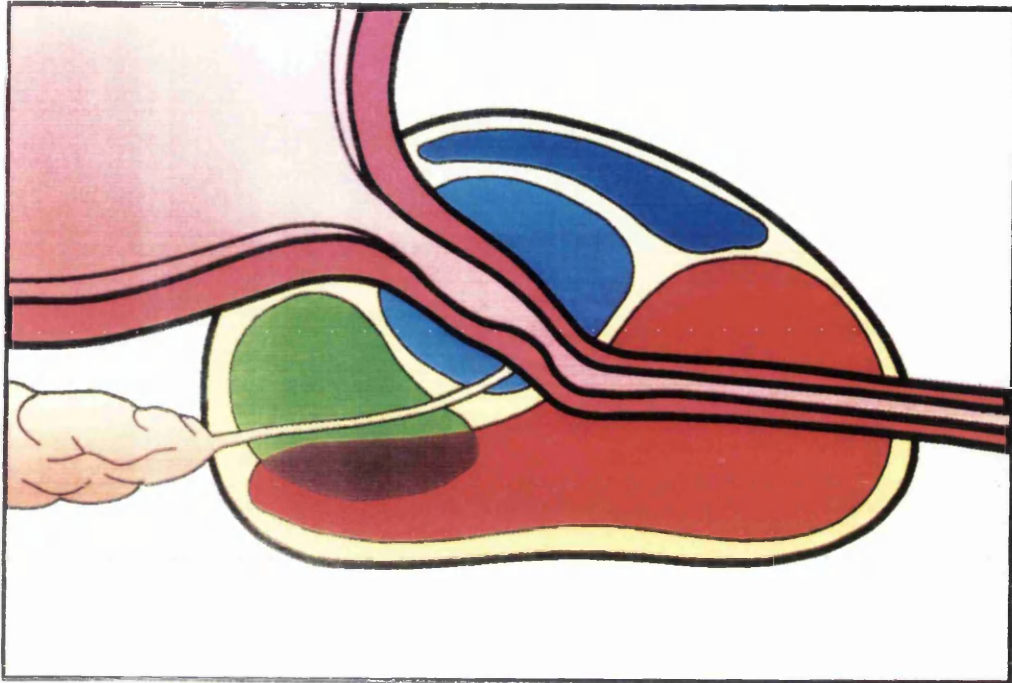


Figure 1. Lobular divisions of the prostate.

Taken from Algaba, 1992.

The prostate contains four different zones: the anterior zone (dark blue/purple area), the central zone (green area), the transitional zone (lighter blue area) and the peripheral zone (red area). These zones are not isolated from each other but do have subtle histological differences.

Prostate cancer

It has been suggested that an imbalance of androgens and growth factors may lead to both BPH and prostate cancer. Increased levels of FGF have been shown to cause prostatic hyperplasia in transgenic mice [Muller *et. al.*, 1990] and increased levels of FGF have been found in patients with BPH [Mori *et. al.*, 1990]. Transforming growth factor- α (TGF α) is expressed in both BPH and prostate carcinoma [LLoydet *al.*, 1992; Yang *et al.*, 1993]. Prostate cancer cells unlike normal prostate cells, do not require the presence of both EGF and FGF but rather the presence of either is sufficient for maximum growth [McKeehan *et. al.*, 1987].

Little is known about the premalignant changes which occur in prostate cancer or why up to 30 % of prostate tumours remain latent and the patient asymptomatic [Griffiths *et. al.*, 1993], but two factors, age and the presence of testes have been shown to be important. Neither BPH nor prostate cancer develops if the testes have been removed before puberty [Griffiths *et. al.*, 1992], and both diseases increase in prevalence after the age of 50 [Griffiths *et. al.*, 1993].

1:4 Treatment of prostate cancer

The treatment of prostate cancer varies according to size of the tumour, local spread, and the possibility of metastatic deposits. It is therefore important to stage prostate cancers accurately (see table 1).

1:4:1 Surgery

Surgery is the first line of therapy for stage T₁A and T₂B (table 1) tumours, where the tumour is confined to the prostate and there is no evidence for metastasis [Brendler, 1992]. Surgery usually involves radical prostatectomy

T ₁ : A	Confined to the Prostate	T ₁ a: A1	Non-Palpable	<5%:3 foci	
		T ₁ b: A2		>5%: Multifocal	
T ₂ : B		T ₂ a: B1	Palpable	Low vol:<1.5cm	
		T ₂ b: B2		High vol:>1.5cm	
T ₃ : C	Capsule Penetration	T ₃	Tumour extension beyond the capsule		
T ₄ : D		T ₄	Tumour fixed to adjoining tissues or invading neighbouring structures		

Table 1:- Classification of the stages of prostate cancer (from Griffiths *et. al.*,1993)

which removes the whole prostate and its capsule thus eliminating the tumour. If, however, the tumour is at a more advanced stage and capsule penetration has occurred surgery may not eradicate the tumour but surgical intervention such as a transurethral resection of the prostate will relieve some of the symptoms until systemic therapies begin to have an effect .

1:4:2 Androgen therapy

For tumours which have advanced beyond the T₃C stage (table 1) some form of systemic therapy is commonly used either alone or in conjunction with surgery. It has been known for some time that androgen ablation is an effective method of treatment [Huggins & Hodges, 1944]. Early androgen ablation involved bilateral orchidectomy (castration). Diethylstilbestrol (DES) was one of the first drugs designed to lower serum testosterone by suppression of hypothalamic LHRH production [Eaton & Griffiths, 1990]. It was effective but has serious side effects including cardiovascular toxicity [Khoury, 1992]. Medical castration is better achieved by administering luteinizing hormone releasing hormone (LHRH) agonists. LHRH agonists often caused a 'tumour flare' due to a transient rise in testosterone levels [Kahan & Delriu, 1984]. This physiological surge of testosterone can be countered by the administration of an antiandrogen given 1 week prior to LHRH agonist and continued for the first month of therapy [Khoury, 1992].

Antiandrogens were originally described as "any compound, independent of feedback mechanisms, that binds to target cell androgen receptors preventing the stimulation by exogenous or endogenous androgens" [Dorfman, 1970]. Several antiandrogens are used clinically in the treatment of prostate cancer and these can be divided into two groups: steroidal and nonsteroidal antiandrogens. Cyproterone acetate and megestrol acetate are both synthetic steroids which compete with androgens for receptor binding. However the main metastatic

suppressive action of these drugs is thought to be exerted through their progestational activities which lowers serum testosterone levels by LH inhibition [Kirby, 1993; McLeod, 1993]. There have been some reports of megestrol acetate and cyproterone acetate activating rather than inhibiting androgenic activity *in vitro* [Poyet & Labrie, 1985]. This may reflect differing mechanisms of action *in vitro* and *in vivo*.

Pure antiandrogens have no effect on hypothalamic pituitary activity [Kirby, 1993; McLeod, 1993]. Three nonsteroidal antiandrogens Flutamide® (α - α - α -trifluoro-2-methyl-4'-nitro-m-propionotoluidine), Nilutamide® (RU23908) and Casodex® (ICI 176,334) are available at present.

Up to 70% of all prostate tumours are androgen sensitive at the time of diagnosis [Whitmore, 1973; Lepor *et al.*, 1982]. The main problem with antiandrogen therapy is the eventual acquisition of androgen insensitivity by the tumour. The reasons tumours become hormone independent are not clear but two main theories exist. Firstly the tumour cells could adapt to the androgen-depleted environment leading to progressive loss of androgen dependence. Growth of a pre-existing androgen insensitive clone within the tumour as the androgen sensitive cells are inhibited would lead to the tumour containing more and more androgen insensitive cells [Gormley, 1992].

5 α - reductase within the prostate cells converts testosterone to 5 α - dihydrotestosterone (DHT) which is the intracellular mediator of androgen action. Therefore 5 α - reductase is a target for treatment of prostatic cancer. It has been suggested that 5 α - reductase inhibitors can produce antitumour effects if the tissue level of DHT is reduced although this may be harder to achieve in tumours than in normal tissue [Isaacs, 1992]. Finasteride is one of the 5 α - reductase inhibitors under investigation. It has been shown to reduce prostate size and suppression of serum and intraprostatic DHT to castrate levels [Gormley, 1992].

1:4:3 Radiotherapy

An alternative to surgery for low-stage prostate cancer (T₁A and T₂B) is radiotherapy. The results obtained by the two treatments are comparable in terms of patient survival [Eklov *et. al.*, 1992a]. Surgeons and radiation oncologists treat two different subsets of patients, the patients treated by radiotherapy tend to be older, less healthy, and often have more advanced disease [Hanks, 1988].

1:4:4 Chemotherapy

Advanced prostatic cancer is often treated by chemotherapeutic agents such as Estracyt® (a nornitrogen mustard attached as a carbonate to the C-3 atom of oestradiol) which accumulates in the prostate and arrests prostatic cancer cell growth and inhibits clonogenic cell survival [Harley-Asp *et. al.*, 1982 ; Eklov *et. al.*, 1992b]. The advantage of this drug lies in the fact that few male tissues have estrogen receptors and therefore it is specific to the prostate but it has been suggested that the liver cleaves the nornitrogen mustard side chain off the estrogen before it has time to enter the prostate.

1:4:5 Thermotherapy

Many types of tumour cells are more sensitive to elevated temperatures than normal cells [Crile, 1963; Giovanella *et. al.*, 1976]. This observation and the accessibility of the prostate gland to an external heat source has led to the development of thermotherapy as a treatment for prostate cancer and BPH [Mendecki *et. al.*, 1980; Servadio *et. al.*, 1987]. Thermotherapy has been used in conjunction with antiandrogen therapy [Linder *et. al.*, 1990] and radiotherapy [Abe & Hiraoka, 1990]. The combination of hyperthermia and chemotherapy produces differing results depending on the type of chemotherapy used. Hyperthermia combined with doxorubicin increases resistance to the drug, which does not involve any known multidrug resistance systems but is

correlated to hsp70 and hsp 27 levels [Ciocca *et. al.*, 1992]. The combination of hyperthermia and lonidamide appears to enhance hyperthermic toxicity [Bloch *et. al.*, 1994]. Lonidamine was originally developed as an antispermatogenic drug and it has been shown to inhibit the mitochondrial energy metabolism of neoplastic cells without significant inhibition of normal cells [Bloch *et. al.*, 1994].

Hyperthermia, thermotherapy and the development of thermotolerance

The observed thermosensitivity of cancer cells and the potential of thermotherapy as an anti-cancer therapy has been known for some time [Cavaliere *et. al.*, 1967; Suit & Schwayder, 1974]. The sequence of events leading to cell death is not well understood and several of the intracellular events that occur after hyperthermia, including cytoskeletal collapse, increasing nuclear protein levels and the decreases in respiration, oxidative phosphorylation and mRNA synthesis could either be due to the elevated temperature itself or a result of impending cell death [Weber, 1992].

Treatment of prostate cancer with local hyperthermia involves heating the gland to between 41°C and 44°C either once or twice a week [Engin *et. al.*, 1993].

This is achieved by several methods including the use of microwaves [Mendecki *et. al.*, 1980]. Temperatures above 42.5°C may result in damage to normal tissue [Lieb *et. al.*, 1986] and therefore have limited use in clinical practice [Lloyd *et. al.*, 1992b]. It has been shown that prostate cells which are subjected to hyperthermia *in vitro* and survive become thermotolerant and then are able to withstand further thermotherapy even at normally lethal temperatures [Lloyd *et. al.*, 1992b].

Thermotolerance has been described as an acquisition of a transient resistance to a thermokilling by pre-exposure of the cells to a sublethal dose of heat or other types of cell stress [Gerner & Schneider, 1975]. The mechanism behind the

acquisition of thermotolerance is unclear at this time although the involvement of a family of proteins, the heat shock proteins, is well documented and will be discussed in section 3:6.

1:5 Androgen synthesis and transport

All steroid hormones have a similar structure and share common synthesis pathways. Minor chemical changes can lead to the striking diversities in the biochemical activity of those steroids e.g. the difference between estrogen and testosterone is that estrogen has one less carbon atom.

There are two types of androgens, adrenal androgens (e.g. androstenedione) and testicular androgens (testosterone). The biosynthesis of testosterone is summarised in figure 2.

Testosterone is produced by the Leydig cells in the testes, after stimulation by luteinizing hormone (LH) [Milgrom, 1990]. Like other steroids, testosterone is derived from cholesterol, a 27-carbon atoms molecule. During steroidogenesis, the number of carbon atoms progressively reduces from cholesterol to the various steroid hormones (glucocorticoids, mineralocorticoids and progestins all have 21 carbons, androgens have 19 and estrogens have 18).

The first stage in the synthesis of steroids conversion of cholesterol to pregnenolone by the cholesterol side chain cleavage enzyme in the mitochondria. Pregnenolone is then converted into progesterone by two enzymes, 3β -hydroxysteroid oxidoreductase and the Δ^5 - Δ^4 -oxosteroid isomerase which transform the 3-hydroxyl group to a ketone group and transfer the double bond from the 5-6 to the 4-5 position. In the microsomes hydroxylation of progesterone (involving cytochrome P₄₅₀) at C17 yields 17α -hydroxyprogesterone, which is in turn converted to androstenedione by C17-

20-desmolase. The final step is the formation of testosterone from androstenedione by 17 β -hydroxy-steroid oxidoreductase.

Testosterone is secreted from the Leydig cells and binds to sex hormone-binding globulin (SHBG) which appears to act as a transport protein but also serves to limit the metabolic clearance of steroids [Hammond, 1993]. It is believed that an equilibrium exists between steroid bound to SHBG and free steroids and that only free steroid is biologically active [Hammond, 1993]. SHBG binds to receptors on the surface of the prostate cells and may play some role in entry of steroids into some cells [Khan, 1990].

Once inside the cell testosterone is converted to DHT by the enzyme 5 α -reductase. DHT has a substantially higher affinity for the androgen receptor than does testosterone.

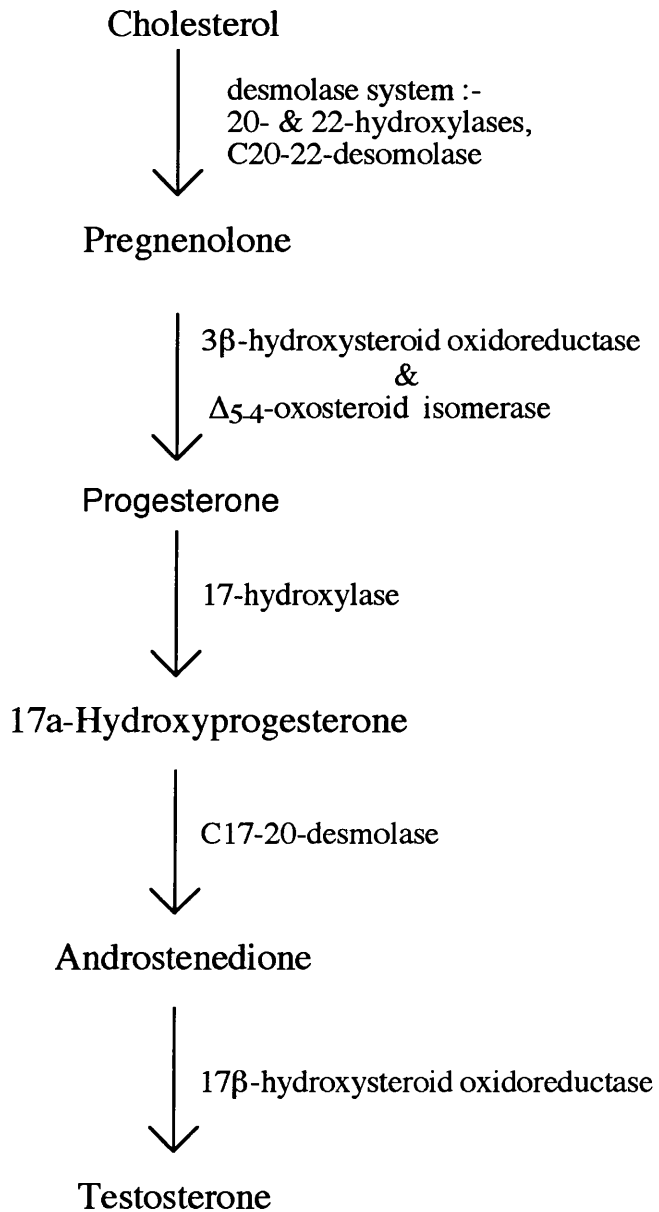


Figure 2:- The synthesis of testosterone from cholesterol in the testes.
Adapted from Milgrom,1990.

2. The mechanism of action of steroids

2:1 Type I and type II steroid receptors

Two classes of binding sites for steroid receptors have been identified by Scatchard analysis of ligand binding assays [Eriksson, 1978; Markaverich & Clark, 1979; Castagnetta *et al.*, 1992]. Type I sites bind ligand with high affinity ($K_d < 1\text{nM}$) and low capacity, the type II receptors bind ligand with a lower affinity but higher capacity [Clark & Peck, 1979; Castagnetta *et al.*, 1992]. The type I receptor is the classical steroid receptor, in that steroid binding to type I receptor causes activation of the receptor, followed by DNA binding and gene activation [Leake & Habib, 1987; Castagnetta *et al.*, 1992]. Little is known about the biological significance of type II receptors [Castagnetta *et al.*, 1992]. It is the type I receptor which will be discussed in the following sections.

2:2 Steroid receptors: a common structure

The ability of a cell and therefore a tissue to respond to a particular steroid hormone has been attributed to the presence of specific hormone receptors within that cell [Strahle, 1989]. Steroids diffuse across the plasma membrane and bind to their receptor. Unligated receptors (see below) are found as part of a larger oligomer which includes several heat shock proteins (see 4:1). Unoccupied steroid receptors are normally located within the nuclear envelope, except for the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) which in most target cells appear to be predominantly cytoplasmic.

All steroid receptors belong to a superfamily of ligand-modulated transcription factors, which also includes thyroid hormone receptor (T₃R), retinoic acid receptor (RAR) and the oncoprotein v-erb A (see Figure 3).

The cDNAs for all major steroid hormone receptors have been cloned [Evans, 1988] and show a high level of homology (see Fig. 3). It is thought that steroid receptors have evolved from a common prototype that contained a ligand binding domain and a DNA binding domain with two non-identical zinc fingers [Amero, 1992].

Amino acid sequence comparisons of the steroid receptor proteins revealed six domains, A-F [Forman, 1990] (Figure 4). Each domain has one or more functional sub-domains. The domain A/B, located at the N-terminal region, is the least conserved, varies greatly in length and contains one of the two transcriptional activation functions (TAFs), TAF-1, which is ligand-independent [Gronemeyer, 1993].

The C (DNA binding) domain, is highly conserved. Two non-identical zinc finger motifs are involved in the binding of receptor to specific nucleotide sequences in the DNA. These zinc fingers are formed by twenty highly conserved amino acids. The receptor superfamily members can be classified by the amino acids present at three positions at the base of the first zinc-finger (N-terminal finger). The GR, AR, MR and PR all contain Gly, Ser, and Val at the discriminatory positions. The second group is characterised by Glu, Gly, and Gly at these positions and includes many of the orphan receptors as well as the T₃R, RAR, and Vit DR [Forman, 1990]. The ER has Glu and Gly at the first and second discriminatory positions but it contains an Ala in the third position. Nevertheless, it recognises the group two DNA binding sequence [Forman, 1990]. The C domain also contains one of the two dimerization sequences and an hsp90 binding region (the association of SR with hsp is discussed in part 4:1).

The ligand-independent nuclear localization signal spans the border of the C and D domains. The D domain is also known as the hinge domain .

The E domain has a highly conserved sequence and contains the ligand binding activity (it is often referred to as the ligand binding domain), a hsp90 binding region, the second dimerization region, the ligand-dependent nuclear localization signal and the second TAF, TAF-2, which is ligand-dependent and cell- and promoter-specific [Gronemeyer, 1993].

Not all receptors have an F domain. The PR lacks this domain and its function in the other receptors is as yet unknown.

2:3 Receptor modification

Steroid receptors display a discrepancy between the molecular weight obtained by SDS-PAGE and that calculated from the amino acid sequence, indicating possible post-translational modification such as phosphorylation, glycosylation and acylation. Phosphoamino acid analysis and proteolysis studies demonstrated serine phosphorylation of SRs, mainly in the N-terminal domain. Phosphorylation on tyrosine residues have been reported in the cases of ER [Auricchio, 1989]. Receptors appear to be phosphorylated in the absence of hormone but become hyperphosphorylated when hormone or other agonists are present [Orti, 1992; Kuiper & Brinkmann, 1994]. In general terms, phosphorylation can increase the negative charge and acidity of a protein and thus have an effect on its interaction with other proteins or with DNA, but its specific role in the case of SR's is not clear at this time [Kuiper & Brinkmann, 1994].

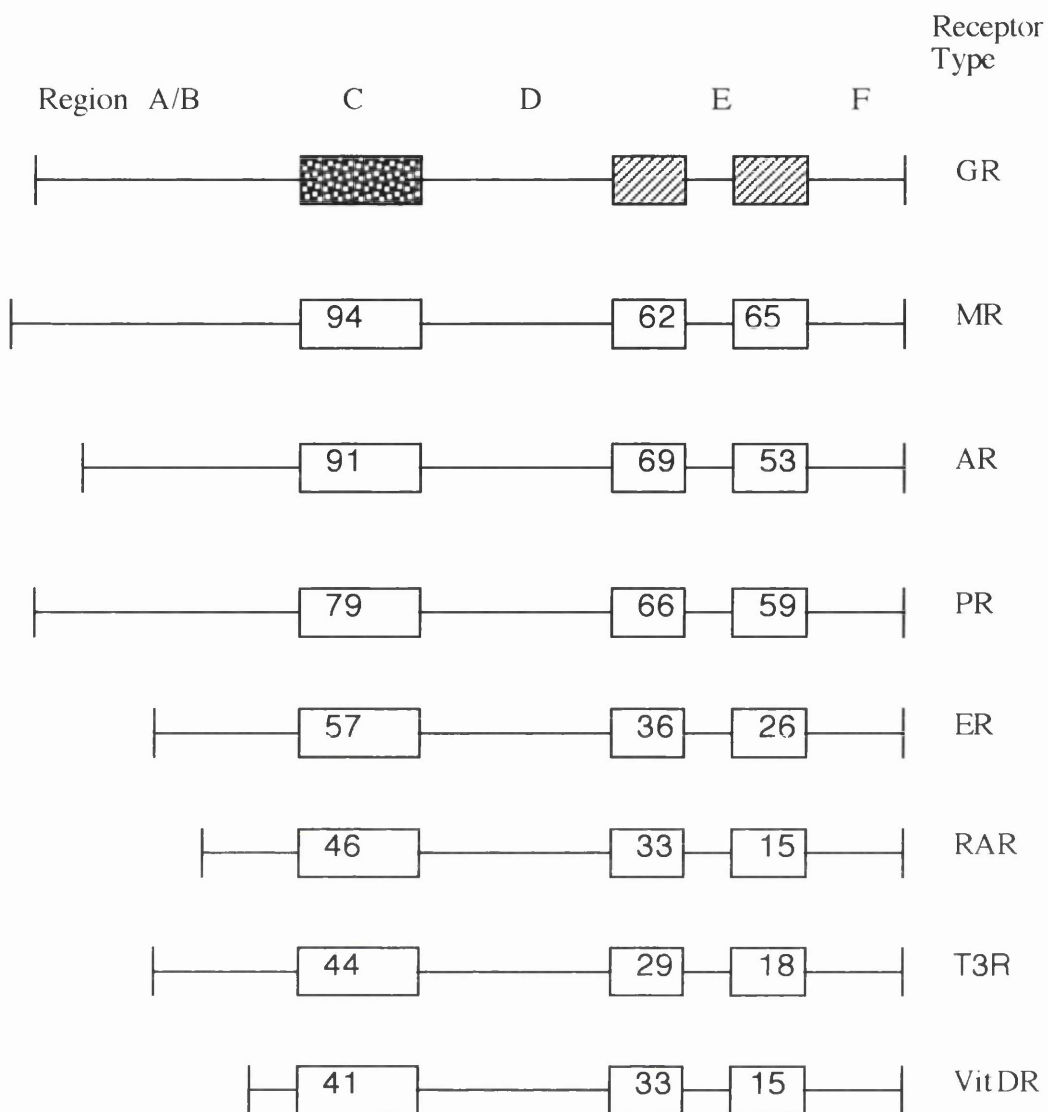


Figure 3:- The steroid receptor superfamily.

Comparison of amino acid sequences, the boxes represent regions of homology within the DNA-binding and steroid binding domains, the figures within show the % homology with the GR sequence. Adapted from O'Malley, 1990.

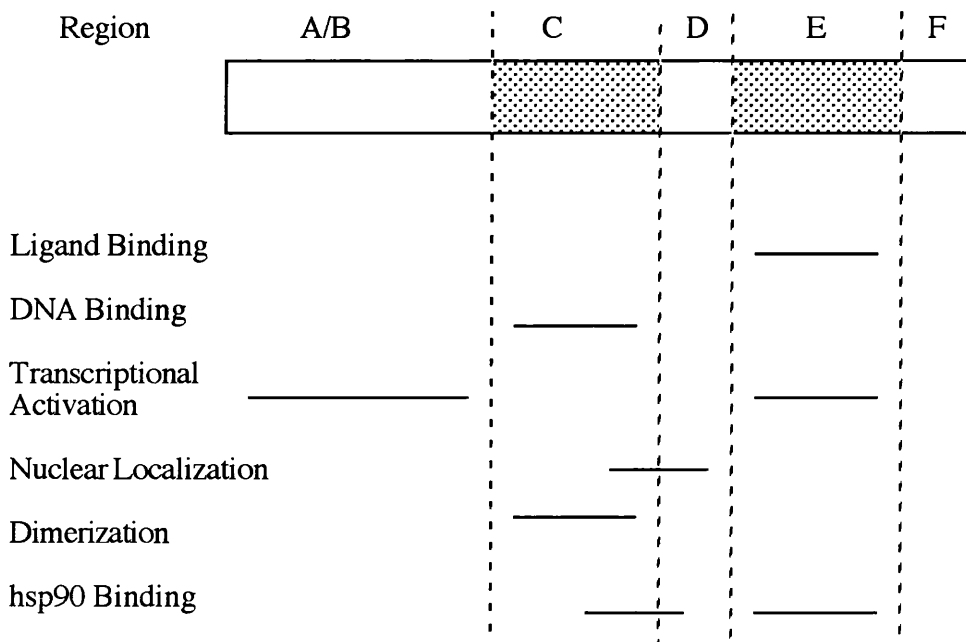


Figure 4. Schematic illustration of structure/function of steroid receptor.

The functions attributed to steroid receptors are listed, the lines indicate the regions of the receptor which are involved in each function.

2:4 Two forms of the steroid receptor exist

Steroid receptors can be found in two states within the cell, either with (transformed receptor) or without (unliganded receptor) ligand bound. The transformed receptor has a sedimentation coefficient of 4S. The unliganded receptor is associated with several other proteins to form a large oligomeric structure with a sedimentation coefficient of 7-10S [Sherman, 1983] which is referred to as the 8S receptor. Purification of the 8S PR receptor [Renoir, 1984] led to the discovery of a 90kDa protein associated with the receptors [Joab, 1984]. This protein was identified as hsp90 [Catelli, 1985]. It was noted that the 8S receptor does not bind to DNA [Beato, 1987]. Hsp90 is believed to mask the DNA binding region of the receptor and thus prevent the unliganded receptor from binding to DNA [Baulieu, 1987]. Other proteins, including hsp27, hsp56, hsp60, and hsp70 have been shown to be associated with steroid receptors and probably form part of the 8S complex. The 8S receptor complex is stabilized by group 6A transition metal oxyanions molybdate, vanadate and tungstate [Dahmer, 1984]. Stabilization of the receptor always coincides with the inability of the receptor to bind DNA [Pratt, 1989].

2:5 Receptor transformation

Steroid hormones bind to the oligomeric 8S receptor causing the dissociation of the complex to generate the 4S form of the receptor which has the ability to bind DNA [Baulieu, 1987; Beato, 1987; Pratt, 1992].

Transformation of the receptor also occurs at high salt concentration, in the presence of high levels of ATP or after dilution, even if no receptor is bound by ligand [Willman, 1986]. DNA binding and transcriptional activation by unliganded, transformed receptor is a matter of controversy. It is clear that

dissociation of the 8S receptor complex is necessary for DNA binding but is it sufficient? The subsequent transcriptional activation might require ligand-receptor interaction. *In vivo*, hormone is required for gene activation by steroid receptors and this has been reproduced *in vitro* [Meshinchi, 1990; Bagchi, 1992] but *in vitro* studies have suggested that hormone independent activation does occur [Bailly, 1986; Willman & Beato, 1986; Elliston, 1990]. Whether hormone binding has effects other than dissociation of the receptor remains unclear. The unliganded transformed receptors which had transcriptional activities may have undergone some, as yet unidentified, structural alteration and activation by the various *in vitro* techniques used to purify the receptors. *In vivo* the receptor may not undergo such conformational change prior to ligand binding and therefore ligand would be necessary for receptor activation. A two step model for receptor activation has been described [Bagchi, 1992; McDonnel, 1991] in which the ligand first causes dissociation of the oligomeric 8S receptor to reveal the 4S receptor (Fig. 5). The ligand then converts the receptor into a transcriptionally active form (Fig. 5). A 5S form of the receptor has been isolated and it was suggested that this 5S form is the active form of the receptor [Muller *et al.*, 1983]. In the case of ER this 5S form has been identified as a dimer of two steroid binding subunits [Miller *et al.*, 1985].

2:6 Steroid receptor dimerization and DNA binding

Dimerization of steroid receptors is required for stable binding of the receptor to DNA and may take place when the receptor is bound to DNA [Kumar, 1988] or prior to DNA binding [DeMarzo, 1991].

Steroid receptor dimers bind to regions of DNA known as hormone response elements (HREs) [Luisi, 1991] within the promoter region of hormone-responsive genes. HREs have an imperfect palindromic structure with a three-

nucleotide non-conserved spacer between the two halves. They can be classified into two main groups according to the consensus sequence within the palindrome. The first group recognise the glucocorticoid response element (GRE) which contains the consensus sequence GGTACAnnnTGYTYCY (where Y= T or C). Members of this group include GR, PR, AR and MR. The second group recognises the estrogen response element (ERE) which has the consensus sequence GGTCAnnnTGACC. Members of this group include ER, T₃R, Vit D₃ and RAR [Beato, 1989]. The region within the receptor responsible for recognising the consensus sequences is known as the P-box and is located in the first zinc finger (N-terminal finger) [Umesono, 1989]. The P-box amino acid consensus sequence for the GRE and ERE groups of receptors are GSCKV and EGCKA, respectively. Only after binding to its HRE can a steroid receptor transcriptionally activate the genes under its control.

2:7 Transcriptional activation by steroid receptors

Two sub-regions of steroid receptors have been identified as transcriptional activation functions and are located within the N-terminal region (TAF-1) and the hormone-binding region (TAF-2) of PR, GR, ER [Gronemeyer, 1993] and AR [Jenster, 1992]. Other regions, such as the DNA binding region have been implicated in transcriptional activation [Gronemeyer, 1993a]. Work carried out by several different groups studying transcriptional activation by steroid receptor hormones have been unable to reach agreement as to the roles of each TAF [Tora, 1989; Meyer, 1990; Berry, 1990] . Each group used a different cell-free system which has led to the theory that transcriptional

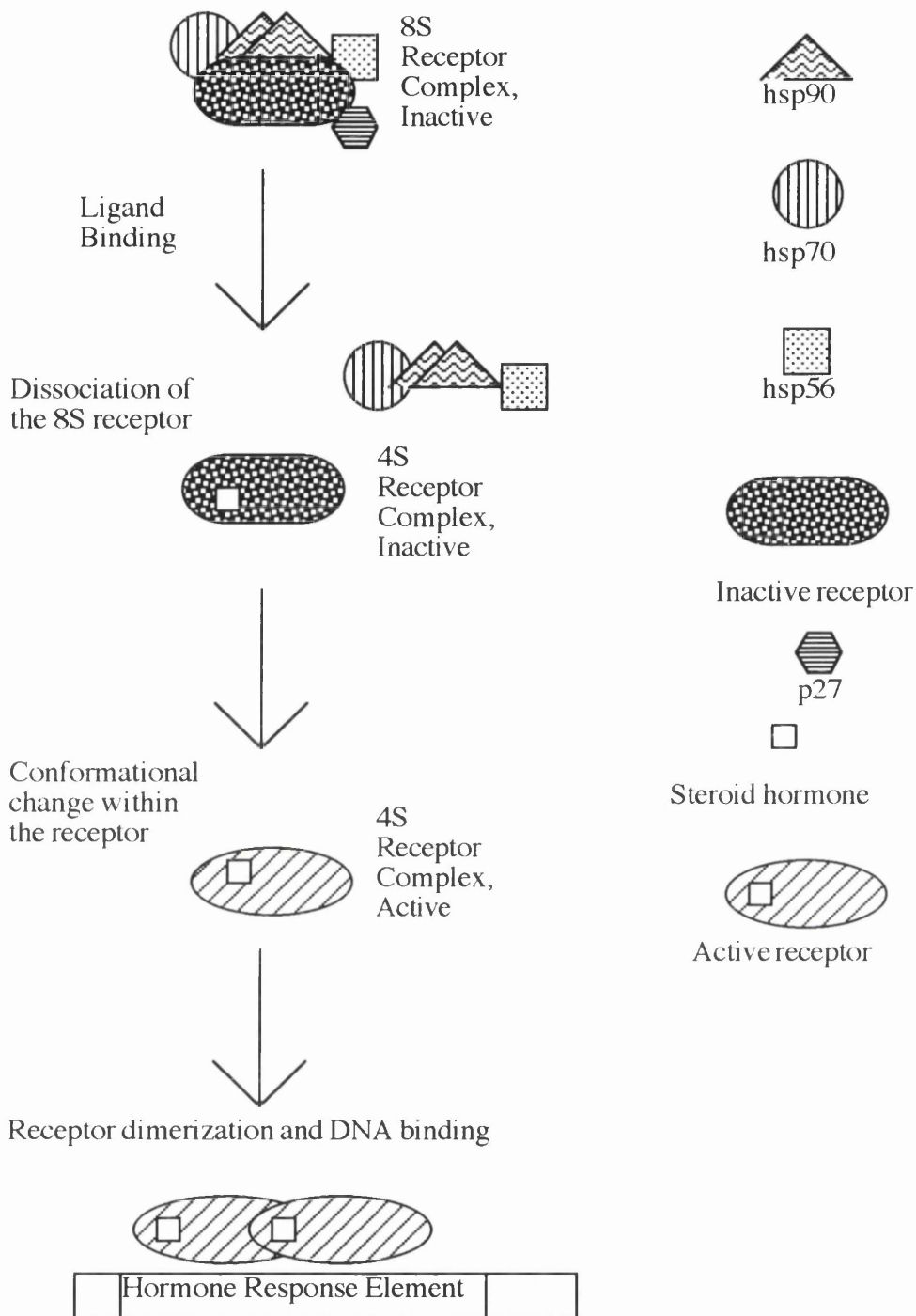


Figure 5:- Schematic model for steroid receptor activation.

Steroid-free receptor exists as part of an 8S receptor complex. Hormone binding causes a two step change to this complex. First the associated proteins dissociate and then a conformational change occurs within the receptor which enables the receptor both to bind to DNA and to dimerise [Bagchi,1992; McDonnel,1991].

activation by a particular TAF is dependent on cell type and promoter context.

This may explain the inconsistency of results obtained so far .

The receptor regulates gene transcription presumably by interaction with other transcription factors. The transcription factor TFIIB has been shown to associate with PR and ER [Ing, 1992] probably via TAF-1 or TAF-2. It has been postulated that TFIIB mediates an interaction between the receptor-DNA complex and the TFIID-DNA complex at the TATA box creating a DNA loop. The subsequent binding of RNA polymerase II together with TF IIF, E, H and J leads to the formation of an active pre-initiation complex [Ing, 1992]. This is supported by observations made on other DNA binding proteins such as AP1 which binds to an enhancer sequence similar to but distinct from HRE as a dimer consisting of either oncogenes Jun and Fos or a dimer of Jun [Gutman & Wasylyk, 1991].

2:8 Repression of transcription by steroid receptors

Several examples of repression of gene expression by steroid hormones have been found. For example glucocorticoids repress the pro-opiomelanocortin (POMC) gene [Drouin, 1989]. In this case repression requires GR binding to a GR-binding site within the POMC promoter region but there are other examples where receptor mediated repression does not require DNA binding of a receptor [Drouin, 1993]. Repression may be due to squelching, tethering, quenching, interference, competition for overlapping binding sites, the existence of null binding sites or direct repressor elements [Drouin, 1993]. More work needs to be done before a true understanding of repression by steroid receptors is obtained.

2:9 Steroid receptor localisation

Originally steroid receptors were believed to be cytoplasmic but in the early 1980's enucleation experiments showed that they were in fact located in the nucleus [Martin &Sheridon, 1982; Welshon *et al.*, 1984] . In the absence of steroid the loose association of receptor with the nuclear elements causes the receptor to partition into the cytosol when the cell is disrupted in a large volume of buffer [Martin &Sheridon, 1982].

In the absence of hormone the AR, PR, and ER are located in the nucleus [King, 1984 ; Husmann, 1990], but the unliganded GR and probably the MR, are found in the cytoplasm [Wikstrom, 1987; Agarwal, 1994] and translocate to the nucleus after hormone binding.

The question of how steroid receptors are transported into the nucleus is still under investigation. Small molecules can diffuse through the nuclear pores but molecules larger than 20-40 kDa must be actively transported through the nuclear pore complex [Feldherr, 1984]. Protein^s which do not randomly diffuse to the nucleus may have a nuclear localisation signal (NLS), such as that of the SV40 large T antigen which has a sequence PKKKPKV [Kalderon, 1984]. A general NLS sequence consisting of two basic regions has been identified [Dingwall, 1991]. The first region contains two basic amino acid residues and is separated from the second by ten random amino acids. The second basic region is a cluster in which three out of five amino acids must be basic [Robbins, 1991]. Steroid receptors contain this sequence located at the boundary of the second zinc finger and the hinge region [Dingwall, 1991; Guiochon-Mantel *et al.*, 1994]. Mutation analysis of these regions, before the NLS was known, had shown these regions to be important in nuclear localisation [Gronmeyer, 1993b].

How the NLS sequences are involved in nuclear localization is not understood. They may interact directly with the nuclear pore structure or additional proteins may be involved.

2:10 Steroid receptor trafficking

It has been suggested that cytosolic steroid receptors are transported along the cytoskeletal system as part of a transportosome [Pratt, 1992], which contains several proteins such as hsp90, hsp56, p50, p23 and p14. In the case of AR, PR and ER, this transportosome enters the nucleus and the receptors remain associated with at least hsp90 in a docking complex until hormone binding. The docking complex is associated with the nuclear skeletal system. The docking complexes of the GR and MR which contain hsp90 are found in the cytoplasm where they are associated with the cytoskeleton [Pratt, 1990]. Hsp 90 can bind actin [Koyasu, 1986] and tubulin [Sanchez, 1988]. The possible role of either of these proteins in receptor transportation or docking has not been established.

There is some evidence which contradicts a possible role of the cytoskeleton in receptor transport. Drugs which disrupt the cytoskeletal systems such as demecolcine (which acts on microtubules and intermediate filaments) and cytochalasin B (which acts on actin-containing filaments) do not prevent nuclear transport of the PR [Perrot-Applanat, 1992; Guiochon-Mantel *et al.*, 1994].

When the mechanism of translocation and docking is fully understood, it may reveal novel ways to prevent receptor-induced transcription by blocking the transport of receptors.

2:11 The androgen receptor

As discussed earlier (1:2), androgens (testosterone (T) and DHT) are essential for the development and normal function of the prostate. Androgens exert their effects through binding to the AR.

The human AR gene has been mapped to the X-chromosome, contains eight exons and spans 54 kilobasepairs of DNA [Marcelli, 1990]. The human AR gene is transcribed from two initiation sites, neither of whose promoters contain canonical TATA or CCAAT boxes [Faber, 1993].

Genes which are regulated by androgens include the prostate specific antigen (PSA) gene. The androgen response element (ARE) for this gene has been identified [Riegman, 1992]. The AREs identified so far are similar to the GRE consensus sequence [Roche, 1992] .

The AR, like all steroid receptors, is a phosphoprotein, phosphorylation occurring within the N-terminal domain, the hormone binding domain and the hinge region [Orti, 1992]. In the absence of hormone, the AR is phosphorylated but becomes hyperphosphorylated on hormone binding [van Laar, 1991].

The 8S AR contains hsp90, hsp70 and hsp56 [Veldscholte, 1992]. Other proteins may well be involved. The first step of receptor transformation after hormone binding may be the dissociation of the hsp90 and hsp56 proteins leaving an intermediate 6S form which contains receptor and hsp70 [Veldscholte, 1992].

It is believed that intermolecular disulfide bonds help stabilise the 8S AR complex. Androgen may cause reduction of these bonds which would result in transformation of the receptor [Wilson, 1986].

Dimerisation of the AR requires androgen binding. Only AR dimers bind to DNA [Wong, 1993].

2:12 Antagonist action on receptor mediated gene transcription

Antagonists are compounds which bind to, but prevent transcriptional activation by the steroid receptor. The molecular mechanism of antagonist action is not well understood, but interference may occur at a number of different stages of receptor activation. Antagonists may inhibit the dissociation of the 8S receptor complex. Alternatively, the 8S complex may dissociate to produce an antagonist-4S receptor complex which is unable to bind to its HRE possibly by inhibition of dimerisation (if this is necessary for DNA binding). Finally the antagonist-4S receptor may bind to the HRE but be unable to activate the transcriptional machinery [Baulieu, 1987]. Different antagonists may act in different ways. In some cases, antagonists can act as partial agonists under certain conditions [Allen, 1992; Berry, 1990; Meyer, 1990]. The group of antagonists which possess agonist activities includes the antiestrogen tamoxifen and the antiprogesterin/antiglucocorticoid RU486. These are probably the best understood antagonists. They do not appear to interfere with DNA binding [Brown, 1990; Meyer, 1990] so must alter the transactivation properties of the receptors . Weak agonists like estriol have a lower affinity for ER and therefore compete with estrogens when they are present but in the absence of estrogen, estriol acts as a weak agonist.

It has been proposed [Berry, 1990; Meyer, 1990] that the two TAFs present in the receptors play a role in the agonistic action of various antagonists. TAF-1, the hormone independent TAF, would be active in the presence of either agonist or antagonist (when antagonist causes dissociation of the 8S complex). When TAF-1 and TAF-2 act independently, the ligand would act as an antagonist if TAF-2 alone was active on a gene promoter, but if TAF-1 alone was active the ligand would be an agonist and if both were active it would act as a mixed agonist/antagonist.

One antagonist ICI182780, has been called a pure antagonist because no steroid agonist action has been associated with it. However recent work [Gronemeyer, 1993] has suggested that ICI182 has a similar mode of action to tamoxifen and RU486 and may be a partial agonist.

A group of pure antagonists has been found which appear to inhibit DNA binding of the receptor . One example ZK98299 may prevent the formation of receptor dimers due to an abnormal conformational change on ZK98299 binding to the receptor [Klein-Hitpass, 1991].

3 . Heat Shock Proteins

3:1 The Heat Shock Response and The Heat Shock Proteins

The cellular response to stress is universal. Part of this response involves the synthesis of a small group of proteins, the heat shock proteins which are also known as stress proteins. Hsps have been defined by two criteria. Firstly, their synthesis is strongly stimulated by stress and, secondly, their genes contain a conserved sequence of 14bp in the 5' noncoding region, the Pelham box [Schlesinger *et al.*, 1986], also known as the heat shock element (HSE).

Induction of hsp synthesis in response to elevated temperatures has been demonstrated in organisms as diverse as *E.coli* [Lemeaux *et al.*, 1978], *Drosophila* [Lindquist, 1980], chickens [Kelley *et al.*, 1978] and humans [Maytin *et. al.*, 1990]. Indeed, it is now understood that cells from all known organisms respond to stress conditions such as heat by producing hsps [Lindquist, 1986].

The DNA sequences of the hsp genes have been extensively conserved throughout evolution [Lindquist, 1986]. The conserved regions include both the protein coding and regulatory sequences. Hsps have been shown to be constitutively expressed in all cell types studied and they appear to have many roles within non-stressed cells, including that of molecular chaperones [Welch, 1991].

The response of cells to stress was first identified by exposing *Drosophila* to elevated temperatures but several other supraphysiological conditions have the same effect on cells [Lindquist, 1986]. Ethanol, zinc, copper, mercury, sulfhydryl reagents, sodium arsenite and amino acid analogues have all been shown to induce the response.

Heat shock induces a rapid change in transcriptional behaviour of the heat shock genes. This occurs on binding of the heat shock factor (see below) to the

HSE. Under stressed conditions, selective translation of hsp mRNAs occurs via increased mRNA stability (due to sequence elements in the 3' untranslated regions) and increased translational efficiency (due to sequence elements within the 5' untranslated regions) [Schlesinger, 1990].

3:2 The heat shock factor

In *E. coli*, stress induces an alternative σ -factor, σ^{32} , which binds to the RNA core polymerase, thus enabling the holoenzyme to recognize the heat shock promoters [Grossman *et al.*, 1984].

As mentioned above, in eukaryotes, a heat shock factor (HSF) binds to the HSE and causes transcriptional activation of the hsp genes. Interactions between the HSF and HSE differ among organisms. In yeast, the HSF is bound to the HSE even in the absence of stress and is transcriptionally activated by stress, but in higher eukaryotes the HSF binds the HSE only under stressed conditions [Sorger *et al.*, 1987]. HSFs form large homocomplexes and it is believed that they bind to the HSE as a trimer. The HSE consist of differently orientated NGAAN repeated sequences [Schlesinger, 1990]. HSF trimerization may explain the ability of the HSF to bind to differently orientated sequences but would require a great deal of flexibility in the trimer structure [Sorger, 1991].

HSFs from several different organisms have been isolated and cloned. Despite the homology between HSEs, there is little sequence similarity between the different HSFs. It has been suggested that what similarity exists occurs in the DNA binding and trimerisation domains [Sorger, 1991].

What regulates the HSFs is unclear. Several different models have been proposed. It has been suggested that a negative regulatory protein may interact with the HSF and that this interaction is disrupted by stress conditions [Clos *et al.*, 1990]. One possibility is that the negative regulatory protein is an hsp in

which case autoregulation of hsp would occur. HSF has been shown to associate with hsp90 [Nadeau *et al.*, 1993]. Dissociation of the proposed HSF-hsp complex might occur when competition for hsp binding from damaged proteins becomes great enough leading to HSF-DNA binding and transcriptional activation of the hsp genes. When the hsp level increases sufficiently due to *de novo* synthesis the HSF-hsp complex reassociates and the HSF is once again repressed [Sorger, 1991]. Another possibility is that HSF may undergo transition to an oligomeric state from an inactive monomer. This is supported by the observations that antibodies to HSF monomers can induce DNA binding in non-stressed cells and that monomers of HSF bind DNA with significantly lower efficiency than do tetramers [Zimarino *et al.*, 1990]. A third possibility is that HSF is held in an inactive form by conformational restraint and that stress causes a conformational change within the HSF leading to its activation.

Which of these possible mechanisms is responsible for the regulation of HSF and therefore the heat shock response is not clear at this time, nor is the mechanism by which the HSF regulates transcription.

3:3 Prokaryotic heat shock proteins

Treatment of *E.coli* with elevated temperatures results in transient acceleration of the expression rate of the heat shock genes. This is due to the activation of an alternative σ -factor which forms σ^{32} RNA polymerase holoenzyme, which specifically recognizes the heat shock promoters [Grossman *et al.*, 1984]. Consensus sequences for σ^{32} RNA polymerase initiation have been identified at positions -35 and -10 within the promoter region of hsp genes [Lindquist, 1986].

Several *E.coli* hsps have been identified and cloned. These are dnaK, dnaJ, grpE, groES, and groEL. dnaK is homologous to eukaryotic hsp 70 [Bardwell & Craig, 1984]. It has roles in phage λ replication and protein translocation and appears to protect cells from denaturing conditions, as well as having ATPase activity [Ang *et al.*, 1990]. dnaJ and grpE also have roles in DNA replication and are required for the ATPase activity of dna K [Ang *et al.*, 1990]. The groEL protein is homologous to the mitochondrial hsp60 protein and binds to nascent polypeptides whose release may require ATP hydrolysis and, in some cases, groES activity [Ang *et al.*, 1990].

3:4 Eukaryotic heat shock proteins

Eukaryotic hsps are expressed under stressed conditions and often at high levels in non-stressed conditions. The role of the constitutively expressed hsps is varied and in many cases is just becoming clear. Eukaryotic hsps can be divided into four classes according to their molecular weight, namely hsp90, hsp70 (this group includes hsp56 & 60), the small hsps (hsp15-30) and ubiquitin. Current understanding of the function of each of the four groups will be discussed below.

3:4:1 Ubiquitin

Ubiquitin is one of the smallest hsps discovered to date. It is approximately 7-8 kDa in size and has a role in protein degradation. Ubiquitin-mediated proteolysis is a major pathway for selective protein degradation in eukaryotic cells [Jentsch, 1992]. This multistep ATP-dependent process involves other proteins which are known as E1, E2 and E3. In the first step a thioester is formed between an internal cysteine residue of E1 and the C-terminus of ubiquitin. This activated ubiquitin is then passed to E2, again via a specific cysteine residue in the enzyme. There are several different E2 enzymes which

are also known as ubiquitin-conjugating enzymes. Ubiquitin then comes into contact with the protein to be degraded. The target proteins are recognised either by the E2 or by E3 which is a substrate recognition protein and is also known as ubiquitin ligase. An isopeptide bond between the C-terminus of ubiquitin and a specific internal lysine residue of the target protein forms which results in a branched conjugate. This ubiquitin-substrate conjugate may undergo further ubiquitinations to create multiubiquitin chains or trees depending on which lysine residue in the ubiquitin molecule is used for ubiquitination. For protein degradation to occur only a single ubiquitin molecule needs to be attached but there are usually multiubiquitin complexes involved [Jentsch, 1992].

The ubiquitin-substrate conjugates are believed to be degraded by a proteasome, which is a multisubunit complex, containing at least twelve different subunits. This 20-26 S complex is arranged in four stacked rings and has at least three distinct endopeptidase activities [Craig *et al.*, 1993].

Ubiquitin-mediated proteolysis is believed to be regulated at the level of protein substrate recognition by E2 and E3 and it is therefore easy to see why there are so many different ubiquitin conjugating-enzymes. There is a high level of primary sequence homology between enzymes from different animals [Jentsch, 1992]. It is not known how the enzymes select which protein needs to be degraded.

Under stressed conditions it has been suggested that ubiquitin would remove damaged or denatured protein and complement the other hsp roles in preventing protein aggregates from forming within the stressed cell [Lindquist, 1986] .

3:4:2 The small hsps

All organisms encode for small hsps with a molecular mass of between 15-30 kDa. The sequences of the small hsps of different organisms are less highly conserved than those of the larger hsps, but they can be considered homologous

on the basis of their limited sequence similarities and the similarities in the predicted protein structure [Lindquist, 1986]. Another common feature is that they are found in high molecular weight complexes.

Less is known about the small hsps than the other hsps. The role of some small hsps as molecular chaperones has been suggested by *in vitro* studies. For example murine hsp25, human hsp27 and bovine α -B-crystallin all refold citrate synthetase and α -glucosidase after urea denaturation [Jakob *et al.*, 1993]. Actin depolymerization was prevented and cytochalasin D cytotoxicity was reduced by hsp27 in chinese hamster ovary cells [Lovoie *et al.*, 1993]. Human hsp27 has been implicated in the resistance of human breast cancer cells to Doxorubicin [Oesterreich *et al.*, 1993]. Hsp27 may be involved in a regulatory loop in estrogen dependent cells. Estrogen induces transcription of hsp27 mRNA and hsp27 interacts with the ER [Mendelsohn., 1991].

In most systems studied to date, phosphorylation of hsp27 occurs rapidly after heat shock [Mendelsohn *et al.*, 1991] and this is believed to play a role in thermotolerance, as is the stimulation of hsp27 synthesis and the translocation of hsp27 to intra- or peri-nuclear sites [Lovoie *et al.*, 1993].

Phosphorylation of hsp27 occurs on serine residues and it may have phosphorylation-activated functions which could effect signal transduction and thereby protect cells from heat-induced damage [Landry *et al.*, 1992].

Phosphorylation of murine hsp25 and human hsp27 occurs at homologous sites [Stokoe *et al.*, 1992]. *In vitro* studies have shown that hsp27 is phosphorylated by several different kinases, including mitogen-activated protein kinase activated protein kinase-2 (MAPKAP kinase 2) [Stokoe *et al.*, 1992], protein kinase C (PKC) [Faucher *et al.*, 1993] and a novel protein kinase which has been called hsp27 kinase [Guesdon *et al.*, 1993; Zhou, 1993]. Whether all of these enzymes play a role in *in vivo* phosphorylation of hsp27 is not clear.

Expression of hsp27 is low in normal breast tissue but is often found at high levels in human breast tumor cells [Thor, 1991]. Hsp27 staining appears to correlate with different biological features in early and advanced breast cancer. High hsp27 staining is linked with a short disease-free survival in node-negative patients but with prolonged survival from first recurrence [Love *et al.*, 1994].

3:4:3 Hsp56, a novel hsp

Until recently very little was known about hsp56, which is also known as p56, p59 FK506-binding protein, FKBP59 and FKBP52 [Tai *et al.* , 1993; Renoir *et al.*, 1990; Sanchez *et al.* , 1990a; Peattie *et al.*, 1992]. It has been isolated in association with the 8S SR, though it does not bind directly to the receptor. Hsp56 is bound to hsp90 which forms a complex with SR [Renoir *et al.* , 1990]. It has been suggested by cross-linking studies that hsp56 and the SR may lie in close proximity to each other in the larger heterocomplex [Alexis *et al.*, 1992]. Rabbit and human hsp56 have significant sequence homology suggesting that the protein is evolutionarily conserved [Sanchez, 1990a]. Hsp56 is found mainly in the nucleus [Gasc *et al.*, 1990]. It does not have a consensus nuclear localisation signal, but consensus sequences for phosphorylation sites, an ATP-binding site and a calmodulin binding site have been identified, although it is not clear if they are functional [Lebeau *et al.* , 1992]. 55% amino acid homology exists between hsp 56 and peptidyl-prolyl isomerase and it has been suggested that hsp56 has rotamase activity. A possible role of hsp56 is the regulation of hsp90. Hsp56 is an immunophilin of the FK506 class [Hutchison *et al.* , 1993]. Immunophilins are proteins which bind immunosuppressive agents like cyclosporin A, FK506 and rapamycin. The binding of these specific agents (in the case of hsp56, FK506) inhibits the peptidylprolyl isomerase activity (rotamase activity) of the immunophilin [Schreiber, 1991]. FK506 does not

affect GR function, transcriptional activation or assembly of the 8S complex. This suggests that the rotamase activity of hsp56 is not required for the 8S complex formation or subsequent receptor function [Hutchison *et al.* , 1993]. One group has recently suggested that hsp56 plays a role in nuclear localisation of the receptor [Pratt *et al.*, 1993]. It is possible that hsp56 forms a core structure with hsp70 and 90 which may be involved in the recognition, folding, assembly and disassembly of regulatory proteins such as SRs [Tai *et al.*, 1993].

3:4:4 The hsp60 family

The study of how proteins fold after synthesis led to the discovery of molecular chaperones. The hsp60 family is involved in protein folding in all organisms so far investigated and family members are known as chaperonins or Cpn60s. Chaperonins are highly conserved and form a two stacked ring structure, each ring containing seven homologous subunits [Craig *et al.*, 1993]. The *E.coli* groEL protein is a member of this family. It binds to denatured peptides and is involved in protein folding and phage head assembly [Ang *et al.*, 1990 ; Welch, 1991]. A protein homologous to groEL has been isolated from mitochondria and chloroplasts. In yeast, the mitochondrial hsp60 protein is encoded by the nuclear genome. The nascent protein is targeted to the mitochondria and requires folding once inside the mitochondria, which appears to involve functional pre-formed hsp60 [Craig *et al.*, 1993]. Mitochondrial hsp60 function is similar to that of groEL in that it is responsible for protein folding and assembly in an ATP-dependent manner [Craig *et al.*, 1993], as is chloroplast hsp60 which was identified by its role in Rubisco complex formation [Welch, 1991]. TCP1 is the cytosolic equivalent of mitochondrial and chloroplastic hsp60. It is believed that they evolved from a common prototype although there is only limited sequence similarity [Craig *et al.*, 1993]. The action of TCP1 is only now

being investigated but there is some evidence to suggest that it functions in a similar way to hsp60 [Craig *et al.*, 1993].

3:4:5 The hsp70 family

The hsp70 family is one of the most highly conserved group of proteins with between 50 and 98% homology between different eukaryotic hsp70 molecules. The *E. coli* protein Dna K has 50% homology with all eukaryotic hsp70s [Craig *et al.*, 1993]. Members of the hsp 70 family have been found in all compartments of the eukaryotic cell and have been shown to be essential for normal growth and development [Gething & Sambrook, 1992].

Mitochondrial hsp70, Ssc1p, is a nuclear encoded protein which plays a major role in the translocation of proteins across the mitochondrial membrane and their correct folding in the mitochondria [Pfanner & Neupert, 1990, Kang *et al.*, 1990]. Endoplasmic reticulum hsp70, known as BiP, is required for transmembrane transport into the endoplasmic reticulum [Vogel *et al.*, 1990]. BiP is also involved in the assembly of multimeric protein complexes [Pfanner & Neupert, 1990]. In the cytoplasm of eukaryotes there are two groups of hsp70s, a stress inducible form, hsp72, and a constitutively expressed form, hsp73, also known as hsc70 [Gething & Sambrook, 1992]. Both cytosolic forms translocate to the nucleus under stressed conditions, where they associate with partially assembled pre-ribosomes [Hightower, 1991]. They also bind partially denatured proteins that would otherwise form insoluble aggregates at elevated temperatures.

All hsp 70s identified to date have the ability to bind ATP [Lindquist, 1986]. They contain two domains, a N-terminal ATP-binding domain and a C-terminal peptide-binding domain. The secondary structure of the C-terminal domain is thought to be similar to the major histocompatibility complex class 1 antigen-presenting molecule which is known to bind to peptides which are in an extended conformation [Rippmann *et al.*, 1991].

A three-stage cycle involving hsp70 for the folding of partially unfolded and denatured proteins has been proposed [Palleros *et al.*, 1991]. Hsp70 has an ADP molecule bound when it associates with an unfolded protein. Replacement of the ADP molecule by an ATP molecule causes the folding of the protein. The correctly folded protein can only dissociate from the complex after the hydrolysis of ATP, thus leaving an hsp70-ADP complex which is able to bind to another unfolded protein. However another possible mechanism has been proposed which suggests that ATP hydrolysis causes conformational changes within the hsp70 molecule which are then passed on to the protein and it is this that causes protein folding [Pelham, 1986]. A third possible mechanism suggests that hsp70 may hold the unfolded protein in a semifolded state and that ATP hydrolysis provides a timing mechanism for the release of the protein which can then continue folding [Rothman, 1989]. Which of the three proposed mechanisms is correct is the subject of much debate.

3:4:6 Hsp 90

Hsp 90 is an abundant, mainly cytoplasmic, protein in unstressed cells and may account for as much as 1-2 % of the total cellular protein content [Lindquist *et al.*, ; 1986; Lai *et al.*, 1984]. The amino acid sequence is highly conserved through evolution [Yonezawa *et al.*, 1988; Lindquist & Craig, 1988] which suggests that it plays an important role in cell function. The functions attributed to hsp 90 will be described below.

Two forms of hsp 90 have been identified, hsp90 α and hsp90 β [Simon *et al.*, 1987]. Multiple sequences have been identified on the human genome which could encode these proteins but only one structural gene has been characterized for each. These genes possess HSEs in their promoter regions [Hickey *et al.*, 1989; Rebbe *et al.*, 1989]. One of the features which distinguishes these structural genes from pseudogenes is the lack of introns in the pseudogenes [Durkin *et al.*, 1993]. The genes encoding hsp90 α and hsp90 β are

differentially regulated [Simon *et al.*, 1987; Yamazaki *et al.*, 1990], the significance of which is unclear. Neither is it clear whether both genes are expressed at the same level under different cell conditions or if both hsp90s are absolutely required.

The amino acid sequences for hsp90 α and hsp90 β are identical in 630 out of 724 possible residue matches: hsp90 α is 8 residues longer than hsp 90 β [Lees-Miller & Anderson, 1989a]. Two serine phosphorylation sites have been identified in both proteins. In hsp90 α , the sites lie 32 amino acids apart, while in hsp90 β they lie 28 amino acids apart, at serines 231/263 and serines 227/255, respectively [Lees-Miller & Anderson, 1989b]. These sites are phosphorylated by casein kinase II *in vitro* [Lees-Miller & Anderson, 1989b]. Casein kinase II has been copurified with hsp90, which suggests a role for casein kinase II in the phosphorylation of hsp90 *in vivo* [Miyata & Yahara, 1992], although an alternative role for hsp90's association with casein kinase II will be discussed below. Hsp90 α has two threonine residues in its N-terminal region which are not present in hsp90 β . These are phosphorylated by human double-stranded DNA-activated protein kinase [Lees-Miller & Anderson, 1989a]. The existence of unique phosphorylation sites within hsp90 α suggests that hsp90 α and 90 β could be independently regulated and may even serve different functions.

Hsp90 possesses a nucleotide binding site and is able to undergo autophosphorylation but only when specific residues are already phosphorylated [Nadeau *et al.*, 1993; Csermely & Kahn 1991]. The overall extent of autophosphorylation is small but only a small subpopulation of hsp90 may undergo autophosphorylation at any one time and thus may be significant. ATP induces an "open-closed" conformational change in hsp90 which has been attributed to an increase of β -pleated sheet and also an increase in the hydrophobic character of hsp90. Similar conformational changes were seen on molybdate and heat treatment [Csermely *et al.*, 1993]

3:5 The possible functions of hsp90

The role of hsp90 as a molecular chaperone does not require nucleotide triphosphates and may involve hsp90 stimulation of protein folding by preventing non-native proteins from participating in unproductive intermolecular interactions [Wiech *et al.*, 1992]. Hsp90 associates with the glucocorticoid receptor at or near the end of receptor translation. This may aid the correct folding of the receptor as well as the well-documented role of receptor inactivation [Dalman *et al.*, 1989]. The ability of hsp90 to aid protein folding does not have a role in transport of proteins into the endoplasmic reticulum suggesting that the different chaperone proteins have differing functional specificity, although most chaperones have the general feature of preventing protein aggregation [Wiech *et al.*, 1993]. A proportion of the cytosolic hsp90 has been found in association with several other proteins including hsp70 [Perdew & Whitelaw, 1991] and p59, which, as previously mentioned, is a peptidyl prolyl isomerase [Lebeau *et al.*, 1992; Nadeau *et al.*, 1993] and cycophilin A [Nadeau *et al.*, 1993]. The different proteins in this heterologous complex may all interact in their functions as chaperones.

Hsp90 is an actin-binding protein and can cross link actin filaments in a Ca^{2+} -calmodulin-regulated manner [Koyasu *et al.*, 1986; Nishida *et al.*, 1986]. Calmodulin binds to hsp90 in the presence of Ca^{2+} and inhibits the binding of hsp90 to actin filaments [Nishida *et al.*, 1986]. Hsp90 has also been shown to be associated with tubulin in intact cells [Sanchez *et al.*, 1988]. The ability of hsp90 to bind to actin and tubulin structures within the cell is consistent with the model that hsp90 is involved in the anchoring of proteins such as the glucocorticoid receptor to the cytoskeletal structures [Miyata & Yahara 1992]. It can be envisaged that both the cytoskeleton and hsp90 play a role in the intracellular transport of proteins. Hsp90 is mainly located in the cytoplasm but

after heat shock nuclear translocation occurs but not along any of the three main cytoskeletal networks [Akner *et al.*, 1992]

In addition to being a substrate for kinase action hsp90 also regulates several kinases. One of the earliest examples studied was hsp90's association with the transforming tyrosine kinase pp60^{v-src}. The association is believed to result in pp60^{v-src} being enzymatically inactive and its transport to the inner side of the plasma membrane where it dissociates from the hsp90 molecules and regains its kinase activity [Brugge, 1981, 1986; Schuh, 1985].

Hsp90 has been implicated in the regulation of protein synthesis through its interaction with the heme-sensitive kinase that is responsible for the phosphorylation of the eukaryotic peptide initiation factor 2 (eIF-2 α) [Rose *et al.*, 1989]. Increased phosphorylation of eIF-2 α leads to inhibition of protein synthesis due to the failure of guanine nucleotide exchange on the initiation factor [Farrell *et al.*, 1977; Rose *et al.*, 1989]. Hsp90 increases the enzymic activity of heme-sensitive eIF-2 α kinase only when hsp90 is phosphorylated. Dephosphorylation of hsp90 results in loss of the kinase activity which can be restored by addition of casein kinase II (CKII) [Rose *et al.*, 1989]. A conformational change in hsp90 is induced by either ATP or increased temperature [Csermely *et al.*, 1993]. This heat-induced conformational change of hsp90 may be a mechanism to control the redirection of translational machinery following stress (see discussion section). Phosphorylation of eIF-2 α occurs during heat shock resulting in reduced translation [De Benedetti & Baglioni, 1986].

CKII phosphorylates hsp90 *in vitro* [Lees-Miller & Anderson, 1989b]. Hsp90 dissociates aggregates of CKII and also increases the activity of CKII which plays a role in the signal transduction involved in cell cycle and growth regulation [Miyata & Yahara, 1992]. Relatively high concentrations of hsp90 are required for optimal CKII activity. An increase in hsp90 expression occurs

after stress and this may result in increased activity of CKII [Miyata & Yahara, 1992].

3:6 The role of heat shock proteins in thermotolerance

Thermotolerance could involve either increased resistance to heat-induced damage or an enhanced capacity to repair heat induced lesions (see figure 6) [Laszlo, 1992; Kampinga, 1993]. The role of heat shock proteins in thermotolerance has been suggested for some time. The idea is supported by kinetic studies of the synthesis and decay of hsps which showed the pattern to be the same as that for thermotolerance gain and loss [McAllister & Finkelstein, 1980; Li & Werb, 1982]. This of course is not direct evidence of a link and other groups have shown that hsp synthesis is not exclusively required for thermotolerance development [Przybytkowski *et al.*, 1986; Widelitz *et al.*, 1984]. There may be more than one mechanism involved in thermotolerance and there is some evidence for the idea that synthesis of hsps is only required at extreme temperatures [Lee & Dewey, 1988]. Perhaps at less extreme temperatures the hsps which exist in normal cells are sufficient to mediate thermotolerance [Weber, 1992].

The role of each group of hsps in stressed cells is only now beginning to be discovered. Hsp70 has been shown to play a critical role in thermotolerance. Although the biological processes protected are still unknown it seems likely that its function in protein folding and transport are involved [Parsell & Lindquist, 1993]. In yeast cells the requirement for hsp 90 is increased dramatically at elevated temperatures and its role in helping cells cope with stress is well observed [Parsell & Lindquist, 1993]. The small hsps have been implicated in microfilament protection [Lavoie *et al.*, 1993].

The level of nuclear-associated protein increases in hyperthermia treated cells and has been correlated with cell killing but the level of nuclear-associated

proteins is reduced if thermotolerance is established [Borrelli *et al.*, 1993]. The nuclear translocation of several hsps has been reported, including hsp90 (Chalmers submitted for publication), hsp70 [Velazquez & Lindquist, 1984]. The role of these proteins within the nucleus could be either to prevent the formation of or break up the nuclear-associated proteins.

4:1 Heat shock protein and steroid receptor interaction

Isolation of the steroid receptors led to the discovery of two forms of the receptors, an 8S form isolated from hormone free cells and a 4S form isolated from hormone treated cells [Sherman, 1984; Renoir *et al.*, 1984]. The discovery that molybdate stabilised the 8S receptor enabled the 8S form to be affinity purified [Dahmer *et al.*, 1984]. It was then revealed that the 8S form was, in fact, a combination of receptor plus a 90kDa protein dimer [Puri *et al.*, 1982; Renoir *et al.*, 1984]. The use of antibodies against this 90 kDa protein led to its identification as a common subunit in all 8S steroid receptors [Gasc *et al.*, 1984; Joab *et al.*, 1984] and its characterization as hsp90 [Schuh *et al.*, 1985; Catelli *et al.*, 1985; Sanchez *et al.*, 1987]. The 8S steroid receptor complex does not consist of the receptor and hsp90 alone but rather it is a large heteromeric complex of several different proteins [Pratt, 1990]. The different protein components of the 8S receptor will be discussed in the following section.

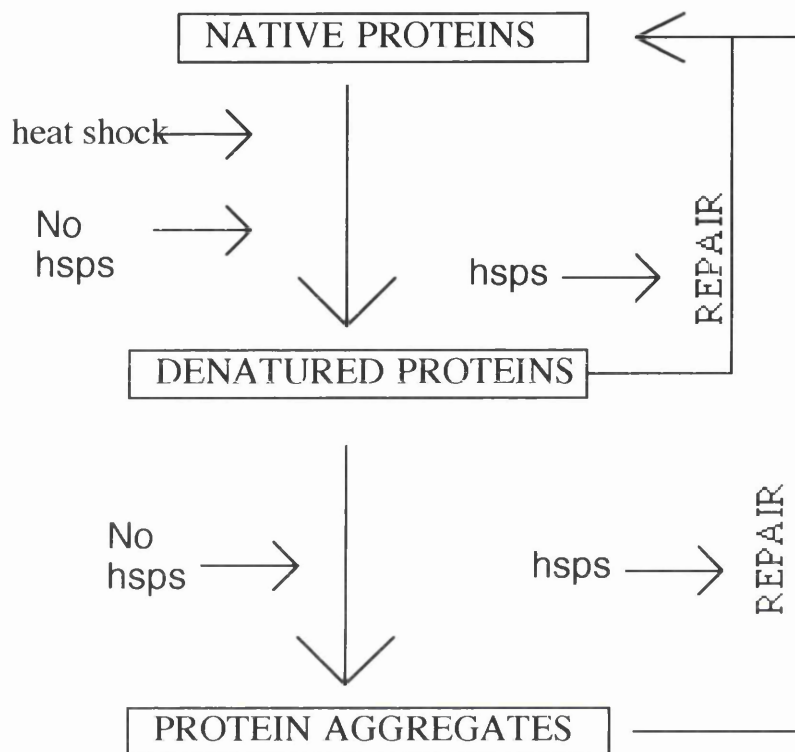


Figure 6:- Possible roles of hsp in thermotolerance
(from Kampinga, 1993)

4:2 Receptor-associated proteins

Hsp90

The discovery that the receptor in the 4S complex (the transformed receptor), which does not contain hsp90, can bind DNA while the receptor in the 8S complex cannot, led to the hypothesis that hsp90 is responsible for the inhibition of DNA binding by the 8S receptor [Baulieu, 1987]. Hsp90 interacts with the hormone-binding domain of the GR [Denis & Gustafsson, 1989; Dalman *et al.*, 1991], PR [Carson-Jurica *et al.*, 1989] ER [Chambraud *et al.*, 1990], and AR [Marivoet *et al.*, 1992]. In the ER, a region within the DNA-binding domain was found to be necessary for hsp90 binding [Chambraud *et al.*, 1990]. It has been suggested, that as a general rule, the attachment of hsp90 to the ligand binding domain is critical for the favorable positioning and interaction of hsp90 with the DNA binding domain of the receptor [Baulieu, 1991]. An acidic region (amino acids 232-266) of hsp90 is involved in the interaction [Tbarka *et al.*, 1993]. The C-terminal half has also been implicated [Sullivan & Toft, 1993]. An endogenous metal ion M^{2+} is believed to play a critical role in the interaction between steroid receptors and hsp90 [Sabbah *et al.*, 1987]. Molybdate may stabilize the untransformed receptor by interaction with the binding site for this metal ion [Meshinchi *et al.*, 1988].

A cell-free system was used to show that hsp90-PR complexes are in a steady state equilibrium ($t_{1/2}$, 5 min) and that ligand binding prevents assembly rather than causes dissociation of hsp90 [Smith, 1993]. This assembly/ disassembly was an hsp70 mediated event.

Hsp90 binding occurs at or near the termination of receptor translation which suggests a dual role for hsp90 as both a chaperone and regulator of steroid hormone action [Dalman *et al.*, 1989]. It has been shown *in vivo* that low levels of hsp90 reduce the level of hormone responsiveness [Picard *et al.*, 1990], supporting the idea that hsp90's association with hormone-free receptors is

required to maintain them in a form with high affinity for steroid. *In vitro* hsp90 is required for the high-affinity steroid-binding of the GR and MR [Nemoto *et al.* , 1993; Bresnick *et al.*, 1989], although this does not seem to be the case for the AR [Nemoto *et al.*, 1992; Ohara-Nemoto *et al.*, 1991].

Hsp70

Several reports have identified hsp70 as part of the untransformed receptor complex [Kost *et al.*, 1989; Smith *et al.*, 1990; Sanchez *et al.*, 1990]. In other cases no such association was found [Bresnick, 1990; Sanchez *et al.*, 1990; Alexis *et al.* , 1992]. It remains unclear why hsp70 is associated with steroid receptors in some instances and not others and it was even thought that hsp70 might be a contaminant of the immunopurification procedure [Rexin *et al.* , 1988]. In one case mouse GR overexpressed in chinese hamster ovary cells associated with hsp70 and was found in the nucleus whereas the endogenous GR in L cells (fibroblast) was not associated with hsp70 nor present in the nucleus [Sanchez *et al.*, 1990]. There is obviously some difference in the GR in the two cell types. The authors suggested that the overexpressed GR in the chinese hamster ovary cells acts like PR in that it is found in a loosely-bound docking complex in the nucleus and that hsp70 binding is a result of its nuclear localization. Hsp70 may play a role in maintaining steroid receptors in an unfolded state for passage across the nuclear membrane. The role of hsp70 in the translocation of proteins across membranes is well documented [Rothman, 1989]. It has also been suggested that recovery of receptors with hsp70 is related to their cellular location. Cytosolic GR seems to be hsp70 free whereas nuclear GR is found associated with hsp70 [Pratt, 1993c].

Hsp70 has been found associated with both the 8S and the 4S forms of the receptor [Rexin *et al.* , 1988; Onate *et al.*, 1991]. Hsp70 was not found to be involved in PR binding to DNA, either directly or indirectly but hsp70 may influence the DNA-binding of GR in an *in vitro* system [Srinivasan *et al.* ,

1994]. ATP mediated dissociation of hsp70 was demonstrated in either receptor form and this was taken to suggest that hsp70's role in the receptor complex may be an early event in receptor folding and recycling [Onate *et al.*, 1991]. Cell-free experiments using reticulocyte lysate have shown that assembly of hsp90-free PR into the complete 8S complex containing both hsp90 and hsp70 is temperature- and ATP dependent, and requires the PR to be in a hormone free state. The presence of hsp70 was paramount [Smith *et al.*, 1990, 1992]. These observations have led to a model for the assembly of steroid receptor complexes which will be discussed in section 4:4.

Hsp56

Hsp56 was first isolated in association with the 8S steroid receptor when antibodies to the 8S form of PR were developed and subsequently it was found to be a component of the PR, ER, AR and GR 8S receptor complex [Tai *et al.*, 1986; Sanchez *et al.*, 1990a].

Hsp56 does not bind directly to the steroid receptor but rather it interacts with hsp90 [Renoir *et al.*, 1990]. A cytosolic complex comprising of hsp90, hsp70, hsp56 and several other proteins has been isolated [Perdew & Whitelaw, 1991]. This complex may have a general chaperone function or perhaps the proteins are arranged in a specific orientation to enable specific tasks to be carried out [Tai *et al.*, 1993].

Other receptor-associated proteins

p23 is a phosphoprotein that has been isolated both with hsp90 and the 8S PR and GR [Johnson *et al.*, 1994]. The function of this protein is unknown but it is thought that p23-associated hsp90 may define a subpopulation of hsp90 with a specific role to play, perhaps in steroid receptor folding [Johnson *et al.*, 1994]. An hsp27 has been immunopurified with ER [Mendelsohn *et al.*, 1991]. This protein could be identical to p23.

The association of pp60^{v-src} with hsp90 and a 50 kDa protein, p50, has been extensively demonstrated [Brugge & Erikson, 1981; Brugge, 1986]. The same 50 kDa protein has been identified in a heterocomplex with hsp90 [Perdew & Whitelaw, 1991; Whitelaw, 1991]. At present, it is unclear whether this p50 protein is the same as that isolated with the chick PR complex [Smith *et al.*, 1990].

4:3 Stoichiometry

Most groups investigating the stoichiometry of the 8S steroid receptors have found a hsp90:SR ratio of 2:1 [Renoir *et al.*, 1984; Mendel & Orti, 1988; Denis *et al.*, 1987; Radanyi *et al.*, 1989]. These figures were obtained using either GR or PR. The only work of this type carried out on the ER suggested a ratio of 2:2 in that a dimer of ER bound to a dimer of hsp90 [Redeuilh *et al.*, 1987]. If the 8S ER does exist as a dimer, prior to hormone binding and hsp90 dissociation, as suggested [Sabbah *et al.*, 1989], it is difficult to explain how a problem of steric hindrance is overcome since it has been shown that both dimerization and hsp90 binding occur within the hormone binding domain. Sequence analysis has shown that the dimerization and hsp90 binding regions do not overlap but they could still be too close together to allow both activities at once [Schlatter *et al.*, 1992]. Another possible argument against the ER stoichiometry of 2:2 suggested by Redeuilh is the evolutionary conservation of the steroid receptor family [Evans, 1988], which would suggest that if they bind the same protein they should do so using similar protein-protein interactions.

The other proteins in the complex are believed to be in monomeric form [Smith *et al.*, 1990a]. This arrangement of one molecule of receptor, two of hsp90 and one each of hsp70, hsp56, p50 and p23 would mean the 8S receptor complex has a molecular weight of 400-450 kDa, which is larger than the 320-350 kDa originally suggested [Sherman & Stevens, 1984]. The association of several of

the proteins may only be a transient event. One solution is that a common heterocomplex core unit may exist with a basic stoichiometry of one steroid-binding protein, two hsp90 and one hsp56.

4:4 Assembly of the heterocomplex

Models for the assembly of receptors into heterocomplex structures have been postulated by two groups [Smith *et al.*, 1992; Smith, 1993; Pratt, 1993b]. The basic ideas of the two models are principally the same and have been combined in this section and figure 7. The model is supported by evidence described in the previous sections.

Stage 1 involves either the newly synthesised receptor molecule or the ligand free 4S receptor becoming associated with the preformed hsp90-hsp70-hsp56-p27 complex [Perdew, 1991]. This event requires hsp70 and ATP and may involve the partial unfolding of the receptor by hsp70 [Onate *et al.*, 1991; Smith *et al.*, 1990, 1992]. The complex is now able to bind ligand but is unable to bind DNA.

It is not clear whether hsp70 now dissociates from the complex as is indicated by the absence of hsp 70 from some purified complexes (4:1, hsp70).

Stage 2 is stimulated by hormone binding. A conformational change occurs within the complex and the hsp90 dimer and its associated proteins dissociate from the hormone-bound receptor, to reveal the DNA binding domain. Prior to DNA binding receptor dimerization occurs [DeMarzo *et al.*, 1991].

Stage 3 occurs after transcriptional activation has occurred. The ligand dissociates from the 4S receptor and is unable to reassociate. The receptor may then diffuse out of the nucleus and can re-enter the cycle at stage 1.

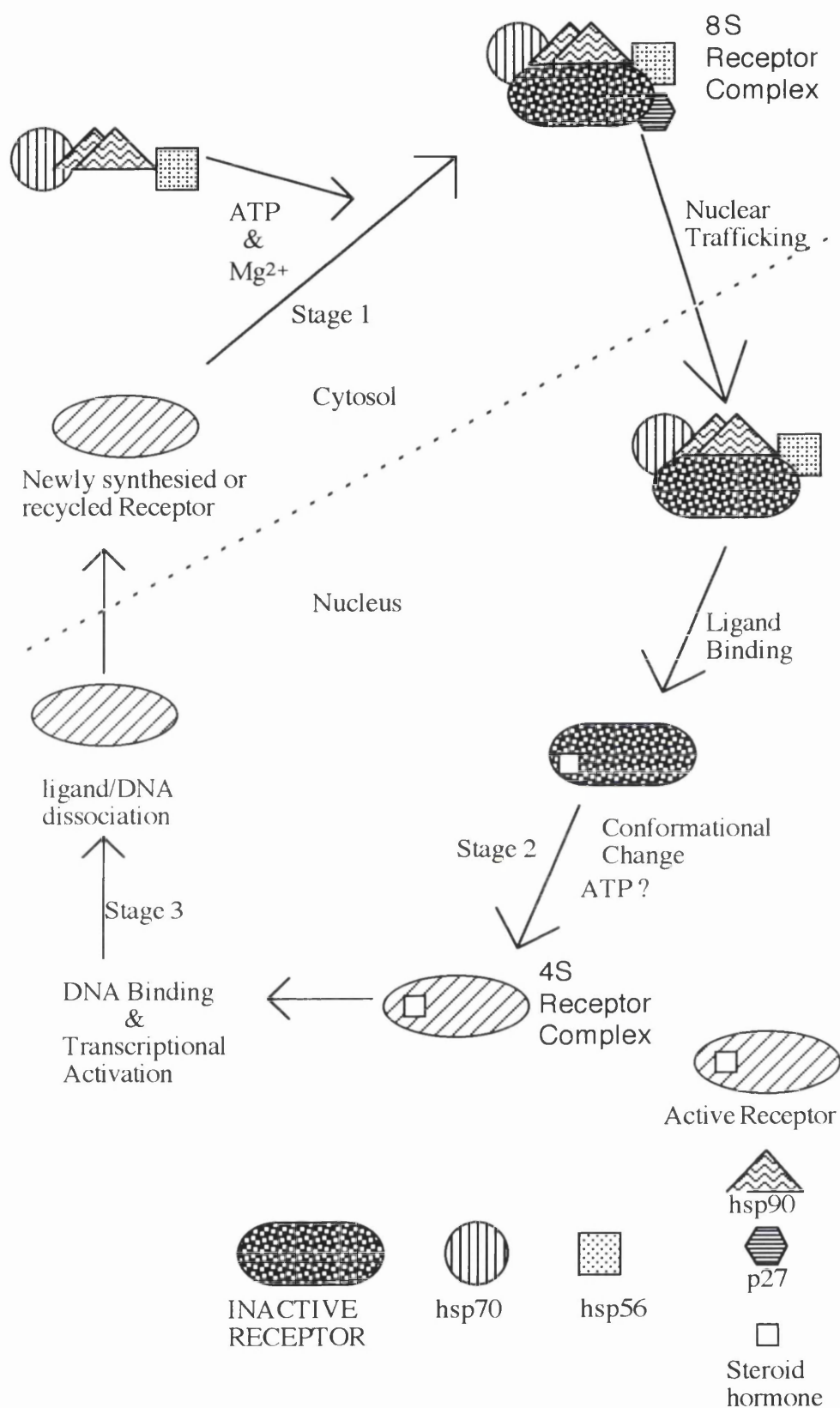


Figure 7:- Steroid receptor assembly and recycling.
See text for details

If the assembly/disassembly concept [Smith, 1993] proves to be correct it would most likely occur at the end of stage 1 due to the availability of the reassembly apparatus

Between stage 1 and stage 2 nuclear translocation occurs in the case of ER, AR, and PR [King & Greene, 1984; Husmann *et al.*, 1990] but nuclear translocation does not happen until after stage 2 in the case of GR and MR [Wikstrom *et al.*, 1987; Agarwal, 1994].

4:5 Role of heat shock proteins in translocation and docking

The first indications that hsps play a role in steroid receptor trafficking came from observations that hsp90 binds to pp60^{v-src} (a member of the SR superfamily) at the site of its synthesis and remains bound while it moves to the cell membrane [Brugge, 1986]. It is believed that hsp90 plays a role in the formation of a transportosome which transports the receptor along the cytoskeletal system [Pratt, 1992, 1993b]. It has long been known that hsp90 associates with actin and tubulin structures and it has been suggested that this interaction plays a role in the docking of the receptor complex to the cytoskeletal complex [Miyata & Yahara 1992]. Recently hsp90 has been suggested to play a role in the nuclear-cytoplasmic shuttling of steroid receptors [Kang *et al.*, 1994]. Hsp56 has been isolated with the motor protein dynein and therefore could play a role in movement towards the nucleus

The different cellular locations of receptors may only reflect the end of the transportation highway at which they dock [Pratt, 1990]. For example at the cytosol end in the case of GR in L cells and at the nuclear end in the case of overexpressed GR [Sanchez, 1990b]. The signals which decide where each receptor complex is docked are not known nor is the mechanism for docking and transportation of receptors fully understood.

5. Aims of this thesis

The administration of sub-lethal temperatures to cells in culture invokes a condition known as thermotolerance, where the cells are able to withstand normally lethal temperatures due to this pre-exposure to an elevated but sublethal temperature. The cells responds to this increased temperature by synthesizing a group of proteins known as the heat shock proteins, one of which is hsp90. In unstressed cells hsp90 plays an important role in several cell processes, including the regulation of steroid receptors.

Administration of heat shock in conjunction with anti-androgen therapy results in a more effective cell kill than either therapy alone [Lloyd *et al.*, 1992]. What is the mechanism behind this phenomenon ? The aims of this thesis was to look at the molecular effect of heat on androgen receptor function and on anti-androgen action.

Chapter 2

MATERIALS & METHODOLOGY

Suppliers

Fine Chemicals

Chemicals were of 'Ana1aR' grade or equivalent and were obtained from Fisons apart from the following:

bovine serum albumin (fraction V), cyanogen bromide-activated Sepharose 4B, Dextran, diphenylamine, dithiothreitol, α -monothioglycerol, sodium molybdate, TEMED, triethanolamine, Tween-20 and urea which were purchased from Sigma Chemical Co. Ltd

Hormones and antiandrogens

1-dehydrotestosterone	Sigma Chemical Co. Ltd
hydroxyflutamide	Schering-Plough
mibolerone	NEN DUPONT
testosterone	Sigma Chemical Co. Ltd
triamcinolone acetonide	Sigma Chemical Co. Ltd

Antibodies

Anti-hsp90 (IgG)	AC88	Stress Gen Biotechnologies
Anti-hsp90 (IgM)	3G3	Affinity Bioreagents
Anti-AR (IgG)	AN1-15	Affinity Bioreagents
Goat anti-mouse IgG		Pierce
Anti-sheep/goat (IgG) peroxidase-linked		Scottish Antibody Production Unit
Anti-mouse Ig peroxidase-linked		Amersham
Anti-rat Ig peroxidase-linked		Amersham

Cell culture

All cell culture media and supplements were obtained from Gibco with the exception of phenol red-free medium and methionine-free medium which were both obtained from Sigma.

All plasticware used in cell culture was purchased from Nunc except for LNCaP culture flasks which were obtained from Bibby

Radiochemicals

Albumin (bovine serum), [methyl- ¹⁴ C]	NEN DUPONT
Globulins, [methyl- ¹⁴ C]	NEN DUPONT
Lactoglobulin A, [methyl- ¹⁴ C]	NEN DUPONT
L-[³⁵ S]methionine	Amersham
Mibolerone, [17 α -methyl- ³ H]	NEN DUPONT

Miscellaneous Materials

Calibration proteins for SDS-PAGE	Boehringer Mannheim
DNA (calf thymus) type XV	Sigma Chemical Co. Ltd
ECL (western blotting detection kit)	Amersham
Hoechst no 33258	Sigma Chemical Co. Ltd
Prestained SDS-PAGE standards	Bio-Rad
Protein assay dye reagent concentrate	Bio-Rad
RibonucleaseA	Sigma Chemical Co. Ltd
Scintillation fluid (Ultima-Flo)	Packard

Suppliers for any other specific materials or apparatus are indicated throughout the text.

1. Buffers and Solutions

1.1 BUFFERS

1.1.1. Phosphate Buffered Saline (PBS) pH 7.2

170 mM NaCl; 1 mM Na₂HPO₄; 2 mM KH₂PO₄.

1.1.2. ETN Buffer pH 7.0

10 mM EDTA; 10 mM Tris-HCl ; 100 mM NaCl.

1.1.3. HE Buffer pH 7.4

20 mM HEPES; 1.5 mM EDTA; 10% Glycerol (v/v).

1.1.4. HED Buffer pH 7.4

HE Buffer plus 0.25 mM Dithiothreitol (DTT)

Make fresh daily.

1.1.5. Low Salt Homogenate Buffer pH 7.4

10 mM Na₂HPO₄; 1.5 mM EDTA; 12 mM α-Thioglycerol;

20 mM Na₂ MO₄; 0.25 mM Leupeptin; 10% Glycerol (v/v).

1.1.6. High Salt Homogenate Buffer pH 7.4

40 mM Tris-HCl; 1.5 mM EDTA; 0.5 M NaCl; 10 mM DTT;

0.25 mM Leupeptin; 10% Glycerol (v/v).

1.1.7. Gradient Buffer pH 7.4

10 mM Na₂HPO₄; 1.5 mM EDTA; 12 mM α-Thioglycerol;

10% Glycerol (v/v).

1.1.8. Extraction Buffer pH 8.5

As High Salt Homogenate Buffer, except pH 8.5

1.1.9. Hank's Modified Buffer pH 7.2-7.4

1.3 mM CaCl_2 ; 5.4 mM KCl ; 0.5 mM MgCl_2 ; 0.5 mM MgSO_4 ;
137 mM NaCl ; 4 mM NaHCO_3 ; 0.4 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$.

1.1.10. HEPES Buffered Saline pH 7.4 (HBS)

10mM HEPES, 150mM NaCl

1.2. CELL CULTURE SOLUTIONS.

1.2.1. Routine Sub-Culture Medium.

RPMI 1640 medium with L-Glutamine; 10% (v/v) Fetal Calf Serum (FCS); 60 mg/ml Tylocine; 100 units/ml Penicillin; 50 mg/ml Streptomycin.

1.2.2 Dextran Coated Charcoal (DCC)

0.5% sieved, prewashed charcoal (w/v); 0.005% Dextran (w/v);
suspended in HE buffer (see 1.1.3), stirred continuously for 30
minutes at room temperature.

1.2.3. Dialysed Heat- Inactivated Dextran-Coated Charcoal Stripped Serum (DHIDCCFCS).

100ml of FCS were dialysed against 1L Hank's modified buffer for 48 hours at 4°C, changing buffer 4 times. Heat inactivation was carried out by incubating dialysed FCS for 45 minutes at 56°C. A pellet of dextran-coated charcoal (derived from 12.5ml of DCC) was added and

the mixture was stirred for 30 minutes at 4°C then centrifuged at 10 000g for 30 minutes at 4°C. Finally the supernatant was filter-sterilised through 0.2 micron filters. This was tested ensure that it contained less than 10^{-12} M of androgen, estrogen and progesterone.

1.2.4. Steroid-Free Medium

RPMI 1640 with L-Glutamate, without Phenol Red and Sodium Bicarbonate; 5% (v/v) DHICCFCS; 60 mg/ml Tylocine; 100 units/ml Penicillin; 50 mg/ml Streptomycin.

1.2.5. Methionine-Free Medium

RPMI without L-Glutamine, L-Leucine, L-Lysine, L-Methionine and Sodium Bicarbonate; 0.3 g/l L-Glutamine; 0.05 g/l L-Leucine; 0.04 g/l L-Lysine.HCl; 60 mg/ml Tylocine; 100 units/ml Penicillin; 50 mg/ml Streptomycin.

1.2.6 Cell Culture Freezing Medium

RPMI 1640 medium with L-Glutamine; 10% FCS (v/v); 10% Dimethyl Sulphoxide (v/v).

1.2.7 Versene

125 mM NaCl; 2.7 mM KCl; 6.3 mM Na_2HPO_4 3.2mM KH_2HPO_4 ; 0.5 mM EDTA

1.3 SDS PAGE SOLUTIONS

1.3.1. 30% Acrylamide Stock

29.2% Acrylamide (w/v); 0.8% Bis-Acrylamide (w/v).

1.3.2. Sample Buffer

62 mM Tris-HCl (pH 6.8); 10% Sucrose (w/v); 5% 2-mercaptoethanol (v/v); 2% SDS (w/v); 3 M Urea; 0.02% Bromophenol Blue (w/v).

1.3.3 Stacking Buffer

0.125 M Tris-HCl (pH6.8); 0.4% SDS (w/v).

1.3.4. Resolving Buffer

0.375 M Tris-HCl (pH8.8); 0.4% SDS (w/v).

1.3.5. Electrophoresis Tank buffer

25 mM Tris-HCl; 192 mM Glycine; 0.01% SDS (w/v).

1.3.6. Tris buffered saline with tween (TBST)

10 mM Tris-HCl (pH8.2); 150 mM NaCl; 0.05% Tween20 (v/v).

1.3.7. Transblot Buffer pH 8.2

25 mM Tris-HCl; 192 mM Glycine; 20% methanol (v/v)

1.4. SILVER STAINING SOLUTIONS

1.4.1. Solution 1

50% Methanol (v/v); 10% Acetic acid (v/v).

1.4.2. Solution 2

5% Methanol (v/v); 7% Acetic acid (v/v).

1.4.3. Solution 3

10% Glut~~ar~~aldehyde (v/v).

1.4.4. Solution 4

5 μ g/ml DTT.

1.4.5. Solution 5

0.1% Ag₂NO₃ (w/v).

1.4.6. Solution 6

3% (w/v) Na₂CO₃ ; 0.0185% Formaldehyde (v/v).

1.4.7 Solution 7

2.3 M Citric acid.

1.5 COOMASSIE BLUE STAINING SOLUTIONS

1.5.1. Coomassie stain

0.1% (v/v) Coomassie brilliant blue R250; 50% (v/v) Methanol;

10% (v/v) Acetic acid.

1.5.2. Coomassie destain

10% Methanol (v/v); 10% (v/v) Acetic acid.

1.6. CNBr-SEPHAROSE IMMUNOPRECIPITATION
SOLUTIONS

1.6.1. Coupling Buffer

0.1 M NaHCO₃; 0.5 M NaCl.

1.6.2. HENG Buffer

25 mM HEPES; 1 mM EDTA; 0.02% NaN₃; 10% Glycerol (v/v).

1.6.3. DMP Solution

20 mM Dimethyl pimelimidate dihydrochloride (DMP) dissolved in
0.2 M Triethanolamine.

1.6.4. Acetate Buffer pH 4.0

0.1M NaAc; 0.5M NaCl

2. CELL CULTURE METHODS

2.1. CELL LINES

Three prostate carcinoma cell lines were used: LNCaP, DU145 and PC3. The LNCaP and DU145 cell lines were a gift from Dr. C. Eaton, Tenovus Institute for Cancer Research, University of Wales College of Medicine, Cardiff. The PC3 cell line was purchased from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, England.

LNCaP cells

LNCaP was isolated and established from a metastatic lesion of human prostatic adenocarcinoma [Horoszwicz *et al.*, 1983]. Prostate-specific acid phosphatase is present in these cells, as is prostatic antigen (PSA), supporting the theory that LNCaP cells originated from prostate tissue [Horoszwicz *et al.*, 1983].

LNCaP contains androgen receptors and shows hormone-responsive growth. Not only androgens but also progesterone and estradiol stimulate LNCaP cell growth. The estradiol-induced increase in LNCaP cell proliferation has been ascribed to the presence of a point-mutated form of the androgen receptor, which possessed an increased affinity for estrogen [Veldscholte *et al.*, 1990]. However recent work using radioligand binding assay, reverse transcriptase-PCR and immunocytochemical studies has shown the presence of normal estrogen receptors in this cell line [Carruba, 1994].

Epidermal growth factor (EGF) receptor is upregulated in androgen-stimulated LNCaP cells which results in the cells having greater sensitivity to EGF [Schuermans *et al.*, 1988b].

LNCaP cells form monolayers which are only loosely attached to the surface of plastic culture dishes and so great care is required when handling this line as the cells are very easily dislodged by tapping, shaking or pipetting. It is not

uncommon to lose 10-20% of cells during media changes if care is not taken. LNCap cells which have been trypsinized tend to form aggregates which are difficult to dissociate and prolonged trypsinization lowers the anchoring potential of the cells [Horoszwicz et al., 1983 and personal observation].

DU145 cells

The DU145 cell line was derived from a brain lesion of metastatic prostatic carcinoma [Stone *et al.*, 1978]. DU145 cells are not growth stimulated by androgens (see results figure 9), although Scatchard plot analysis of ligand binding has indicated that significant levels of androgen receptors are present within these cells and it has been suggested that these cells do show a metabolic response to androgens [Carruba 1994]. The prostate-specific acid phosphatase has been detected in DU145 cells, albeit at low levels [Stone *et al.*, 1978].

PC3 cell line

This cell line was established from a bone metastasis of a human prostatic carcinoma and has the characteristics of a poorly-differentiated adenocarcinoma [Kaighn, 1979]. Prostate-specific phosphatase levels in these cells are similar to those obtained from normal prostatic epithelial cultures [Kaighn, 1979]. This cell line is hormone insensitive (see results figure 9). Scatchard plot analysis of ligand binding has shown no or only minimal levels of androgen receptor present in PC 3 cells [Carruba, 1994].

2.2.1. ROUTINE GROWTH AND SUBCULTURE.

All cell lines were routinely grown at 37°C in atmospheric air enriched with 5% CO₂. All manipulations were carried out in a laminar air flow cabinet using aseptic techniques. 180 cm² plastic cell culture flasks were used for routine growth. These required approximately 30ml of medium per flask. The size of flasks or plates used for experimental work depended on the number of cells required. These included 75 cm² flasks requiring 15 ml of medium, 180 cm² flasks requiring 30 ml of medium, 6 well tissue culture plates (each well has a surface area of 12.5 cm²) requiring 2 ml/well. All routine and experimental media were changed every 48-72 hours.

2.2.2. SUBCULTURE TECHNIQUE.

Cells were passaged when cultures occupied 80-90% of the surface area of the culture dish. The medium was removed and the cells washed twice with 20 ml PBS at 37°C. 0.05% trypsin/versene solution (pre-warmed to 37°C) was added to the culture flasks; 0.5 ml per well of a 6 well plate, 4 ml per 75 cm² flask and 8ml per 180 cm² flask. The cells were incubated at 37°C in the trypsin solution for either 4 minutes for DU145 and PC3 cells or 2 minutes for LNCaP cells. The trypsinization reaction was stopped by adding 2 volumes of fresh medium (37°C) followed by rapid pipetting to ensure an even cell suspension. The cell suspension was then dispensed into 3-5 sterile culture flasks to which growth medium had been added before a final rapid pipetting ensured even distribution of the cells.

Seeding of experimental plates and flasks was carried out using the technique described above with the following differences. After the trypsinization was stopped, the cell number per ml was counted using a hemocytometer and the

suspension was diluted to the required concentration. Routine medium was used at this stage as LNCaP cells do not seed in serum-free medium [Schuurmans *et al.*, 1988b] or in DHIDCCFCS supplemented medium (S. Thomas, personal communication). After 24-48 hours the routine medium was removed and the cells washed twice with pre-warmed PBS before the experimental medium was added (see individual experiments for details of experimental media and supplements added).

2.2.3. CRYOPRESERVATION OF CELL LINES

To ensure that all experiments could be carried out on cells of a similar passage number and to protect against loss of a cell line due to contamination, frozen stocks of cells were prepared. One 180 cm² flask of cells which were 60-70% confluent was trypsinized as in section 2.2.2. and the reaction was stopped by adding 10 ml of routine medium. The cell suspension was transferred to a sterile universal container. The cells were pelleted by centrifugation at 1000g for 2 minutes at 4°C and the supernatant removed. The cells were resuspended in 4ml of freezing medium and 1ml volumes were transferred to sterile Biofreeze vials prior to being frozen at -70°C. They were stored under liquid nitrogen . To grow cells from frozen stocks the vials were thawed by placing in a 37°C waterbath for 5-10 minutes. The vials were removed from the waterbath before all the ice had melted, and the cell suspension was transferred to a universal container to which 10 ml of routine medium (37°C) had been added. This was then centrifuged for 2 minutes at 1000g before discarding the supernatant and resuspending the cells in medium at 37°C. The cell suspension was placed in a 75 cm² flask and incubated at 37°C, as described in section 2.2.1.

2.2.4. CELL LYSIS

The cell membrane must be ruptured before many of the assays and procedures could be carried out. This was achieved in two different ways depending on the procedure for which the sample was required .

SDS lysis

In the first method the cells were trypsinised as in section 2.2.2. and 10ml of routine medium was added. The cell suspension was transferred to a sterile universal container. This was then centrifuged for 2 minutes at 1000g before discarding the supernatant and resuspending the pellet in 0.2% SDS in ETN buffer (section 1.1.2.) followed by incubation at 37°C for 30 minutes. This technique solubilises the DNA and denatures proteins, as well as solubilising the membranes.

Freeze/thaw lysis

If the proteins were required in the native form or the use of SDS is contraindicated in the procedure to be applied, a method involving freeze/thawing of the cells was applied to lyse the membranes. The pelleted cells were resuspended in 1ml of either PBS, HED or low salt homogenate buffer depending on the procedure to be carried out. The cell suspension was then transferred to an Eppendorf tube which was closed and sealed with a strip of Nescofilm and then floated in a bath of liquid nitrogen for 10 minutes. The tubes were then carefully removed and placed at 37°C for 10 minutes or until the samples were totally thawed. This freeze/thawing procedure was repeated 3 times and a sample was examined under the microscope to ensure cell lysis had occurred.

2.3. MYCOPLASMA TESTING OF CELL LINES

Mycoplasmas are a common and serious contamination of cell culture which cannot be detected with the naked eye [Russel, 1975]. They cause changes in metabolism, growth and viability which lead to variability of results. Routine mycoplasma testing is therefore important to ensure early detection and treatment of any infection. An immunoassay mycoplasma detection kit (Boehringer Mannheim) was used for detection. The cells were incubated in antibiotic- and anti-PPLO-free medium for 72 hours prior to the immunoassay being carried out to increase the sensitivity of the assay. Firstly, microtiter plates were coated with antibodies to one of the four commonest mycoplasma strains (*Mycoplasma arginini*, *M.hyorhinis*, *A.laidawii*, *M. orale*) by incubating plates for 2 hours at 37°C in coating solution. Nonspecific binding sites were blocked and the plates washed before cell samples and controls were added and incubated overnight at 4°C. Detection was carried out by incubating plates for 2 hours at 37°C with biotin-conjugated antibodies against the mycoplasma strains mentioned above, followed by a 1 hour incubation with streptavidin-AP and a final 1 hour incubation with 4-nitro-phenylphosphate. Evaluation was carried out visually, a mycoplasma positive plate being yellow in colour whereas a mycoplasma negative plate is colourless.

This procedure was carried out simultaneously on all the cell lines used in the laboratory every three months. If any cell lines were infected all the growing cells were destroyed as was any opened medium, supplement, buffer or plastic ware. The cell culture unit and incubators were cleaned with Decon and 70% ethanol prior to fresh frozen stocks (frozen down prior to the last totally negative mycoplasma testing) being defrosted and seeded down.

To help prevent mycoplasma infection, all medium was supplemented with anti PPLO

3. HOECHST DNA ASSAY

3.1. DNA STANDARDS

DNA standards were prepared from a 100 μ g/ml solution of calf thymus DNA dissolved in ETN buffer. The standards used were 0, 10, 20, 30, 40, 50 and 60 μ g/ml DNA. The concentrations of these solution were verified by measuring their absorbances at 260nm as 1 unit of absorbance at this wavelength corresponds to 50 μ g/ml DNA. The standards were stored for up to a month at -20°C.

3.2. ASSAY PROTOCOL

The assay methodology was modified from that of Leake and Habib [Leake & Habib, 1987] and involves the intercalation of Hoechst 33258 with solubilised DNA resulting in a complex which fluoresces with a maximum emission at 450nm.

Cell culture monolayers were harvested and the DNA was solubilised using 0.2% SDS in ETN buffer as described in section 2.2.4.

100 μ l aliquots of each standard and sample (and 1:10 and 1:100 dilutions of samples) were transferred to RT-30 tubes and 3ml of ETN buffer containing Hoechst 33258 (100ng/ml) and RNase (5.0 μ g/ml) were added. After vortexing the tubes were incubated in the dark for 30 minutes at room temperature. The fluorescence enhancement at 450nm was measured using an Hitachi Perkin-Elmer MPF-2A fluorescent spectrophotometer with an excitation wavelength of 360nm and both slit widths at 5mm.

The fluorescence enhancement of each standard was then plotted against the DNA concentrations of the standards to create a calibration curve. The concentrations of DNA present in the samples were extrapolated from the calibration curve.

4. BRADFORD PROTEIN ASSAY

4.1. Protein standards

Bovine serum albumin (BSA) was used to create protein standard solutions ranging from 0-500 μ g/ml. This was achieved by diluting a stock of 1mg/ml BSA with PBS or the same buffer as the samples to create standards of 0, 10, 20, 50, 100, 200 and 500 μ g/ml protein content. The standards were stored at 4°C for up to two weeks.

4.2. ASSAY PROTOCOL

This assay is based on the principle that there is a shift in maximum absorbance from 465nm to 595 nm when protein binds to a solution of Coomassie Brilliant Blue G-250 [Bradford, 1976]. Cells were lysed using the freeze thaw method (section 2.2.4.) as the detergent SDS is incompatible with this assay. Dilutions of the samples were made (1:10 and 1:100). Biorad Bradford reagent was prepared by diluting 1:6 and filtering through Whatman filter paper No. 1. The assay was carried out by placing 0.8ml of each sample and standard into RT-30 tubes in duplicate and adding 0.2ml of Bradford reagent. These were mixed gently and incubated at room temperature for 30 minutes before the absorbance was read at 595nm using a spectrophotometer (LKB ultrospec 4050). The absorbance of each standard was then plotted against the protein concentration of the standards to create a calibration curve, which was used to calculate the protein content of each of the samples.

5. STEROID RECEPTOR LIGAND BINDING ASSAY

These assays can determine the total specific steroid receptors present in a variety of tissues and cells [Leake, 1981]. The principle used is based on the calculations of Scatchard [Scatchard, 1949].

5.1. Preparation of radioactive steroid solutions for androgen receptor assay

The concentration range of radiolabeled steroid ([17 α -methyl-³H] mibolerone) used was from 0.1-5nM. This enables the detection of type I and type II binding sites [Eriksson, 1978; Markaverich & Clark, 1979; Castagnetta *et al.*, 1992]. Determination of nonspecific binding was achieved by adding 100-fold excess of unlabeled mibolerone to duplicates of the three highest radiolabeled concentrations. Unlabelled steroid must be added first to these vials and the solvent evaporated using compressed nitrogen. To hamper the mibolerone binding to glucocorticoid receptor triamcinolone acetate (1×10^{-7} M) was added to all the vials and the solvent evaporated using compressed nitrogen. The appropriate volume of ethanol containing [17 α -methyl-³H] mibolerone was then added to each vial before equalising the alcohol content by adding ethanol alone. HED buffer was added in order to bring the final volume of all solutions to 2.5ml. These were stored at 4°C for up to two months.

5.2. Assay protocol

Approximately 20×10^6 cells were allowed to "plate down" for 24hr in routine conditions to provide a 50% confluent monolayer in a 175cm² plastic tissue culture flask. The cells were then harvested by trypsinisation as described in section 2.2.2. The cells were resuspended in 4ml of HED buffer (section 1.1.4.). Cell lysis was carried out by the freeze/thaw method described in section 2.2.4. and the cell extracts were centrifuged at 800g for 5 minutes at

4°C to separate the cytosol (supernatant) from the nuclear pellet fraction. The supernatant was further centrifuged at 2000g for 15 minutes at 4°C to pellet the cell debris. The nuclear pellet was washed three times in ice-cold HBS and resuspended in 4 ml of ice cold HBS. 50 µl of each of the radiolabeled steroid standards was added to 150 µl of each cell fraction and these were incubated for 18 hours at 4°C. Free ligand was removed from the soluble fraction by incubation with 200 µl DCC (final concentration 0.25% (w/v)) for 15 minutes on ice with occasional mixing, before centrifuging at 1000g for 5 minutes at 4°C. 200µl of each supernatant was then transferred to scintillation vials and 4 ml of scintillation fluid added.

To remove free ligand from the nuclear fraction 100 µl was added to 5 ml of ice-cold HBS immediately prior to pouring onto a prewetted Whatman GF/C filter held in a Millipore filter apparatus under vacuum. The tube which had contained the saline plus nuclear suspension was then washed out with 5 ml of ice-cold HBS which was poured onto the filter. The chimney apparatus was washed with a further three volumes ice-cold HBS prior to removal and the edges of the filter were also washed before the filters were placed in scintillation vials and 4 ml scintillation fluid added.

50 µl of each standard was also added to a set of scintillation vials and 4 ml scintillation fluid (Ultima-Flo) added, to establish total cpm added to samples. All the vials were counted for tritium using a scintillation counter (LKB 1209 Rackbeta counter).and analysed as described below.

5.3. Analysis of receptor data

The ligand concentrations used (0.1-5nM) allow definition of an experimental window which covers both type I (high affinity, low number/ cell) and type II (lower affinity, greater number/ cell) [Clark & Peck, 1979; Leake *et al.*,1981; Castagnetta *et al.*,1989].

Receptor assay data was processed using Scatchard analysis and a modification of a regression analysis best fit routine (Rosenthal-Feldman method for the resolution of curved Scatchard plots[Rosenthal, 1967]) [Leake *et al.*, 1987] (Oncolog2.2®), run on an IBM-PC, yielding both dissociation constant (Kd) and concentration values (fmol/ml homogenate) for each class of binding site. Receptor concentration was expressed as fmol/mg DNA. Data was analysed using a model for two binding sites to assess whether there was one or two ligand binding sites (Carruba, 1994).

6. APPLICATION OF HEAT SHOCK TO CELL MONOLAYERS

Cells were subcultured as in section 2.2.2. allowed to "plate down" for 24hr in routine conditions (section 2.2.1.) so that a 20% confluent monolayer was formed. The number of cells and the type of flask used varied depending on the experimental conditions. The medium was changed either to experimental or routine medium depending on the experiment.

Heat shock was achieved in the early experiments using a thermostatically controlled water bath [Lloyd *et al.*, 1992]. Tissue culture flasks were placed within a glass trough containing a layer of glycerol 1mm deep which was floated in the water bath; multi-well plates were sealed water tight (using Nescofilm) and allowed to float on the surface of the water. The efficiency of heat transfer from the water to the cells was assessed by measuring the temperature inside vessels containing only culture medium.

After optimum heat shock temperatures were established, an humid incubator with atmospheric air enriched with 5% CO₂ (LEEC) was used. Again the transfer of heat to the cells was measured and a direct comparison was made between the two methods to ensure that there was similar heat transfer and effect.

7. SUCROSE DENSITY GRADIENT CENTRIFUGATION

Sucrose density gradient analysis (SDGA) was originally used as a method for determining the molecular nature of proteins [Martin & Ames, 1961].

Sedimentation of a protein through a gradient depends on the size and shape of that protein or complex. Sedimentation coefficients are usually expressed in Svedberg units (S). 1S is equal to 10^{-13} seconds.

7.1. Radioactive sedimentation markers

The sedimentation coefficients of several proteins are well documented [Fazekas & Mac Farlane, 1980] and these were used as standards to enable the calculation of unknown coefficients. [^{14}C]-labelled BSA, 4.6S and [^{14}C]-labelled human- γ -globulins, 7.1S were used as standards in SDGA.

7.2. Sedimentation pattern of steroid receptors in low salt buffers

Cells were sub-cultured as in section 2.2.2. and allowed to plate down in a 75cm² tissue culture flask for 24hr in routine conditions so that a 50% confluent monolayer was formed. The routine medium was removed and the cells washed twice with PBS and 30ml of steroid-free medium was added. The cells were grown in this medium for 48hr. The cells were then harvested by trypsinisation as described in section 2.2.2. The cells were resuspended in 2ml of low salt homogenate buffer and cell lysis was carried out by the freeze/ thaw method described in section 2.2.4.. The cell lysate was then centrifuged at 105000g for 30 minutes at 4°C to pellet the nucleus and cell debris, a protein assay was carried out to ensure that the total protein content of the supernatant was between 5-10 mg/ml. 1 ml aliquots of the supernatant were then incubated for 1 hour at 4°C with either ³H-mibolerone with a final concentration of 5×10^{-9} M alone or ³H-mibolerone at a final concentration of 5×10^{-9} M plus a cold

competitor of mibolerone (not radiolabelled) at a final concentration of 5×10^{-7} M.

Unbound mibolerone was removed by incubating the samples with DCC at a final concentration of 0.5% w/v for 15 minutes at 4°C, mixing every 5 minutes. The DCC-bound mibolerone was then pelleted by centrifugation at 2000g at 4°C for 15 minutes. 200 μ l of supernatant was then gently layered on top of a 5-20% (5ml) sucrose gradient.

Sucrose density gradients were made by mixing 5% sucrose, in gradient buffer and 20% sucrose in gradient buffer in a two chamber gradient forming device. The gradient was formed in 5ml cellulose nitrate tubes at 4°C and allowed to settle for at least 4 hours before use. The formation of the sucrose gradient was checked periodically using a refractometer.

After layering the cytosol on top of the gradient the tubes were centrifuged at 50,000 rev/min (250000g) for 20 hours at 4°C using a Beckman SW 55Ti rotor in a Beckman L2-65B ultracentrifuge. Each sample contained ^{14}C -labelled BSA, 4.6S and ^{14}C -labelled human- γ -globulins, 7.1 S as internal markers. After centrifugation, the bottom of the tube was punctured with an 21 gauge needle and two-drop fractions were collected in Eppendorf tubes (28-33 fractions from each SDG). 10 μ l of each sample was then placed in scintillation vials and 3ml of scintillation fluid was added. The amounts of ^3H and ^{14}C in each vial were counted in a scintillation counter (LKB 1209 Rackbeta).

Spectrum plots of both ^{14}C and ^3H showed that an overlap exists between the two radioactive emission profiles. The counter windows were changed to eliminate the ^3H counts from the ^{14}C spectrum. It would be impossible to eliminate the ^{14}C counts from the ^3H spectrum but these were minimised and the % overlap was calculated and the ^3H counts adjusted accordingly.

Graphs were plotted showing the number of counts as a function of the fraction number and arrows used to indicate the position of the sedimentation markers. The positions of the 4S and 8S [^3H] peaks were determined by comparison

with the migration of the [^{14}C]-labelled standard proteins. The relative areas under the sedimentation peaks were calculated and the ratio of 4S/8S peaks obtained.

8. [^{35}S]-METHIONINE LABELLING OF PROTEINS

Cells were sub-cultured as in section 2.2.2. and allowed to plate down in 6-well tissue culture plates for 24hr in routine conditions so that a 50% confluent monolayer was formed. 45 minutes prior to labelling, the medium was removed and the cells washed twice with PBS and once with methionine-free medium. The cells were incubated for 40 minutes with a further volume of methionine-free medium supplemented with 2% DHIDCCFCS. The cells were then washed with methionine-free medium and incubated for 3 hr with methionine-free medium supplemented with 2% DHIDCCFCS and $5\mu\text{Ci/ml}$ [^{35}S]-methionine. The medium was removed and the cells washed twice with low salt homogenate buffer. The cells were removed from the wells using a small cell scraper in 0.5ml of low salt homogenate buffer. The samples were placed in an Eppendorf tube and stored at -70°C . Cell lysis was achieved by freeze/thawing the samples (section 2.2.4.).

The polypeptides were resolved by SDS/PAGE (section 10.) and Western blotting carried out (section 11.). The blots were air-dried and exposed for one week to X-ray film (Amersham hyper film MP) with intensifying screens at -70°C . The X-ray film was developed using an automated machine (Kodak X-Omat Processor Model ME3).

9. IMMUNOPRECIPITATION OF HSP90

The method was adapted from that of Perdew and Whitelaw [Perdew & Whitelaw, 1991] and uses a mouse monoclonal anti-hsp90 antibody of the IgM class (3G3) obtained from Affinity Bioreagents. A goat anti-mouse (GAM) IgM antibody was used to link the 3G3 antibody with Sepharose gel as follows

9.1. Preparation of GAM-sepharose complex

0.3g of CNBr-Sepharose (1ml final gel volume) was resuspended in 1mM HCl and incubated for 15 minutes at room temperature. The CNBr-Sepharose gel was then washed for 15 minutes on a sintered glass filter with a further 60ml of 1mM HCl (a tight-fitting piece of glass filter paper (Whatman glass microfibre filter GF/C) was used to prevent loss of gel volume). The gel was then placed in a glass bijou bottle.

The GAM-IgM was diluted in coupling buffer to a final concentration of 0.5mg/ml and 2ml of this was added to 1 ml of washed CNBr-Sepharose. The GAM and CNBr-sepharose were incubated, rotating at 4°C overnight. The GAM linked CNBr-Sepharose (GAM-Seph) was then pelleted by centrifugation for 2 minutes at 1000g at 4°C and the supernatant discarded. The GAM-Seph was washed twice with coupling buffer and pelleted as before. The remaining active sites on the CNBr-Sepharose were blocked by incubating the GAM-Seph with 1M ethanolamine pH 8.0 for 2 hours at 4°C. This was followed by one wash with coupling buffer and one wash with acetate buffer and a final wash with coupling buffer.

To ensure that GAM was bound to the CNBr-Sepharose prior to 3G3 binding, a small amount of the GAM-Seph was incubated, rotating for 1 hour at room temperature with a horseradish peroxidase linked anti-goat antibody (5mg/ml). The GAM-Seph/ anti-goat conjugate was washed twice with 1 ml of coupling buffer followed by a 1 minute incubation with ECL reagent (section 11.2.). Aliquots of this mixture were spotted onto nitrocellulose paper and this was then exposed to X-ray film (Amersham hyper film MP) for 15 seconds in the dark. The X-ray film was developed using an automated machine (Kodak X-Omat Processor Model ME3). The intensity of the spots indicated the level of GAM bound to the CNBr-Sepharose.

9.2. Binding of 3G3 antibody with GAM-Sepharose complex

The 3G3 antibody (100 μ g) was dissolved in 200 μ l HENG buffer and incubated with 100 μ l GAM-Seph (actual volume of GAM-Seph, the coupling buffer having been removed) for 1 hour, shaking on ice. The GAM-Seph-3G3 was pelleted by centrifugation for 2 minutes at 1000g at 4°C and the supernatant discarded. The GAM-Seph-3G3 was washed with HENG buffer and once with 0.2 M triethanolamine (pH 9.0) before the GAM and the 3G3 antibodies were crosslinked. Crosslinking was achieved by incubating the GAM-Seph-3G3 with 20 mM DMP for 45 minutes, rotating at room temperature, followed by washing with one volume of 0.2 M ethanolamine pH 8.0 then incubating with a further volume of 0.2 M ethanolamine at room temperature, rotating, for two hours. The GAM-Seph-3G3 was finally washed twice with HENG buffer and stored in HENG buffer at 4°C.

9.3. Purification of hsp90 using GAM-Seph-3G3

Cells were harvested, as appropriate for the experiment (see results chapter 5), and suspended in HENG buffer, lysed by freeze/ thawing and centrifuged for 30 minutes at 18000 rpm at 4°C to pellet the membranes and nuclei. ^{As a result of this} separation method all soluble proteins in the nucleus will be found in the cytosol fraction (cell extract). The supernatant (cell extract) was removed and a protein assay carried out (section 4.2.). The cell extract was diluted with HENG buffer to a protein concentration of 400 μ g/ml. 1 ml of diluted cell extract was added to 100 μ l GAM-Seph-3G3 and incubated for 90 minutes in ice, on an orbital shaker (Luckman R100). The GAM-Seph-3G3-hsp90 was pelleted by centrifugation for 2 minutes at 1000g at 4°C and the supernatant stored at -70 °C. The pellet was washed twice with HENG, once with 50mM NaCl and once with 20 μ l HENG buffer. 20 μ l sample buffer was added and the sample boiled for 5 minutes before SDS/PAGE (section 10.) followed by Western blotting (section 11.).

10 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

10.1 Preparation of discontinuous SDS-PAGE gels

The methodology for discontinuous SDS-PAGE is based on that of Laemmli [Laemmli, 1970]. Discontinuous gels consist of a resolving (lower) gel and a stacking (upper) gel, resulting in better band resolution than a continuous gel which has no stacking section [Laemmli, 1970].

The gel apparatus used was a Mini-protean® II dual slab cell (BIORAD). Assembly of the kits was carried out as per manufacturer's instructions, using 1mm spacers and comb. Before the resolving gel was poured between the plates, the comb was inserted and the plate marked 1 cm below the teeth of the comb. This is the level to which the resolving gel is poured. Preparation of 7.5% resolving gel monomer solution was as follows: 5ml resolving buffer, 10ml 30% acrylamide buffer, 22ml distilled water and 3ml 1% ammonium persulfate were added together and immediately before pouring 300 µl TEMED was added. After the resolving gel was poured it was immediately overlaid with isopropanol to ensure an even surface on the top of the resolving gel. After the gel had polymerized, the isopropanol was rinsed off thoroughly with distilled water and the area above the resolving gel was dried with filter paper before the stacking gel was poured. The stacking gel was made by mixing 5 ml stacking buffer, 2.5 ml 30% acrylamide buffer, 11 ml distilled water and 1.5 ml 1.0% ammonium persulfate and, immediately before pouring, 300 µl TEMED. The stacking gel monomer was poured on top of the resolving gel and immediately the comb was inserted. Care was taken to prevent air bubbles forming within the wells. The gel was allowed to polymerise at room temperature. The upper buffer chamber was assembled as per manufacturer's instruction and was filled with electrophoresis tank buffer. The combs were

removed by pulling straight up slowly and gently. Before sample loading the wells were washed with electrophoresis tank buffer to ensure removal of any monomer polyacrylamide solution. The lower buffer chamber was filled with electrophoresis tank buffer so that at least 1 cm of the gel was covered and any air bubbles removed from the bottom of the gel so that good electrical contact was achieved.

10.2 Loading and electrophoresis of samples and standard proteins

Samples and protein standards (unless Biorad pre-stained SDS-PAGE markers were used) were mixed 1:1 with sample buffer and boiled for 5 minutes, then centrifuged for 20 seconds at 1000g before being loaded. The samples were loaded into the wells under the electrode buffer with a Hamilton syringe placed about 1.5mm from the well bottom. Care was taken to prevent overspill into neighbouring wells.

The unit was then connected to a power supply (Biorad constant voltage power supply). A constant voltage of 200 volts was applied. Electrophoresis was carried out until the bromophenol blue tracker dye was at the bottom of the gel. The gels were then removed from the apparatus as per the manufacturer's instruction and either stained for total protein content (section 12) or western blotting (section 11) carried out.

10.3 Determination of protein size by SDS-PAGE

To assess the molecular weight of observed proteins, one lane of each well contained calibration standards (kit obtained from Boehringer Mannheim Biochemica), which had been dissolved in sample buffer. A linear calibration curve was obtained by plotting the logarithm of the molecular weight of the calibration protein against the distance which that protein had migrated into the gel. The standards used were α_2 -macroglobulin (Mr 170,000 in reduced state),

β -galactosidase (Mr 116,353), fructose-6-phosphate kinase (Mr 85,204), glutamate dehydrogenase (Mr 55,562) and triose phosphate isomerase (Mr 26,626).

11. WESTERN BLOTTING OF SDS-PAGE SAMPLES

11.1 Transfer of proteins unto nitrocellulose paper

The use of an electrical current to transfer proteins from polyacrylamide gels onto a nitrocellulose membrane was first described by Towbin [Towbin, 1979]. The apparatus used was a Mini trans-blot® electrophoretic transfer cell (Biorad). Proteins were separated by SDS-PAGE as described in section 10. The gel and the nitrocellulose membrane (Amersham ECL) were equilibrated in transblot buffer for 20 minutes. The assembly of the transblot sandwich was carried out as described in the manufacturer's instruction manual ensuring correct orientation of the gel and nitrocellulose paper (anode-fiberpad-filter paper-nitrocellulose-gel-filter paper-fiber pad-cathode). The transblot sandwich and the cooling unit were placed in the buffer chamber which was filled with transblot buffer. Electrophoretic transfer was carried out at 100 volts for 1 hour using a Biorad constant voltage power supply. Once transfer was complete the nitrocellulose was cut to the size of the gel and rinsed in TBST. The remaining active sites on the nitrocellulose were blocked by incubating the blot in 5% non-fat milk in TBST at 4°C for 48hr. The blot was then washed five times with TBST over a period of 1 hour at room temperature. Exposure to the primary antibody (Ab) was achieved by incubating the blot in 10 μ g/ml Ab in 5% non-fat milk/TBST for either 2 hours at room temperature or overnight at 4°C. The blot was again washed five times with TBST over a period of 1 hour at room temperature.

11.2 Detection of protein using enhanced chemiluminescence (ECL)

SDS-PAGE and western blotting were carried out as in sections 10 and 11.1..

A horseradish peroxidase-labelled secondary Ab is used to detect the primary Ab on the western blot.

The secondary antibody was diluted 1:1000 in TBST . The blot was then incubated in 10ml of the diluted second antibody for 1hr at room temperature. The blot was washed five times with TBST over a period of 1 hour at room temperature before being transferred to the dark room. The ECL developer solutions (Amersham) were mixed 1:1 and 4 ml of the mixture was gently poured on top of the blot. After 1 minute the developer mixture was removed and the blot wrapped in Saran wrap (Dow). Care was taken to ensure that no air bubbles or wrinkles were formed before the blot was exposed to X-ray film (Amersham hyper film MP) for 15 seconds in the dark. The X-ray film was developed using an automated machine (Kodak X-OMat Processor Model ME3). The intensity of the resultant bands could be varied by altering the film exposure time.

12. STAINING AND PRESERVING OF SDS-PAGE GELS

12.1. Silver staining of SDS-PAGE gels

Gels were silver stained according to the method of Morrissey [Morrissey, 1981]. Gels were prefixed in solution 1 for 30, minutes followed by a further 30 minutes in solution 2. They were then placed in solution 3 for 30 minutes, after which time they were rinsed in distilled water and left in a large volume of distilled water for at least 2 hours. The gels were soaked in solution 4 for 30 minutes, which was then replaced with solution 5 for a further 30 minutes before rinsing first with distilled water and then solution 6. The gels were allowed to sit in solution 6 until the desired level of staining was achieved. The

reaction was stopped by adding 12 ml of solution 7 before the gels were stored in distilled water.

12.2. Coomassie blue staining of SDS-PAGE gels

Gels were stained by incubating in Coomassie staining solution for 1 hour at room temperature. Destaining of the gel was then achieved by soaking in five washes of destaining solution over a period of about 5 hours.

13. Statistical analysis

All statistical analyses was carried out using GraphPad Instat Mac® version 2.0 (GraphPad Software). The means and standard deviations of repeated experiments were also calculated using GraphPad Instat Mac®. The experimental and control means were compared using an unpaired, two-tailed student *t* test unless otherwise stated.

Instat reports exact P values and states the "significance" of the results using the following arbitrary scheme.

P> 0.10	"not significant"
P<0.10	"not quite significant"
P<0.05	"significant"
P<0.01	"very significant"
P<0.001	"extremely significant"

The *t* test assumes that the data populations are scattered according to a gaussian distribution, and that the standard deviation of the two populations are equal. Instat tests this assumption of equal variances with an F test and unless stated the standard deviations were found to have equal variances.

14. Computer software

This thesis was written using Microsoft® Word version 5.1, all graphs and tables were created using CA-Cricket Graph III® (Computer Associates Limited) and diagrams were created using Clarisworks® (Claris Corporation). Bibliographic references (citations) were organised and managed using EndNote Plus® (Niles & Associates Inc.). All these programs were run on an Apple Macintosh computer.

Scatchard analysis of steroid receptors was carried out using Oncolog® which runs on an IBM computer.

Chapter 3

The effect of hyperthermia on prostate cancer cell lines

3.1 Correlation of Cell number with DNA content

Introduction

Throughout this thesis DNA synthesis is used as a means of assessing cell survival and growth. Therefore, it is important to ensure that the level of DNA will reflect cell number. An initial experiment was carried out to show the correlation between DNA content and cell number.

Methods

The three cell lines were subcultured as in section 2.2.2. of the methods chapter so that a 20% monolayer was formed within 2x 6-well tissue culture plates per cell line. The cells were then allowed to "plate down" for 24 hours before the medium was renewed. Every 24 hours, the culture medium was removed from 2 wells and the cell monolayers were washed twice with PBS. The cells were harvested by trypsinisation, a sample of the harvested cells was removed and the number of cells counted using a haemocytometer. The remaining cells were lysed and a DNA assay carried out as described as in section 3. of the methods chapter.

Results

The relevance of using DNA concentration as a measure of cell number or cell proliferation can be validated by the production of linear correlation when cell number is plotted against DNA concentration. Linear correlation between the two is seen in figure 8 ($r = 1.00$).

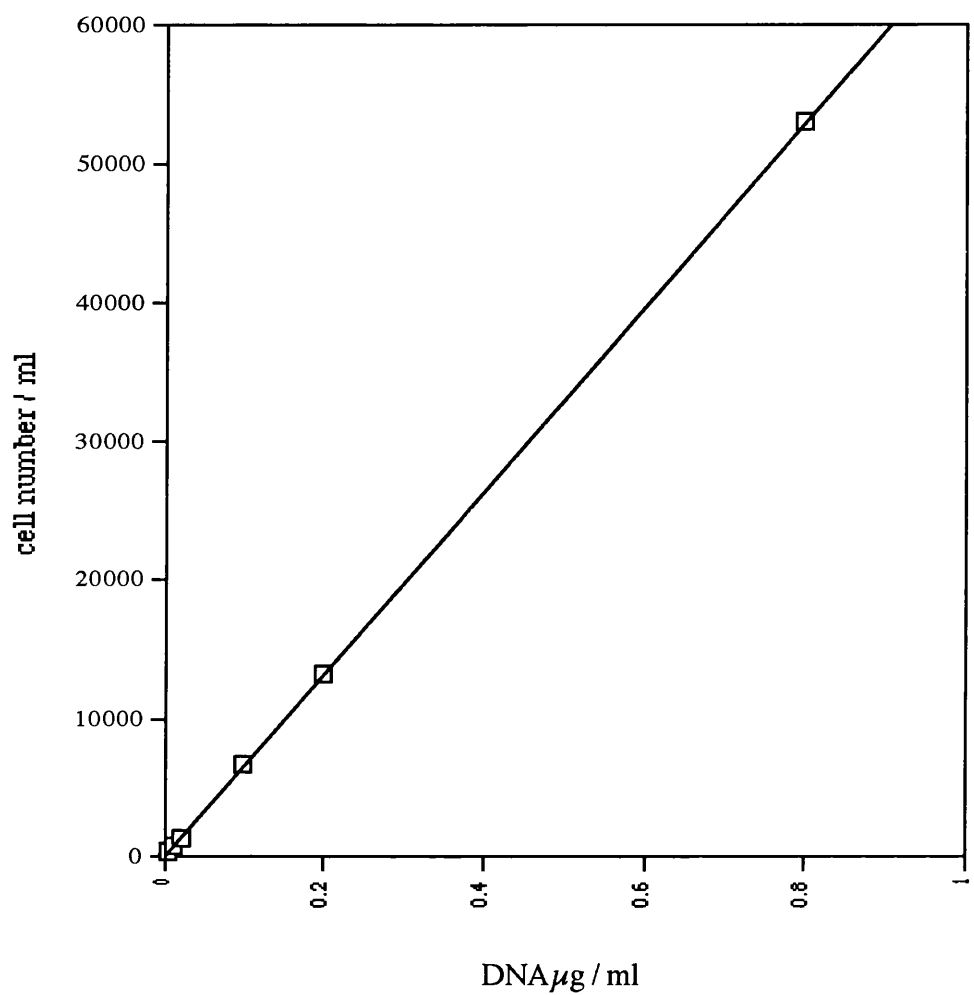
Conclusions

Figure 8 shows that there is good correlation between the concentration of DNA in a sample and the number of cells in the same sample.

Figure 8. Correlation of DNA concentration with cell number.

This graph is an example of correlation between cell number and DNA concentration. Each plot represents one sample, half of which was counted using a haemocytometer, the remaining half was assayed for DNA using the Hoechst method as described in methods section 3..

DNA concentration versus cell number



$y = 66231.337x - 8.761 \quad r = 1.000$

3.2. Androgen sensitivity of prostate cancer cell lines

Introduction

Androgens are essential for normal prostate development and maintenance [Davies & Eaton, 1991]. Therefore, it was important to characterise the growth response of the three prostate cancer cell lines used (LNCaP, DU145, PC3) to androgens under normal growth conditions. The ligand used was 5 α -dihydrotestosterone (DHT) which is the normal intracellular mediator of androgen action.

Methods

The three cell lines were subcultured as in section 2.2.2. of the methods chapter so that a 20% monolayer was formed within 2x 6-well tissue culture plates per cell line. The cells were then allowed to "plate down" for 24 hours before the routine medium was removed and the cells washed twice with PBS. Steroid free medium containing different concentrations of DHT (concentration range 1x10⁻⁶M- 1x10⁻¹²M) was added to each well. The DHT was dissolved in absolute alcohol to form a stock solution of 1x10⁻³M which could then be diluted into culture medium to produce working concentrations with negligible alcohol content. Duplicate wells were used for each experimental and control concentration. Control cells were incubated in steroid free medium with no hormone additive but with the same final absolute alcohol concentration as the experimental medium.

The cells were incubated at 37°C for 72 hours. The culture medium was removed and the cell monolayers were washed twice with PBS. The cells were harvested by trypsinisation, a sample of the harvested cells was removed and the number of cells counted using a haemocytometer. The remaining cells were lysed and a DNA assay carried out as described as in section 3. of the methods chapter.

Results

The results of the androgen sensitivity studies are expressed in the form of dose response curves. Figure 9 illustrates the dose response to all three cell lines, these are shown as means of 3 independent experiments each done in duplicate and the standard deviations are indicated by the error bars.

Conclusions

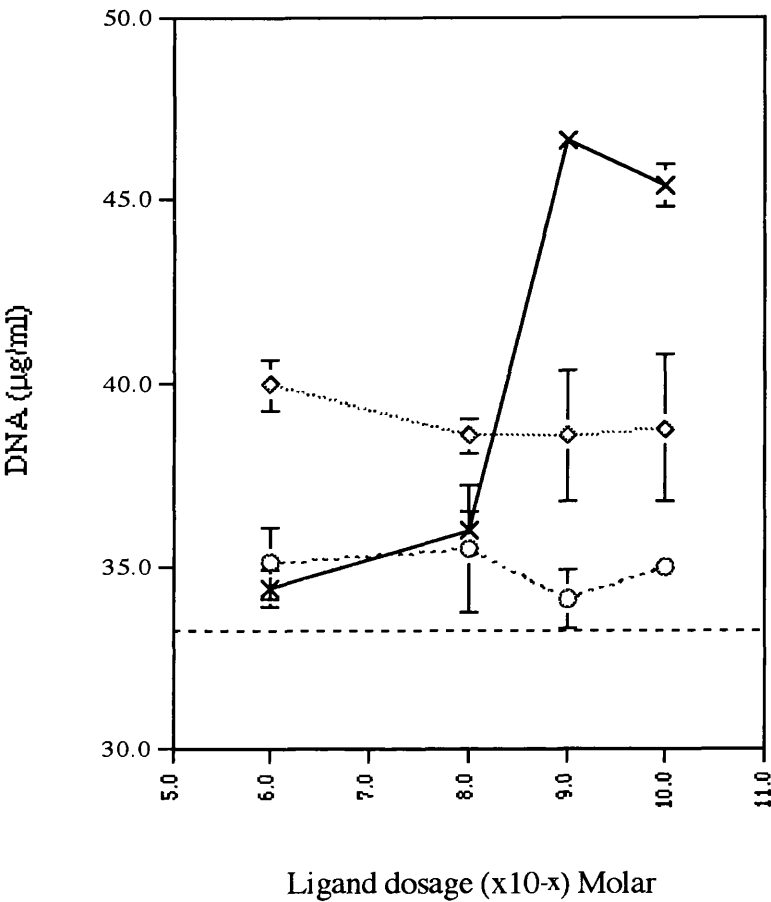
Figure 9 shows that LNCaP cell growth responds positively to the influence of DHT with maximum stimulation at 10^{-9} M DHT. This result supports previous reports that LNCaP is an androgen responsive cell line [Horoszwicz *et al.*, 1983; Lloyd, 1992]. Neither DU145 nor PC3 cell growth responds to DHT. Again this corresponds to earlier results obtained by this group and others [Stone *et al.*, 1978; Lloyd, 1992; Carruba 1994].

Although DU145 cells are not growth stimulated by androgens, they do have androgen receptors present as shown by Scatchard analyses and it has been suggested that these cells do show a metabolic response to androgens [Carruba 1994].

Figure 9. Dose response curve for DHT in all three prostate cancer cell lines. All three cell lines (LNCap, DU145 & PC3) were exposed to DHT over a range of concentrations from $1 \times 10^{-6} \text{M}$ to $1 \times 10^{-11} \text{M}$ for 72 hours. The dotted line at $34 \mu\text{g/ml}$ DNA represents the LNCaP control (no DHT) DNA level at 72 hrs, the DU145 control level was $35 \mu\text{g/ml}$ and the PC3 's level was $40 \mu\text{g/ml}$. Data points represent means of means of 3 independent experiments each done in duplicate and the standard deviations are indicated by the error bars.

Where no error bars are visible, the standard error is too small to be shown.

The effect of DHT on prostate cancer cell growth



—x— LNCaP
.....◇..... PC3
-----○----- DU145

3.3. The effects of heat on prostate cancer cell lines

Introduction

The thermosensitivity of different cell types varies, depending on the normal range of environmental exposure to heat [Lindquist, 1986]. Therefore, it was important to characterise the normal range of thermal tolerance of prostate cell lines and to define the temperatures required to establish thermotolerance in these cells.

The experiment was designed so that the cells would receive a measured dose of sub lethal heat to shock the cells. The experimental conditions were altered to establish the optimal heat shock temperature which would induce thermotolerance.

Methods

The cells were subcultured as described in method section 2.2.2., so that a 20% confluent monolayer was formed within 6-well plastic tissue culture plates. The cells were allowed to "plate down" for 24 hours and the culture medium was changed prior to commencing "heat shock". Heat shock was achieved using a thermostatically controlled water bath [Lloyd *et al.*, 1992]. The plates were sealed water tight (using Nescofilm) and allowed to float on the surface of the water. The efficiency of heat transfer from the water to the cells was assessed by measuring the temperature inside vessels containing only culture medium. The ability of a particular temperature to induce thermotolerance on a cell line was determined by incubating the cells at that temperature (38°C, 39°C and 40°C) for two hours followed by a 12 hour recovery period (37°C). The cells were then incubated at a normally lethal temperature (42°C and 43°C) for 2 hours. The incubation period of two hours was used as earlier experiments had shown this time to be sufficient to achieve maximal thermotolerance (data not shown). To determine whether or not the

heat shock treatment had positively changed cell growth potential, cells were left to recover for 24 hr after the second heat shock treatment, then one sample was taken. Remaining wells were allowed to grow for a further 24 hours and then sampled. DNA assays were carried out as described in the methods section 3. to assess whether the cells continued to grow between 24 and 48hrs post heat shock. Thermotolerance is defined as the ability to continue growth between 24 and 48 hours post 2nd heat shock.

Results

The conditions required to induce maximum thermotolerance were investigated using LNCaP cells. Figure 10 shows the effect of altering the 1st sub-lethal temperature on the induction of thermotolerance as a % of DNA synthesis at constant 37°C. Figure 11 shows the experimental time course for maximal thermotolerance induction.

Figures 12,13 and 14 demonstrate the acquisition of thermotolerance by LNCaP, DU145, and PC3 cells.

Control experiments are as follows: one set of cells were grown at 37°C throughout the experiment to show normal cell growth; another set of cells were kept at 37°C until exposure to the normally lethal temperatures (42°C and 43°C) to show the effects of hyperthermia on cells; and a third set of cells were incubated at the sub lethal temperature but not the lethal temperature to show the effects of the sub lethal temperature alone.

Each experimental point is the mean of triplicate experiments \pm standard deviation.

Figure 10. The induction of thermotolerance in LNCaP cells.

The cells were incubated with different elevated temperatures (38°C, 39°C and 40°C) to establish the temperature required to induce thermotolerance. The induction of thermotolerance was assessed by incubating the cells at a normally lethal temperature (42°C and 43°C) 12 hours after the initial heat shock.

The ability of the different temperatures to induce thermotolerance was assessed by measuring the DNA synthesis between 24 and 48 hours.

Each column represents the mean of 3 independent experiments each done in duplicate and the standard deviations are indicated by the error bars

Establishing thermotolerance in LNCaP cells

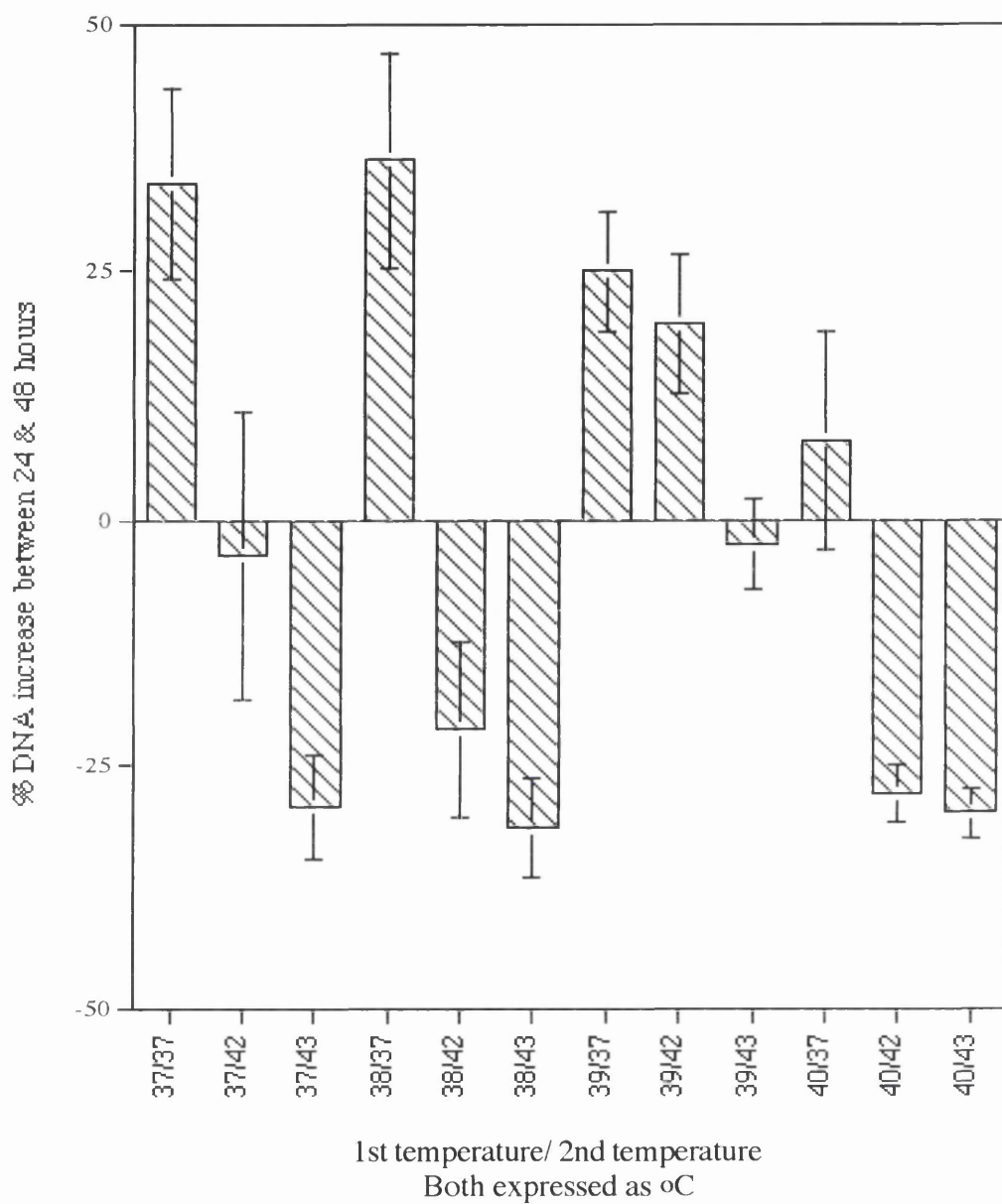


Figure 11. Graphical representation of timing and temperature required to establish thermotolerance.

The cells were incubated at 39°C for 2 hrs then returned to 37°C for a 12hr recovery period before exposure to 42 °C for 2 hrs. Cell samples were taken 24 and 48 hrs after the end of the second heat shock.

Heat shock time course for maximal thermotolerance establishment

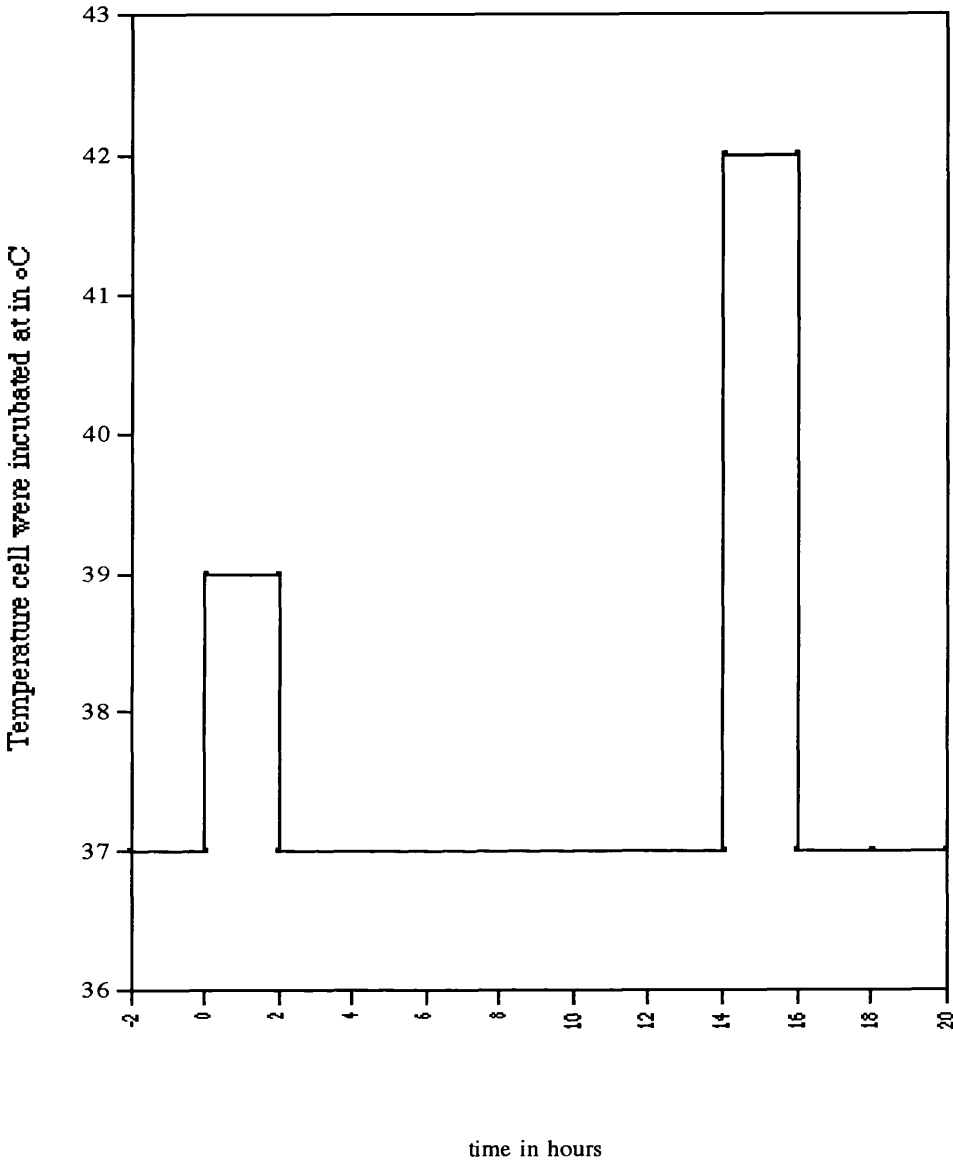


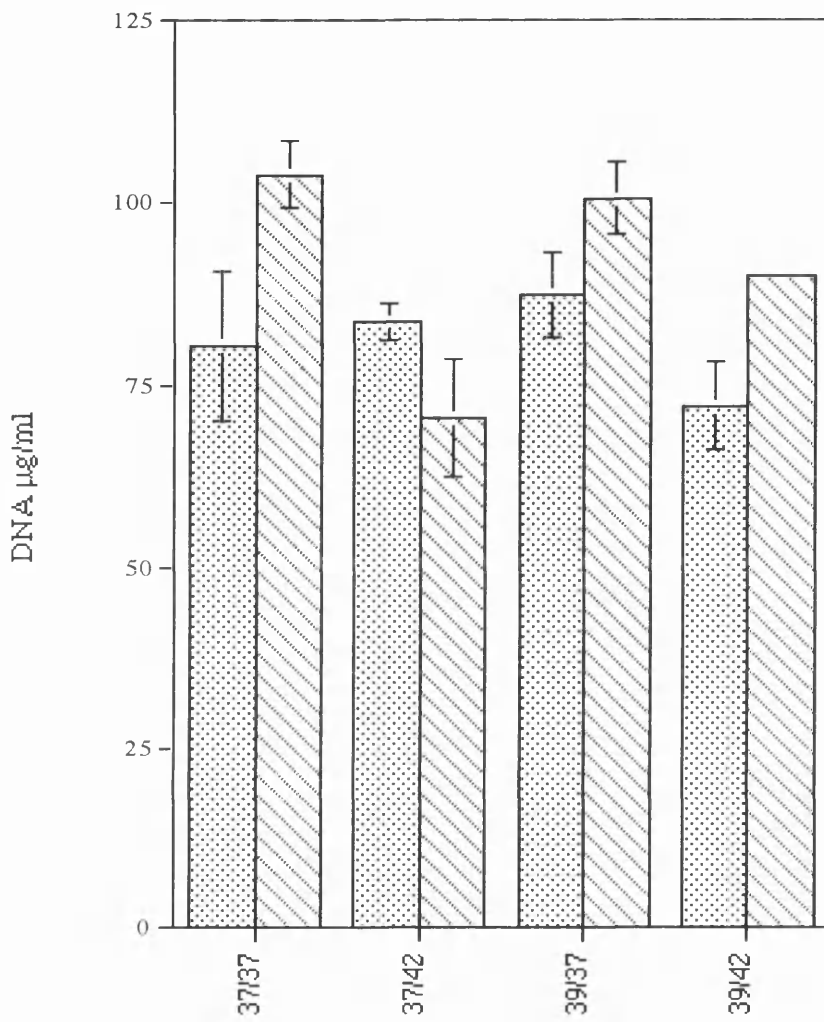
Figure 12. The induction of thermotolerance in LNCaP cells.

The cells were exposed to the first temperature for 2 hours followed by a 12 hour recovery period at 37°C before being exposed to the second temperature. The effects of the different heat shock treatments on the cell growth were assessed by measuring the amounts of DNA present 24 and 48 hours after the second heat shock treatment.

Each column represents the mean of triplicate experiments, the error bars are the standard deviations .

Where no error bars are visible, the standard error is too small to be shown.

LNCap Cell Thermotolerance



Heat Shock Temperatures in °C



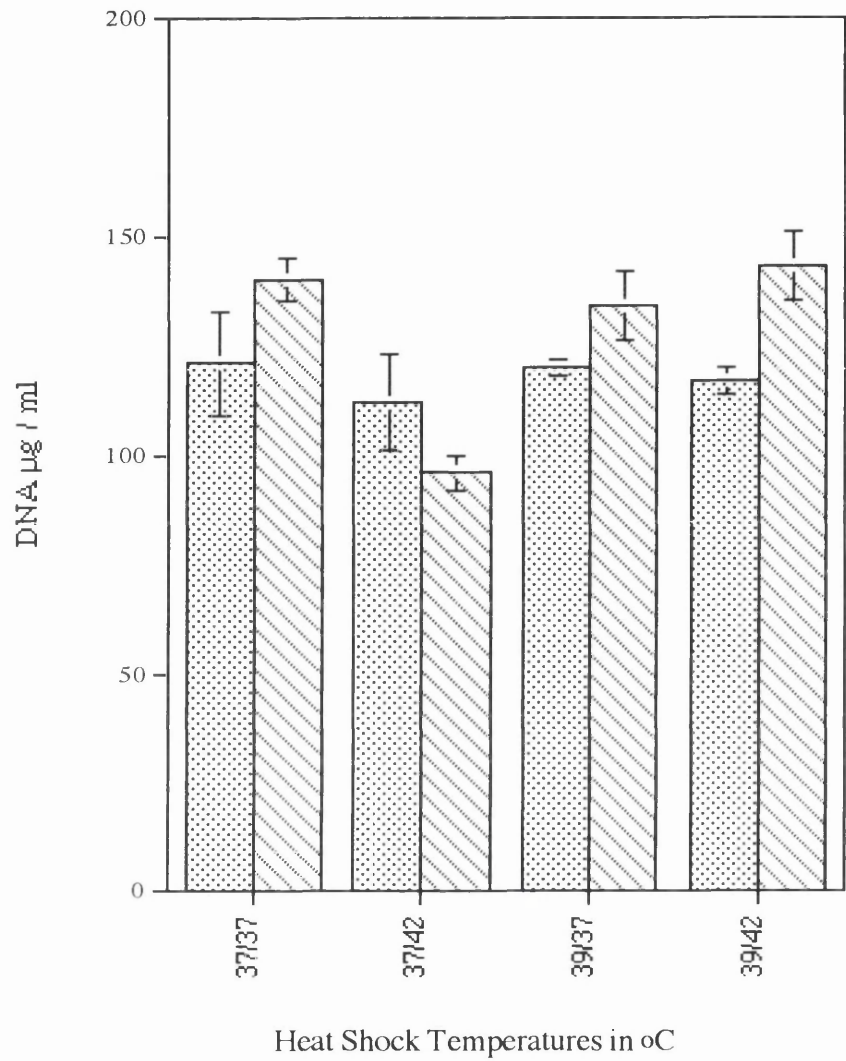
-  24 hr sample
-  48 hr sample

Figure 13. The induction of thermotolerance in DU145 cells.

The cells were exposed to the first temperature for 2 hours followed by a 12 hour recovery period at 37°C before being exposed to the second temperature. The effects of the different heat shock treatments on the cell growth were assessed by measuring the amounts of DNA present 24 and 48 hours after the second heat shock treatment.

Each column represents the mean of triplicate experiments, the error bars are the standard deviations .

DU 145 Cell Thermotolerance



■ 24 hr sample
▨ 48 hr sample

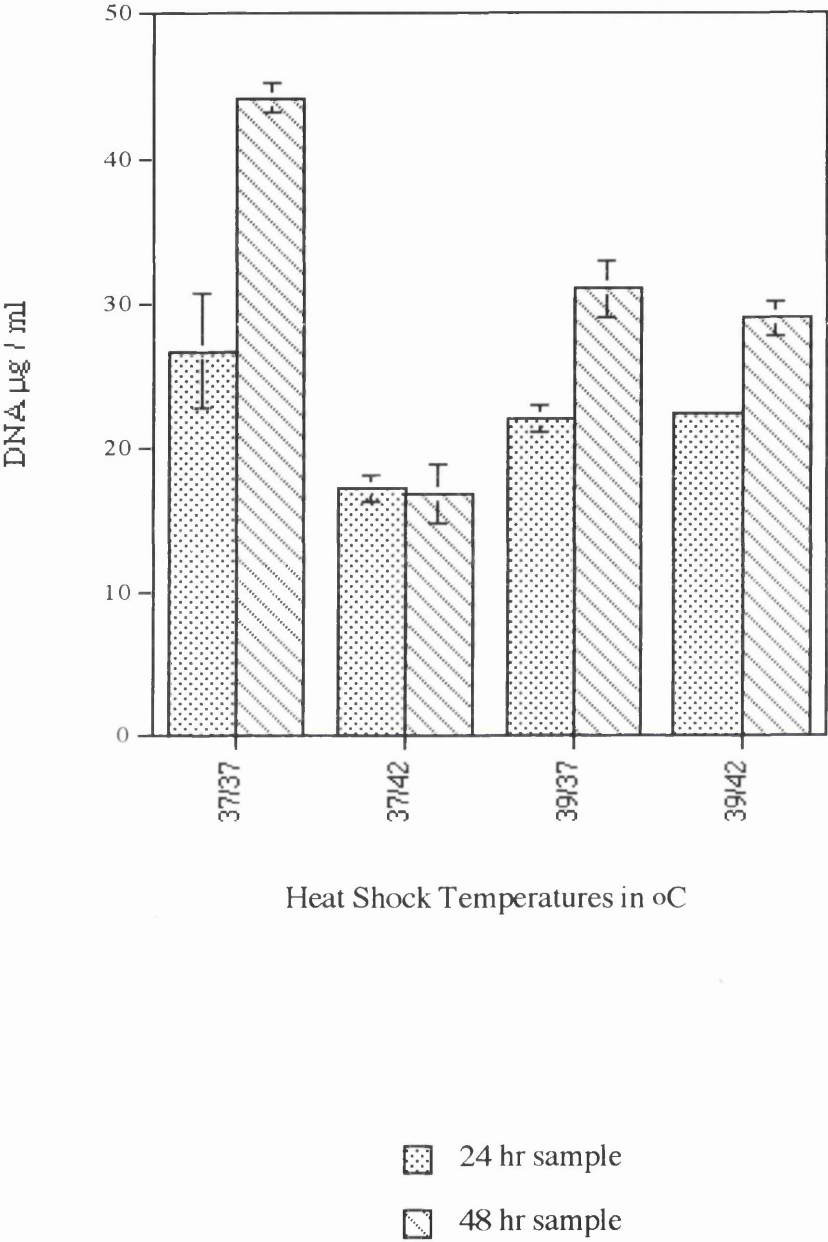
Figure 14. The induction of thermotolerance in PC3 cells.

The cells were exposed to the first temperature for 2 hours followed by a 12 hour recovery period at 37°C before being exposed to the second temperature. The effects of the different heat shock treatments on the cell growth were assessed by measuring the amounts of DNA present 24 and 48 hours after the second heat shock treatment.

Each column represents the mean of triplicate experiments, the error bars are the standard deviations .

Where no error bars are visible, the standard error is too small to be shown.

PC3 Cell Thermotolerance



Conclusions

A temperature of 42°C is high enough to induce cell kill in LNCaP cells. This is seen in figure 10 columns 37/42 and 37/43. This is prevented if the cells are first exposed to 39°C (figure 10 column 39/42 and 39/43). 38°C does not appear to induce thermotolerance which suggests that there is a threshold temperature under which the cells will not implement the stress response. 40°C is not a lethal temperature (figure 10 column 40/37) nor does it induce thermotolerance (figure 10 40/42 and 40/43). It may be that the cells are so damaged by the initial 40°C exposure that they are unable to induce the stress response or survive the lethal temperatures.

Prior exposure to 39°C enable cells to survive exposure to 42°C and still continue to grow. Cells pre-treated at 39°C are still not capable of surviving and growing after a second exposure to 43°C, at least not in so far as the test for overall cell growth indicates between 24 and 48 hours after the 2nd heat shock treatment. Therefore it was decided to use a combination of 39°C followed by 42°C to establish thermotolerance in LNCaP cells (figure 12). Having established these conditions for the induction of thermotolerance in LNCaP cells, it was important to show that these temperatures would also induce thermotolerance in DU145 and PC3 cells.

The results demonstrated in figures 13 and 14 show that DU145 and PC3 cell lines are able to establish thermotolerance under this regimen.

Comparing 37/37 with 37/42 in figures 12, 13 and 14, all three cell lines showed a significant decrease in DNA present at 48 hours after heat shock at 42°C alone (LNCaP $p=0.0087$; DU145 $p=0.0465$; PC3 $p=0.0026$) compared with 37°C controls. In the case of LNCaP and DU 145 cells there was also a further decrease in the amount of DNA present after 48 hours compared with 24 hours and this may be due to cell kill at 42°C (LNCaP cells are not well attached to the surface of the culture plates and therefore quickly detach once dead). The

decrease in DNA synthesis after exposure to 42°C is prevented if the cells are first exposed to the elevated but sub lethal temperature of 39°C. Comparing the level of DNA synthesis (the increase in DNA content between 24 and 48 hours) in columns 37/37 and 39/42 shows that the relative increase in DNA is the same in the control (37/37) as in the experimental 39/42 for both LNCaP (see figure 13) and DU145 cells (see figure 13). This can not be said for PC3 cells (see figure 14) the level of DNA synthesis in the control cells (37/37) is far greater than in the experimental 39/42 cells although growth does occur in the 39/42 cells.

3.4. The effect of time on thermotolerance

Introduction

Treatment of prostate cancer with local hyperthermia involves heating the gland either once or twice a week [Engin *et. al.*, 1993]. Thermotolerance in some cases is a short lived phenomenon, lasting only 2 to 3 days [Nielsen & Overgaard, 1982]. Therefore it is important to see if the thermotolerance in prostate cancer cells will vary with time.

Little is known about the mechanism of thermotolerance, but it is clear that it depends on a pre-exposure to an elevated but sub-lethal temperature [Gerner & Schneider, 1975]. The persistence of thermotolerance and whether thermotolerance is an all or nothing event is also poorly understood. Therefore it was decided to investigate the minimum time required to establish thermotolerance (the time between the sub-lethal and the lethal dose) and whether it is an all or nothing event or if there are degrees of thermotolerance.

Methods

The cells (LNCaP's, DU 145's, PC3's) were subcultured as described in methodology section 2.2.2., so that a 20% confluent monolayer was formed within 6-well plastic tissue culture plates. The cells were allowed to "plate down" for 24 hours and the culture medium was changed prior to commencing "heat shock". Heat shock was achieved by placing the tissue culture plates in an humid incubator with atmospheric air enriched with 5% CO₂ (LEEC) which had been preset to either 39°C or 42°C. The efficiency of heat transfer was assessed by measuring the temperature inside vessels containing only culture medium. Incubation at the sub lethal temperature of 39°C was administered for 2 hours as was the normally lethal temperature of 42°C. The period of time between the

two heat shock temperatures was varied from 0-72 hours. The culture medium was changed every 48 hours. Cell samples were taken at 24 and 48 hours after the second heat shock incubation. Cell lysis and DNA assays were carried out as described in the methods section 3.. The level of thermotolerance was measured as a percentage of cell proliferation between the two samples.

Results

Figure 15 shows the time course of acquisition of thermotolerance over time for all three cell lines. Figure 16 shows the loss of thermotolerance over time. In both graphs each point is the mean of three separate experiments \pm standard deviation.

As in earlier experiments the level of DNA synthesis between 24 and 48 hours post heat shock is taken to reflect the level of thermotolerance. The increase in amount of DNA detected between 24 and 48 hours after the second heat shock is given as $\mu\text{g/ml}$.

Figure 15. The time required to establish thermotolerance in all 3 cell lines. The cells were incubated at the sublethal temperature of 39°C followed by a recovery period at 37°C which was varied from 0-14 hours. Finally the cells were exposed to 42°C for 2 hours. Cell samples were taken at 24 and 48 hours after the second heat treatment and the level of DNA synthesis calculated. Each point represents the mean of three separate experiments \pm standard deviation.

Where no error bars are visible, the standard error is too small to be shown.

Acquisition of thermotolerance over time

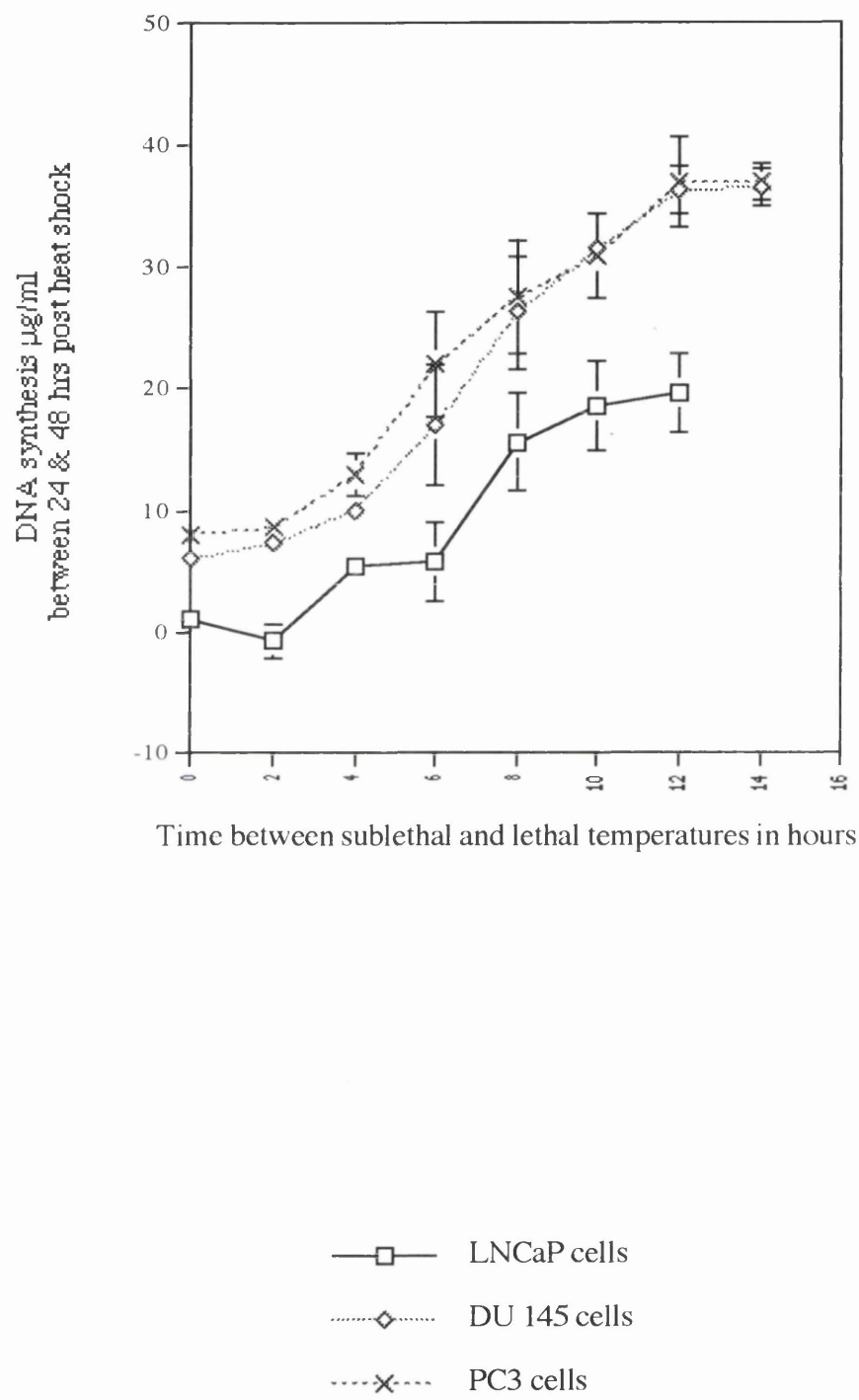
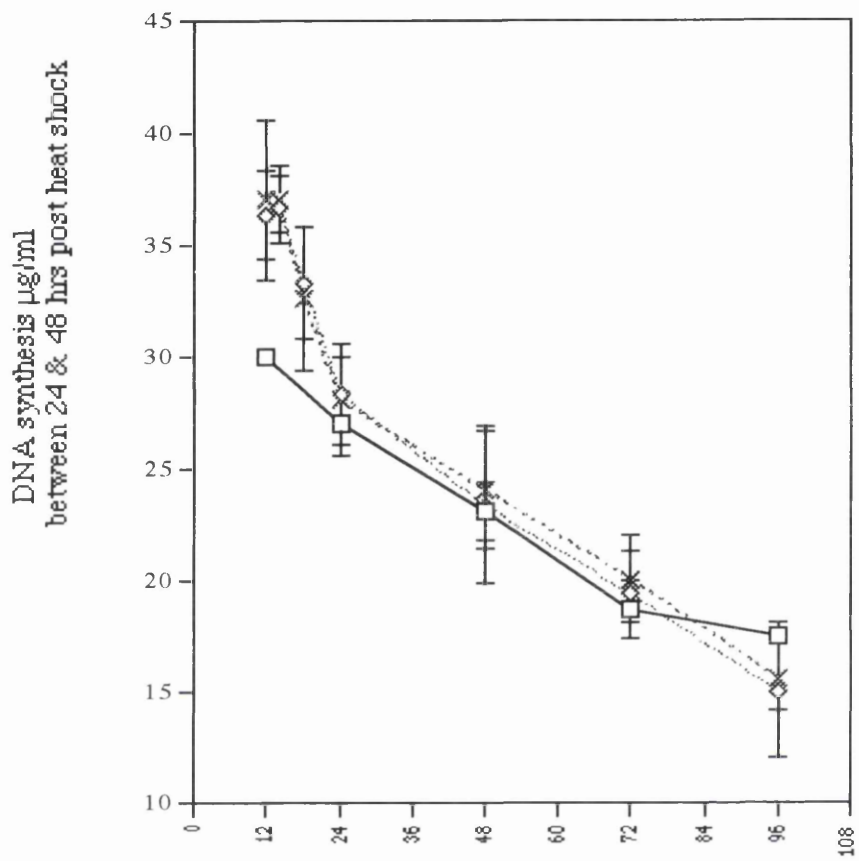


Figure 16. The loss of thermotolerance over time in all 3 cell lines.

The cells were incubated at the sublethal temperature of 39°C followed by a recovery period which was varied from 12-72 hours. Finally the cells were exposed to 42°C for 2 hours. Cell samples were taken at 24 and 48 hours after the second heat treatment and the level of DNA synthesis calculated. Each point represents the mean of three separate experiments \pm standard deviation.

Where no error bars are visible, the standard error is too small to be shown.

The loss of thermotolerance over time



Time between sublethal and lethal temperatures in hours

- LNCaP
-◇..... DU 145
- x--- PC3

Conclusions

Figure 15 demonstrates the time required to induce thermotolerance in the 3 prostate cancer cell lines (LNCaP, DU145 & PC3). Thermotolerance does not occur immediately the cell are exposed to 39°C but rather takes up to 8 hours before it becomes significant (comparing DNA levels at 0hr & 8hr, DU145 $p= 0.0017$; PC3 $p=0.0023$; LNCaP $p= 0.0036$). LNCaP cells appear to be more sensitive to 39°C as their DNA synthesis is nearly stopped by the exposure to the sub lethal temperature. They also take longer to recover than the other two lines which show some degree of thermotolerance after 6 hours whereas it is 8 hours before the LNCaP cells show any significant thermotolerance. LNCaP cells also have a longer doubling time and so their level of DNA synthesis never reaches that of PC3 or DU145 in this experiment. Thermotolerance is not an all or nothing response. Figure 15 shows that the first significant level of thermotolerance is seen after 6 hours (post second heat shock treatment) but it is 12 hours before the maximum levels of thermotolerance is achieved.

Thermotolerance is lost over time as seen in figure 16. Maximal thermotolerance, measured as an increase in DNA synthesis observed between 24 and 48 hours after the 2nd heat shock treatment, was obtained with a gap of 12 hours between the first and second heat shock treatment. The loss of thermotolerance when this gap was increased beyond 12 hours (figure 16) was exponential, as expected and returned to control levels when a 96 hour gap was used. It occurs more rapidly in the DU 145 and PC 3 cell lines initially but the rate of loss of thermotolerance levels out for all three cell lines

Comparison figures 15 and 16 indicates that the loss of thermotolerance is slower than its acquisition. It takes only 12 hours for all three cell lines to express maximal thermotolerance but as much as 96 hours for the levels of thermotolerance to fall to control levels again.

These results show that thermotolerance takes time to be established, reaches a peak about 12 hours after the initial insult, and once established it is slowly lost.

3.5. The re-establishment of thermotolerance

Introduction

Thermotolerance can be gained and lost over a period of 4 days as seen in figures 15 and 16. This would suggest that thermotolerance will not alter the effect of treatment of prostate cancer with local hyperthermia, if the doses of heat were given at least 4 days apart, if thermotolerance has an advantage in sensitising cells to anti-androgen, then this feature may also be lost. This leaves the question: would thermotolerance be as quickly re-established once lost by a further sub-lethal heat shock ?

Methods

LNCap cells were subcultured as described in methodology section 2.2.2., so that a 20% confluent monolayer was formed within 6-well plastic tissue culture plates. The cells were allowed to "plate down" for 24 hours before the first heat shock treatment was applied. In this experiment the sublethal temperature was 39°C and the normally lethal temperature was 42°C. Further heat shock treatments were applied at 74 and 88 hours. The different conditions for the various experiments are summarised in figure 17. All heat shock treatments were carried out for 2 hours

Heat shock was achieved by placing the tissue culture plates in an humid incubator (LEEC) with atmospheric air enriched with 5% CO₂ which had been preset to either 39°C or 42°C. Routine culture medium was used and it was changed every 48 hours during the experiment.

Cells were harvested at 24 and 48 hours after the final heat shock treatment (88-90 hrs), then lysed and DNA assays carried out as described in methods section 3.. The amount of DNA synthesis between 24 and 48 hrs was calculated and the difference was used as a measure of thermotolerance.

Results

Figure 18 shows the level of thermotolerance gained, after the heat shock combinations, as $\mu\text{g/ml}$ DNA synthesis between 24 and 48 hrs. Although the graph shows the results for LNCaP only, the data obtained for DU 145 and PC3 cells followed the same pattern. They have not been shown to simplify interpretation.

Each column represents the mean of three separate experiments \pm standard deviation.

The statistical significance of the increase in thermotolerance is calculated using a two tailed student t test comparing each column with the control 37/37/37/42 column.

Figure 17. Time course of heat shock therapies in re-establishment experiments

The heat shock timing shown in figure 11 was altered to allow the loss of thermotolerance and possible regain of thermotolerance, the incubation times referred to were 0-2 hr/14-16 hr/74-76 hr/88-90 hr.

37/37/37/42:- 37°C from 0-88 hrs then 2 hrs at 42°C

37/37/39/42:- 72 hrs at 37°C then 12 hrs between 39°C and 42°C incubations

37/39/37/42:- 72 hrs between 39°C (14-16 hrs) and 42°C (88-90hrs) incubations

39/42/37/42:- 12 hrs between 39°C (0-2hrs) and 42°C (14-16hrs) followed by another 42°C (88-90hrs) 72 hrs after the 1st 42°C

39/37/39/42:- 39°C incubation at 0 hrs which was repeated at 74 hrs followed by a final 42°C incubation 12 hrs later (84hrs)

39/37/42/42:- 72 hrs between 39°C and the first 42°C, a second 42°C incubation was given 12 hrs after the first

Incubation times & temperatures for re-establishment experiment

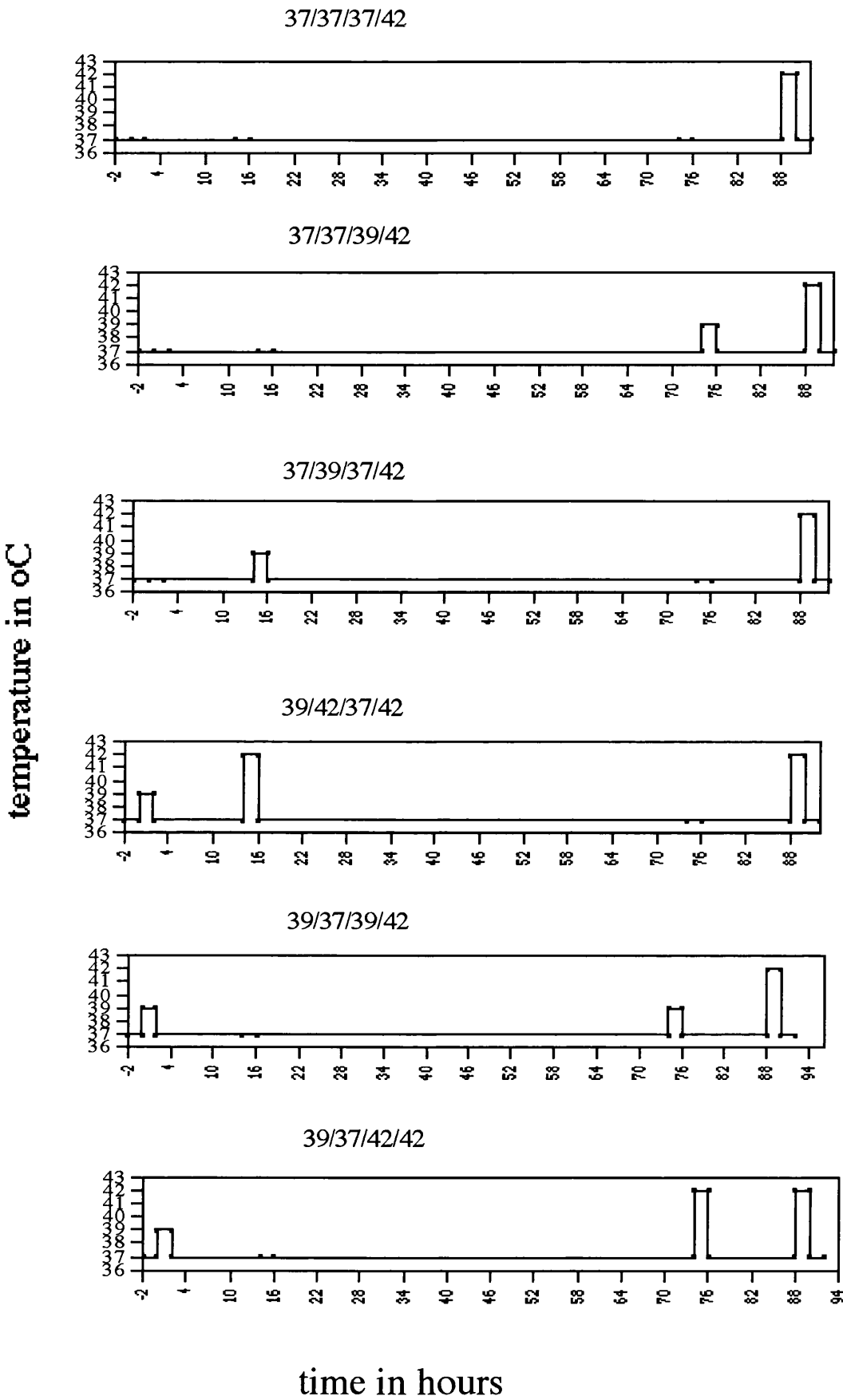
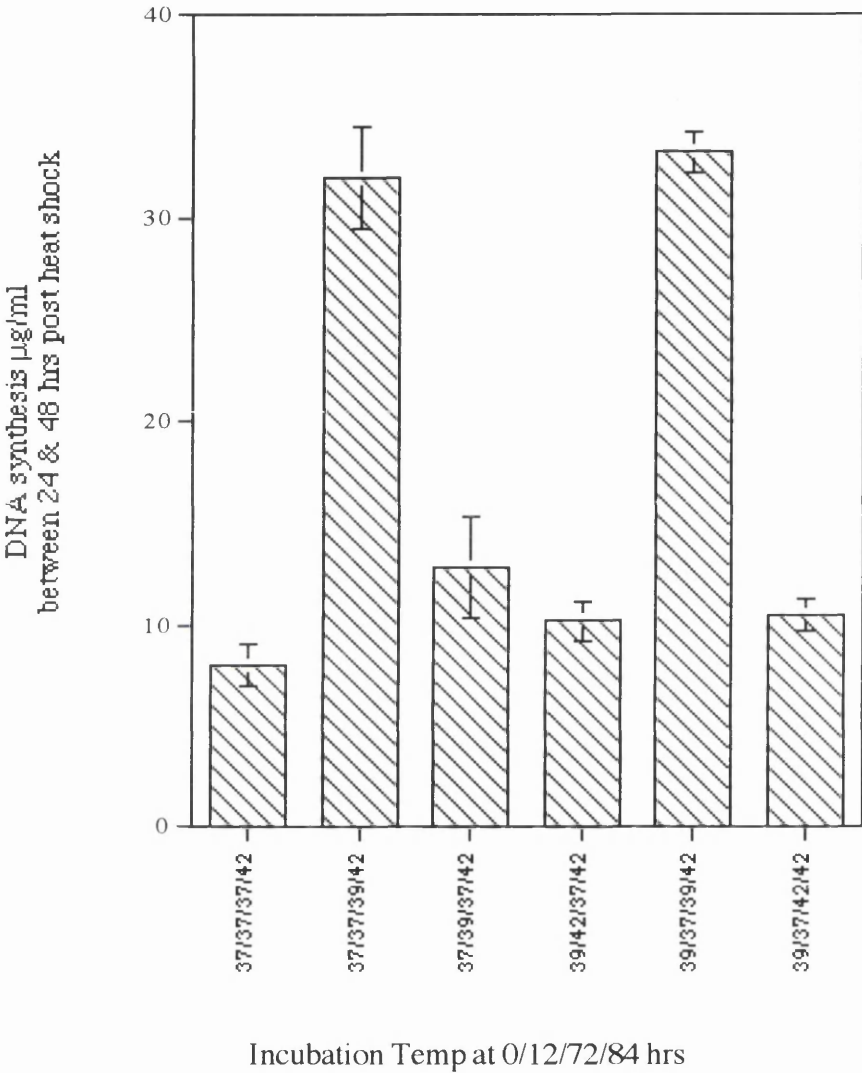


Figure 18. The re-establishment of thermotolerance in LNCaP cells. The cells were exposed to different combination of nonlethal or lethal temperatures at 0hrs, 12hrs, 72 hrs and 84 hrs as indicated on the graph. Cell samples were taken at 24 and 48 hours after the final heat treatment (84hrs and the level of DNA synthesis calculated. Each column represents the mean of three separate experiments \pm standard deviation.

Re-establishment of thermotolerance in LNCaP cells



Conclusions

Figure 18 shows the re-establishment of thermotolerance in LNCaP cells. In the first column (37/37/37/42) there is no thermotolerance as the cells were not exposed to a sublethal temperature to induce thermotolerance. This is used as a control column to which all other columns in the graph are compared. A low level of DNA synthesis was observed in the control cells.

Column 2 (37/37/39/42) shows the maximal levels of thermotolerance as there is a 12 hour period between the sub-lethal and lethal heat shocks (see figure 15).

DNA synthesis in column 3 (37/39/37/42) is greatly reduced. This is because the incubation period between the sub-lethal and lethal heat shocks was 72 hours, which has been shown in figure 16 to greatly reduce the levels of thermotolerance in these cells.

In column 4 (39/42/37/42), the cells were exposed to sub-lethal and lethal temperatures with 12 hours between the two and this was followed by a final lethal incubation 72 hours after the first. Again there is a little thermotolerance (a p value of 0.0600 suggests that the level of DNA synthesis is not significantly higher than column 1).

Column 5 (39/37/39/42) shows that thermotolerance can be re-established in these cells by a further incubation at the sublethal temperature. The level of DNA synthesis is significantly greater than in column 1 ($p=0.0001$ extremely significant difference), the levels of DNA synthesis in column 2 and 5 are comparable ($p=0.7147$, no significant difference between the two groups).

The final column (39/37/42/42) shows that thermotolerance can not be re-established by an exposure to a lethal temperature if the sublethal dose occurred 72 hours before the 1st lethal dose. The level of DNA synthesis is comparable with level in column 3 where there is a 72 hour period between the sub-lethal and the lethal temperatures ($p=0.1893$, no significant difference between column 3 and 6).

This experiment was carried out on all three cell lines. All three lines showed the same patterns of establishing and re-establishing thermotolerance. Only the results for the LNCaP cells are shown, to simplify the graph and the interpretation of the results. The statistical significance of each column compared to column 1 was similar in all three cell lines. These results give direct evidence for the ability of these cell lines to re-establish thermotolerance once it has been lost.

3.6. The combined effect of anti-androgen and hyperthermia therapy on prostate cancer cells.

Introduction

Figure 9 demonstrates that LNCap cells respond positively to DHT. They are also positively stimulated by the anti-androgen hydroxyflutamide (OH-Flut) [Lloyd *et al.*, 1992; Veldscholte *et al.*, 1992]. This stimulation of LNCaP cells by OH-Flut conflicts with the antiandrogenic effect of this drug *in vivo* [Poyet & Labrie, 1985] and may reflect differing mechanisms of action *in vitro* and *in vivo*.

Both DU145 and PC3 cells are not growth stimulated by DHT as seen in figure 9, nor are they affected by OH-Flut [Lloyd *et al.*, 1992; G.Carruba personal communication].

Hyperthermia has been used in conjunction with endocrine therapy as a treatment for prostate cancer. It has even been reported that their combined use *in vivo* results in a more effective treatment compared with the individual use of either method [Servadio *et al.*, 1987; Linder *et al.*, 1990]. The *in vitro* studies combining hyperthermia and endocrine therapies seem to support this *in vivo* effect [Lloyd *et al.*, 1992].

It was decided to investigate the role of thermotolerance in the combined effect of OH-Flut and hyperthermia on all three cell lines. It was also decided to investigate the effect of the timing of each treatment ^{sequence} to determine whether it is more effective to start the anti-androgen therapy prior to or after the heat shock dose.

Methods

The cells were subcultured as described in method section 2.2.2., so that a 20% confluent monolayer was formed within 6-well plastic tissue culture plates (8 plates per cell line) and the cells were allowed to "plate down" for 24 hours.

This experiment was carried out in two parts (*summarised in figure 19*) which were run simultaneously. In part A the anti-androgen OH-Flut was given 48 hours prior to the first heat shock treatment. In part B the heat shock treatment was given first, followed by the OH-Flut 12 hours after the normally lethal temperature.

Prior to adding the OH-Flut, the culture medium was removed from the wells and the cells washed twice with prewarmed PBS. The medium was replaced with steroid-free medium supplemented with OH-Flut (final concentration 1×10^{-9} M). The steroid free medium \pm OH-Flut was replaced every 48 hours.

The heat shock treatment was designed to induce maximal thermotolerance as seen in figure 10. An initial incubation for 2 hours at the sublethal temperature of 39°C was followed by a 12 hour recovery period before the administration of the normally lethal dose of 42°C for a final 2 hours. Heat shock was again achieved by placing the tissue culture plates in an humid incubator (LEEC) with atmospheric air enriched with 5% CO₂ which had been preset to either 39°C or 42°C.

Cells were harvested at 24 and 48 hours after the second heat shock treatment in the case of the first part of the experiment and 48 and 72 hours after the addition of OH-Flut in the second part.. Cell lysis and DNA assays were carried out as in section 3. of the methods chapter and a comparison made between the levels of DNA synthesis.

Results

Figures 20 and 21 show the effect of combining antiandrogen therapy and heat shock on the survival of all three cell lines. DNA synthesis between 24 and 48 hours (post heat shock) or 48 and 72 hours (after OH-Flut addition) is used as a measurement of cell survival.

Each column represents the mean of triplicate experiments \pm standard errors

Figure 20 represents the first part of this experiment in that the cells are incubated in OH-Flut for 48 hours prior to the heat shock treatment.

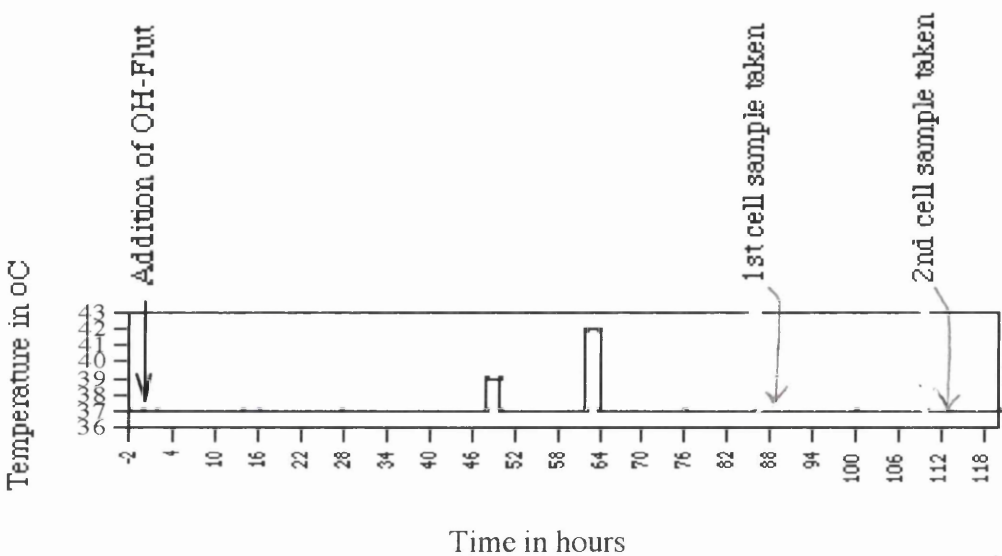
Figure 21 represents the second part of this experiment in that the cells have been heat shocked prior to the administration of OH-Flut

Figure 19. Time course of experiments to see the combined effects of antiandrogen and hyperthermic treatment on prostate cells.

In part A. cells were given 1×10^{-9} M OH-Flut 48 hrs before they were heat shocked. Cell samples were taken 24 and 48 hours after the 2nd heat shock.

In part B. cells were heat shocked first and 1×10^{-9} M OH-Flut was added 12 hours after the 2nd heat shock. Cell samples were taken 48 and 72 hours after the addition of 1×10^{-9} M OH-Flut.

Part A. Anti-androgen effect on thermotolerance



Part B. The effect of heat shock on anti androgen action

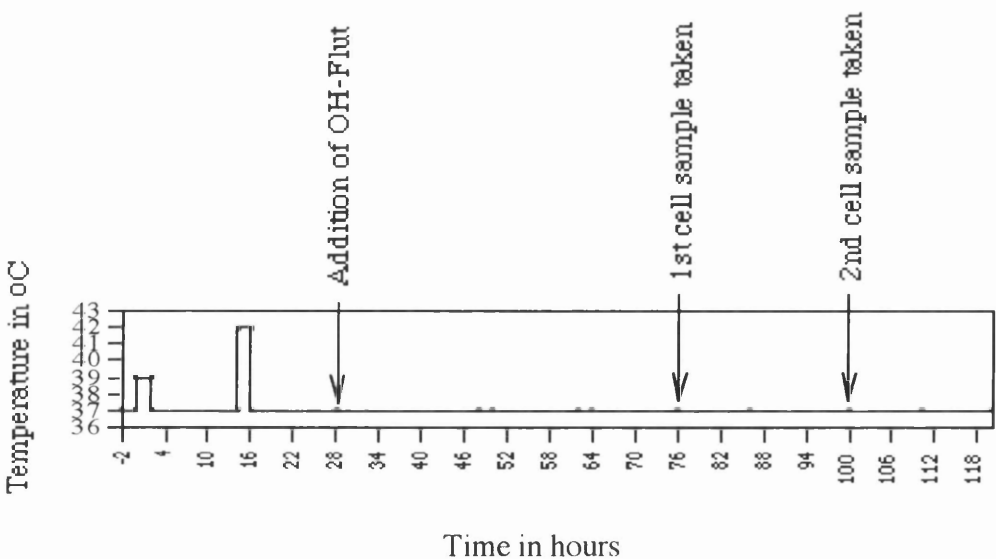


Figure 20. The effect of antiandrogens on thermolerance of all three prostate cell lines.

The cells were exposed to $1 \times 10^{-9} \text{M}$ HO-Flut for 48 hours prior to heat shock, which was achieved by incubating the cell at 39°C for 2 hours followed by a 12 hour recovery period before a final heat shock of 42°C .. Cell samples were taken at 24 and 48 hours after the second heat shock treatment and the DNA synthesis between 24 and 48 hours is expressed as a % of maximum for each cell line.

Each column represents the mean of three separate experiments \pm standard deviation .

Where no error bars are visible, the standard error is too small to be shown.

The effect of antiandrogens on prostate cell thermotolerance

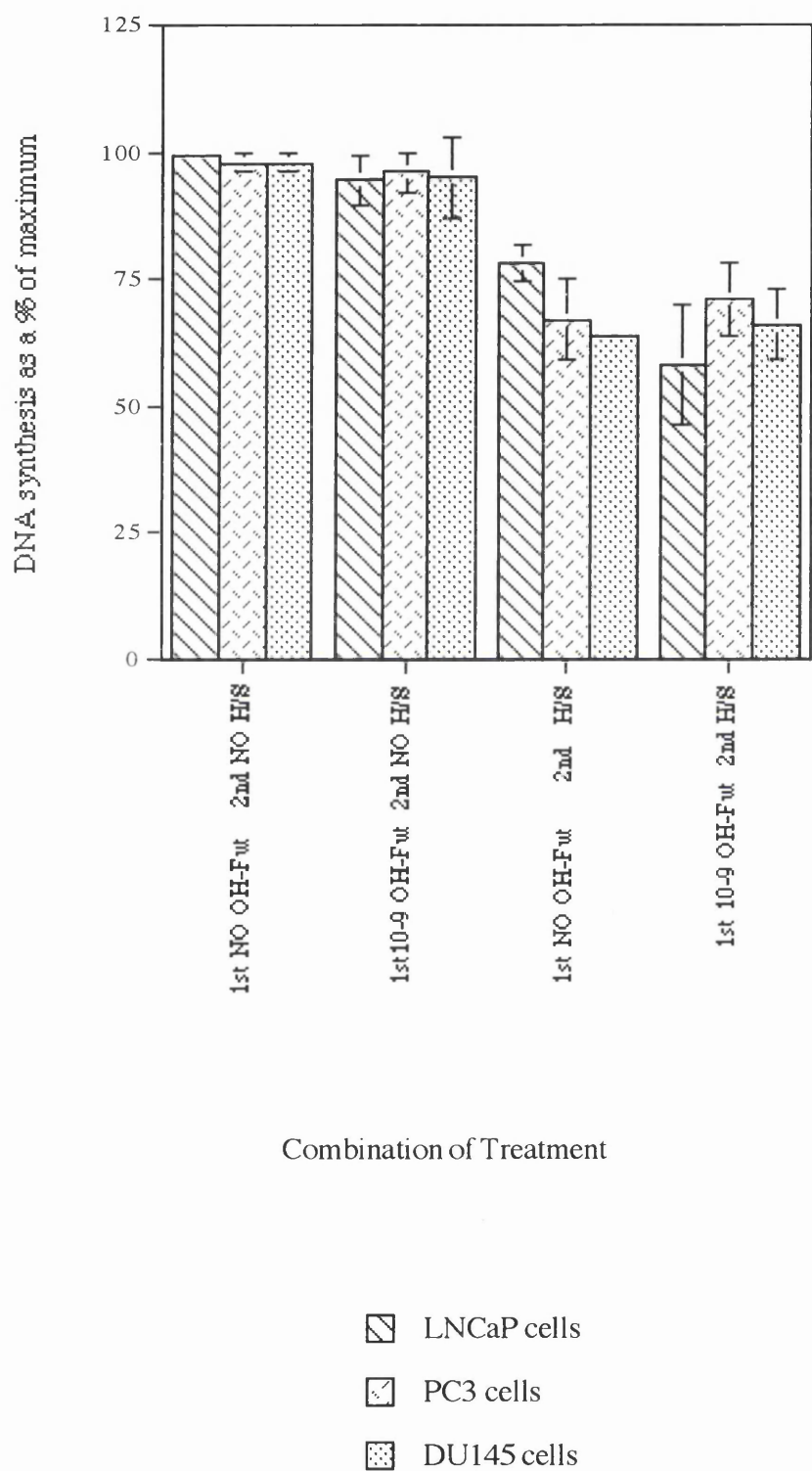
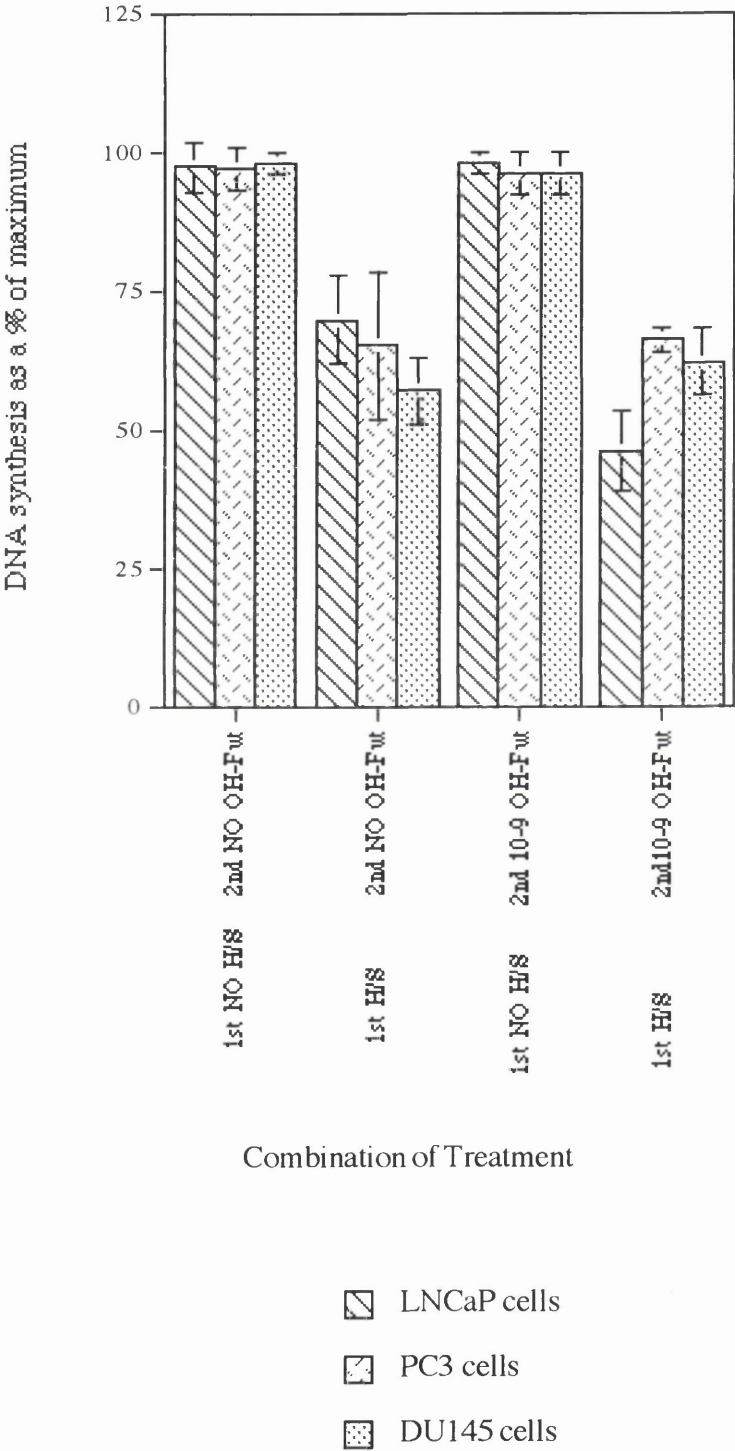


Figure 21. The effect of heat shocking all three cell lines prior to treatment with antiandrogens.

Thermotolerance was induced in the cells prior to incubation with 1×10^{-9} M OH-Flut. Thermotolerance was achieved by incubating the cell at 39°C for 2 hours followed by a 12 hour recovery period before a final heat shock of 42°C . Cell samples were taken at 48 hours and 72 hours after commencing 1×10^{-9} M OH-Flut and the DNA synthesis between these points was expressed as a % of maximum for each cell line.

Each column represents the mean of three separate experiments \pm standard deviation .

The effect of thermotolerance on antiandrogen action in prostate cells



Conclusions

Figure 20 shows all three cell lines' responses to heat shock after prior exposure to the antiandrogen OH-Flut, assessed in terms of cell growth between 24 and 48 hours after the 2nd heat shock. There is no significant difference between the first two columns ($p=0.2158$ for LNCaPs, $p=0.5072$ for PC3s and $p=0.530$ for DU145s) which suggests that in this instance none of the cell lines respond to OH-Flut in contrast to earlier work done in this lab. In the third column the cells were exposed to heat shock without prior exposure to OH-Flut. In this instance the cell survival is reduced as would be expected from previous results. When the cells are exposed to OH-Flut for 48 hours prior to the heat shock treatment (see column 4) both PC3 and DU145 cells show the same survival as if heat shock was given alone. This is not surprising considering both cell lines are unresponsive to hormone and antiandrogens. There is reduced cell survival in the LNCaP cells on exposure to OH-Flut prior to heat shock but due to the large standard deviation seen in column 4 LNCaP it is of limited value ($p=0.0618$, not quite significant).

Figure 21 shows the different cells response to OH-Flut after prior heat shock treatment. As in part 1 (figure 17) OH-Flut treatment alone has no effect on the three cell lines and the heat shock treatment alone reduces cell survival (comparing column 1 and 2 $p=0.008$ for LNCaPs, $p=0.015$ for PC3s and $p=0.006$ for DU145s). Exposure to heat shock which would induce thermotolerance prior to treatment with OH-Flut does have a significant effect on cell survival for LNCaP cells only compared to either treatment alone (heat shock alone compared to heat shock and OH-Flut $p=0.0233$ and the combination of heat shock and OH-Flut compared to OH-Flut alone has a p value of $p=0.004$).

These results suggest that the combination of heat shock and anti-androgen represents a synergistic partnership capable of suppressing prostate cell growth, but this phenomenon is confined to the androgen/"antiandrogen"-responsive cell line LNCaP and is more evident if thermotolerance induction precedes OH-Flut exposure.

3.7. Long term effects of combining antiandrogen therapy and heat shock on LNCaP cell.

Introduction

In the last section it was found that combining antiandrogen and heat shock therapies led to a more effective cell kill of LNCaP cells than either treatment alone and that this synergistic effect could be due to the establishment of thermotolerance in these cells.

Thermotolerance is achieved by exposing the cells to an elevated but sublethal temperature, which is 39°C in the case of the prostate cell lines LNCaP, DU145 and PC3. Once the cells have acquired thermotolerance it does not become inherent in these cells but is rather slowly lost (see figure 15.) but can be regained by a further exposure to 39°C (see figure 16).

If the combined effect of antiandrogen and heat shock is due to the existence of thermotolerance within the cells it would be logical to assume that as thermotolerance is lost so would the synergistic effect of the combination. The experiment described below was designed to test this.

Methods

The cells were subcultured as described in methods section 2.2.2., so that a 20% confluent monolayer was formed in 24 25cm² plastic tissue culture flasks and the cells were allowed to "plate down" for 24 hours. Just before the start of the experiment, the culture medium was removed from the flasks and the cells washed twice with prewarmed PBS. The medium was replaced with steroid-free medium supplemented with OH-Flut (final concentration 1x10⁻⁹M). The steroid free medium supplemented with OH-Flut was replaced every 48 hours. The cells were then either heat shocked once or twice as shown in figure 22. (control cells were cultured in OH-Flut but not heat shocked). The first group of

cells were incubated at 39°C for 2 hours at time 0, the second group were exposed to 39°C for 2 hours at time 0 and again for 2 hours at 72 hours. Heat shock was achieved by placing the tissue culture plates in an humid incubator (LEEC) with atmospheric air enriched with 5% CO₂ which had been preset to either 39°C.

Cell samples were taken every 24 hours. Cell lysis and DNA assays were carried out as described in the methods chapter section 3..

Results

Figure 23 shows the long term effects of combining antiandrogen therapy with heat shock therapy. DNA synthesis is taken to be a measure of cell survival and is given as a percentage of DNA present at the beginning of each 24 hour period (e.g. DNA at 0 hrs =20 µg/ml, DNA at 24 hrs=28.5 µg/ml therefor % growth is $8.5 \div 20 \times 100 = 42.5\%$).

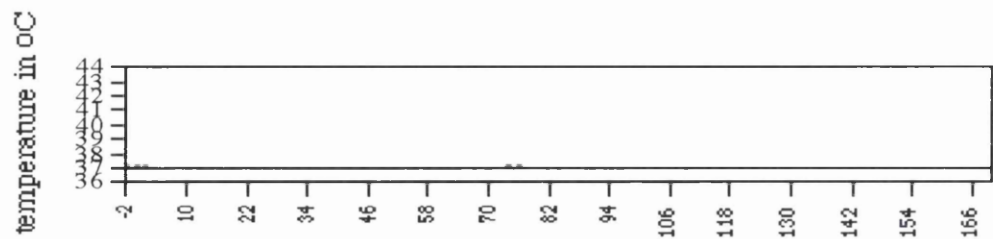
Each experiment was carried out in triplicate and the means and standard deviations calculated.

Figure 22. Graph showing heat shock treatment, timing and temperature.

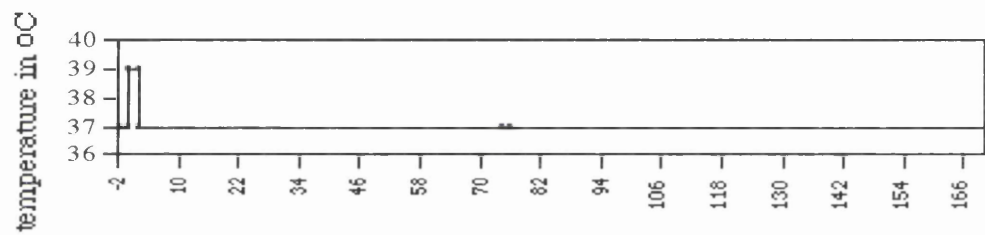
All cells (LNCaP) were incubated in steroid-free medium supplemented with 1×10^{-9} M OH-Flut throughout. The control cells were not heat shocked. One group of experimental cells were heat shocked once for 2 hours at time 0. The other group was heat shocked twice once at time 0, and again at 72 hours. DNA samples were taken every 24 hours through out.

Time course of heat shock treatments in long term effects of combination therapies

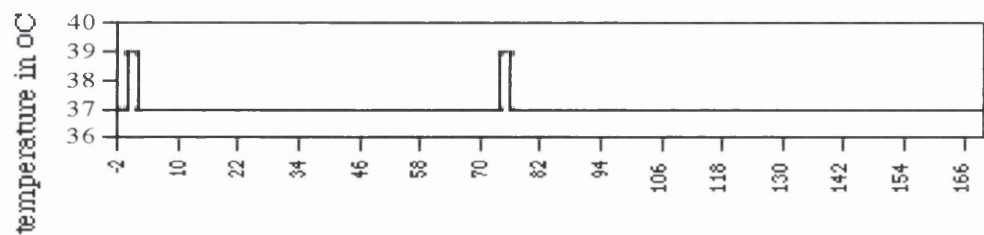
Control



Single heat shock



Repeated heat shocks



time in hours

Figure 23. The effects of time on the combined therapies of heat shock and anti-androgen.

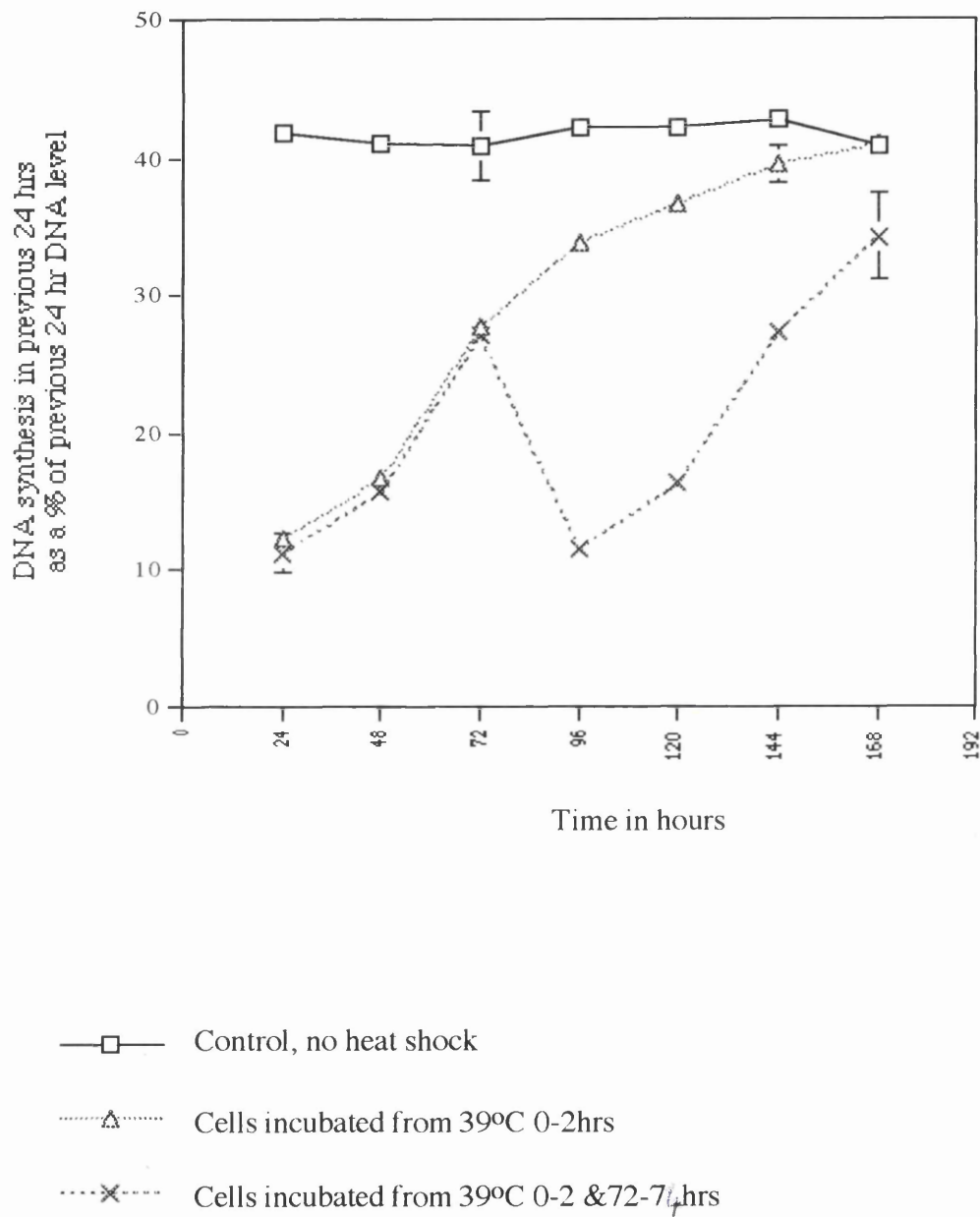
All cells (LNCaP) were incubated in steroid-free medium supplemented with 10^{-9}M ϵ

OH-Flut throughout. The experimental cells were subjected to heat shock by incubating them at 39°C for 2 hours either once (Δ) at time 0 or twice, at time 0 and again at 74 hours (X), see figure 22 for graphical representation. DNA samples were taken every 24 hours and DNA synthesis is given as a percentage of the DNA level at the beginning of each 24 hour period.

Each point represents the mean of triplicate experiments \pm the standard deviation.

Where no error bars are visible, the standard error is too small to be shown.

Long Term Effects of combining Antiandrogen therapy
and Heat Shock on LNCaP cells



Conclusions

The long term effects of combining antiandrogen and heat shock treatments are seen in figure 23. The effects of a single heat shock treatment on antiandrogen action can be seen to decrease with time until after 7 days they are at control levels. At 24 hours there is statistical^{ly} significant difference between the heat shocked samples and the control ($p < 0.0001$), but by 168 hrs (7 days) there is no statistical^{ly} significant difference between the cells which received one heat shock and the control cells ($p = 0.5185$).

A second heat shock treatment 72 hours after the first reintroduces the synergistic effect. There is no statistical^{ly} significant difference between the level of DNA synthesis after the first and second heat shock ($p = 0.7415$) but comparing the two heat shock samples at 96 hours there is a statistically significant reduction in DNA synthesis between the cells that have been heat shocked once and twice ($p < 0.0001$).

The time course of the loss of the combined effect is similar to that of the loss of thermotolerance (see figure 16) and the ability of a second heat shock to re-establish the combined effect is similar to the re-establishment of thermotolerance (see figure 17). This supports the idea that thermotolerance (and therefore the heat shock response and hsp's) is responsible for the increased effectiveness of OH-Flut.

Chapter 4.

The effect of hyperthermia on the androgen receptor

4.1. The effect of heat shock on the binding affinity of the androgen receptor in prostate cancer cell lines.

The results in the previous chapter suggest that the combination of heat shock and anti-androgen therapies has a synergistic effect on prostate cell growth, but that this phenomenon is confined to the androgen/anti-androgen-responsive cell line, LNCaP. Therefore it would seem logical to assume that the androgen receptor played a role in this synergistic effect and to investigate the effect of heat treatment on the binding affinity and overall structure of the androgen receptor in both cell lines which possess androgen receptors (LNCaP and DU145 cells).

Two classes of binding sites for steroid receptors have been identified by Scatchard analysis of ligand binding assays [Eriksson, 1978; Markaverich & Clark, 1979; Castagnetta *et al.*, 1992]. Type I sites bind ligand with high affinity ($K_d > 1 \text{ nM}$) and low capacity, while type II sites bind ligand with a lower affinity but higher capacity [Castagnetta *et al.*, 1992]. The type I sites are assumed to reflect the molecular mediators of steroid responses whereas the type II sites are perhaps storage or transport proteins. The experiment described below was designed to look at both classes of binding sites.

Methods

The cells were subcultured as described in methods section 2.2.2., so that a 20% confluent monolayer was formed in 175cm² plastic tissue culture flasks and the cells were allowed to "plate down" for 24 hours. The culture medium was changed prior to commencing heat shock. Heat shock was achieved by placing the tissue culture plates in an humid incubator with atmospheric air enriched with 5% CO₂ at 39°C. The cells were incubated for two hours at this temperature followed by a recovery period of between 0-72 hours at 37°C. The cells were harvested after the recovery period and the separation of nuclear and

cytosol fractions carried out as described in method section 5. Androgen receptor ligand binding assays carried out as described in the method section 5.. The concentration range of radiolabeled steroid ([17 α -methyl-³H] mibolerone) used was from 0.1-5nM. This enables the detection of type I and type II binding sites [Eriksson, 1978; Markaverich & Clark, 1979; Castagnetta *et al.*, 1992].

Receptor assay data was processed using Scatchard analysis and a modification of a least^{square}/fit routine [Leake *et al.*, 1987] (Oncolog2.2®), run on an IBM-PC, yielding both dissociation constant (Kd) and concentration values (fmol/ml homogenate). Receptor concentration was expressed as fmol/mg DNA. Data was analysed using a model for two binding sites to assess whether there was one or two ligand binding sites [Carruba, 1994].

Results

Figures 24 and 26 show typical Scatchard plots of nuclear and cytosol androgen binding obtained from ligand binding assays for AR in non-heat shocked LNCaP (figure 24) and DU145 (figure 26) cells and give the Kd and concentrations of the AR within those cells.

Tables 2 and 3 show the Kd and concentrations of type I and type II receptors in the cytosol (table 2) and nuclear (table 3) fractions of LNCaP cells at increasing time after heat shock. Tables 4 and 5 give the cytosol (table 4) and nuclear (table 5) concentrations of type I and type II receptors and their Kds for DU145 cells at increasing time after heat shock.

Figures 25 (LNCaP) and 27 (DU145) show the changes to the Kd of type I AR after the cells have been heat shocked and allowed to recover for increasing periods of time (0-72 hours).

One-way analysis of variance (ANOVA) test was carried out to determine if the differences in the means of the Kds and receptor concentrations at different time points were due to chance or if the values were significantly different from each

other. In the cases where the ANOVA test showed a significant difference ($p < 0.05$) unpaired t tests comparing each experimental point with the control were carried out.

Figure 24. Ligand binding assay of androgen receptor in LNCaP cells.

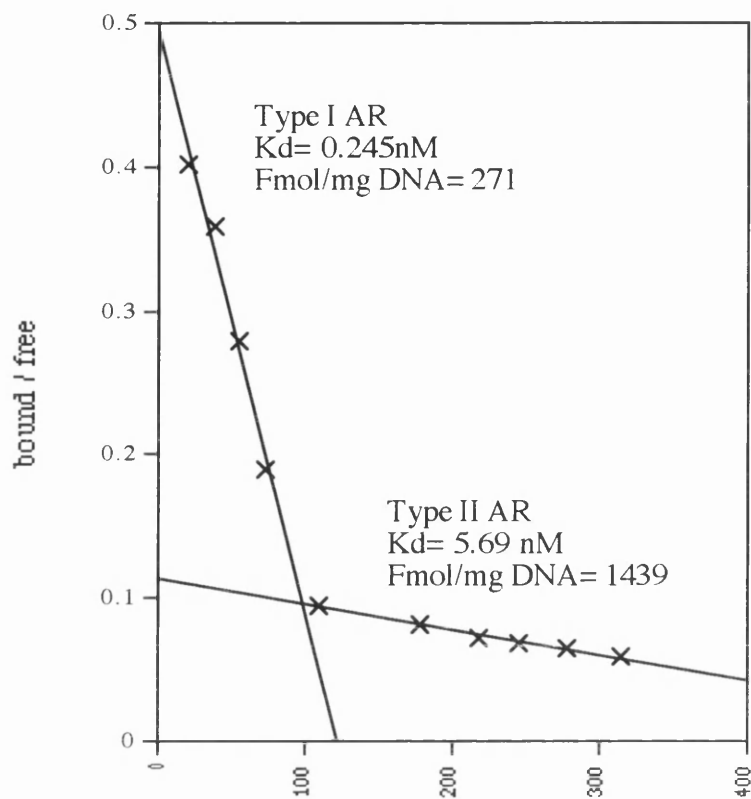
A typical Scatchard plot of cytosol and nuclear androgen binding is shown.

The concentration range of radiolabeled steroid ([17 α -methyl-³H] mibolerone) used was from 0.1-5nM. The presence of two androgen binding sites was determined according to a significantly better fit for the two-sites model.

K_d =dissociation constant

BpM= bound picomolar

Cytosol Fraction



Nuclear Fraction

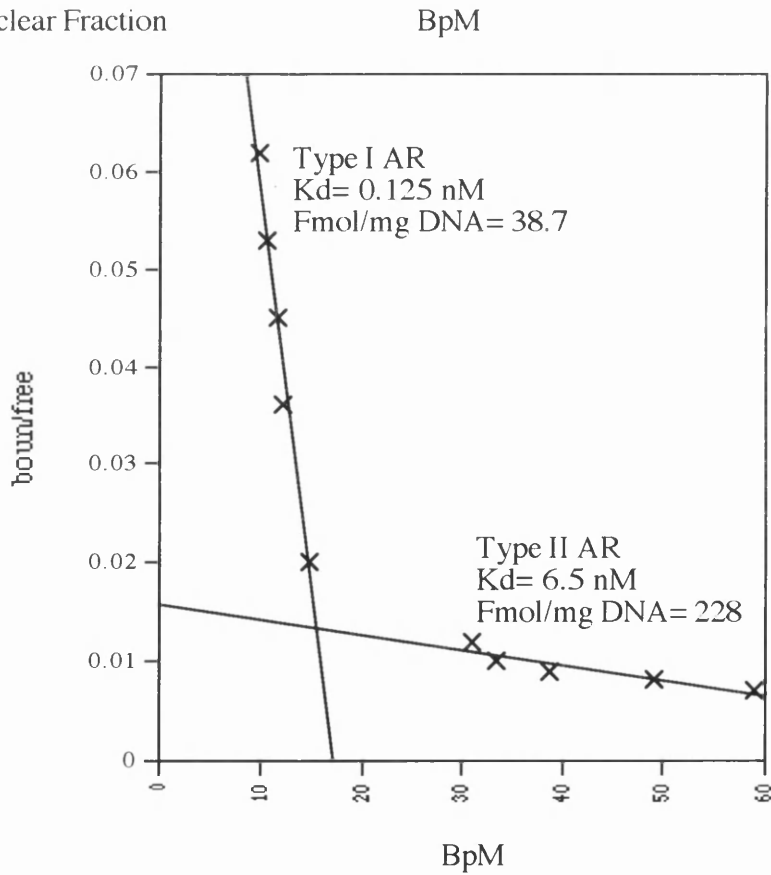


Table 2. Dissociation constants and concentrations of androgen receptors in heat shocked LNCaP cell cytosol fraction.

Table 2 shows the dissociation constants (K_d) of the type I and type II androgen receptor in the cytosol fraction of LNCaP cells. The receptor concentration is expressed as fmol/ mg DNA.

The dissociation constants were obtained by carrying out steroid receptor ligand binding assays on cells which had been heat shocked and allowed to recover for increasing lengths of time. Scatchard analysis was then carried out using Oncolog2.2® and the existence of one or two binding sites established. Each experiment was carried out in triplicate and the results shown are the means obtained \pm the standard deviations (S.D.)

Sample taken X hours after Heat Shock	Type I Kd (nM) (mean \pm S.D)	Type I (fmol/ mg DNA) (mean \pm S.D)	Type II Kd (nM) (mean \pm S.D)	Type II(fmol/ mg DNA) (mean \pm S.D)
Non H/S	0.232 \pm 0.050	278 \pm 38	4.67 \pm 0.886	1331 \pm 207
0	0.248 \pm 0.037	304 \pm 26	4.32 \pm 0.789	1377 \pm 270
2	0.258 \pm 0.041	354 \pm 34	4.81 \pm 0.851	1708 \pm 244
4	0.248 \pm 0.032	391 \pm 24	3.56 \pm 0.473	1450 \pm 99
6	0.309 \pm 0.035	249 \pm 15	4.01 \pm 0.344	1100 \pm 78
8	0.391 \pm 0.047	327 \pm 10	3.87 \pm 0.055	1169 \pm 62
10	0.512 \pm 0.019	367 \pm 14	5.10 \pm 0.415	1270 \pm 114
12	0.523 \pm 0.035	360 \pm 8	3.33 \pm 0.076	1027 \pm 29
24	0.403 \pm 0.023	343 \pm 5	3.40 \pm 0.219	1112 \pm 109
48	0.339 \pm 0.024	288 \pm 16	3.44 \pm 0.370	1143 \pm 76
72	0.252 0.027	371 \pm 22	3.77 \pm 0.280	1299 \pm 143

Table 3. Dissociation constants and concentrations of androgen receptors in heat shocked LNCaP cell nuclear fraction.

Table 3 shows the dissociation constants (K_d) of the type I and type II androgen receptor in the nuclear fraction of LNCaP cells. The receptor concentration is expressed as fmol/ mg DNA.

The dissociation constants were obtained by carrying out steroid receptor ligand binding assays on cell which had been heat shocked and allowed to recover for increasing lengths of time. Scatchard analysis was then carried out using Oncolog2.2® and the existence of one or two binding sites established. Each experiment was carried out in triplicate and the results shown are the means obtained \pm the standard deviations (S.D.)

Sample taken X hours after Heat Shock	Type I Kd (nM) (mean \pm S.D)	Type I (fmol/ mg DNA) (mean \pm S.D)	Type II Kd (nM) (mean \pm S.D)	Type II(fmol/ mg DNA) (mean \pm S.D)
Non H/S	0.139 \pm 0.12	63.3 \pm 24.8	6.46 \pm 0.901	355 \pm 144
0	0.143 \pm 0.12	72.3 \pm 22.3	6.40 \pm 2.9	322 \pm 202
2	0.140 \pm 0.02	66.5 \pm 9.2	6.29 \pm 2.03	334 \pm 77
4	0.13 \pm 0.14	59.0 \pm 18.3	6.38 \pm 0.57	316 \pm 96
6	0.13 \pm 0.098	56.1 \pm 6.1	6.6 \pm 0.6	335 \pm 153
8	0.143 \pm 0.098	62.3 \pm 2.8	6.38 \pm 0.47	365 \pm 134
10	0.127 \pm 0.176	55.5 \pm 7.1	6.08 \pm 1.8	376 \pm 140
12	0.133 \pm 0.007	60.5 \pm 1.2	5.83 \pm 1.0	337 \pm 102
24	0.132 \pm 0.03	61.5 \pm 1.5	5.95 \pm 0.636	452 \pm 50
48	0.128 \pm 0.19	64.5 \pm 2.1	5.55 \pm 0.49	349 \pm 126
72	0.125 \pm 0.014	56.0 \pm 9.9	6.6 \pm 0.283	384 \pm 140

Figure 25. The effect of hyperthermia on the type I AR Kd in LNCaP cytosol fraction over time.

The cells were heat shocked and allowed to recover for increasing lengths of time (0-72 hours). Androgen receptor ligand binding assays were carried out and Scatchard analysis was used to calculate the Kds of the receptors.

The dotted line at 0.232nM represents the mean Kd of non heat shocked cells.

Each point is the mean of three separate experiments \pm the standard deviation.

Student t tests comparing the Kd at each time point with the control was carried out. ϕ beside a point represents a statistical difference between that point and the control (no heat shock).

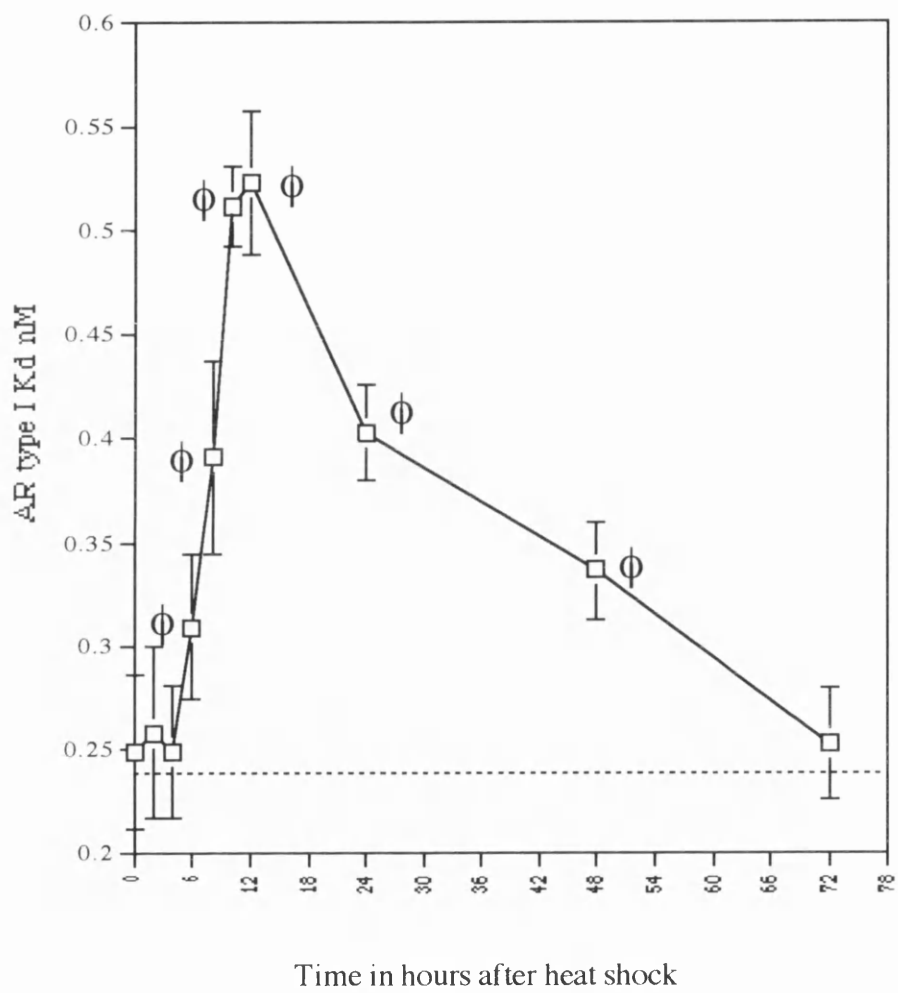


Figure 26. Ligand binding assay of androgen receptor in DU145 cells.

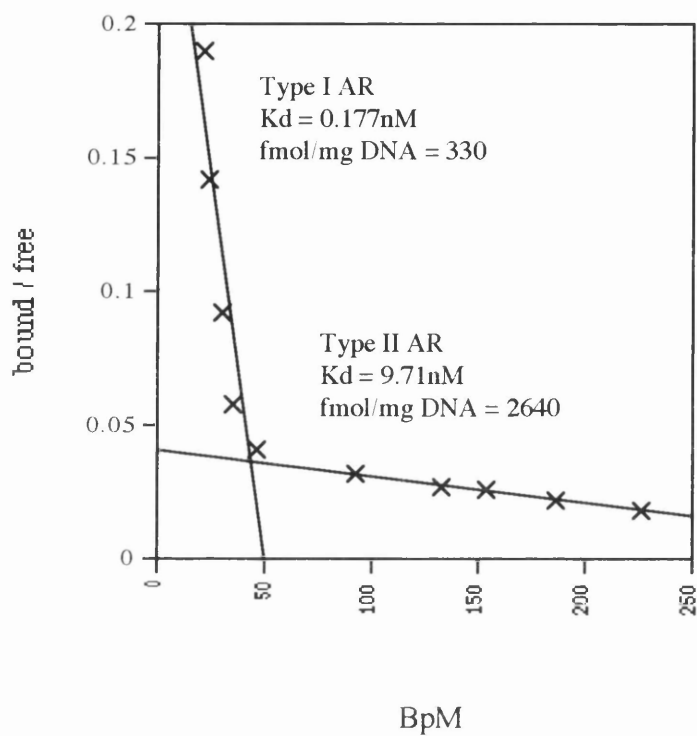
A typical Scatchard plot of cytosol and nuclear androgen binding is shown.

The concentration range of radiolabeled steroid ([17 α -methyl-³H] mibolerone) used was from 0.1-5nM. The presence of two androgen binding sites was determined according to a significantly better fit for the two-sites model (Oncolog2.2®).

K_d =dissociation constant

BpM= bound picomolar

Cytosol



Nuclear

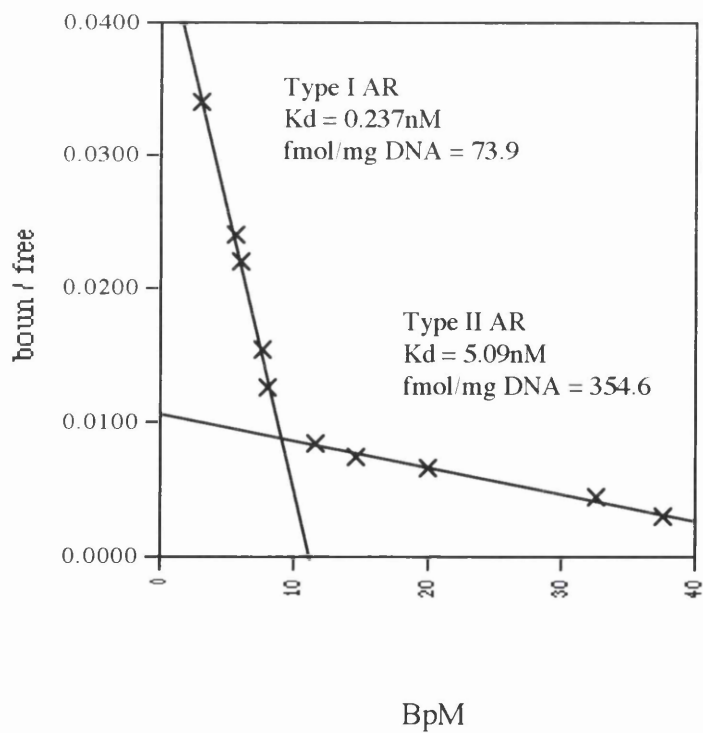


Table 4. Dissociation constants and concentrations of androgen receptors in heat shocked DU145 cytosol fraction.

Table 4 shows the dissociation constants (K_d) of the type I and type II androgen receptor in the cytosol fraction of DU145 cells. The receptor concentration is given as fmol/ mg DNA.

The dissociation constants were obtained by carrying out steroid receptor ligand binding assays on cells which had been heat shocked and allowed to recover for increasing lengths of time. Scatchard analysis was then carried out using Oncolog2.2® and the existence of one or two binding sites established. Each experiment was carried out in triplicate and the results shown are the means obtained \pm the standard deviations (S.D.)

Sample taken X hours after Heat Shock	Type I Kd (nM) (mean \pm S.D)	Type I (fmol/ mg DNA) (mean \pm S.D)	Type II Kd (nM) (mean \pm S.D)	Type II(fmol/ mg DNA) (mean \pm S.D)
Non H/S	0.163 \pm 0.19	334.5 \pm 20.5	9.7 \pm 0.9	2936 \pm 419
0	0.155 \pm 0.056	328 \pm 26	7.6 \pm 1.7	2459 \pm 233
2	0.154 \pm 0.049	303 \pm 10	8.7 \pm 0.4	2432 \pm 62
4	0.140 \pm 0.049	278 \pm 3	8.6 \pm 0.6	2343 \pm 126
6	0.207 \pm 0.084	349 \pm 54	8.2 \pm 1.1	2472 \pm 412
8	0.332 \pm 0.035	399 \pm 33	7.7 \pm 0.9	1785 \pm 169
10	0.470 \pm 0.035	389 \pm 12	7.5 \pm 1.4	1893 \pm 79
12	0.575 \pm 0.049	393 \pm 5	7.3 \pm 0.7	1972 \pm 420
24	0.363 \pm 0.098	406 \pm 6	8.0 \pm 0.9	2067 \pm 339
48	0.376 \pm 0.026	378 \pm 27	6.1 \pm 0.7	1859 \pm 122
72	0.141 \pm 0.034	321 \pm 5	9.1 \pm 0.3	2271 \pm 558

Table 5. Dissociation constants and concentrations of androgen receptors in heat shocked DU145 nuclear fraction.

Table 5 shows the dissociation constants (K_d) of the type I and type II androgen receptor in the nuclear fraction of DU145 cells. The receptor concentration is expressed as fmol/ mg DNA.

The dissociation constants were obtained by carrying out steroid receptor ligand binding assays on cells which had been heat shocked and allowed to recover for increasing lengths of time. Scatchard analysis was then carried out using Oncolog2.2® and the existence of one or two binding sites established. Each experiment was carried out in triplicate and the results shown are the means obtained \pm the standard deviations (S.D.).

Sample taken X hours after Heat Shock	Type I Kd (nM) (mean \pm S.D)	Type I (fmol/ mg DNA) (mean \pm S.D)	Type II Kd (nM) (mean \pm S.D)	Type II(fmol/ mg DNA) (mean \pm S.D)
Non H/S	0.251 \pm 0.19	73.3 \pm 31.7	4.85 \pm 0.33	377 \pm 33.2
0	0.247 \pm 0.13	86 \pm 6.8	5.07 \pm 0.15	369 \pm 17.7
2	0.219 \pm 0.1	60 \pm 18.3	4.94 \pm 0.21	348 \pm 15.5
4	0.213 \pm 0.18	66 \pm 3.2	4.73 \pm 0.70	349 \pm 31.8
6	0.24 \pm 0.05	80 \pm 7.2	5.1 \pm 1.40	387 \pm 41.7
8	0.226 \pm 0.05	69 \pm 3.5	5.18 \pm 0.36	356 \pm 54.4
10	0.230 \pm 0.70	73 \pm 15.6	5.76 \pm 0.20	383 \pm 10.6
12	0.200 \pm 0.19	80 \pm 29.7	5.56 \pm 1.20	383 \pm 72.1
24	0.260 \pm 0.09	76 \pm 5.5	5.32 \pm 0.50	344 \pm 62.9
48	0.270 \pm 0.03	71 \pm 5.4	4.92 \pm 0.47	364 \pm 68.5
72	0.240 \pm 0.17	78 \pm 5.2	5.71 \pm 0.28	378 \pm 30.4

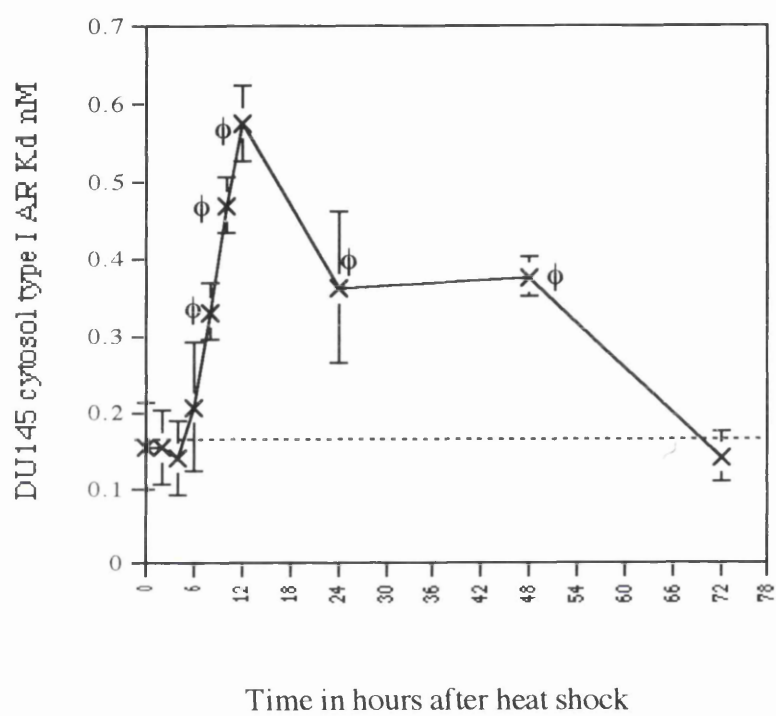
Figure 27. The effect of hyperthermia on the type I AR Kd in DU145 cytosol fractions over time.

The cells were heat shocked and allowed to recover for increasing lengths of time (0-72 hours). Androgen receptor ligand binding assays were carried out and Scatchard analysis was used to calculate the Kds of the receptors.

The dotted line at 0.163nM represents the mean Kd of type I AR in non heat shocked cells.

Each point is the mean of three separate experiments \pm the standard deviation.

Student t tests comparing the Kd at each time point with the control was carried out. ϕ beside a point represents a statistical difference between that point and the control (no heat shock).



Conclusions

The two classes of binding sites for the androgen receptor in LNCaP and DU145 cells appear to react differently to heat shock.

In LNCaP cells the cytosol type I receptor (high affinity, low capacity) under non-stressed conditions has a K_d of $0.232 \pm 0.05 \text{ nM}$ but 12 hours after the cells were heat shocked (2hrs at 39°C) the K_d had risen to $0.523 \pm 0.035 \text{ nM}$. By 72 hour post heat shock, the K_d had fallen again to $0.252 \pm 0.027 \text{ nM}$ (see table 2 and figure 25). An ANOVA test showed that the variation was significantly greater than would be expected by chance ($p < 0.0001$) and so Student *t* tests comparing the K_d at each time point with the control (non heat shocked cells) was carried out. There was no statistically significant difference between the control K_d and those in the first 4 hours after heat shock ($p > 0.01$). In figure 25, the increase in K_d seen between 6 and 12 hours post heat shock is statistically significantly different from the control group ($p < 0.001$). The K_d decreases to control levels by 72 hours post heat shock. Figure 27 and table 4 show that the type I binding sites in DU145 also displays an increase in the K_d values after heat shock. In non-heat shocked cells the K_d is $0.163 \pm 0.19 \text{ nM}$. Twelve hours after heat shock, the type I K_d is $0.575 \pm 0.049 \text{ nM}$, which is significantly higher than in non-heat shocked cells ($p = 0.0082$). By 72 hours post heat shock the K_d had fallen again to the non-heat shocked level.

The K_d of the type II receptor in the cytosols of both LNCaP and DU145 did not vary significantly after heat shock (see tables 2 and 4). An ANOVA test showed that there was no statistically significant variation between the mean K_d means ($p = 0.5642$ for LNCaP; $p = 0.1105$ for DU145) for each group of experiments.

Neither the type I nor type II K_d s varied in the nuclear fractions of either cell line as shown in table 3 (LNCaP) and table 5 (DU145). ANOVA tests confirmed that there was no significant variation in the mean K_d of each

experimental group (LNCaP type I $p=0.5170$, type II $p=0.9981$; DU145 type I $p=0.4327$, type II $p=0.3393$).

ANOVA tests were carried out to see if the differences in the concentration of receptor present at each time point after heat shock were statistically significant.

There does not appear to be any statistically significant variations in the concentration of receptors in DU145 cells as all the ANOVA p values were greater than 0.05. In LNCaP cells the receptor concentrations in the nuclear fraction showed no significant variation ($p>0.05$).

The LNCaP cytosol type II receptor concentrations also did not vary significantly ($p=0.5642$). The type I AR concentrations in heat shocked LNCaP cells were different from those in non-heat shocked LNCaP cells ($p<0.0001$).

The receptor concentration increases for the first 4 hours after heat shock and falls sharply between 4 and 6 hours post heat shock but rises again until 24 hours, then there is a second dip followed by a return to the highest level by 72 hours (see table2). The biological significance of this variation in receptor concentration is unclear.

The type I receptor is the classical steroid receptor, in that steroid binding to type I receptor causes activation of the receptor DNA binding and gene activation [Leake & Habib, 1987; Castagnetta *et al.*, 1992; Gronmeyer, 1993b]. The increase in the K_d reflects a decrease in the affinity of the type I binding site for the ligand and therefore the affinity of the AR for its ligand.

4.2.The effect of hyperthermia on the size of the AR complex in LNCaP and DU145 cells

Introduction

The change in the affinity of the AR after heat shock could reflect a change in the untransformed 8S steroid receptor complex. It is well understood that cells from all known organisms respond to stress conditions such as heat by producing hsps [Lindquist, 1986]. One of these hsps, hsp90 is a component of the untransformed 8S steroid receptor complex in non-stressed conditions [Puri *et al.*, 1982; Renoir *et al.*, 1984]. Several other hsps have been implicated in the structure or synthesis of the 8S complex. It is possible that the association of one or more of the hsps with the 8S complex is altered under stressed conditions and that this leads to the observed increase in receptor K_d.

Any change in 8S receptor composition may be reflected in a change in the size of the steroid receptor complex or the ratio of 8S to 4S receptors. Therefore, it was decided to investigate the size of the receptor complexes in the cytosol fraction of LNCaP and DU145 cells after heat shock.

The cell fraction that is known as the cytosol fraction is more accurately the low salt extracted fraction and contains proteins that are normally loosely associated with cell structures as well as purely cytosolic proteins. In the absence of hormone the AR is located in the nucleus [Husmann, 1990] possibly loosely associated with the cytoskeleton [Pratt, 1992]. The process of cell lysis and separation of the nuclear from the cytosol fraction normally means that most of the steroid receptor is recovered in the cytosol fraction . The steroid receptor which remains in the nuclear fraction is tightly bound to the nuclear framework and requires high salt to separate it.

It is the AR that is found in the cytosol/ low salt extracted fraction that shows a change in K_d after heat shock. Therefore, it is this low salt extracted fraction that was used to investigate the effects of heat on AR size and ratio.

Method

The cells were subcultured as described in method section 2.2.2., so that a 20% confluent monolayer was formed in 175cm² plastic tissue culture flasks and the cells were allowed to "plate down" for 24 hours. The routine medium was removed and the cells washed twice with PBS and 30ml of steroid-free medium was added. The cells were grown in this medium for 48 hours prior to heat shocking the cells. Heat shock was achieved by placing the tissue culture plates in an humid incubator with atmospheric air enriched with 5% CO₂ (LEEC) which had been preset to 39°C. The cells were heat shocked for two hours at this temperature followed by a recovery period (varied between 0-72 hours) at 37°C. The cells were harvested and the AR analysed using SDGA as described in method section 7. 5×10^{-9} M [³H]-mibolerone was used to radioactively label the ARs as this gave maximum binding to type I sites with only a small amount of binding to type II sites [Carruba, 1994]. Non specific binding was identified by incubating the cell extracts with 5×10^{-9} M [³H]-mibolerone plus unlabelled mibolerone (not radiolabelled) at a final concentration of 5×10^{-7} M. Graphs were plotted showing the number of counts as a function of the fraction number and arrows used to indicate the position of the sedimentation markers. The positions of the 4S and 8S [³H] peaks were determined by comparison with the migration of the [¹⁴C]- labelled standard proteins. The relative areas under the [³H] peaks were calculated and the ratio of 4S : 8S peaks obtained.

Results

Figures 28 and 29 show typical sucrose density profiles of the cytosol fraction of LNCaP cells before heat shock, and 12 and 72 hours post heat shock. The profiles of DU 145 cell cytosol extracts show a similar pattern and are not shown here.

Table 6 gives the ratio of 4S to 8S AR concentrations in cells which were heat shocked for 2 hours at 39°C and then returned to 37°C for increasing periods of time (0-72 hours).

Figure 30 shows the effect of heat shock on the ratio of 4S to 8S AR concentrations in the cytosol fractions of both LNCaP and DU145 cells.

The 8S peak is the large oligomeric complex which comprises the androgen receptor and hsp90 as well as other hsps. To aid interpretation the 8S peak is taken to be any specific [³H] peak which is larger than the 7.2S [¹⁴C] marker protein.

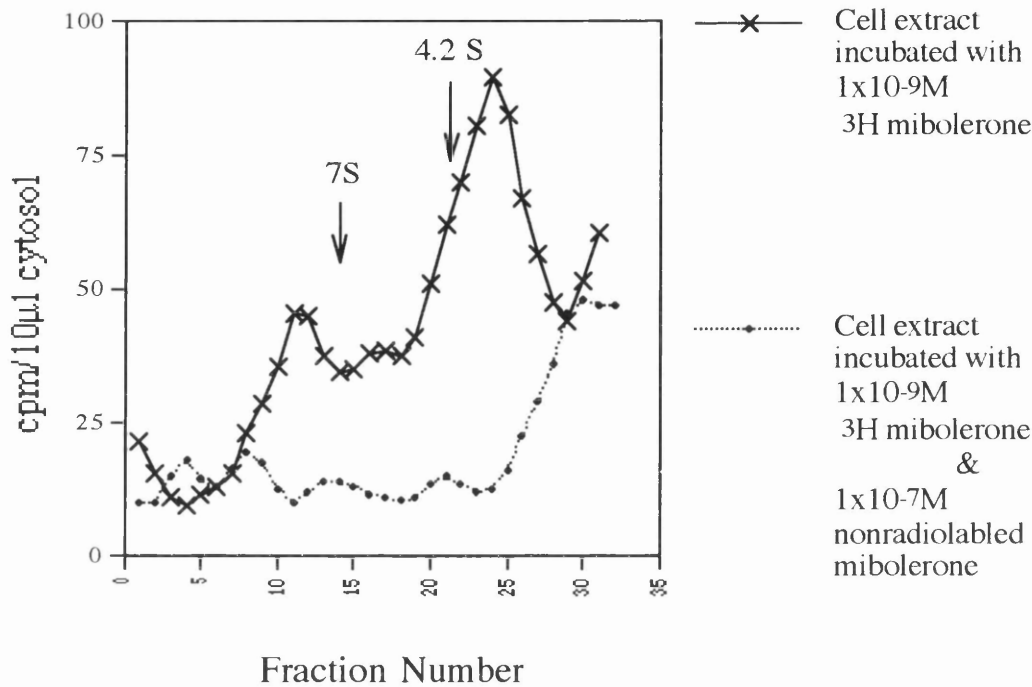
Non-specific binding was determined by incubating the cell extracts with 5×10^{-9} M [³H]-mibolerone plus a cold competitor (unlabelled mibolerone at a final concentration of 5×10^{-7} M). In figures 28 and 29 non-specific binding is shown. In both table 6 and figure 30 the non-specific counts were subtracted from the total counts to give the specific [³H]-ligand binding.

Each separate experiment was repeated three times and the results shown are the means \pm standard deviations.

Figure 28. Typical sucrose density gradient profiles of cytosol AR from control and heat shocked LNCaP cells.

Cells were either incubated at 37°C throughout the experiment or heat shocked for 2 hours at 39°C and then allowed to recover for 12 hours. The cytosol fraction was then incubated with either 5×10^{-9} M [^3H]-mibolerone alone or with 5×10^{-7} M nonradiolabelled mibolerone for 1 hour at 4°C before the unbound ligand was removed and the cytosol layered on top of a 5-20% sucrose gradient. The gradients were centrifuged at 250,000xg for 20 hours at 4°C. Each sample contained ^{14}C -labelled BSA, 4.6S and ^{14}C -labelled human- γ -globulins, 7.1 S as internal markers. After centrifugation, the bottom of the tube was punctured and two-drop fractions were collected. The amounts of ^3H and ^{14}C in 10 μl of each fraction were assessed by scintillation counting and the ^3H cpm per 10 μl was then plotted against the fraction number and the ^{14}C -labelled marker protein positions shown as arrows.

SDGA of Non Heat Shocked LNCaP cytosol



SDGA of LNCaP cytosol 12 hours Post Heat/Shock

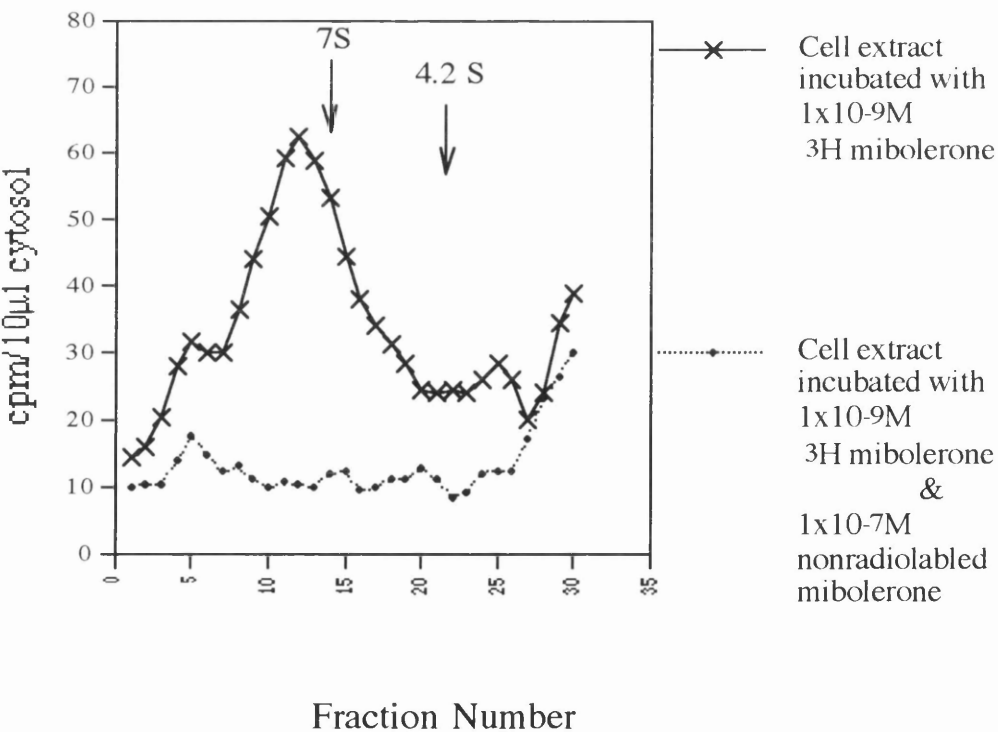
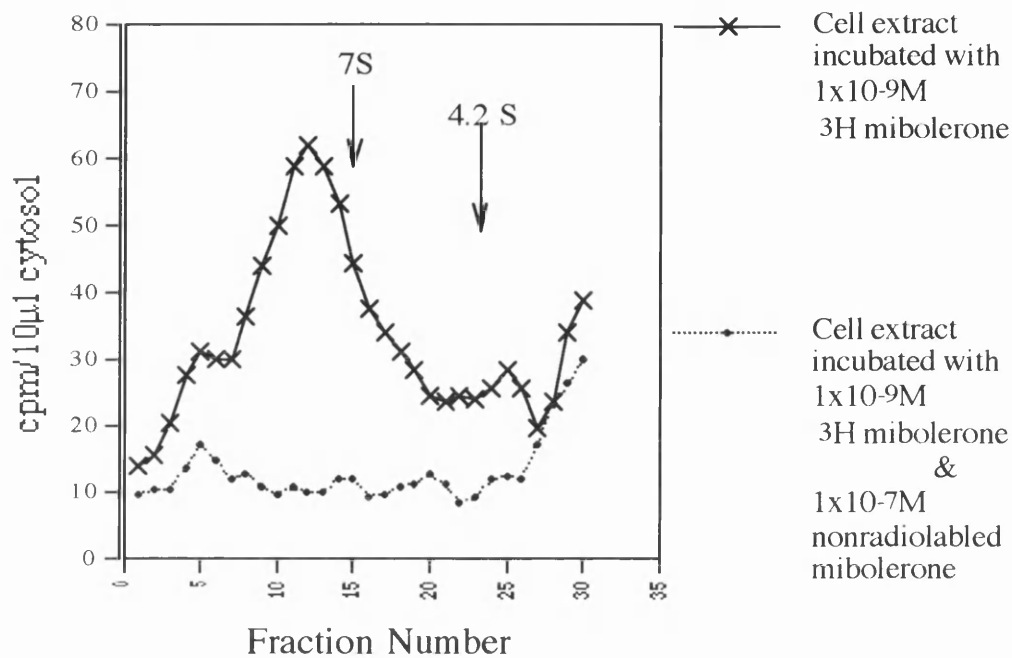


Figure 29. Comparison of typical sucrose density gradient profiles of cytosol AR heat shocked LNCaP cells allowed to recover for 12 and 72 hours. Cells were heat shocked for 2 hours at 39°C and then allowed to recover for 12 or 72 hours. The cytosol fraction was then incubated with either 5×10^{-9} M ^3H -mibolerone alone or with 5×10^{-7} M nonradiolabelled mibolerone for 1 hour at 4°C before the unbound ligand was removed and the cytosol layered on top of a 5-20% sucrose gradient. The gradients were then centrifuged at 50,000 rev/min (250000g) for 20 hours at 4°C. Each sample contained ^{14}C -labelled BSA, 4.6S and ^{14}C -labelled human- γ -globulins, 7.1 S as internal markers. After centrifugation, the bottom of the tube was punctured and two-drop fractions were collected. The amounts of ^3H and ^{14}C in 10 μl of each fraction was assessed and the ^3H cpm per 10 μl was then plotted against the fraction number and the ^{14}C -labelled marker protein positions shown as arrows.

SDGA of LNCaP cytosol 12 hours Post Heat/Shock



SDGA of LNCaP cytosol 72 hours Post Heat/Shock

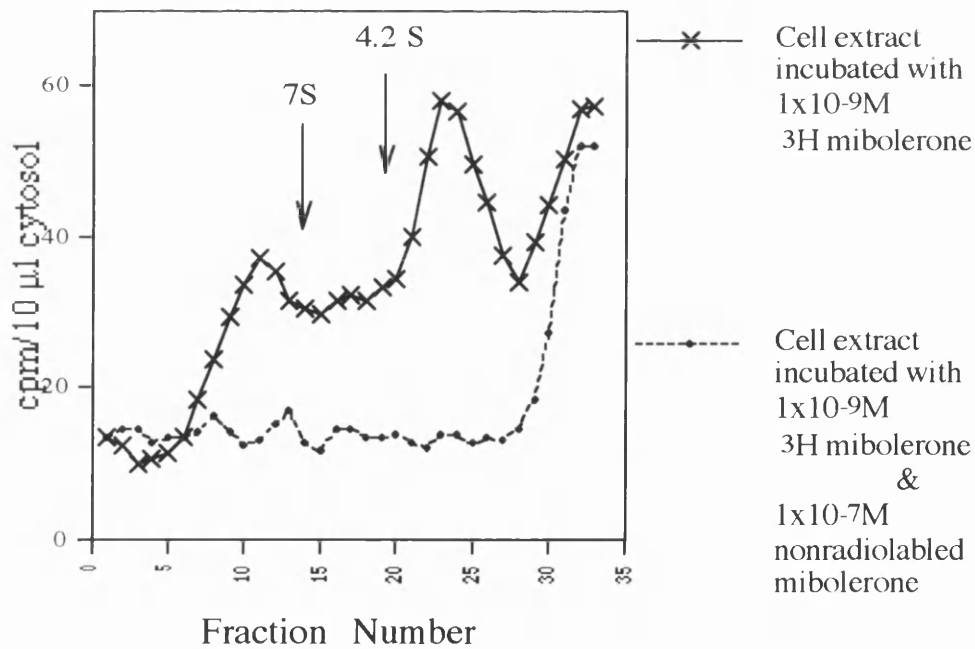


Table 6. The changes in AR 4S and 8S ratios in heat shocked LNCaP and DU145 cells.

The cells were heat shocked for 2 hours at 39°C and then allowed to recover for increasing periods of time (0-72 hours) before SDGA was carried out as described in method section 7. The cytosol fraction was then incubated with either 5×10^{-9} M [^3H]-mibolerone alone or with 5×10^{-7} M nonradiolabelled mibolerone for 1 hour at 4°C before the unbound ligand was removed and the cytosol layered on top of a 5-20% sucrose gradient. The gradients were centrifuged at 250000g for 20 hours at 4°C. Each sample contained ^{14}C -labelled BSA, 4.6S and ^{14}C -labelled human- γ -globulins, 7.1 S as internal markers. After centrifugation, the bottom of the tube was punctured and two-drop fractions were collected. The amounts of ^3H and ^{14}C in 10 μl of each fraction collected was assessed by scintillation counting and the ^3H cpm per 10 μl was then plotted against the fraction number. The levels of both 4S and 8S receptors was determined by calculating the areas under the appropriate peak and the ratio of 4S to 8S calculated. The 8S peak represents the large oligomeric receptor complex and includes the larger peak seen in the 12 hour sample (see conclusions for details).

Each experiment was carried out in triplicate and the results shown are the means obtained \pm the standard deviations (S.D.).

Hours post heat shock	LNCaP cells RATIO 4S : 8S AR mean \pm S. D.	DU145 cells RATIO 4S : 8S AR mean \pm S. D.
Non Shocked	1 : 0.26 ± 0.19	1 : 0.42 ± 0.01
0	1 : 0.51 ± 0.37	1 : 0.49 ± 0.07
2	1 : 0.60 ± 0.15	1 : 0.49 ± 0.09
4	1 : 1.03 ± 0.20	1 : 1.08 ± 0.13
6	1 : 1.70 ± 0.43	1 : 1.37 ± 0.25
8	1 : 4.10 ± 0.28	1 : 1.83 ± 0.11
10	1 : 3.74 ± 0.62	1 : 2.43 ± 0.29
12	1 : 7.45 ± 2.33	1 : 4.68 ± 0.50
24	1 : 3.85 ± 0.49	1 : 3.33 ± 0.57
48	1 : 1.45 ± 0.15	1 : 1.11 ± 0.39
72	1 : 0.53 ± 0.10	1 : 0.86 ± 0.15

Figure 30. Comparison of the change in 4S to 8S AR after heat shock after heat shock in LNCaP and DU145 cells.

The cells were heat shocked for 2 hours at 39°C and then allowed to recover for between 0-72 hours before SDGA was carried out as described in methods section 7.

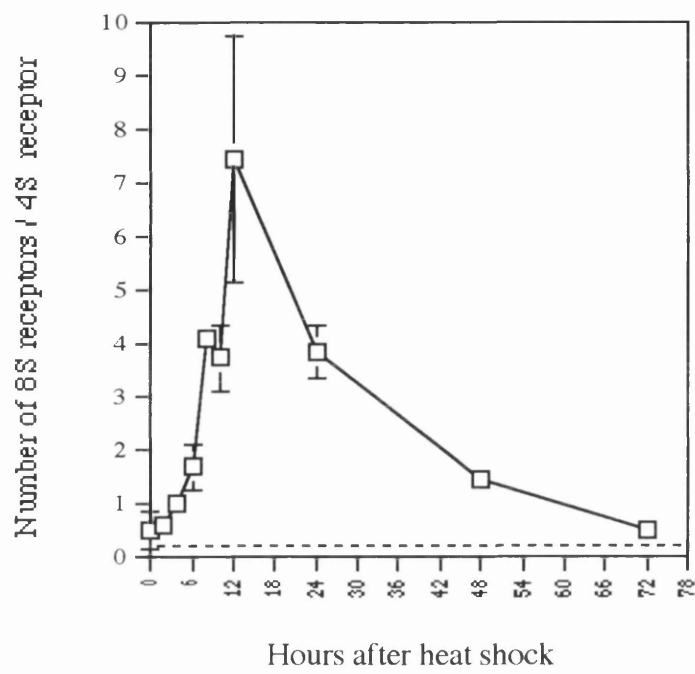
The levels of both 4S and 8S receptors was determined by calculating the areas under the appropriate peak and the ratio of 4S to 8S calculated (see table 6).

The 8S peak represents the large oligomeric receptor complex and includes the larger peak seen in the 12 hour sample (see conclusions for details).

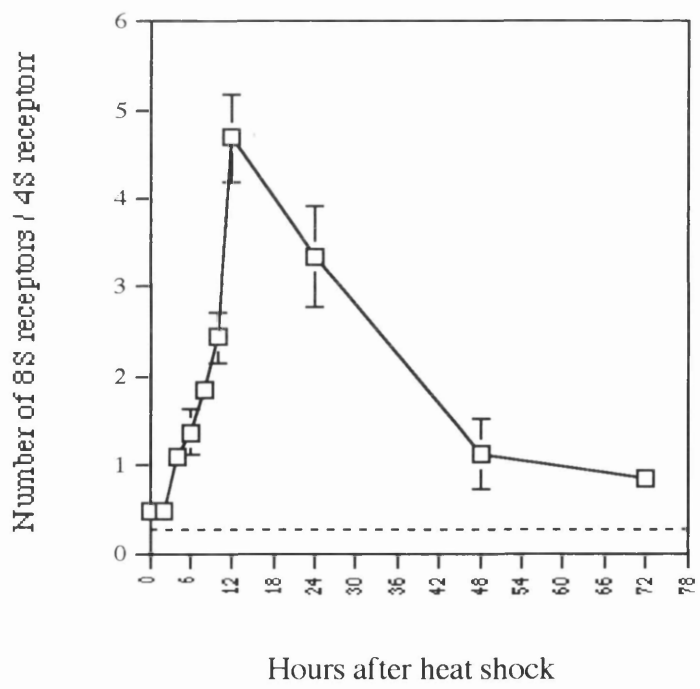
This experiment was carried out in triplicate and the results shown are the means \pm the standard deviations.

Where no error bars are visible, the standard error is too small to be shown.

LNCaP cell AR 4S to 8 S ratio



DU145 cell AR 4S to 8 S ratio



Conclusions

Comparison of the sucrose density gradient profiles of the cytosol fraction AR shown in figures 28 and 29 suggests that there is a change in the amount of active 4S receptor after heat shock. There also appears to be an increase in the amounts of untransformed 8S receptor. 12 hours after heat shock a larger ligand binding complex with the approximate size of 10S is visible. This is also seen in the DU145 cells 12 hours after heat shock.

Table 6 gives the 4S : 8S ratios following heat shock in both LNCaP and DU145 cell cytosols. The 8S receptor numbers include the larger complex seen after 12 hours (figures 28 and 29) as this is taken to represent the androgen receptor oligomer with possibly extra hsps associated with it (see discussion section). Figure 30 clearly shows the increase in the amount of 8S receptor present in the cell after heat shock. This increase coincides with a decrease in the active 4S receptor level within the cell cytosol. Total receptor levels remain the same.

This alteration in the ratio of 4S : 8S AR does not appear to be permanent but rather it slowly reverts and by 72 hours post heat shock the ratio is similar to that of the control.

The time course for this alteration of 4S:8S receptor is similar to the time course for the alteration in K_d of the type I receptor and for gain and loss of thermotolerance as shown in figures 15 and 16.

Chapter 5.

The induction of hsp90 in prostate cancer cells.

5.1. Induction of hsp90 by heat in DU145 cells

Introduction

Hsp90 belongs to a group of proteins (the hsps) whose synthesis is strongly stimulated by cellular stresses such as hyperthermia [Lindquist, 1986]. The temperature at which induction of the hsps occurs varies from organism to organism and reflects stress conditions for that organism [Parsell & Lindquist, 1993]. Thermotolerance is induced in human prostate cell lines by pre-exposure to the sublethal temperature 39°C (see chapter 3). This temperature also induces an increase in the proportion of AR in the 8S form. This raises the question as to whether the 39°C temperature induces hsp synthesis ?

Due to the association of hsp90 with the 8S steroid receptor in unstressed cells, it was decided to assess whether the levels of hsp90 synthesis were altered by exposure to 39°C. This was achieved using [³⁵S]-methionine to label proteins as they were being synthesised for different periods during and after heat shock. Immunopurification of hsp90 , followed by autoradiography was then used to assess the levels of hsp90 synthesis under different conditions.

Method

DU145 cells were subcultured as in section 2.2.2. of the methods chapter so that a 50% monolayer was formed within 6-well tissue culture plates. The cells were then allowed to "plate down" for 24 hours before the routine medium was removed and the cells washed twice with PBS and once with methionine-free medium. The cells were incubated for 45 minutes in methionine-free medium with 2% DHIDCCFCS before being incubated for 3 hours with methionine-free medium supplemented with 2% DHIDCCFCS and 5 μ Ci/ ml [³⁵S]-methionine. The cells were heat shocked in the normal way and sets of tissue culture wells were incubated with [³⁵S]-methionine for 3 hour periods either during heat

shock or for appropriate periods throughout the first 12 hours after heat shock. Immediately after the incubation with [^{35}S]-methionine the cells were harvested and immunoprecipitation of hsp90 carried out as described in methods section 9.. The immunopurified material was analysed by SDS/PAGE (methods section 10.). Two identical gels were run. One was stained with Coomassie blue (methods section 12.) and the other was used in Western blotting (methods section 11.) The blots were probed with an anti-hsp90 monoclonal antibody (AC88). ECL (methods section 11.2) was used to visualise the bands. The blots were then washed in TBST to remove ECL reagent and were air-dried, then exposed for one week to X-ray film (Amersham hyper film MP) with intensifying screens at -70°C . The X-ray film was developed using an automated machine (Kodak X-Omat Processor Model ME3).

Results

The results of this experiment are shown in the following figures.

Figure 31 shows the total protein content, (detected by Coomassie blue staining) of cells which were heat shocked and cells allowed to recover for between 0 and 12 hours. There appears to be little change in the total protein concentration or pattern observed by this method. Detection of [^{35}S]-methionine incorporation, by Western blotting and autoradiography, produced no clear bands (not shown). All the lanes were very faint which probably suggests that the amount of protein loaded onto the gel was insufficient to allow detection of newly synthesised proteins by [^{35}S]-methionine incorporation.

Figure 32 shows the products of immunoprecipitation of hsp90 using GAM-Seph-3G3. The Coomassie blue stain of total protein immunoprecipitated is not shown as the protein levels were too low to be detected by this method.

Both parts of figure 32 are derived from the same western blot. Part A was visualised using the antibody AC88 followed by ECL detection. Part B is an

autoradiograph which indicates the [^{35}S]-methionine incorporation into newly synthesised proteins.

Figure 31 The effects of heat on the total protein concentrations of DU 145 cells.

Cells were incubated at 39°C for 2 hours, and allowed to recover for between 0 and 12 hours before SDS/PAGE of whole cell lysate was carried out.

Coomassie blue staining of the 7.5% gel was carried out to visualize the protein. Molecular weight size markers (194, 000; 116,000; 85,000 & 49,000) were used to calculate the sizes of proteins stained.

The 8 lanes represent different timing of [³⁵S]-methionine incubation as follows from right to left:

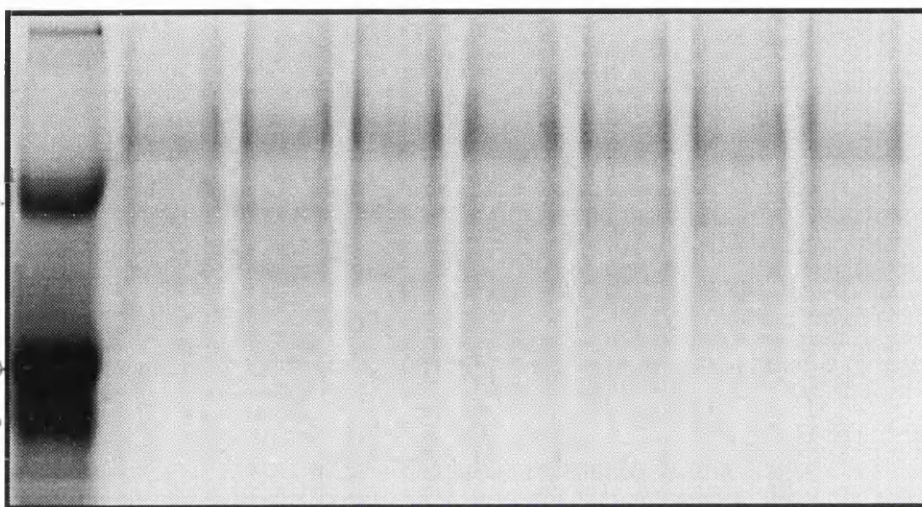
1. [³⁵S]-methionine incubation for the 3 hours prior to heat shock
2. [³⁵S]-methionine incubation during heat shock (2 hour at 39°C)
3. [³⁵S]-methionine incubation 0-3 hours after heat shock
4. [³⁵S]-methionine incubation 3-6 hours after heat shock
5. [³⁵S]-methionine incubation 6-9 hours after heat shock
6. [³⁵S]-methionine incubation 9-12 hours after heat shock
7. No [³⁵S]-methionine incubation, no heat shock
8. Immunoprecipitation procedure carried out substituting PBS for cell extract

Molecular weight

194000 →

116000 →

85000 →



M

1

2

3

4

5

6

7

Figure 32 The effects of hyperthermia on hsp90 synthesis in DU145 cells. Cells were incubated at 39°C for 2 hours, and allowed to recover for between 0 and 12 hours. The cells were incubated in [³⁵S]-methionine for 3 hours at different periods throughout heat shock and recovery time. Cells were harvested immediately after the [³⁵S]-methionine incubation was stopped. Hsp90 was immunopurified as described in methods section 9. SDS/PAGE of the immunopurification products were then carried out followed by Western blotting.

The 8 lanes represent different timing of [³⁵S]-methionine incubation as follows from right to left:

1. [³⁵S]-methionine incubation for the 3 hours prior to heat shock
2. [³⁵S]-methionine incubation during heat shock (2 hour at 39°C)
3. [³⁵S]-methionine incubation 0-3 hours after heat shock
4. [³⁵S]-methionine incubation 3-6 hours after heat shock
5. [³⁵S]-methionine incubation 6-9 hours after heat shock
6. [³⁵S]-methionine incubation 9-12 hours after heat shock
7. No [³⁵S]-methionine incubation, no heat shock
8. Immunoprecipitation procedure carried substituting PBS for cell extract

A. The western blot was probed with the anti-hsp90 antibody AC88, and the bands visualised by ECL. This shows the total amount of hsp90 immunoprecipitated.

B. The blot was exposed to X-ray film for one week at -70°C. This allows the detection of the hsp90 synthesised during the [³⁵S]-methionine incubation

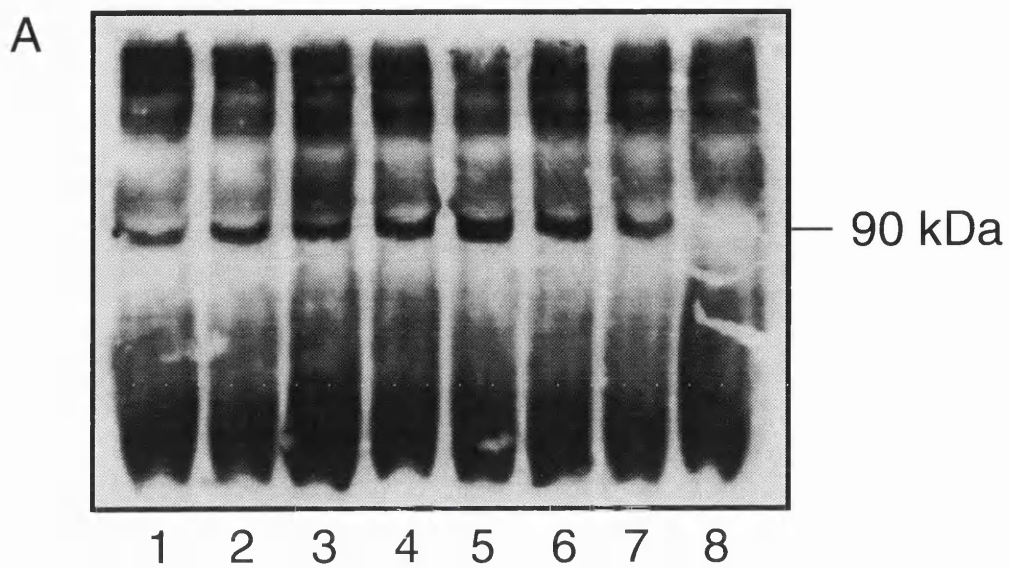


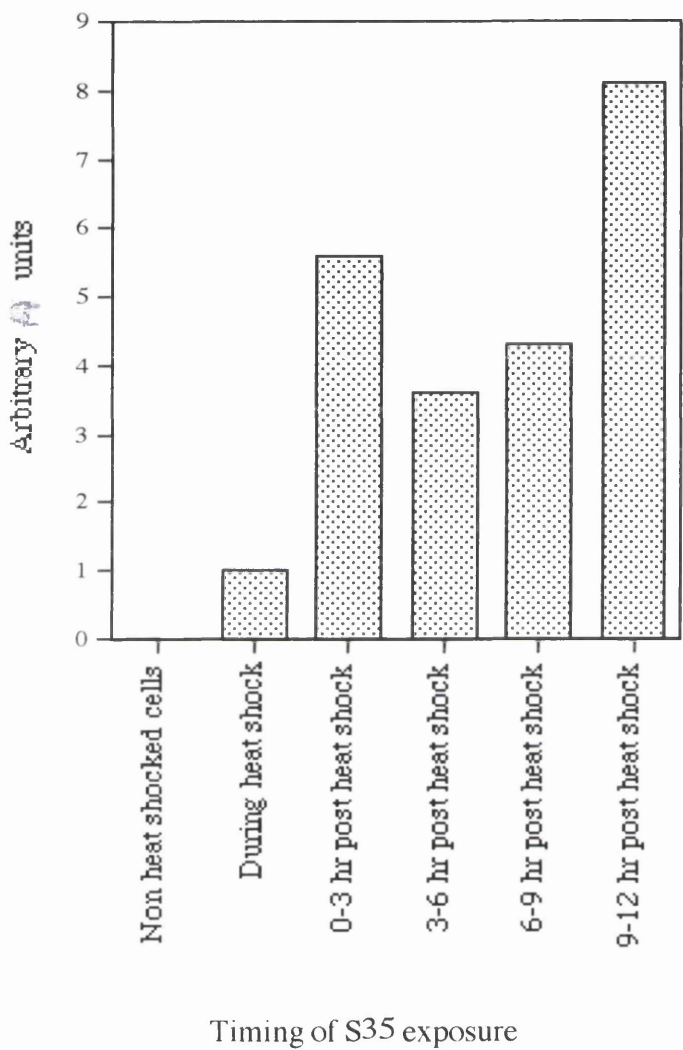
Figure 33. Quantification of hsp90 synthesis following heat shock.

This histogram shows the relative amounts of hsp synthesised during DU145 cell recovery from heat shock.

The intensity of bands visualised in figure 32 part B were assessed using a densitometer and the arbitrary values shown here.

Absorbance (A)

Effect of heat on hsp90 synthesis



Conclusions

In figure 32 part A a 90 Kd band is clearly visible in lanes 1-7 and this is taken to be hsp90 visualised by ECL of the AC88 antibody. The other bands which are present in all of the lanes including the negative control (lane 8; no cell extract) are due to the detection of the mouse Ig components of GAM-Sepharose 3G3 by the ECL goat anti-mouse antibody.

The intensity of the 90 kD band was assessed using a densitometer (BioRad Imaging Densitometer model GS 670). The total level of hsp90 immunoprecipitated gradually increased in the 12 hours after heat shock, figure 32A lanes 2 to 6 (from 1 to 8.1, arbitrary O.D. values). This increase in hsp90 concentrations reflects an increase in synthesis of hsp90 after heat shock as shown in figure 32 part B.

The level of hsp90 synthesis in non-stressed cells was too low to be detected, (see lane 1 figure 32 part B), but a band is visible in lane 2 which suggests that the synthesis of hsp90 is increased during heat shock. In the first 3 hours following heat shock there is a noticeable increase in synthesis of hsp90 (arbitrary O.D. values obtained using a densitometer, lane 3 = 5.6 compared with 0 in lane 1). Between 3-9 hours post heat shock (lanes 4 and 5) the levels of synthesis seem to fall slightly (arbitrary O.D. values 3.6 and 4.3).

Hsp90 synthesis appears to be at greatest in the period between 9-12 hours post heat shock (arbitrary O.D. value 8.1) (lane 6).

Figure 32 part B and figure 33 shows that the heat shock conditions (39°C for 2 hours) used in this thesis does induce hsp90 synthesis in the prostate cell line DU145.

Chapter 6

Discussion

Many types of tumour cells are more sensitive to elevated temperatures than normal cells [Crile, 1963; Giovanella *et. al.*, 1976]. This observation led to the development of thermotherapy as a form of cancer treatment. Prostate cancer, due to its accessibility, is a prime candidate for thermotherapy [Mendecki *et al.*, 1980; Servadio *et.al.*, 1987].

Treatment of prostate cancer with local hyperthermia involves heating the gland to between 41°C and 44°C either once or twice a week [Engin *et.al.*, 1993].

Temperatures above 42.5°C may result in damage to normal tissue [Lieb *et al.*, 1986] and therefore have limited use in clinical practice [Lloyd *et.al.*, 1992].

It has been shown that prostate cells which are subjected to hyperthermia *in vitro* and survive become thermotolerant and then are able to withstand further thermotherapy even at normally lethal temperatures [Lloyd *et.al.*, 1992].

The role of heat shock proteins in thermotolerance has been suggested for some time. The synthesis and decay of hsp90 shows a similar pattern to thermotolerance [McAllister & Finkelstein, 1980; Li & Werb, 1982].

It has been shown that, the acquisition of thermotolerance in breast cancer cells (MCF-7s) was accompanied by the abolition of the oestrogen binding capacity in those cells [Chalmers, 1991]. This fact, taken together with the association of hsp90 with steroid receptors in normal conditions, has led to the idea that heat shock may alter the relationship between the steroid receptor and hsp90 and that this complex has an altered ligand binding pattern.

The implication that there could be an interaction between thermal and endocrine effects on prostate cancer cells *in vitro* and *in vivo* remains uninvestigated, yet it is of potentially great importance due to the combination of antiandrogen and thermal-therapies being developed for the treatment of prostate disease.

The experiments described in this thesis used *in vitro* techniques to investigate the effects of heat and antiandrogen therapies on prostatic cells and studied at the effects of heat on the AR in these cells.

6.1. The response of prostate cell lines to androgens and antiandrogens.

In order to understand the response of prostate cells to combined endocrine and thermotherapies, it is important to identify the growth effect of androgens on all three cell lines.

In this study, LNCaP cells show growth stimulation by exogenous androgen (DHT) in a dose dependent manner (figure 9.). Maximal growth occurred when a final concentration of 1×10^{-9} M DHT was used. This result is similar to results obtained for the same cell line by other groups [Schuurmans *et al.*, 1988a; Wilding *et al.*, 1989]. LNCaP cells do not appear to respond to OH-Flut (figure 20). This result supports the work of others who have shown that OH-Flut is not inhibitory to LNCaP cells *in vitro* although in some cases they have shown OH-Flut actually growth stimulates LNCaP cells growth [Wilding *et al.*, 1989; Olea *et al.*, 1990; Chalmers, 1991; Veldscholte *et al.*, 1992]. Flutamide (OH-Flut is the activated derivative of flutamide) acts as a non-steroidal antiandrogen *in vivo*.

The different effects of OH-Flut *in vivo* and *in vitro* suggests differing mechanisms of action in the two cases.

Some other authors have shown partial agonistic effect of OH-Flut in LNCaP cells [Chalmers, 1991; Veldscholte *et al.*, 1992]. This could be a similar effect to that seen with tamoxifen-ER complexes [Baulieu, 1987 ; Ham & Parker, 1989; Dauvois & Parker, 1993]. This partial agonist action of OH-Flut could be due to the mutated AR in LNCaP cells [Veldscholte, 1992] or it could be an intrinsic property of the compound. If it is an intrinsic property, OH-Flut would act as a antiandrogen *in vivo* if there were endogenous androgens present, but in cases where there is little or no androgen present (due to androgen ablation or castration), OH-Flut would act as an agonist. This type of action has already

been described for tamoxifen (an antiestrogen used in the treatment of hormone sensitive breast cancer) [Dauvois & Parker, 1993].

It has been also been suggested that the androgenic action of OH-Flut, is mediated through binding of OH-Flut to androcolyne (an extra cellular inhibitory factor), relieving androcolyne-inhibition of LNCaP cell proliferation [Sonnenschein *et al.*, 1989; Olea *et al.*, 1990]. This extra-cellular steroid binding inhibitory factor has still to be identified.

DU145 and PC3 cells were not growth stimulated by either DHT or OH-Flut . However our DU145 cells do contain ARs. Scatchard plot analysis of ligand binding has indicated that significant levels of androgen receptors are present within these cells . DU145 cells show a metabolic response to androgens [Carruba 1994].

6.2. Thermotolerance in cultured prostatic cells.

Thermotolerance is a general term used to refer to the transient, non-heritable state of resistance to normally lethal temperatures by the pre-exposure to non-lethal heat treatment. The degree of resistance and the temperatures which induce thermotolerance vary greatly from organism to organism [Carper, 1987] It was important to characterise the normal range of thermal tolerance of prostate cell lines and to define the temperatures required to establish thermotolerance in these cells before any studies of the underlying mechanisms could be carried out.

All three prostate cell lines investigated (LNCaP, DU145, PC3) showed marked thermosensitivity to 42°C as shown in figures 12,13and 14. The observed thermosensitivity can be prevented by pre-exposing the cells to 39°C for 2 hours as seen in figures 12,13 and 14. This survival of prostate cells to

normally lethal temperatures following pre-exposure to a sublethal temperature fits the original description of thermotolerance [Gerner & Schneider, 1975]. Thermotolerance is a transient phenomenon, little work has been done on the time course of thermotolerance. It was decided to use the establishment of thermotolerance in prostate cell lines to investigate the transient aspects of this phenomenon.

In this study, the prostate cell lines did not become thermotolerant immediately after exposure to the sublethal temperature but rather it takes up to 8 hours before significant levels of thermotolerance are seen and 12 hours before maximal thermotolerance is reached. Thermotolerance has also been induced in mammalian fibroblasts in monolayer cell culture using a 12 hour recovery period between the two doses [Welch & Suhan, 1986]. This time lapse could be due to the time required to up-regulate the synthesis of hsps and for them to translocate to their site of action, where they can exert their effect.

It has been shown that a nuclear translocation of hsp90 occurs following heat shock [Chalmers, 1991]. This nuclear translocation is time-dependent and reaches a plateau in human fibroblasts after 15 to 20 hours [Akner *et al.*, 1992]. Once established, thermotolerance is slowly lost over time (see figure 16). 72 hours after the sublethal dose the level of thermotolerance is almost completely lost in all three prostate cell lines.

This loss of thermotolerance could be due to two cellular events. Firstly when cells divide there is a dilution factor as the hsps are split between the daughter cells. If the cells are not in stressed conditions their hsp synthesis may have fallen to nonstressed levels. Secondly the heat shock proteins may be undergoing normal protein degradation which would lead to a gradual decrease in cellular levels of hsps if their synthesis had returned to nonstressed levels. In all three prostate cell lines, thermotolerance can be easily re-established once lost, by re-exposing the cells to the sublethal temperature. The level of thermotolerance achieved by re-exposure is similar to the original level of

thermotolerance (figure 18) . This would suggest that the same process is involved in both cases. The earlier heat shock does not prime the cell to react quicker to the second heat shock which would mean that the transcriptional and translational changes within the cell are short lived.

6.3. The combined effects of hyperthermia and endocrine therapy on prostate cell growth.

Hyperthermia has been used in conjunction with endocrine therapy as a treatment for prostate cancer. It has been reported that their combined use *in vivo* results in a more effective treatment compared with the individual use of either method [Servadio *et al.*, 1987; Linder *et al.*, 1990]. This phenomenon is also seen when LNCaP cells are treated with the combination of $1 \times 10^{-9} \text{M}$ OH-Flut and heat shock (see figures 20 and 21).

The response of the LNCaP cells to the combination therapies was more obvious when heat shock was administered prior to OH-Flut (figure 21).

Heat shock alters the response to OH-Flut seen in earlier experiments using LNCaP cells. This would suggest that the partial agonistic effect, reported by others, of OH-Flut is unlikely to be due to the mutated androgen receptor and more likely to be an inherent property of the compound.

The mechanism behind the conversion of OH-Flut activity from that of an androgen to that of an antiandrogen could be due to several possibilities.

The antiandrogen could sensitise the androgen responsive cell to heat shock.

This could occur due to the OH-Flut-AR complex retaining its hsp90 dimer and therefore the amount of nuclear hsp90 would be reduced which may in turn lead to the cells increased susceptibility to heat. Although hsp90 a common protein it is a mainly cytoplasmic with only trace amounts being found in the nucleus [Lindquist *et al.*, 1986] but after heat shock hsp90 levels in the nucleus increase dramatically [Chalmers, 1991].

If the increased effect is due to the decrease in free nuclear hsp90 the timing of heat shock would be important. As shown in this thesis the effect is greatest if heat shock is given first. This would mean that nuclear hsp90 levels would be much higher than in non stressed cells. Any reduction in free hsp90 caused by the OH-Flut-AR complex retaining its hsp90 dimer would be masked by the large influx of hsp90 into the nucleus.

Another possibility is that heat shock enhances the response of the cells to OH-Flut. On hormone binding the hsp90 dimer dissociates from the hormone-receptor complex revealing the DNA binding domain of the receptor [Baulieu, 1987]. One possible action of antiandrogens could be to prevent the dissociation of the hsp90 dimer from the complex thereby preventing DNA binding [Baulieu, 1987]. After heat shock there is an increase in the level of nuclear hsp90. Consequently the increased hsp90 levels may reduce or prevent the dissociation of the receptor complex even in the presence of androgen. It may prevent the agonistic effect of OH-Flut by preventing the OH-Flut-AR complex from releasing its hsp90 dimer preventing OH-Flut-AR binding to DNA. Of course as the ^{2,1,2} is now a positive effect of OH-Flut-AR complex in inhibiting growth some of this complex, possibly modified by additional hsps, must interact with the genes involved in cell growth

6.3.1. The long term effects of combined hyperthermia and endocrine therapy on prostate cell growth.

The effect of combining heat shock and antiandrogens is clearly seen (figures 20 and 21). The temperatures used have been shown to induce thermotolerance in LNCaP cells (figure 12) and the synergistic effect is greater if the cells are heat shocked prior to OH-Flut administration. This supports the idea that several of the events involved in the development of thermotolerance are responsible for the enhanced antiandrogen effect of OH-Flut. If this were the

case the synergistic effect would be lost with time in a similar way to the loss of thermotolerance seen in figure 16.

Figure 23 shows that the repression of cell growth by the combination of heat shock and OH-Flut is slowly lost with time but can be re-established by a further heat shock treatment.

These results supports the idea that thermotolerance (and therefore the heat shock response and hsps) is responsible for the increased effectiveness of OH-Flut.

The synergistic effect appears to be lost more slowly than thermotolerance (see figures 17 and 23). This could be due to the decrease in cell division slowing down the hsp90 dilution effect (discussed above).

6.4. Induction of hsp90 by heat in DU145 cells

Hsp90 has been shown previously to be a inducible protein [Catelli *et al.*, 1985] and its gene promoter is known to contain the appropriate heat shock response element that confers heat inducibility on hsp90 transcription [Rebbe *et al.*, 1989].

The results shown in chapter 5 show that in DU145 cells the synthesis of hsp90 is strongly stimulated by exposure to 39°C for 2 hours. This induction has an initially fast rate which tails off between 3 and 9 hours post heat shock.

Maximal hsp90 synthesis appears to be between 9-12 hours post heat shock.

The reason for this is unclear. Perhaps the initial burst of synthesis utilizes the hsp90 mRNA which is already available and the latter burst involves newly synthesised hsp90 mRNA. Little is known about the stability or half life of hsp90 mRNA. The two periods of increased hsp90 synthesis could also reflect synthesis of both hsp90 forms (hsp90 α and β) or alternatively each could represent synthesis of only one of the forms. The genes encoding hsp90 α and hsp90 β are differentially regulated [Simon *et al.*, 1987; Yamazaki *et al.*, 1990]

but it is unclear whether both genes are expressed at the same level under different cell conditions or if both hsp90s are absolutely required.

One study on the effects of heat on hsp mRNA synthesis suggests that there is a delay in the induction of hsp mRNA synthesis after sublethal heat shock and that if the temperature is increased the synthesis of hsp mRNA is halted and that this prevents the establishment of thermotolerance [Van Wijk *et al.*, 1994]. If this is the case then it could explain the decrease in synthesis between 3-9 hours post heat shock seen in figure 32, if the endogenous hsp90 mRNA has a short half-life it could well be degraded before the heat shock stimulated mRNA becomes available for translation.

This delay in hsp induction following heat shock could also help explain the time taken to establish thermotolerance.

6.5. The effect of heat shock on the binding affinity of the androgen receptor in prostate cancer cell lines.

The synergistic effect of combining heat shock and OH-Flut administration is confined to the androgen/ antiandrogen-responsive cell line LNCaP. The androgen receptor must play a role in the effect.

Two classes of binding sites for steroid receptors have been identified by Scatchard analysis of ligand binding assays [Eriksson, 1978; Markaverich & Clark, 1979; Castagnetta *et al.*, 1992]. Type I sites binds to its ligand with high affinity ($K_d < 1\text{nM}$) and low capacity; Type II receptors bind ligand with a lower affinity but higher capacity [Castagnetta *et al.*, 1992]. Type I receptors are the classic steroid receptors but the role of type II receptors are unknown [Carruba, 1994]. Ligand concentrations ranging from 0.1nM to 5nM allows the identification of both type I and type II receptors [Clark & Peck, 1979; Carruba, 1994], enabling proper studies into the effects of heat shock on both receptor types.

Many studies of steroid receptors have used a ligand concentration from 1 to 25nM and as such will result in the identification of principally type II receptors with only a small amount of the type I receptor being detected [Horoszwicz *et al.*, 1983; Sonnenschein *et al.*, 1989; Olea *et al.*, 1990]. This will alter the K_d value obtained and lead to a higher receptor concentration if assessed as a single type of binding site[Carruba, 1994].

The two types of androgen receptor in the soluble and chromosome-bound fractions of LNCaP and DU145 cells appear to react differently to heat shock. There is a decrease in the affinity of cytosol type I receptor (the "classic AR") for its ligand in the soluble fraction of heat shocked cells. This is shown by an increase in the K_d of type I binding, as determined by Scatchard analysis (see tables 2 and 4). The type I receptors which are tightly associated with the nuclear framework do not alter their binding affinity as a result of heat shock (see tables 3 and 5) nor does the type II receptor in either the nuclear or cytosol fractions. It can be concluded that only the high affinity cytosolic receptor alters as a result of heat shock.

This decrease in binding affinity could be due to a subtle change in the conformation of the steroid receptor (possibly within the ligand binding domain). Another possibility is the association of extra hsps after heat shock, leading to the alteration in the binding affinity. Perhaps the influx of hsp90 into the nucleus after heat shock results in the hsp90 dimer associating more tightly to the receptor and this may lead to the change in binding affinity.

The levels of type I androgen receptors fluctuate after heat shock. Receptor concentration increases for the first 4 hours after heat shock and fall sharply between 4 and 6 hours post heat shock but rises slowly until 24 hours, then drops again and rises until 72 hours post heat shock. The scientific significance of this variation in receptor concentration is unclear.

Previous studies on the oestrogen binding capacity of breast cancer cells (MCF-7) cells following heat shock showed an almost total abolition of estrogen-

binding capacity over a period of 24 hours [Wolffe *et al.*, 1984; Chalmers, 1991]. In the latter case an immunoassay method was used to detect ER and it is possible that additional hsps associating with the ER masked the epitope

The only study to date on the composition of steroid receptors after heat shock was carried out using the progesterone receptor [Edwards *et al.*, 1992]; there was a partial reduction in cellular levels of PR but the binding affinity of the PR remained unchanged. They also suggest that heat shock treatment did not effect PR-hsp90-hsp70 complexes that pre-existed under normal cellular conditions but that newly formed complexes did alter. Samples were taken one hour post heat shock, which may have been too soon to allow any changes to become visible. In LNCaP cells it takes up to 8 hours before a significant change in the binding affinity is seen. There is also a time lapse between heat shock and thermotolerance establishment which suggests that there would be a delay before the effects of heat shock are seen.

The different effects of heat shock described may be due to differences in cell lines, species, or possibly the different steroid receptors studied.

6.6. The change in size of the "empty" AR complex following heat shock in LNCaP and DU145 cells.

In normal cells two forms of the type I steroid receptor can be identified. The transformed receptor which has a sedimentation coefficient of 4S and the untransformed oligomeric complex with a sedimentation coefficient of 7-10S [Sherman, 1983] which is referred to as the 8S receptor. In unstressed LNCaP and DU145 cells most of the receptor is found in the 4S form (see figure 28 and table 6).

After heat shock of LNCaP cells, there is a gradual shift in the ratio of receptor forms until after 12 hours very little 4S receptor remains and the amount of 8S receptor has greatly increased. This apparent shift in receptor size from 4S to 8S

is also seen in heat shocked DU145 cells. It is not a sudden change but rather it happens gradually during the first 12 hours after heat shock. The ratio does not return to pre-shocked levels until 72 hours after the heat shock treatment.

The timing of this shift from the 4S to the 8S receptor is similar to that seen in the change in the K_d of the receptor. It also follows a similar time course as the induction and loss of thermotolerance.

The nuclear translocation of hsp90 has reached significant levels by 12 hours post heat shock [Chalmers, 1991]. This increase in nuclear hsp90 could affect the ratio of 4S to 8S receptors. Hsp90 may account for as much as 1-2 % of the total cellular protein content [Lindquist *et al.*, 1986; Lai *et al.*, 1984] which is in vast excess of the total amounts of steroid receptors. It seems unlikely that the changes in hsp90 levels would alter the ratio of 4S to 8S complexes. However one important point needs to be considered. Hsp90 is predominantly a cytoplasmic protein whereas the steroid receptors are found in the nucleus. The level of hsp90 in the nuclei of unstressed cells is minute compared to the level found in the cytosol (Chalmers submitted for publication) and so it is possible that the levels of hsp90 in the nucleus could affect the ratio of 4S to 8S receptors. This could explain the increase in the levels of the large 8S complex seen following heat shock.

A 11 S ^ushoulder was observed after 10-12 hours and this was included in the overall 8S complex as time did not allow further investigation.

6.7. Possible mechanisms for antihormone action and the effect of heat on those mechanism.

This supposition is based on the work of Etienne-Emile Baulieu, who has produced a model for antisteroid action [Baulieu, 1987].

There is a wide variation in the ability of hormone antagonists to inhibit different physiological responses *in vivo* [Dauvois & Parker, 1993] and therefore several different mechanisms for their action may exist.

1) Antihormones may stabilize the receptor-hsp90 hetero-oligomer, preventing the release of the active receptor. RU 486 (an antiglucocorticoid) has been shown to stabilize the 8S form of the GR [Baulieu, 1987] although not totally as RU486 has partial agonist activity [Dauvois & Parker, 1993].

This stabilisation effect may be increased after heat shock when more hsp90 is translocated to the nucleus and the level of 4S receptor is reduced. This could explain the synergistic effect of heat shock on antiandrogen action described in this thesis.

2) The binding of antihormones may be followed by the release of antihormone-4S receptor which may possess reduced affinity for the HRE, as compared to agonist-receptor complexes. This model could explain the promotion of DNA binding of the ER and PR by tamoxifen and RU486, respectively [Berry *et al.*, 1990; Meyer *et al.*, 1990].

3) A steroid receptor dimer is required for stable binding of the receptor to DNA [Kumar *et al.*, 1988; DeMarzo *et al.*, 1991] and the pure antiestrogen ICI 164384 may sterically interfere with dimerisation [Fawell *et al.*, 1990].

4) The antihormone-4S receptor complex interacts with the HRE in an identical manner to agonist-4S receptor but these may be unable to promote gene transcription. Cells may contain specific proteins or co-activators that determine the transcriptional activity of hormone responsive genes and the antihormone-4S complex may not be able to activate them. This could explain the variety of results obtained for the effect of tamoxifen on different cells from different animals [Dauvois & Parker, 1993]. TAF-1 is hormone-independent and may still be active in the antihormone-4S receptor, leading to stimulation of promoters where TAF-1 contributes most activity but where TAF-2 is

predominant the antihormone-4S receptor will act as an inhibitor of transcription [Berry *et al.*, 1990; Meyer *et al.*, 1990].

In reality the mechanism of action of any given antihormone may combine any of the mechanisms mentioned and their mechanism of action may alter from tissue to tissue in different animals.

Heat shock and the induction of thermotolerance could enhance several of these mechanisms of action *in vitro* and *in vivo*. Most likely as mentioned above the increased nuclear hsp90 could help to stabilise the antihormone-8S-receptor complex. Any increase in the efficiency of this stabilisation process would mean that less antihormone-4S receptor would be present and the other mechanisms would have a less significant role.

In the longer term it is hoped that the results of this *in vitro* work may help to guide the combination of hyperthermia and OH-Flut in repeated therapies in patients with prostate cancer.

6.8. Future work

The results in this thesis have increased the understanding of thermotolerance and of the synergistic effect of combining heat shock and antiandrogen therapies on prostate cells.

Analysis of the 8S receptor seen in the low salt fractions of heat shocked cells would reveal if this receptor complex was identical to the nonstressed 8S complex. It would also give some insight into the composition of the larger 11S steroid binding complex visible 12 hours after heat shock.

A number of questions regarding the role of hsp90 remain unanswered:

- 1) which of the 2 forms of hsp90 is associated with the steroid receptor;

- 2) whether this alters after heat shock (it may help to explain the change in K_d of the heat shocked receptor);
- 3) is one hsp90 molecule predominantly expressed in nonstressed cells and the other induced by stress;
- 4) does nuclear translocation involve both hsp90 forms.

These will be difficult questions to answer because the amino acid sequences for hsp90 α and hsp90 β are identical in 630 out of 724 possible residue matches and therefore antibodies would need to be very specific. The trypsin digestion patterns of hsp90 α and hsp90 β differs and this may be used as a method of discriminating between the two forms.

It would also be interesting to see the fate of the proteins whose synthesis is stimulated by stress.

Much more work needs to be carried out before the mechanisms of antiandrogen action and thermotolerance is fully understood.

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