M. D. THESIS

1997

Elijah O Kehinde

UMI Number: U106335

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M.D. THESIS

1997

ELIJAH O. KEHINDE

REGULATION OF PROSTATIC CARCINOMA BY GROWTH FACTORS

BY

ELIJAH OLADUNNI KEHINDE

M.B.B.S., F.M.C.S. (Nig), F.R.C.S. (Eng), Dip. Urol(Lond)

Department of Surgery and Urology,

Leicester General Hospital NHS Trust,

Leicester. LE5 4PW.

United Kingdom.

Thesis submitted for the degree of:

Doctor of Medicine

University of Leicester 1997

ABSTRACT : M.D. THESIS

REGULATION OF PROSTATIC CARCINOMA BY GROWTH FACTORS

Elijah O Kehinde

New insights regarding the biology of metastatic hormone resistant prostate cancer have demonstrated that, apart from testosterone, various non-androgenic growth factors are major mitogens for the cellular proliferation of prostate cancer cells. These growth factors may behave in an endocrine, paracrine or autocrine fashion to promote the growth of hormone independent prostate cancer cells. The present study was designed to test the hypothesis that non-androgenic growth factor inhibitors have a role to play in the management of patients with metastatic hormone-resistant prostate cancer.

The effects of the growth factor inhibitors Suramin, Somatostatin 201-995 (SMS 201-995), Estramustine phosphate (EMP) alone and in combinations (Suramin and SMS, Suramin and EMP, and SMS 201-995 and EMP) on the cellular proliferation of established human prostate cancer cell lines LNCaP, DU145 and PC3 and on cells obtained by primary culture from patients with various stages of prostate cancer were assessed using ^{3}H thymidine incorporation assay.

Suramin (0-270 ug/ml), SMS 201-995 (0-20 ug/ml), and EMP (0-50 ug/ml) produced dose dependent growth inhibition on both hormone dependent (LNCaP) and hormone independent (PC3) prostate cancer cells and cells obtained by primary culture of prostate cancer epithelial cells. Suramin and EMP combination produced statistically significant synergism on established prostate cancer cell lines, but not on prostate cancer cells obtained by primary culture.

In a preliminary clinical trial, patients with metastatic hormone-resistant prostate cancer were randomised into two treatment groups. Group A received EMP (280 mg twice daily) while Group B received EMP (280 mg twice daily) and low dose intravenous Suramin (1 gm weekly for 6 weeks). There were 6 patients in each treatment group. The control group, made up of 6 patients continued on maximal androgen blockade namely castration and Flutamide (250 mg three times daily) or Cyproterone acetate (100 mg three times daily) At the end of 6 months of treatment, there was statistical significant reduction of serum levels of prostate specific antigen and better pain control for patients on EMP and Suramin compared to patients who received EMP alone ($P < 0.01$) or patients on EMP and Suramin compared to controls ($P < 0.001$) (Kruskal-Wallis test).

Suramin, a non-androgen growth factor inhibitor has been shown in this study to produce significant in vitro and in vivo inhibition of the proliferation of prostate cancer cells. Its use as a novel chemotherapeutic agent for hormone-resistant prostate cancer patients alone or in combination with EMP deserves further study.

Acknowledgements

I would like to record my gratitude to:

Professor Peter Frank Robert Bell, Head of Department of Surgery for his encouragement, assistance and vital support which made this project a reality.

Dr. Terry Horsburgh for his moral support on numerous occasions when I felt like giving up because my cells were becoming persistently infected at the onset of my project in 1991! He also provided invaluable guidance during my project.

Professor Peter K Donnelly, now of Queensland Medical School, Australia who also provided advice on my project and who put pressure on me to present my data at scientific conferences!!

Mr. Timothy Terry for his general and financial support for this work.

Mr. Natu Mistry - a gifted tissue and cell culture specialist, for helping to passage repeatedly the established cell lines while I was working on separate samples. He also carried out all the work involving the ^{3}H thymidine incorporation assay.

Dr. Javed Iqbal who measured acid phosphatase in the supernatant of confluent cells - free of charge.

Dr. Fouad Habib of the University of Edinburgh for allowing me to be initiated into tissue and cell culture techniques in his laboratory at the University of Edinburgh. He also provided our unit with free samples of established prostate cancer cell lines DU145, LNCaP and PC3.

Dr. John Masters of the Institute of Urology, University of London, for supplying our unit with prostate cancer cell lines and for providing useful hints on tissue culture techniques.

Statement of Originality

The in vitro culture of tissues or cells is a recognised method of testing response to chemotherapeutic agents and for investigating the mechanism of drug resistance.

However, many established cell lines have lost their specific tissue characteristics due to de-differentiation as a result of passage (Otto et al 1988). In this study, we have shown that for cancer of the prostate, it is better to use epithelial cells derived from primary tissue culture for testing response to chemotherapeutic agents. Analysis by electron microscopy indicated that established human cancer of prostate cell lines (LNCaP and PC3) with passage numbers greater than 25, differ greatly from freshly isolated tumour cells. These cells had characteristics like deformed mitochondria that may make the cell lines highly susceptible to the effect of chemotherapeutic agents. Results obtained using these cells may therefore not be truly representative of what obtains in vivo.

The synergistic activity of Suramin (a growth factor inhibitor) and Estramustine phosphate (a microtubule associated protein inhibitor), has not hitherto been described in prostate cancer cell lines nor in cells obtained by primary culture of prostate cancer epithelial cells. This combination may prove useful in the management of patients with hormone resistant cancer of the prostate.

From our preliminary prospective clinical trial, we also report for the first time that EMP and low dose Suramin produced greater bone pain relief and statistically significant reduction in PSA maintained for at least 6 months compared to EMP alone in patients with metastatic hormone resistant prostate cancer.

Dedication

This work is dedicated to my parents who both developed cancers in their seventh decade of life and thus sparked my life-long research interest in oncology, and particularly urological oncology.

Ethical Considerations

For our clinical trials, we obtained approval to use Suramin for patients with hormone resistant prostate cancer from Medicines Control Agency, Department of Health, in London, Leicestershire Ethics Committee and Bayer U.K. Ltd., Newbury. Patients recruited into the study were given a patient information sheet to read, and gave written signed informed consent in the presence of the principal investigator (E.O. Kehinde) and a witness.

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Prostate Cancer: Literature Review

Section A

1.1 Introduction

> *"When the hair becomes grey and scanty, when specks of earthy matter*
> *begin to be deposited in the tunic of the arteries and when a white zone*
> *is formed at the margin of the cornea, at this same period the prostate*
> *gland usually - I might perhaps say invariably - becomes increased in*
> *size."*
>
> *(Benjamin Brodie 1862)*

The above description of the relationship between prostatic enlargement and the process of ageing was made by Sir Benjamin Brodie over a century ago. In 1832, he also first appreciated that the prostate gland could be involved in a cancerous process. However, it was not until after another period of about 100 years, when prostate cancer (CaP) was treated mostly by surgical extirpation, that the classic work by Huggins and Hodges established the pivotal role that androgens play in the development and treatment of prostate cancer. Consequently, hormonal ablation has remained the standard therapy for metastatic prostate cancer (Huggins and Hodges 1941, Huggins et al. 1941). About 80% of men with metastatic CaP will respond to hormonal manipulation, suggesting that a portion of their tumour cell population is androgen-responsive (Murphy et al. 1983). However, given time, the vast majority of patients will relapse into a hormonally unresponsive state for which no effective treatment exists at present (Brendler 1985, Warner et al. 1991). Such patients will invariably succumb to their disease with a median survival time of less than one year (Schutz and Crawford 1995).

Work by a series of investigators has suggested that even in the untreated state, prostate cancer is rarely homogeneous but probably represents a heterogeneous mix of androgen-dependent (dependent on androgen for survival), androgen-sensitive (stimulated by androgens but not dying in their absence) and androgen-independent (not stimulated by androgens and not dying in their absence) cells (Kasterdieck 1980). Treatment

by androgen withdrawal would be expected to impact only on androgen-dependent clones without eliminating any androgen-sensitive or androgen-independent cells.

Despite extensive evaluation of cytotoxic chemotherapy in hormone-insensitive CaP, no effective treatment regimen has been identified. Recently, there has been cautious optimism due to an improved understanding of the tumour biology of hormone-resistant CaP which might lead to new non-endocrine approaches to treating hormone-resistant disease. There is now ample proof that androgens are not the sole, or major, factor in the pathogenesis of benign prostatic hypertrophy (BPH) or of CaP. Increasing evidence suggests that other mitogens might be involved as regulators of prostate growth. Some of these modulators act in synergy with steroid hormone to ensure the normal development and function of the gland, whilst others bypass the androgens and imprint their own characteristics on the tissue, thus rendering the target cell androgen-independent.

One major aspect of tumour biology gaining increased understanding is the role of a unique group of polypeptide growth factors which control tumour growth. These growth factors are frequently produced by non-glandular tissues and they do not need to be transported via the blood stream in order to produce their biological effects (Habib 1990, Steiner 1995).

It is now believed that prostate cancer cells that survive after androgen ablation appear to be capable of providing their own fuel in the form of proteins or growth factors, which stimulate tumour growth in the absence of circulating androgens (Steiner 1995). Growth factors that are released by the cancer cells bind to the cell surface via specific receptors which act to stimulate cell division. This offers a potential opportunity to interfere with tumour progression by disruption of this autocrine loop. If it is possible to stop all cell division by blocking the interaction between the growth factor and the cell receptor, it would be possible to palliate further all patients with CaP once their tumours have become androgen-insensitive. Such agents will be attacking tumours at another site in the growth pathway and therefore open new avenues of therapy totally different in their mechanism of action from conventional chemotherapy.

1.1.2   Incidence of prostate cancer

Clinical CaP is rare before the age of 50 years, but thereafter it occurs at an increasing rate with age, reaching a peak incidence during the eighth decade. The disease occurs in varying forms and includes tumours with widely different activities (Franks 1954).

The worldwide incidence of CaP derived from data published by the Union Internationale Contre le Cancer (UICC) and the International Red Cross Committee, has been estimated at 600,000 new cases each year. The incidence was highest in the United States of America (U.S.A.) at 75 cases/100,000 population/year, the tumour being particularly prevalent in the black populations (Young et al. 1981), with Alameda County, California, having an exceptionally high incidence of 100.2/100,000 population/year. The incidence among Caucasians in the same region was about 40-60/100,000 population/year. A similarly high incidence has been observed in Australia and Northern Europe, especially Scandinavia (Fig.1). The incidence was lowest in Asian countries, e.g. India 6.2-6.8/100,000 population/year, Japan 3.1/100,000 population/year and Shanghai (Peoples' Republic of China) only 0.8./100,000 population/year. When considering the incidence in Africa, there is a striking contrast between relatively low incidence of 4.3/100,000 population/year in Senegal, and the high incidence of 32.4/100,000 population/year found in the black population of Bulawayo, Zimbabwe. This may be explained by the fact that an especially high percentage of autopsies were performed in Bulawayo during the period when the survey was carried out (Jackson et al. 1977, Kehinde 1995). In Nigeria, CaP is the commonest tumour of the genito-urinary tract (Lawani et al. 1982).

CaP is the second most common cause of cancer in males in the U.S.A., and the 4th most common cause in the U.K. Similarly, CaP represents 10% of all deaths from cancer in the U.S.A. The incidence of CaP has been increasing progressively in the Western world since the beginning of the 20th Century, but seems to have become stable since about 1960. Mortality has also been increasing for the past few years, but less than the incidence. This may be due to an improvement in diagnosis, particularly of the early forms or even latent disease, as well as an improvement in treatment (Debre et al. 1990).

Fig 1   The worldwide incidence of prostate cancer in 1990
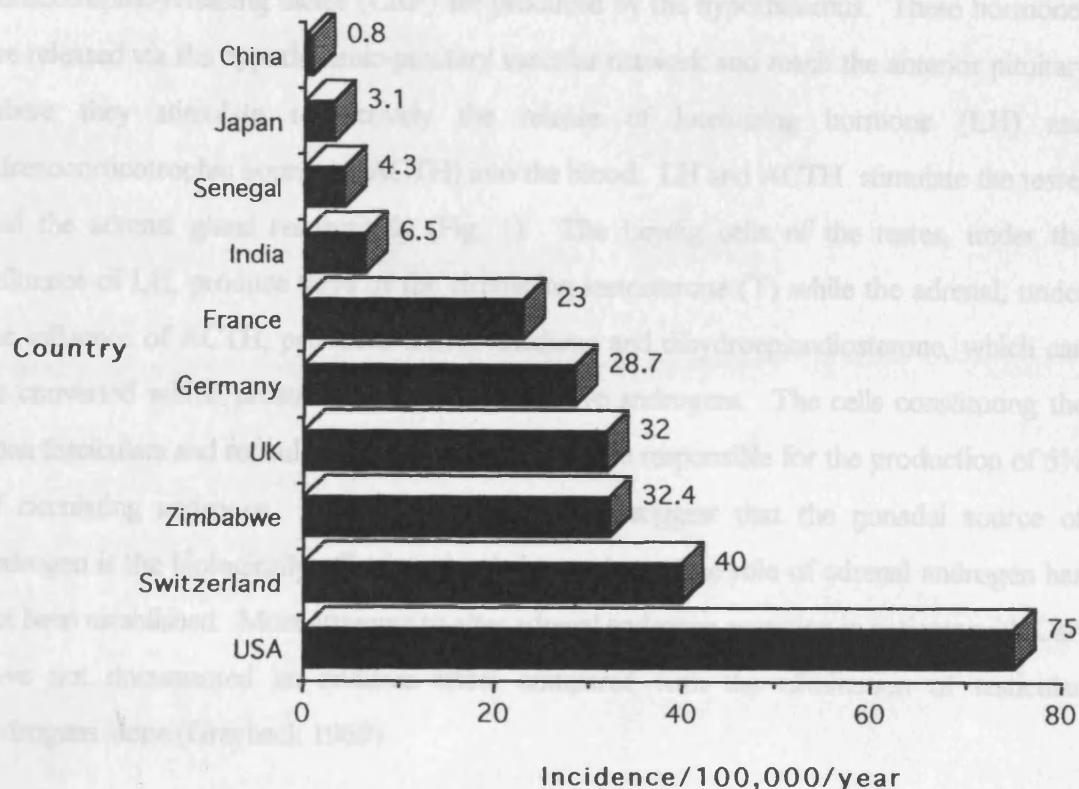
After Debre et al.1990.

The incidence of CaP is high in Europe and America and low in Africa (except Zimbabwe) and the Far East.

5

1.1.3 Endocrine control of the prostate gland

Besides prostatic stroma, androgen is the main factor that promotes growth and function of the prostate during embryonal, adolescent and adult life. The organs involved in the regulation of androgen production are the hypothalamus, pituitary, testes and adrenal glands (Fig 1). The peptide hormones, luteinizing hormone-releasing hormone (LHRH) and corticotrophin-releasing factor (CRF) are produced by the hypothalamus. These hormones are released via the hypothalamic-pituitary vascular network and reach the anterior pituitary where they stimulate respectively the release of luteinizing hormone (LH) and adrenocorticotrophic hormone (ACTH) into the blood. LH and ACTH stimulate the testes and the adrenal gland respectively (Fig. 1). The Leydig cells of the testes, under the influence of LH, produce 95% of the circulating testosterone (T) while the adrenal, under the influence of ACTH, produces androstenedione and dihydroepiandiosterone, which can be converted within prostatic tissue to more active androgens. The cells constituting the zona fasciculata and reticularis of the adrenal gland are responsible for the production of 5% of circulating androgen. Our present perceptions suggest that the gonadal source of androgen is the biologically effective circulating moiety. The role of adrenal androgen has not been established. Most attempts to alter adrenal androgen secretion in patients with CaP have not documented an additive effect compared with the elimination of testicular androgens alone (Grayhack 1969).

More than 95% of circulating T is bound to T-oestradiol-binding globulin and plasma albumin. Feedback loops serve to modify the secretion of the anterior pituitary and the hypothalamus. Thus, serum T exerts a negative feedback in both the hypothalamus and pituitary on the release of LHRH and LH respectively. Serum cortisol feeds back on the hypothalamus and pituitary and inhibits further production of CRF and ACTH. Adrenal androgens appear to be quite weak and do not exert any negative feedback on the hypothalamic-pituitary axis (Fig 2).

At the target level, in this case the prostatic cell T is converted to dihydrotestosterone (DHT) by the enzyme 5-alpha reductase which has been shown to be localised in

Fig 2

Hypothalamic-pituitary - testicular axis (from Schulz M J, Crawford E D.1995).

Adrenal Glands

LHRH and CRF

Cortisol Level

Adrenocorticotropic Hormone (ACTH)

Kidneys

Hypothalamus

Pituitary Gland

Adrenal Androgen and Estrogen

Prolactin

Prostate

Luteinizing Hormone (LH)

Testosterone and Estrogen

Testosterone Level

Testes

—— Feedback   – – –.– Stimulation
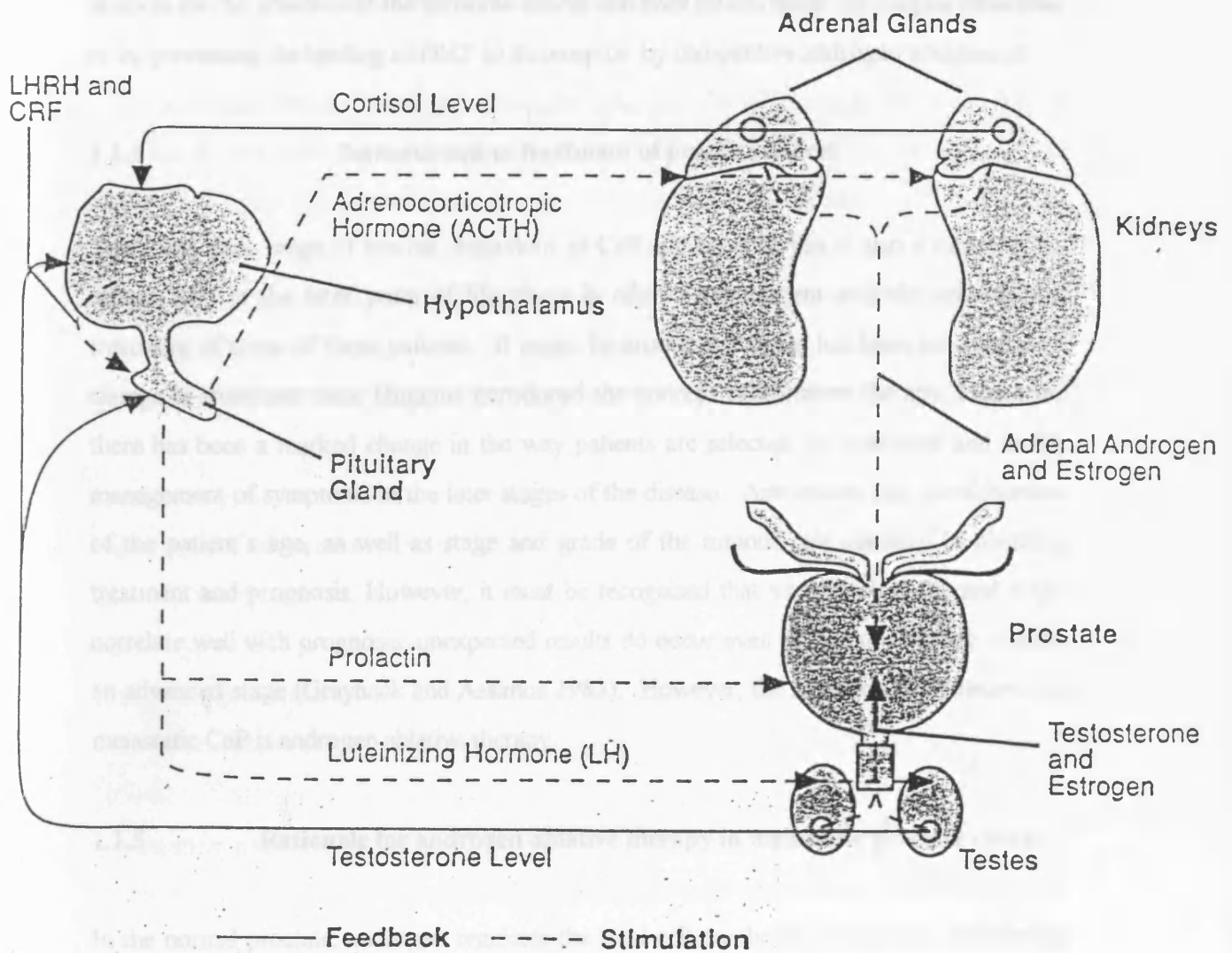
Androgenic Hormonal Axis: Stimulation, Production and Regulation

the nuclear membrane. In addition, DHT may also enter the prostate from the plasma in which the level of DHT (approximately 2 nmol/l) is less than 10% of the concentration of T. Inside the cell, DHT binds to the intracellular androgen receptor (Anderson and Liao 1968). It is this DHT-androgen receptor binding which results in the trophic stimulation and proliferation of the hormone dependent CaP cells (Kyprianou et al. 1990). Thus androgen ablation can be achieved at the prostatic cancer cell level by inhibition of 5-alpha reductase or by preventing the binding of DHT to its receptor by competitive androgen antagonists.

1.1.4   Introduction to treatment of prostate cancer

There is a wide range of clinical behaviour of CaP and because this is also a tumour that affects men in the later years of life, there is often a complacent attitude towards the treatment of some of these patients. It might be argued that there has been no significant change in treatment since Huggins introduced the concept of hormone therapy. However, there has been a marked change in the way patients are selected for treatment and in the management of symptoms in the later stages of the disease. Assessment and consideration of the patient's age, as well as stage and grade of the tumour, are essential in planning treatment and prognosis. However, it must be recognised that while both grade and stage correlate well with prognosis, unexpected results do occur even with a poor grade or with an advanced stage (Grayhack and Assimos 1983). However, the mainstay of treatment for metastatic CaP is androgen ablative therapy.

1.1.5   Rationale for androgen ablative therapy in metastatic prostate cancer

In the normal prostate, androgen regulates the total cell number by chronically stimulating the rate of cell proliferation while simultaneously inhibiting the rate of cell death (Isaacs, 1984). Like the normal prostate, CaP cells often retain the ability for androgenic regulation of their rate of cell proliferation and death (Kyprianou et al. 1990) and therefore often respond to androgen ablation therapy (Scott et al. 1980). Such androgen ablation therapy can be achieved by either surgical or pharmacological approach. Surgical removal of the testes (bilateral orchidectomy) eliminates testicular androgens. Second line hormonal ablation involves hypophysectomy or adrenalectomy to eliminate adrenal androgens.

At present, the 'gold standard' for androgen ablation therapy is bilateral orchidectomy, as was first suggested by Huggins et al. 1941. Surgical removal of the testes results in a 95% reduction of circulating T. The advantages of this simple surgical approach include known efficacy, assurance of patient compliance, cost effectiveness, minimal morbidity and rapidity of symptomatic response. The disadvantages are side effects like loss of libido, erectile impotence, hot flushes, occasional breast tenderness and psychological trauma. Because of these disadvantages, substantial numbers of men with metastatic CaP prefer pharmacological means to achieve androgen ablation. Presently, there are a number of pharmacological agents for androgen ablation. These agents can be divided into 2 basic groups. The first group produces androgen ablation indirectly by means of lowering the level of androgens in the blood. Such agents include oestrogenic compounds, progestational agents and LHRH analogues. The second group of agents produces androgen ablation by inhibiting the intracellular uptake of 5 DHT at the nuclear receptors of CaP cells.

It has been estimated that more than half of patients with newly diagnosed CaP will present with evidence of metastatic disease (Chisholm, 1981). However, only half of these will have tumour related symptoms other than those of outflow tract obstruction. Therefore, it has been suggested that hormonal treatment be withheld until needed for treating these symptoms, due to the belief that this gives optimum benefit to the patient (Byar, 1973). This may be acceptable in the very elderly or medically ill patients but it has been shown that patients will progress more rapidly from a locally advanced stage to metastatic stage when treatment is withheld (Byar, 1977). It may be correct to say that the optimum effect of hormonal treatment is in symptomatic patients, but since there is no useful alternative primary treatment for metastatic disease, either hormone treatment or orchidectomy must remain the first choice for all of these patients.

1.1.6   Methods of hormone therapy

1.1.6.1   Orchidectomy.

Orchidectomy was considered by Huggins and Hodges in 1941 to be equivalent to stilboestrol in hormone manipulation of CaP. Huggins noted a clinical improvement in 15 of his 21 original patients treated by orchidectomy. Orchidectomy has become increasingly used as the first choice for hormonal treatment; there is no debate about its role in those patients for whom oestrogens are definitely contra-indicated, that is, patients over 75 years, those with a past history of cardiovascular disease and possibly those who have undergone a recent operation. The advantages include the avoidance of pill - taking, lack of cardiovascular complications and gynaecomastia while impotence is not inevitable. Some patients however experience unpleasant "hot flushes", but this can be treated with cyproterone acetate (CPA) 25 mg once daily. For surgical castration, it does not matter whether the testes are removed completely or a subcapsular orchidectomy is carried out. Plasma testosterone and LH values have been shown to be identical after both operations. (Clark and Houghton, 1977).

1.1.6.2   Oestrogens

Together with castration, Huggins and Hodges, (1941) introduced the newly synthesised oestrogen dimethyl stilboestrol (DES) into the management of metastatic CaP to achieve medical castration. Numerous clinical trials by the Veterans Administration Co-operative Urological Research Group (VACURG) have shown that DES 1 mg t.d.s. should be regarded as the 'standard' dose as DES 1 mg once daily (o.d) does not suppress serum testosterone fully for 24 hours. Bishop, (1996) has argued for the continued evaluation of low dose DES (1 mg o.d.) in patients with advanced prostate cancer because the results in terms of response, progression and survival rates compare favourably with more conventional, but expensive treatment. However, DES 1 mg o.d. can be the cause of considerable cardiovascular hazard to the patient. To ameliorate the cardiovascular side-effects of DES, most urologists still using this drug combine it with low-dose Aspirin 75-150 mg o.d. DES remains the first choice for oestrogen treatment of CaP because it is cheap and also because none of the other oestrogenic preparations have been shown to be superior. Adverse effects of DES include feminisation, exacerbation of heart failure, hypertension, vascular complications (such as increased risk of myocardial infarction), painful breasts, water retention, nausea and impotence.

1.1.6.3 Progestogens

Several progestogens have been studied with the aim of avoiding the feminisation and cardiovascular side-effects of oestrogen therapy. Some progestogens have the ability to suppress gonadotrophin production, to inhibit testicular steroid production and to inhibit directly the intracellular prostatic effects of testosterone. Medroxyprogesterone acetate (MPA) 100 mg t.d.s. was used in the VACURG study but found to have no advantage over 1 mg of DES o.d.

1.1.6.4 Anti-Androgens

There are 2 types of anti-androgens, namely: steroidal (e.g. CPA) and non-steroidal (e.g. Flutamide and Casodex). These drugs act by blocking testosterone synthesis in the testes. The early results from several clinical trials in patients with metastatic disease show no particular advantage over DES with perhaps fewer side-effects. It is, however expensive. CPA is also useful as an adjuvant in other forms of hormonal manipulation. It is useful in the prevention of "hot flushes" induced by castration or LHRH analogues like Zoladex. It is also used to reduce the initial flare reaction caused by the administration of LHRH analogues. Flutamide on the other hand, is regarded as a pure anti-androgen because it is non-steroidal. Its usage is not associated with the development of impotence, a serious side-effect of virtually all other methods of hormonal manipulation. It is, therefore, very useful for men with metastatic CaP who require hormonal manipulation, but who are also keen to retain their potency. Flutamide, like CPA, is also used for treating "hot flushes" induced by castration or LHRH analogues and as a second line method of hormonal manipulation for patients with metastatic CaP not responding to DES. Flutamide produces its pure anti-androgenic effect by binding and blocking DHT receptor protein.

1.1.6.5 Anti-Adrenals

Drugs such as aminogluthetamide, ketoconazole and spironolactone interfere with enzymes that are involved in the synthesis of testosterone. They have been tested in clinical studies, mainly as secondary forms of endocrine treatment; the responses are brief and of little clinical value. Toxicity is also a major drawback to the widespread usage of these agents.

1.1.6.6   Luteinizing hormone releasing hormone (LHRH) analogues

The structure of LHRH was first described by Schally and co-workers (1971) who also accomplished the synthesis of the substance and of several active analogues and antagonists (Coy et al. 1975). For this achievement Schally and Guillemin were awarded the Nobel Prize in 1981. LHRH analogues, when applied to humans, produce an initial rise of plasma testosterone reaching a maximum after 3-4 days. Plasma testosterone will then decrease to castration levels within 3-4 weeks. LHRH agonists are effective in continuously suppressing plasma testosterone over long periods of time. Flare up of very advanced disease can be associated with the initial rise of plasma testosterone which can be minimised by preceding treatment with LHRH agonists by a 1-2 weeks course of an anti-androgen such as CPA (Labrie, 1987).

The disadvantages of LHRH agonists are that they are expensive and require monthly injections - although 3 monthly preparations are currently under trial. Their main advantage is in the reduction of side-effects, especially cardiovascular (Schroder et al. 1987). However, in prospective studies, LHRH agonists have been compared to standard treatment by orchidectomy and no differences in response and progression rates were observed by Parmar et al. (1985).

1.1.6.7   Maximal or Total Androgen Blockade (MAB)

The adrenal androgens dehydroepiandrosterone (DHEA) and androstenedione are converted peripherally to T and DHT. Adrenal androgens contribute 6% to 20% of the total DHT in the prostate (Harper, 1984). However, after orchidectomy, or oestrogen or LHRH therapy, up to 50% of intraprostatic DHT remains. A significant level of DHT remains in the prostate tissues even though serum T has been reduced by 90%.

The possible role of adrenal androgens in metastatic prostate cancer was first investigated by Huggins and Scott in 1945 when they performed bilateral adrenalectomy on four patients. Unfortunately, all four died of adrenal insufficiency. The first hypophysectomy for metastatic prostate cancer was done in 1948. Hypophysectomy has been shown to

produce objective remissions in previous non-responders (Brendler, 1973).

Animal studies have been used to study the effects of adrenal androgens in prostate cancer growth. Van Weerden and colleagues, studying a transplantable human prostate cancer cell line in mice, found that androstenedione exerted a stimulating effect on prostate cancer cells when its conversion resulted in intracellular T and DHT levels exceeding threshold levels of tumour growth (Van Weerden et al. 1991).

Most patients experience progression after conventional androgen deprivation with orchidectomy, LHRH agonist or oestrogen therapy. Two theories exist to explain disease progression. One proposes that prostate cancer cells are heterogeneous in their growth requirements for androgens (polyclonal theory; vide infra). Relapse occurs when the population of androgen-insensitive cells grows to a critical size after hormone deprivation. This suggests that further androgen blockade, specifically adrenal androgen blockade, would have little effect on response or survival.

Coffey and Isaacs (1981) studied Dunning R-3327 rat prostate adenocarcinomas and found a heterogeneous cell composition within the tumour. Eighty per cent of cells were found to be androgen-sensitive, 20% androgen insensitive. They theorized that relapse of prostate cancer after androgen ablation is the result of continued growth of the androgen insensitive clone. Mechanisms by which cells may develop androgen insensitivity include genetic instability or multifocal origin (monoclonal theory; vide infra).

The monoclonal theory is the basis for total androgen suppression. One attempts to reduce the adaptation of hormone-resistant clones by lowering the serum T below castrate level. After orchidectomy or oestrogen treatment, substantial amounts of DHT are found in prostate cancer cells, and this is of adrenal origin. Thus, the rationale for maximal androgen blockade (MAB) is to remove or block the effects of the gonadal and adrenal androgens on the prostate cancer cell to inhibit further growth.

Labrie, one of the strongest advocates of complete androgen blockade, used medical or surgical castration and a non-steroidal anti-androgen to achieve complete androgen suppression. Many investigations have been undertaken to test this combination in patients with Stage D_2 prostate cancer. In 1987, Labrie reported encouraging results in an uncontrolled trial in which the combination of leuprolide and flutamide was used in 154 patients with Stage D_2 prostate cancer (Labrie 1987). The average length of treatment was 22 months. He reported complete response rates of 29.2% according to National Prostate Cancer Project (NPCP) criteria, a result 6.3 times greater than the response rate in five previously published studies used as control (4.6%). Of the combined-therapy patients, 4.5% did not respond, in contrast to 18% non-responders in the monotherapy group. The duration of response was longer in the patients receiving combination therapy.

The National Cancer Institute (NCI) sponsored a large double-blind, randomised intergroup trial for patients with Stage D_2 CaP that compared leuprolide to leuprolide and flutamide (Crawford and Eisenberger 1989, Crawford 1990). The combination arm showed a statistically significant increase in time to progression and survival. Patients with minimal disease and a good performance status had the greatest benefit from combination therapy. However, in another study, 262 patients with untreated advanced CaP were treated with orchidectomy or goserelin and flutamide. Median follow-up in the study was 39 months. Time to progression and survival were similar for the two groups (Iversen 1990).

Finally, the results of another multi-centre study comparing orchidectomy and nilutamide with orchidectomy and placebo involving more than 350 patients have shown a significant increase in time to progression and cancer-related survival in the active combination arm. Median survival time was 28.3 months in the nilutamide group versus 23.2 months in the placebo group. These results are encouraging and other multi-centre randomized trials are underway to clarify the issue.

1.1.6.8   Conclusion - Hormone therapy

The long term results from endocrine treatment in patients with metastatic disease are disappointing. Approximately 70-80% will respond, but the mean survival time is less than 18 months. Up to 25% of patients may survive 5 years with no more than 10% surviving at 10 years.

**1.1.7   TREATMENT OPTIONS FOR FAILED PRIMARY HORMONE
                  TREATMENT THERAPY**

1.1.7.1	Additional hormone therapy or manipulations
1.1.7.2	Chemotherapy
1.1.7.3	Radiotherapy
1.1.7.4	Bone-seeking isotopes - Strontium 89
1.1.7.5	Pain relief

The details of the mechanisms leading to failed primary hormonal treatment or to the emergence of hormone resistance will be discussed in Section B. The concept of hormone escape or resistance is relevant to management, as it indicates that further hormonal manipulation is unlikely to be of benefit. However, it is essential to ensure that treatment failure is not due to other factors, such as compliance with medication.

The choice of secondary treatment is made on the basis of the nature of the symptoms, the general state of the patient, and sometimes, on the available expertise. The main problem to the patient is pain and it is usually this that becomes his principal complaint.

1.1.7.1   Additional hormone therapy or manipulations

Once a patient has failed to respond to first line hormonal manipulation as detailed above, it is illogical to expect that either another oestrogen or a larger dose will have any further effect. The occasional response that has been recorded with second line hormone treatment is always brief and subjective and probably indicates that the diagnosis of failed primary treatment was incorrect. The addition of oestrogens after orchidectomy is ineffective. The reverse, orchidectomy after oestrogen therapy, is often carried out, but in a study of 20 such patients Stone et al. (1980), found no objective response to secondary orchidectomy

and only 20% subjective responses of short duration. At present, Flutamide is undergoing clinical trials as a second line hormone manipulation that is, after failed initial hormone therapy. The results of these trials are awaited.

Adrenalectomy to remove extragonadal sources of androgen was originally theoretically attractive, but in the few early studies the results were so poor that this treatment fell into disrepute (Brendler, 1973). Attempts to achieve a similar effect pharmacologically have shown very little benefit.

Hypophysectomy, in contrast has remained a relatively neglected procedure. The mechanism of action of hypophysectomy in relieving bone pain is not well understood. While it does not improve survival, it does have a dramatic symptomatic benefit in up to 80% of those with severe metastatic bone pain and long pain-free survival can occur (O'Donoghue, 1980). The results of hypophysectomy do not appear to depend on prior hormone responsiveness or length of hormone remission.

1.1.7.2 Chemotherapy

The results from a range of studies with chemotherapeutic agents have been disappointing (Torti and Carter 1982, Tannock 1985, Schutz and Crawford 1995). The assessment of these drugs to see if they have an anti-prostate cancer effect has continued for more than a decade, yet many of the results are unconvincing and the value of any of these agents in clinical practice is very limited. Much of the difficulty lies in assessing the results in a disease which can have a slow course and which has few reliable objective measures of response. Perhaps more importantly, there is lack of understanding of the mechanisms involved in the emergence of hormone resistant prostate cancer cells. This will be discussed in Chapter 1 Section C.

A group of agents has been studied in adequate numbers to establish that they do not have anti-tumour activity: - nitrogen mustard, lomustine, methotrexate, cisplatin and dacarbamazine (Scott et al 1975, Slack and Murphy 1983). However, combinations of nitrogen mustard with oestradiol (Estramustine Phosphate - EMP) and chlorambucil with Prednisone (Prednimustine) have shown modest response rates, with their main attraction being low toxicity. Cyclophosphamide, methotrexate and EMP offer the best

compromise between side-effects and anti-cancer activity and these have been most studied. The combination of cyclophosphamide with 5-fluorouracil or with doxorubicin (adriamycin) has overall response rates ranging from 20-40%. There is, however, no definite evidence of the superiority of any combination over single agent treatment. Ever optimistic, some centres are studying three or more agent combinations! (Straus et al. 1982).

There are many reasons for the dismal experience with chemotherapy. The patients have extensive disease and are heavily pre-treated. They have less physiologic reserve to withstand full-dose chemotherapy; the doses may be attenuated to compensate for the diminished physical activity. Performance status is also usually impaired in these patients. Extensive pre-treatment, extensive disease and diminished performance status have been shown to be adverse prognostic factors for outcome in other more chemosensitive tumours, and there is no reason why these factors should have a different outcome in prostate cancer (Eisenberger and Abrams 1988, Schutz and Crawford 1995). Identifying patients who will have poor responses to hormonal therapy will allow other therapies to be instituted earlier, when these patients can better withstand the side effects of chemotherapy.

The main clinical problem is to balance effectiveness with toxicity in these relatively elderly patients. At present, there is no justification for using the majority of these drugs except within clinical trials. In the future, more effective and less toxic drugs will be needed and their optimum role may be earlier in the course of management rather than for secondary treatment only.

1.1.7.3 Radiotherapy

The use of radiotherapy for localised bone pain is well established. In some cases of pulmonary metastases causing haemoptysis or shortness of breath, palliative radiotherapy may be effective. Techniques of half-body irradiation have been used with success for diffuse bony pain (Fitzpatrick and Ryder, 1976). Either the upper trunk or lower trunk or whole body in split courses can be treated. A maximum dose of 750 c Gy is given to the upper half of the trunk and after 4-5 weeks, 1000 c Gy to the lower half. Using this regimen, Rowland et al. 1981 achieved an 80% response within 24-48 hours and 67% of patients had pain relief until death.

1.1.7.4 Bone-seeking isotopes : Strontium-89

Radiation may also be delivered by bone-seeking isotopes intravenously infused, and many patients with painful bony metastases from hormone-resistant prostate cancer have derived symptomatic relief with this treatment. Strontium-89 emits beta particulate radiation and is selectively taken up by metastases in bones; it is as effective as local or hemi-body external beam irradiation, and is significantly more effective than conventional radiotherapy in preventing the appearance of new sites of pain (Robinson et al. 1987).

1.1.7.5 Pain Relief

When both primary and secondary treatments have been tried and have failed, the treatment of symptoms will remain an essential part of management. Non-steroidal anti-inflammatory agents such as Ibuprofen, by their inhibition of prostaglandins, can help very effectively with metastatic bone pain. When non-narcotics fail to relieve symptoms, a weak narcotic such as Codeine or dihydrocodeine should be prescribed. In severe cases, opiates may be required.

Section B

1.2   **Background to potential novel drug treatment of hormone resistant prostate cancer**
     1.2.1   Tumour biology of prostate cancer - a new hypothesis
     1.2.2   Apoptosis and prostate cancer
     1.2.3   Oncogenes and tumour suppressor genes in prostate cancer
     1.2.4   Growth factors - what are they?
     1.2.5   Growth factors as targets for therapy in hormone resistant prostate cancer
     1.2.6   Growth factor inhibitors in the therapy of hormone-resistant prostate cancer
     1.2.7   Conclusion

1.2.1   **Tumour biology of prostate cancer - a new hypothesis**

Despite advances in earlier diagnoses, the mortality of CaP has increased at 0.7% per year for white and 1.7% for black males within the US (NCI 1985). Since the pioneering studies of Charles Huggins in the 1940s androgen ablation therapy has been the standard treatment for metastatic CaP. This is because nearly all men with metastatic CaP have an initial, often dramatic, beneficial response to this hormonal therapy. This initial response demonstrates that at least a portion of the cancer cells are androgen responsive. Unfortunately while this initial response is of substantial palliative value, nearly all treated patients eventually relapse to an androgen-insensitive state and succumb to the progression of their cancer; cures if any, are rare (Scott et al. 1980). Because of this, the annual death rate from CaP has not decreased at all over the 56 years since hormonal treatment has been the standard therapy (Silverberg and Lubera, 1988). In fact, over the last 56 years, the superficially benign nature of androgen ablation therapy has tended to disguise the fact that CaP is still a fatal disease for which no effective therapy is presently available. (Lepor et al. 1982). This does not mean that hormonal therapy is not useful in the treatment of metastatic CaP. It only indicates that such hormonal therapy would have been used in combination with additional forms of therapy if the cure rate for metastatic CaP was ever going to be substantially increased (Isaacs, 1988).

In order to develop new and more effective forms of therapy for the treatment of metastatic CaP, an understanding of the basic mechanism involved in the development of androgen insensitivity is required.

Four distinct mechanisms have been proposed:

a)   Inadequate elimination of androgen produced by standard androgen ablation.

b)   Adaptation of initial androgen-dependent prostatic cancer cells to become androgen-independent, induced by the low androgen environment (environmental adaption model or monoclonal theory).

c)   Clonal selection of androgen - independent prostatic cancer cells heterogenously present within the prostatic cancer before therapy is begun (environmental selection model or polyclonal theory)

d)   Changes associated with androgen receptor secondary to androgen ablation therapy.

Each of these 4 mechanisms will now be considered in detail.

1.2.1.1   Mechanism (a): Inadequate androgen suppression model

This mechanism proposes that prostatic cancers are homogeneously composed of androgen-dependent cancer cells which vary widely with regard to the absolute concentration of androgens needed to stimulate their proliferation and prevent their death (Labrie and Veilleux, 1986). The standard forms of androgen ablation presently used (i.e. surgical or medical castration) eliminate testicular androgen, but do not eliminate adrenal androgens and provide only partial androgen ablation.   CaP cells requiring low levels of T would therefore survive and proliferate.

If some of the cancer cells within a prostatic cancer can respond to any level of androgen stimulation, then the complete elimination of all serum and prostatic androgen would be absolutely required in order to kill all the cancer cells. If this concept is correct, then the presently used forms of partial androgen withdrawal would not be optimal (Labrie et al. 1983).   While these standard therapies do eliminate testicular androgen, adrenal androgens are not eliminated (Geller and Albert, 1987).   Thus in the human male, as in the rat, castration reduces DHT in the cancer cells by greater than 80%, but it does not completely eliminate all androgens (Kyprianou and Isaacs, 1987). The adrenals in males of both rats and humans thus supply 10-20% of the prostatic DHT (Geller and Albert, 1987).   It has been postulated that a more complete form of androgen ablation could be achieved by combining a direct acting anti-androgen, to block any possible effects of adrenal androgen, with surgical or medical castration to eliminate the testicular androgens.   Such a combinational approach theoretically could be more effective in the treatment of prostatic

cancer than castration alone, if indeed the 10-20% of DHT remaining in prostatic cancer cells would be shown to have a stimulatory effect on the proliferation of these cancer cells.

In addition, if all the cells in a CaP were androgen-dependent but varied widely with regard to the absolute concentration of androgens needed to stimulate their growth, then it should be possible to castrate a man and eliminate all of the cancer cells dependent upon higher levels of androgen and then later, at relapse, either remove or block the adrenal androgens to completely eliminate all the remaining highly sensitive cancer cells.

If this model were correct, it predicts that theoretically all men treated initially with partial androgen ablation and who subsequently relapse, should respond to secondary forms of androgen ablation targeted at eliminating or blocking the adrenal androgens. Clinically, it is well established, however, that only a very small population (<10%) of men treated with secondary forms of androgen ablation at relapse respond with an objective response (Schulz et al. 1987).

These results are completely incompatible with the concept that insufficient androgen suppression is the major mechanism for relapse of CaP to testicular androgen withdrawal. Thus, the scientific basis for complete androgen withdrawal as the only acceptable form of androgen ablation (Labrie et al. 1983), does not appear tenable based upon the scientific facts (Isaacs, 1988).

1.2.1.2   Mechanism (b):   Environmental adaption or monoclonal theory
One way in which the relapse to androgen withdrawal can occur is that prostatic cancers initially could be composed of tumour cells that are homogenous, at least in regard to their dependence on androgenic stimulation for their maintenance and continuous growth (i.e. androgen-dependent cancer cells). Following castration, most of these dependent cells stop proliferating and die, thus producing an initial positive response to withdrawal therapy. Some of these androgen-dependent cells, however, under environmental pressure, could randomly adapt to become androgen independent. These androgen-independent cells once formed, proliferate without the requirement for androgenic stimulation and thus repopulate the tumour producing a relapse after castration. In such an explanation, the changing

environmental conditions following castration are assumed to be critically involved in inducing the adaptive transformation of an initially androgen dependent to an androgen independent tumour cell, hence the name "the environmental adaption model" or "the monoclonal theory" (Fig 3).

1.2.1.3 Mechanism (c): Environmental Selection model or Polyclonal theory

This model proposes that initially CaP are heterogeneous, being composed of pre-existing clones of androgen-dependent cells and independent tumour cells (Fig 4). The androgen-independent cancer cells can be of 2 types: cells which are neither dependent nor sensitive to androgenic stimulation for their growth (i.e. androgen-independent insensitive cells) or cells which grow faster in the presence of adequate androgen levels but which can still grow continuously even when no androgen is present (i.e. androgen-independent sensitive cancer cells) (Nowell 1976, Isaacs, 1982, 1988). Regardless of what type of androgen-independent cells (i.e. insensitive or sensitive) are present, castration in such a context would result in the death of only the androgen-dependent cells without stopping the continuous growth of the androgen-independent ones. While this would produce an initial positive response, these independent cells would continue to proliferate following castration. Even if these androgen-independent cells initially represented only a small fraction of the starting tumour, their continuous growth would eventually not only replace completely any tumour loss due to the death of androgen-dependent cells, but progressively re-expand the tumour producing a relapse (Fig.4).

The environmental adaptation or selection models further state that the growth of androgen - independent CaP cells depend on polypeptide growth factors (Figs 3 and 4) (Steiner 1995). Regardless of whether environmental adaption or selection is the mechanism for relapse to androgen withdrawal therapy, eventually clones of androgen-independent CaP grow to kill the patient. Because of this, it is important to resolve whether environmental adptation or selection is actually responsible for relapse. The importance of resolving whether adaptation versus clonal selection is the mechanism responsible for relapse is that the optimal therapy for CaP is very dependent upon the answer. If the environmental adaptation model is the mechanism for the relapse to standard androgen ablatation, then

Figure 3

Monoclonal theory of the emergence of androgen independent prostate cancer cells and implications for treatment.

Rx Options

MALIGNANT TRANSFORMATION

⟵ Unicellular origin

ANDROGEN DEPENDENT
100%

⟵ *Anti-Androgens
only*

⟵ Genetic instability

⟵ Adaptation/Clonal Selection

ANDROGEN INDEPENDENT / ANDROGEN DEPENDENT
↑ ↑
(GROWTH FACTORS ++++)

**Anti-Androgens
+
Anti-growth factors

Survival ± 2 years

**Survival ? > 2 years
duration**

Death
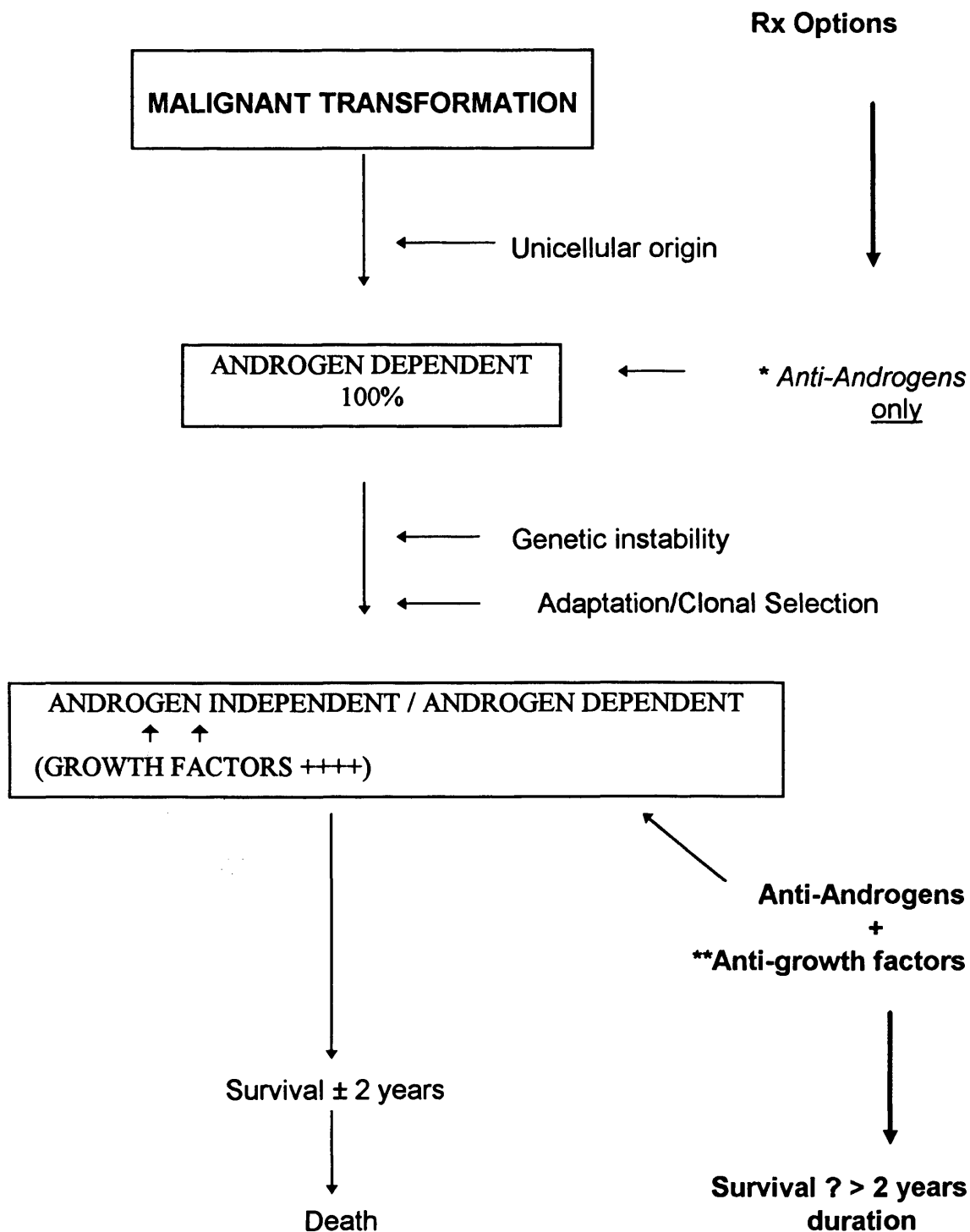
Rx = Treatment
*   CURRENT treatment - Huggins et al 1941, ie hormone manipulation
**   FUTURE treatment modification - if monoclonal theory is correct
.+++ Growth Factors - stimulate growth of androgen independent cells

Figure 4

Polyclonal theory of the emergence of androgen independent prostate cancer cells and implications for treatment.

Rx Options (proposed)

MALIGNANT TRANSFORMATION

← multi-cellular origin

ANDROGEN DEPENDENT/ ANDOGEN INDEPENDENT

← *Anti-Androgens

+
**Anti-growth factors

← X - never occurs or
← > 2years

ANDROGEN INDEPENDENT / ANDROGEN DEPENDENT   (GROWTH FACTORS +++)

DEATH

? prolonged survival
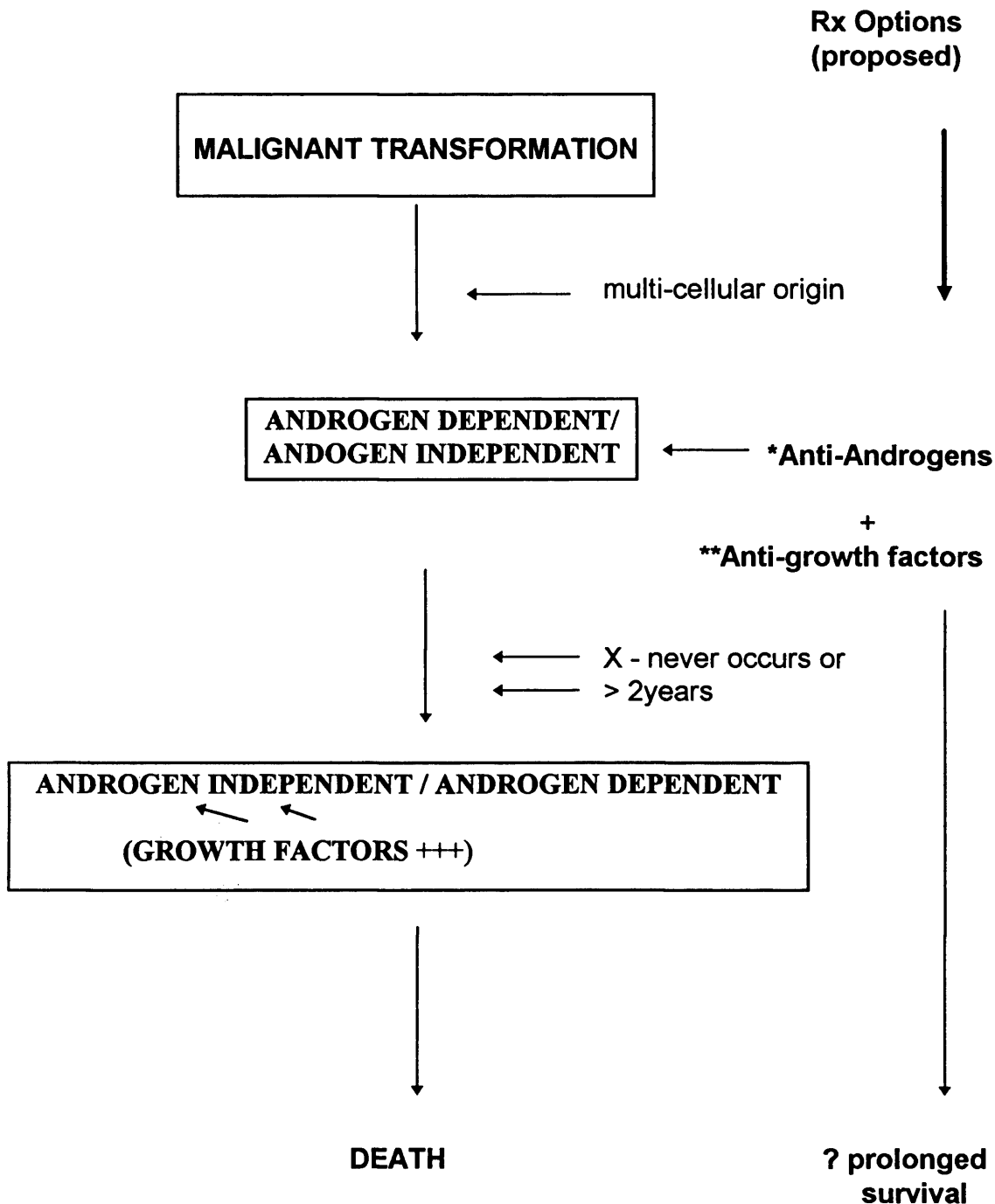
Rx = treatment

*   Current treatment
**   Future treatment modification - if polyclonal theory is correct
++++ Growth Factors - stimulate growth of androgen independent cells

chemotherapy targeted at the androgen-independent CaP cells should be initiated only after relapse to androgen ablation (i.e. chemotherapy given sequentially after androgen ablation) as indicated in Fig. 3. This is because only after relapse would such androgen-independent cells be present within the tumour (Fig. 3). In direct contrast, if CaP are heterogenously composed of clones of both androgen-dependent and independent cancer cells even before therapy is begun as shown in Fig 4, then chemotherapy must be combined simultaneously with androgen ablation to affect the growth of both populations of tumour cells, at the smallest tumour burden (Isaacs 1988). Animal models have clearly demonstrated not only the fact that CaP can be heterogenously composed of androgen-dependent and independent CaP cells before hormonal therapy is begun (Mostofi et al. 1981, Viola et al. 1986), but also the fact that an increase in survival above that produced by castration alone cannot be produced no matter how complete the androgen withdrawal therapy is utilised (Smoley et al. 1977, Isaacs and Coffey, 1981, Kyprianou and Isaac, 1987). Using these animal models, it can be shown that an increase in survival above that produced by castration alone can only be produced if non-hormonal chemotherapy is given simultaneously in combination with castration, as early as possible in the course of the disease (Isaacs, 1984).

Furthermore, studies involving the androgen-dependent Shionogi mammary tumour have shown that endocrine management leads to a large increase in the tumorigenic stem cell to progeny cell ratio and that this increase is associated with a 500-fold increase in the number of androgen-independent stem cells. These androgen-independent stem cells, which are thought by Rennie et al. (1990) to arise through adaptation and not through selection, as propounded by Isaacs and Coffey (1981), of initially androgen-dependent cells, because they have an extremely low incidence in regressing parent tumours. As if this picture is not confused enough, studies into the cellular and molecular events leading to hormone escape are proving to be even more difficult and it is yet to be established what the role of the androgen receptor (AR) is in the acquisition of the androgen independent phenotype (Habib and Grant, 1996).

1.2.1.4   **Mechanism (d). Changes associated with androgen receptors secondary to androgen ablation therapy**

This mechanism which seeks to explain cellular and molecular events involving the AR leading to the androgen independence or hormone escape phenomenon states that

androgens act antagonistically as well as agonistically on prostatic secretory epithelia, balancing the rate of cell division with that of apoptosis. Additionally, the suggestion is that the AR are also intrinsic to epithelial cell dynamics, both potentiating and inhibiting normal cell cycling in normal hormonal environments. As a consequence, under the androgen-limiting conditions associated with the endocrine management of prostate tumours, the AR is present in an unliganded form unable to give the appropriate growth-stimulation signal but instead only giving the system messages for cell death. In hormone escape these cell-death signals no longer occur, with resulting tumour relapse and the eventual rapid death of the patient. In view of this, the question must be whether the AR is directly involved with this crucial event in the disease progression and if so, how is its involvement manifest?

There are three potential mechanisms by which the AR can directly influence the switch to androgen-independence in prostatic tumours: (i) down-regulation of the AR, thereby removing the cell-death signal; (ii) alteration of the responses of target genes to AR interaction and; (iii) alteration of the AR through somatic mutation (Habib and Grant 1996).

To date, attempts to show the loss or down-regulation of AR gene expression as the precursor of tumour relapse have been very erratic, with the most conclusive data coming from studies in vitro in which androgen-independence is heavily associated with altered cellular AR content.

It has been shown that AR gene expression in the androgen-independent prostate carcinoma cell lines DU145 and PC3 is markedly down-regulated compared with their androgen-sensitive counterpart, the LNCaP cell line (Tilley et al. 1990). Indeed, in DU145 cells, transcription of the AR gene appears to be completely switched off (Culig et al.1993).

Furthermore, the inherent androgen-independence of primary epithelial cell cultures derived from both benign and malignant prostate specimens is also associated with a lack of functional receptor protein. Analysis of several tumour models *in vivo* has corroborated these findings, with at least two groups reporting altered AR levels in Dunning tumours displaying the androgen-independent phenotype (Quarmby *et al* 1990). In addition,

Rennie *et al.* 1990, in their examination of the stem cells and resulting progeny associated with the post-androgen ablation Shionogi model, have been unable to detect AR. In contrast to the studies in cancer models, immuno-staining of human prostatic adenocarcinoma specimens has produced a very confusing picture. Masai *et al.* 1990 observed that in untreated human prostatic carcinomas, clusters of cancer cells contain both receptor-positive and -negative clones and that the endocrine management of tumours induces a reduction in the percentage of AR-positive cells. One year later, Van der Kwast *et al.* 1991 presented some very convincing data contradicting the earlier observations by Masai *et al.* 1990 and more recently, Hobisch *et al.* 1995 have shown that in all patients studied, endocrine therapy does not lead to the preferential out-growth of AR-negative cells in distant metastasis.

Evidence to support the theory that hormone escape relies upon the alteration of target gene responses to AR binding is, to say the least, sparse. Yuan *et al.* 1993 observed that transfection of the supposed AR-negative cell line PC3 with an AR cDNA-bearing expression vector produced unexpected results in terms of growth response to exogenous androgens. DHT in the range 0.1 nmol/L to 10 umol/L produced a surprising 40-50% decrease in the proliferative rate compared to mock transfected and untransfected cells. Subsequently, it has been shown that this cell line is not strictly receptor-negative and is in fact a heterogeneous mixture of AR-positive and AR-negative cells. Treatment with ß-interferon markedly increases the number of receptor-bearing cells, with concomitant decreases in growth rate. Furthermore, the increased expression of the AR results in an increased growth rate after administration of the anti-androgen hydroxyflutamide. Whether these paradoxical effects are true in all androgen-independent cells and whether they represent a change in the population of genes regulated by the AR or a change in the transcriptional responses of androgen-dependent sequences has yet to be established (Sica et al. 1993, Habib and Grant, 1996).

Is it possible that a mutation within the AR gene alters the protein such that it no longer requires a ligand for activation, or is activated by an alternative molecule?

The LNCaP model of hormone-sensitive advanced metastatic prostate cancer expresses high levels of AR (Brinkmann et al. 1991). It is well documented that the LNCaP AR

carries a point mutation (A --> G: thr^{868} --> ala) in its ligand-binding domain which dramatically alters its binding characteristics such that it displays a higher affinity for progestagenic and oestrogenic steroids in addition to altered responses to flutamide/hydroxyflutamide and cyproterone acetate (Yuan et al. 1993). Veldscholte *et al.* 1992 reported the growth stimulatory effects of oestradiol and of the anti-androgens on the LNCaP cell line and, in the case of cyproterone acetate and hydroxyflutamide, showed that these effects are mediated by the activation of the AR.

Gaddipati *et al.* 1994 observed the LNCaP mutation in six of 24 patients with stage C to D 2 prostate cancer. However, to date, the incidence of detection of the AR $^{thr868 \rightarrow ala}$ mutation in androgen-independent tumours has been very low, with only a single case having been reported (Suzuki *et al.* 1993). A similarly low incidence AR mutation has been observed by Culig *et al.* 1993, who observed a G ---> A transition locating to nucleotide 2671 of the AR open-reading frame in one of seven patients receiving androgen ablation therapy. The consequence of this somatic mutation is an alteration of the functionality of the resulting protein such that it displays increased transactivation potential with adrenal androgens and progesterone, with no marked increases in affinity for these ligands. Clearly, if AR mutations such as those described appear in advanced prostatic tumours there is potential for carrier cells to have a growth advantage within endocrine environments generated as a consequence of androgen ablation therapies (Habib and Grant, 1996).

Another significant recent finding by the Cancer Genetic Group at Tampere, Finland, showed that AR gene amplification during endocrine therapy enabled prostate cancer cells to survive in the presence of low androgen concentrations. The androgen receptor gene lies at X_q11 - 12 and this region was amplified in 28% of prostate cancers which recurred after androgen-depletion therapy but not in any paired untreated primary specimens. Significantly, patients with AR amplification had no mutations of codon 877 of the AR gene. (Visakorpi *et al.* 1995).

1.2.2 Apoptosis and Prostate cancer

For any organ, including the prostate, normal structure and function is only maintained if the number of cells undergoing mitosis is balanced by an equal number being cleared by

apoptosis. Cancers can arise when changes in DNA result in unbalanced rates of mitosis and apoptosis, which cause the cancer to grow through anomalous accumulation of cells with an increased life span. In the normal rat ventral prostate, the rate of apoptosis is very low, approximately 2%, and is balanced by an equal cell proliferation rate (Isaacs 1984). In these animals, castration results within a week in the loss of about 70% of the prostatic epithelial cells by apoptosis, because androgen 'survival' factors are withdrawn (English et al. 1989). A minority of the cells is androgen-independent and survives (Montpetit et al. 1988). As the prostate involutes, there is enhanced expression of the genes for testosterone-repressed prostatic message 2 (TRPM-2), transforming growth factor-β (Kyprianou and Isaacs, 1989), and the oncogenes *c-myc* and *c-fox* (Buttyan et al. 1988). The significance of this new gene expression is uncertain, but it may represent a useful marker of exposure to 'apoptotic stress'. Inhibition by calcium channel blockers demonstrates that castration-induced prostatic apoptosis is mediated via an increase in intracellular calcium ion concentration, which activates a Ca^{2+}/Mg^{2+}-dependent endonuclease, resulting in the fragmentation of nuclear DNA into the characteristic oligonucleosomal fragments. The cell death programme has not yet been identified in its entirety, but it has been shown that prostatic apoptosis occurs by a pathway of intracellular events distinct from cell proliferation, which involves expression of a different pattern of genes (Furuya et al 1993).

Cell lines from human prostate cancer have been established and are useful for studying the effects of androgen withdrawal. The androgen-responsive PC-82 line inoculated into nude mice was found to retain the ability to activate programmed cell death, and the cells underwent apoptosis after androgen ablation by castration. This was associated with a rise in TGF-β_1 and TRPM-2 gene expression. A similar response was seen after treatment with luteinizing hormone-releasing hormone (LH-RH) analogues (Kyprianou et al 1990).

Although androgen-insensitive prostate cancer cells do not undergo apoptosis in response to androgen withdrawal, they do have the potential to engage this programme of death. Thus, androgen-independent rat prostate cancer cells retain the ability to undergo apoptosis in vitro in response to 5-fluorouracil and trifluorothymidine. These agents exert their effect by depleting intracellular thymidine-5-phosphate pools, resulting in a toxic 'thymineless' state (Grem, 1990). Interestingly, in common with normal rat prostate and androgen-responsive cancer cell lines, androgen-independent human prostate cancer cell lines undergoing

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apoptosis after exposure to these drugs also exhibit increased TGF-β_1 and TRPM-2 gene expression (Kyprianou et al. 1994). Furthermore, calcium ionophore induced sustained rises of intracellular calcium ion concentration, leading to apoptotic cell death. However, in these experiments the associated increase in TRPM-2 expression, which is usually observed in prostatic cell apoptosis, was not demonstrated. A possible explanation for this discrepancy is that TRPM-2 may be involved in triggering the rise in calcium induced by cytotoxic drugs that was, in this instance, by-passed by the use of ionophore, which directly increases intracellular calcium ion concentration. A related, if not identical, substance, sulphated glycoprotein-2, has a similar calcium-elevating role in the acrosomal reaction of sperm. However, data from other cell types suggest that TRPM-2 (also called clusterin) expression may represent an attempt to resist the apoptotic stress. Nevertheless, a rise in intracellular calcium ion concentration is clearly important in the initiation of programmed cell death in prostatic cells, because cell death in normal prostate cells and androgen-sensitive prostate cancer cells induced by androgen withdrawal is associated with elevated intracellular calcium (Kyprianou et al. 1990).

Therefore, in vitro at least, prostatic cancer cells retain the ability to engage apoptosis, whether androgen-dependent or -independent. Furthermore, apoptotic cells are easily recognised in different types of high grade prostate cancer and in prostatic intra-epithelial neoplasia (Gaffney, 1994). It has also been shown that increased programmed cell death is a feature of the increasing malignant potential that is associated with higher Gleason grades (Aihara, 1994). These findings suggest that, although high grade and androgen-insensitive elements of prostate cancer are less responsive to current treatments, they do retain the potential to undergo apoptosis. This has obvious implications for future therapy of hormone-resistant disease.

How might androgen-insensitive prostate cells resist withdrawal of exogenous survival factors? Clearly, up-regulated expression of bcl-2 would be expected to confer such resistance. Interestingly, bcl-2 is expressed in the basal epithelial cells of the normal human prostate. Furthermore, increased expression of bcl-2 has been reported in prostate cancer and is particularly pronounced in hormone-refractory disease. This is an exciting finding, because there is already in vivo evidence that antagonism of bcl-2 expression can retard growth of lymphoma. Antisense oligonucleotides that interfere with expression of bcl-2 by

binding to the 'sense' DNA strand of the gene clearly have therapeutic potential in prostatic malignancy (Colombel et al. 1993).

1.2.3   Oncogenes and tumour suppressor genes in prostate cancer

A number of genetic changes have been documented in prostate cancer, ranging from allelic loss to point mutations and changes in DNA methylation patterns (summarised in Fig 5). To date, the most consistent changes are those of allelic loss events, with the majority of tumors examined showing loss of alleles from at least one chromosomal arm. The short arm of chromosome 8, followed by the long arm of chromosome 16, appear to be the most frequent regions of loss, suggesting the presence of novel tumor suppressor genes. Deletions of one copy of the Rb and p53 genes are less frequent as are mutations of the p53 gene, and accumulating evidence suggests the presence of an additional tumor suppressor gene on chromosome 17p, which is frequently inactivated in prostate cancer. Alterations in the E-cadherin/α catenin mediated cell-cell adhesion mechanism appear to be present in almost half of all prostate cancers, and may be critical to the acquisition of metastatic potential of aggressive prostate cancers. Finally, altered DNA methylation patterns have been found in the majority of prostate cancers examined, suggesting widespread alterations in methylation-modulated gene expression. The presence of multiple changes in these tumors is consistent with the multistep nature of the transformation process. Finally, efforts to identify prostate cancer susceptibility loci are underway and will hopefully elucidate critical early events in prostate carcinogenesis (Bookstein et al. 1990, Visakorpi et al. 1992, Isaacs et al. 1994).

Fig 5

Genetic alterations associated with progression of prostate cancer.

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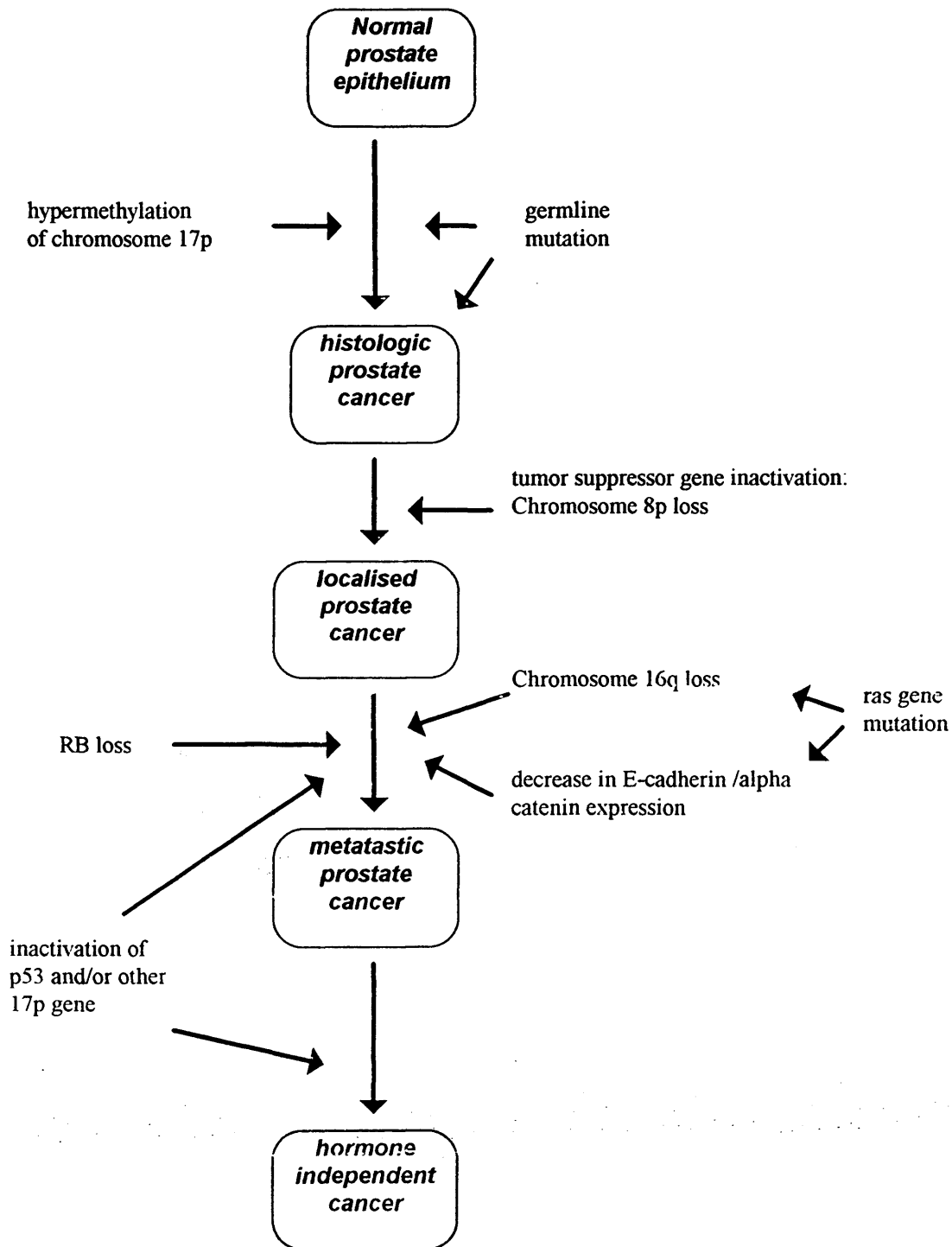
1.2.4 GROWTH FACTORS - WHAT ARE THEY?

1.2.4.1   What are growth factors ?

One of the most exciting achievements of the medical research community has been the characterisation of a diverse family of signal molecules, the growth factors which are involved in the control of cell growth and differentiation and play a crucial role in cancer and other diseases. The origin of this research lies in the studies of Cohen, Levi-Motalchini, Metcalf and others who first used complex biological assays to characterise epidermal, nerve growth factors and the colony stimulating factors respectively, and then stimulated many groups to establish methods for the elucidation of both their biological role and mechanism of molecular action through specific receptors (Schally, 1988, Steiner, 1995).

The availability of pure growth factors, together with modern cellular techniques for their assay has firmly established a central role of these signal molecules in all aspects of biology. Growth factors trigger through specific receptors, the generation of second intracellular messengers activating intracellular enzyme cascades. These can be subverted by oncogenes or tumour promoters at particular sensitive points, providing a unifying mechanism which may be operating in malignant diseases. In addition, these studies have stimulated the introduction of novel methods for manipulating growth factor induced signal cascades which could be important in many diseases. It seems clear that a large number of growth factors remain to be characterised and it is to be expected that the understanding of their receptor signal transduction mechanisms will allow the design of specific antagonists. It remains to be seen whether the selectivity of these antagonists will be useful for therapeutic manipulation because many distinct differentiated cell types may respond to the same

factor with fundamentally different consequences. Table 1 lists some important polypeptide growth factors, their sources and functions.

1.2.4.2   Mechanism of action of growth factors

Growth factors may operate in 3 general ways namely a) endocrine b) paracrine c) autocrine. Their effects may be endocrine, that is to say, like hormones, they may be synthesised elsewhere in the body and pass in the circulation to their target cells. They may be synthesised in certain cells and secreted from them to affect neighbouring cells. However, the cells that synthesise the growth factors are not themselves affected, because they lack suitable receptors. This mode of action is called paracrine. Certain growth factors can affect the cells that synthesise them. This third mode of action is called autocrine. That is, a factor may be secreted and then attach to its cell of origin, provided that the cell possesses appropriate receptors. (Fig 6).

1.2.4.3   Peptide growth factors in the prostate as mediators of stromal epithelial interaction

There are five principal peptide growth factors that are presently known to be involved in normal and abnormal prostatic growth. They are transforming growth factors α and β (TGF-α and TGF-β), epidermal growth factor (EGF), insulin-like growth factors (IGF-I and IGF-II), and some members of the fibroblast growth factor family (FGF).

1.2.4.4   EGF and TGF-α

EGF is a 53-amino acid polypeptide that is mitogenic for prostatic epithelial cells *in vitro*. Castration in adult mice results in prostatic involution, accompanied by a marked reduction in the amount of EGF in the prostate. Conversely, replacement of testosterone in these animals stimulates prostatic growth and restores the tissue levels of EGF. This is in contrast to the role of TGF-β_1 which plays the principal role in castration-induced prostatic cell death. (Sutkowski et al. 1992).

Table 1

Polypetide Growth Factors - Sources and Functions

Growth Factor	Source	Function
Epidermal growth factor (EGF)	Mouse salivary gland	Stimulate growth of many epidermal and epithelial cells
Erythropoietin	Kidney Urine	Regulates development of early erythropoietic cells
Insulin-like growth factors I & II (Somatomedins C and A)	Serum	Stimulate sulfate incorporation into cartilage, are mitogenic for chrondrocytes, exert insulin-like effect on many cells
Interleukin - 1 (IL-1)	Monocytes Conditioned medium	Stimulates production of IL-2
Interleukin - 2 (IL-2)	Lymphocyte Conditioned medium	Stimulates growth of T-cells
Nerve growth factor (NGF)	Mouse salivary gland	Trophic effect on sympathetic and certain sensory neurons
Platelet derived growth factor (PDGF)	Platelets	Stimulates growth of mesenchymal endothelial cells
Transforming growth factor (TGF Alpha.)	Conditioned medium of transformed or tumour cells	Is similar to EGF
Transforming growth factor (TGF Beta)	Kidney and platelets	Exerts both stimulatory and inhibitory effects on certain cells
Fibroblast growth factor	Brain, pituitary submaxillary gland	Similar to EGF

Fig 6

Mechanisms for delivery of growth factors and hormones to target cells.
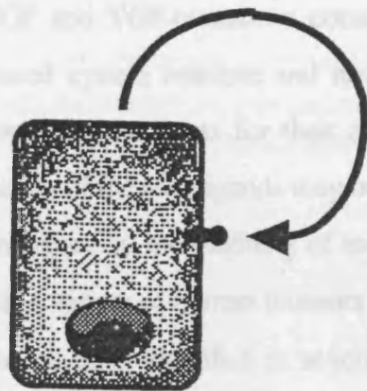
Autocrine

A cell produces and can respond to a Growth factor through specific receptors.
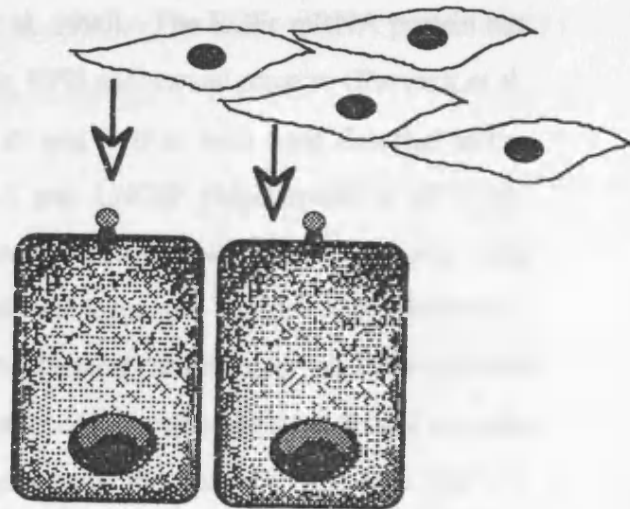
Paracrine

One cell type produces a Growth factor to which another cell type located close by can respond to through specific receptors.
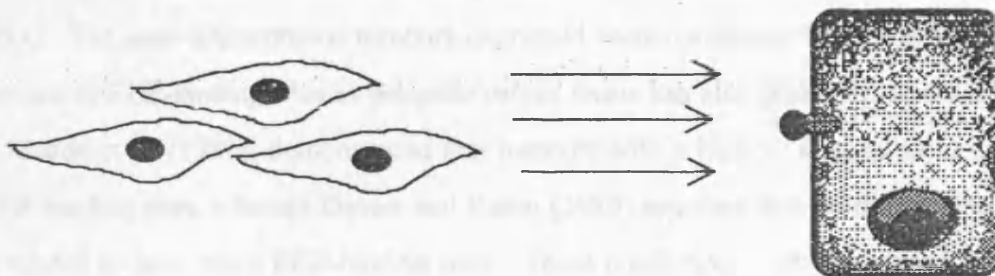
Endocrine

Distribution through the circulation.

EGF and TGF-α exert their effects through members of the EGF-receptor family (a group of transmembrane glycoprotein receptors with shared structure) especially the EGF-receptor (EGFr) (Fig 7). EGF and TGF-α share a common amino acid sequence containing six characteristically spaced cystein residues and have a sequence homology of about 35%. This structural conservation accounts for their ability to interact with the same receptor. The differences in action of the two ligands may be due to different conformational changes induced within the receptor by the bindning of each ligand. Elevated levels of EGFr have been demonstrated in a variety of human tumours and cell lines (Derynck et al. 1987). The EGFr ligand TGF-α has been identified in several tumour cell lines (Todaro et al. 1980, Byrne et al. 1996).

EGF has been identified in extracts of prostatic tissue from BPH and prostate cancer (Jacobs and Lawson, 1980, Elson et al. 1984, Shaikh et al. 1990). The EGFr mRNA protein has been demonstrated in human prostatic carcinomas, BPH and normal prostate (Derynck et al. 1987, Ching et al. 1993). In addition both EGF and TGF-α have been detected in the human prostate cancer cell lines DU145, PC-3 and LNCaP (MacDonald et al. 1990, Connolly and Rose, 1989, 1990). Increased levels of EGFr mRNA are associated with greater tumour extent and with poor differentiation (Morris and Dodd, 1990). However, this conflicts with findings using immunohistochemical techniques, where significant decreases in EGFr expression were found in prostate cancer compared with normal prostate or BPH (Mellon et al 1992). Ware (1993), suggested that this discrepancy may be due to a modification of the EGfr in prostate cancer that prevents its detection by standard immunohistochemical techniques, or that the secretion of TGF-α by the tumours may downregulate the receptor. Immunocytochemistry for TGF-α has shown low amounts in the epithelium of BPH and an increased intensity of staining in prostatic carcinoma (Harper et al. 1993). The least differentiated tumours expressed immunoreactive TGF-α the most. Measurement of EGF-binding sites in prostatic cancer tissue has also produced conflicting results. Maddy et al. (1989), demonstrated that tumours with a higher Gleason score had fewer EGF-binding sites, whereas Davies and Eaton (1989) reported that de-differentiated tumours tended to have more EGF-binding sites. These conflicting results may be due to differing assay techniques and sources of tissues.

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Fig 7

Effects of peptide growth factors on normal prostatic epithelial cells and fibroblasts.

Stromal Cells

IGF-II

bFGF

TGF-β

KGF

bFGF

Epithelial Cells

EGF

TGF-α

☒ bFGF Receptor

◼ EGF Receptor

⬭ Type 1 I GF Receptor

→ Stimulatory pathway

➡ Inhibitory pathway

KGF = Keratinocyte growth factor
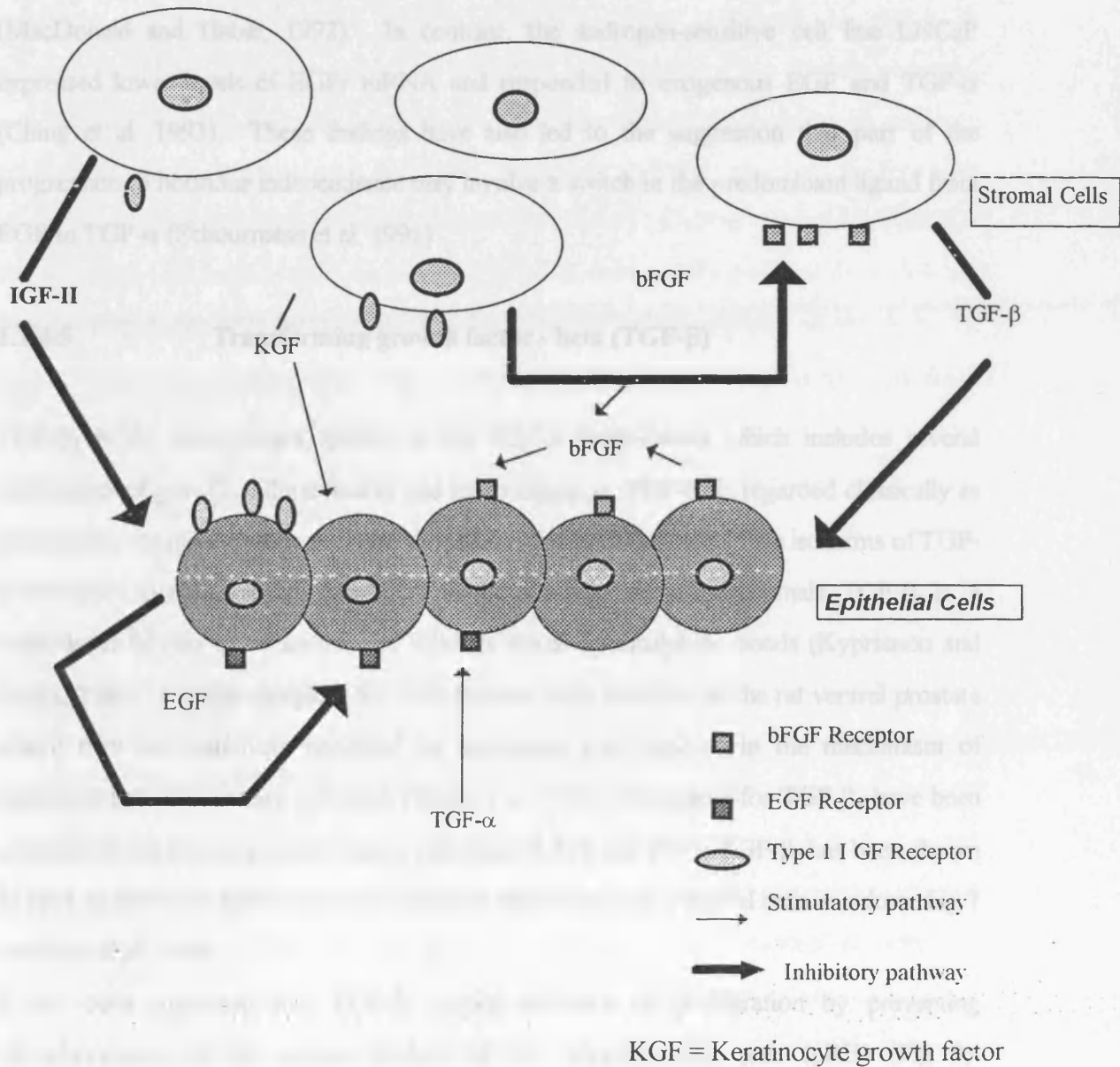
The highest levels of expression of EGFr mRNA have been found in the human androgen-independent prostate cancer cell lines DU-145 and PC-3 (Morris and Dodd, 1990). However, these cell lines showed no significant proliferative response when treated with EGFr ligands (Hofer et al. 1991). Both these cell lines produced TGF-α (MacDonald et al. 1990). The autologous production of growth factors may be linked to the loss of steroid responsiveness in these two cell lines and reduced response to exogenous growth factors (MacDonald and Habib, 1992). In contrast, the androgen-sensitive cell line LNCaP expressed lower levels of EGFr mRNA and responded to exogenous EGF and TGF-α (Ching et al. 1993). These findings have also led to the suggestion that part of the progression to hormone independence may involve a switch in the predominant ligand from EGF to TGF-α (Schuurmans et al. 1991).

1.2.4.5   Transforming growth factor - beta (TGF-β)

TGF-β_1 is the predominant species in the TGF-β super-family which includes several modulators of growth, differentiation, and morphogenesis. TGF-β_1 is regarded classically as a stimulator for mesenchyme cells and an inhibitor for epithelial cells. Five isoforms of TGF-β have been identified so far, of which only the first three occur in mammals. TGF-β_1 is a homodimer of two 112 - amino acid subunits linked by disulphide bonds (Kyprianou and Isaacs, 1989). Cellular receptors for TGF-β_1 have been identified in the rat ventral prostate where they are negatively regulated by androgens and involved in the mechanism of castration-induced prostate cell death (Shain et al. 1990). Receptors for TGF-β_1 have been identified in the human prostate cancer cell lines DU145 and PC-3. TGF-β_1 has been shown to have an inhibitory effect on human prostatic fibroblasts and epithelial cells in culture Fig 7 (Wilding et al. 1989).

It has been suggested that TGF-β_1 causes inhibition of proliferation by preventing phosphorylation of the protein product of the retinoblastoma gene (pRB) (Fig 8). Phosphorylated pRB normally interacts with a cis acting element in the regulatory region of the growth-related gene c-myc (which it induces) leading ultimately to cell proliferation.

Thus, inhibition of phosphorylation of pRB by TGF-β would decrease the expression of *c-myc* causing a reduction in cellular proliferation. The cell line DU145 contains an abnormal RB protein which is associated with loss of sensitivity to inhibition by TGF-β_1 (Laiho et al. 1990, Zentella et al. 1991).

Immunohistochemical studies have identified TGF-β_1 in histological sections of human prostate. Significantly greater staining was shown in prostate cancer compared with BPH specimens, supporting a role for TGF-β_1 in prostate cancer. However, as TGF-β_1 is secreted in a latent, inactive form, the biological significance of the protein detected immunocytochemically is unclear. Indeed, the bioactivity of the TGF-β_1 in cancer cell lines has been shown to vary between 5 and 50%. Wilding et al. (1989) examined the response of the human prostate cancer cell lines DU145, PC-3 and LNCaP to exogenous TGF-β_1. The androgen-independent cell lines DU145 and PC-3 were initially inhibited dependent on dose by TGF-β; however the androgen-sensitive LNCaP cell line showed no response. In addition, the androgen-independent cell lines were able to secrete TGF-β_1 in its active form and contained a high-affinity receptor for TGF-β_1. Thus, in androgen-independent cell lines, there exists an inhibitory autocrine loop for control of cellular proliferation by TGF-β_1. However, such inhibition by TGF-β_1 was lost after 7 days (Wilding et al. 1989), which shows that this mechanism is not sustained. The failure of such an autocrine loop may be due to the development of unresponsive phenotypes and may be important in the progression of prostate cancer. Indeed, there is evidence that TGF-β_1 may even have a growth-promoting effect in prostate cancer. Although increasing levels of TGF-β_1 initially inhibit cell proliferation, further administration causes an increase in the growth rate in rat prostate cancer cell lines. Overexpression of TGF-β_1 in a different rat prostate cancer cell line led to the development of tumours that were at least 50% larger, more anaplastic and caused more metastases than controls. Thus, the effect of TGF-β_1 is dependent on the cell type examined. Other functions of TGF-β_1 that would support growth of tumour cells and may encourage metastasis include its association with extracellular matrix, its ability to inhibit T and B lymphocytes leading to defects in host immunity, and the ability to act as a potent angiogenic factor. Understanding of the function of TGF-β_1 in both normal and malignant prostate is further complicated by its interaction with other growth factors which influence cell proliferation (Steiner and Barrack, 1994, Byrne et al. 1996).

Fig 8

Suggested mechanism of TGF-β₁ inhibition of cellular proliferation. TGF-β₁ prevents phosphorylation of RB protein thus decreasing RB-induced *c-myc* expression leading to reduced cellular proliferation.
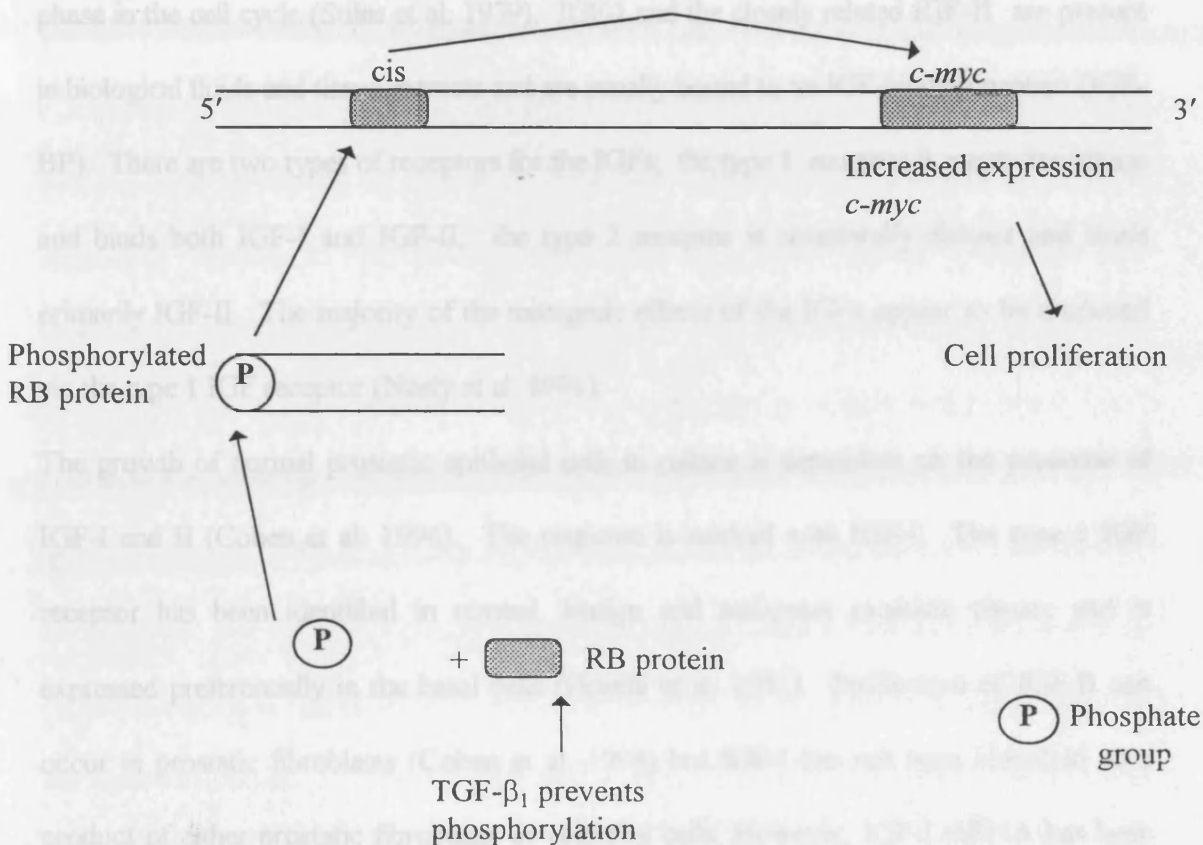
1.2.4.6   **Insulin-like growth factors**

IGF-I is a 70-amino acid polypeptide with functional homology to insulin. The mitogenic effect of IGF-I is due to its ability to facilitate the transfer of cells from the G_1 phase to the S phase in the cell cycle (Stiles et al. 1979). IGF-I and the closely related IGF-II are present in biological fluids and tissue extracts and are usually bound to an IGF-binding protein (IGF-BP). There are two types of receptors for the IGFs; the type 1 receptor is a tyrosine kinase and binds both IGF-I and IGF-II; the type 2 receptor is structurally distinct and binds primarily IGF-II. The majority of the mitogenic effects of the IGFs appear to be mediated via the type 1 IGF receptor (Neely et al. 1991).

The growth of normal prostatic epithelial cells in culture is dependent on the presence of IGF-I and II (Cohen et al. 1994). The response is marked with IGF-I. The type 1 IGF receptor has been identified in normal, benign and malignant prostatic tissues and is expressed preferentially in the basal cells (Fiorelli et al. 1991). Production of IGF-II can occur in prostatic fibroblasts (Cohen et al. 1994) but IGF-I has not been identified as a product of either prostatic fibroblasts or epithelial cells. However, IGF-I mRNA has been identified in stromal cells from specimens of BPH (Barni et al. 1994). The mechanism of IGF-II-mediated stimulation of prostatic epithelial cells in BPH appears to be similar to that in a normal prostate, although the expression of IGF-II mRNA is 10-fold higher in fibroblasts from BPH tissues (Cohen et al. 1992). This led to the hypothesis that such cells may be 'reverting to a fetal-like state' where IGF-II expression is normally high, causing stimulation of proliferation and the development of BPH (Cohen et al. 1994).

IGF-I has a stimulatory effect on the human androgen-independent cell lines DU145 and PC-3. No effect on proliferation has been shown in the androgen-dependent cell line LNCaP (Schuurmans et al 1991). Undetectable levels of IGF-I have been recorded in the conditioned media of these cell lines by some authors (Iwamura et al 1993); whereas

abundant amounts of IGF-I, as measured by radioimmunoassay, have been recorded in the

same cell lines by others (Pietrzkowski et al. 1993). The androgen-independent cell lines

DU145 and PC-3 expressed more IGF-I mRNA than did the androgen-dependent LNCaP

cell line. Further evidence supporting an autocrine role of IGF-I in prostate cancer is that

blockage of the receptor for IGF-I caused inhibition of proliferation in DU145 and PC-3 cell

lines. This is in contrast to normal prostatic cells where IGF-II, produced by prostatic

fibroblasts, appears to be the dominant factor stimulating prostatic epithelial cell

proliferation probably via the type 1 IGF receptor. IGF-1 is not commonly secreted by

tumour cell lines of epithelial cell origin. In normal and neoplastic breast tissue IGF-I

mRNA is present but is expressed by stromal, not epithelial cells (Iwamura et al. 1993).

Media conditioned by human non-malignant prostatic epithelial cells contain two IGF-BPs,

IGF-BP2 and IGF-BP4. The culture medium from the cell line PC-3 has been shown to

contain IGF-BP4 but not IGF-BP2. However, the significance of the lack of IGF-BP2 has

not been established. In contrast, IGF-BP2 levels in the serum of patients with prostatic

cancer have been shown to be elevated by up to twofold, the degree of elevation being

related to the clinical stage of the tumour (Cohen et al 1992, 1994).

1.2.4.7 The fibroblast growth factor (FGF) family

The family of FGFs consists of nine cloned members, ie FGF-1 to FGF-9. They are small

polypeptides with 35-55% homology in their amino-acid sequences, sharing similar exon-

intron structures. FGF-1 (acidic FGF) and FGF-2 (basic FGF) were identifed first and

have been investigated most intensely. Basic FGF has been identified in both benign and

malignant human prostate. Immunohistochemistry has identified FGF-2 predominantly in

prostatic stroma but also epithelial cells. Expression of FGF-1 is also present in the

prostate but at a lower level than FGF-2. In addition, using a prostate stromal cell

culture model, there is evidence for the interaction of FGF-2 and TGF-β_1 , resulting in

positive and negative proliferation effects, respectively. Besides potential activation of the

autocrine loop, FGFs may also contribute to the pathophysiology of the prostate through

paracrine activity FGF-7, or keratinocyte growth factor (KGF), is thought to be synthesized

and secreted by stromal cells to act on FGF-receptor (FGFr)-bearing epithelial cells,

resulting in cellular proliferation. It is also interesting that in a transgenic mouse system

using FGF-3 or the int-2 gene, changes in the prostate of the male progeny were dramatic

and involved only hyperplasia, with no evidence of malignant transformation (Story. 1987,

Muller et al. 1990).

Preliminary work has revealed that the receptor for FGF-2 is present in prostatic epithelial

and stromal cells in culture (Story et al. 1987), and it is now known that there are four

cloned members of the high-affinity tyrosine kinase receptors for FGFs. The FGFrs share

structural similarities in both the extracellular domain that binds FGFs and the intracellular

domain that activates the signal transduction pathway. FGFr-1 to FGFr-4 display different

binding affinities for the different FGFs. FGF-1 binds to all four receptors while FGF-2 is

more restricted and only binds to the first three receptors. Receptor/receptor reactions are

known to occur and may have a role in the development of both benign hyperplasia and

carcinoma of the prostate. To date, the expression of FGFs and their receptors is not fully

understood, but there does appear to be a major difference between benign and malignant

prostate in the activity of the FGF system. It is likely that subtle changes occur in the FGF

system during malignant progression. This has been demonstrated elegantly in a mouse

prostate   cancer model (Yan et al. 1993). During progression from the benign to the

malignant phenotype, the prostate epithelial cells displayed a switch of expression of FGFr

splice variants, conferring a swtich of high-affinity binding from FGF-7 to FGF-2. FGF-2

was also concomitantly upregulated along with FGF-3 and FGF-5. Further evidence supporting a role for FGFs in the development of malignancy is that transformation of BHK-21 (baby hamster kidney) cells with plasmids carrying the bFGF coding sequence resulted in cells which exhibited the transformed phenotype. The prostatic cancer cell lines DU145 and PC-3 produce active FGF-2 and express large amounts of FGF-2 receptors. However, only the DU145 cell line and not the PC-3 cell line has been shown to respond to exogenous FGF-2 (Nakamato et al. 1992).

The interaction between the androgen receptor and the growth factor systems is important. It is known that certain growth factors, including FGF-7/KGF, IGFs and EGF, may activate androgen-dependent gene expression in the absence of androgen, which may represent a mechanism for the development of hormone-insensitive tumours (Culig et al 1994).

1.2.4.8 Growth factors and hormone resistance

There is now much circumstantial and direct evidence to support the hypothesis of autocrine secretion originally proposed by Sporn and Roberts in 1985, whereby many types of tumour cells release polypeptide molecules into their conditioned medium and these same tumour cells often possess functional receptors for their secreted polypeptides. Autocrine secretion would therefore allow tumour cells to evade normal cellular growth control by producing and responding to the secreted growth factor.

Further evidence suggests that minimal response in the androgen-insensitive prostate cells to exogenous growth factors may be caused by autologous production of EGF-like molecules, the levels of which are also stimulatory. In contrast to this, the androgen sensitive cells appear to have retained their capacity to respond to exogenous EGF possibly because of lower secretion of bioactive EGF-like peptides (Habib and Chisholm, 1991). The detection of these peptide molecules suggests an involvement in cell transformation since these cells are autoregulated by virtue of possessing receptors for the secreted molecule. Using a specific EGF radiolabelled-receptor competition assay, EGF-like molecules were detected in the media 45 conditioned by the androgen-insensitive cell line DU145 (MacDonald et al 1990). Although the androgen sensitive prostate cells also

produce EGF-like molecules, these are stimulated by androgens (Wilding et al. 1989), are secreted at a significantly lower capacity and may of course represent different forms of TGF. The progression to an androgen independent state may, in part be due to loss of androgen regulation of growth factor irrespective of the presence of androgens. The increase in growth factor expression may in turn increase the expression of EGF receptors, resulting in down regulation of the androgen receptor. Studies on the androgen-responsive (SC3) and androgen independent (SC4) cell lines which are derived from a mouse mammary tumour (Nonomura et al, 1988) suggest that such mechanisms occur in the progression from androgen-dependent to -independent cell status. The progression to androgen independence may occur by a mechanism in which the cells obtain the ability to respond to growth factors and autonomously produce growth factor receptors (Habib and Chisholm 1991, Steiner 1995).

1.2.4.9 Summary

Peptide growth factors play a role in the maintenance of normal prostatic growth and differentiation (Fig 7). It seems likely that the androgen sensitivity of human prostate is mediated by the production of peptide growth factors from stromal cells which act as the direct intermediate of androgen action on epithelial cells. TGF-β_1 inhibition of epithelial cells is opposed by the stimulatory action of EGF, IGF and FGFs to maintain an equilibrium of epithelial cell numbers. The indirect mitogenic action of androgens appear to act by down-regulation of TGF-β_1 and possibly EGF receptors. There is also interaction with the effects of IGF-II, produced by prostatic stromal cells and acting on epithelial cells to increase proliferation. The growth of normal prostatic fibroblasts is under the control of bFGF and TGF-β_1. However, although our understanding of the actions of these growth factors in the normal prostate has improved over the last decade, their role in the development and maintenance of prostate cancer is less clearly defined. TGF-β_1, classically considered to be inhibitory for epithelial cells may be up-regulated in prostatic tumours, stimulating growth. Alternatively, autocrine production of such growth factors by tumour cells may lead to loss of inhibitory effects from exogenous TGF-β_1, a mechanism also witnessed with TGF-α and bFGF.

The role of EGF in the development of prostate cancer is confusing because results from the use of different cell type and experimental conditions is contradictory. It may be that a switch in the production of the predominant EGFr ligand from EGF to TGF-α is an important feature in the development and maintenance of the malignant phenotype. The presence of TGF-α autocrine loops has been shown clearly in some tumour cell lines. This switch in the production of a particular ligand may also be a feature of IGFs in prostate cancer. IGF-II may be replaced by IGF-I during malignant progression, both of which are able to act via the type 1 receptor. This change in IGF expression appears to be accompanied by altered expression of the IGF-BP2, while less detectable within prostatic tissues but with elevated serum levels. Basic FGF is normally produced by prostatic fibroblasts but is also produced by some prostatic cancer cell lines. However, as with all growth factors, the expression of the bFGF protein and its receptor is dependent on the cell line examined. The autocrine and paracrine control of normal and abnormal prostatic growth by growth factors is important in determining their role in the development and maintenance of prostate cancer. Better understanding of such mechanisms is essential for the development of novel therapeutic strategies in the control and treatment of prostate cancer (Nakamoto et al. 1992).

1.2.5.1   Growth factors as targets for therapy of prostate cancer

From the above discussions on the role of growth factors in the regulation of prostatic cell growth, particularly androgen independent cells, a therapeutic use for growth factors may be imagined, as growth factors and their receptors are now believed to be involved in tumour progression. Thus, normal and abnormal cell growth is regulated by peptide growth signals which provide a framework for the design of therapeutic intervention. Interruption of the effect on polypeptide receptor interaction could be accomplished by blocking the growth factor at its active site with a specific antibody, blocking the receptor with antibodies specific for the binding site, or by the use of extracellular antagonist that would compete for receptor sites. For example, it is known that cells of the A431 epidermoid carcinoma cell lines express an increased number of EGF receptors on their cell surfaces and antibodies that block the binding of EGF to its receptor produce a marked inhibition of cell growth in culture and of tumour growth in nude mice (Hamui et al. 1984).

1.2.5.2   Model 'curative' therapy for metastatic prostate cancer

From the discussions above on the new tumour biology of cancer of the prostate, it is possible to theorise a model for future curative therapy of prostate cancer. This model combination therapy will consist of the use of:

(a)   Androgen deprivation by medical or surgical castration, which will destroy androgen sensitive cells; and

(b)   Growth factor inhibitors (e.g. Somatostatin 201-995, Suramin, Tumour Necrosis Factor, etc.) which will destroy rapidly dividing androgen-insensitive cells; and

(c)   Chemotherapeutic agents (like Cyclophosphamide, Methotrexate, EMP, etc.) which will destroy slowly dividing or non-proliferating androgen insensitive cells.

1.2.6   Growth factor inhibitors in the therapy of hormone resistant prostate cancer

1.2.6.1   Suramin

1.2.6.1.1	Mechanisms of Suramin action
1.2.6.1.2	Toxicity profile of Suramin
1.2.6.1.3	Cell lines studies in vitro
1.2.6.1.4	Results of clinical trials involving Suramin monotherapy

Suramin and Somatostatin are 2 drugs known to inhibit growth factors.

Suramin, the hexasodium salt of 8, 8'- [carbonylimin] bis-1, 3, 5 -naphthalenesulfonic acid (Fig 9) was synthesised in 1916 and was used to treat African trypanosomiasis and onchocerciasis in the 1920s (WHO Technical Report, 1962, Hawkins, 1978). Interest in the drug was rekindled in 1979 when it was found to reduce viraemia in patients with Acquired Immunodeficiency Syndrome (AIDS) and also inhibit reverse transcriptase (De Clercq, 1979, Mitsuya et al. 1984). Subsequently, Suramin was shown to inhibit growth factor binding and growth factor-induced mitogenesis in vitro at concentrations achievable in vivo (Williams et al. 1984). This led to clinical trials in both haematological malignancies and solid tumours. CaP was targeted for evaluation due to Suramin's inhibition of growth factor action, its effects on human prostate derived cell lines in vitro and its known adrenal inhibitory effects (Hosang, 1985, Ashby et al. 1989).

1.2.6.1.1   **Mechanisms of Suramin action**

The ability of Suramin to inhibit growth factor binding in vitro correlates well with its ability to inhibit cell growth as measured by inhibition of DNA synthesis, by assays of cell number, and by its effects on colony-forming ability. The effects vary with the concentration of Suramin, duration of Suramin exposure, concentration of serum in the medium, and the number and type of cells plated. For example, a Suramin concentration that had a minimal effect on EGF induced mitogenesis of AKR-2B cells and blocked EGF mitogenesis on LNCaP cells by >90% (Berns et al. 1990). The drug is extensively protein bound, and its action can be inhibited by albumin. Other growth factors inhibited in vitro include basic and acidic FGF and TGF α and β (Knabbe et al. 1989, Morton et al. 1990, Pollack and Richard, 1990).

The actions of Suramin are not restricted to growth factor inhibition. Other actions include inhibition of lysosomal enzymes, including hyaluronidase, iduronate sulfatase, β-glucuronidase, and sphingoid hydrolases. The effect on lysosomal enzyme synthesis results in a systemic accumulation of glycosoaminoglycans (GAGs) and sphingolipids mimicking the pathology associated with mucopolysaccharidosis. GAGs, primarily heparan and dermatan sulfate, accumulate in the serum, urine, kidney, liver, lung, and spleen. This explains some of the unique toxicities of the compound. Suramin also inhibits nucleic acid polymerases, membrane-associated ion pumps such as Na^+, K^+, Ca^{++} and H+-ATPases, protein kinase C, and glucolysis, and induces differentiation of HT29 colon carcinoma cell lines (Ono et al. 1988, Fantini et al. 1990). A summary of known biologic effects and possible mechanisms of action of Suramin is shown in Table 2. (Hofer et al. 1991, Daugherty et al. 1992)

1.2.6.1.2   **Toxicity profile of Suramin**

Use of Suramin contrasts significantly with classical chemotherapy because the agent has several unique toxicities. Many adverse effects are mediated by the inhibition of glycosaminoglycan synthesis and growth factors. Among the most worrisome is a polyradiculopathy progressing to a Guillain-Barré syndrome. An inhibitory effect on FGF, a growth factor for Schwann cells, has been postulated. Risk of toxicity correlates with

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Fig 9

The Structure of Suramin Sodium
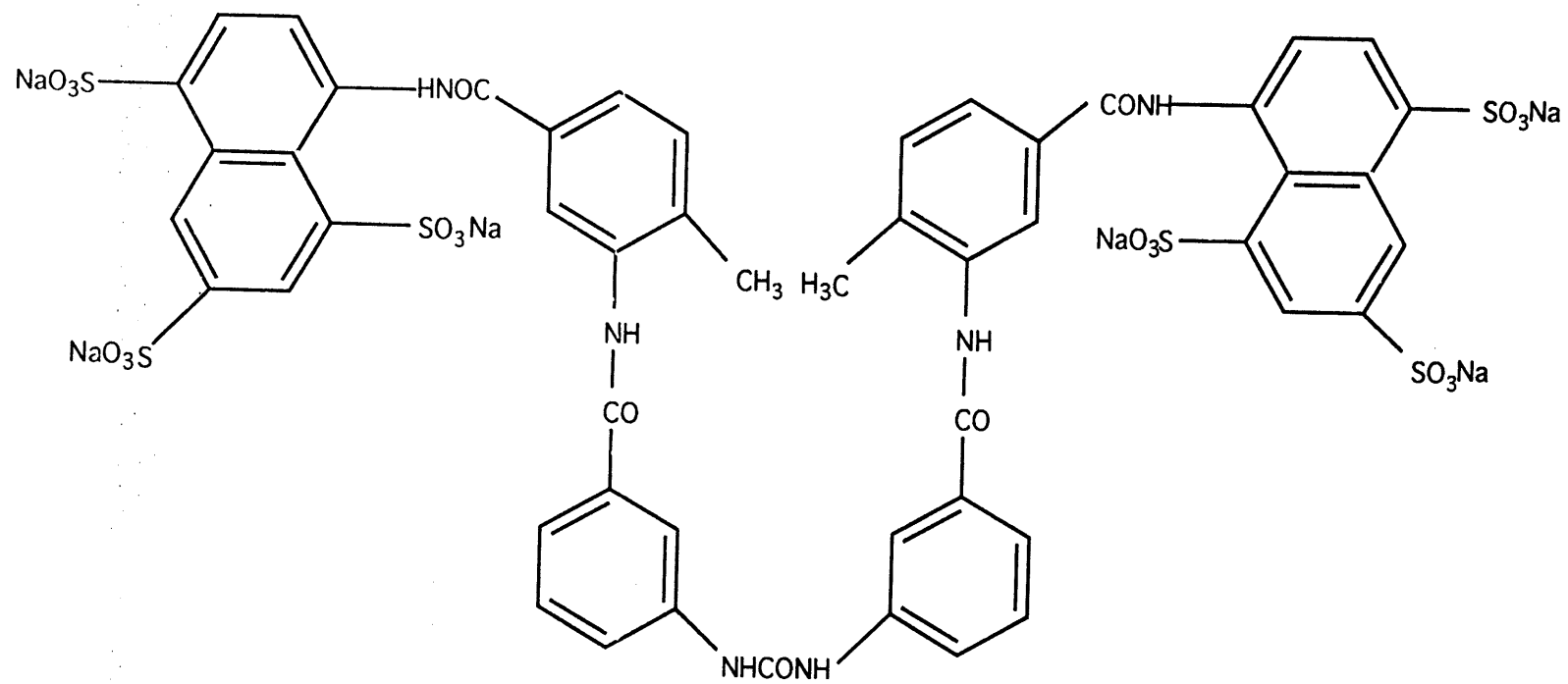
peak Suramin levels, exceeding 40% at > 350 ug/ml plasma concentrations (La Rocca et al, 1990). It is unclear, however, whether longer duration of treatment at lower plasma concentrations will have the same effect on nerve cells.

A coagulopathy, the result of accumulation of heparan and chondroitan sulfate, which function as circulating anticoagulants, has been reported. This can be particularly severe in patients with pre-exisiting liver dysfunction. As a result, protocols contain provisions to stop infusions when the prothrombin time is >17 sec. Discontinuance of the drug leads to normalization of the coagulation parameters in several days. Increases in serum creatinine and decreases in creatinine clearance and proteinuria have also been reported. This may be related to the selective accumulation of Suramin in the kidney.

Glycosoaminoglycan inhibition can also result in a vortex keratopathy in about 25% of cases. Slit lamp examinations reveal intraepithelial deposits in the cornea, conjunctiva, and lens epithelia. Electron microscopy shows lipid inclusion lamellar membranous bodies similar to those in Fabry's disease. Symptoms, which include blurring of vision, photophobia and tearing generally resolve when Suramin is discontinued.

An increased incidence of severe and/or life-threatening infections has been documented in patients with normal granulocyte counts. No consistent effect on neutrophil function has been observed. Hypocalcemia, liver function abnormalities, pericardial effusion, anaphylaxis, nausea, vomiting, urticaria, fever, rash, lymphopenia and thrombocytopenia have also been reported (Senderowicz et al. 1991).

1.2.6.1.3 Cell line studies in vitro

Suramin has been shown to produce growth inhibition by at least 50% of the PC-3, DU145 and by >97% using LNCaP human-derived prostate cancer cell lines at a concentration of

Table 2

Biologic effects and possible mechanisms of action of Suramin

- **Interference with glycosaminoglycan metabolism**
- **Growth factor inhibition**

    Transforming growth factor α

    Transforming growth factor β

    Insulin-like growth factor I

    Insulin-like growth factor II

    Bombesin

    Platelet-derived growth factor

    Colorectum-derived growth factor

    Epidermal growth factor

    Basic fibroblast growth factor

    Interleukin 2

- **Inhibition of intracellular enzymes**

    DNA polymerase α

    Topoisomerase II

- **Disruption of cell motility and adhesion**
- **Disruption of mitochondrial energy balance**
- **Induction of differentiation**

    Human colon adenocarcinoma

    Human promyelocytic leukemia

    Human neuroblastoma

    Rat glioma

- **Interference with P_{2x} and P_{2y} purinergic signal transduction**
- **Immune system effects**

    Interference with the delayed-type hypersensitivity reaction

    Impairment of T helper cell function

100 uM (150 ug/ml) (Stein et al 1988, Ewing et al 1989). With DU145 cells, growth-inhibitory effects were partially reversed by exogenous androgens or EGF/TGF α, and in LNCaP cells by bFGF, EGF, and testosterone. Greater inhibition of PC3 cell growth was seen with increased concentrations and/or longer durations of exposure. However, if cells are rinsed free of Suramin after 5 days, cell growth continues. This implies a cytostatic as opposed to cytocidal effect. However, if Suramin (100 uM) is maintained for 10 days, an 87% inhibition of growth is observed, suggesting a cytocidal effect. The relative effect of different serum concentrations was shown by comparing the IC_{50} in 2% versus 10% serum. An increase from 30 to 300 uM was noted. In this system, cytotoxicity was observed after 3 days' exposure, with evidence of mitochondrial damage on histological examination (Ewing et al. 1989, Rago et al. 1990).

The effects have also been examined in human prostate primary epithelial cultures. Again, an effect on growth factor-induced mitogenesis was reversible up to 6 days. Thereafter, cytotoxicity was observed. Of potentially greater importance was the observed differential effects at various serum concentrations. Levels above 10^{-4} M were inhibitory, while stimulation was observed in a range of 5×10^{-7} to 10^{-5} M in 9 out of 14 cultures (Mitchen et al. 1990, Peehl et al. 1991). A similar outcome was observed in the Dunning rat tumor model. Although Suramin inhibited growth of the R3327-AT-2 variant, it stimulated growth of the R3327-AT-3 and MAT-LYLU variants (Morton et al 1990). The differential inhibition may reflect Suramin's blockade of growth factors known to inhibit cell growth such as TGF β and may have important clinical implications (Martikainen et al 1990, Morton et al. 1990).

1.2.6.1.4   Results of clinical trials involving Suramin monotherapy in prostate cancer

As previously noted, the impetus to evaluate Suramin in patients with prostatic cancer was based on its antitumor activity against prostatic cancer cell lines in vitro and in vivo, its inhibitory effects on growth factor-induced mitogenesis and proliferation and its effects on the adrenal steroidogenesis. In the first study at the National Cancer Institute (NCI) a

decrease of ≥ 50% was observed in 7/11 (63%) cases, whereas 4/8 patients showed >50% regression in measurable disease (Myers et al. 1989). Subsequently, a confirmatory trial using the same dose schedule was started at the University of Texas M D Anderson Cancer Center, the Mayo Clinic, and the Memorial Sloan-Kettering Cancer Centre. Of the first 17 evaluable patients, partial remission (PR) was noted in 2 (12%, 95% confidence limits 0-27%), and decreases in PSA (>50%) in 3/14 evaluable cases.

The NCI later updated its experience with 35 patients - 15 with measurable lesions, 21 with painful skeletal lesions, and 29 with a baseline PSA >5 times normal. Overall response in measurable disease was observed in 6 (40%); 3 had no change in PSA, 6 (21%) had a normalization; whereas a total of 16 (55%) had at least a 50% decrease from the baseline. The median survival was 9 months for those with soft-tissue disease and 15 months for those with disease limited to the skeleton (Myers et al. 1989).

Ahmann et al. (1991), noted partial response (PR) in measurable disease in 6/14 evaluable cases, with a > 80% decrease in PSA in 6 (43%, 95 confidence limits 17-69%). In 2 other studies in which a slightly different infusion schedule was used, response in PSA was observed in 3/5 and 4/9 cases respectively (Van Oosterom et al. 1990). More recently, Eisenberger et al. (1992), using an intermittent schedule based on a modified priori Bayesian pharmacokinetic parameter (MAP-PK) estimator, noted regression in soft-tissue disease in 3/3 and >50% decrease in PSA in 8/9 evaluable cases. In most studies, response durations are generally short (Van Oosterom et al 1990, Eisenberger et al. 1992).

The reported results show that Suramin has activity in hormone-refractory prostatic cancer. Considering measurable disease sites, a 30% response proportion (95% confidence limits 20-40%), a ≥ 50% decrease in PSA was observed in 54% of cases (95% confidence limits 44-64%). This is higher than reported for other chemotherapeutic agents or secondary hormonal manipulations. Further investigation of Suramin is warranted, but practical and reproducible dosing regimens need to be developed. In this regard, the report by Eisenberger et al. (1992) is of interest; responses were observed in 8/9 cases using an intermittent schedule designed to maintain levels in a therapeutic range.

Examination of reported results in several centers does show that Suramin has activity in this disease and that future studies are warranted. It also provides the rationale for investigating Suramin in combination. In vitro studies have shown synergy with adriamycin and tumor necrosis factor at concentrations that are well below those associated with significant toxicities (Sherwood et al 1990). Further, the lower limit of 200 ug/ml, may be different for different tumor types. A lower concentration range may improve patient tolerance (Freuhauf et al. 1990).

1.2.6.2   Somatostatin (SMS 201-995)

        1.2.6.2.1 Mechanism of action of somatostatin
        1.2.6.2.2 Somatostatin analogues
        1.2.6.2.3 Somatostatin and prostate cancer

Somatostatin (SMS) is a naturally occurring cyclic tetradecapeptide (Fig 10) that inhibits release of growth hormone and all gastrointestinal hormones. The beneficial effect of SMS in the treatment of certain hypersecretory disorders of hormone excess is well recognised; however, its clinical usefulness has been limited in the past by its extremely short plasma half life. The development of long acting SMS analogues has provided clinically useful agents for treatment of hormone-producing tumours.

In addition to well known inhibiting effects on hormone release and actions, recent studies using experimental tumour models, have demonstrated an anti-proliferative effect of SMS and its analogues on the growth of a variety of neoplasms. The exact role of SMS analogues in cancer therapy has yet to be established; however, studies suggest that these agents would provide a useful and relatively non-toxic adjuvant therapy in the treatment of certain tumours. In this section, the urological application of SMS and a possible mechanism of action are examined.

Somatostatin has been identified by a variety of immunocytochemical and radioimmunoassay techniques in multiple sites throughout the nervous system including cerebral cortex, pituitary infundibular process and spinal cord. (Newman et al. 1987, Reichlin 1987). In the gastrointestinal tract, SMS has been found in the stomach, duodenum, ileum, colon and pancreas; the greatest amount is present in the stomach and pancreas (Reichlin. 1987). More than 90% of the SMS immunoreactivity in the human

Fig 10

Amino Acid sequence of long-acting somatostatin octapeptide analogue (SMS 201-995)

$$\text{S} — \text{S}$$

H - (D)PHE - CYS - PHE - (D)TRP - LYS - THR - CYS - THR(ol)
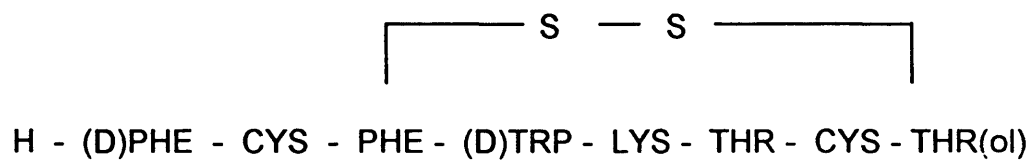
gut is present within endocrine cells called D cells. SMS in the pancreas is located in the D cells at the periphery of the islets of Langerhans (Newman at al. 1987).

1.2.6.2.1 Mechanism of action of somatostatin

SMS is characterised as a regulatory-inhibitory peptide with exocrine, endocrine, paracrine and autocrine activity. The general inhibitory function of SMS is wide-ranging and affects a number of organ systems. Many have characterised it as the universal endocrine off-switch. SMS inhibits the release of growth hormone and somatomedin C and all known gastrointestinal hormones. SMS also inhibits gastric acid secretion and motility, intestinal absorption, pancreatic bicarbonate and enzyme secretion and selectively decreases splanchnic and portal flow in dogs and humans without affecting mucosal blood flow (Price et al. 1985). The exact mechanism of the antitumour effects of SMS is not known, but some possibilities include one (or a combination) of the following: a) direct anti-proliferative effect mediated through specific, high activity SMS receptors; b) inhibition of secretion of gastrointestinal hormones thought to be important in tumour growth (that is, gastrin, secretin, cholecystokinin and insulin); and c) inhibition of release of growth hormone and other growth factors (that is, EGF and Somatomedin C) (Stiles et al. 1979).

Mascardo and Sherline (1982) demonstrated direct antiproliferative effects of SMS by noting in vitro inhibition of EGF-induced DNA synthesis and cell replication by blocking centrosomal separation in gerbil fibroma and HeLa cells. Direct effects of SMS may not be mediated through its interaction with specific SMS receptors found on normal cells and on cancer cells.

Hierowski et al. (1985), found that binding of SMS to its membrane receptors located on the pancreatic cancer cell line MIA Pa Ca-Z activates dephosphorylation of the EGF receptor, which prevents EGF induced growth.

1.2.6.2.2 Somatostatin analogues

The clinical usefulness of native SMS is limited greatly by its short plasma half-life (1 to 3 minutes) in humans (Longnecker, 1988). SMS analogues were developed that are long-acting and more potent than native SMS 14. The first such analogue was SMS

201-995 (Sandostatin) L-Octreotide acetate (Fig 10). SMS 201-995 is a synthetic octapeptide with a prolonged circulating half-life of approximately 41 to 58 minutes in humans when administered intravenously (Longnecker, 1988). The elimination half-life of SMS 201-995, when given subcutaneously, is even longer (approximately 113 minutes) (Bauer et al. 1982). This analogue was found to be 3 times more potent than native SMS in suppressing glucose-stimulated insulin secretion and 19 times more potent than native SMS in inhibiting growth hormone secretion. In recent years, major strides were made in the synthesis of newer, longer acting analogues specifically designed for anti-tumour activity. The SMS analogues have a wide therapeutic index and seem to be free of major side-effects (Schally, 1988). Most of the reported side-effects are gastrointestinal in nature and include nausea, bloating, diarrhoea, constipation or steatorrhoea. Currently, SMS 201-995 is approved only for the control of symptoms associated with metastatic carcinoid or vaso-active-intestinal polypeptide (VIP) secreting tumours. However, use of these analogues may prove beneficial in the future as novel adjuvant agents in cancer chemotherapy, because of the ability of SMS to inhibit EGF- induced centrosomal separation in a number of cancer cells.

1.2.6.2.3　　　　　　Somatostatin and prostate cancer

Investigators have studied the effect of two long-acting SMS analogues (RC 160 and RC 121) combined with a luteinizing hormone releasing hormone agonist, in the growth of the hormone-dependent CaP, Dunning R-3327H (Schally and Redding, 1987). LHRH, when given alone suppressed pituitary and gonadal function and inhibited growth of Dunning R-3327H tumour (Schally et al. 1971). The combination of LHRH and a long-acting SMS analogue (RC121 or RC 160) was more effective in inhibiting tumour growth than either agent alone. A possible explanation suggested by the authors for the enhanced effect seen with combination treatment is that the SMS analogues inhibit release of growth hormone and prolactin, which are thought to be important for growth of the normal prostate and possibly play a role in the proliferation of malignant cells. Whatever the mechanism, this study suggests that inhibition of hormonally sensitive CaP may be enhanced with a combination of SMS analogues.

1.2.6.3   Estramustine Phosphate (EMP)

Estramustine Phosphate or Estracyst (EMP) is not a growth factor inhibitor. However, it is one of a few agents known to be effective against androgen-independent cells.

1.2.6.3.1 Mechanisms of action of EMP

EMP is a drug with a dual mode of action: hormonal and cytotoxic. As seen in Figure 11, the preparation that is used in clinical situations is the sodium salt of EMP which is an oestradiol connected to non-nitrogen mustard, an alkylating agent, via a carbamate bridge. The phosphate group was attached to it to render it water soluble. EMP is rapidly dephosphorylated in the body, releasing estramustine (Fig 11). This is partially oxidised to estromustine, and these two are broken down into oestrone and oestradiol which are responsible for its antigonadotropic qualities. EMP inhibits the assembly and causes the disassembly of microtubules. This is similar to the effect of colchicine and the vinca alkaloids. The analogues are used in chemotherapy and both are classical microtubule inhibitors. EMP binds to microtubule associated proteins (MAP) (Stearns and Tew, 1988). This is in contrast to classical antimitotic agents such as colchicine and vincristine, which bind to tubulin and not MAPS. Prostate cancer is a slowly dividing tumour and if one only affects microtubules in the mitotic spindle during cellular division, a dramatic tumour response would not be expected. Microtubules, however have other functions that are essential to the cell, which if disrupted will cause cell death. Vinca alkaloids are often used in adjuvant chemotherapy because they interfere with microtubule assembly. Microtubules are essential for cell movement which is required for invasion, that is metastasis. It is possible therefore, that EMP might have a similar type of effect.

Fig 11

The Structure of Estramustine Phosphate

Major routes of metabolism of Estramustine Phosphate

Estramustine Phosphate

[1] = Dephosphorylation

[2] = Oxidation

[3] = Hydrolysis

Estramustine

Estromustine

Oestradiol

Oestrone
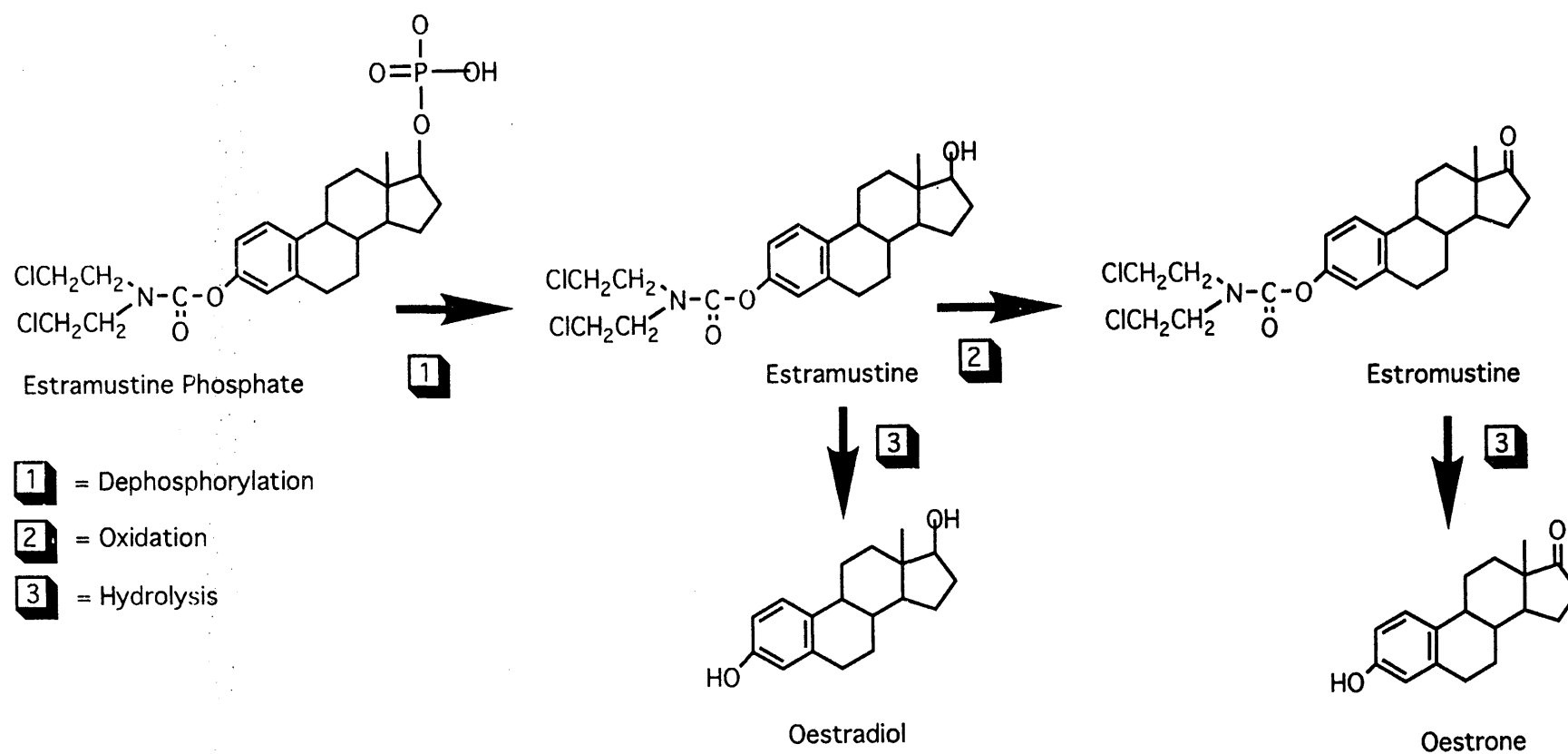
For EMP to be effective in treating hormone refractory CaP, it would be advantageous if the drug reaches the prostatic tumour in a patient who has been given the drug orally. Experiments have been carried out to study the uptake of EMP and its metabolites in patients. TURP samples from the tumour and samples from the plasma of the same patient, have been examined for the levels of EMP and its metabolites, and it does appear that EMP is present in the prostatic tumour after oral treatment with the drug (Fritjofsson et al. 1983). An additional interesting observation emerged from these studies. It was found that the levels of EMP were much higher in the tumour compared with the level in plasma of each patient than those of oestradiol, indicating facilitated uptake. This increase in intratumoural drug level could be due to the presence of a protein called prostatic binding protein which originally was found in the rat ventral prostate. This protein binds estramustine with a high affinity and has also been found in human tumours (Bjork et al. 1982). Thus, it may be that this protein is responsible for the increased level of estramustine found in tumour tissues.

From the experimental data available,it appears that EMP is a drug with both hormonal and cytotoxic action which appears to be mediated through an interaction with microtubules.

1.2.6.3.2 Clinical studies using EMP on hormone resistant prostate cancer

EMP was first employed in the treatment of hormonally unresponsive patients with prostatic cancer. An examination of 18 Phase II studies of EMP involving a total of 634 hormone refractory patients performed from 1971 onwards reveal an objective response rate averaging 37% (Benson and Gill, 1988). These data suggest that EMP is at least comparable to any other agent available for retreatment of hormonally unresponsive disease (Benson and Hartley-Asp, 1990). The other advantage of EMP is that it can be given to patients who have had previous radiotherapy (DXT) to treat skeletal metastasis (Soloway et al 1981). Other chemotherapeutic agents used for hormone resistant CaP, such as methotrexate and CIS platinum, produce severe side-effects in patients previously treated with DXT and their use has been limited in this disease because of this (Benson and Hartley-Asp, 1990). The other advantage of EMP over CIS platinum and methotrexate is the fact that it is given orally, whereas the other 2 agents require intravenous administration. However, significant toxicity and limited duration of response limit the use of

EMP in clinical practice. Hence, it would be very useful if agents with a synergistic or additive activity could be found to improve the therapeutic profile of EMP.

1.2.6.3.3 Clinical studies using EMP in primary treatment of metastatic prostate cancer

The effectiveness of EMP in the treatment of hormone refractory prostatic cancer led to investigations of its efficacy in the primary treatment of the disease. As was true for secondary treatment, the clinical efficacy of EMP in the primary treatment was initially examined in numerous Phase II studies. An objective response could be registered in about 80% of the treated patients (Edsmyr et al. 1982). This is a response rate of the same magnitude as that reported with treatment with orchidectomy or oestrogens. Numerous investigations have confirmed no statistically significant difference in effect between full dose EMP, that is 280 mg t.d.s., DES 1 mg t.d.s., or orchidectomy in the primary treatment of metastatic prostate cancer (Murphy et al. 1986).

In these studies, the investigators found no statistical difference with regard to response, progression-free survival or overall survival. However, in one study (Hedlund et al. 1980), although survival differences were not demonstrated between standard oestrogen therapy and EMP treatment, in primary metastatic prostate cancer, the time to progression in this randomised double-blind study was significantly increased in patients who received EMP.

1.2.6.3.4 Adverse effects of EMP

Treatment with EMP is associated with certain side-effects. These complications include fluid retention, reversible thrombocytopenia, venous thromboembolism, aggravation of ischaemic heart disease, impotence, gynaecomastia, nausea, vomiting and skin rashes. These side effects are dose dependent (Chisholm et al. 1977).

1.2.7   **Conclusion on Growth Factor Inhibitors**

From the literature review there is every reason to be optimistic that growth factor inhibitors will have a role to play in the treatment of tumours whose growth depends on growth factors. It is conceivable that a combination of growth factor inhibitors will be more effective than single inhibitors. Similarly, the combination of growth factor inhibitors and some chemotherapeutic agents may produce synergistic activity, thus allowing lower doses of toxic chemotherapeutic agents to be used without loss of anti-tumour activity. It is the purpose of this thesis to explore various combination therapies involving known growth factor inhibitors with chemotherapeutic agents that are known to be effective against CaP cells, especially the hormone-independent CaP cells.

Section C

1.3   **Models for studying prostate cancer**

For experimental control and simplicity, models are a *sine qua non* in medical research. This is because clinical trials involving human subjects have limitations such as cost, ethical and moral considerations. For studying prostate cancer, there are 2 models in use at present:

          1.3.1   Tissue culture

          1.3.2   Laboratory animals.

1.3.1   **Advantages and disadvantages of cell cultures**

          1.3.1.1   Advantages of cell culture
          1.3.1.2   Disadvantages of cell culture
          1.3.1.3   Major differences between cells in Vitro and in Vivo
          1.3.1.4   The development of prostate cancer cell lines

Tissue culture was first devised at the beginning of this century (Harrison, 1907, Carrel, 1912) as a method of studying the behaviour of animal cells free of systemic variations that might arise in the animal both during normal homeostasis and under the stress of an experiment. As the name implies, this technique was elaborated first with undisaggregated fragments of tissue, and growth was restricted to the migration of cells from the tissue fragments, with occasional mitoses in the outgrowth. Since culture of cells from such primary explants of tissue dominated the field for more than 50 years, it is not surprising that the name 'tissue culture' has stuck in spite of the fact that most of the explosive expansion in this area since the 1950s has utilised dispersed cell cultures. "Cell culture" refers to cultures derived from dispersed cells taken from the original tissue, from a primary culture, or from a cell line or cell strain, by enzymatic, mechanical or chemical disaggregation. The demonstration that human tumours could also give rise to continuous cell lines (e.g. HeLa; Grey et al. 1932), encouraged interest in human tissue, helped later by Hayflick and Moorhead's (1961) classical studies with normal cells of a finite life-span. The study of cellular activity in tissue culture may have many advantages, however considerable emphasis must also be placed on its limitations in order to maintain some sense of perspective.

1.3.1.1   Advantages of cell culture

Control of the environment

This includes the control of the physio-chemical environment (pH, temperature, osmotic pressure, oxygen, carbon dioxide tension), which may be controlled very precisely and the physiological conditions which may be kept relatively constant but cannot always be defined. Most cell lines will require supplementation of the medium with serum or other poorly defined constituents. These supplements are prone to batch variation (Honn et al. 1975) and contain undefined elements such as hormones and other regulatory substances. Gradually the essential components of serum are being identified, making replacement with defined constituents more practicable (Maurer, 1986). It is also possible today to get serum-free media with obvious advantages.

Characterisation and homogeneity of sample

Tissue samples are invariably heterogeneous. Replicates, even from one tissue, vary in their constituent cell types. After one or two passages, cultured cell lines assume a homogeneous or at least uniform constitution as the cells are randomly mixed at each transfer and the selective pressure of the culture conditions tends to produce a homogeneous culture of the most vigorous cell type. Hence, at each subculture each replicate sample will be identical and the characteristics of the line may be perpetuated over several generations or indefinitely if the cell line is stored in liquid nitrogen. Since experimental replicates are virtually identical, the need for statistical analysis of variance is reduced.

Economy

Cultures may be exposed directly to a reagent at a lower and defined concentration, and with direct access to the cell. Consequently, less is required than for injection in vivo where greater than 90% is lost by excretion and distribution to tissues other than those under study. Screening tests with many variables and replicates are cheaper, and the legal, moral and ethical questions of animal experimentation are avoided.

1.3.1.2   Disadvantages of cell culture

Expertise

Culture techniques must be carried out under strict aseptic conditions, because animal cells grow much less rapidly than many of the common contaminants such as bacteria, moulds and yeasts. Furthermore, unlike micro-organisms, cells from multicellular animals do not exist in isolation, and consequently are not able to sustain independent existence without the provision of a complex environment simulating blood plasma or interstitial fluid. This requires a level of skill and understanding to appreciate the requirements of the system and to diagnose problems as they arise.

Instability

This is a major problem with many continuous cell lines resulting from their unstable aneuploid chromosomal constitution. Even with short term cultures, although they may be genetically stable, the heterogeneity of the cell population, with regard to cell growth rate, can produce variability from one passage to the next.

1.3.1.3 Major differences between cells in vitro and in vivo

Many of the differences in cell behaviour between cultured cells and their counterparts in vivo stem from the dissociation of cells from a 3-dimensional geometry and their propagation on a 2-dimensional substrate. Specific cell interactions characteristic of the histology of tissue are lost, and, as the cells spread out, become mobile, and in many cases, start to proliferate, the growth fraction of the cell population increases. When a cell line forms, it may represent only one or two cell types and many heterotypic interactions are lost. The culture environment also lacks the several systemic components involved in homeostatic regulation in vivo, principally those of the nervous and endocrine systems. Without this control, cellular metabolism may be more constant in vitro than in vivo, but may not be truly representative of the tissue from which the cells were derived. Recognition of this fact has led to the inclusion of a number of different hormones in culture media and it seems likely that this trend will continue. Energy metabolism in vitro occurs largely by glycolysis, and although the citric acid cycle is still functional it plays a lesser role. There are more differences between the environmental conditions of a cell in

vitro and in vivo and this has often led to tissue culture being regarded in a rather sceptical light. Although the existence of such differences cannot be denied, it must be emphasised that many specialised functions are expressed in culture and as long as the limits of the model are appreciated, it remains a valuable tool (Freshney, 1987).

1.3.1.4   The development of prostate cancer cell lines

The task of growing in vitro epithelial prostatic cells either from animals or from man is a difficult one, as evidenced by numerous publications focused on short-term organ and cell cultures (Lasnitski et al. 1966, Chen and Heidelberger, 1969). Prostatic cell lines MA160 (Fraley et al. 1970) and EB33 (Okada and Schroder, 1974) which were frequently used experimentally and widely quoted in the literature, were found in fact to be HeLa cell contaminants (Nelson-Rees and Flandermeyer, 1976). New techniques for initiation of cultures from human prostates described by Stonington and Hemmingson, (1971), Brehmer et al. (1972), grew for approximately 3 months, cells from CaP and BPH. Kaighn and Babcock, (1975), established a cell line from BPH which at 3 weeks showed intracellular tartrate-inhibited acid phosphatase. Webber and Stonington, (1975), were successful in propagating human prostatic epithelium as explant cultures over a period of a few weeks, and they were also able to make valuable observations on the morphology and properties of prepubertal prostate in vitro. Stone et al (1978), isolated an epithelial cell line DU145, from an individual with leukaemia and CaP metastasis to the brain. Similarly, Kaighn et al. (1979), were able to grow epitheloid cells (line PC3) from a CaP metastasis to bone. Both cell lines are tumourigenic in nude mice. Neither line, however, appears to be responsive to sex hormones, no significant levels of acid phosphatase in cultures were observed, and organ-specific prostatic antigens were not reported. An androgen-dependent cell line LNCaP was successfully cultured by Horoszewicz et al. in 1980. Culture was obtained from metastasis in the supraclavicular lymph node. The unique property of the LNCaP cell line is the continuous production of acid phosphatase, both in vitro and in vivo in the nude mice. The acid phosphatase was shown to be biochemically similar and immunologically identical to human prostatic acid phosphatase (PAP) by counter-immunoelectrophoresis and double-diffusion gel precipitation with monospecific antiserum. The acid phosphatase activity is also proportional to the cell density of tested cultures. PC3, DU145 and LNCaP human CaP cell lines have been tested and found not to be contaminated with the HeLa

virus, thus making these lines new and relevant models for studies in the areas of aetiology, biology, diagnosis and treatment of human CaP (Horoszewicz et al. 1979 and 1980). These in vitro cell models derived from human cells are particularly suited for studies on the aetiology of cancer and on the mechanism of carcinogenesis. Normal cell systems further provide a basic understanding of the physiology of normal cells and of various growth-regulating mechanisms. Models using malignant human cells are useful for testing different models of treatment and for examining a possible relationship between tumour resistance or sensitivity and related cell characteristics. Such a relationship, if established, can help in predicting the response of a tumour to a particular treatment.

1.3.2   Advantages and disadvantages of animal models

For the investigation of basic biological patterns of tumours, animal models are essential. Knowledge about basic functions and properties of malignant tumours cannot be obtained by clinical studies alone. On the other hand in vitro experiments allow the study of single aspects of the tumour without all the various influences of its natural environment. Few culture cell lines were successfully derived from human prostate cancer as stated above. In many cases, overgrowth by fibroblasts was observed (Okada and Schroder, 1974; Reid, 1978). Therefore many attempts have been made to establish suitable animal models for the investigation of CaP. Due to different reasons, these models are unable to answer most basic questions about the pathophysiology of prostatic tumours (Murphy, 1980). For example, two very well known animal models are the Dunning tumour in rats (Dunning, 1963), and the Noble rat CaP (Noble, 1977), which have been used to evaluate many biochemical modalities in malignant prostate tissue, but both are not of human origin. Therefore, their usefulness is limited. The investigation of human tumours requires viable, growing human tumour tissue containing all different cell types in their natural environment and the possibility to manipulate its environmental conditions. This has led to many efforts to establish human tumour cell lines by transplantation into animals. The chief limitation of this is whether it is reasonable to extrapolate data derived from animals directly to human disease. Hence, it is obvious that animal models alone are not sufficient in themselves to assess the full clinical importance of a therapeutic approach. However, animal

models do provide a unique opportunity to develop new concepts that are very difficult to obtain through available clinical studies and within reasonable cost, time and ethical restraints. Appropriate animal studies and careful clinical trials must work in concert to assist in the search for a new and more effective management of human CaP. Experimental studies on the available models of rodent prostatic carcinoma reviewed by Coffey et al. (1979), are highly useful in providing us with a better understanding of the biology of prostatic malignancy. These transplantable and easily-grown in vitro tumours, represent a collection of neoplasms originating in rat prostate. Because of preserved biological and biochemical markers, hormonal responsiveness, malignancy and drug sensitivity, they are very attractive as animal model systems. Their limitations are rooted in restrictions imposed by difficulties in direct translation of data obtained in an animal model system into the language of human disease.

Heterotransplantation of human prostate tumours into nude mice has been attempted by several investigators, but the results, with few exceptions, were disappointing. Reid and co-workers, (1978), reported that only one out of over 100 tumour specimens developed into a somewhat differentiated transplantable tumour in the nude mice. The properties of this tumour are modulated by androgens, and trace amounts of tartrate were observed to inhibit acid phosphatase.

Section D

1.4   **The scope of the present study**

        1.4.1   Treatment of prostate cancer: an overview and rationale for current study.

        1.4.2   Aims of study

        1.4.3   Study design

1.4.1   **Treatment of prostate cancer: an overview and rationale for current study.**

From the above discussion, it is obvious that CaP is heterogeneous and that growth factors appear involved in the growth of the tumour. Androgen-dependent CaP cells within an individual prostatic cancer can be effectively eliminated by various forms of androgen ablation such as castration, DES, LHRH analogues or anti-androgens. In order to increase survival, the clones of androgen independent CaP cells, whose growth depend on growth factors and are probably already present even before therapy is begun, must be eliminated. By combining effective androgen ablation treatment with inhibition of growth factor production, a better treatment for CaP with increased patient survival may be attained.

Suramin and SMS 201-995 appear to have anti-growth factor properties. The aim of this study is to establish whether Suramin and SMS 201-995 will affect growth factors involved in androgen-independent CaP growth. This hypothesis will be tested on established CaP cell lines like LNCaP, PC3 and DU145 and subsequently on prostate cancer cells obtained by primary culture from patients in Leicester. A key objective of this study is to find out whether Suramin and SMS 201-995 alone, or in combination with other chemo-hormonal agents, will produce inhibition of growth-factor-induced growth of prostate cancer cells. Lastly, the effect of any combination of growth factor inhibitor and chemohormonal agents showing promise in vitro, will be tried on patients with hormone-resistant prostate cancer in Leicester.

1.4.2   **Aims of study**

a]   Do growth factor inhibitors Suramin and Somatostatin inhibit the proliferation of

- androgen-dependent prostate cancer cell line LNCaP
- androgen-independent prostate cancer cell line PC3
- prostate cancer cells obtained by primary culture?

b]   Is the effect of Suramin on the above cell types potentiated by SMS or EMP?

c]   Are the effects, if any, of Suramin, SMS; Suramin + EMP and Suramin + SMS cytotoxic or cytocidal?

d]   To carry out a preliminary clinical trial in patients with hormone resistant prostate cancer to find out their response to treatment with EMP + low dose Suramin or EMP alone.

1.4.3.1   **Study design - Theoretical considerations**

a]   To find out if growth factor antagonists have any influence on prostate cancer cell function by looking at their effects on established prostate cancer cells in culture and on prostate cancer cells obtained by primary culture.

b]   To find out if combinations of growth factor inhibitors or growth factor inhibitor with other chemotherapeutic agents will produce additive or synergistic effects on CaP cell growth, thus allowing the use of lower doses of the agents and reduce toxicity.

1.4.3.2   **Study Design - Practical considerations.**

To test for inhibition of established CaP cell lines and CaP cells obtained by primary culture, the ability of the cells to proliferate was established as follows:

a)   cultures of established cell lines and cells obtained by primary culture were established

b)   the cells were passaged to generate cells for culture

c)   a proliferation assay was then established.

d)   assay was used to establish toxicity or synergy

e)   the results of in vitro studies were used to design a clinical trial.

CHAPTER 2 : MATERIALS AND METHODS OF EXPERIMENTS ON ESTABLISHED PROSTATE CANCER CELL LINES

Section A :   Introduction to established human prostate cancer cell lines

Section B :   Methods of determining cell growth or methods of counting
                       cells in culture

Section C: Determination of the effects of drugs or drug combinations
                       on proliferation of prostate cancer cell lines PC3 and LNCaP

Section D :   Determination of whether the enhanced effect of Suramin
                       and EMP was synergistic or additive

Section E :   Determination of whether the effect of the drug(s) was
                       cytostatic or cytotoxic on LNCaP and PC3 cell lines

Section F :   The effect of exogenous epidermal growth factor on the
                       proliferation of LNCaP, PC3 and DU145 prostate cancer cell
                       lines

Section G: Statistical methods

MATERIALS AND METHODS OF EXPERIMENTS ON ESTABLISHED PROSTATE CANCER CELL LINES

Section A

2.1	**Introduction to established human prostate cancer cell lines**

	2.1.1	LNCaP cell line - androgen dependent
	2.1.2	PC3 and DU145 - androgen independent
	2.1.3	Removing attached cells from the tissue culture flask or passaging
	2.1.4	Media for experiments

2.1   **Introduction to established human prostate cancer cell lines**

The following established human prostatic carcinoma cell lines: PC3 (androgen-independent), DU145 (androgen independent) and LNCaP (androgen-dependent) were generously provided by Dr F K Habib, University Department of Surgery and Urology, Western General Hospital, Edinburgh and Dr J Masters (Institute of Urology, London). These lines were maintained in RPM1-1640 medium containing 10% foetal calf serum (FCS), Glutamine (2 mM), Penicillin (100 ug/ml) and Streptomycin (100 ug/ml) (see appendix for full complements of media used). Cells were grown in an incubator maintained at an air: CO_2 atmosphere (95%: 5% v/v) and 37^0C in 50 or 200 cm^2 flasks.

2.1.1   **LNCaP cell line - androgen dependent**

The LNCaP cell line is an androgen-dependent prostate cancer cell line, derived from CaP metastatic to supraclavicular lymph nodes. LNCaP is androgen-sensitive in that its proliferative activity is amplified in the presence of exogenous dihydrotesterone (DHT) (Schuurmans et al. 1989). It is also capable of basal growth in the absence of androgens. In concert with its demonstrated androgen sensitivity, LNCaP has maintained its ability to express, - albeit in heterogeneous fashion, both prostatic acid phosphatase (PAP) and prostate specific antigens (PSA) in culture (Kozlowski et al. 1989). Morphologically, LNCaP cells appear stellate shaped with long 3-4 processes (Fig 12).

Fig 12

Typical LNCaP prostate cancer cells. The cells appear stellate shaped with 3-4 long processes.

75
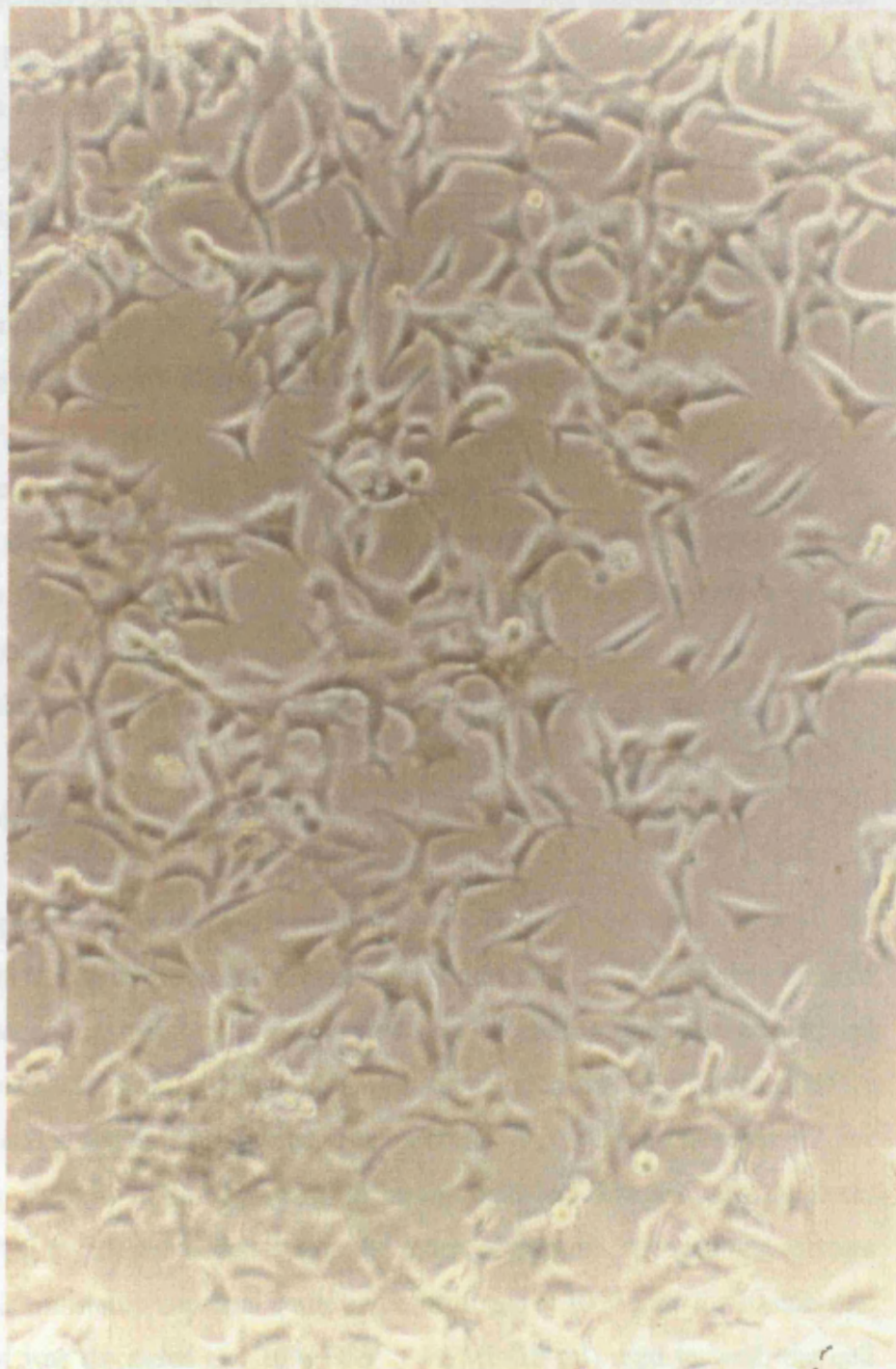
2.1.2   DU145 and PC3 - androgen independent cell lines.

These two lines are completely androgen-independent. DU145 was derived from metastatic foci in the brain, while PC3 was derived from metastatic foci in the lumbosacral bone marrow. The two lines are capable of sustained proliferation in the absence of exogenous DHT. Moreover, they neither express nor secrete the biochemical markers prototypic of prostatic epithelium, namely PAP and PSA. Despite their origins from aggressive tumour systems, only PC3 appears to be highly tumourigenic in nude mouse model (Brothman et al. 1989). PC3 has also been vigorously assessed and shown to maintain a highly invasive and metastatic phenotype in vivo (Keer et al. 1990). PC3 also synthesises and secretes TGF and other growth factors into their conditioned media that are highly mitogenic for the more biologically indolent cell lines DU145 and LNCaP. Morphologically PC3 appears ellipsoidal in shape with 1-2 filaments (Fig 13).

These 3 long term cell lines have been used as models for studying cytogenetics, the immunological basis of, as well as the response to, chemotherapeutic agents of CaP. Recent changes in research methodologies and identification of new nutrients or growth promoting factors of these cells have made it possible for other long term cell lines to be developed and these cell lines continue to contribute to prostatic cancer research.

2.1.3 Removing attached cells from the tissue culture flask or passaging

Once cells became confluent in the tissue culture flasks, they were passaged by dissociation with 0.25% trypsin solution containing 1 mM EDTA. Working in a laminar flow sterile hood, culture medium from each flask was discarded aseptically and attached cells washed twice with 5 ml of Hanks balanced salt solution (HBSS). Trypsin/EDTA (5ml) was then added and the flask placed in an incubator at 37° C for 4 minutes. After checking with the aid of a microscope to see that cells had detached from the flask, 10% FCS + RPMI (5 ml) was added to stop the action of trypsin. The resulting mixture was put into a 25 ml sterile universal bottle and centrifuged at 1000 rpm. for 10 minutes. The supernatant was discarded and 10% FCS + RPMI (10 ml) used to wash the cell pellets twice. The cell pellet was then re-suspended in 10% FCS + RPMI and a small aliquot (1-2 ml) put in T-25 flasks containing 10% FCS to initiate the growth of the cells again.

Fig 13

Typical PC3 prostate cancer cells. The cells appear ellipsoidal with 1-2 filaments.
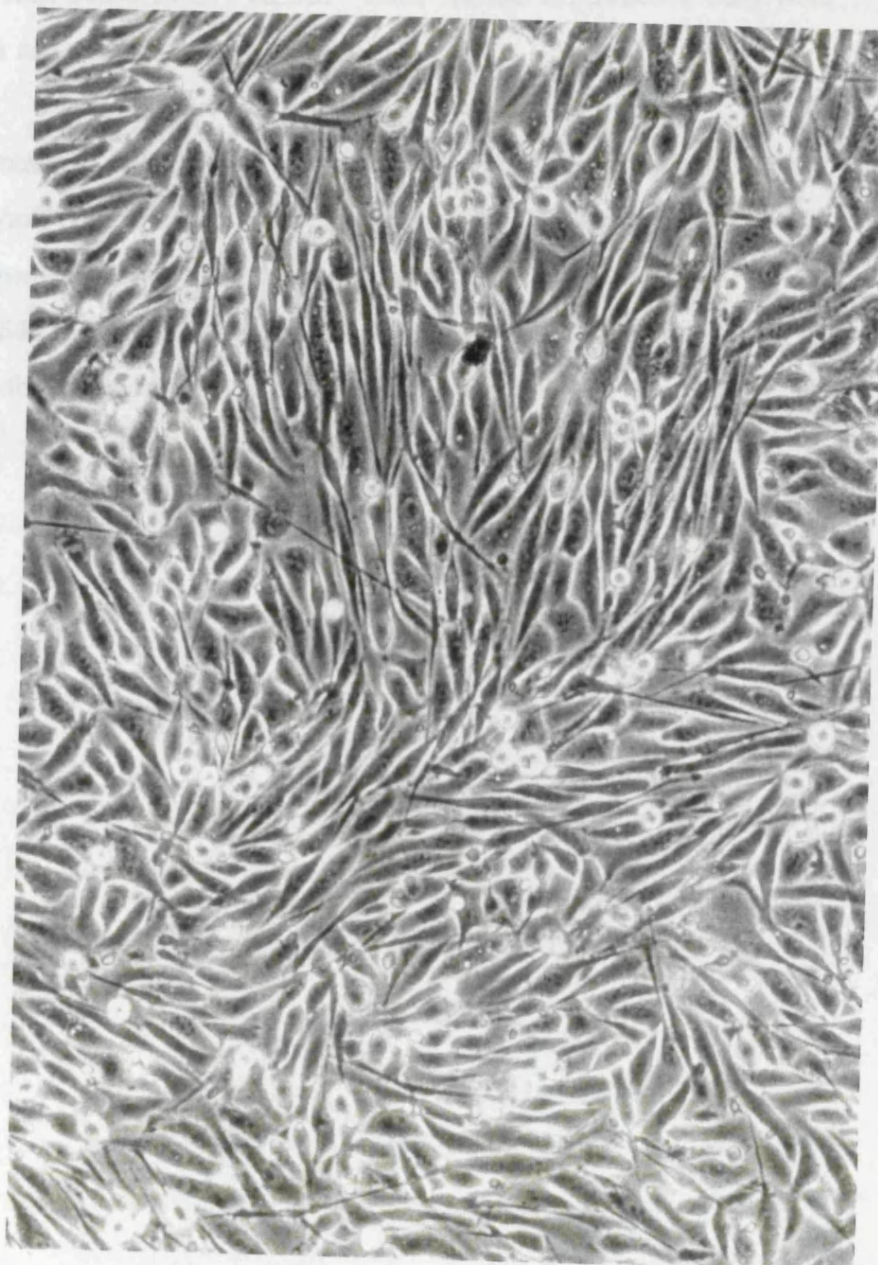
2.1.4 Media for experiments

Cells were passaged with 100% FCS + RPMI. Then, for the experiments, cells were grown in low serum or serum free media as follows:

Serum-free media containing RPMI-1640 with insulin (5 ug/ml), transferrin (5 ug/ml), sodium selenite (5 ng/ml) (ITS obtained from Sigma Chemical Co., Dorset, Product No. 11884) for all experiments involving androgen-independent cell lines PC3 and DU145, and 2.5% FCS in RPMI-1640 for androgen-dependent cell line LNCaP. Cells were adapted for growth in these media for all experiments as described in Appendix A.

Section B

2.2   **Methods of determining cell growth or cell numbers in culture**
2.2.1   Haemocytometer (total cell count)
2.2.2   MTT growth determination assay
2.2.3 ^{3}H thymidine incorporation assay
2.2.4   Prostatic acid phosphatase activity in culture supernatants.
2.2.5   Establishment of clonal growth or proliferation of cells

2.2.1.   **Haemocytometer (total cell count)**

The normal haemocytometer was first used to check that the cells in culture were proliferating. Cell counting was done every other day from samples of cells in tissue culture plates after cells had been detached using 0.25% Trypsin/EDTA solution as previously described.

Cells (100 uL) in suspension were diluted with an equal volume of 0.5% trypan blue in saline. The coverslip was attached firmly to the haemocytometer. The solution was added to either side of the haemocytometer and the number of cells in 4 large corner squares of the haemocytometer counted under high power. Cells that appeared clear were viable, while cells staining blue were regarded as dead. The number of cells in each well was calculated using the formula:

$$\text{Total Count} = X/4 \times 2 \times 10^4 = X/2 \times 10^4$$

Where X represents total number of cells counted in 4 corner squares of the haemocytometer.

This traditional method of counting viable cells, after staining, is time-consuming and considering the number of experiments to be carried out, it was used only once at the beginning of each stage of the experiments to ensure that the cells were proliferating. This method was used as the "gold standard" for verifying the accuracy of other methods of counting cells, namely MTT growth determination assay, ^{3}H thymidine incorporation assay and prostatic acid phosphatase level in the supernatant of confluent cells. Automated cell counters (Coulter B2 Dickenson Laboratory, California, USA, Model CBZ 7167, 1992) used in the Haematology Department, was also used to count the number of cells in a given well.

2.2.2   MTT growth determination assay

(Cell growth determination kit MTT based M-0283, M-0408
Sigma Immunochemical)

The MTT system is a simple, accurate, reproducible means of measuring the activity of living cells via mitochondrial dehydrogenase activity. The key component is (3-[4,5-dimethylthiazol-2 -yl] - 2, 5-diphenyltetrazolium bromide) or MTT. Solutions of MTT solubilized in tissue culture media or balanced salt solutions, without phenol red, are yellowish in colour. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals (Fig 14) which are insoluble in aqueous solutions. The crystals can be dissolved in isopropanol, dimethyl sulfoxide (DMSO) and other solvents. The absorbance of the resulting purple solution (Fig 15) is spectrophotometrically measured at a wavelength of 570 nm. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance.

2.2.2.1   The experiments

Confluent cells in 24 well plates were used. Fig 16 shows a typical 24 well plate used in all the experiments described in this study. Cultures were removed from the incubator into the laminar flow hood.

0.1 ml of MTT solution was added to cells in a single well containing 1 ml of feeding medium (2.5% FCS for LNCaP, or ITS and RPMI for PC3 and DU145 and primary epithelial growth medium (PEGM) for cells obtained by primary culture).

The cultures were then returned to the incubator and incubated for 3-4 hours. After the incubation period, the cultures were removed from the incubator and the formazan crystals dissolved by adding 1 ml of MTT solvent to each well of the 24 well plate. This was because the cells detached on the addition of MTT solution.

Fig 14

Mitochondrial dehydrogenase of viable cells cleave tetrazolium ring, yielding purple MTT formazan crystals.
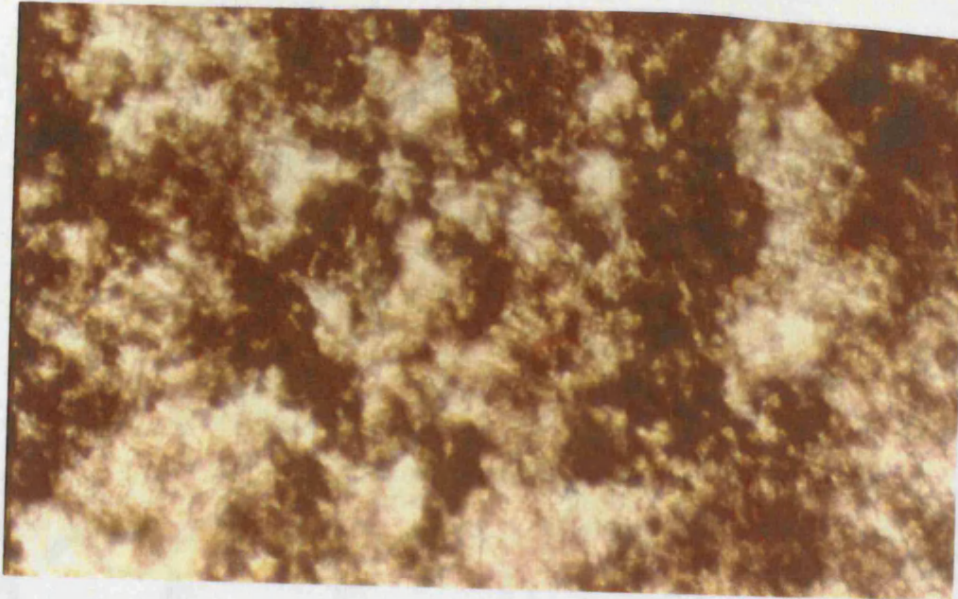
Fig 15

Dissolution of formazan crystals in isopropanol dimethyl sulfoxide (DMSO) yield purple solution which can be spectrophotometrically measured.
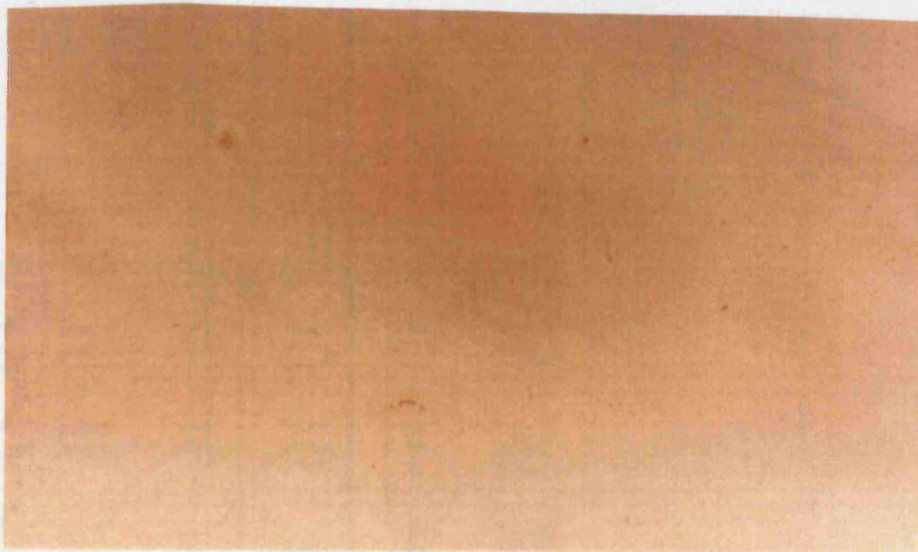
Fig. 16

Typical 24 well plate used in all experiments described.
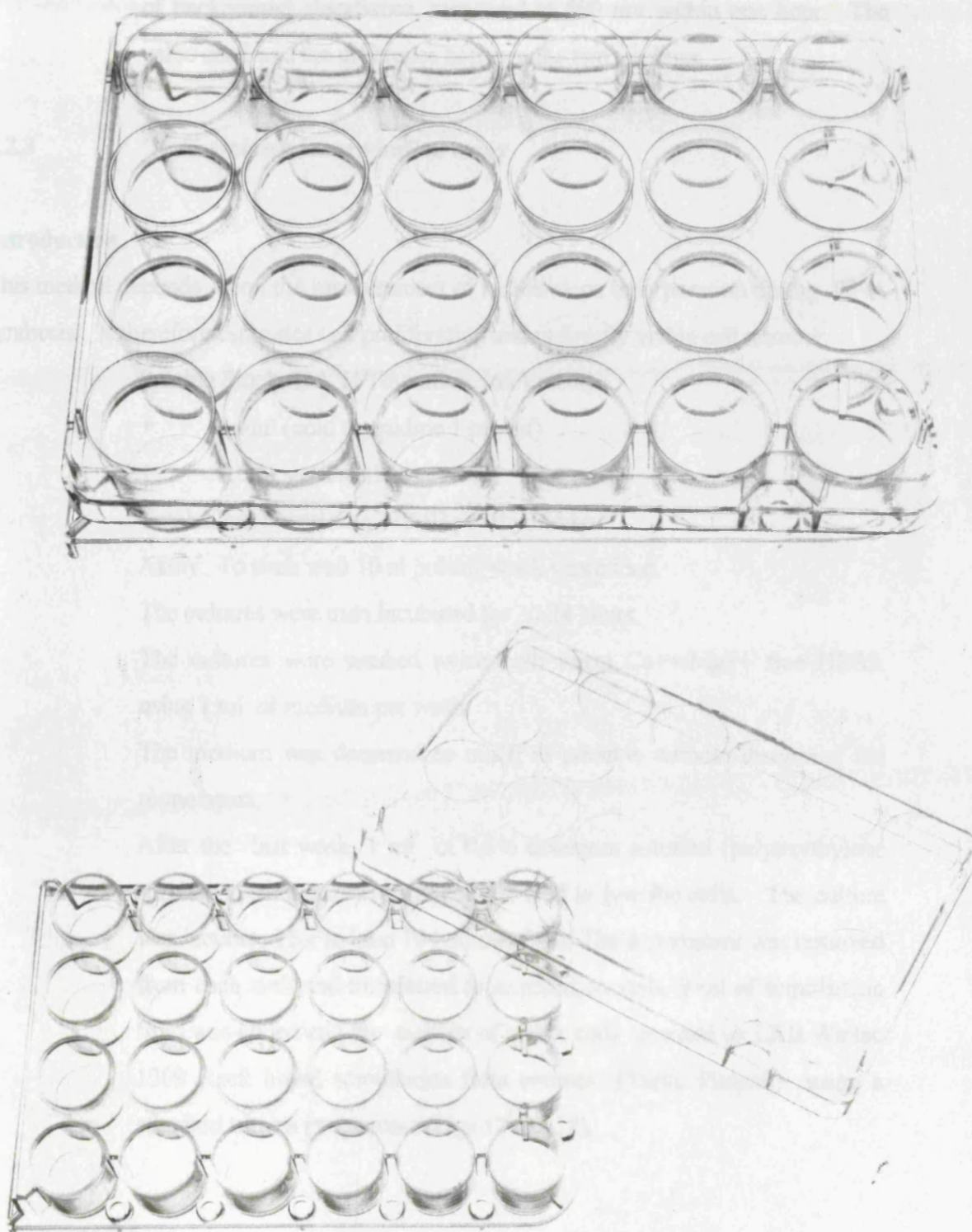
Gentle trituration was required to completely dissolve the MTT formazan crystals.

The optical density was measured spectrophotometrically, by measurement of absorbance at a wavelength of 570 nm with subtraction of background absorbance, measured at 590 nm within one hour. The value used was the difference between the two readings.

2.2.3 ^{3}H Thymidine incorporation assay

Introduction

This method depends upon the measurement of radioisotope incorporation during DNA synthesis. It therefore estimates cell proliferation and indirectly viable cell number.

- Pulsing Stock: 1M ^{3}H Thymidine stock =1ml.
- + 0.6ml (cold thymidine 1 mg/ml)
- + 0.6ml sterile saline
- Total 2.2ml containing 37 MBq of thymidine
- Assay: To each well 10 ul pulsing stock was added.
- The cultures were then incubated for 20-24 hours.
- The cultures were washed twice with warm Ca++/Mg++ free HBSS. using 1 ml of medium per wash.
  The medium was decanted as much as possible without disturbing the monolayers.
- After the last wash, 1 ml of 0.5% detergent solution (polyoxythylene sorbitan monolantate) was added per well to lyse the cells. The culture was incubated for at least 10 hours at 37°C. The supernatant was removed from each well and transferred to scintillation vials. 3 ml of scintillation fluid was added and the number of viable cells counted on LKB Wallac 1209 Rack liquid scintillation Beta counter (Turku Finland) using a standard tritium programme (Figs 17 and 18).

Fig 17

LKB Wallac 1209 Rack Beta-counter (Turku Finland) used for ^{3}H thymidine incorporation assay in all experiments carried out.

Figure 18 Computer printout of Beta-counter reading


```
CH 3  008-060
CH 4  060-110
STD TIME 030
PRINT 01,02,03,04,06,15
REP 01
EFF1%  RATIO

36.50  .705
31.73  .425
26.37  .287
20.28  .192
15.31  .115
13.89  .099
11.54  .078
10.07  .061
```

S TIME	C1	CPM1	DPM1	CLM%
1 0060	2872	2868.0	7748.9!	.02
2 0060	4055	4051.0	10914.4!	.02
3 0060	2244	2240.0	6120.9!	.03
4 0060	2664	2660.0	7108.5!	.02
5 0060	3712	3708.0	9911.4!	.01
6 0060	3012	3008.0	8041.5!	.02
7 0060	1002	998.0	2744.2!	.04
8 0060	978	974.0	2523.5!	.05
9 0060	2460	2456.0	6625.1!	.02
0 0060	2272	2268.0	6184.9!	.02
1 0060	4388	4384.0	11643.5!	.02
2 0060	4071	4067.0	10944.0!	.02
4 0060	3391	3387.0	9242.2!	.03
5 0060	3790	3786.0	10351.6!	.03
6 0060	1536	1532.0	4104.7!	.06
7 0060	1730	1726.0	4724.3!	.07
8 0060	4156	4152.0	11268.0!	.03
9 0060	5515	5511.0	15053.8!	.03
0 0060	961	957.0	2568.7!	.12
1 0060	872	868.0	2350.3!	.16
2 0060	2792	2788.0	7726.6	.06
3 0060	3544	3540.0	9567.5!	.04
4 0060	5308	5304.0	14226.0!	.02
5 0060	4724	4720.0	12756.6!	.03
7 0060	1246	1242.0	3374.9!	.12
8 0060	1466	1462.0	3965.0!	.10
9 0060	2235	2231.0	6017.8!	.06
0 0060	2103	2099.0	5642.8!	.07
1 0060	1477	1473.0	3869.6!	.17
2 0060	1395	1391.0	3697.1!	.14
3 0060	2078	2074.0	5427.2!	.08
4 0060	2014	2010.0	5414.8!	.10
5 0060	2155	2151.0	5743.3!	.06
6 0060	1982	1978.0	5359.2!	.08
7 0060	1603	1599.0	4291.6!	.08
8 0060	1438	1434.0	3750.9!	.11
0 0060	2664	2660.0	7147.4!	.09
1 0060	2403	2399.0	6535.7!	.11
2 0060	4149	4145.0	11056.1!	.05
3 0060	4882	4878.0	13474.8!	.04
4 0060	2815	2811.0	7645.0!	.06
5 0060	2841	2837.0	7727.3!	.05
6 0060	3619	3615.0	9768.7!	.05

DPMI = distintegration per minute used as a measure of radioisotope incorporation during DNA synthesis

All cell cultures were done in duplicate. The experiments were repeated three times on three different days. The average disintegration per minute (DPM) was calculated for each set of experiments, that is the mean of triplicate experiments.

2.2.4 Prostatic acid phosphatase activity in culture supernatant

Some malignant prostate cells, especially androgen-dependent cells, retain their ability to produce prostatic acid phosphatase (PAP) in culture. We used this fact to assess the mitogenic or cytotoxic effects of chemotherapeutic agents on LNCaP and CaP cells obtained by primary culture.

Our method was as follows:

In order to preserve the acid phosphatase activity, serum and culture supernatant fluids were buffered with 3 M acetate buffer pH 5.5. The buffer (50 ul) was added to 1 each millilitre of the supernatant.

The acid phosphatase activity was measured using the dri-stat reagent kit for acid phosphatase produced by Beckman Instruments, Carlsbad, California 92008-4836 USA. The method utilises alpha naphthylphosphate 3 mmol/ in acetate buffer pH 5.0, 5 mol/l using fast red TR 1 mmol as the colour-forming reagent. Inhibition was carried out with L-tartrate at 2 mmol/l. The analysis was carried out on the Monarch centrifugal analyser (Instrumental Laboratories Limited, Warrington, UK). The enzyme activity was measured at 30°C using standard settings of the instrument.

We used the haemocytometer and ^{3}H thymidine incorporation assay - *vide supra* - to count the number of cells in each well whose PAP was determined.

2.2.5   **Establishment of clonal growth or proliferation of prostate cell line**

On receiving samples of established human CaP cell lines from London and Edinburgh, half were frozen away in liquid nitrogen to retain a sample at a lower passage number, while the remaining half were passaged repeatedly for our experiments. All experiments were carried out in a hood with laminar flow and an incubator at $37^{\circ}C$ and 5 per cent CO_2 as described before. Cells were plated in 24 well plates at an initial concentration of 2×10^4 cells/well. Cells were fed on days 2, 4 and 6. ^3H thymidine incorporation assay and the haemocytometer were used to estimate the number of cells every other day.

Section C

2.3   **Determination of the effects of drugs or drug combinations on proliferation of prostate cancer cell lines LNCaP and PC3**

> 2.3.1   Suramin or Somatostatin SMS 201-995
> 2.3.2   Estramustine phosphate
> 2.3.3   Suramin and SMS 201-995 combination
> 2.3.4   Suramin and EMP combination
> 2.3.5   SMS 201-995 and EMP combination

2.3.1   **Suramin or Stomatostatin SMS 201-995**

The effect of Suramin or Somatostatin 201-995 (SMS 201-995) on LNCaP and PC3 prostate cancer cell lines.

Aim: To find out the effect of Suramin or Somatostatin on the cellular proliferation of LNCaP and PC3 prostate cancer cell lines.

Method: Growth of cells maintained in RPMI 1640 /10% FCS as previously described. Tumour cells were adapted to growth in 2.5% FCS for LNCaP and serum free media containing RPMI 1640 and insulin, transferrin and sodium selinite (ITS) for PC3 cell line. Cells were plated in 24 well plates at a density of 2×10^4 cells/well on day 0. Cells were exposed to the following concentrations of:

-        Suramin (0, 10, 30, 90, 270, 810 ug/ml) and;

-        SMS 201-995 (0, 0.002, 0.02, 0.2, 2 and 20 ug/ml) after 24 hours of initiation of cell growth following plating. (See appendix on how various drug concentrations were prepared.)

Cells exposed to Suramin were fed with a medium containing Suramin on days 2 and 4, while cells exposed to SMS 201-995 were fed every day with a medium containing SMS 201-995. The number of cells in each well were counted on days 2, 3, 4, 5 and 6 using the haemocytometer after detaching cells from each well using 0.25% trypsin/0.1% EDTA. Triplicate assays (one experiment per week) were performed for each experimental condition.

The percentage inhibition of cell growth was calculated using this formula:

$$\% \ INHIBITION \ = \ 1 - \ \frac{No. \ of \ cells \ in \ experimental \ well}{No. \ of \ cells \ in \ control \ well} \ x \ 100\%$$

or $\% \ INHIBITION = 100 - \% \ surviving \ cells$

2.3.2 The effect of Estramustine phospate on proliferation of LNCaP and PC3

Aim: To evaluate the ability of EMP to inhibit the proliferation of the following CaP of cell lines, PC3 and LNCaP.

Method: LNCaP and PC3 cell lines were adapted to growth in 2.5% FCS and RPMI 1640 and ITS respectively as indicated above. Cells were plated in 24 well plates at a density of 2×10^4 cells/well on day 0. After 24 hours of initiation of cell growth, after plating, cells were fed with media containing EMP (0, 3.1, 6.25, 12.5, 25, 50) ug/ml. Fresh media containing EMP were added to the wells on day 3. The number of cells in each well was counted on day 4 using the ^3H thymidine incorporation assay. Triplicate assays were performed for each experimental condition.

The percentage inhibition of cell growth was calculated using the standard formula previously described.

2.3.3 The effect of combined administration of Suramin and SMS 201-995 on proliferation of CaP cell lines LNCaP and PC3.

Aim: To find out whether the combined anti-proliferative effects of SMS 201-995 and Suramin is synergistic (or additive), antagonistic or of no effect on CaP cell line PC3 and LNCaP i.e. when both drugs are administered simultaneously.

Method: LNCaP and PC3 were adapted to growth in 2.5% FCS and RPMI 1640 and ITS as stated previously. After 24 hours of initiation of cell growth, after plating at 2×10^4 cells/well, the cells were exposed to the following concentrations of:

-             Suramin (0, 10, 30, 90, 270, 810 ug/ml) only;

                 Suramin (0, 10, 30, 90, 270, 810 ug/0.5ml) and SMS 201-995 (0.02mg/0.5 ml) and Suramin (0-810 ug/0.5 ml) and SMS 201-995 (2.0 ug/0.5 ml.) (i.e. for wells containing mixture of Suramin and SMS total volume per well = 1 ml).

The media containing the drug(s) were changed on day 2 and 4 with fresh media with drug(s). The number of cells proliferating in each well was assessed using ^3H thymidine incorporation assay on day 6. The percentage inhibition was then calculated using the standard formula as indicated previously. The Student t-test was used to find out if there were any differences between cells exposed to Suramin only and those exposed to Suramin and SMS 201-995.

2.3.4   The effect of Suramin and EMP on proliferation of prostate cancer cell lines LNCaP and PC3

Aim: To find out if the antiproliferative effects of Suramin can be enhanced by combination with EMP and the effect of combination therapy using Suramin and EMP on the cellular proliferation of prostate cancer cell lines LNCaP and PC3.

Method: Cells were adapted to growth in RPMI and ITS and 2.5% FCS for PC3 and LNCaP respectively. Cells were plated at a density of 2×10^4 cells/well on day 0. 24 hours after initiation of cell growth, cells were exposed to the following concentrations of drugs Suramin only, Suramin + EMP (3.1 ug/0.5ml) or Suramin + EMP (12.5 ug/0.5 ml).

The number of cells surviving in each well was assessed using ^{3}H thymidine incorporation assay 48 hours after exposing the cells to the test drug or drug combinations. Experiments were carried out in triplicates, that is, one set of experiments per week.

The percentage surviving cells was calculated using the standard formula previously stated.

2.3.5 The effect of combination therapy using EMP and Somatostatin 201-995 on the proliferation of prostate cancer cell lines LNCaP and PC3.

Aim: To find out whether the anti-proliferative effects of EMP can be enhanced by combination with SMS 201-995.

Method: Cells were adapted to growth in RPMI and ITS and 2.5% FCS for PC3 and LNCaP respectively. Cells were plated at a density of 2×10^4 cells/well on day 0. 24 hours after initiation of cell growth, the cells were exposed to the following concentrations of SMS 201-995 (0-20 ug/ml) + EMP (3.1 ug/0.5ml or 12.5 ug/0.5ml) as shown in Table 3. The percentage inhibition of cell growth was calculated using the previously stated standard formula.

Three separate experiments were carried out, one experiment performed over an 8-day period with the medium changed on days 2 and 4, and final results read on day 6. ^{3}H thymidine incorporation assay was used to determine the percentage surviving cells and the percentage growth inhibition produced by the drugs on the cell lines.

Table 3

The layout of drugs added to wells to test for the response of prostatic cancer cells to SMS 201-995 alone or SMS + EMP 3.1 or 12.5 ug/0.5 ml, combination.

A.   **Somatostatin 201-995:**   0, 0.002, 0.02, 0.2, 2, 20 ug/ml

B.

SMS 201-995 + EMP	0 + 0	0.002 + 3.1	0.02 + 3.1	0.2 + 3.1	2 + 3.1	20 ug/0.5 ml + 3.1 ug/0.5 ml

C.

SMS 201-995 + EMP	0 + 0	0.002 + 12.5	0.02 + 12.5	0.2 + 12.5	2 + 12.5	20 ug/0.5 ml + 12.5 ug/0.5 ml

D.

SMS 201-995 + EMP	0 + 0	0.002 + 25	0.02 + 25	0.2 + 25	2 + 25	20 ug/0.5 ml + 25 ug/0.5 ml

Section D

2.4 **Determination of whether the enhanced effect of Suramin by EMP was synergistic or additive.**

2.4.1 **Introduction**

Of all the 3 combination therapies analysed in Section 2.3.3 - 2.3.5, only EMP and Suramin had possible synergistic or additive activity [see results in Chapter 3, Section C, 3.3.1 - 3..3.6]. The differences in the effect of Suramin alone on LNCaP and PC3 compared to the effect of Suramin and EMP was statistically significant in all concentrations of EMP tested. The aims of the following experiments were:

(a)   to find out the concentrations of Suramin and EMP producing less than 5% growth inhibition on LNCaP, PC3 and DU145 cell lines.

(b)   to find out the effect of Suramin 3 ug/ml, EMP 1.5 ug/ml and combinations of Suramin 3 ug/0.5 ml and EMP 1.5 ug/0.5 ml on proliferation of prostate cancer cell lines PC3 and DU145 and (EMP 1.2 ug/0.5ml + Suramin 4.5 ug/0.5ml) on LNCaP cell line

2.4.2 **Materials/Methods**

In a series of preliminary experiments, the concentrations of Suramin and EMP producing less than 5% growth inhibition on CaP cell lines DU145, PC3 and LNCaP were determined. These were found to be Suramin 3.0 ug/ml and for EMP 1.5 ug/ml for PC3 and DU145; and EMP 1.2 ug/ml and Suramin 4.5 ug/ml for LNCaP. Cell lines were adapted to growth in RPMI and ITS and 2.5% FCS for PC3 and DU145 and LNCaP cell lines respectively. Cells were plated at a concentration of 2×10^4 cells/well on day 0. On day 1, the media were changed and media containing the above mentioned drug concentrations were added to the wells. The layout of the wells and drug concentrations added was as shown in Table 4.

The combination of EMP and Suramin at low concentrations produced marked inhibition of growth compared with either drug alone, hence, the results were read 48 hours after

Table 4

The layout of drug concentrations added to wells containing prostate cancer cell lines to test for additive or synergistic effects of EMP and Suramin

A	EMP	1.5	1.5	1.5 ug/ml
B	Suramin	3.0	3.0	3.0 ug/ml
C	EMP + Suramin	1.5 + 3.0	1.5 + 3.0	1.5 ug/0.5 ml + 3.0 ug/0.5 ml

Concentrations of Suramin (3.0 ug/ml) and EMP (1.5 ug/ml) producing less than 5% growth inhibition on prostate cancer cell lines were determined. The effect of combining these 2 drugs at the above concentrations on growth inhibition was then assessed. The above layout was for PC3 cell line. A similar layout was used for LNCaP cell line, although different concentrations of EMP and Suramin were used as explained in the text.

adding media containing the drugs. The experiments were repeated 3 times - one experiment per week.

The percentage of growth inhibition produced in each well by the drugs was calculated using the previously stated standard formula.

Section E

**2.5   Determination of whether the effect of the drug(s) is
            cytostatic or cytotoxic on LNCaP and PC3 prostate cancer cells**

The aim of this section of the study was to determine whether Suramin, SMS 201-995,
EMP or their combinations namely Suramin and SMS 201-995, Suramin and EMP, and
SMS 201-995 and EMP produce their anti-proliferative effects on LNCaP, and PC3
prostate cancer cell lines by producing cytotoxicity or cytostasis.

LNCaP cells were adapted to growth in 2.5% FCS and PC3 in RPMI and ITS as
described previously. LNCaP and PC3 cells were plated (2 x 10^4/cells/well in 24 well
plates). On day 1, the media were changed to media containing either 270 ug/ml of
Suramin, 25 ug/ml of EMP, 2 ug/ml of SMS 201-995, or a combination of Suramin 135
ug/0.5 ml and EMP 12.5 ug/0.5 ml - in combinations. There were also control wells
containing no drugs but LNCaP and PC3 cells in 2.5% FCS or RPMI and ITS
respectively. On day 3, media containing drugs were removed in half the number of
wells and in the remaining half of the wells, fresh media with appropriate drug
concentrations were added to the cells in the wells. This was to find out the ability of the
cell lines to re-initiate proliferation following the removal of Suramin, EMP, SMS
201-995 or EMP and Suramin combination. On days 3, 5, 6 and 7, cells surviving in each
well (control, those with drugs removed on the third day, and those with drugs added on
the third day) were estimated using ^3H thymidine incorporation assay.

Section F

2.6.1 **The effect of exogenous epidermal growth factor on the proliferation of LNCaP, PC3 and DU145 prostate cancer cell lines.**

2.6.1.1 **Introduction**

A postulated mechanism of action of Suramin is that it interrupts the interaction between growth factors and growth factor receptors on the cell membrane of prostate cancer cells. The aim of this subsection of experiments was to find out the effect of exogenous epidermal growth factor (EGF) on the proliferation of prostate cancer cell lines PC3, DU145 and LNCaP, and to find out if Suramin interferes with the growth stimulatory effect of EGF.

2.6.1.2 **Materials/Methods**

Androgen-independent prostate cancer cell lines PC3 and DU145 were adapted to growth in RPMI and ITS, while LNCaP was adapted to growth in 1.25% FCS. Cells were plated in 24 well plates at a concentration of 1×10^4 cells per well. Cells were allowed to initiate growth over the next 24 hours - as previously described. On Day 1, EGF at a concentration of 0, 1, 5, 12.5, 25 and 125 ng/ml was added to 6 different wells in quadriplicates in a 24 well plate. The media were replaced on days 3 and 5. The number of cells in each well was estimated using ^3H thymidine incorporation assay on day 6 using the method described above. The layout for the experiment was as shown in Table 5.

In the second set of experiments, each row of the 24 well plate had media containing various agents singularly or in combination added to the wells, as shown in Table 6. That is, the first row of wells in Table 24 had Suramin at a concentration of 270 ug/ml added, row 2 had EGF 5 ug/ml added, row 3 had a mixture of EGF 2.5 ug/0.5 ml and Suramin 135 ug/0.5 ml added and to the 4th row, no drugs were added, that is, the control wells.

Media were changed on days 3 and 5 and the results read on day 6.

Table 5

The Layout of EGF concentrations added to different wells containing either LNCaP, PC3 or DU145 cells.

ng /ml					
0	1	5	12.5	25	125

Cells per well = 2 x 10^4 cells/well. Total volume of media in each well = 1 ml.

Table 6

The layout of growth stimulatory or inhibitory effects of EGF, Suramin and their combination on LNCaP or PC3 cell lines.

Suramin 270 ug/ml						→
EGF 5 ng/ml						→
Suramin 135 ug/0.5 ml + EGF 2.5 ng/0.5 ml						→
No Drugs CONTROL						→

Suramin and EGF in above concentrations were added to 2 x 10^4 cells/well of either LNCaP or PC3 cells. Total volume of media in each well = 1 ml.

2.6.2 The effect of anti-epidermal growth factor receptor on proliferation of LNCaPand PC3 prostate cancer cell lines

2.6.2.1 Introduction

The postulated mechanism of action of Suramin is to interfere with the binding of growth factors onto their receptors on cell membranes of prostate cancer cells. If this was to be the correct explanation for the action of Suramin, then one would expect anti-epidermal growth factor receptors to produce inhibition of proliferation of prostate cancer cells like Suramin. The aim of this subsection of the study was therefore to find out the effect of anti-EGF receptor on the rate of proliferation of PC3, DU145, and LNCaP prostate cancer cell lines.

2.6.2.2 Materials/Methods

LNCaP and PC3 and DU145 prostate cancer cell lines were adapted to growth in 2.5% FCS and RPMI and ITS respectively as previously described. On day 0, cells were plated at a concentration of 2×10^4/cells per well. After 24 hours of plating cells, medium containing anti-EGF receptor in the following concentrations 0, 0.1, 1, 10, 100 ug/ml was added to cells in different wells. Media were changed on days 2 and 4. On day 6, the number of cells surviving in each well was determined by ^3H thymidine incorporation assay as previously described.

The percentage growth inhibition produced by varying concentrations of anti-EGF receptor was calculated using the previously stated standard formula.

2.7 Section G: Statistical Methods

Appropriate statistical methods or tests were used to find out differences between results or the correlation between corresponding observations. For details of biostatistical analysis, see Appendix B (page 260).

CHAPTER 3 :   **RESULTS OF EXPERIMENTS ON ESTABLISHED PROSTATE CANCER CELL LINES - LNCaP, PC3 and DU145**

Section A :   Results of determination of cell growth

Section B :   Results of experiments to establish clonal growth of PC3, DU145 and LNCaP

Section C :   Results of experiments to determine the effects of drug or drug combinations on prostate cancer cell lines PC3 and LNCaP

Section D :   Results of experiments to determine whether the enhanced effect of Suramin by EMP was synergistic or additive

Section E :   Results of experiments to determine whether the effect of the drugs on prostate cancer cells was due to cytostasis or cytotoxicity

Section F :   Results of experiments to determine the effects of epidermal growth factor and anti-epidermal growth factor receptors on proliferation of LNCaP, PC3 and DU145 prostate cancer cell lines.

RESULTS OF EXPERIMENTS ON ESTABLISHED PROSTATE CANCER CELL LINES - LNCaP, PC3 AND DU145

3.1 Results of determination of cell growth using:

3.1.1. Haemocytometer

Table 7 shows the result of counting viable cells using the haemocytometer. As would be expected the cells in culture continued to proliferate in a linear fashion being 2.7×10^4 cells/well on day 2 and 8.7×10^4 cells on day 6.

Because the haemocytometer takes longer to use to count viable cells, we explored the possibility of using the Coulter counter used in our department of haematology to count the prostate cells. However, there was very poor correlation between the actual cell number in culture as determined by the haemocytometer and that given by the Coulter counter as shown in Table 7.

3.1.2 MTT growth determination assay

Carmichael et al. (1987), and Mossman, (1983), have shown that there are differences in absorbency for comparable cell numbers when different cells are tested, hence the MTT conversion to MTT formazan is cell type specific. Consequently it was necessary to produce conversion curves for each cell type studied. The conversion curves for LNCaP and PC3 cells are shown in Figs 19 and 20, respectively. The 2 curves show very good correlation (correlation square: $r^2 = 0.97$) between the number of cells in each well as determined by the haemocytometer and the absorbance of the wells using the MTT method as shown in Tables 8, 9, and Figs 19 and 20.

3.1.3 ^{3}H thymidine incorporation assay

Table 10 and Fig 21 show the good correlation ($r^2 = 0.8$) between the number of cells in a well as determined by the haemocytometer

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Table 7

Cell counting using the haemocytometer compared with automated Coulter counter (B2 Dickensen Laboratory California, Model (B7167-1992).

No of cells (per well x 10⁴) using haemocytometer (LNCaP cell line)					No of cells (per well x 10⁴) using Coulter counter				
Days in Culture	Expt 1	Expt 2	Expt 3	Mean ± SEM	Expt 1	Expt 2	Expt 3	Mean ±SEM	P Value
0	2	2	2	2±0	0.5	1.0	1.2	0.9±0.2	P = 0.03
2	2.8	2.7	2.6	2.7±0.06	1.4	1.1	1.6	1.4±0.07	P = 0.007
4	4.4	4.7	4.5	4.54±0.09	2.1	3.1	1.6	2.3±0.4	P = 0.03
6	8.3	8.8	8.9	8.7±0.2	3.1	2.9	2.7	2.9±0.1	P = 0.003

P = 0.003 - statistically significant (T - test).

There was statistical significant difference between cells counted on day 6 using the haemocytometer and the automated Coulter counter. The wells contained the same number of cells.

Table 8

Correlation between 2 methods of assessing the number of LNCaP cells per well in culture: MTT assay (based on differences in optical density) versus the haemocytometer.

No of cells per well using haemocytometer x 10^4				Differences in Optical density (MTT Assay) at 570 nm and 590 nm			
Days in Culture	Expt 1	Expt 2	Expt 3	Mean ± SD	Expt 1	Expt 2	Expt 3	Mean ± SD
2	0.18	0.18	0.18	0.18±0	0.06	0.07	0.05	0.06±0.01
4	0.24	0.26	0.22	0.24 ± 0.02	0.16	0.12	0.14	0.14±0.02
6	0.44	0.44	0.46	0.45±0.01	0.20	0.21	0.16	0.19±0.02
8	1.3	1.2	0.89	1.1±0.03	0.30	0.26	0.28	0.28±0.03
10	1.44	1.46	1.39	1.43±0.04	0.32	0.34	0.30	0.32±0.01
12	2.45	2.47	2.44	2.45±0.02	0.39	0.37	0.38	0.38±0.02

The same number of cells [0.2 x 10^4 cells/ well] were plated on day 0 for both sets of experiments.

Figures under experimental sections represent total cell count in wells.

Correlation square: r^2 = 0.98, indicating a good correlation between the number of cells in each well as determined by the haemocytometer and in another corresponding well using the MTT method.

nm = unit of measurement of wavelength
570 nm - measurement of absorbance of test solution

590 nm - blank background wavelength at which reading of optical density was measured

Fig 19

Graph showing correlation between the MTT assay and the haemocytometer used to assess the number of LNCaP cells/well in culture.
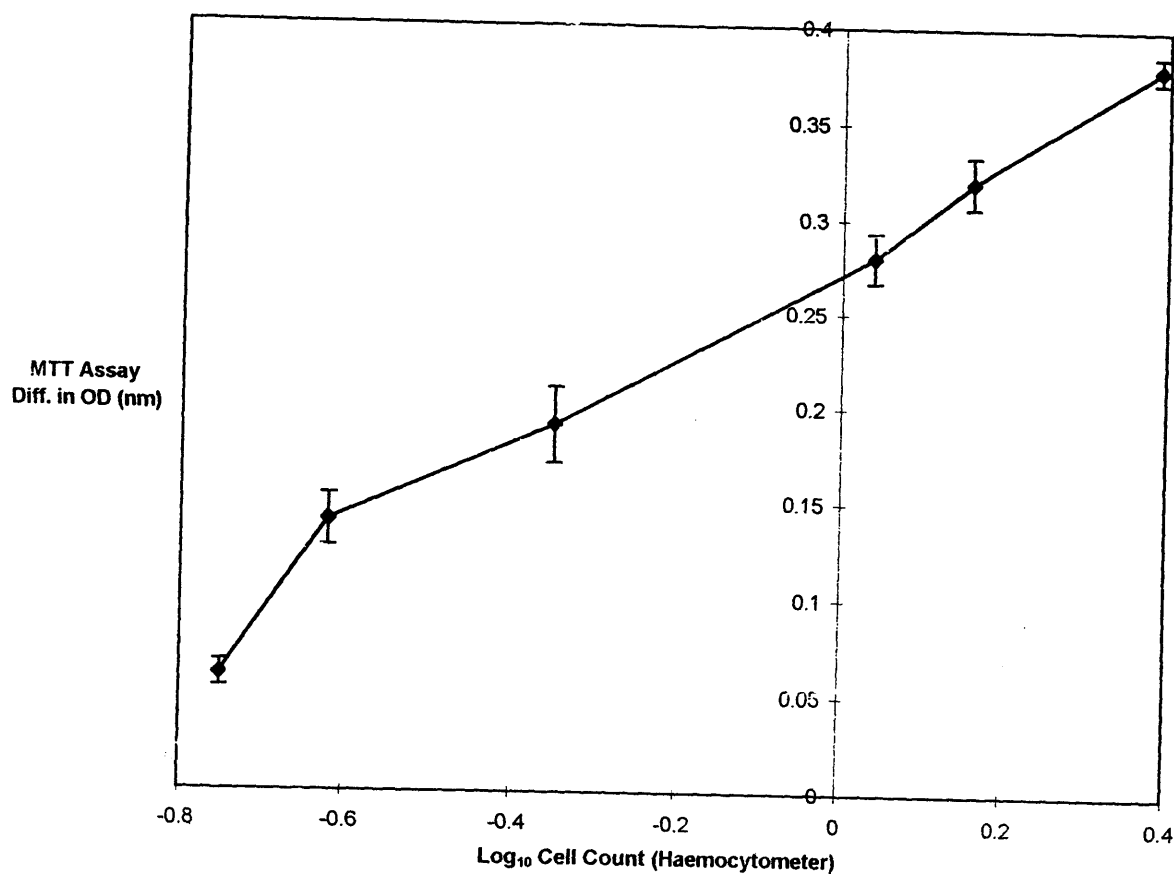
$r^2 = 0.98$ indicating good correlation between the 2 methods of assessing the number of cells per well.

The MTT assay uses differences in optical density (O.D.) of test solution at 570 nm and of background at wavelength of 590 nm

Table 9

Correlation between 2 methods of assessing the number of PC3 cells per well in culture: MTT assay (based on differences in optical density) versus the haemocytometer..

No of cells per well using haemocytometer x 10^4				Differences in Optical density (MTT Assay) at 570 nm and 590 nm			
Days in Culture	Expt 1	Expt 2	Expt 3	Mean ± SD	Expt 1	Expt 2	Expt 3	Mean ± SD
2	0.20	0.25	0.20	0.20±0	0.060	0.059	0.059	0.059±0.01
4	0.44	0.44	0.46	0.45±0.01	0.085	0.087	0.083	0.085±0.02
6	0.75	0.76	0.77	0.75±0.01	0.13	0.14	0.15	0.13±0.01
8	1.5	1.6	1.4	1.5±0.01	0.21	0.22	0.20	0.21±0.02
10	2.66	2.7	2.6	2.6±0.06	0.27	0.27	0.26	0.27±0.01
12	3.6	3.5	3.7	3.6±0.01	0.29	0.29	0.28	0.29±0.03

The same number of cells were plated (0.2 x 10^4 cells/well) on day 0 for both sets of experiments.

Correlation square = r^2 = 0.97 - indicating a good correlation between number of cells in each well as determined by the haemocytometer and the absorbance of the cells in another corresponding well using the MTT method.

Fig 20

Graph showing correlation between the MTT assay and the haemocytometer used to assess the number of PC3 cells/well in culture.

Log_{10} Cell Count (Haemocytometer)
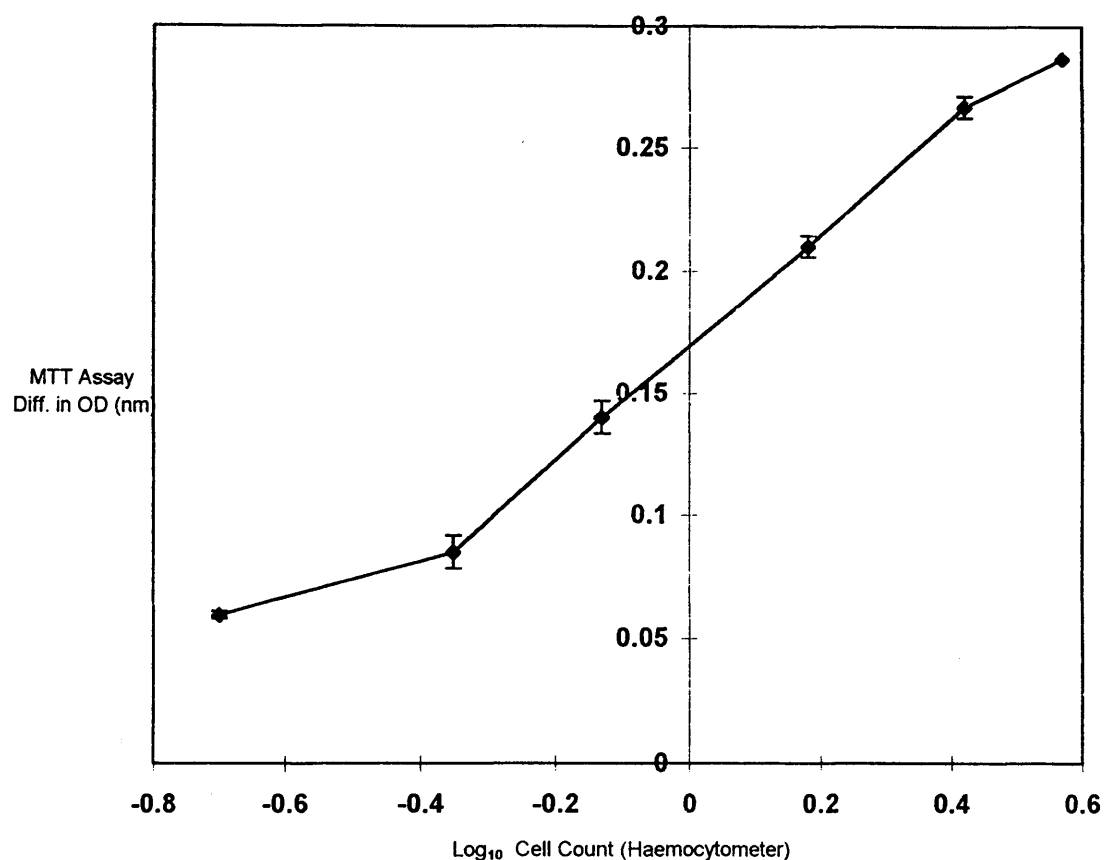
0.2 x 10^4 cells/well were plated on day 0 for both sets of experiments.

r^2 = 0.97 indicating good correlation between the 2 methods of assessing the number of cells per well.

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Table 10

Correlation between 2 methods of assessing the number of LNCaP cells in culture per well: haemocytometer versus ^3H thymidine incorporation assay.

Cells in Culture (Haemocytometer) x 10^4				Incorporation of ^3H thymidine (DPM) per well			
Expt 1	Expt 2	Expt 3	Mean ± SEM	Expt 1	Expt 2	Expt 3	Mean ± SEM
2.1	1.92	1.98	2±0.05	2007	1814	1681	1834±164
2.8	2.65	2.6	2.7±0.06	3481	3500	3669	3600±67
4.4	4.7	4.50	4.5±0.06	5000	5000	4810	4900±63
8.3	8.7	8.9	8.7±0.09	6000	4500	5621	5374±450
16.1	17.2	13.8	15.7±0.01	6800	7800	7900	7500±351

2×10^4 cells/well plated on day 0 for both sets of experiments.

Cells were counted on days 2, 4, 6 , 8, 10 after plating.
$r^2 = 0.85$ - good correlation existed between the number of cells estimated using the haemocytometer and ^3H thymidine incorporation.

DPM = disintegration per minute

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Fig 21

Graph showing correlation between the number of LNCaP cells in culture using the haemocytometer and ^3H thymidine incorporation.
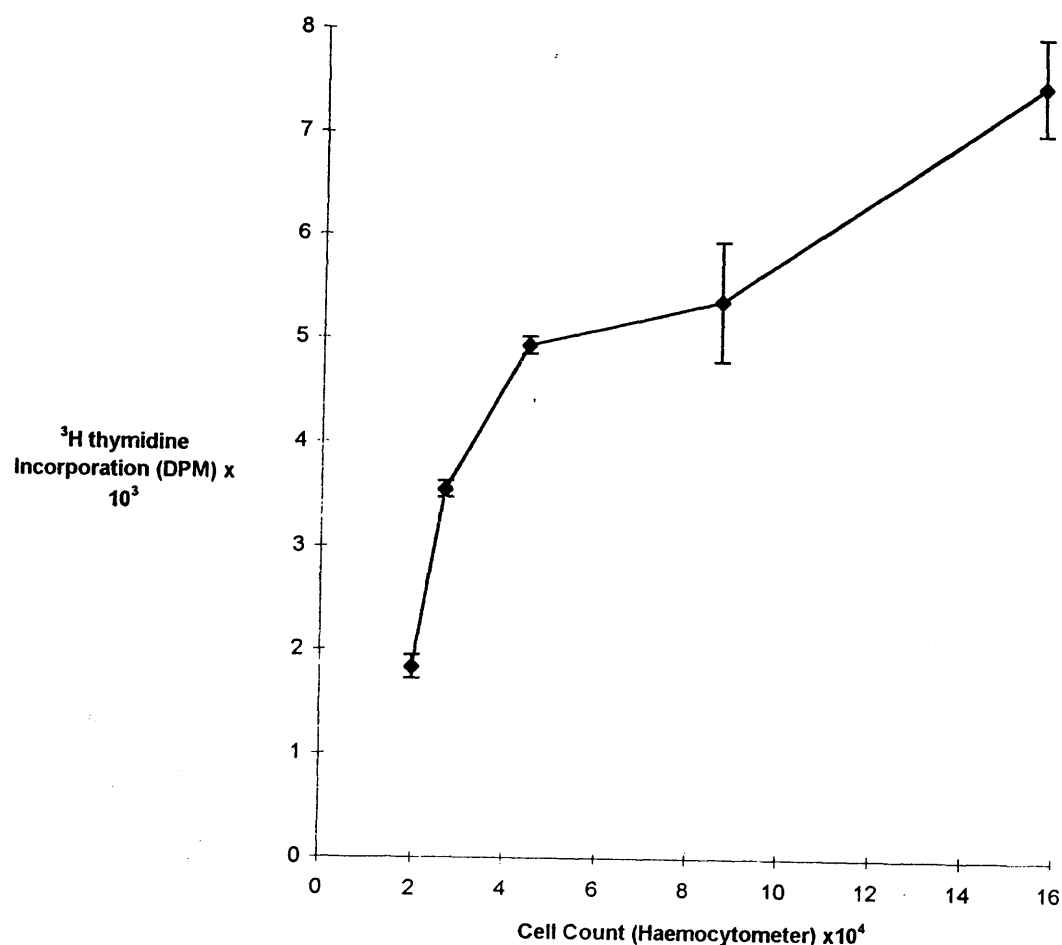
2 x 10⁴ cells/well were plated on day 0 for both sets of experiments. Cells in each well counted on days 2, 4, 6, 8, and 10 after plating.

r^2 = 0.85 = good correlation established between the number of cells in culture estimated using the haemocytometer and ^3H thymidine incorporation.

and the ^{3}H thymidine incorporation assay reading of disintegration per minute as produced by the beta counter.

3.1.4 Measurement of prostatic acid phosphatase activity in the supernatant of cultured cells versus the number of cells in culture as measured by ^{3}H thymidine incorporation.

Table 11 and Fig 22 show there was good correlation ($r^2 = 0.96$) between the prostatic acid phosphatase activity in the supernatant of cultured cells and the total number of cells in culture as determined by ^{3}H thymidine incorporation assay.

Section B

3.2.1 Results of experiment to establish clonal growth of PC3, DU145 and LNCaP

Cells plated at 2×10^4 cells/well in 24 well plates continued to proliferate for the next 8 days (Fig 23). After 8 days, the wells became overgrown and cell growth became haphazard (Table 12).

Table 11

Correlation between prostatic acid phosphatase activity in the supernatant of LNCaP cells in culture and the number of cells in culture as determined by ^3H thymidine incorporation assay.

Cells per well (DPM) by ^3H thymidine				PAP IU/L			
Expt 1	Expt 2	Expt 3	Mean ± SEM	Expt 1	Expt 2	Expt 3	Mean ± SEM
2007	1814	1681	1800±95	1.4	1.1	1.1	1.2±0.1
3481	3500	3692	3600±67	1.4	1.9	2.1	1.8±0.2
5000	5000	4810	4900±63	4.1	4.1	2.9	3.7±0.4
6000	4500	5621	5700±450	4.8	2.9	4.9	4.2±0.7
6800	7800	7900	7500±351	5.8	5.9	5.6	5.8±0.1

Cells were counted on days 2, 4, 6, 8 and 10 after culture.

There was a good correlation (r^2 = 0.96) between prostatic acid phosphatase (PAP) activity in the supernatant of LNCaP cells and the number of cells in culture as determined by ^3H thymidine.

Fig 22

Correlation between prostatic acid phosphatase (PAP) activity in the supernatant of LNCaP cells and the number of cells in culture as determined by ^{3}H thymidine incorporation.
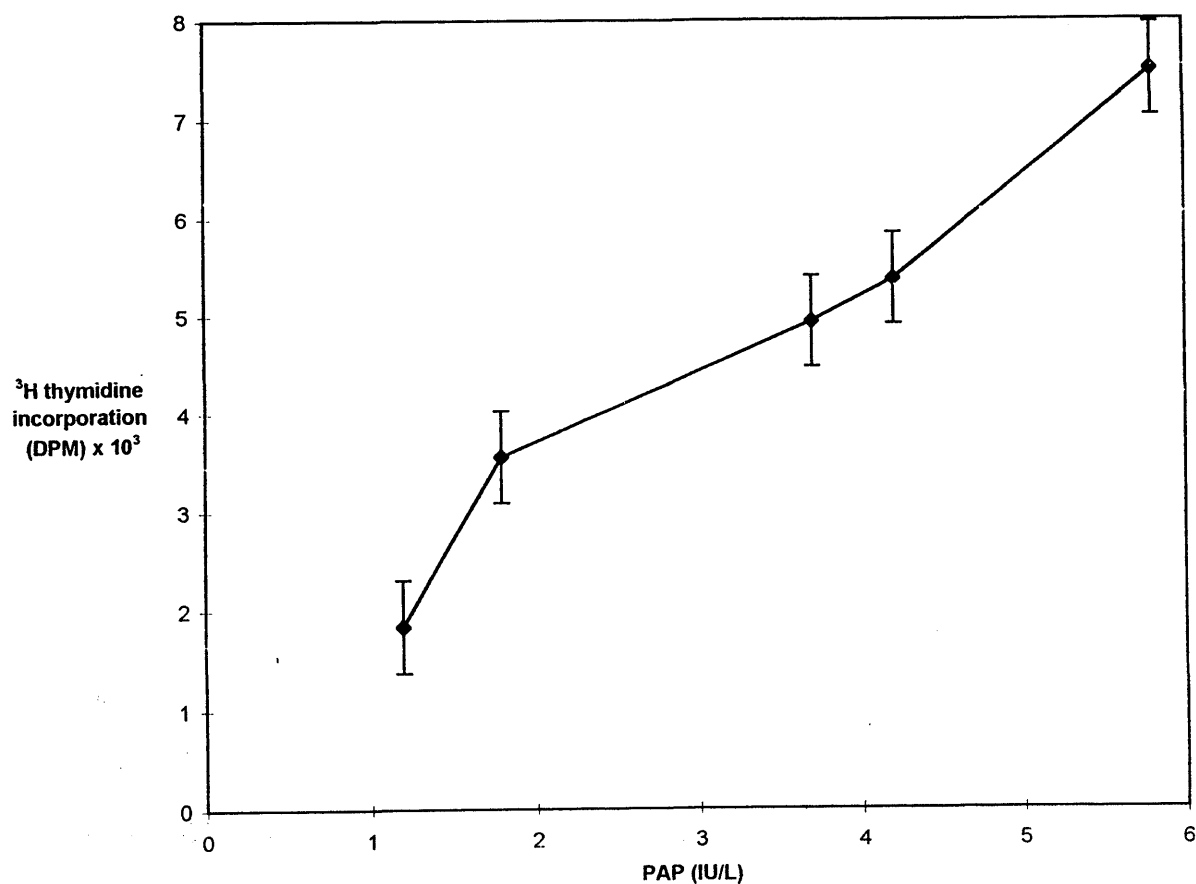
Results represent the mean of 3 experiments.

DPM = Disintegration per minute = number of cells determined by ^{3}H thymidine incorporation assay.

r^2 = 0.96, indicating a good correlation between the number of LNCaP cells in culture as determined by ^{3}H thymidine incorporation and PAP activity in supernatant of LNCaP cells.

Table 12

Serial growth of LNCaP and PC3 cell lines from Day 0 to Day 8 - as determined by ^{3}H thymidine incorporation assay.

	No of Cells (DPM/well) LNCaP				No of Cells (DPM/well) PC3			
Day	Expt 1	Expt 2	Expt 3	Mean ± SEM	Expt 1	Expt 2	Expt 3	Mean ± SEM
0	1600	1695	1634	1640±28	1800	1900	1860	1841±29
2	1814	1681	2007	1884±95	1700	1900	1750	1800±60
4	3462	3500	3692	3581±71	3000	2900	2950	2931±29
6	4000	5000	4810	4912±307	4000	4211	3711	3924±145
8	6000	4500	5621	5686±450	4567	4800	4700	4684±67
10	Cells overgrown in well				Cells overgrown in well			

DPM   =   Disintegration per minute

Mean of 3 experiments (one experiment per week)

Cells plated at a concentration of 2 x 10^4 cells per well on day 0. Cells continued to proliferate in the wells for the next 8 days. By day 10, the cells became overgrown in the wells and counting of cells became impossible. Cells were fed with fresh media on days 2, 4, 6, 8 and 10.

Fig 23

Growth curves for prostate cancer cell lines, LNCaP (passage no. 36) and PC3 (passage no. 53).
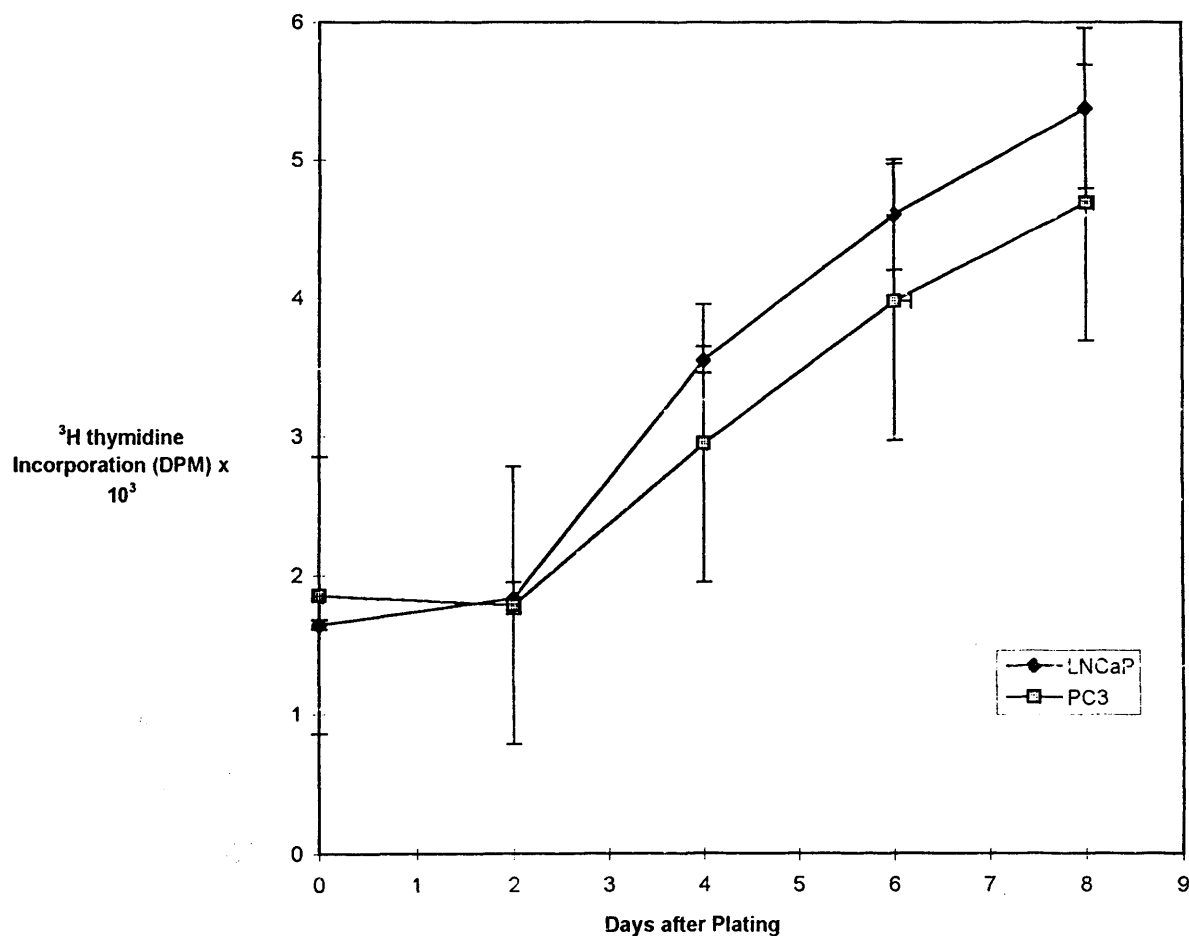
Cells plated at an initial concentration of 2×10^4 cells per well on day 0, continued to proliferate over the next 8 days.

Results represent the mean of 3 experiments.

Section C

3.3   **Results of experiments to determine the effects of drug or drug combinations on proliferation of prostate cancer cells PC3 and LNCaP**

3.3.1   **Suramin or Somatostatin 201-995**

Suramin and Somatostatin 201-995 inhibited the proliferation of LNCaP and PC3 in a dose dependent fashion as shown in Tables 13 and 14 and Figs 24 and 25 respectively. Compared to Suramin free control, LNCaP and PC3 cell growth was significantly inhibited by Suramin (270 ug/ml) by 50.1% and 44.7% respectively, while cell growth was similarly significantly inhibited by SMS (0.2 ug/ml) by 44% and 37% respectively, after 6 days of exposure to the drugs. SMS 201-995 was more effective at inhibiting the growth of PC3 at lower doses being 19.3% for PC3 and 6% for LNCaP at a SMS 201-995 concentration of 0.002 ug/ml. Similarly, Suramin was more effective at inhibiting the growth of PC3 at lower doses being 28.8% for PC3 and 12.5% for LNCaP at a Suramin concentration of 30 ug/ml.

3.3.2   **Estramustine phosphate**

The results are as shown in Table 15 and Fig 26. EMP, like Suramin and SMS 201-995, produced dose-dependent growth inhibition of the 2 cell lines. EMP was more effective at inhibiting the proliferation of PC3 cells compared to LNCaP cells as shown in Table 15.

3.3.3   **Suramin and SMS 201-995 combination**

The results are shown in Table 16 and 17 (for the purpose of clarity only highlights of the results have been shown), however, the entire raw data appear in the appendix section. The experiments were carried out in triplicate, and the figures shown represent the mean of 3 experiments.

Table 13

The inhibition of proliferation of LNCaP and PC3 cell lines by Suramin.

% Inhibition LNCaP				% Inhibition PC3			
Dose of Suramin ug/ml	Expt 1	Expt 2	Expt 3	Mean ± SEM	Expt 1	Expt 2	Expt 3	Mean Expt ± SEM
0 (control)	0	0	0	0±0*	0	0	0	0±0**
10	4.5	5.0	7.0	5.5±0.8	29	10	16	18.3±5.6
30	9	15	13.4	12.5±1.8	38	16.4	32.1	28.8±6.5
90	47.8	43.7	49.7	47.1±1.8	46.9	25.9	45.2	39.3±6.8
270	49	51.1	52.3	50.1±1.0*	48.7	35	50.3	**44.7±4.9
810	58	62	61	60.3±1.2	69.1	91	75.1	78.4±6.5

One set of experiments was carried out per week. See appendix for raw data

p* = 0.001 (T - test) Suramin at 270 ug/ml compared to control LNCaP
p** = 0.001 (T - test) Suramin at 270 ug/ml compared to control PC3
P value: statistically significant.

Suramin inhibited proliferation of LNCaP and PC3 prostate cell lines in a dose dependent fashion. Media containing appropriate fresh concentrations of Suramin were added to the cells on days 3 and 5. Final results were read on day 6 using ^3H thymidine incorporation assay.

Fig 24

The inhibition of proliferation of PC3 and LNCaP cells by Suramin.
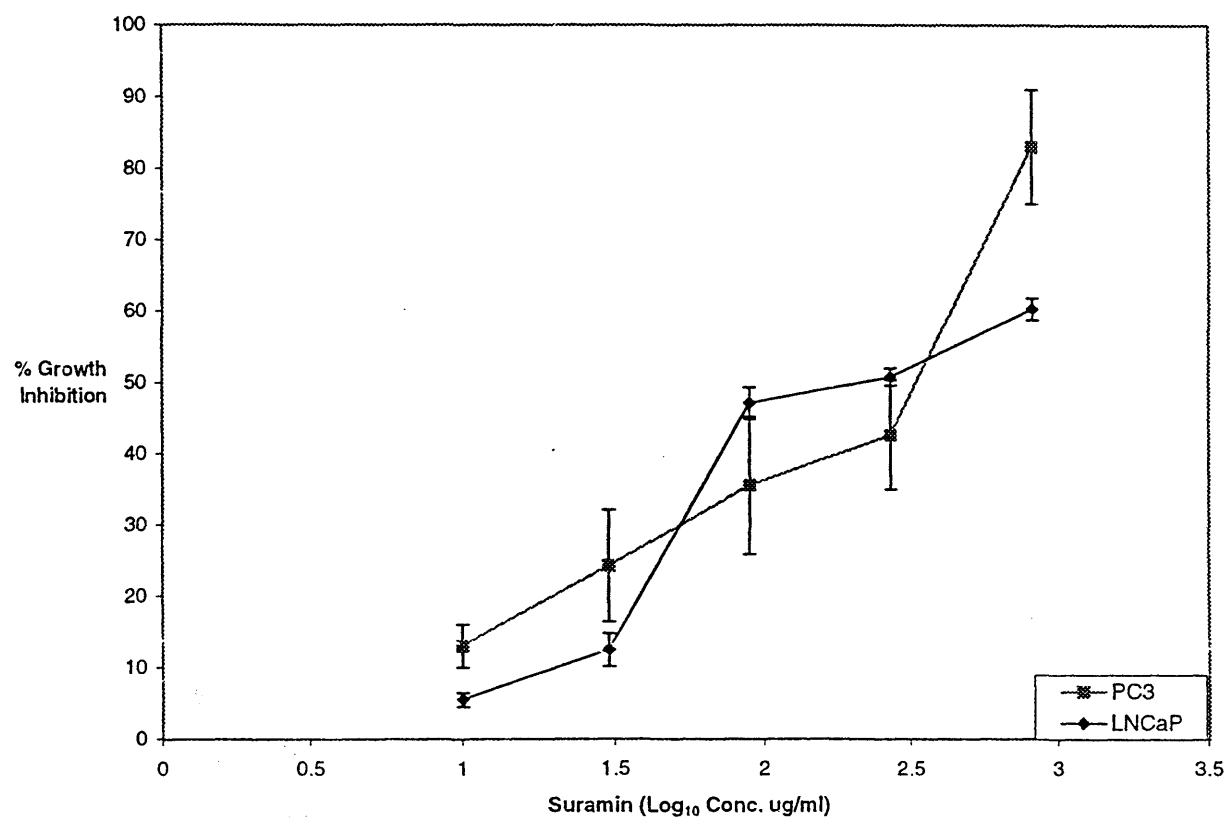
Results represent the mean of 3 experiments.

Suramin inhibited the growth of LNCaP and PC3 cell lines in a dose dependent manner

Table 14

The inhibition of proliferation of LNCaP and PC3 cell lines by somatostatin 201-995.

	% Inhibition LNCaP				% Inhibition PC3			
Dose of SMS 201-995 ug/ml	Expt 1st	Expt 2nd	Expt 3rd	Mean ± SEM	Expt 1st	Expt 2nd	Expt 3rd	Mean ± SEM
0	0	0	0	0±0*	0	0	0	0±0**
0.002	10	2	6	6±2.3	11	28	19	19.3±4.9
0.02	14	3	6	7.7±3.28	18	37	22	25.8±5.8
0.2	58	34	40	44*±7.2	31	48	32	37**±5.5
2.0	78	91	87	85±3.8	46	59	46	50.3±4.3

p*   = 0.001 (T - test) SMS at 0.2 ug/ml compared to control (LNCaP)

p** = 0.001 (T - test)   SMS at 0.2 ug/ml compared to control (PC3)

See appendix for raw data

Somatostatin 201-995 inhibited proliferation of LNCaP and PC3 prostate cancer cell lines in a dose dependent fashion. Media containing fresh concentration of SMS 201-995 were added to the cells on days 3 and 5. Final results were read on day 6 using ^{3}H thymidine incorporation assay.

Fig 25

The Inhibition of proliferation of LNCaP and PC3 cell lines by Somatostatin 201-995.
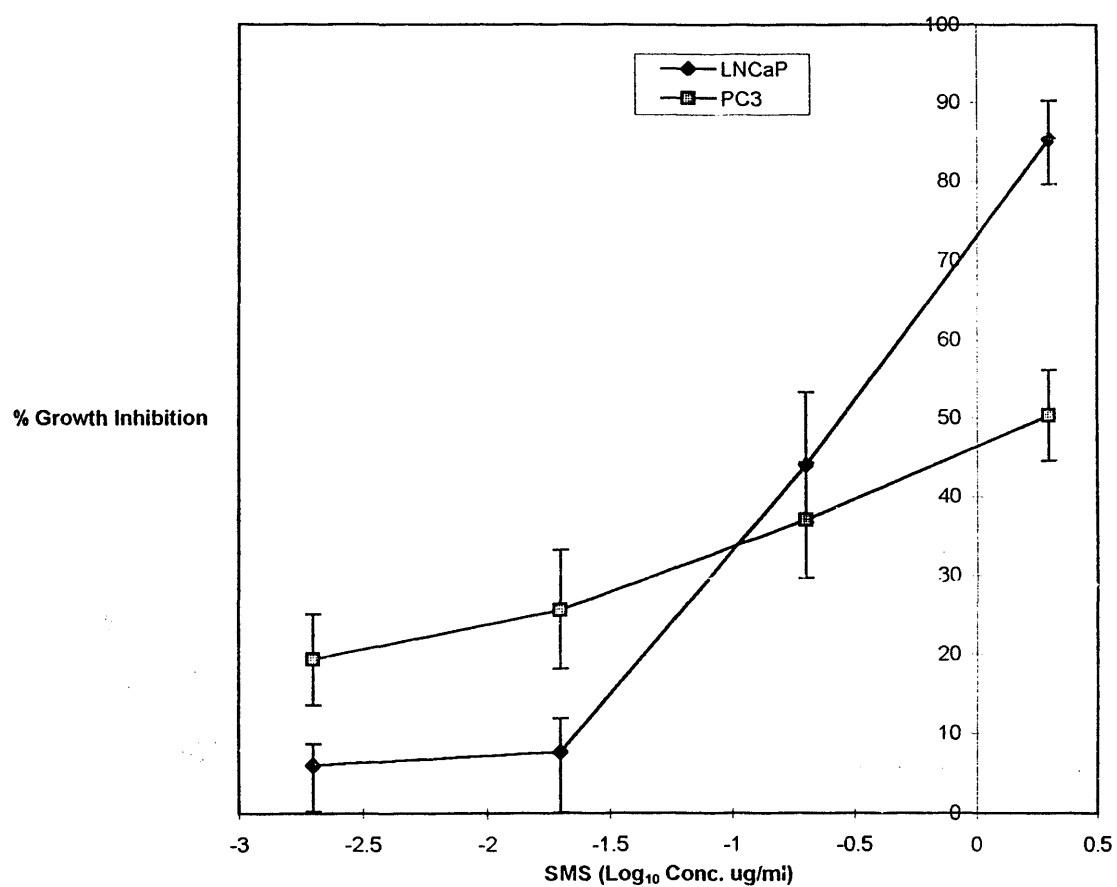
Results represent the mean of 3 experiments. SMS 201-995 inhibited the growth of LNCaP and PC3 prostate cancer cell lines in a dose dependent manner.

Table 15

The inhibition of proliferation of LNCaP and PC3 cells by EMP.

	% Inhibition LNCaP				% Inhibition PC3			
Dose of EMP ug/ml	Expt 1st	Expt 2nd	Expt 3rd	Mean ± SEM	Expt 1st	Expt 2nd	Expt 3rd	Mean ± SEM
0 (control)	0	0	0	0±0*	0	0	0	0±0**
3.1	30	32	30	31±0.9	28	33	11	24±1.2
6.25	51	53	51	52±0.8	42	48	36	42±0.8
12.5	86	85	89	87*±0.8	58	61	60	60**±0.3
25	98.7	96	97	97±0.3	69	65	77	70±0.5
50	99.1	98	98	98±0.4	79	75	83	79±2.3

p*   = 0.05 (T - test) EMP (12.5 ug/ml) compared with control for LNCaP

P** = 0.05 (T-test) EMP (12.5μg/ml) compared with control for PC3

EMP produced dose dependent growth inhibition of LNCaP and PC3 prostate cancer cell lines. Media containing fresh EMP were added to the wells on days 3 and 5. Final results were read on day 6 using ^{3}H thymidine incorporation assay. See appendix for raw data.

Fig 26

The inhibition of proliferation of LNCaP and PC3 cell by EMP.
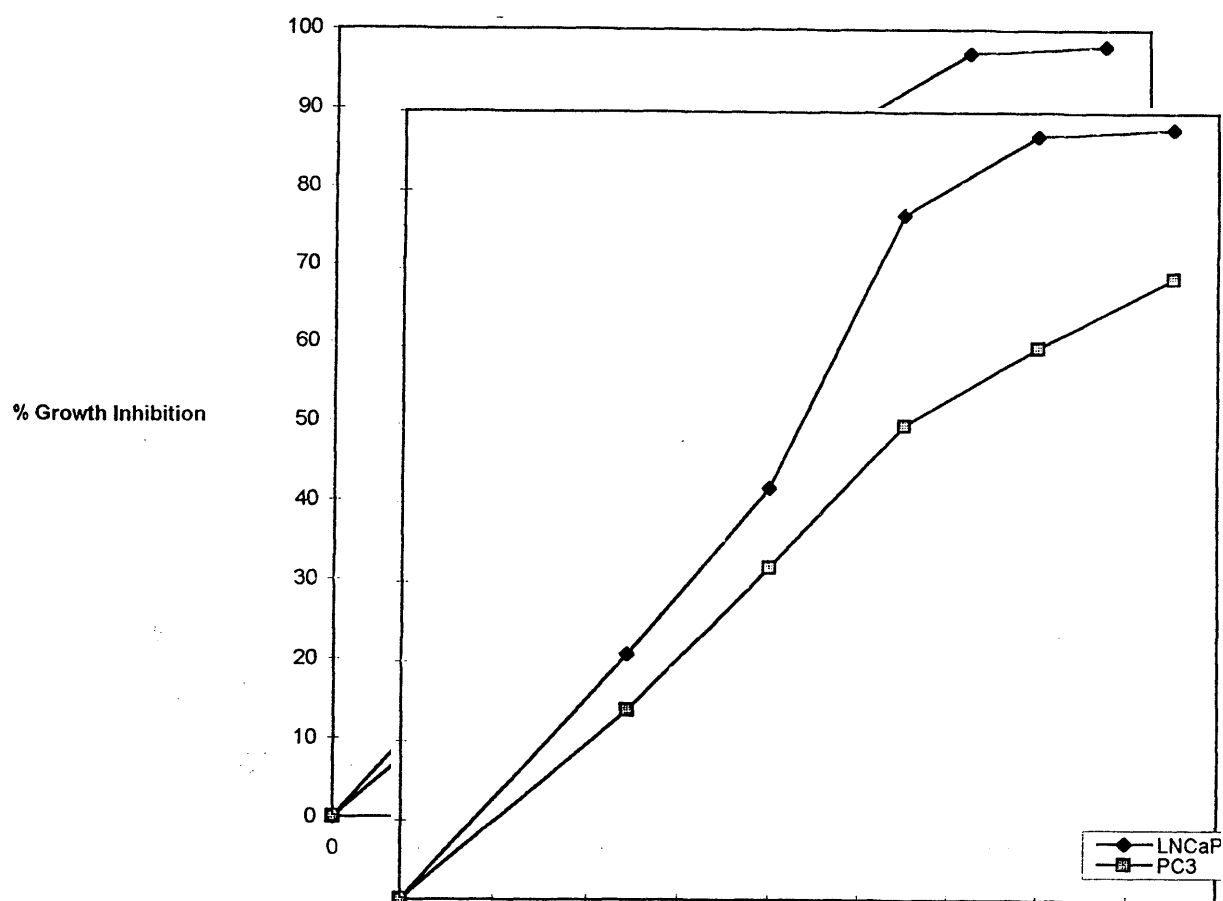
Results represent the mean of 3 experiments.

EMP produced dose dependent growth inhibition of LNCaP and PC3 prostate cancer cell lines.

Table 16

The effect of Suramin (0-810 ug/ml) or Suramin (0-405 ug/0.5ml) and Somatostatin (0.01 ug/ml) combination on cellular proliferation of prostate cancer cell lines PC3 and LNCaP.

% Inhibition	Sur 10 ug/ml	Sur 5 + SMS 0.01 ug/0.5ml	Sur 90 ug/ml	Sur 45 + SMS 0.01 ug/0.5 ml	Sur 270 ug/ml	Sur 135 ug/0.5 ml + SMS 0.01 ug/0.5 ml
LNCaP	5.6	8.8	49.2	58.5	54.1*	59.7*
PC3	20.1	22	40.1	41.2	45.0**	46.0**

* $P < 0.2$ NS (T - test) ** $P < 0.8$ NS (T - test)   NS = not significant

Sur = Suramin   SMS = Somatostatin 201-995.

See appendix for raw data including S.E.M. differences

The addition of SMS 0.02 ug/ml to Suramin 0-810 ug/ml did not result in statistically significant growth inhibition of LNCaP or PC3 prostate cancer cell lines.

Table 17

The effect of Suramin (0-810 ug/ml) or Suramin (0-405 ug/0.5 ml) and Somatostatin 201-995 (1 ug/ 0.5 ml) combination on cellular proliferation of prostate cancer cell lines PC3 and LNCaP.

% Inhibition	Sur 10 ug/ml	Sur 5 + SMS 1 ug/0.5 ml	Sur 90 ug/ml	Sur 45 + SMS 1 ug/0.5 ml	Sur 270 ug/ml	Sur 135 ug/0.5 ml + SMS 1 ug/0.5 ml
LNCaP	9.12	19.1	48.0	58.0	55.1*	68.2
PC3	19.4	15.5	43.7	38.2	44.4**	48.3

* $P = 0.2$ - NS (T - test) ** $P = 0.35$ - NS (T - test) NS = not significant

See appendix for raw data including changes in S.E.M. The addition of SMS 2 ug/ml to Suramin 0-810 ug/ml did not result in statistically signifcant growth inhibition of LNCaP or PC3 prostate cancer cell lines. % inhibition shown on Tables 16 and 17 represent the mean of triplicate experiments.

The addition of SMS 201-995 (0.02 ug/ml or 2.0 ug/ml) to Suramin (0-810 ug/ml) did not result in a significantly increased inhibition of cell growth of either PC3 or LNCaP. Suramin alone at a concentration of 270 ug/ml produced 54.1% and 45% growth inhibition on LNCaP and PC3 respectively. The addition of 0.02 ug/ml of SMS 201-995 to Suramin 270 ug/ml, produced 59.7 and 46.0% growth inhibition on LNCaP and PC3 cell lines respectively, a difference that was not statistically significant. The use of higher concentrations of SMS 201-995 (2.0 ug/ml), did not result in any significant increase in inhibition of LNCaP or PC3 cell lines.

3.3.4   Suramin and EMP combination

The results of the response of the LNCaP and PC3 cells to Suramin or Suramin + EMP combination are as shown on Tables 18, 19, 20 and 21 and Fig 26.

Suramin alone at 10 ug/ml produced 14% growth inhibition on PC3 cell line, but combining this with 12.5 ug/0.5 ml of EMP, the percentage growth inhibition rose to 64% which was statistically significant, $P < 0.01$ (analysis of variance with Fischer's correction) (Fig 27).

EMP 12.5 ug/ml produced 56% growth inhibition on PC3 cell line. For purposes of clarity and possible therapeutic implications, only parts of the experiments where serum levels that can be achieved in vivo have been highlighted in Tables 19 and 20. However, the raw data (see appendix) contain all the details of the results. As Fig 27 show Suramin and EMP combination produced growth inhibition of PC3 cell lines in very small concentrations.

EMP at a concentration of 25 ug/ml produced 97% and 70% growth inhibition on LNCaP and PC3 respectively. However, with the addition of EMP at a concentration of 12.5 ug/0.5ml to Suramin (0-405 ug/0.5ml), even though it resulted in improved growth inhibition rate (Table 21), the differences were not statistically significant from the effect of adding 6.25 ug/0.5ml of EMP (Table 20).

Table 18

The response of cells to Suramin (0-810)ug/ml or Suramin (0-810)ug/0.5ml +
EMP (3.1 or + 12.5) ug/0.5ml. Format of drug(s) added to culture wells.

Row	Drugs	Concentration						
A	Suramin	0	10	30	90	270	810	ug/ml
B.	Suramin + EMP	0 + 0	10 + 3.1	30 + 3.1	90 + 3.1	270 + 3.1	810 + 3.1	ug/0.5 ml ug/0.5 ml
C.	Suramin + EMP	0 + 0	10 + 12.5	30 + 12.5	90 + 12.5	270 + 12.5	810 + 12.5	ug/0.5 ml ug/0.5 ml

Throughout this study, for experiments where the effect of one drug on
cells in a well was being assessed, the concentration of the test drugs has
been expressed as /ml, e.g. as indicated in row A above.

For those experiments where the effect of 2 drugs in combination on cells
in a well was being assessed, the final concentrations of the test drugs
have been expressed as /0.5ml as shown in rows B and C above, such that
the total volume of medium per well for all experiments (test and control
wells) was 1 ml.

Table 19

The effect of Suramin (0-270 ug/ml) or Suramin (0- 135 ug/0.5 ml) + EMP (1.5 ug/0.5 ml) combination on LNCaP and PC3 cells

% Growth Inhibition	Sur 10 ug/ml	Sur 5 + EMP 1.5 ug/0.5 ml	Sur 90 ug/ml	Sur 45 + EMP 1.5 ug/0.5 ml	Sur 270 ug/ml	Sur 135 ug/0.5 ml + EMP 1.5 ug/0.5 ml	EMP 3.1 ug/ml
LNCaP	33	30	59*	60.2*	76	82.1	31
PC3	14+	43.1+	51++	71.2++	63	85.1	24

"Control" wells contained Suramin alone e.g. 10 ug/ml i.e. double the concentration in the test well which contained half the concentration of Suramin in "Control" well e.g. 5 ug/0.5 ml + EMP (1.5 ug/0.5 ml). Total volume of media in each well ("control" or test) was 1 ml.

Results represent the mean of 3 experiments, each experiment was performed weekly.

+   P < 0.05   (significant) (Analysis of Variance)   *p < 0.11 (not significant)
++   P < 0.06   (not significant)

See appendix for raw data.

EMP (1.5 ug/0.5 ml) potentiated the effect of Suramin at low concentrations on prostate cancer cell line PC3, but has no significant effect on LNCaP.

Table 20

The effect of Suramin (0-270 ug/ml) or Suramin (0-135 ug/0.5 ml)+ EMP (6.25 ug/0.5 ml) combination on LNCaP and PC3 cells

% Growth Inhibiti on	Sur 10 ug/ml	Sur 5 + EMP 6.25 ug/0.5 ml	Sur 90 ug/ml	Sur 45 + EMP 6.25 ug/0.5 ml	Sur 270 ug/ml	Sur 135 ug/0.5 ml + EMP 6.25 ug/0.5 ml	EMP 12.5 ug/ml
LNCaP	33*	75*	59	89	76	93	48
PC3	14**	64**	49.0	86	63	92	56

Results represent the mean of 3 experiments.

*   P < 0.01   (significant)
** P < 0.01   (significant)

(Analysis of Variance)

See appendix for raw data

EMP (6.25 ug/0.5 ml) potentiated the effect of Suramin at low concentration on prostate cancer cell lines LNCaP and PC3, but the differences were not statistically significant (p < 0.11) compared to when EMP 1.5 ug/0.5 ml was used (compare results in Table 19 with Table 20).

Table 21

The effect of Suramin (0-270 ug/ml) or Suramin (0-135 ug/0.5 ml) + EMP (12.5 ug/0.5 ml) combination on LNCaP and PC3 cell lines.

% Growth Inhibition	Sur 10 ug/ml	Sur 5 + EMP 12.5 ug/0.5 ml	Sur 90 ug/ml	Sur 45 + EMP 12.5 ug/0.5 ml	Sur 270 ug/ml	Sur 135 ug/0.5 ml + EMP 12.5 ug/0.5 ml	EMP 25 ug/ml
LNCaP	33*	78*	59	91	76	92	97
PC3	14**	65**	51	88	63	91	70

Results represent the mean of 3 separate experiments. One experiment per week.

* P < 0.01 (significant) - Analysis of Variance
** P < 0.001 (significant)

See appendix for raw data.

EMP (12.5 ug/0.5 ml) potentiated the effect of Suramin at low concentration on prostate cancer cell lines LNCaP and PC3, but the difference was not statistically significant for both cell lines compared to when EMP 6.25/0.5 ml was used (compare results in Table 20 with Table 21).
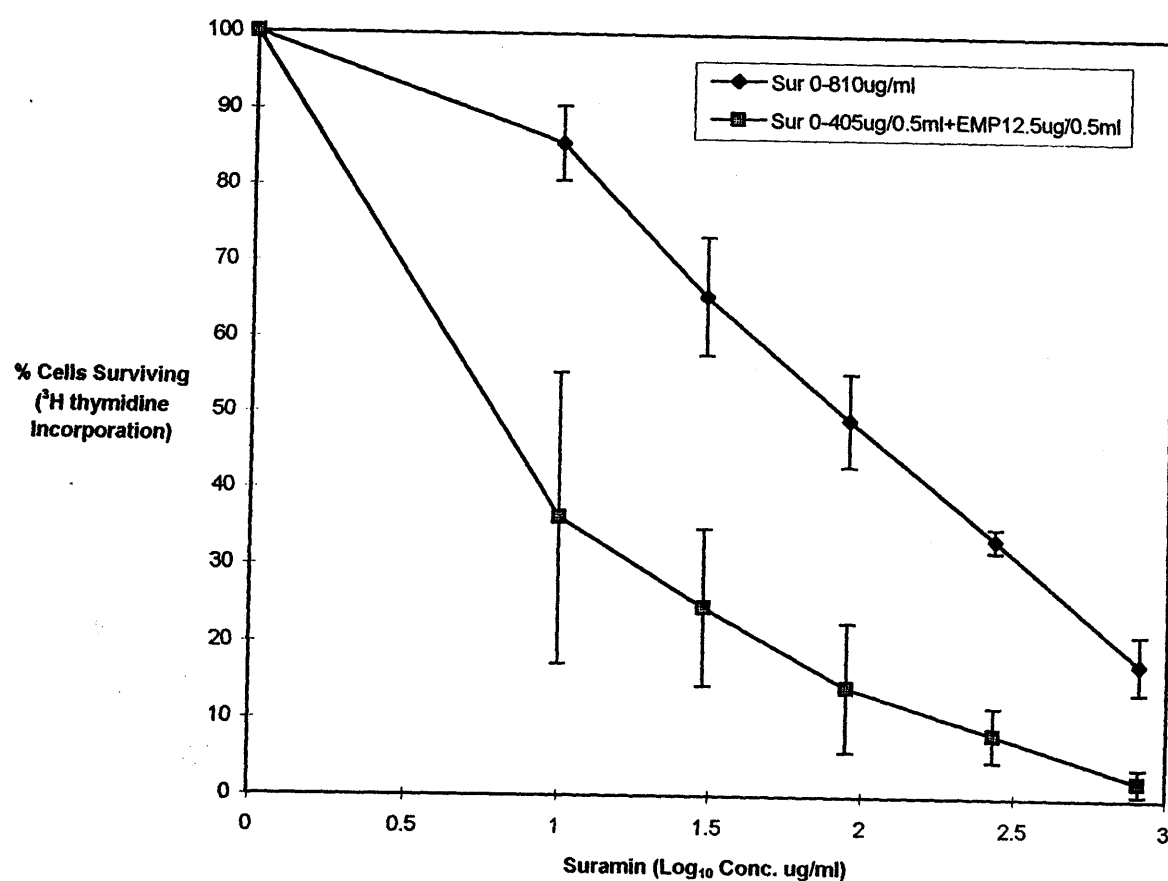
The addition of EMP at a concentration of 1.5 ug/0.5 ml to Suramin (0-405 ug/0.5ml) resulted in significantly higher growth inhibition rate with EMP added (Table 19). However the differences were not statistically significant (Table 20) when the EMP concentration was increased to 6.25 ug/0.5 ml.

3.3.5   SMS 201-995 and EMP combination

SMS 201-995 at a concentration of 0.2 ug/ml produced 41.2% and 36% growth inhibition respectively on LNCaP and PC3 cell lines, the addition of EMP (1.5 ug/0.5 ml) to SMS 201-995 (0.2 ug/ml) increased the growth inhibition rates to 42.3% and 39.3% for LNCaP and PC3 cell lines respectively, a difference that was not statistically significant (P < 0.1). EMP alone at a concentration of 3.1 ug/ml produced 31% and 24% growth inhibition on LNCaP and PC3 prostate cancer cell lines respectively, as shown in Table 22. Increasing the dose of EMP to 25 ug/0.5 ml and adding it to SMS 201-995 (0-20 ug/0.5ml) did not similarly increase the growth inhibitory effect of SMS 201-995 (Table 23). In fact, high dose EMP was counter productive as EMP (25 ug/ml) alone on LNCaP and PC3 cell lines produced 70% and 97% inhibition of growth respectively, while when the same dose of EMP was combined with SMS 201-995 (0.2 ug/0.5 ml), the growth inhibitory effect was reduced to 45% and 44.8% for LNCaP and PC3 respectively. It was possible therefore that EMP interfered with the effectiveness of SMS 201-995.

These results show that when EMP was combined with SMS 201-995, it did not result in a more potent anti-neoplastic regimen for LNCaP and PC3. There was a slightly increased percentage inhibition of cell growth on PC3 cell lines using EMP and SMS 201-995, but this was not statistically significant for all the ranges of EMP used i.e. 3.1, 12.5 or 25 ug/0.5ml.

Table 22

The effect of SMS 201-995 (0-20 ug/ml) or SMS 201-995 (0-10 ug/0.5 ml) + EMP (1.5 ug/0.5 ml) on prostate cancer cell lines PC3 and LNCaP.

% Growth Inhibition	SMS 0.02 ug/ml	SMS 0.01 + EMP 1.5 ug/0.5 ml	SMS 0.2 ug/ml	SMS 0.2 ug/ml + EMP 1.5 ug/0.5 ml	SMS 20 ug/ml	SMS 10 ug/ml + EMP 1.5 ug/0.5 ml	EMP 3.1 ug/ml
LNCaP	7.9	8.3	41.2*	42.3*	78.1	77.8	31
PC3	18.1	20.1	38.0**	39.3**	50.1	60.1	24

Results represent the mean of 3 experiments

*   P < 0.8   (not significant) - Analysis of Variance
**   P < 0.8   (not significant)

See appendix for raw data

EMP (1.5 ug/0.5 ml) when combined with SMS (0.10 ug/0.5 ml) did not result in significant inhibition of growth of PC3 and LNCaP cells.

Table 23

The effect of SMS 201-995 (0-20 ug/ml) or SMS 201-995 (0-10 ug/0.5 ml) + EMP (12.5 ug/0.5 ml) combination on prostate cancer cell lines LNCaP and PC3.

% Growth Inhibition	SMS 0.02 ug/ml	SMS 0.01 + EMP 125 ug/0.5 ml	SMS 0.2 ug/ml	SMS 0.1 ug/ml + EMP 12.5 ug/0.5 ml	SMS 20 ug/ml	SMS 10 ug/ml + EMP 12.5 ug/0.5 ml	EMP 25 ug/ml
LNCaP	7.9	10.2	43.1*	45.0*	78.1	84.4	97
PC3	18.1	20.4	38.1**	44.8**	50.1	64.2	70

Results represent the mean of 3 experiments.

*   P < 0.7   (not significant) - Analysis of Variance
**   P < 0.9   (not significant)

See appendix for raw data.

EMP (25 ug/0.5 ml) when combined with SMS (0-10 ug/0.5 ml) did not result in significant increased growth inhibition of LNCaP or PC3 cell lines.

Section D

3.4   **Results of experiments to determine whether the enhanced effect of Suramin by EMP was synergistic or additive**

The results are as shown in Tables 24 and 25 and Figures 28 and 29.

These results showed that for concentrations of EMP and Suramin producing less than 5% growth inhibition on either PC3, DU145 or LNCaP, the combination of these 2 drugs produced greater than 10% growth inhibition in all the three cell lines indicating a synergistic effect. The effect of the drug combination was tested on DU145 cell line to find out whether this drug combination produced an effective result on another androgen independent prostate cancer cell line.

Section E

3.5   **Results of experiments to determine whether the effect of the drug(s) on prostate cancer cells was due to cytostasis or cytotoxicity**

The results are shown in Table 26 and Figs 30, 31, 32 and 33. These results showed that the inhibitory activity of Suramin, EMP, SMS 201-995, and a Suramin + EMP combination were reversed by removal of the drugs from the culture media. Incubation of PC3 cells and LNCaP cells with Suramin (270 ug/ml) for 6 days resulted in a significant (P = 0.004) 81% and 78% inhibition of cell growth, compared to Suramin-free control on day 7. Removal of Suramin from the culture medium on day 3 resulted in a significant (P = 0.04) increase in PC3 and LNCaP cell counts compared to cells exposed to the drug for 6 days, that is, for LNCaP 30 x 10^3 cells/well for exposure to Suramin 270 ug/ml for 3 days, and 22 x 10^3 cells/well for exposure to Suramin 270 ug/ml for 6 days. However for PC3 it was 33 x 10^3 cells/well and 19 x 10^3 cells/well respectively for exposure to Suramin 270ug/ml for 3 and 6 days respectively.

As with cells cultured with Suramin for 6 days a similar recovery of cell proliferation was observed for cells exposed to SMS 201-995 (2 ug), EMP (25 ug/ml), as well as EMP (12.5 ug/0.5 ml) and Suramin (135 ug/0.5 ml) combination. However, the EMP and

Table 24

The effect on growth inhibition of combining drug concentrations producing less than 5% growth inhibition on prostate cancer cell lines PC3 and DU145.

% Growth Inhibition on cell line		EMP 1.5 ug/ml	Suramin 3.3 ug/ml	EMP 0.8 ug/0.5 ml & Suramin 1.7 ug/0.5 ml
PC3 Experiment	a	5	2	9
	b	8	4	21
	c	7	6	15
MEAN ± SEM		6.7 ± 0.9	4 ± 1.2	15 ± 3.5
DU145 Experiment	a	6	7	10
	b	7	6	12
	c	4	3	11
MEAN ± SEM		5.7 ± 0.9	5.3 ± 1.2	11 ± 0.6

See appendix for raw data.

Results represent the mean of 3 experiments. EMP produced a synergistic effect when combined with Suramin on PC3 and DU145 cells.

Table 25

The effect on growth inhibition of combining drug concentrations producing less than 5% growth inhibition on prostate cancer cell line LNCaP.

% Growth Inhibition on cell line		EMP 1.2 ug/ml	SUR 4.5 ug/ml	EMP 0.6 ug/0.5 ml & Sur 2.25 ug/0.5 ml
LNCaP Experiment	a	4	6	14
	b	3	3	12
	c	4	5	11
MEAN ± SEM		3.7 ± 0.3	4.7 ± 0.9	12.3 ± 0.9

See appendix for raw data.

Results represent the mean of 3 experiments. EMP produced a synergistic effect when combined with Suramin on LNCaP cells.

Fig 28

The effect of combining drug concentrations producing less than 5% growth inhibition on CaP cell lines PC3 and DU145.
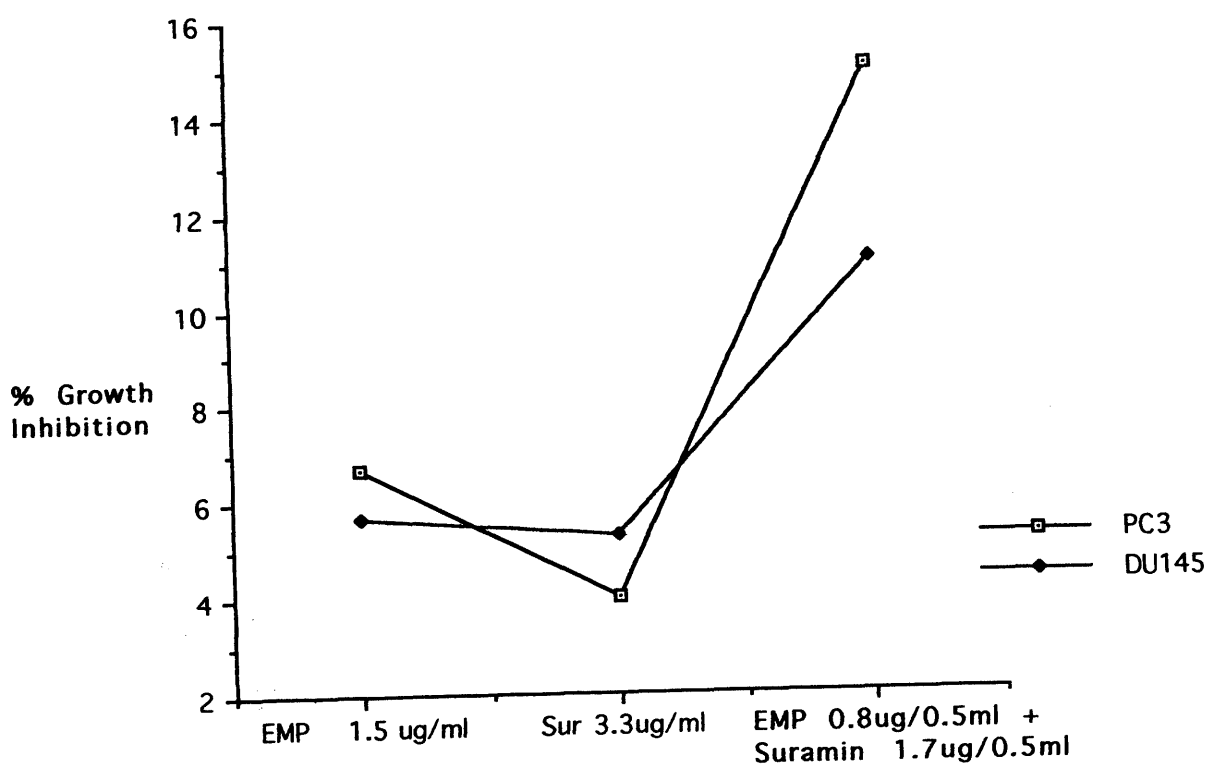
Results represent the mean of 3 experiments.

EMP produced a synergistic effect when combined with Suramin on PC3 and DU145 cells.

Fig 29

The effect of combining drug concentrations producing less than 5% growth inhibition on CaP cell line LNCaP.
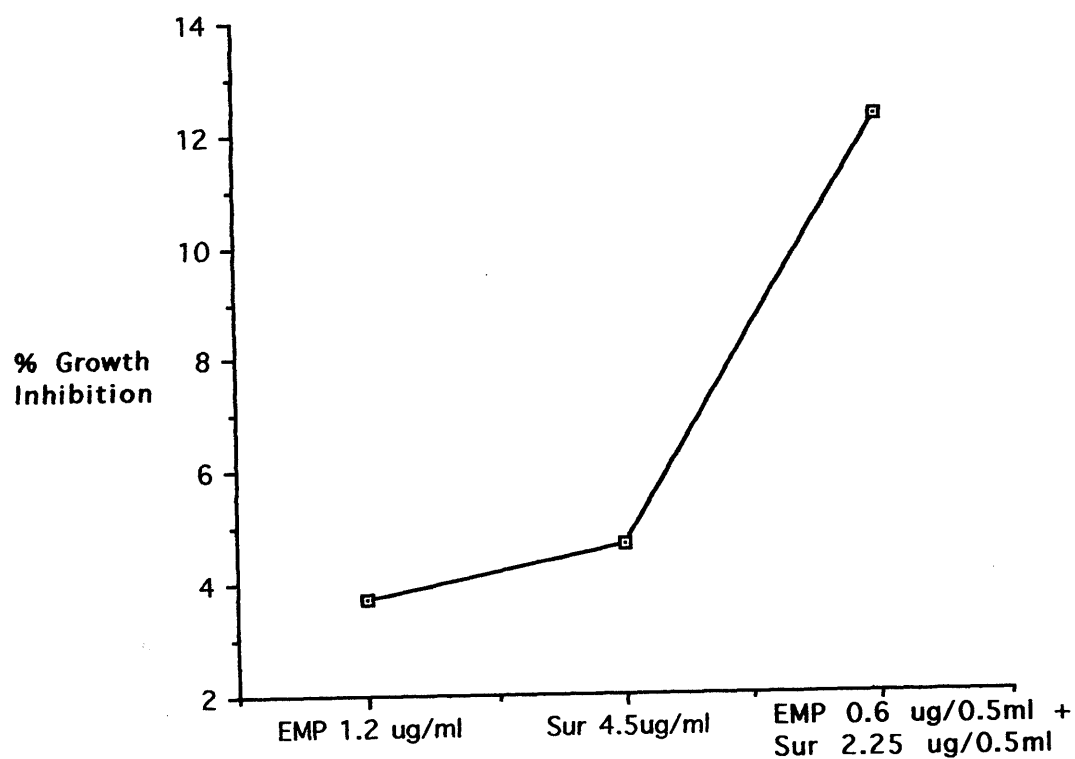
Results represent the mean of 3 experiments.

EMP produced a synergistic effect when combined with Suramin on LNCaP cells.

Table 26

The reversal of Suramin, EMP or (Suramin and EMP) induced inhibition of growth of prostate cancer cell lines PC3 and LNCaP.

Cells/well $\times 10^4$	Days	Suramin 270 ug/ml			EMP 25 ug/ml			Sur 135 ug/0.5 ml + EMP 12.5 ug/0.5 ml		
		Control	x 3/7	x 6/7	Control	x 3/7	x 6/7	Control	x 3/7	x 6/7
LNCaP	0	2	2	2	2	2	2	2	2	2
	3	12	7	7	12	5	5	12	3	3
	5	19	12	9	19	8	6	19	4	6
	6	33	25	16	33	12	8	33	10	8
	7	49	30	22	49	25	15	49	19	10
PC3	0	2	2	2	2	2	2	2	2	2
	3	8	4	4	8	4	4	8	3	3
	5	15	10	8	15	7	6	15	4	3
	6	32	28	17	32	11	8	32	8	6
	7	42	33	19	42	26	18	42	15	10

Cells were incubated with media containing no drugs (control) or Suramin 270 ug/ml, SMS2 ug/ml, EMP 25 ug/ml or EMP 12.5 ug/0.5 ml and Suramin 135 ug/0.5 ml (combination) for 6 days.

On day 3, media containing drugs were removed from half of the wells and replaced with fresh media containing no drugs.

Cells were counted on days 3, 5, 6 and 7. Values represent the mean of 3 experiments. One set of experiments lasted 10 days.

Data for SMS 201-995 not included for clarity. See appendix for full raw data.

3/7 - Cells exposed to drug concentration for 3 days.
6/7 - Cells exposed to drug concentration for 6 days.

The growth inhibitory activity of Suramin, EMP and (Suramin + EMP combinations) were reversed by removal of the drugs from the culture media. The earlier the removal of the drugs from the culture media, the greater the reversal of growth of the cells in culture.

Fig 30

Reversal of suramin (270 ug/ml) induced growth inhibition of CaP cell line PC3.
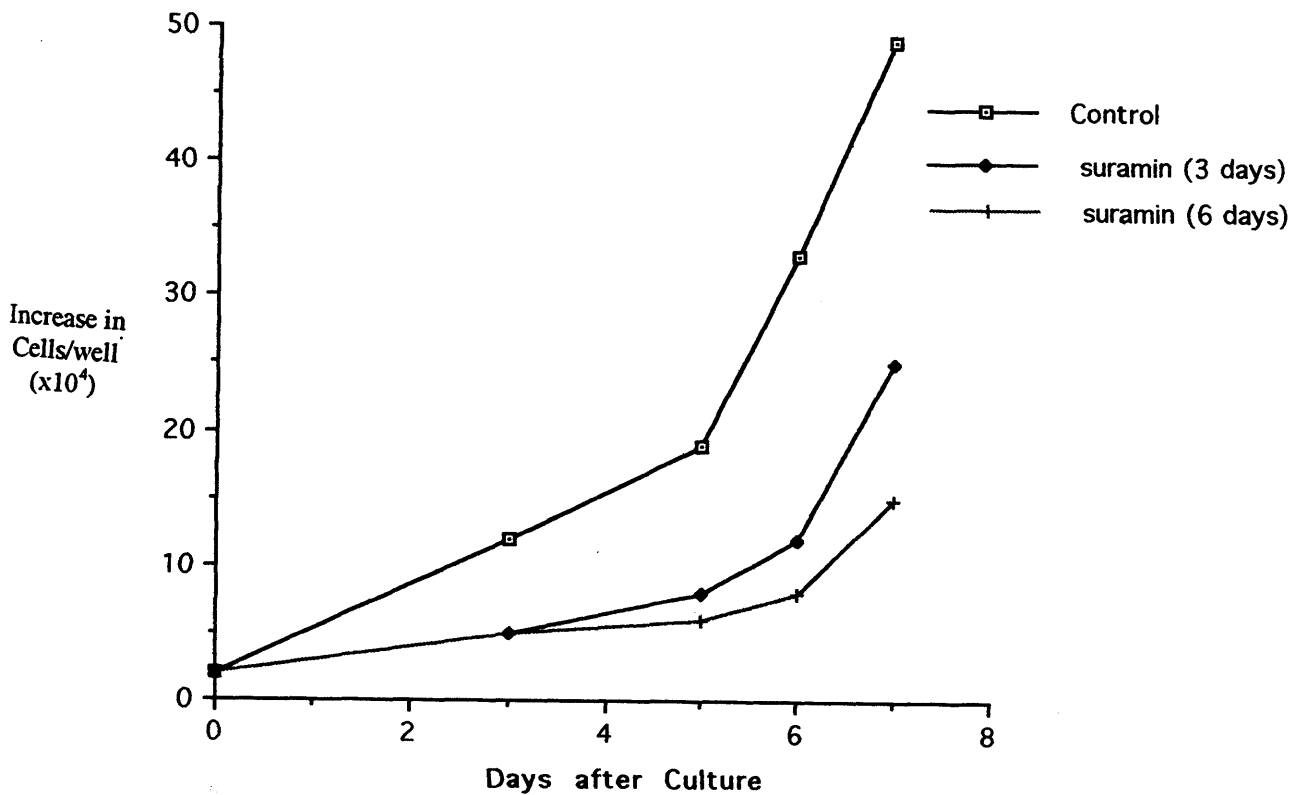
Cells were incubated with medium containing no drugs (Control) or Suramin 270 ug/ml.

On day 3, medium containing Suramin 270 ug/ml was removed from half of the wells and replaced with fresh medium containing no drugs.

Cells were counted on days 3, 5, 6 and 7.
Values represent the mean of 3 experiments.

+ Suramin (3 days) = cells exposed to Suramin for 3 days only..
+ Suramin (6 days) = cells exposed to Suramin for 6 days.

The growth inhibitory effect of Suramin 270 ug/ml on PC3 cells was reversed by removal of Suramin from the culture medium after 3 or 6 days of continuous exposure to Suramin 270 ug/ml.

Fig 31

Reversal of EMP (12.5 ug/0.5 ml) + Suramin (135 ug/0.5 ml) induced growth inhibition of CaP cell line PC3.
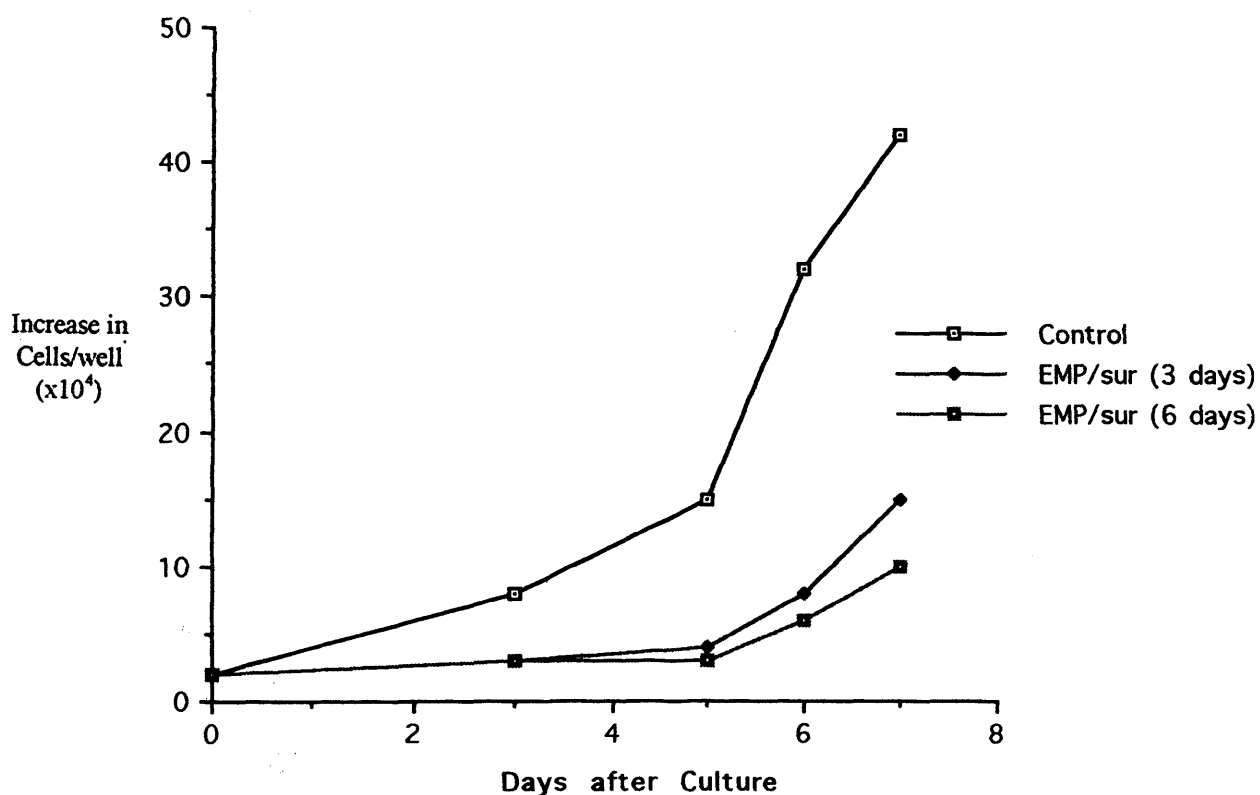
Values represent the mean of 3 experiments.

+EMP/Sur (3 days) = cells exposed to drug combination for 3 days only
+EMP/Sur (6 days) = cells exposed to drug combination for 6 days.

The growth inhibitory effect of EMP (12.5 ug/0.5ml) + Suramin (135 ug/0.5ml) on PC3 cells was reversed by removal of the drugs from the culture medium after 3 or 6 days of continuous exposure to this combination.

Fig 32

Reversal of Suramin (270 ug/ml) induced growth inhibition of CaP cell line LNCaP
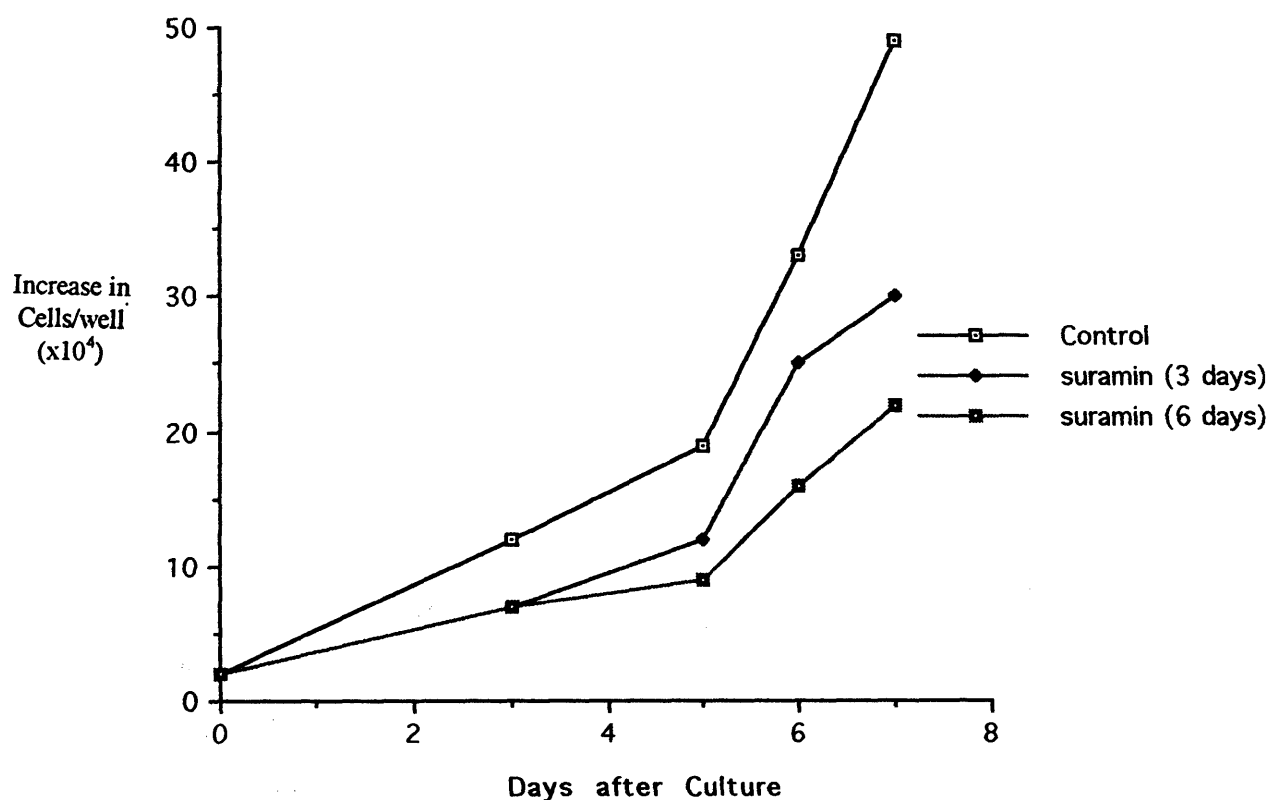
Values represent the mean of 3 experiments.

Suramin (3 days) = cells exposed to Suramin for 3 days only.
Suramin (6 days) = cells exposed to Suramin for 6 days.

The growth inhibitory effect of Suramin 270 ug/ml on LNCaP was reversed by removal of Suramin from the culture medium after 3 or 6 days of continuous exposure to Suramin.

Fig 33

Reversal of EMP (12.5 ug/0.5 ml) + Suramin (135 ug/0.5 ml) induced growth inhibition of CaP cell line LNCaP.
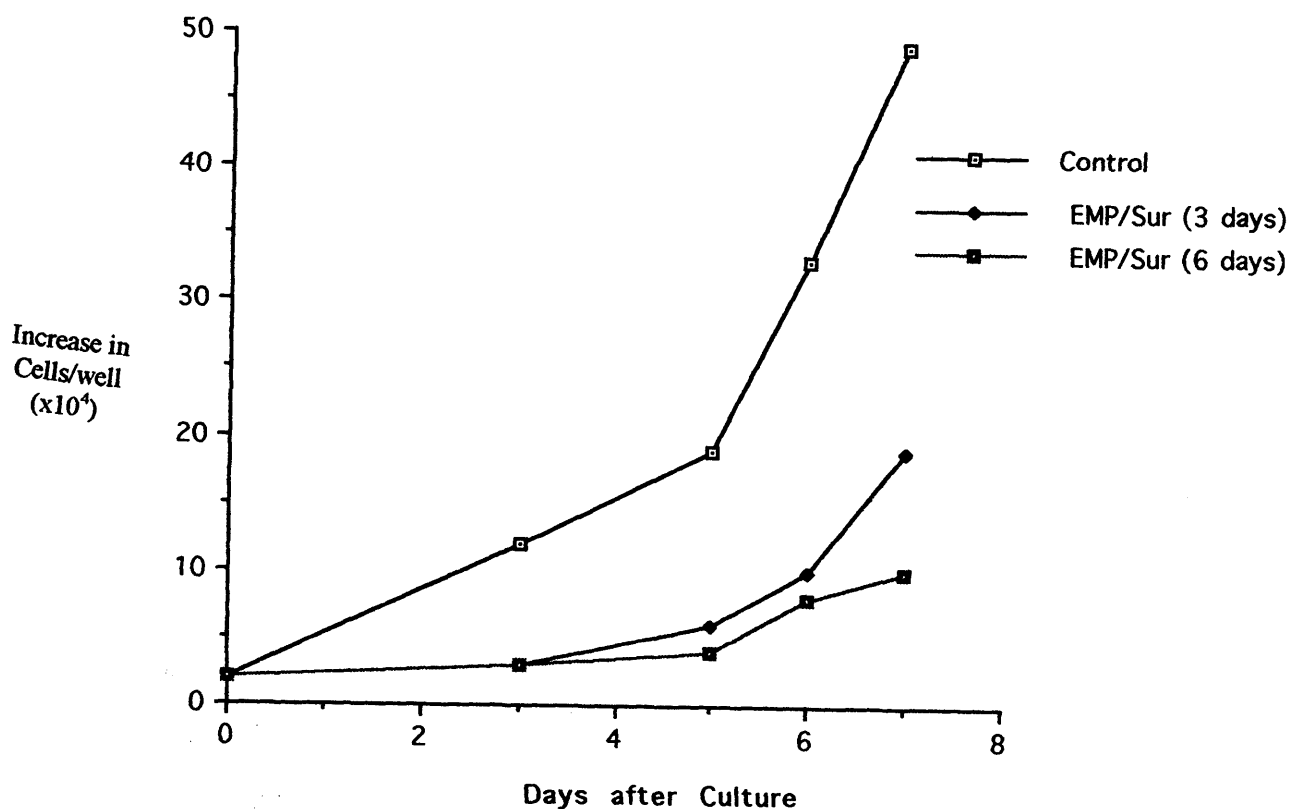
Values represent the mean of 3 experiments.

+EMP/Sur (3 days) = cells exposed to drug combination for 3 days only.
+EMP/Sur (6 days) = cells exposed to drug combination for 6 days.

The growth inhibitory effect of EMP 12.5 ug/0.5 ml + Suramin 135 ug/0.5 ml on LNCaP cells was reversed by removal of Suramin + EMP from the culture medium after 3 or 6 days of continuous exposure to the drug combination .

Suramin combination produced greater inhibition of growth and a greater reduction of the ability of the cells to re-initiate growth after the media containing drugs were withdrawn as shown in Table 26 and Figs 30, 31 and 32 and 33. Light microscopic appearance of cells exposed to Suramin or Suramin and EMP revealed cells that assumed a spherical shape suggesting cytostasis (Figs 34 and 35) as opposed to lysed cells which would have suggested cytotoxicity.

Section F

3.6   **Results of experiments to determine:**

3.6.1   **The effect of epidermal growth factor on proliferation of LNCaP, PC3 and DU145**

EGF between a concentration of 1 to 5 ng/ml stimulated the growth of LNCaP and DU145 but not that of PC3. Compared to control, 1 ng/ml of EGF stimulated growth of LNCaP and DU145 by 16% and 21% respectively. Maximal stimulation of growth of LNCaP and DU145 cells occurred at an EGF concentration of 5 ng/ml when EGF produced 46% and 62% growth over control for LNCaP and DU145 cell lines respectively. Increasing the concentration of EGF to over 5 ng/ml did not result in a further increase in growth-stimulatory rate for LNCaP and DU145 cell lines as shown in Fig 36 and Tables 27 and 28.

When we did not observe a growth stimulatory effect by EGF on PC3, we decided to test the effect of exogenous EGF on another androgen independent prostate cancer cell line to see whether the effect would be the same. We were surprised to find that EGF stimulated the growth of DU145 but not that of PC3 prostate cancer cell lines. Electron microscopic analysis of DU145 and PC3 (Figs 37 and 38) showed that DU145 has numerous rough endoplasmic reticulum and mitochondria compared to PC3. This might explain the differences in the effect of EGF on the 2 cell lines.

Figures 39 and 40 and Table 29 show the effect of Suramin on EGF stimulated growth of LNCaP and DU145. As stated in the above paragraph, EGF at a concentration of 5 ng/ml caused maximal growth stimulation of LNCaP (46%) and DU145 (62%).

Fig 34

LNCaP Cells exposed to Suramin (270 ug/ml) or EMP (12.5 ug/0.5 ml) + Suramin (135 ug/0.5 ml) assumed a spherical shape suggesting cytostasis and were not lysed as seen with cytotoxicity.
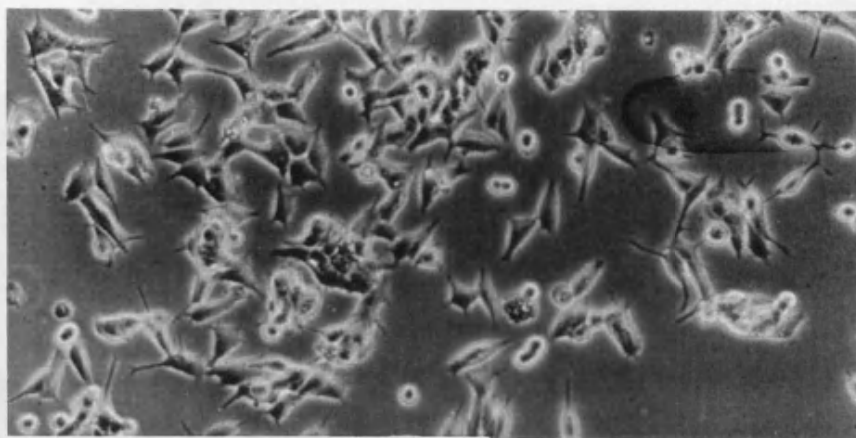
Control
x
48 Hrs
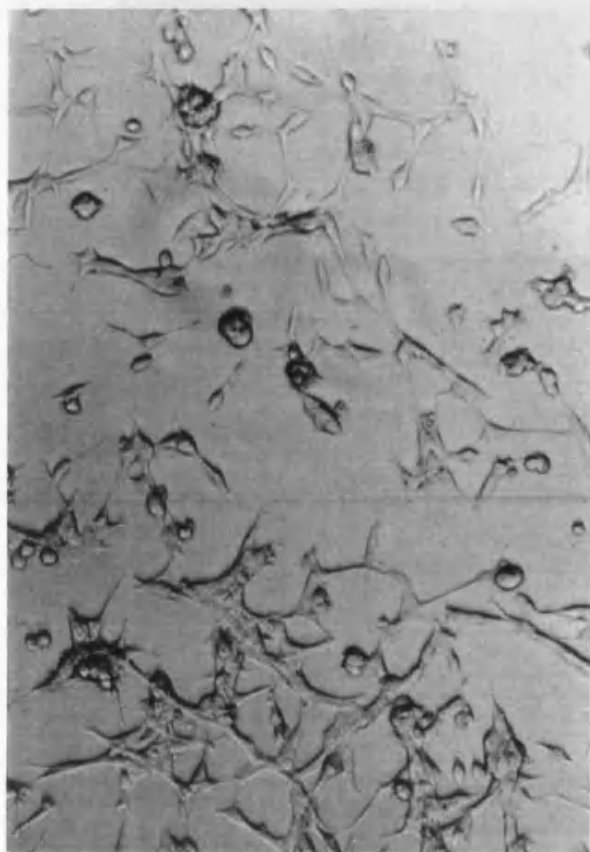
Suramin 270 ug/ml
x 48 Hours
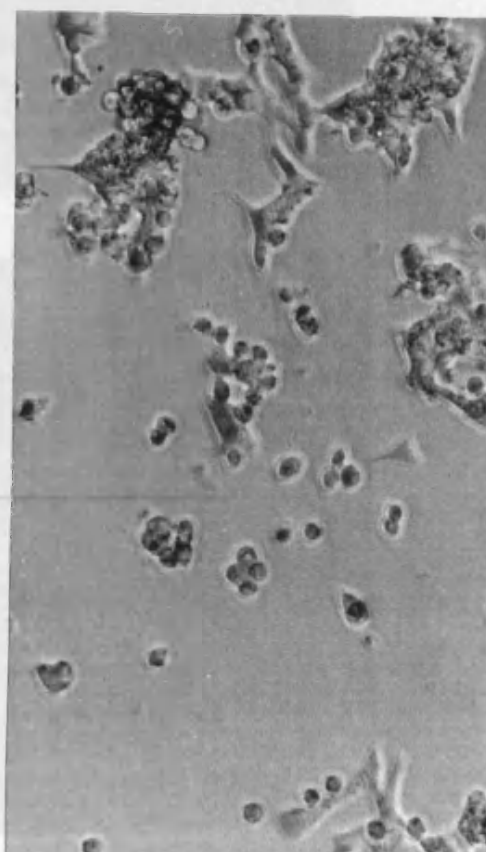
Suramin 135 ug/0.5 ml +
EMP   12.5 ug/0.5 ml   x 48 Hours

Fig 35

PC3 prostate cancer cells exposed to Suramin (270 ug/ml) or Suramin (135 ug/0.5 ml) + EMP (12.5 ug/0.5 ml) cells assumed a spherical shape and were not lysed on exposure to the drugs, suggesting cytostasis as opposed to cytotoxicity as possible mechanism of action of the drug combination.
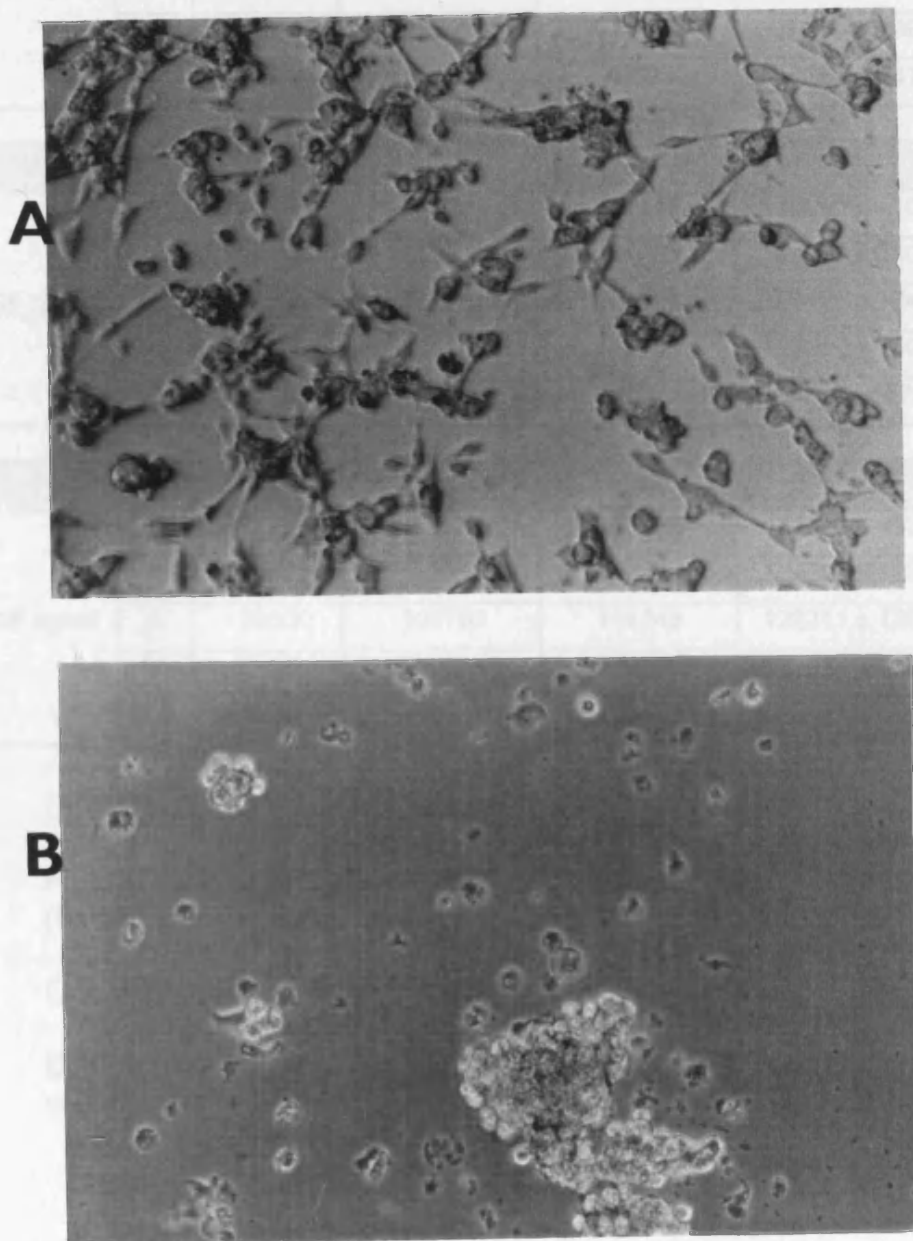
A = Suramin 270 ug/ml x 48 Hours
B = Suramin 135 ug/0.5ml + EMP 12.5 ug/0.5 ml   x 48 Hours

Table 27

Growth stimulatory effect of EGF on LNCaP, DU145 and PC3 prostate cancer cell lines.

LNCaP		1st Expt	2nd Expt	3rd Expt	Mean ± SEM	% Growth
	0	13597	16937	19916	16817 ± 1827	100%
	1	14864	21263	17488	17872 ± 1859	116
EGF ng/ml	5	16654	113863	19347	19955 ± 1537	146
	25	16465	19626	19538	18543 ± 1292	125
	125	14879	12185	30803	19289 ± 5816	136

DU145		1st Expt	2nd Expt	3rd Expt	Mean ± SEM	% Growth
	0	34967	158263	157747	116990 ± 41061	100%
	1	100700	140005	184005	141570 ± 24089	121%
EGF ng/ml	5	650790	570012	955398	725400 ± 17478	162%
	25	9600	227150	150700	157950 ± 38078	135%
	125	155001	16200	177905	164970 ± 6783	141%

PC3		1st Expt	2nd Expt	3rd Expt	Mean ± SEM	% Growth
	0	35029	161627	166581	121079 ± 43099	100%
	1	100572	132750	140877	124733 ± 12320	103%
EGF ng/ml	5	122000	100790	144143	122311 ± 12531	101%
	25	7100	159566	140000	123522 ± 26893	102%
	125	140000	106240	120660	122300 ± 9792	101%

EGF 0-125 ng/ml added to 2×10^4 cells/well.

Figures represent cells/well as measured by ^3H thymidine incorporation (DPM).

One experiment was performed per week.

EGF stimulated growth of LNCaP, DU145, but had no effect on PC3 cellular proliferation.

Table 28

The effect of Suramin on EGF stimulated growth of LNCaP prostate cancer cell line.

Drugs added	No of cells per well on days				LNCaP Cells
	0	2	4	6	
EGF 5 ng/ml	6000	7270 13165 16902 12446 ± 2807	14551 26892 26425 22623 ± 4043	57114 55578 30340 47644 ± 8690	1st Expt 2nd Expt 3rd Expt Mean ± SEM
Suramin 270 ug/ml	6000	5589 10646 6927 7720 ± 1515	6508 21952 14169 14210 ± 4464	21221 25208 17113 21181 ± 2340	1st Expt 2nd Expt 3rd Expt Mean ± SEM
EGF 2.5 ng/0.5 ml + Suramin 135 ug/0.5 ml	6000	5995 12383 12476 10285 ± 2148	9196 24100 22025 18440 ± 4666	23351 31421 19555 24776 ± 3503	1st Expt 2nd Expt 3rd Expt Mean ± SEM
CONTROL	6000	6978 12394 15406 11593 ± 2469	1169 22872 23199 15747 ± 7298	39461 41184 26026 35557 ± 4797	1st Expt 2nd Expt 3rd Expt Mean ± SEM

EGF stimulated LNCaP cell growth, while Suramin inhibited LNCaP cell growth compared to control. A mixture of EGF and Suramin produced a cell growth curve similar to that of the control. EGF counteracted some of the growth-inhibitory effect of Suramin.

Results represent the mean of triplicate experiments.

Figures represent cells in wells as measured by ^{3}H thymidine incorporation assay. (DPM)

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Table 29

The effect of EGF (5 ng/ml) or Suramin (270 ug/ml) and EGF (2.5 ng/0.5 ml) + Suramin (135 ug/0.5 ml) combination on the proliferation of DU145 prostate cancer cell line.

		No of cells per well (DPM) on days			
Days	0	2	4	6	
EGF 5 ng/ml	4000	5612 12000 33245 23619 ± 6721	31480 12704 43824 29336 ± 9058	39051 16980 61783 39271 ± 12949	1st Expt 2nd Expt 3rd Expt Mean ± SEM
Suramin 270 ug/ml	4000	7941 2139 7639 6524 ± 2282	12211 8089 16941 12414 ± 2560	18609 9098 23428 17045 - 4215	1st Expt 2nd Expt 3rd Expt Mean ± SEM
EGF 2.5 ng/0.5 ml + Suramin 135 ug/0.5 ml	4000	18358 10560 9879 12932 ± 2723	24140 11989 37028 24386 ± 7238	25431 15789 38371 26530 ± 6560	1st Expt 2nd Expt 3rd Expt Mean ± SEM
CONTROL	4000	23488 12783 18094 18122 ± 3094	23844 15676 26791 22104 ± 3329	31906 16091 56426 34808 ± 11748	1st Expt 2nd Expt 3rd Expt Mean ± SEM

EGF stimulated cell growth, while Suramin inhibited cell growth of DU 145 cells compared to the control. A mixture of EGF and Suramin produced less cell growth compared to the control.

Number of cells per well as determined by ^{3}H thymidine incorporation (DPM).

Results represent the mean of triplicate experiments.

Fig 36

Growth stimulatory effect of EGF on LNCaP, DU145 and PC3 prostate cancer cell lines.
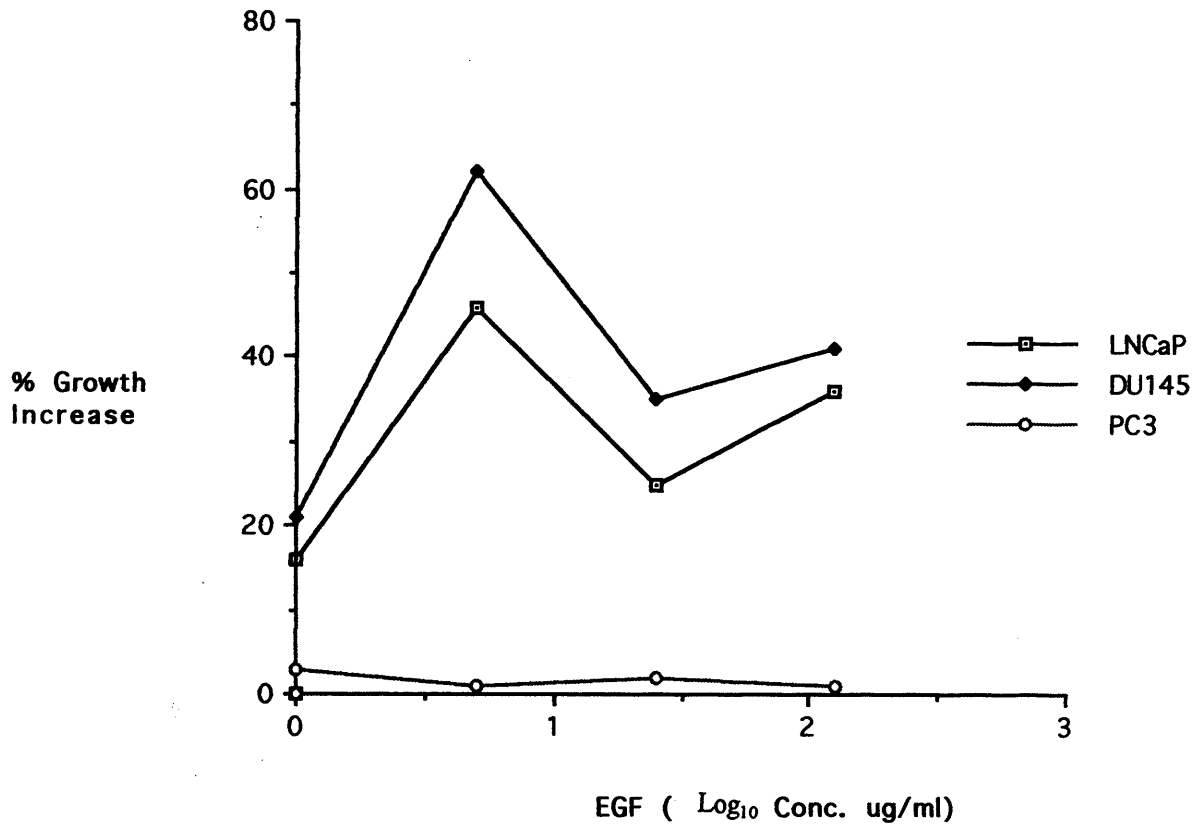
Results represent the mean of triplicate experiments.

EGF stimulated the growth of LNCaP and DU145 prostate cancer cell lines, but not that of PC3.

Maximal stimulation of LNCaP and DU145 occurred at an EGF concentration of 5 ng/ml.

Fig 37

Electron microscopic appearance of DU145 prostate cancer cell line at passage number 25 showing numerous rough endoplasmic reticulum and mitochondria compared to PC3 cell line at passage number 86, Fig 37.
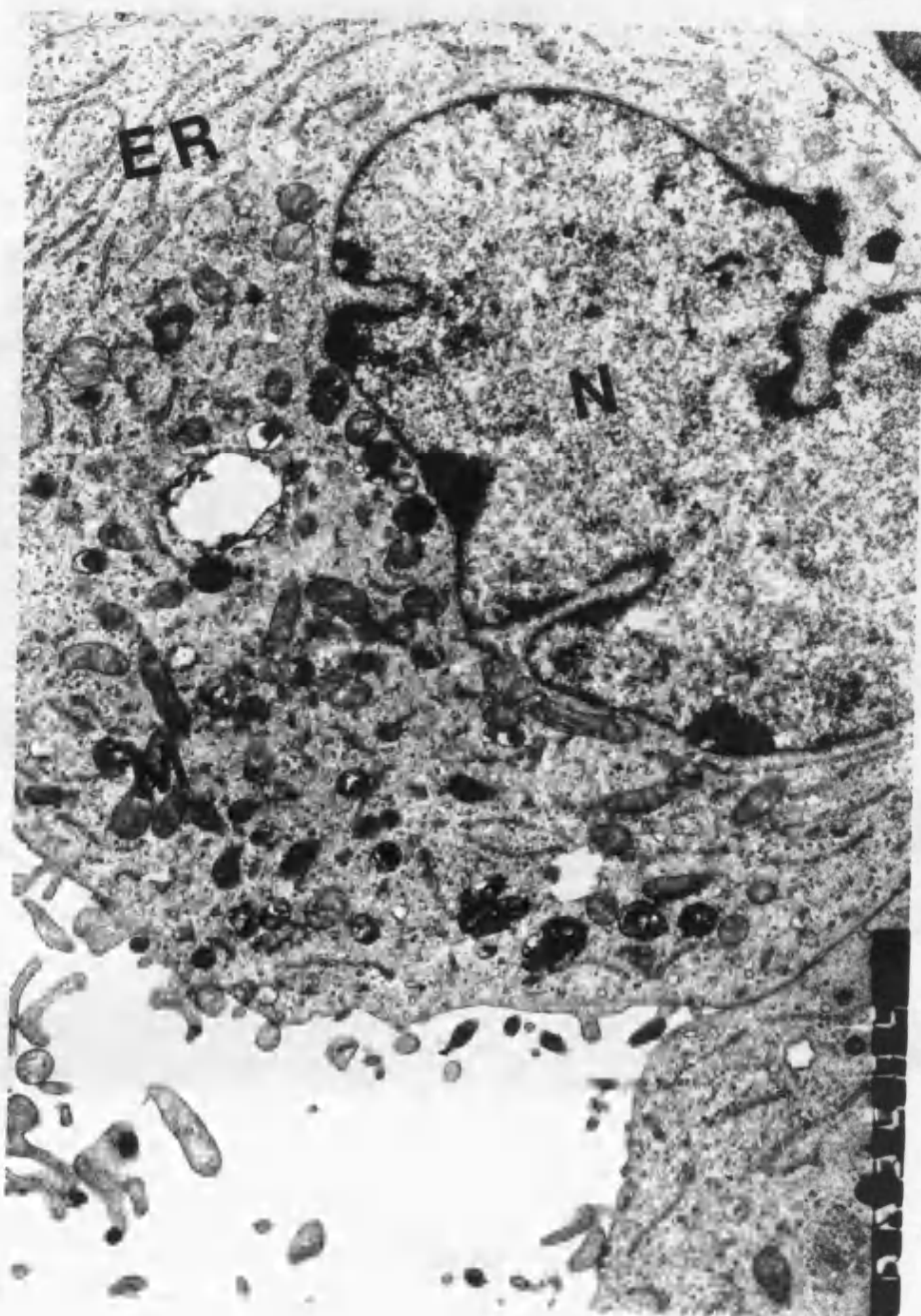
ER = Endoplasmic reticulum   M = Mitochondria   N = Nucleus
Magnification x 4,100

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Fig 38

Phase contract electron microscopic appearance of PC3 cell line at passage no 86. Note the irregular disposition of organelles in the cytoplasm, few interdigitating cytoplasmic projections, senescent and fragmented mitochondria. Magnification x 4,100.
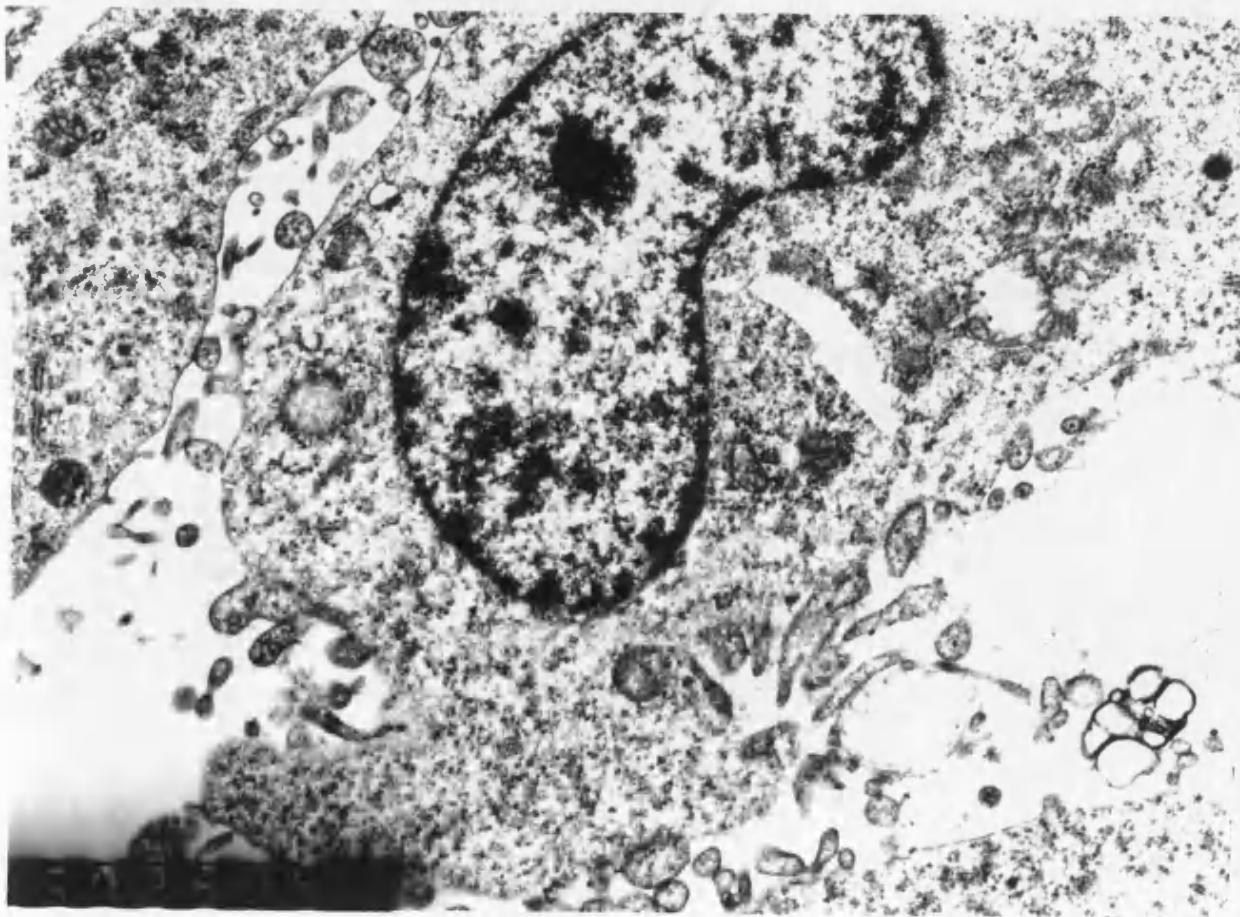
Repeated passaging of established cell line PC3 affected its morphological and possibly functional characteristics, making PC3 cell line of high passage numbers an unsuitable in vitro CaP model.

Fig 39

The effect of Suramin on epidermal growth factor (EGF) stimulated growth of LNCaP.
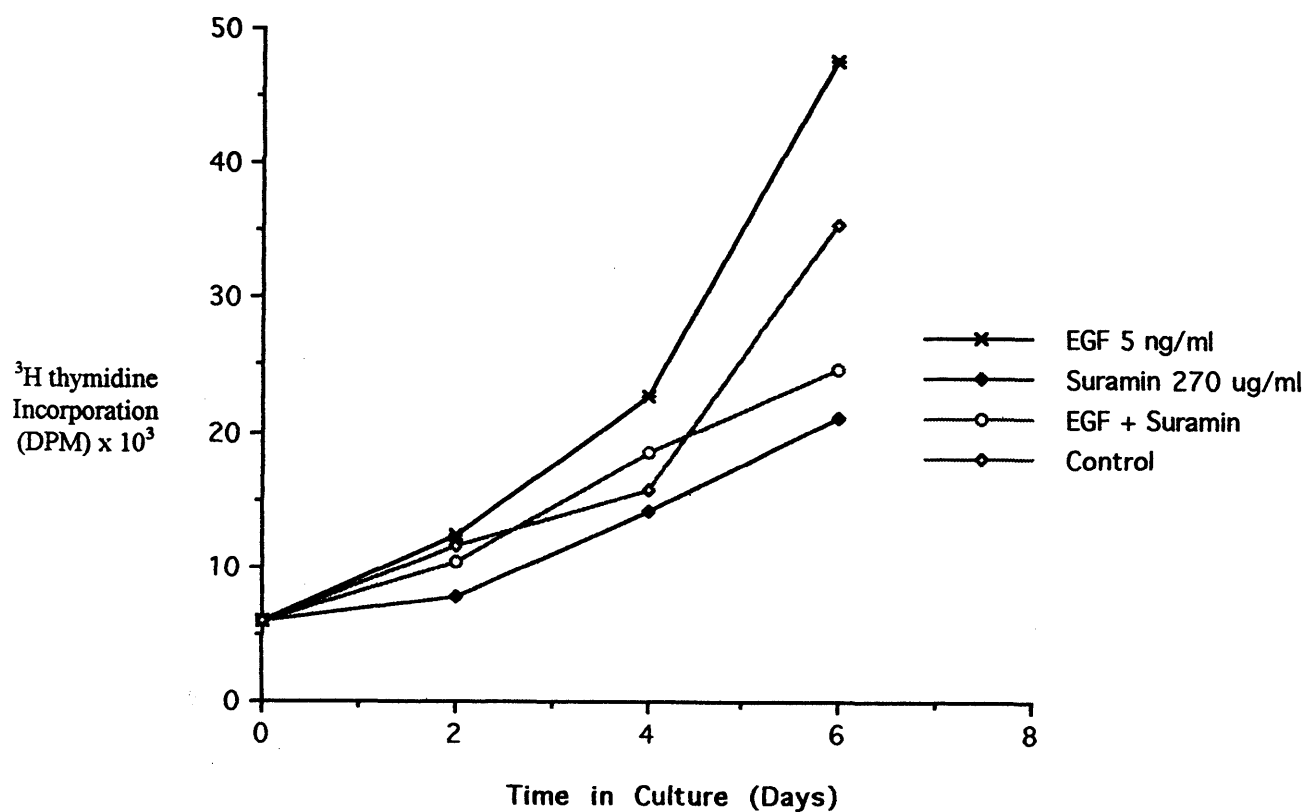
Results represent the mean of 3 experiments.

EGF stimulated LNCaP cell growth and Suramin inhibited cell growth compared to control without additions.

A combination of EGF and Suramin produced a cell growth curve similar to that of the control. EGF counteracted some of the growth inhibitory effect of Suramin on LNCaP cells.

Fig 40

The effect of Suramin on EGF stimulated growth of DU145.
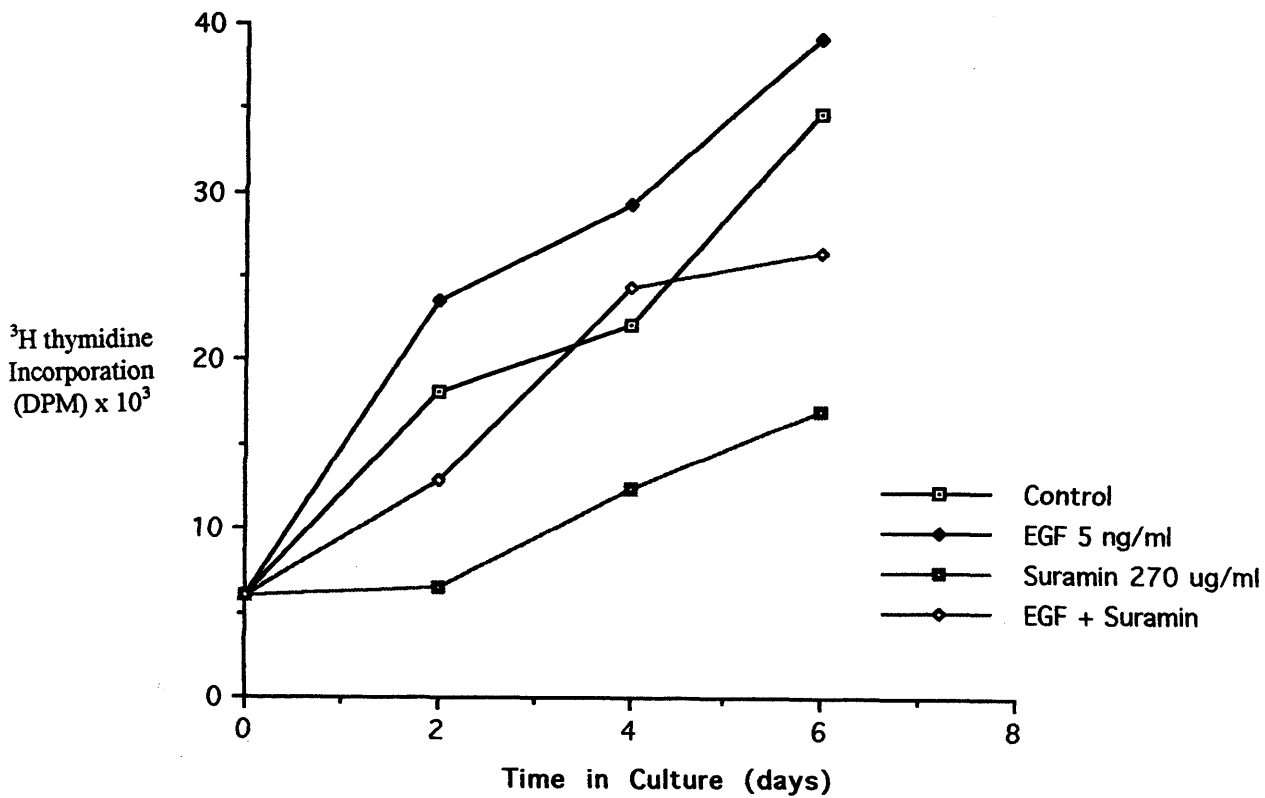
Results represent the mean of 3 experiments.

EGF stimulated DU145 cell growth and Suramin inhibited cell growth compared to control.

A combination of EGF and Suramin produced less cell growth than the control. EGF counteracted some of the growth inhibitory effect of Suramin on DU145 cells.

Table 30

The effect of EGF (5 ng/ml) or Suramin (270 ug/ml) and (EGF 2.5 ng/0.5 ml + Suramin 135 ug/0.5 ml) combination on the proliferation of PC3 prostate cancer cell lines.

Days	No of cells/well (DPM) on:			
	0	2	4	6
EGF 5 ng/ml	6000	6520	6471	8731
Suramin 270 ug/ml	6000	5589	6572	11831
EGF 2.5 ng/0.5 ml + Suramin 135 ug/0.5 ml	6000	6120	6481	9785
Control	6000	12000	17865	32000

EGF did not stimulate the proliferation of PC3 cell lines, even though Suramin inhibited the proliferation of PC3 cell growth.

Results represent the mean of 3 experiments. Figures represent the number of cells in culture as measured by ^{3}H thymidine incorporation assay.

See appendix for raw data.

Fig 41

The effect of EGF 5 ng/ml or Suramin 270 ug/ml and (EGF 2.5 ng/0.5 ml + Suramin 135 ug/0.5 ml) combination on the proliferation of PC3 prostate cancer cell line.

^{3}H thymidine Incorporation (DPM) x 10^3

Control
EGF 5ng/ml
Suramin 270ug/ml
EGF 2.5ng/0.5ml +Sur135ug/0.5ml

Time in Culture (days)
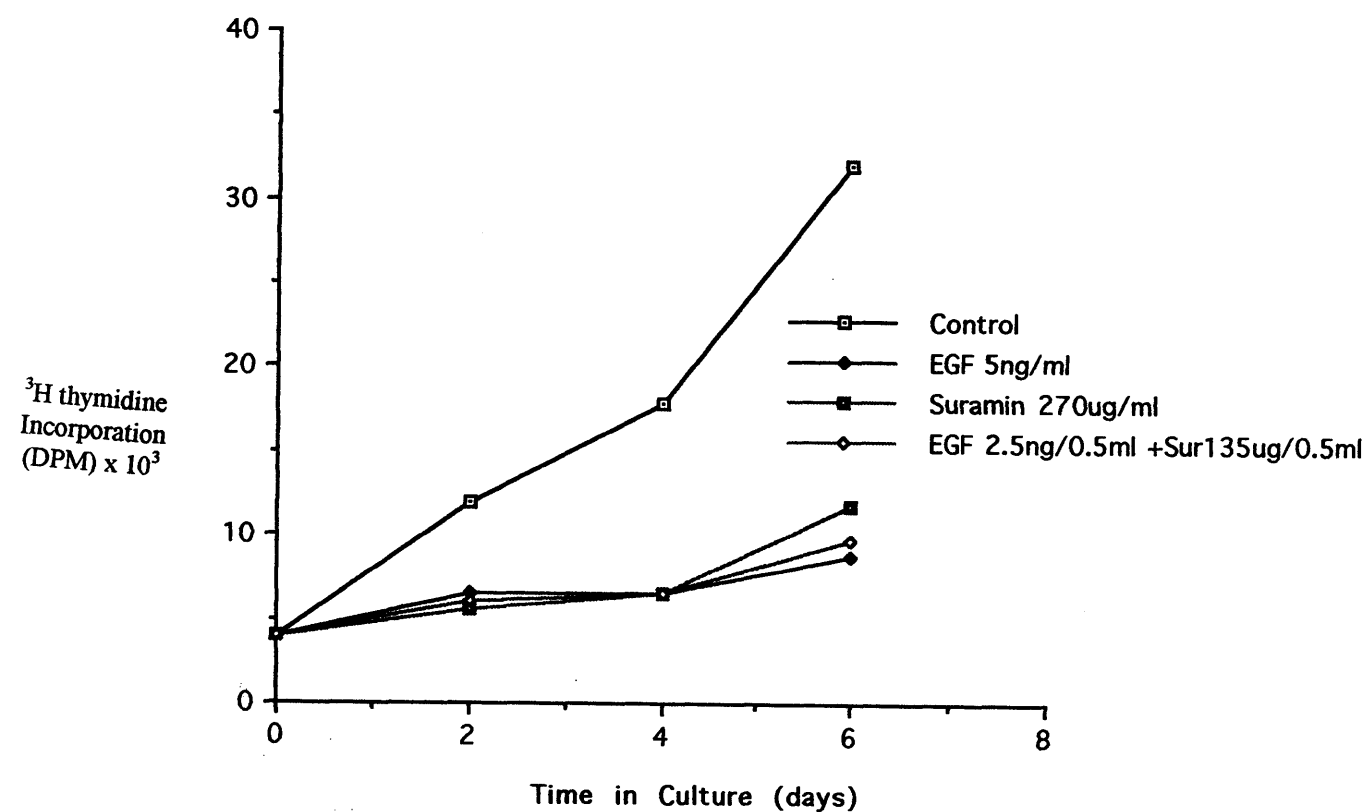
EGF did not stimulate the proliferation of PC3 prostate cancer cell line.

Suramin inhibited the proliferation of PC3 prostate cancer cell line.

EGF and Suramin in combination had no effect on the proliferation of PC3 prostate cancer cell line.

Results represent a mean of 3 experiments.

Similarly, Suramin at a concentration of 270 ug/ml produced 48% and 62% growth inhibition of LNCaP and DU145 respectively. The combination of EGF 2.5 ng/0.5 ml and Suramin 135 ug/0.5 ml resulted in less cell growth compared to control cells and cells being maximally stimulated by EGF alone (Fig 39). This meant that EGF was counteracting some of the growth inhibitory effect of Suramin. Moreover, the growth rate of cells exposed to Suramin and EGF was higher than to those exposed to Suramin alone for both cell lines as shown in Figs 39 and 40.

EGF, up to a concentration of 125 ng/ml, did not stimulate PC3 prostate cancer cell line as shown in Figs. 36 and 41 and Tables 27 and 30. However, Suramin inhibited the growth of PC3 prostate cell line. Hence, compared to the control as shown in Table 30 the EGF and Suramin combination produced less cell growth. Compared to the effect of Suramin alone which inhibited PC3 cell growth, the Suramin and EGF combination resulted in less cell growth.

3.7 The effects of anti-epidermal growth factor receptor on proliferation of LNCaP, PC3 and DU145 prostate cancer cell lines

Compared to antibody-free control, anti-EGF receptor produced dose dependent growth inhibition of LNCaP, PC3 and DU145 cell lines respectively. However, the results reached statistical significance (P < 0.01) only for inhibition of growth produced by anti-EGF receptor on PC3 and DU145.

For LNCaP, the degree of growth inhibition produced by anti-EGF receptor in a concentration range of 0.1-10 ug/ml did not reach statistical significance (P < 0.65) compared to the control. Hence, 1 ug/ml of anti-EGF receptor produced 19.6%, 36.1% and 45.6% growth inhibition of LNCaP, PC3 and DU145 cell lines respectively. Incubation of all cell lines with anti-EGF receptor at 10 ug/ml or 100 ug/ml did not induce significantly higher levels of growth inhibition than 1 ug/ml as shown in Table 31.

Table 31

The effect of anti-EGF receptor on growth of LNCaP, DU145 and PC3 prostate cancer cell lines.

% Growth inhibition on cell line	Concentration of anti-EGF receptor in ug/ml				
	0	0.1	1	10	100
LNCaP	0	11.8	19.6	20.7	22.7
PC3	0	23.7	36.1*	37	37
DU145	0	29.1	45.6*	49.1	48.1

Anti-EGF receptor (0-100 ug/ml) added to 2×10^4 cells/well on day 1. The number of cells surviving in each well was estimated using ^3H thymidine incorporation assay.

Results represent the mean of three experiments. See appendix for raw data.

* P < 0.01 (analysis of variance) - statistically significant inhibition of growth compared to control.

Anti-EGF receptor produced dose-dependent growth inhibition of LNCaP, PC3 and DU145.

CHAPTER 4 :   DISCUSSION OF RESULTS OF EXPERIMENTS ON
                 ESTABLISHED PROSTATE CANCER CELL LINES

Section A :   Methods of counting cells in culture

Section B :   Establishment of clonal growth of PC3, DU145 and LNCaP

Section C :   The effect of the following drugs or drug combinations on
                 established prostate cancer cell lines PC3 and LNCaP

Section D :   Discussion on whether the effect of Suramin and EMP was
                 synergistic or additive

Section E :   Discussions on the mechanism of anti-proliferative effects of
                 Suramin, Somatostatin 201-995, EMP and combinations of
                 Suramin and EMP on prostate cancer cell lines

Section F :   The effect of epidermal growth factor on proliferation of
                 LNCaP, PC3 and DU145

Section G: The effect of anti-epidermal growth factor receptor on LNCaP,
                 PC3 and DU145 prostate cancer cells

Section A

4.1   Methods of counting cells in culture

4.1.1   Haemocytometer

The haemocytometer is the "gold standard" for counting the number of viable cells. However the method is time consuming. The Coulter counter would have been a good substitute; however, we were unable to adapt the Coulter counter used for routine haematological work to count prostate cancer cells in culture. The prostate cancer cells were larger than the red and the white blood cells which the Coulter counter was designed to count. Hence, we explored the other possible methods of counting viable cells in wells.

4.1.2   MTT/growth determination assay

The use of the MTT does have limitations influenced by the physiological state of cells and variance in mitochondrial dehydrogenase activity in different cell types (Mossman, 1983). Nevertheless, the MTT method of cell determination is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors, other cell growth promoting reagents, cytotoxicity studies and in the derivation of cell growth curves. The MTT method of cell determination is most useful when cultures are prepared in 24 well plates. For best results, cell numbers can be determined during log growth stage. Bacteria, mycoplasma and other microbial contaminants may also cleave the MTT tetrazolium ring and formation of MTT formazan, yielding erroneous results.

However, we found that the MTT cell growth determination assay method of counting viable cells in wells was time-consuming. It was therefore not our favoured method of

finding out the mitogenic or cytotoxic effects of various agents that were used subsequently in our numerous experiments. We found that established cell lines PC3, LNCaP and DU145 and less so prostate cancer cells obtained by primary culture, detached after adding MTT solution, which led to some loss of formazan crystals; hence the MTT may not be a very accurate method of counting prostate cancer cells in culture. However, the MTT method may be an ideal method for experiments involving cells that are adherent to the culture flask after the addition of MTT solution, such that the culture fluid can be decanted –without the loss of cells- before MTT solvent is added to dissolve the formazan crystals.

4.1.3 ^{3}H thymidine incorporation assay

In our experience, this method was very accurate in counting cells in culture. There was very good correlation with cells counted using the haemocytometer. This method also saves a lot of time as many plates could be processed at the same time. For this reason, this was the method that we used in over 95% of experiments described in this work.

4.1.4 Measurement of prostate acid phosphatase in the supernatant of culture medium.

Even though this was a good method of estimating the number of cells in culture, we did not use it a great deal because the method was expensive and also time-consuming. Moreover, 2 of the cell lines that we used in these experiments, DU145 and PC3, being androgen independent, did not produce PAP in culture, making this method impossible to use for assessing the growth of these 2 types of cell. We used this method to confirm that cells growing from tissue collected for primary culture were mostly malignant, as CaP tissue produced PAP while BPH tissues produced negligible amounts of PAP in culture.

Section B

4.2.1 **Establishment of clonal growth of PC3, DU145 and LNCaP**

The cells that we obtained were viable as shown by proliferation over an 8-day period. Feeding the cells at regular intervals allows for maximum proliferation of cells. From this initial experiment, we realised that we could follow the inhibitory or stimulatory effects of drugs on the cells for about 8 days when 2×10^4 cells per well were plated on day 0. We chose to experiment with cells at an initial density of 2×10^4 cells/well, because other investigators have found that, this number of cells will continue to proliferate in a 24 well plate for about 8 days without becoming overgrown (La Rocca et al. 1991). Our experience confirms these previous observations. However, the culture medium plays an important role in the rate of proliferation of the cells. Consequently, our observations hold true for LNCaP grown in 2.5% FCS and DU145 and PC3 grown in ITS and RPMI.

Section C

4.3 **The effect of the following drugs or drug combinations on established prostate cancer cell lines PC3 and LNCaP**

4.3.1 **Suramin or Somatostatin 201-995**

From these experiments, Suramin and SMS 201-995 produce dose-dependent growth inhibition of PC3 and LNCaP cell lines, a finding that is in accordance with previous observations (Taneja et al. 1990, La Rocca et al. 1991, Kim et al. 1991). Suramin and SMS 201-995 produce more inhibition of PC3 (44.7%) cell line growth compared to LNCaP (50.1%) at Suramin concentration of 270 ug/ml. A possible explanation for this is that both Suramin and SMS 201-995 are potent inhibitors of growth factors which are believed to be important for the proliferation of androgen-independent cells such as PC3 but not for androgen-dependent cells such as LNCaP. Similarly SMS 201-995 produced 44% and 37% growth inhibition of LNCaP and PC3 prostate cancer cell lines respectively. La Rocca et al. (1991), have shown in preliminary clinical trials that the toxic, but also the tumoricidal serum Suramin level is between 250 and 300 ug/ml.

4.3.2   **Estramustine phosphate (E.M.P.)**

EMP produces dose dependent inhibition of growth of CaP cell lines PC3 and LNCaP. This finding is in keeping with the observations of Hartley-Asp and Gunnarsson in 1984, who showed that EMP induced mitotic arrest in PC3 and DU145 prostate cancer cell lines. Unlike Suramin which antagonises the effect of growth factors on CaP cell lines, EMP's mechanism of action is by combination with microtubule associated proteins which are important during cell replication (Stearns and Tew 1988). The usual dosage of EMP in clinical practice is 280 mg tds orally which is designed to achieve a serum concentration of about 12.5 ug/ml. From Table 15, EMP at a concentration of 12.5 ug/ml will inhibit growth in 87% and 60% of LNCaP and PC3 cells respectively. At a dosage of EMP 280 mg tds, EMP produces dose dependent side effects which often require discontinuation of the drug in about 20% of patients (Johansson et al. 1988, Slack and Murphy, 1984). To improve the therapeutic efficacy of EMP and to reduce its toxicity, it will be necessary to find another drug with which it produces a synergistic effect or at worst, an additive effect. This was the aim of subsequent experiments. The other advantage of finding a drug which has synergistic activity with EMP will be to increase the kill rate of androgen-independent cells.

4.3.3   **Suramin and Somatostatin (SMS 201-995)**

The structures of Suramin and SMS 201-995 are quite different as shown in Figs 9 and 10. Yet both are believed to be effective against the proliferation of CaP cell lines by interruption of interaction between growth factors and receptors for growth factors on cell membrane receptors (Kim et al. 1991, Stein et al. 1989, La Rocca et al. 1991). There are also several growth factors implicated in the autonomous growth of androgen-independent cells (Isaacs, 1987, Taneja et al. 1990). These findings have prompted us to investigate the possible synergistic or additive effects of combination therapy using Suramin and SMS 201-995 on CaP cell lines LNCaP and PC3.

Combination therapy using Suramin and SMS 201-995 has a slight beneficial effect (not statistically significant) in low doses for LNCaP, while this combination does not result in a more potent anti-neoplastic regimen for PC3. It is, therefore, possible that SMS 201-995 and Suramin are displacing the same type of growth factor from their receptors even though they have different chemical structures. Growth factors are believed to be important in the growth of androgen-independent cells; hence it was surprising that the combination of SMS 201-995 and Suramin produced more growth inhibition than Suramin alone in an androgen dependent prostate cancer cell line LNCaP (not statistically significant) and not in PC3 cell line which is an androgen independent prostate cancer cell line. However, Suramin and SMS 201-995 combination has other possible theoretical advantages, possibly in patients with metastatic hormone resistant CaP with high prolactin. This group of patients is known to have a very poor prognosis (Mee and Mashinter, 1984). SMS 201-995 is a very potent inhibitor of prolactin (Schally et al. 1988). However, Dupont et al. (1990), reported that in a clinical trial when they used SMS 201-995 on patients with stage D2 disease in combination with LHRH analogues, all 19 patients had progressive disease. Similarly, Schally and Redding, (1987) showed that the combination of LHRH analogue and SMS 201-995 was more effective in inhibiting the growth of hormone dependent prostatic cancer Dunning R-3327H than either agent alone. They explained their observation by stating that SMS analogues inhibit the release of growth hormone and prolactin, which are thought to be important for growth of the normal prostate and possibly play a role in the proliferation of malignant cells. Consequently, at the present time, data from our laboratory, the work of Schally and Redding, (1987) and Dupont et al's. (1990) clinical findings, do not support the use of SMS in advanced hormone-resistant CaP, even though the results of our experiments showed a beneficial effect of SMS and Suramin combination on LNCaP cells - results similar to that of Schally and Redding, (1987). However, we believe that there are more effective methods of hormonal manipulation for androgen-dependent CaP cells than this combination, except of course, if this combination can be shown in clinical practice to prevent or delay the emergence of androgen independent cells better than other simpler first line methods of hormonal manipulations.

4.3.4   Suramin and Estramustine phosphate

Suramin and EMP have been shown in clinical practice to be effective in the treatment of hormone resistant prostate cancer (Chisholm et al. 1977; Johansson et al. 1988; Kim et al. 1991; Manyak et al. 1992; and Eisenberger et al, 1992). The use of Suramin is limited in clinical practice because of side-effects like; polyradiculoneuropathy and coagulopathy when serum levels exceed 300 ug/ml (Freuhauf et al,1990, La Rocca et al. 1990). Suramin is active against prostate cancer in vivo when serum concentration is between 200-300 ug/ml. On the other hand, EMP has some effect against hormone resistant CaP (response rate between 27-36%) but relapse occurs within 12-18 months, and significant adverse effects like fluid retention, nausea and vomiting has limited its use in elderly patients (Benson and Hartley-Asp, 1990).

Moreover, EMP also increases the risk of patients to cardiovascular side effects of oestrogen therapy such as increased risk of myocardial infarction, hypertension and deep vein thrombosis (Edsmyr et al. 1982, de Voogt et al. 1986). Furthermore, EMP and Suramin have different chemical structures and mechanisms of actions. Hence the search for agents that act in synergy (or produce additive effect) with any of these 2 agents remains a worthwhile effort to further palliate patients with metastatic hormone resistant (Stage D2 or D3) CaP without producing significant toxicities or side effects. In Chapter 3, Sections 3.3.1 and 3.3.2, we showed that Suramin and EMP independently produced dose dependent growth inhibition of CaP cell lines LNCaP and PC3.

Consequently the current sets of experiments were carried out, to find out whether the anti-proliferative effects of Suramin on PC3 and LNCaP can be enhanced by combination with EMP. The rationale for these experiments is that the combination of 2 anti-neoplastic agents with potentially different mechanism of actions may result in a more potent anti-neoplastic regimen than either agent alone. Similarly, lower doses of drugs producing synergistic effects, when combined, will reduce toxicity without loss of anti-tumour activity.

The results of the combination of Suramin and EMP shows that the activity of Suramin against CaP cell lines can be enhanced to a significant extent by giving Suramin with EMP. As shown in Fig. 24 the in vitro median doses of Suramin were 250 ug/ml for PC3 and 230 ug/ml for LNCaP. These concentrations can be achieved in vivo; however they approach to the 270 ug/ml level reportedly associated with coagulopathy and polyradiculoneuropathy in clinical practice. (La Rocca et al. 1990, Freuhauf et al. 1990). However, when combined with EMP at concentrations of EMP easily achieved in vivo (less than 12.5 ug/ml) the median dose of Suramin for PC3 and LNCaP cell lines was reduced to a much safer level of 90 and 70 ug/ml respectively (Fig 27). Such levels of EMP are achieved routinely in the clinical setting. EMP of 280 mg twice or three times daily is designed to give a serum level of EMP of between 15 and 25 ug/ml (Gunnarsson et al, 1981). More important is the fact that both Suramin and EMP are taken up by prostatic tumour tissue after oral administration (Fritjofsson et al. 1983). It would therefore appear that an EMP and Suramin combination has great therapeutic potentials in the treatment of patients with hormone-resistant CaP as the two drugs not only act peripherally but also at the prostatic cell level to inhibit the proliferation of CaP cells.

In conclusion, the results obtained from the use of Suramin in recent clinical trials for the treatment of hormone-resistant CaP have been encouraging, although significant neurotoxicity has been dose-limiting. We report here that the action of Suramin is enhanced when Suramin is combined with EMP. The median dose for Suramin in combination was reduced about 4-fold in our assay system. The Suramin and EMP combination was highly effective for both the hormonally dependent and independent CaP cell lines, suggesting that the clinical utility of Suramin and EMP may be more fully exploited when these agents are used in combination. The other major advantage of this finding is that EMP is taken orally and can be given to patients who have had previous radiotherapy, which is in contrast to other chemotherapeutic agents used in the treatment of patients with hormone-resistant (stage D3) CaP where the method of administration is often parenteral and bone-marrow toxicity is an additional complication to worry about (Benson and Hartley-Asp, 1990). Finally, this study suggests that clinical trials employing low doses of a combination of Suramin and EMP may produce increased efficacy and

minimise toxicity of Suramin in patients with metastatic hormone resistant CaP.

In subsequent experiments we will attempt to find out the mechanism of action of a combination therapy using Suramin and EMP and also establish whether the increased efficacy of the drug combination produces synergy or an additive effect only.

4.3.5 Somatostatin SMS 201-995 and Estramustine phosphate

As with the combination of SMS 201-995 and Suramin, the combination of SMS 201-995 and EMP does not result in enhanced inhibition of growth of prostate cancer cells, even though the combination of SMS 201-995 and EMP produced slightly increased growth inhibition on PC3. Even then, this holds true only when one considers the percentage growth inhibition produced by the combination of SMS 201-995 and EMP with that produced by SMS 201-995. For example, SMS 201-995 at 0.2 ug/ml produced 38.1% growth inhibition on PC3 cell line whereas the combination SMS 201-995 (0.1 ug/0.5 ml) and EMP (12.5 μg/0.5 ml) produced 44.8% ($P < 0.7$). EMP alone at 25 μg/ml produced 70%. This would suggest some interference in the activity of EMP against PC3 and LNCaP prostate cancer cell lines by SMS 201-995.

In a way these results are not quite as surprising as it may seem at first since SMS 201-995 and EMP have about the same mechanism of action, namely SMS 201-995 prevents centrosomal separation induced by epidermal growth factor during cell division (Scambia et al. 1988); while EMP acts on microtubule associated proteins to prevent cell replication. The 2 drugs act on spindle formation during cell division (Benson and Hartley-Asp, 1990, Kozlowski, 1992).

Section D

4.4.1   Discussion on whether the effect of Suramin and EMP is synergistic or additive

The combination of EMP and Suramin producing less than 5% growth inhibition individually produced greater than 15% growth inhibition when the 2 drugs were used together on LNCaP and PC3 cell lines. This implies that the activity of Suramin against CaP cell lines can be enhanced in a synergistic fashion when Suramin is given in combination with EMP. Moreover the in vitro median doses for Suramin was 210 ug/ml for PC3 and 240 ug/ml for LNCaP. These concentrations can be achieved in vivo, but they are close to the 250 ug/ml level reportedly associated with coagulopathy and polyradiculoneuropathy. When combined with EMP, the median dose for PC3 and LNCaP cell lines was reduced to a much safer level of 92 and 72 ug/ml respectively in the presence of 12.5 ug/ml of EMP (Fig 27 and Table 21). Such levels of EMP are achieved routinely in the clinical setting (Guannarsson et al, 1981).

The mechanism of the observed synergy is unclear. However the 2 drugs act at different points in the growth pathway of CaP cells as stated before. We used the combination of drugs producing less than 5% growth inhibition on the cell lines as opposed to the Chou and Talalay method of proving synergism or additivity, because their method is open to errors in analysis and requires very complex analytical methods of specially designed computer-programmed analysis (Chou and Talalay, 1983). Enquiries from Apple Macintosh Computers Ltd. in Leicester reveal that such a programme can be designed, but would cost about £10,500! However, personal communications with Dr I Lawrence (1993), of the Department of Pharmacology, University of Leicester, indicated that the method which we used is also an acceptable method of analysis.

Section E

4.5   Discussion on the mechanism of anti-proliferative effects of Suramin, Somatostatin 201-995, EMP and combinations of Suramin and EMP on prostate cancer cell lines

The ability of the cell lines to re-initiate proliferation following removal of the drugs from the culture media suggests that all the drugs when used alone or in combinations act through cytostatic rather than cytotoxic mechanisms. The lack of drug-induced direct cytotoxicity was also confirmed by microscopic appearance of the cells (Figs 34 and 35), when cells exposed to these drugs assumed a round or spherical shape and were not lysed. The lack of drug related cytotoxicity was also confirmed by ^{3}H thymidine release studies. The combination of EMP and Suramin also produced cytostasis and not cytotoxicity. These results are in agreement with those of Berns et al. (1990) and Kim et al. (1991). If one considers that among the desirable properties of an optimal chemotherapeutic agent might be irreversibility of cytotoxic effects on the target cells or induction of terminal differentiation in the target cells, then Suramin, SMS 201-995 or Suramin and EMP combination would appear to be less than optimal, even though the activity of Suramin and EMP produced a greater degree of inhibition of growth and the least response to irreversibility (Fig 31 and 33). However, in clinical usage it is possible that the other properties of Suramin may compensate. For example, its ability to inhibit angio-genesis may result in a decreased rate of tumour growth (Morton et al. 1990). Suramin also suppresses androgen production by inhibition of gonadotrophin binding to its receptors and suppression of pituitary gonadotrophin levels in serum. This inhibition of androgen production by Suramin may be useful in the treatment of advanced prostate cancer (Daugherty et al. 1992). Another way to improve the therapeutic profile of Suramin will be to continue the search to find other chemotherapeutic agents with which Suramin has synergistic activity and also with which it produces tolerable clinical cytotoxicity.

Section F

4.6 The effect of epidermal growth factor on proliferation of LNCaP, PC3 and DU145

The cellular proliferation of LNCaP and DU145 prostate cancer cell line was modulated in a dose-dependent manner by exogenous EGF from a concentration 1-5 ng/ml. Maximal cellular proliferation occurred for both cell lines at 5 ng/ml in our experiments. It is possible that, there are limited numbers of growth factor receptors on the cells and once these receptors have been occupied by EGF, further increments in EGF concentrations would not result in higher cellular proliferation. The growth inhibition produced by Suramin on DU145 and LNCaP can be reduced by excess exogenous EGF. A possible explanation for this is probably that excess EGF can displace Suramin from its receptors on cell membranes, as the growth of cells exposed to Suramin and EGF was higher than those of cells exposed to Suramin alone.

Suramin, while causing growth inhibition in PC3 cell lines, is not significantly affected by exogenous administration of EGF, which would imply that Suramin's growth-inhibitory properties may result from other mechanisms including perhaps interaction with an as yet undetermined growth factor common to all prostate cancer cells, or blockage of other intracellular enzymes, (La Rocca et al. 1991, Kim et al. 1991). Suramin is believed to be extremely protein bound; it is therefore possible that it exerts part of its antitumour activity by this mechanism as this may affect other enzyme systems in vivo. Recently, Kim et al. (1991), have shown that even though PC3 does not respond to exogenous EGF, TGF alpha stimulates its growth in vitro. The results of these experiments therefore demonstrate that different growth factors may be effective in stimulating different androgen-independent CaP cell. Since Suramin is protein-bound, the differences in the morphology of the cells (Figures 37 and 38) could also account for the differences in the stimulating effects of EGF on androgen independent PC3 and DU145 prostate cancer cell lines.

Section G

4.7   The effect of anti-epidermal growth factor receptor on LNCaP, PC3 and DU145 prostate cancer cells

The results of these experiments show that the effect of anti-EGF receptor on LNCaP, PC3 and DU145 is to produce dose dependent growth inhibition of all 3 cell lines, even though the growth inhibitory effect reached statistical significance for PC3 and DU145 cell lines only. This finding confirms that, apart from other possible mechanisms of actions, Suramin, Somatostatin SMS 201-995 and possibly EMP produce dose dependent growth inhibition of prostate cancer lines by interfering with the growth stimulatory effects of growth factors especially on androgen independent prostate cancer cells (Schally, 1988). These findings and the findings of growth stimulatory effect of EGF on LNCaP and DU145, (although not on PC3), suggest that prostate cancer cells have receptors on their membranes for growth factors and prevention of interaction between these growth stimulatory growth factors and the receptors on the cells will have therapeutic implications (Berns et al, 1990, Chodak, 1991, Kim et al, 1991). As anti-EGF receptor concentration higher than 1 ug/ml did not increase the percentage inhibition of growth of LNCaP, PC3 and DU145, just as EGF (Table 27) did not increase the growth stimulatory effect of the cells above 5 ng/ml, it is likely that there are limited numbers of receptors on the cell membranes, and that once these receptors have been blocked by anti-EGF receptors, further increases in concentration of anti-EGF receptors will not result in an increase in growth inhibition (Kim et al. 1991).

In summary, the results of these experiments demonstrate that Suramin, EMP and SMS 201-995 are inhibitory to monolayer proliferation of the androgen-independent prostate cancer cell lines PC3 and DU145 and androgen dependent prostate cancer cell line LNCaP. These cell lines possess EGF receptors especially DU145 and LNCaP, but less so PC3. PC3 has been shown to possess FGF and TGF-alpha receptors (Kim et al. 1991, La Rocca et al. 1991). Suramin and SMS 201-995 appear to interfere with the functional interaction between growth factors and their receptors on cell membrane, hence reducing their cellular proliferation.

The anti-proliferative effect of Suramin on LNCaP and DU145 cell lines was reversible by the addition of exogenous EGF.

CHAPTER 5 :   MATERIALS AND METHODS FOR EXPERIMENTS ON
PROSTATE CANCER CELLS OBTAINED BY PRIMARY
CULTURE

Section A :   Introduction to primary culture of prostate cancer epithelial cells

Section B :   Determination of the effects of Suramin, Somatostatin and EMP on
cells obtained by primary culture of prostate cancer epithelial cells

Section C :   Determination of the effects of drug combinations using EMP and
Suramin on prostate cancer cells obtained by primary culture

Section D :   Determination of whether the effect of Suramin and EMP is
synergistic or additive on prostate cancer cells obtained by primary
cultures

Section E :   Determination of whether the effects of Suramin and EMP was
cytostatic or cytotoxic on prostate cancer cells obtained by
primary culture of CaP epithelial cells

Section F :   The effect of epidermal growth factor on proliferation of prostate
cells obtained by primary culture

MATERIALS AND METHODS FOR EXPERIMENTS ON PROSTATE CANCER EPITHELIAL CELLS OBTAINED BY SHORT TERM PRIMARY CULTURE

Section A

5.1   Introduction to primary culture of prostate cancer epithelial cells

Cell lines are useful in the evaluation of the biological defects involved in carcinogenesis and testing response to chemotherapeutic agents (Peehl et al. 1991). Initial attempts at long term culture of prostate epithelial cells were disappointing due to contamination with He La cells (Nelson-Rees and Flandermeyer, 1976, Webber et al. 1977) and overgrowth by fibroblasts (Reid 1978, Okada and Schroder, 1974). Serial passaging of established prostate cancer cell lines like PC3 and LNCaP can lead to loss of tumour characteristics (Reid et al. 1978 and Otto et al. 1988) thus a reliable method for short term culture of primary tumour tissue might provide a more reliable in vitro cancer model.

To be a useful prostate cancer in vitro model, there is the need to establish a relevant method of characterisation by light and electron microscopy, tumour marker production such as prostate specific antigen or prostatic acid phosphatase, and karyotyping of the tissue. Since prostate specimens can be obtained from transurethral resection of the prostate gland (TURP) chips, radical prostatectomy, Biopty gun biopsies or immediately post mortem, it is important to identify the best tissue source for primary culture.

One of the aims of this section was to establish a reliable short term culture of prostate cancer epithelial cells to be used as an "in vitro" model. We compared the rate of clonal growth from cells obtained from different sources in an attempt to identify the best source of specimen for CaP cell culture.

The response of prostate cancer epithelial cells obtained by primary culture to the following drugs or drug combinations was assessed as in Chapter 2. The drugs or their combinations were:

-         effect of Suramin
-         effect of SMS 201-995
-         effect of EMP
-         effect of Suramin and EMP

The response of cancer cells obtained from the following category of patients to the above drugs or drug combinations was also compared:

(a)   patients with newly diagnosed CaP with no previous hormonal treatment (NDCaP), cells from such patients were assumed to represent hormone-sensitive CaP; and

(b)   patients with hormone-resistant prostate cancer (HRCaP). These were patients who had had maximal androgen blockade in the form of orchidectomy, or medical castration using LHRH analogue, e.g. Zoladex and anti-androgen, e.g. Flutamide, but whose PSA has risen on two successive occasions.

5.1.1   Materials/Methods

Specimens for primary culture were obtained from the following sources:

- Patients undergoing T.U.R.P. because of bladder outflow obstruction due to benign prostatic hyperplasia (BPH). These patients had normal PSA and transrectal ultrasound showing no evidence of malignancy in the prostate gland. Histological examination also confirmed BPH only. Cultures obtained from BPH chips acted as control.

- Patients undergoing channel TURP for obstructing and advanced CaP (Stage C and D).

- Radical prostatectomy specimens performed for early (T1 or T2 NoMo) Stage A and B CaP in our unit.

- Prostatic biopsies from patients with CaP (Stage A, B or C) using a Biopty gun.

- Lymph nodes containing metastases removed during pelvic lymphadenectomy for radical prostatectomy or excised supraclavicular lymph nodes (Stage D disease).

One histopathologist (K.O'Reilly) examined a representative sample of specimens cultured and confirmed the presence of cancer in more than 90% of the specimens submitted for histology. For radical prostatectomy specimens, the histopathologist confirmed the presence of cancer from the edge of the specimen where a wedge section of tissue had been taken for culture. For biopty gun biopsies, a tiny section of each end of the specimen confirmed the presence of cancer before primary cell culture took place. We did not attempt to obtain any specimens from a patient immediately post mortem because of ethical considerations.

The following patients were excluded from our analysis: patients who were on urethral catheters before surgery or patients whose histological analysis revealed less than 90% of chips from TURP specimen contained cancer or whose resection margin from a radical prostatectomy specimen or Biopty gun specimen did not contain cancer.

5.1.2   Media Used

Source: all chemicals and reagents listed were obtained from Sigma Chemical Co Ltd, Dorset, United Kingdom except where otherwise stated.

(a)   Transport medium: 200 mls of transport medium made of 188 mls of RPMI (GIBCO) containing 20 mm Hepes buffer (no L-glutamine) + 10 mls of 22% foetal calf serum (FCS) (equivalent to 5% FCS) and 2 ml of penicillin 100 ug/ml and streptomycin 100 ug/ml. Specimens were kept in transport medium for up to 5 days at 4°C before initiation of dissociation and culture.

(b)   Hanks balanced salt solution (HBSS) prepared in the usual manner (See Appendix A) was used to wash the cells to reduce bacterial contamination.

(c)   Worthington Collagenase solution (600 u/ml) was obtained from Lorne Labs Ltd, Reading, United Kingdom. A working solution was made by dissolving 20 mg collagenase in 5 ml of transport medium. This solution was used for tissue dissociation.

(d)   One litre of primary epithelial growth medium (PEGM) was made up as follows;

    i)   WAJC 404 medium (Kyokuto Pharmaceuticals, Kyoto,Japan) 11.04 gm

    ii)   HEPES 6.7 g = 28.15 ml

    iii) $NaHCO_2$ (1.2g) = 8 mls of 8.4% $NaHCO_2$ solution.

    iv)   Zinc stabilised insulin 2 ml of a working solution of 0.25 mg/ml.

    (v)   Dexamethasone 100 ul of a 10 mM working solution dissolved in 0.1% ethanol.

    (vi)   Epidermal growth factor (EGF) 1 ml of a 10ug/ml working solution.

    (vii)   2 ml of penicillin/streptomycin 100 ug/ml each.

    (viii)   Add distilled water up to 1 litre.

    (ix)   Bring pH up to 7.6 by adding 1 ml of 4N NaOH.

        Each component of the above solutions was filtered using a 0.2 micron sterile filter and the PEGM stored in aliquots of 200 mls at 4^0C for up to three months. Checks were made regularly to ensure that PEGM maintained a constant pH of 7.6.

(e)   Preparation of 50% PEGM. PEGM prepared as in (d) above was regarded as 100% or full strength PEGM. For all experiments to test growth inhibitory or stimulatory culture, the PEGM was diluted using sterile normal saline solution in a ratio of 1:1 to give 50% PEGM. The total volume of medium in each well was 1 ml. Cells were plated at an initial concentration of 2×10^4 per well on day 0.

5.1.2.1   Secondary epithelial growth medium

PEGM was used for the secondary and other subcultures of cells.

The medium for detaching cells to initiate secondary epithelial growth was Worthington Collagenase. A working solution of 200 u/ml was made by dissolving 30 mg collagenase in 22.5 ml transport medium.

10 ml liquid nutrient broth was used to test for the sterility of samples of specimens before proceeding with primary culture.

5.1.3   Tissue dissociation and initiation of primary and secondary epithelial cell culture

Specimens were treated as follows:

Washed twice in HBSS to reduce bacterial contamination.

Stored in transport medium for the next 48 hours at $4^\circ C$. Tested for sterility by placement of a sample in liquid nutrient broth and incubated for 48 hours at $37^\circ C$. If liquid nutrient broth became cloudy, the sample was regarded as contaminated and was discarded.

Histological confirmation of the presence of cancer in the sample was also provided within 48 hours while the test for sterility was going on.

Specimens fulfilling the above criteria were minced and dissociated for 20 hours in Worthington Collagenase (prepared as described above) in an incubator maintained at $37^\circ C$ and 5% CO_2. Fig 42 shows the initial layout of mediums used in primary culture of prostate cancer cells.

At the end of the 20 hours of tissue dissociation, the resulting cell suspension was washed twice with HBSS and cells separated by centrifuging at 2400 rpm for 10 minutes.

Cells were then plated in 200 ml culture flask to which 6 ml of PEGM and 4 ml of cell suspension was added, and it was placed in an incubator maintained at $37^\circ C$ and 5% CO_2.

Cells were also plated in tissue culture flasks containing 10% FCS in RPMI.

Chips digested in
1 Worthington Collagenase
x 20 Hours

Chips in
Transport
Medium

HBSS

Nutrient
Broth

Figure 42

Primary culture of prostate cancer: preliminary stages of primary culture.

Chapter 5

After 5 days, 8 mls of supernatant from a growing colony of cells was removed and replaced with 8 mls of fresh PEGM. After 10-12 days with good clonal growth, the cells were passaged to initiate secondary epithelial growth. The method of passaging was similar to that used for established cell lines (Appendix A).

Secondary Epithelial Cell Culture

Briefly, once there was a good spread from the original, acini and islands of basal cells formed and multiplied (this took about 10-12 days in our experience), the cells were subcultured by detaching cells in tissue culture flasks using collagenase I (200 U/ml). The cells were incubated with 5 ml collagenase I in 200 ml flasks for 1-2 hours at $37^{\circ}C$ by gently shaking them in an orbital shaker until the cells detached. 25 ml of HBSS was added to the tissue culture flasks and the cells collected in a universal plastic tube by centrifugation for 10 minutes at 2400 rpm.

Following the centrifugation, the collagenase was decanted and the cell pellet washed using HBBS solution. The cell pellet was resuspended in PEGM to re-initiate secondary epithelial growth. The above process of initiating secondary epithelial cell culture or passaging, unlike in established prostate cancer cell lines unfortunately, resulted in the loss of about 50% of the cells obtained by primary culture. This made it difficult to obtain enough cells except in cases where a large tissue material was available initially from one patient for the experiments described in Sections B-F. Hence, pooled cells at the first passage from not more than 2 patients were used for most of the experiments.

Cells obtained as above were diluted with half-strength (50%) PEGM (*vide supra*) to obtain a cell density of 2×10^4 cells per ml, ie. to give 2×10^4 cells per well in tissue culture plates. That is, cells obtained at the first passage were used for all experiments described in Section B-F. Cells obtained by primary cell culture also differed from cells of established cell lines as they normally take 48 hours to attach to tissue culture plates as opposed to 24 hours for cells of established prostate cancer cell lines. Hence, test drugs were added 48-hours after plating. The media (with or without test drugs) were changed at 48 hour intervals. Most of the experiments described in Section B-F ran for approximately 8 days.

For biopty gun specimens, the collagenase digestion step was omitted. Instead, 0.5 ml of collagenase (600 u/ml) was added to the specimen placed in a 50 ml tissue culture flask and 2.5 ml of PEGM added to the mixture in the flask.

5.1.4   **Characterisation of prostatic cells obtained by primary culture**

5.1.4.1   **Clonal growth**

4 ml of PEGM was placed in a 50 ml tissue culture flask. 50×10^4 cells obtained by secondary epithelial detachment (passage number 1) using Worthington collagenase (200 U/ml) were placed in 50 ml tissue culture flasks and growth estimated using ^{3}H thymidine incorporation assay on days 2, 4, 6 and 8. The cell doubling times were determined through quantitative dose - response kinetic assays - while cellular proliferation was measured by ^{3}H thymidine incorporation assay. The experiments were carried out in triplicates. One experiment was performed per week.

5.1.4.2   **Tumour marker - prostatic acid phosphatase measurement**

In order to preserve the acid phosphatase activity, culture supernatant fluids in tissue culture plates as described in subsection 5.1.4.1 were buffered with 3M acetate buffer pH 5.5. 50 ul of buffer was added to 1 ml of the sample. The acid phosphatase activity was measured using the dri-stat reagent kit for acid phosphatase produced by Beckman Instruments, Carlsbad, California, 92008-4836. The method utilises alpha napthylphosphate 3M/L in acetate buffer pH 5.5, using fast red TR 1 M/l as the colour forming reagent. Inhibition was carried out with L-tartrate at 2 M/l. The analysis was carried out on the Monarch centrifugal analyser, Instrumental Laboratories Limited, Warrington, UK. The enzyme activity was measured at 30°C using standard settings of the instrument.

5.1.4.3 Presence of keratin

The epithelial nature of the cells was confirmed by staining representative samples of tumour tissue with monoclonal antibody to keratin. Keratin 8 and 18 expression is specific for prostatic epithelial cells. Therefore staining reaction to monoclonal antibody to keratin 8 and 18 was performed to confirm that the cells were of prostatic epithelial origin.

5.1.4.4 Cell morphology using electron microscopy

The malignant nature of the cells obtained was confirmed by phase contrast electron microscopy on cells obtained by secondary epithelial detachment. The morphology of cells obtained by primary culture was compared with the morphology of PC3 cell line at passage number 86 and DU145 at passage number 25. T Deharo of the Electron Microscopic Unit, carried out all the electron microscopic analysis described in this study. Magnification used was x 20,000.

Method of transmission electron microscopy (TEM)

Protection

All tissues were received fixed in formalin or 4% glutaldehyde.

Block selection and processing were performed in a fume cupboard with gloved hands.

Equipment and Reagents

1. Single edge razor blade
2. Dental wax
3. 60°C LTE incubator
4. Formalin
5. 4% glutaldehyde
6. 1% Osmium tetroxide
7. Sorensons phosphate buffer pH 7.4
8. Ethanol
9. Acetone

10.   EMIX resin kit

11.   37° incubator

Method

Samples were centrifuged prior to processing and after each processing step.

1.   Cut tissue into 1mm blocks

2.   Wash in buffer   10 min x 2

3.   Post fix in 1% Osmium tetroxide   60 min

4.   Wash in buffer   10 min x 2

5.   Dehydrate in graded alcohols

        a)   50% ethanol   10 min

        b)   70% ethanol   10 min

        c)   90% ethanol   10 min

        d)   Absolute ethanol   10 min x 2

6.   Acetone   10 min x 2

7.   Equal parts EMIX/acetone without lid 37°C   1 hour

8.   EMIX resin without lid   30°C   2 hour

9*.   Embed tissue in fresh resin

10.   Polymerise   60°C   overnight.

11.   0.5 μm survey sections are cut on a Reichert Jung Ultracut E Microtome
                and stained with 1% toluidine blue.

12.   Ultrathin sections are then cut off the area of interest at 90nm, and
                mounted on a copper grid.

13.   The sections are stained with a saturated alcoholic solution of uranyl
                acetate followed by lead citrate (Reynolds version).

14.   Sections are viewed on a Jeol 100cx electron microscope.

*   Since the specimen needed centrifuging they were embedded in a single
                eppendorf and excess resin removed.

5.1.4.5   **Features of cells used for experiments in primary epithelial cell culture work**

Cells obtained by primary epithelial culture all behaved as androgen-dependent cells as shown by the production of acid phosphatase (vide infra) even when the cells were from patients who were judged clinically to have hormone resistant prostate cancer. Hence, all experiments described below were from pooled cells from not more than 2 patients. The cells used were all obtained from secondary epithelial detachment, that is passage number 1 using Worthington collagenase 200 U/ml. The cells had been growing (that is, period of culture) for about 12 days, before they became confluent in the 50 ml flasks, and therefore were passaged. .

To confirm that the cells were all behaving as androgen-dependent cells, it would have been ideal to test the response of the cells to dihydrotestosterone (DHT). Unfortunately, we were unable to obtain DHT. Consequently, we had to deduce that the cells were androgen-dependent by virtue of their production of prostatic acid phosphatase in culture. Cells obtained from patients with BPH did not produce prostatic acid phosphatase in culture - vide infra - another piece of indirect evidence showing that the prostate cancer cells that we used were most likely to be or behaved as androgen-dependent.

Section B

5.2. **Determination of the effects of Suramin, Somatostatin and EMP on cells obtained by primary culture of prostate cancer epithelial cells**

5.2.1. **Introduction**

Most of the experiments performed in Chapter 2, were carried out on cells with passage numbers between 36-87 for LNCaP, 52-95 for PC3 and 18-36 for DU145. Since the changes in the morphological and possibly functional activities of established cell lines with repeated passaging can affect the response of the cells to chemotherapeutic agents, it was felt that a more accurate response of prostate cancer cells would be obtained by using cells obtained by primary culture of prostate epithelial cells. Hence, most of the experiments described in Chapter 2 were repeated using cells obtained by primary culture of prostate cancer tissues from patients in Leicester whose prostate glands were removed because of obstructive symptoms.

5.2.2 **Materials/Methods**

Cells obtained by primary culture of prostate cancer - as described above (subsection 5.1.4.5) - were plated (at a concentration of 2×10^4 cells/well) in 24 well plates and allowed to initiate growth over the next 48 hours in 50% PEGM. After 48 hours, the media were changed to media containing the following concentrations of :

Suramin	0	10	30	90	270	810 ug/ml
SMS 201-995	0	0.002	0.02	0.2	2	2 ug/ml
EMP	0	3.125	6.25	12.25	25	50 ug/ml

Media were changed on days 4 and 6, and the results read on day 8. The number of cells surviving in each well was assessed using ^{3}H thymidine incorporation assay as previously described. The percentage growth inhibition produced by the 3 drugs was calculated using the previously stated standard formula.

Section C

5.3 **Determination of the effects of drug combinations using EMP and Suramin on prostate cancer cells obtained by primary culture**

The aim of this section was to find out whether EMP and Suramin would produce a synergistic effect or an additive effect as this combination did on established prostate cancer cell lines PC3 and LNCaP.

Prostate cancer cells obtained using the method previously described [Chapter 5, Section (a)] were adapted to growth in 50% PEGM. The cells were then plated in 24 well plates at a concentration of 2×10^4 cells per well and allowed to initiate growth over the next 48 hours. Cells were then exposed to the following concentrations of Suramin 0-810 ug/ml; or Suramin 0-405 ug/0.5 ml and EMP 6.25 ug/0.5ml; or Suramin 0-405 ug/0.5ml and EMP 12.5 ug/0.5ml

The set-up for this experiment on day 3 is as shown on Table 32.

The effects of Suramin and SMS or SMS and EMP in varying concentrations on the proliferation of prostate cells were also analysed.

Table 32

The layout of the drug(s) added to prostate cancer cells obtained by primary culture.

Drugs	Concentrations					
Suramin	0	10	30	90	270	810 ug/ml
Suramin + EMP	0 0	5 6.25	15 6.25	90 6.25	135 6.25	405 ug/0.5 ml 6.25 ug/0.5 ml
Suramin + EMP	0 0	5 12.5	15 12.5	45 12.5	135 12.5	405 ug/0.5 ml 12.5 ug/0.5 ml

Cells plated at 2 x 10^4 cells/well on day 0. On day 2, drug(s) added to triplicate wells as shown in table.

Media were changed on days 4 and 6 and the number of cells surviving in each well was assessed using ^{3}H thymidine incorporation assay on day 8.

Volume of medium in each well = 1 ml.

Section D

5.4   **Determination of whether the effect of Suramin and EMP is synergistic or additive on prostate cancer cells obtained by primary culture**

The aim of this subsection was to determine whether the decrease in the percentage of surviving prostate cancer cells obtained by primary culture, seen when Suramin and EMP were used in combination compared to either agent alone, was due to an additive or synergistic effect.

Prostate cancer cells obtained by primary culture as described above, were adapted to growth in 50% PEGM. The minimum dose of Suramin and EMP which produced less than 5% growth inhibition each, was determined by using smaller concentrations of the drugs. This was found to be 2 ug/ml of Suramin and 1.5 ug/ml of EMP. The effect on the percentage growth inhibition of these 2 drugs combined was then determined. Cells were plated at 2×10^4 cells/well on day 0. Cells were allowed to initiate growth over the next 48 hours. Media containing Suramin (1 ug/0.5 ml) and EMP (0.8 ug/0.5 ml) were then added to the cells in the wells. Media were changed on days 4 and 6 and the results read on day 8. ^3H thymidine incorporation assay was used to calculate the number of surviving cells in each well from which the percentage inhibition of growth was calculated as stated previously.

Section E

5.5 **Determination of whether the effects of Suramin and EMP was cytostatic or cytotoxic on prostate cancer cells obtained by primary culture of CaP epithelial cells.**

The aim of this subsection was to find out more about the mechanism of action of Suramin, that is, whether it produces cytotoxicity or cytostasis and whether the addition of EMP to Suramin resulted in a more cytotoxic or cytostatic agent; in other words, to determine if the effect of Suramin on prostate cancer cells obtained by primary culture is reversible, and whether the addition of EMP to Suramin will result in irreversibility, that is, a more potent combination.

Materials/Methods

Prostate cancer cells obtained by primary culture were plated in 24 well plates at a concentration of 2×10^4 cells/well in 50% PEGM. The cells were allowed to initiate growth for the next 48 hours. At 48 hours, Suramin 270 ug/ml, EMP 25 ug/ml or Suramin (135 ug/0.5 ml) and EMP (12.5 ug/0.5ml) combination was added to separate wells. After 4, 8, 16, and 24 hours triplicate wells were rinsed and fed with media containing no drugs. In another 3 sets of wells, the different drug concentrations were replaced on days 5 and 7; that is, cells were exposed continuously to the drugs for 5 or 7 days. On day 8, ^{3}H thymidine incorporation assay was used to assess the number of cells surviving in cells exposed to no drugs (control), exposed to drugs for 4 hours, 8 hours, 16 hours, 24 hours, and exposed to drugs continuously for 5 and 8 days. Similarly, aliquots of media in each well were assessed for the levels of PAP, to see whether the cells were still producing acid phosphate. The gelatin method was used to measure PAP as described before.

Section F

5.6   Determination of the effect of epidermal growth factor (EGF) on proliferation of prostate cancer cells obtained by   primary culture

The aim of the experiment was to find out if excess EGF influenced the rate of proliferation of prostate cancer cells obtained by primary culture and whether excess EGF blocked-Suramin-induced growth inhibition of prostate cancer cells obtained by primary culture.

Cells were adapted to growth in 50% PEGM. Cells were plated at 2×10^4 cells/well in 24 well plates. After 48 hours, a medium containing EGF (0-125 ng/ml) was added to the cells. Fresh media were added to the cells on day 4 and the number of cells in each well quantitated using ^3H thymidine incorporation assay on day 5.

In a second set of experiments designed to find out if EGF was capable of reversing Suramin-induced growth inhibition, cells were exposed to the following drugs:

(a)   Control - no drugs added

(b)   EGF (25 ng/ml)

(c)   Suramin (90 ug/ml)

(d)   EGF (12.5 ng/0.5ml) and Suramin (45 ug/0.5ml) combination.

Cells surviving in each well were counted on days 4, 6 and 8 using ^3H thymidine incorporation assay.

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CHAPTER 6 :   *RESULTS OF EXPERIMENTS ON PROSTATE CANCER CELLS OBTAINED BY PRIMARY CULTURE*

Section A :   Results of primary culture using prostate cancer specimens from TURP chips, radical prostatectomy biopsies, biopty gun and lymph node metastasis.

Section B :   Results of cell characterisation using phase contrast electron microscopy

Section C :   Results of primary culture of prostate cancer cells from different sources

Section D :   Results of the effects of Suramin , SMS 201-995, EMP and (Suramin + EMP) combination on cells obtained by primary culture of CaP epithelial cells

Section E :
Suramin   Results of experiments to determine whether the effect of and EMP was synergistic or additive

Section F :   Results of experiments to determine whether the effect of the drugs or drug combinations was cytostatic or cytotoxic on prostate cancer cells obtained by primary culture

Section G :   Results of experiments to determine the effect of epidermal growth factor on proliferation of cells obtained by primary culture.

Section A

Table 33 shows the profile of specimens collected for primary prostatic epithelial cell culture. Of the 37 specimens collected, 12 specimens failed the initial sterility test. Of the 12 specimens failing the initial sterility test, 6 were from patients on urethral catheter drainage for a mean duration of 5 (range 2-14) days prior to prostatectomy.

One of the 3 specimens obtained by biopty gun method failed the sterility test while no identifiable reason was found for 5 specimens that failed the initial sterility test. Four specimens on histological analysis contained less than 90% cancer and were not cultured.

Fig 43 shows primary epithelial cells (acini) without fibroblasts, growing from a prostate cancer specimen when the cells were grown in PEGM, while a representative sample of the same specimen grown in 10% FCS (Fig 44) shows growth of a mixture of epithelial cells and fibroblasts. Secondary culture of cells growing in PEGM continued to yield epithelial cells while that grown in 10% FCS produced mostly fibroblasts.

Fig 45 shows typical prostate cancer specimen staining positive to the presence of Keratin 8 using monoclonal antibody to Keratin 8, that is, the cells stained positive to the presence of keratin, confirming the epithelial nature of cells cultured.

Section B
6.2.   Results of cell characterisation using phase contrast electron microscope
Phase contrast electron microscopy of epithelial cells obtained by primary culture of CaP confirmed the malignant origin of the cells as shown by the presence of organelles such as rough endoplasmic reticulum, golgi bodies, ribosomes that are arranged in a regular pattern around an irregular nucleus, the presence of numerous normal mitochondria, few tonofilaments and interdigitating cytoplasmic projections or intercellular junctions (Fig 46). In contrast, phase contrast electron microscopy of epithelial cells obtained

Table 33

Profile of Specimens for Prostatic Epithelial Cell Culture

Total number of specimens collected	37
Number of specimens contaminated	12
- Number on urethral catheter	6
- Biopty gun specimen	1
Number of specimens processed	25
Number without CaP involvement > 90%	4
Number of specimens analysed	21

Histology
   -    well differentiated
   -    moderately differentiated
   -    poorly differentiated
	3
	2
	3

Initial sterility test prevented wastage of resources on culturing specimens that would not have grown due to bacterial contamination "ab initio".

Fig 43

Epithelial cells (acini) without fibroblasts growing from CaP specimens when cells were cultured in PEGM (WAJC 404 & supplements). WAJC 404 medium suppresses the growth of fibroblasts.
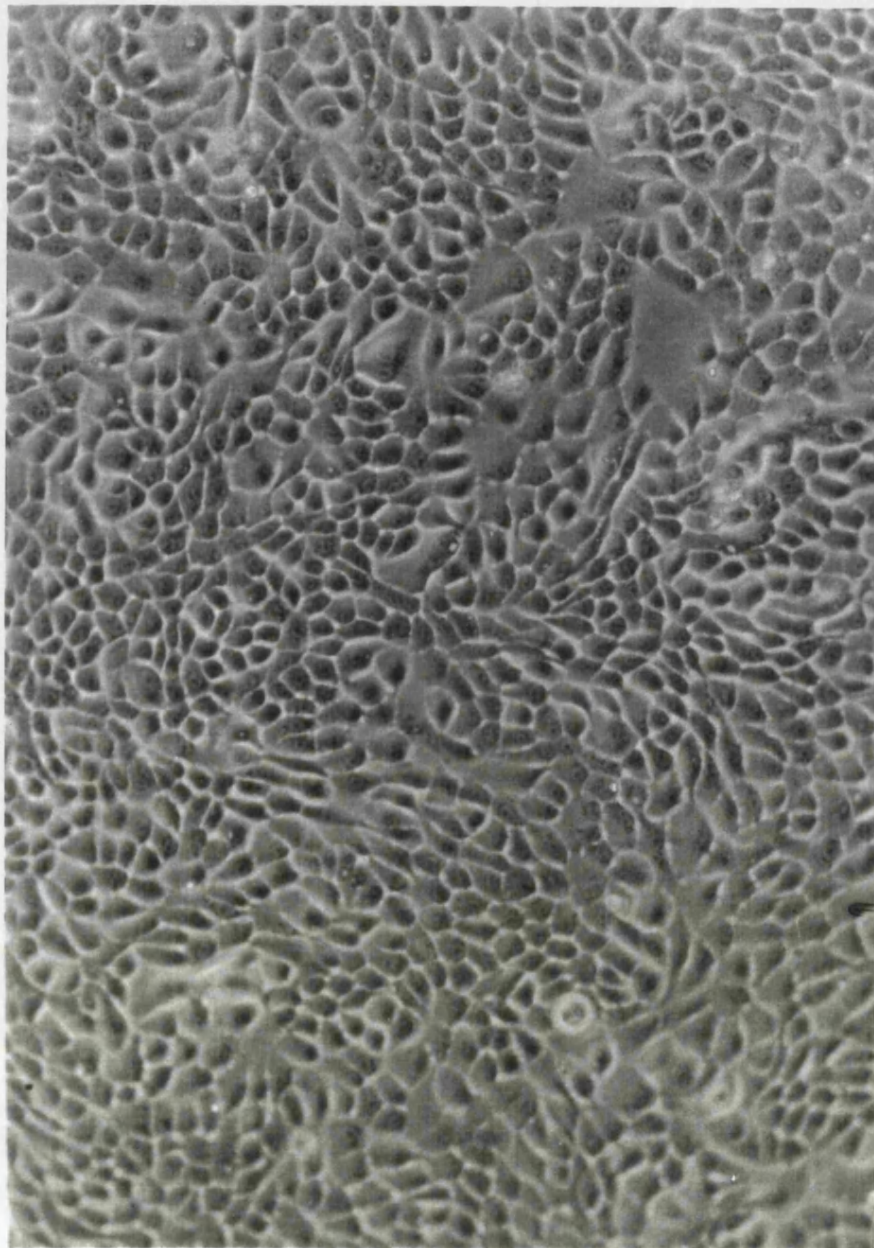
Fig 44

Epithelial cells and fibroblasts growing from CaP specimens when cells were cultured in 10% FCS.
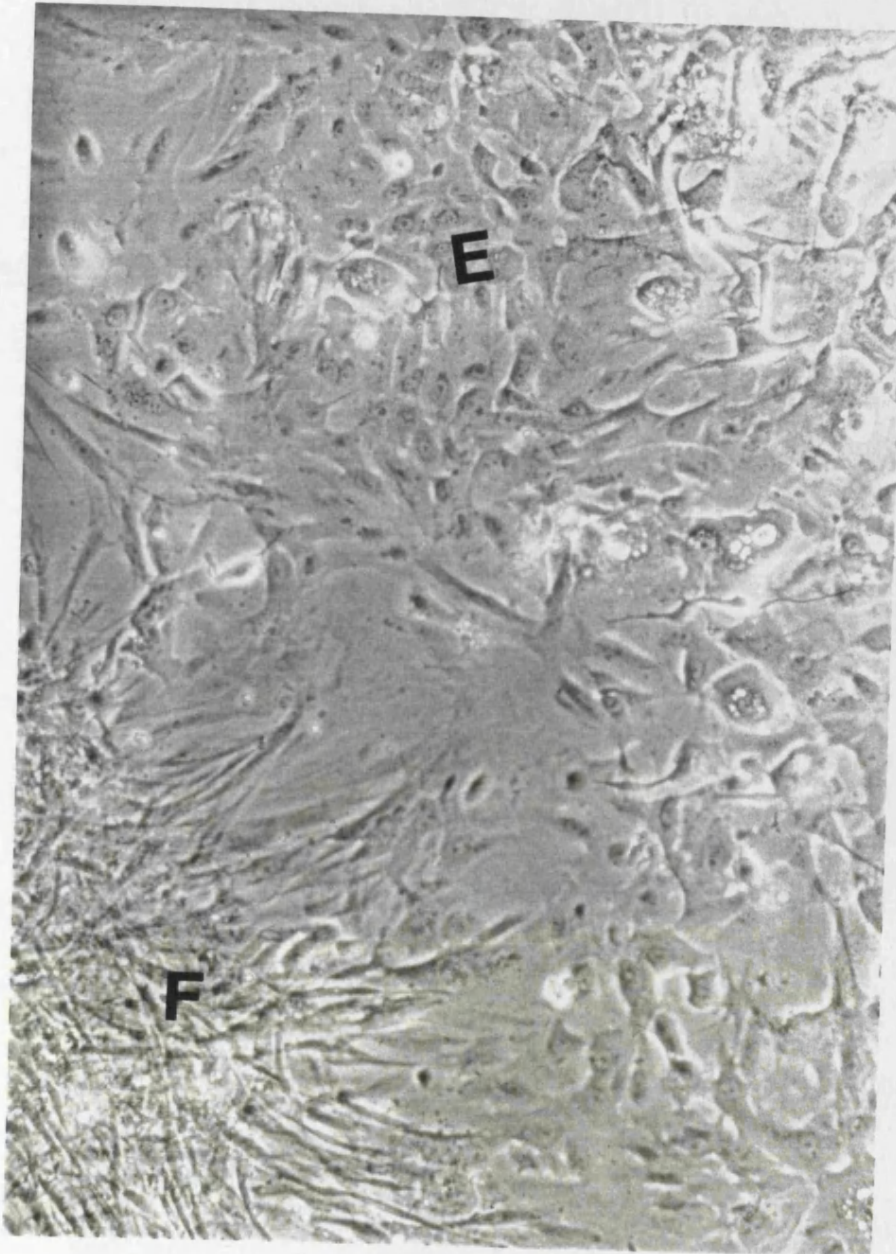
E = Epithelial cells   F = fibroblasts

Fig 45

Prostate cancer specimen staining positive to the presence of keratin 8 (using monoclonal antibody to keratin 8).
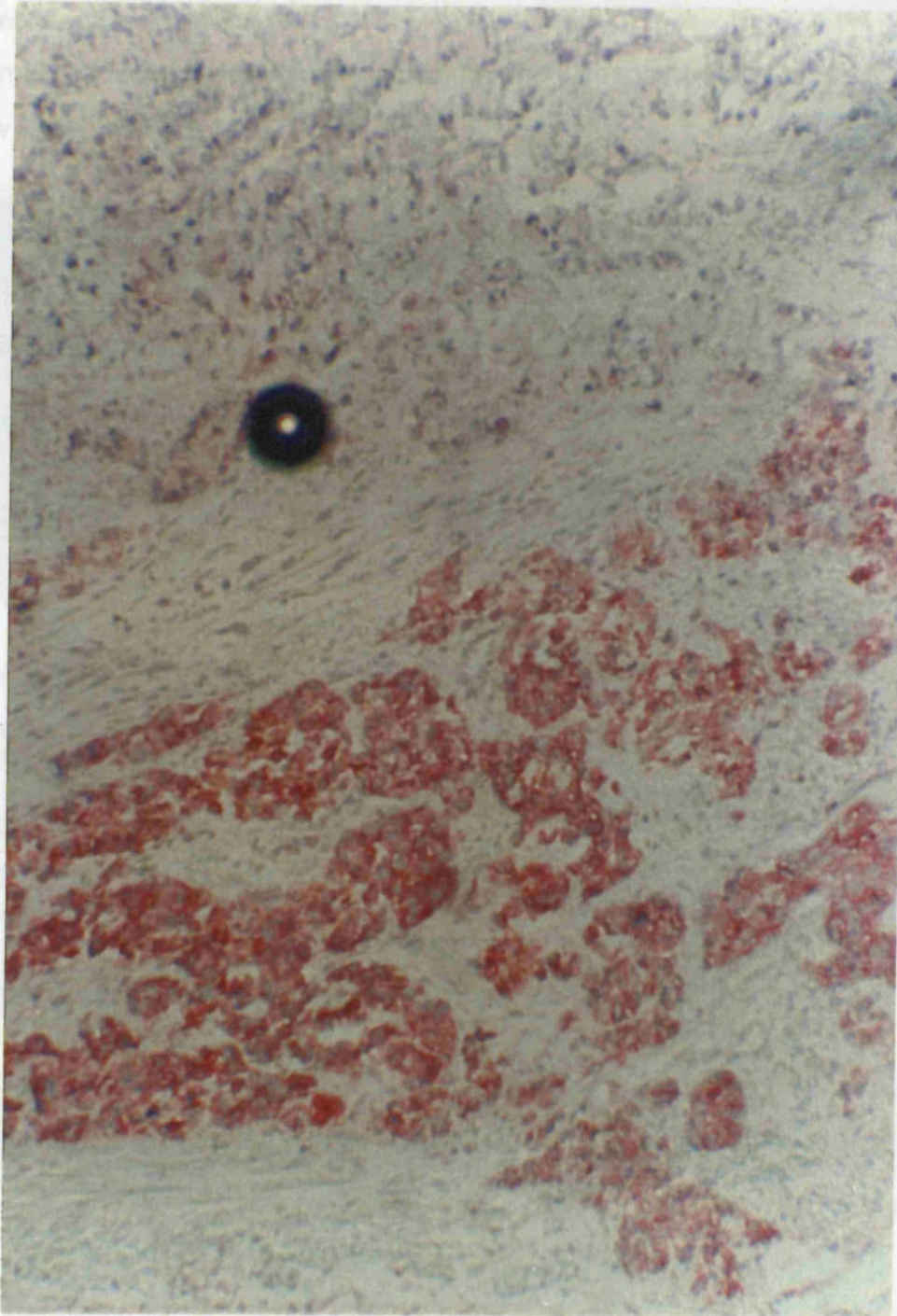
This confirms the epithelial nature of the cells cultured.

Fig 46

Phase contrast electron microscopy of epithelial cells obtained from culture of cancer of prostate cells (well differentiated). Note the following features:

- organelles are arranged in a regular pattern, around the irregular nucleus
- normal mitrochrondria
- numerous rough endoplasmic reticulum and lysosomes with lipids
- few tonofilaments
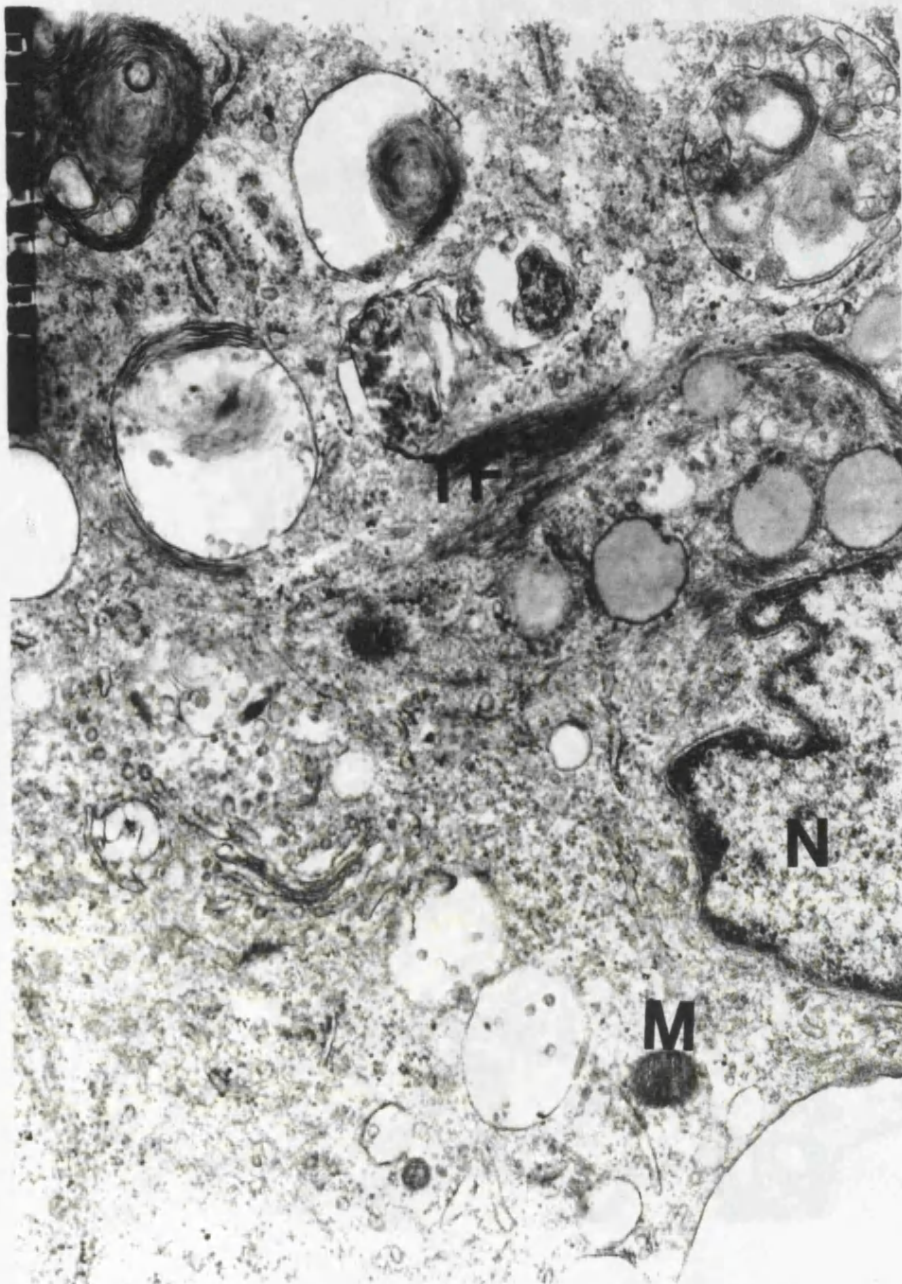
TF   =   tonofilament, M = mitochondria, N = nucleus

Magnification x 20,000

Fig 47

Phase contrast electron microscopic appearance of epithelial cells obtained from culture of benign prostatic hyperplasia showing numerous tonofilaments (cytoskeleton).
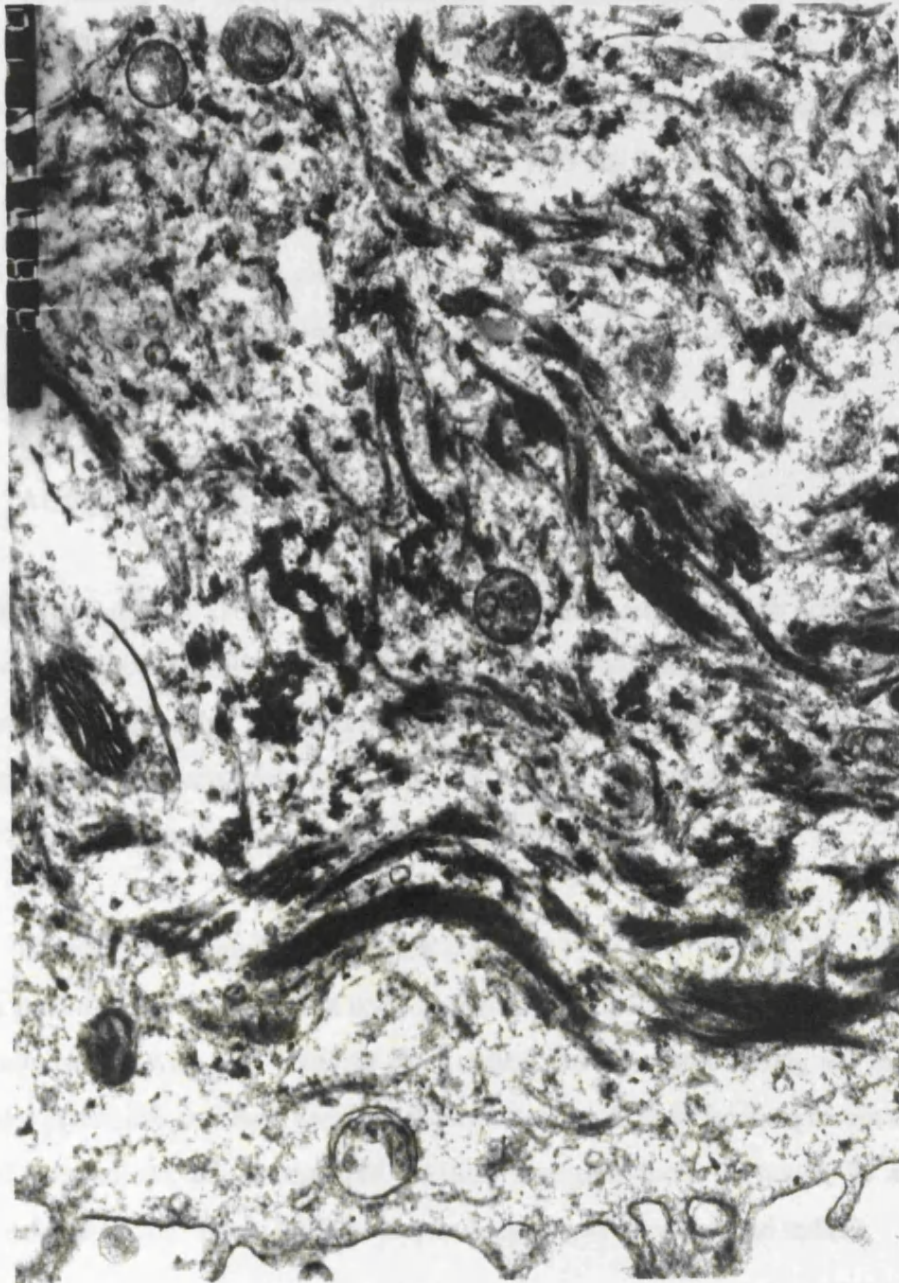
Magnification x 20,000

from BPH (Fig 47), showed the following features: numerous tonofilaments, organelles that were arranged in a regular pattern around a regular nucleus; and more interdigitating cytoplasmic projections holding the cells together. Similarly, electron microscopy of PC3 at passage no. 86 (Fig 38) showed that the mitochondria were swollen, elongated, distorted and had a dense matrix and with organelles not arranged in a regular pattern around the nucleus. The organelles were spread throughout the cytoplasm and there were also few interdigitating cytoplasmic projections or intercellular junctions; no tonofilaments were seen. Electron microscopy of DU145 prostate cancer cell line at passage number 25 (Fig 37) revealed more healthy looking organelles, such as normal mitochondria, numerous rough endoplasmic reticulum and more intercellular junctions compared to PC3 cell line at passage number 86.

The only distinguishing feature on electron microscopy between cells from newly diagnosed prostate cancer (NDCaP) and hormone-resistant prostate cancer (HRCaP) was the presence of fewer tonofilaments (cyto-skeleton) in cells from HRCaP.

6.2.1   Acid phosphatase production

As shown in Figure 48 and Table 34, the cells in culture produced prostatic acid phosphatase and there was a good correlation (r^2 = 0.96) between the amount of acid phosphatase produced and the total number of cells in culture. Cells from patients with hormone-resistant prostate cancer also produced acid phosphatase in culture, almost at the same rate as cells from newly diagnosed prostate cancers, as shown in Fig 49 and Table 35.

6.2.2   Clonal growth and cell doubling time

The in vitro growth rate was dependent upon the initial cell concentration plated, as well as the source of cells plated, with cells obtained from radical prostatectomy having the most rapid rate of cellular proliferation. Using linear regression analysis of the individual growth curves of differing cell populations, the average cell doubling time was about 36 hours, 33 and   28 hours for cells obtained from TURP chips, biopty gun biopsies and radical

Table 34

Prostatic Acid Phosphatase (PAP) production in the supernatant fluid of confluent CaP epithelial cells obtained by primary culture.

	PAP U/L				Cells in well (DPM)			
Day	Expt 1	Expt 2	Expt 3	Mean ± SEM	Expt 1	Expt 2	Expt 3	Mean ± SEM
0	0	0	0	0	1782	1631	1510	1641±25
2	1.6	1.5	1.4	1.5 ± 0.06	2561	2783	1808	2384±39
4	5.2	5.9	4.8	5.3 ± 0.32	3961	3779	3003	3581±87
6	6.6	7.9	8.3	7.6 ± 0.51	4862	4374	5500	4912±21
8	8.6	5.1	10.6	8.1 ± 1.61	5831	5204	6023	5686±56

Cells plated at 5 x 10^5 cells per 50 ml tissue culture flasks containing 4 mls of PEGM as the growing medium from day 1 to 8.

The corresponding number of cells/well using ^3H thymidine incorporation, whose culture supernatant has been removed to estimate PAP level is shown on the right hand side of the table.

r^2 = 0.96 (indicating a good correlation between total number of cells in culture and the amount of acid phosphatase produced).

Results represent the mean of 3 experiments.

Fig 48

Prostatic acid phosphatase in the supernatant of confluent CaP epithelial cells obtained by primary culture.
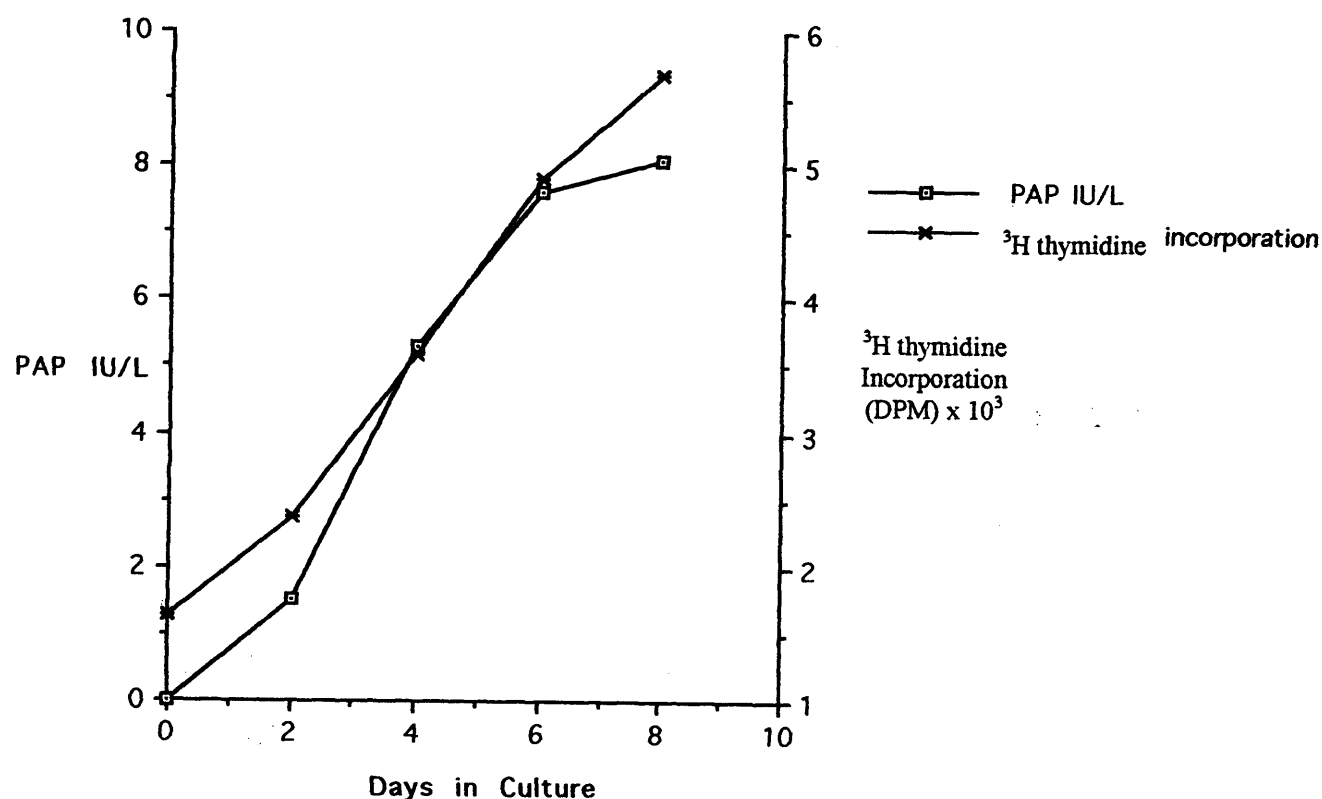
Cells plated at 5 x 10^4 cells per 50 ml tissue culture flasks containing 4 mls of PEGM as the growing medium on day 0.

The concentration of PAP in the supernatant of growing cells versus the number of cells in culture as measured by ^{3}H thymidine incorporation (DPM) from day 1 to 8.

r^2 = 0.96 indicating a good correlation between the number of cells in culture and the amount of PAP produced.

Results represents the mean of 3 experiments.

Table 35

Prostatic acid phosphatase levels in the supernatant of culture media of cells derived from BPH, NDCaP, HRCaP specimens.

Days in culture	PAP IU/L		
	BPH	NDCaP	HRCaP
2	0.1	2.1	2.6
4	0.15	3.7	3.0
6	0.19	4.1	5.0
8	0.18	5.2	5.3

Cells plated at 5×10^5 cells per 50 ml tissue culture flasks containing 4 mls of PEGM as the growing medium. PAP in supernatant of growing cells estimated on days 2 to 8 as shown.

Results represent the mean of 3 experiments using cells from different patients 3 times. One experiment was carried out per week.

See appendix for raw data.

BPH   =   Benign prostastic hyperplasia

NDCaP   =   Newly diagnosed cancer of the prostate

HRCaP   = Hormone resistant cancer of the prostate

Cells from patients with HRCaP produced PAP in culture at about the same rate as cells from patients with NDCaP.

BPH cells produced minimum PAP in culture.

Fig 49

Prostatic acid phosphatase levels in the culture supernatants of cells derived from BPH, NDCaP and HRCaP.

Cells plated at 5 x 10^5 cells/50 ml tissue culture flasks containing 4 ml of PEGM. PAP in supernatant of growing cells estimated on days 2 to 8.

Cells from patients with HRCaP produced PAP in culture at about the same rate as cells from NDCaP.

BPH cells produced minimal PAP in culture.

BPH = Benign prostatic hyperplasia.
NDCaP = Newly diagnosed cancer of the prostate.
HRCaP = Hormone resistant cancer of the prostate.

prostatectomy specimens respectively. Cells growing from metastatic specimens proved difficult to culture compared to cells obtained from other sources, with a cell doubling time of about 44 hours. Cells from biopty gun specimens yielded small quantities of slow-growing cells, surviving no more than three passages.

Section C

6.3   Result of primary culture of prostate cancer cells from different sources

Table 36 summarises the rate of successful culture of CaP epithelial cells using PEGM whose main ingredient was WAJC 404 medium. We successfully cultured proliferating CaP cells from three patients with stages A and B and five patients with Stages C and D prostate cancer diseases.

Section D

6.4.   Results of the effects of Suramin, SMS 201-995 and EMP on cells obtained by primary culture of CaP epithelial cells

The growth inhibitory effect of Suramin, SMS 201-995 and EMP on prostate cancer cells obtained by primary culture are shown in Table 37 and Figs 50, 51 and 52. Suramin, EMP and SMS 201-995 produced dose-dependent growth inhibition on cells obtained by primary culture. EMP produced 79% inhibition at a concentration of 12.50 ug/ml, while Suramin produced 60.7% at a concentration of 270 ug/ml (highest serum levels achievable in vivo).

Fig 50 shows that the half-maximal inhibitory dose of Suramin on prostate cancer cells obtained from primary culture was 90 ug/ml in 50% PEGM. These results are similar to those obtained using established prostate cancer cell lines PC3 and LNCaP.

6.4.1   Results of the effects of EMP and Suramin combination on prostate cancer cells obtained by primary culture of CaP epithelial cells

The results are as shown in Table 38, Fig 53.

Table 36

The rate of successful culture of prostatic epithelial cells from specimens obtained from different sources using WAJC 404.

Diagnosis	Type of specimen	No	Clonal growth	Reason for failure
BPH	TURP	6	5	unknown x 1
CaP	Channel TURP (Stage C and D)**	6	2	unknown x 3 infection x 1
CaP	Radical prostatectomy (Stage A and B)**	3	3	0
CaP	Biopty Gun (Stage C and D)	3	2	infection x 1
CaP	Lymph node metastases (Stage D)*	3	1	unknown x 1 infection x 1
Total		21	13	8

*   Lymph node metastasis from:
-        Pelvic obturator lymph nodes x 2
-        Supraclavicular metastasis x 1

**   Successful culture of cells from patients with Stage A, B, C and D disease.

Table 37

The effect of Suramin (0-810 ug/ml), EMP (0-50 ug/ml) and Somatostatin 201-995 (0-20 ug/ml)on prostate cancer cells obtained by primary culture.

Suramin ug/ml	% Growth inhibition	EMP ug/ml	% Growth inhibition	SMS 201-995 ug/ml	% Growth inhibition
0	0	0	0	0	0
10	19, 8 , 14	3.12	33, 29, 26	0.002	6, 4, 5
Mean ± SEM	13.6 ± 2		29 ± 6		5 ± 2
30	42, 48, 36	6.25	52, 62, 63	0.02	6, 8, 7
Mean ± SEM	42 ± 8		59 ± 7		7 ± 1
90	44, 49, 54	12.5	81, 84, 72	0.2	19, 18 23
Mean ± SEM	49 ± 4		79 ± 7		20 ± 3
270	58, 60, 64	25	84, 89, 85	2	40, 36, 29
Mean ± SEM	60.7 ± 3		86 ± 2		35 ± 8
810	59, 65, 69	50	88, 92, 84	20	42, 40, 41
Mean ± SEM	64.3 ± 4		88 ± 3		41 ± 1
Suramin		EMP		SMS	

Suramin (0-810 ug/ml), EMP (0-50 ug/ml), SMS (0-20 ug/ml) added to 2×10^4 cells/well on day 2 of study. Total volume of medium per well = 1 ml.

Results represent the mean of 3 experiments. See Appendix for raw data. Suramin, EMP and SMS 201-995 produced dose dependent growth inhibition of cells obtained by primary culture.

Fig 50

The effect of Suramin (0-810 ug/ml) on prostate cancer cells obtained by primary culture.
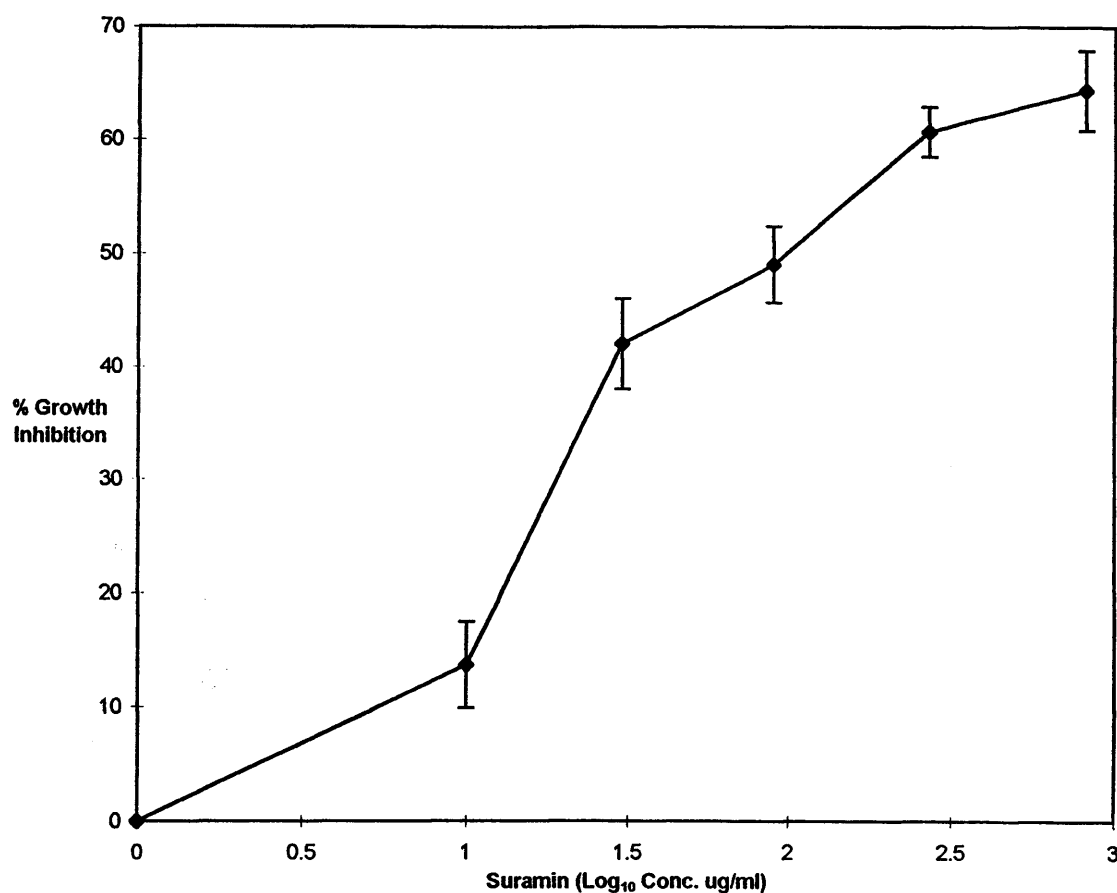
Suramin 0-810 ug/ml added to 2×10^4 cells/well on day 2. The number of cells surviving in each well determined on day 8.

Values represent the mean of 3 experiments. (See appendix for raw data).

Suramin produced dose dependent growth inhibition of prostate cancer cells obtained by primary culture.

Fig 51

The effect of Estramustine phosphate (0-50 ug/ml) on cancer cells obtained by primary culture.
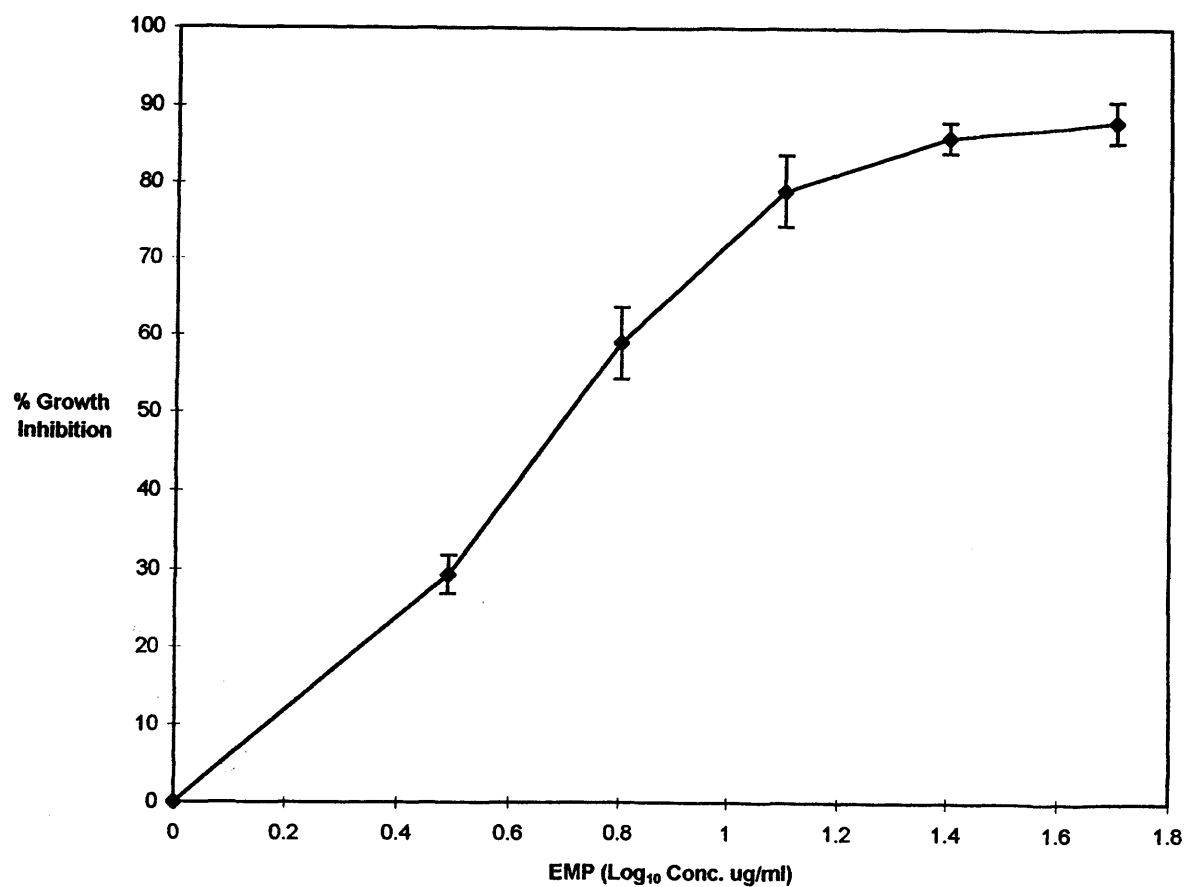
EMP (0-50 ug/ml) added to 2×10^4 cells/well on day 2.

The number of cells surviving in each well determined on day 8.

EMP produced dose dependent growth inhibition of prostate cancer cells obtained by primary culture.

Results represent the mean of 3 experiments.

See Appendix for raw data.

Fig 52

The growth inhibitory effect of SMS 201-995 (0-20 ug/ml) on prostate cancer cells obtained by primary culture.
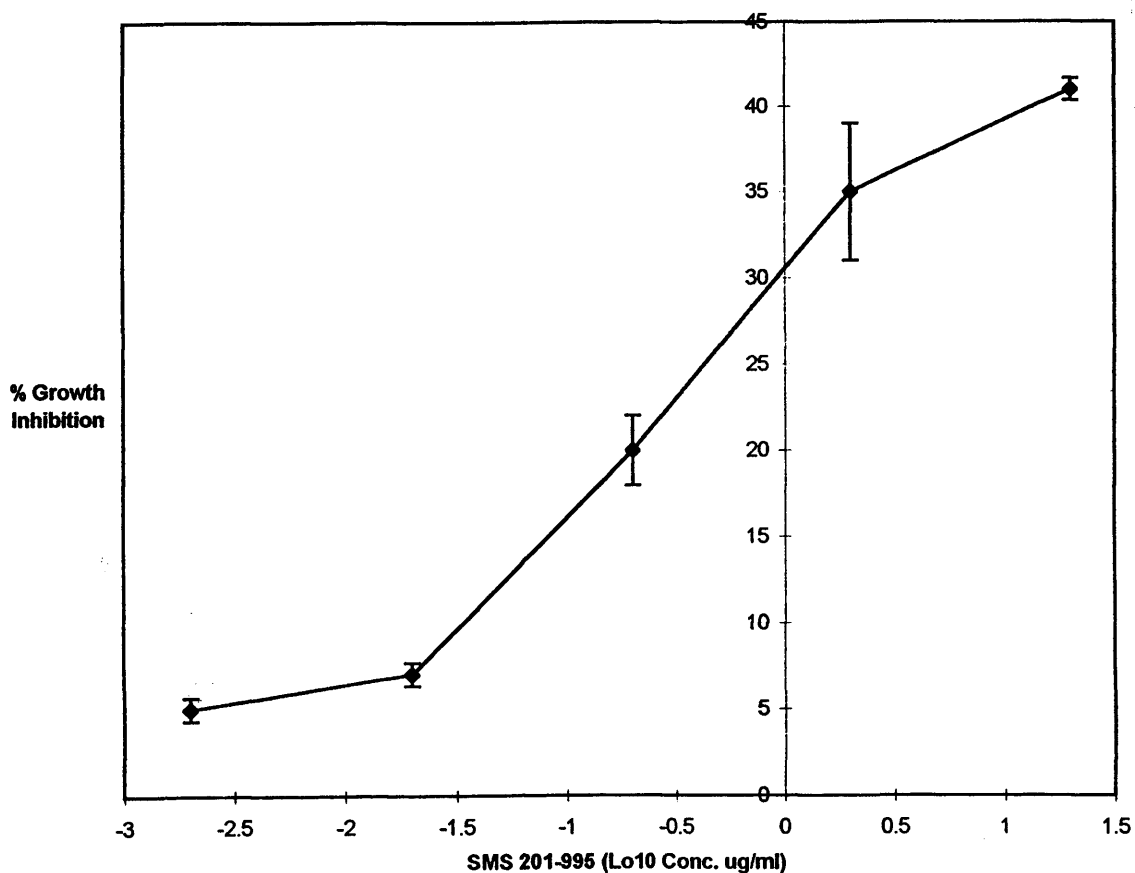
SMS (0-20 ug/ml) added to 2 x 10^4 cells/well on day 2.

The number of cells surviving in each well was determined on day 8.

Results represent the mean of 3 experiments. See Appendix for raw data.

SMS 201-995 produced dose dependent growth inhibition of prostate cancer cells obtained by primary culture.

Table 38

The effect of Suramin (0-270 ug/ml) or EMP (6.25 ug/0.5 ml) and Suramin (0-135 ug/0.5 ml) combination on prostate cancer cells obtained by primary culture.

Drugs	Sur 10 ug/ml	Sur 5 + EMP 6.25 ug/0.5 ml	Sur 90 ug/ml	Sur 45 + EMP 6.25 ug/0.5 ml	Sur 270 ug/ml	Sur 135 + EMP 6.25 ug/0.5 ml	EMP 12.5 ug/ml
% Surviving cells	61%*	37%*	51%	30%	40%+	24%+	42%

Drugs	Sur 10 ug/ml	Sur 5 + EMP 12.5 ug/0.5 ml	Sur 90 ug/ml	Sur 45 + EMP 12.5 ug/0.5 ml	Sur 270 ug/ml	Sur 135 + EMP 12.5 ug/0.5 ml	EMP 25 ug/ml
% Surviving cells	61%**	34%**	51%	25%	40% ++	21%++	39%

Suramin (0-270 ug/ml) or Suramin (0-135 ug/ 0.5ml) + EMP (6.25 ug/0.5 ml or 12.5 ug/0.5 ml) added to 2×10^4 cells/well on day 2. Media containing fresh drug concentration(s) added to cells on days 2, 4 and 6. The number of cells surviving in each well assessed on day 8.

Suramin 0 = Control (100% cells surviving)

Analysis of Variance:

* $P < 0.6$ - difference not significant
** $P < 0.8$ - differences not significant
+ $P < 0.7$ - differences not significant
++ $P < 0.5$ - differences not significant

Results represent the mean of triplicate experiments. See appendix for raw data.

The combination of EMP and Suramin did not result in a statistically significant decrease in the percentage of surviving prostate cancer cells obtained by primary culture

Fig 53

The effect of EMP + Suramin combination on prostate cancer cells obtained by primary culture.
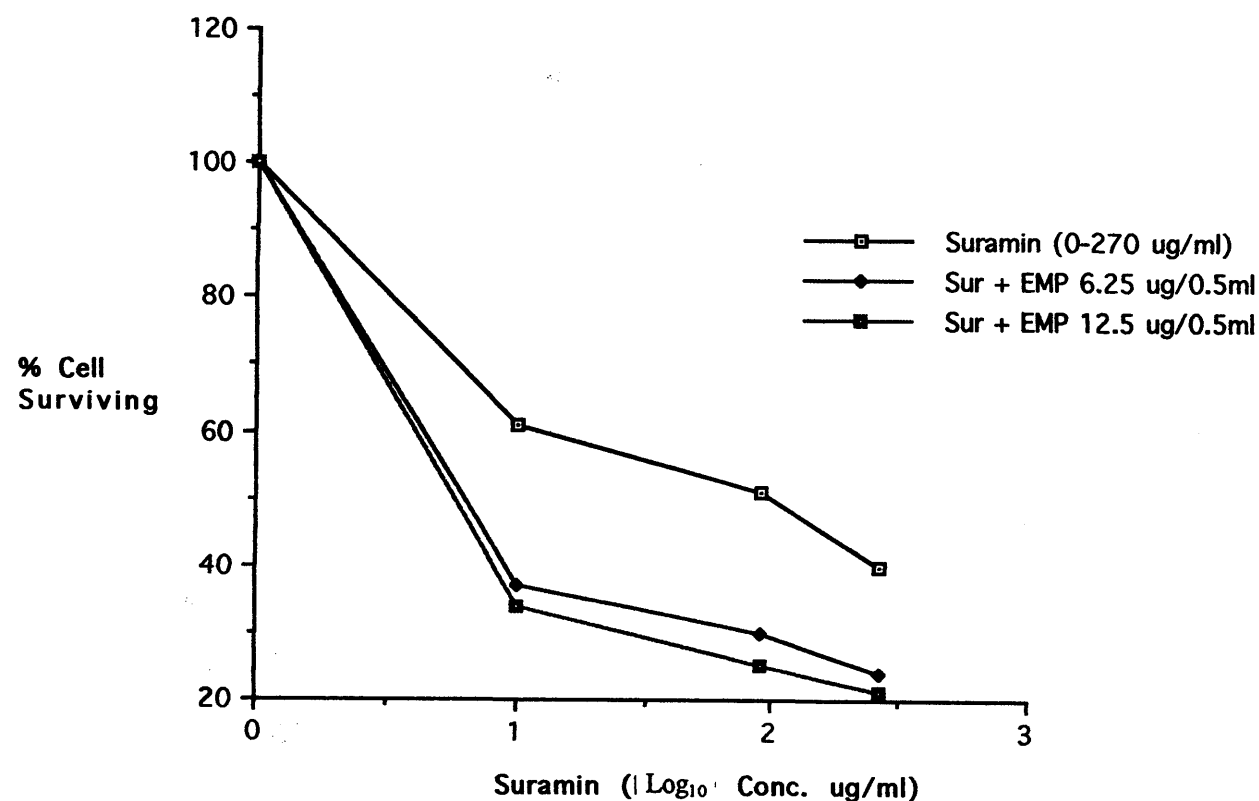
Suramin (0-270 ug/ml) or Suramin (0-135 ug/0.5 ml) + EMP (6.25 ug/0.5 ml or 12.5 ug/0.5 ml) added to 2 x 10⁴ cells/well on day 2. Media containing fresh drug(s) concentration added to cells on days 2, 4 and 6. The number of cells surviving in each well was assessed on day 8.

Results represent the mean of 3 experiments. (See Appendix for raw data).

Suramin 0 ug/ml = control (100% surviving cells).
The combination of EMP and Suramin did not produce a statistical significant decrease in the percentage of surviving cells.

The percentage of surviving cells with a Suramin concentration of 10 ug/ml was 61%, with EMP 12.5 mg/ml added; the percentage of surviving cells was 37% (P<0.6 - not statistically significant) even though, the effects were at least additive in nature. Increasing doses of EMP to 25 ug/ml and Suramin to 270 ug/ml did not result in a significant decrease in the percentage of surviving cells.

The combinations of Suramin + or SMS 201-995, or EMP + SMS 201-995 and EMP did not result in a significant inhibition of proliferation of prostate cancer cells obtained by primary culture either.

Section E

6.5 Results of experiments to determine whether the effect of Suramin and EMP combination was synergistic or additive

The inhibitory effects of a combination of Suramin 2 ug/0.5ml and EMP 1.5 ug/0.5 ml was as shown in Table 39.

Suramin and EMP at a dose of 2.0 ug/ml and 1.5 ug.ml each produced a mean growth inhibition of 4.7% and 4.8% respectively on prostate cancer cells obtained by primary culture, while the 2 drugs combined in the same doses produced a mean growth inhibition of 6.0% with no statistically significant difference between the means (P < 0.9).

Section F

6.6 Results of experiments to determine whether the effects of the drugs or drug combination was cytostatic or cytotoxic on prostatic cancer cells obtained by primary culture

The results are as shown in Table 40 and Fig 54, which shows the percentage of cells surviving in each well after exposure of the cells to the drugs for the length of time specified.

Fig 54 shows that the growth inhibitory effects of Suramin, EMP, SMS 201-995; as well as EMP and Suramin combination were reversible to a varying extent up to 24 hours after exposure to the drugs. However, the percentage of cells surviving after 24 hours of

Table 39

The effect of combining doses of Suramin and EMP producing less than 5% growth inhibition on prostate cancer cells obtained by primary culture.

% Growth inhibition		EMP 1.5 ug/ml	Sur 2 ug/ml	EMP 0.8 ug/0.5 ml + Sur 1.0 ug/0.5 ml
Experiments	a	4.1	3.9	6.1
	b	5.1	4.9	6.8
	c	4.9	5.2	5.2
Mean ± SEM		4.7* ± 0.3	4.7* ± 0.4	6.0* ± 0.5

EMP 1.5 ug/ml, Sur 2 ug/ml, EMP 0.8 ug/0.5 ml + Sur 1.0 ug/0.5 ml added to 2 x 10^4 cells/well on day 2. Fresh media containing appropriate doses of drugs added to wells on days 4 and 6. Number of cells surviving in each well assessed on day 8.

* $P < 0.9$ - not significant - analysis of variance.

Results represent the mean of 3 experiments.

The combination of EMP and Suramin resulted in a higher growth inhibition which is due to an additive effect and not a synergistic effect.

Table 40

Reversibility of the effects of Suramin 270 ug/ml, EMP 25 ug/ml, SMS 201-995 2 ug/ml, EMP (12.5 ug/0.5ml) and Suramin (135 ug/0.5 ml) combination on proliferation of prostate cancer cells obtained by primary culture.

Drug concentration	Hours of continuous exposure					
	0	4	8	16	24	144
	% Surviving cells					
Suramin 270 ug/ml	100	90	85	60	53	9
EMP 25 ug/ml	100	67	50	42	34+	8
EMP 12.5 ug/0.5 ml + Suramin 135 ug/0.5 ml	100	47	35	29	17+	5
SMS 201-995 2 ug/ml	100	95	94	88	82	16

Control wells were not exposed to any drugs.

After 4, 8, 16 and 24 hours of exposure to the drugs, fresh media containing no drugs replaced media with drugs. All results read on day 8.

+ $P < 0.35$ (not significant)   Analysis of variance

Results represent the mean of triplicate experiments. See appendix for raw data.

The growth inhibitory effect of Suramin, EMP, Suramin + EMP and SMS 201-995 on prostate cancer cells obtained by primary culture was reversed more than 24 hours after exposure to the drugs.

Fig 54

Reversibility of the effects of Suramin (270 ug/ml), EMP (25 ug/ml), SMS 201-995 (2 ug/ml), and EMP (12.5 ug/0.5 ml) + Suramin (135 ug/0.5 ml) combination on proliferation of prostate cancer cells obtained by primary culture.
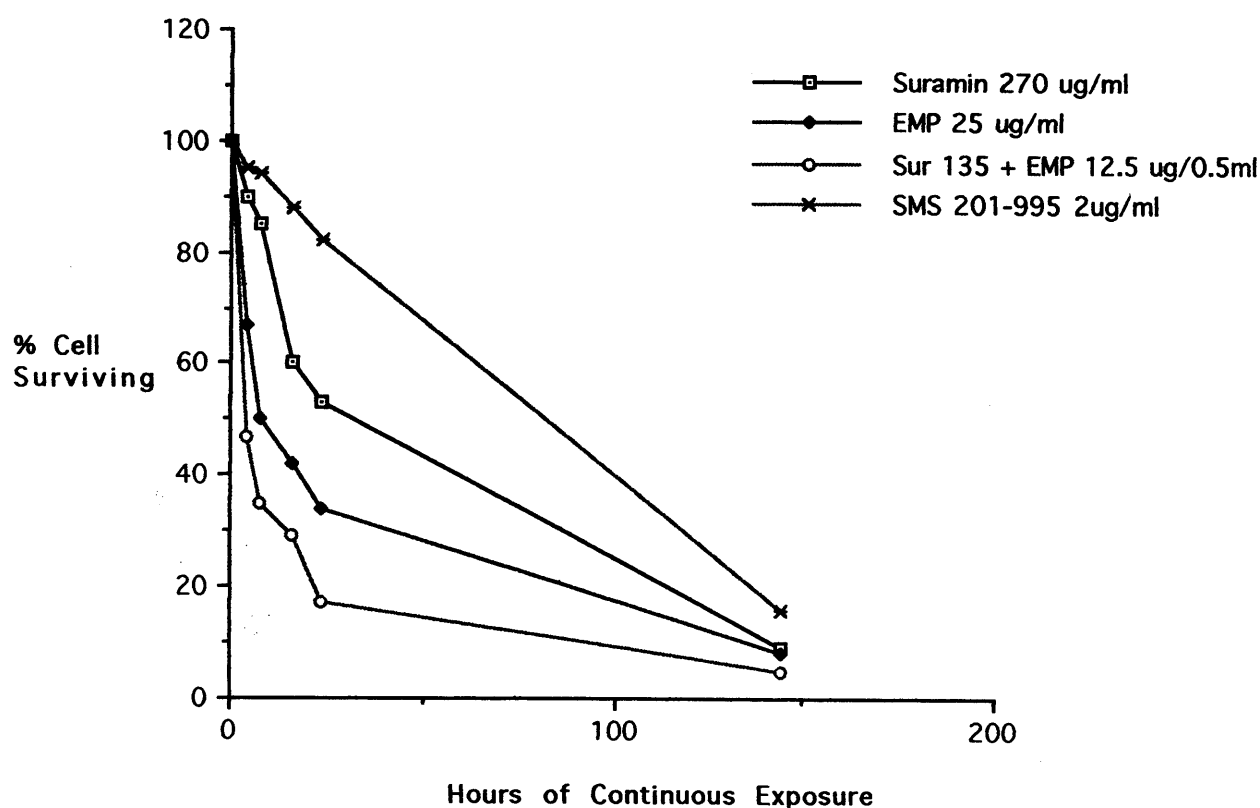
After 4, 8, 16 and 24 hours, fresh medium containing no drugs was used to replace the previous incubation medium which contained various drug concentrations.

Results represent the mean of 3 experiments.

The growth inhibitory effect of Suramin, EMP, Suramin + EMP and SMS 201-995 on prostate cancer cells obtained by primary culture was reversed more than 24 hours after exposure to the drugs. The degree of reversibility was least for Suramin + EMP combination. Ideally, the percentage of surviving cells after 144 hours of continous exposure should be 0%.

exposure was least for the EMP and Suramin combination: 17% compared to 53% for Suramin alone and 34% for EMP. The effect of SMS 201-995 was more easily reversible compared to that of the other drugs or drug combinations tested. Similarly, the expression of PAP by prostate cancer cells obtained by primary culture was not significantly altered by Suramin (0.4 iu/l) compared to 0.35 iu/l for the Suramin and EMP combination after 24 hours exposure. PAP in control wells was 0.6 iu/l.

Section G

6.7 Results of experiments to determine the effect of epidermal growth factor on proliferation of prostate cancer cells obtained by primary culture

EGF in a concentration range of 0-125 ng/ml did not significantly increase the growth of prostate cancer cells obtained by primary culture as shown in Table 41. EGF at a concentration of 1 ng/ml produced 1% growth over the control, while 50 ng/ml produced 8% growth over the control - a difference that is not statistically significant (P < 0.9).

Similarly, excess EGF did not reverse the growth inhibitory effect of Suramin on prostate cancer cells obtained by primary culture as shown in Table 42 and Fig 55.

Table 41

The effect of EGF excess on growth of prostate cancer cells obtained by primary culture.

	Cells per well (DPM) ^{3}H thymidine				% Growth			
EGF ng/ml	Expt 1	Expt 2	Expt 3	Mean ± SEM	Expt 1	Expt 2	Expt 3	Mean
0	4351	6538	6711	5867 ± 760	100	100	100	100
1	4358	6590	6845	5931 ± 791	100	101	102	101*
5	4482	6669	6778	5976 ± 749	103	102	101	102
25	4525	6930	6845	6100 ± 789	104	106	102	104
50	4743	6865	7382	6330 ± 808	109	105	110	108*
125	4612	7061	6979	6217 ± 804	106	108	104	106

Results represent the mean of 3 experiments as analysed by ^{3}H thymidine incorporation assay.

Cells plated on day 0. EGF added after 48 hours. Fresh media containing EGF concentrations as shown added on days 4 and 6. Number of cells in various wells estimated on day 8.

*   P < 0.9 - not statistically significant.   (Analysis of variance.)

Table 42

The effect of EGF on Suramin induced growth inhibition of prostate cancer cells obtained by primary culture.

Drugs	Number of cells using ^{3}H thymidine incorporation (DPM) versus number of days in culture				
	0	2	4	6	8
Control	6000	8852	12918	20925	83061
EGF 5 ng/ml	6000	8852	13059	18721	81000
Suramin 270 ug/ml	6000	8852	6571	7624	8721
EGF 2.5 ng/0.5 ml + Suramin 135ug/0.5 ml	6000	8852	5742	8743	9316

2×10^4 Cells/well plated on day 0. Drugs added after 48 hours, fresh media containing drug(s) added on day 4 and 6. Number of cells/well assessed on day 8 using ^{3}H thymidine (DPM) incorporation

Results represent the mean of triplicate experiments. See appendix for raw data.

Excess EGF did not reverse the growth inhibitory effect of Suramin on prostate cancer cells obtained by primary culture.

Fig 55

Effect of EGF on Suramin (Sur) induced growth inhibition of prostate cancer cells obtained by primary culture.
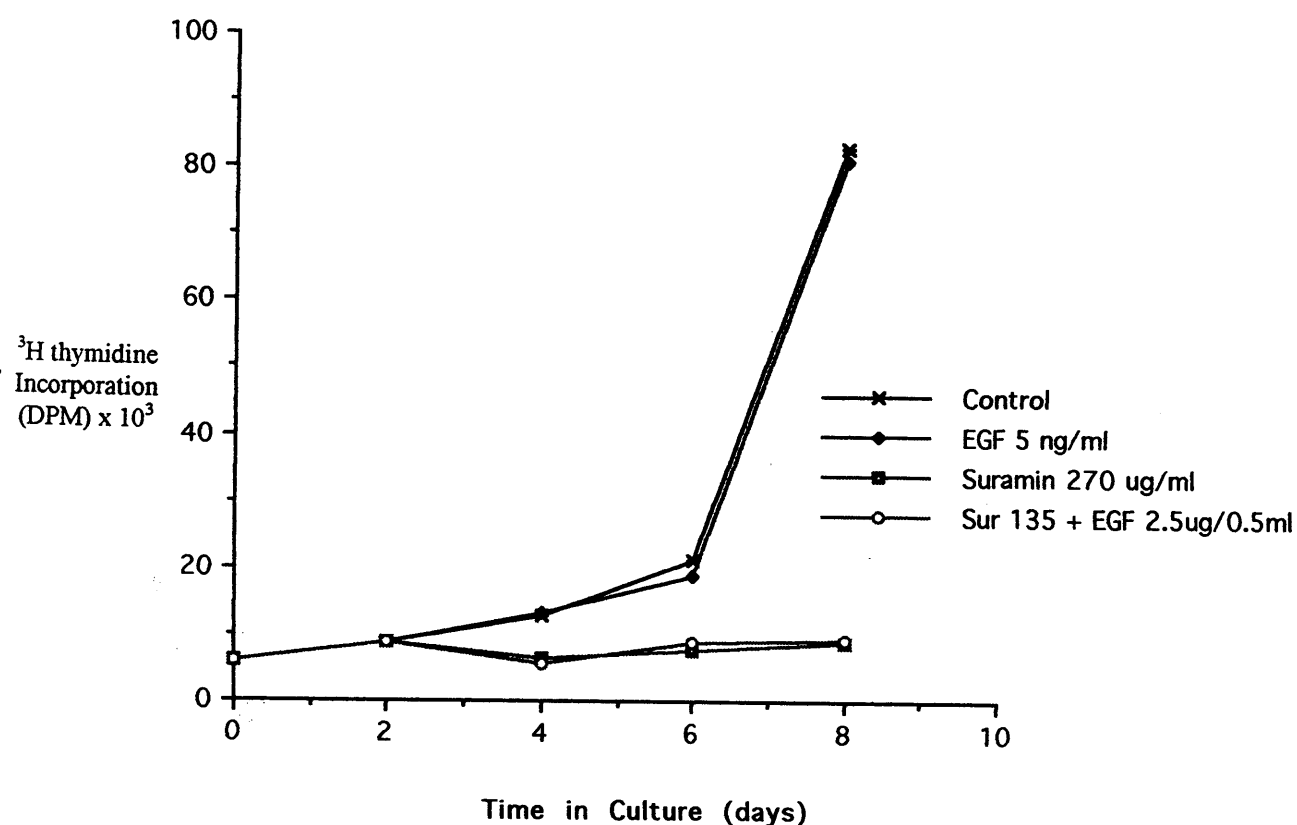
^{3}H thymidine Incorporation (DPM) x 10^3 vs Time in Culture (days)

Legend:
— Control
— EGF 5 ng/ml
— Suramin 270 ug/ml
— Sur 135 + EGF 2.5ug/0.5ml

2×10^4 cells/well plated on day 0. Drug(s) added after 48 hours, fresh media containing appropriate drug concentration added on day 4 and 6. Number of cells/well assessed on days 4, 6 and 8 using ^{3}H thymidine (DPM) incorporation

Results represent the mean of 3 experiments.

Excess EGF did not reverse the growth inhibitory effect of Suramin on prostate cancer cells obtained by primary culture.

CHAPTER 7 :   **DISCUSSION OF RESULTS OF EXPERIMENTS ON PROSTATE CANCER CELLS OBTAINED BY PRIMARY CULTURE**

Section A :   Patients from whom specimens were collected

Section B :   Discussion on the method of characterising cells obtained by primary culture

Section C :   Discussion of the results of primary culture using specimens from TURP, radical prostatectomy specimens and Biopty gun biopsy and lymph node metastasis

Section D :   Differences between cells obtained by primary culture from patients with newly diagnosed prostate cancer (no previous hormonal treatment) and patients with hormone resistant prostate cancer

Section E :   The effects of Suramin, SMS 201-995 or EMP on cells obtained by primary culture of prostate cancer epithelial cells

Section F :   The effect of Suramin and EMP combination on prostate cancer cells obtained by primary culture.

Section G :   Experiments to determine whether the effect of Suramin and EMP was cytostatic or cytotoxic on prostate cancer cells obtained by primary culture

Section H :   The effect of epidermal growth factor on proliferation of prostate cancer cells obtained by primary culture.

Section I: Summary of experiments on prostate cancer cells obtained by primary culture.

DISCUSSION : EXPERIMENTS ON PROSTATE CANCER CELLS OBTAINED BY PRIMARY CULTURE

Sections A, B and C

Section A :

7.1   Patients from whom specimens were collected

Section B :

7.2   Discussion on the method of characterising cells obtained by   primary
                             culture :
                             -        histology
                             -        acid phosphatase production
                             -        detection of keratin 8 and 18
                             -        clonal growth by ^{3}H thymidine incorporation assay

Section C :

7.3   Discussion of the results of primary culture using specimens from
                             TURP, radical prostatectomy specimens and Biopty gun biopsy and
                             lymph node metastasis

7.1 - 7.3   DISCUSSION OF SECTIONS A.B.C.

It has long been recognised that the in vitro cell models derived from human CaP cells are particularly well suited for studies on the aetiology of cancer, mechanisms of carcinogenesis, mechanisms of hormone-resistant CaP and testing the response of CaP to chemotherapeutic agents (Webber, 1980). This led to the development of "immortal" well characterised, established human CaP cell lines such as PC3 (an androgen independent cell line), and LNCaP (an androgen dependent cell line) (Kaighn et al. 1979, Horoszewicz et al. 1980).

However, it is now recognised that repeated passaging of established cell lines can lead to loss of tumour characteristics as a result of de-differentiation (Otto et al. 1988). This has been confirmed by us as shown by the electron microscopic appearance of PC3 at passage no. 86 showing cells with irregularly arranged and senescent organelles, compared to the appearance

of DU145 cells at passage number 25, where the organelles looked nearly normal (Figs 37 and 38). There were many similarities in the morphology of the cells that we obtained by primary culture (Fig. 46) and those at an early passage number of established CaP cell lines PC3 and LNCaP as originally described by Kaighn et al. in 1979, and Horoszewicz et al in 1980. From the foregoing, short term primary culture of tumour tissue should provide a more reliable in vitro model for prostate cancer. The other advantage of short term culture is that it is not expensive to produce, thus making it more easily available to researchers.

In our experience, an initial sterility test using liquid nutrient broth within the first 48 hours of procuring the specimens saved money and time, and prevented the wasting of resources on contaminated specimens that would not grow due to infection. In this experiment, we failed to obtain growth in 4 out of 12 specimens that failed the initial sterility test, in spite of the addition of gentamicin to the culture medium. Patients who had had urethral catheterisation prior to prostatectomy for less than 48 hours all failed initial sterility tests and should be excluded from short term culture of prostate specimens.

WAJC 404 medium with supplements (PEGM) supports serial passaging and clonal growth (up to 4 times in 4-6 weeks duration) of CaP cells obtained by primary culture. More importantly, PEGM actively suppresses the proliferation of fibroblasts as shown in Fig 43 unlike 10% FCS which supports the growth of epithelial cells as well as fibroblasts as shown in Fig 44 (Brehmer et al. 1973). We obtained the best clonal growth from radical prostatectomy specimens and specimens obtained by biopty gun. Specimens from TURP chips gave the least cell proliferation rate presumably because of the excessive heat that was used to obtain the chips, which might have damaged the tissues (Peehl et al. 1992). The ability to culture cells from specimens obtained from lymph node biopsies and biopty gun biopsies will allow assessment of patients with primary CaP of high grade malignancies and stages C and D CaP who are not usually candidates for radical prostatectomy in most centres. This method will also allow specimens to be collected on an out-patient basis. This will make it possible to follow patients at various stages of the disease so as to study the mechanisms of hormone resistance and metastasis, since all that is required is a minimally invasive procedure to obtain specimens for laboratory analysis.

Our primary objective was to develop a reliable short term culture of CaP epithelial cells to be used for in vitro models to test the response of CaP cells to various chemotherapeutic agents. We decided to use methods such as prostatic acid phosphatase production in a supernatant of confluent cells, electron microscopy and staining with a monoclonal antibody against keratin 8 and 18 to characterise the cells. Since we can culture and sub-culture confluent cells for up to 4 times in a 4-6 week period in patients with stages A to D CaP, the method that we have described should be very useful for testing for response of CaP cells to chemotherapeutic agents and should allow for the study of factors involved in tumour progression, metastasis and hormone resistance at cellular level.

Section D

7.4.   **Differences between cells obtained by primary culture from patients with newly diagnosed prostate cancer (no form of hormonal treatment) and patients with hormone resistant prostate cancer**

The aim of this section is to discuss differences, if any, between cells obtained from patients with newly diagnosed prostate cancer (NDCaP) and those from patients with hormone-resistant CaP (HRCaP), as well as cells from patients with well differentiated prostate cancer (Fig 46) and those with poorly differentiated prostate cancer.

On haematoxylin and eosin staining of cells from NDCaP and HRCaP, there were no observed differences in the 15 prostate cancer samples analysed. Similarly, electron microscopic analysis failed to show any remarkable morphological differences between NDCaP and HRCaP, except that cells derived from well differentiated prostate cancer cells had more tonofilaments compared to cancer cells from poorly differentiated tumours.

Fig 47 shows electron microscopy of epithelial cells obtained from culture of BPH specimens. These cells show organelles that are arranged in a regular pattern around a regular nucleus with numerous tonofilaments. This appearance is similar to those observed for well differentiated prostate cancer on electron microscopy. Poorly differentiated cancer of the prostate cells had fewer tonofilaments.

Cells derived from NDCaP and patients with presumably clinical HRCaP both produced prostatic acid phosphatase in culture, which means that the cells were all behaving as androgen-dependent cells in culture (Kozlowski et al. 1989). This finding is in agreement with that of Peehl et al. (1991), who also found that prostatic cancer cells obtained by primary epithelial culture of the prostate gland behaved like androgen-dependent prostate cancer cells by the production of PAP. Cells obtained from BPH which acted as controls for this part of our experiment produced negligible PAP in culture as shown in Fig 49 and Table 36. Equal

numbers of cells from patients with NDCaP, HRCaP and BPH (50×10^4 cells/50 ml tissue culture flasks) were plated on day 0, and acid phosphatase activity measured in the supernatant of culture medium (PEGM) on days 2, 4, 6, and 8.

In experiments described in Chapter 5 Section C, the cells used were from patients with both NDCaP and HRCaP, as from the point of view of function, there were no significant differences between the cells. Possible explanations for this observation may include the following: our method of isolating prostate epithelial cells produced acini only (Fig 43) which, by removing the cells from the stroma, may result in the cells behaving in a similar way i.e. producing PAP. It is now known that the stroma of the prostate gland plays an important role in prostate carcinogenesis (Chung and Cunha, 1983, Chung et al. 1988). There is now sufficient evidence suggesting that mesenchymal-epithelial interactions play a strategic role in the androgen-induced morphologies and growth of embryonic and adult prostatic tissue. Moreover, extracellular matrix plays a fundamental role in the anchorage of epithelial cells to the underlying substratum and determines their shape and polarity. The extracellular matrix may dynamically affect the ability of epithelial cells to respond to various growth factors; and may also play an active role in the control of gene expression which may have a profound impact upon cellular morphology and function (Bissel et al. 1982, Story et al. 1987, Byrne et al. 1996). The other possible explanation is that clinical progression may not equate to changes in biological progression or morphological changes but to functional and biological behaviour, requiring more sophisticated means of analysis to differentiate between the cells, for example cytogenetic analysis using p53 gene expression or mutations. Unfortunately, time did not allow me to pursue this line of investigation to a conclusive end! This may be a worthwhile line of investigation for the future.

Finally, from the foregoing, it is obvious that there is an inadequacy of existing in vitro and in vivo models of human prostate cancer. However, it is fair to say that primary epithelial cells from prostate cancer specimens give a better assessment of response of CaP cells to chemotherapeutic agents compared to established CaP cell lines of high passage numbers for reasons stated above.

Section E

7.5   The effects of Suramin, SMS 201-995 and Estramustine phosphate on cells obtained by primary culture of prostate cancer epithelial cells

Suramin, EMP and SMS 201-995, as on established CaP cell lines, also produced dose dependent growth inhibition on prostate cancer cells obtained by primary culture. EMP is more effective than Suramin in inhibiting the proliferation of prostate cancer cells using 50% PEGM. To date, there are very few reports in the literature assessing the effect of Suramin on prostate cancer cells obtained by primary culture (Morton et al. 1990, Peehl et al. 1991). Our findings especially on the effect of Suramin on the proliferation of prostate cancer cells confirmed the findings of Peehl et al. (1991). However, Peehl et al. (1991), reported that they obtained a half-maximal inhibitory effect of Suramin using a dose of 10 ug/ml. They used serum-free medium which is PFMR-4A based. This medium is not as rich in nutrient as 50% PEGM which we used. This may account for our half-maximal inhibitory dosage being higher - 90 ug/ml of Suramin. This is because Suramin, being a polyanionic compound, can bind to serum proteins which has been shown previously to reduce its efficacy (Hawkins, 1978, Myers et al. 1989). To correct for these observed differences, we attempted to initiate cancer cell growth in 30 and 40% PEGM but the cells did not proliferate when grown in less than 50% PEGM concentration.

Somatostatin 201-995 produced the least growth inhibition of prostate cancer cells obtained by primary culture. The drug failed to produce up to 50% growth inhibition even at a dose of 2 ug/ml. We were unable to find any explanation for this observation.

7.5.1   The effect of EMP and Suramin combination on prostate cancer cells obtained by primary culture

Unlike the effect of Suramin and EMP, which produced statistically significant degrees of inhibition of cell growth on established prostate cancer cell lines, this combination on prostate cancer cells obtained by primary culture, even though the extent of inhibition was higher with the combination, did not reach statistical significance on analysis of variance. However, the

effect of Suramin and EMP combination on percentage surviving cells of 34-37% was lower than the 39-81% produced by EMP or Suramin alone. The implication of these results is that the Suramin and EMP combination produces an additive effect on prostate cancer cells obtained by primary culture. However, in vivo, it is possible that this two-drug combination will be more effective against prostate cancer cells as Suramin has been shown to inhibit testicular androgen production as well as LHRH production from the hypothalamus (Daugherty et al. 1992). These two in vivo effects of Suramin cannot be incorporated into an in vitro assay system. Hence, there can be cautious optimism that on both established CaP cell lines and prostate cancer cells obtained by primary culture, Suramin is not only effective in reducing cellular proliferation, but also combining EMP with Suramin might produce greater response rates in patients with prostate cancer. Lastly, a possible cause of the Suramin and EMP combination not producing statistically significant effects on prostate cancer cell-growth may be because of the media used in these experiments as mentioned briefly before.

Section F

7.6 Suramin and EMP combination produced additive but not synergistic effect on prostate cancer cells obtained by primary culture.

Unlike the effect of the Suramin and EMP combination on established CaP cells lines which produced synergistic growth inhibition, this drug combination on prostate cancer cells obtained by primary culture did not produce such an effect. At best, these results indicate that for CaP cells obtained by primary culture, EMP and Suramin produced an additive effect only. As stated before, it is possible that the media we used might be responsible for this effect, and that in vivo, other actions of Suramin like depression of adrenal androgen and LHRH production might make the combination more effective in treating patients with hormone resistant prostate cancer.

A possible explanation for the differences in the response of established prostate cancer cell lines to that of prostate cells obtained by primary culture, may be our previous observation that, in established prostate cancer cell lines, the organelles, particularly the rough

endoplasmic reticulum and the mitochondria of cells of high passage numbers, are unhealthy, whereas the organelles in the prostate cancer cells obtained by primary culture are well preserved and healthy. These differences may make the established prostate cancer cell lines more susceptible to the effects of drugs since the mitochondria play a crucial role in the oxidative and metabolic process of cells.

Section G

7.7 **Experiments to determine whether the effect of Suramin and EMP is cytostatic or cytotoxic on prostate cancer cells obtained by primary culture**

The results of these experiments indicate that the effects of Suramin, EMP, SMS 201-995 or Suramin and EMP combination are cytostatic and not cytotoxic on prostate cancer cells obtained by primary culture - even though the effect of Suramin and EMP was least reversible (18%). However, there was no statistical significant difference in this effect of Suramin and EMP combination compared with Suramin or EMP ($P < 0.35$). The degree of reversibility of inhibition produced by SMS 201-995 (82%) at 24 hours was statistically significant compared to that produced by EMP + Suramin (18%) ($P < 0.01$). Hence, neither Suramin, nor Suramin and EMP can be considered ideal drugs for producing cytotoxicity to prostate cancer cells, even though the effect of Suramin and EMP produced greater cytostasis. As stated earlier, it is possible that the other properties of Suramin and EMP in vivo will compensate for this defect and render the drug combination more cytostatic if not cytotoxic. Peehl et al. in 1991, reported that the growth inhibition produced on prostate cancer cells obtained by primary culture by TGF β-1 and retinoic acid are non-reversible, that is, they are cytotoxic even when the exposure has been for a period of less than 10 hours.

Similarly Suramin, SMS 201-995 or the Suramin and EMP combination did not appear to alter the phenotype of prostate cells in terms of the expression of PAP production, that is, they did not produce cytotoxicity on the cells, as the cells continued to produce PAP after exposure to the drugs for 24 hours.

Section H

7.8 **The effect of EGF on the proliferation of prostate cancer cells obtained by primary culture**

Many factors are required for the growth of prostate cancer cells in culture, and these include choleratoxin, EGF, pituitary extract, insulin, hydrocortisone and prolactin (Chaproniere and McKeehan, 1986). The PEGM used to culture prostate cancer cells contained most of these factors.

However, the results of this experiment showed that EGF did not increase the proliferation of prostate cancer cells obtained by primary culture, unlike its effect on established prostate cancer cells lines like LNCaP or PC3. Similarly, the effect of Suramin on prostate cancer cells obtained by primary culture was not reversed to any significant extent by excess growth factor. Hence, the half maximal inhibitory dose of 90 ug/ml of Suramin was not appreciably raised by excess EGF at 5 ng/ml. It is possible that there are numerous growth factors in the PEGM used to culture the cells such that the excess of any one growth factor will not affect the overall cell growth substantially. Similarly, neither will the inhibitory effect of Suramin be expected to be reversed by the addition of one growth factor, as the cells in culture may be secreting other autocrine growth factors. These other growth factors may have mitogenic effect on prostate cancer cells. Unfortunately, no measurements of all the growth factors produced by prostate cancer cells obtained by cell culture have been undertaken before. However, it has been postulated that unlike established cell lines whose proliferation and transformed state depends almost exclusively on one or two growth factors, cells obtained by primary culture may be heterogenous in nature and may depend on many interacting factors for optimal growth. (Betsholtz et al. 1986, Huang and Huang, 1988, Peehl et al. 1991).

Section I

7.9   Summary of experiments on prostate cancer cells obtained by primary culture

7.9.1   Summary: Primary culture of prostate cancer cells

Cell lines are useful in the evaluation of the biological defects involved in carcinogenesis and in testing response to chemotherapeutic agents. However, serial passaging of cells can lead to loss of tumour characteristics due to de-differentiation.

To establish a reliable short term primary culture of prostate cancer epithelial cells to be used as an in vitro model for studying response of CaP to novel chemotherapeutic agents, we used WAJC 404 medium with supplements, primary epithelial growth medium (PEGM) to culture prostate cells obtained from radical prostatectomy x 3, TURP chips x 6, lymph node biopsies x 3, and biopty gun biopsies x 3. Tissues were dissociated with Worthington collagenase and grown in tissue culture flasks containing PEGM in an incubator maintained at 5% CO_2 and 37°C. All samples were tested for initial sterility (using liquid nutrient broth), the presence of cancer cells in more than 90% of the specimens, the presence of keratin (using monoclonal antibody against keratin 8 and 18), and prostatic acid phosphatase production in the supernatant of confluent cell cultures.

In PEGM the rate of successful establishment of primary epithelial cell cultures without fibroblasts was 5/6 of BPH obtained from TURP chips, 2/6 CaP from TURP chips, 3/3 CaP obtained from radical prostatectomy, 1/3 of CaP in lymph node metastasis and 2/3 CaP from biopty gun biopsies. Histological examination confirmed the malignant nature of the cells. The prostatic acid phosphatase in the supernatant of confluent cells was proportional to the cell density in culture. The cells tested positive for keratin 8 and 18 using monoclonal antibody. [3]H thymidine incorporation assay confirmed cell proliferation in sub-cultures on 4 different occasions in a 4-6 weeks period. The best clonal growth was obtained from specimens derived from radical prostatectomy while specimens obtained by

TURP chips yielded the least proliferating cells. However, this method of isolating prostate cancer cells resulted in cells behaving as androgen-dependent cells irrespective of the pattern of disease in the patients. This may be the result of a lack of the influence of prostatic stroma on the acini isolated.

PEGM supports short term serial passaging of CaP epithelial cells without the growth of fibroblasts. The ability to derive cultures from lymph node metastases and biopty gun specimens allows the characterisation of cells from primary CaP of high grade malignancy, or those of hormone-resistant CaP patients for whom radical prostatectcmy or TURP is not routinely performed.

7.9.2: **Summary: Effects of Suramin, EMP, SMS 201-995, or (EMP + Suramin) combination on prostate cancer cells obtained by primary culture.**

Suramin , EMP, SMS 201-995 and the Suramin + EMP combination produced dose dependent growth inhibition of prostate cancer cells obtained by primary culture. However, the Suramin and EMP combination failed to produce statistically significant inhibition compared to Suramin or EMP alone. This is unlike the action of the Suramin + EMP combination on established CaP cell lines like LNCaP and PC3, where the combination produced a statistically significant difference. Similarly, the EMP + Suramin combination produced only an additive rather than a synergistic effect on prostate cancer cells obtained by primary culture. Furthermore, the Suramin + EMP combination produced a cytostatic rather than a cytotoxic effect, as the growth inhibitory effect of the combination was reversible up to 24 hours after the cells had been exposed continuously to the 2 drugs. However, other effects of Suramin in vivo, such as inhibition of testicular androgen production and LHRH production may make the EMP + Suramin combination an effective treatment regimen for patients with hormone-resistant prostate cancer.

CHAPTER 8 :   **CLINICAL TRIAL**

Suramin and Estramustine Phosphate versus Estramustine Phosphate in patients with Hormone Resistant Prostate Cancer. Phase II Clinical Trials: Preliminary Report

Section A :   Introduction

Section B :   Patients and methods

Section C :   Results

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SURAMIN AND ESTRAMUSTINE PHOSPHATE VERSUS ESTRAMUSTINE
PHOSPHATE IN PATIENTS WITH HORMONE RESISTANT PROSTATE
CANCER. PHASE II CLINICAL TRIAL : PRELIMINARY REPORT

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8.1   Introduction

Metastatic hormone-resistant prostate cancer (Stage D3) may respond to Estramustine Phosphate (EMP) and Suramin. However, both drugs have significant toxicities which are dose-dependent and have limited the use of the 2 drugs in this disease. Moreover, the duration of response to either agent is less than one year (Stone et al. 1985, Benson and Hartley-Asp 1990, La Rocca et al. 1990, Eisenberger et al. 1992). As described in Chapters 4 and 7 Suramin and EMP in combination have synergistic and additive cell growth inhibitory activity against established human prostate cancer cell lines LNCaP, PC3 and DU145 and prostate cancer cells obtained by primary culture respectively. This synergy may allow less toxic doses of Suramin and EMP if the combination regimen is used clinically compared to either drug as monotherapy. We therefore decided to carry out a preliminary Phase II clinical trial on patients with hormone-resistant prostate cancer using this drug combination.

8.1.1   Aim of study

To investigate the value of EMP with low dose Suramin in patients with hormone-resistant prostate cancer.

Section B

8.2. Patients and Methods

Patients with known metastatic prostate cancer, treated by maximal androgen blockade (medical or surgical castration + an anti-androgen) e.g. orchidectomy or Zoladex with Cyproterone acetate or Flutamide and who have subsequently had 2 successive rises in PSA (that is, Stage D_3 disease), the last level being greater than 40 ug/l, were randomised into 2 treatment arms, namely:

Group A :   Received EMP 280 mg b.d. orally for 3 months

Group B :   Received EMP 280 mg b.d. orally plus weekly intravenous infusion of 1 gm Suramin for 6 weeks.

Group C :   Suitable patients who refused consent and were therefore allowed to continue with their current treatment, that is, maximal androgen blockade (castration + anti-androgen).

The following baseline investigations were performed: full blood count, urea, electrolytes, liver function tests, glucose screen, chest x-ray, PSA, bone scan, ultrasound of the bladder (for residual urine) and para-aortic lymph nodes, urinalysis to exclude albuminuria and quality of life assessment using the Karnofsky Score. Patients completed a quality of life questionnaire on day 1 of randomisation and on day 90 of treatment. (See Appendix for details).

8.2.1 Exclusion criteria

Excluded from this clinical trial were patients older than 80 years, patients with PSA greater than 120 ug/l, patients with a previous history of cardiovascular disease, myocardial infarction, cerebrovascular disease, renal impairment, diabetes mellitus, anaemia and patients with a Karnofsky score less than 6.

8.2.2 Approvals obtained

We obtained approval for this clinical trial from Medicines Control Agency (MCA) in London since Suramin is not licensed for treating CaP, and from Leicestershire Health Authority Ethics Committee (See Appendix for letters of approval and detailed protocol). The MCA in London gave approval for using Suramin in 6 patients only to start with; hence the small number of patients in each treatment arm of this preliminary study.

We also obtained a written informed consent from patients agreeing to participate in this clinical trial, in accordance with the Helsinki declaration, of the nature of the treatment regimens.

8.2.3 Follow-up of patients

Patients in group B receiving Suramin were seen weekly for the first 8 weeks. All patients in group A, B and C were seen once a month in the Out-patients Department, and blood samples were taken for full blood count, liver function tests and PSA estimation. A repeat bone scan was obtained after six months of treatment. Also, a repeat ultrasound of the para-aortic lymph nodes was obtained, if these were found to be enlarged before treatment was commenced.

8.2.4 Measurement of Serum Suramin levels

To prevent toxicity due to Suramin, the Suramin trough level was determined before the next dose was given. We used the high pressure liquid chromatography (H.P.L.C.) method to measure serum Suramin levels. For full details of the HPLC assay, see Appendix A and figures 58 and 59.

Section C
8.3 The Results

Details of the patients in this clinical trial as well as the treatment the patients had received before developing hormone-resistant CaP are shown in Table 43. Response to treatment is shown in Table 44 and Fig 56. The mean baseline PSA for patients in groups A and B was 54

Table 43

Profile of patients with hormone resistant prostate cancer (Stage D₃)
undergoing clinical trials with Estramustine Phosphate (EMP) or EMP and
Suramin.

Patient Characteristics	Treatment Groups		
Number	A (n = 6)	B (n = 6)	C (n = 6)
Mean age and (range)	68 (65 - 74)	71 (62 - 74)	70 (60 - 73)
Mean duration of previous therapy (months)	72	79	74
Previous hormonal manipulation			
• BSCO ± CPA ± flutamide	2	3	1
• LHRH analogue ± CPA ± flutamine	4	3	5
• EMP	0	1	1
• Previous DXT	2	2	1
• Stilboestrol ± Aspirin	2	1	2
Extent of disease after Soloway et al 1988 I	2	2	1
II	3	2	2
III	0	1	2
IV	1	1	1

Extent of metastasis on bone scan after Soloway et al 1988.

I   =   <6 sites of metastases
II   =   6-20 sites of metastases
III   =   > 20, less than superscan
IV   =   superscan
All 18 patients had Stage D₃ disease.

BSCO =   Bilateral subcapsular orchidectomy
CPA   =   Cyproterone acetate
LHRH =   Luteinising hormone releasing hormone
DXT   =   Radiotherapy

Table 44

Response to treatment based on changes in PSA in patients with hormone resistant prostate cancer in Groups A, B, C.

Patients Group	No of Patients	PSA ug/L					
		Before Rx		3/12 Rx		6/12 Rx	
			Mean + SEM		Mean + SEM		Mean + SEM
A	6	50, 48, 54, 61, 71, 40	54 ± 4.41	21, 14, 10, 15, 9, 21	15* ± 2.12	59, 60, 51, 44, 81, 71	61* ± 5.47
B	6	42, 48, 69, 72, 59, 58	58 ± 4.74	6, 4, 3, 6, 1, 4	4.1** ± 1.64	5, 6, 5, 1, 3 +	4** ± 0.89
C	6	73, 55, 66, 72, 75, 79	70 ± 3.47	146, 180, 190, 125, 137, 296	159 ± 37.30	250, 141, 164, 178, 216, 137	181 ± 18.11

Rx = treatment
* P < 0.01 - (Group B vs A) - statistically significant
** P < 0.001 - (Group B vs C) - statistically significant
[using Kruskal-Wallis non-parametric one-way Anova test]

3/12 = after 3 months of treatment
6/12 = after 6 months of treatment

+ One patient in Group B died suddenly at home before 6 months of treatment. Cause of death was presumed to be myocardial infarction as relatives refused a post mortem examination. He was 74 years old.

Fig 56

Changes in PSA over a 6 month period in patient groups A, B and C.
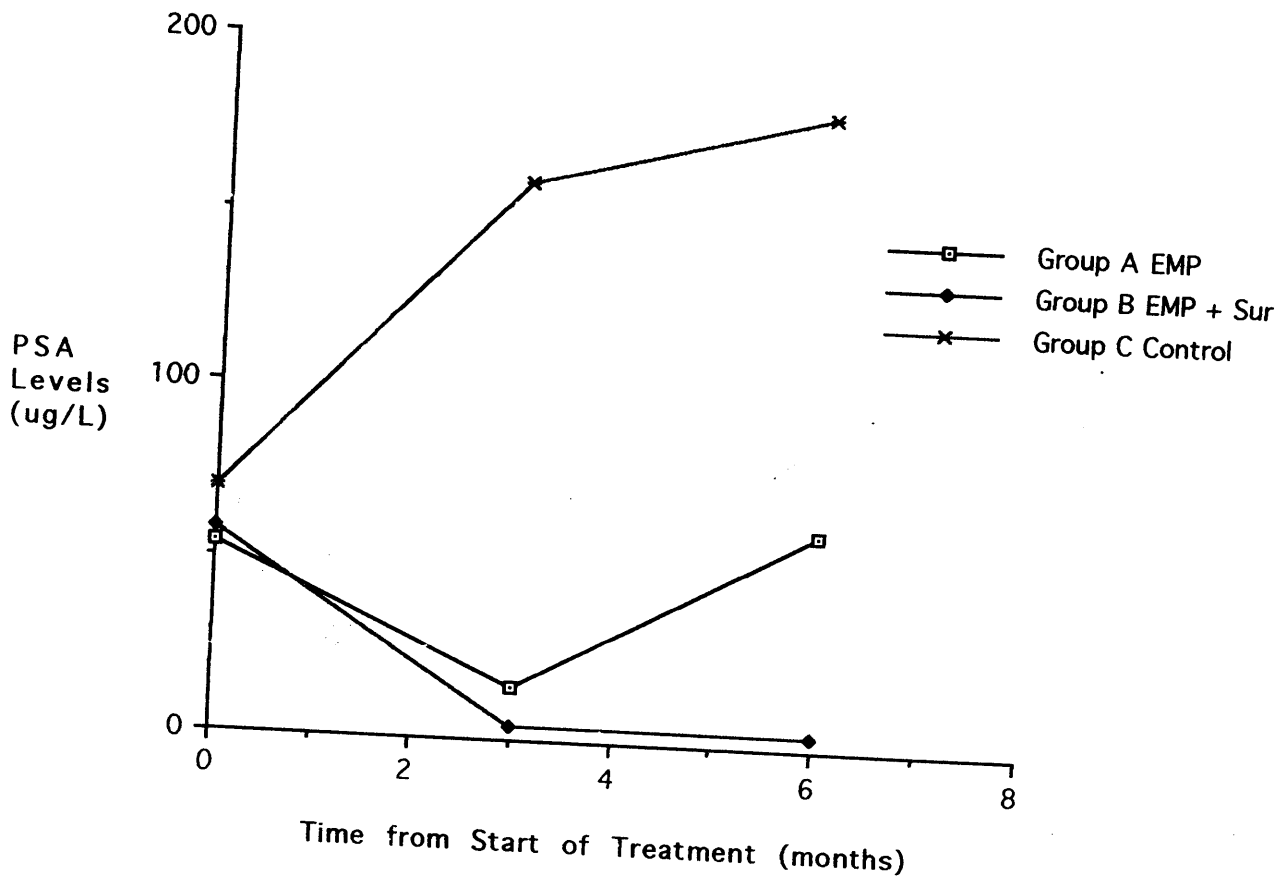
Results represent the mean of PSA for all patients in each group at 0, 3 and 6 months from start of treatment.

Statistically significant reduction of PSA between groups B and C (p < 0.001), and between groups A and C (p < 0.01), after 3 months of treatment. Reduction in PSA maintained for more than 6 months for group B patients, whereas for group A patients, PSA started to rise again after 3 months.

(p - calculated using Kruskal-Wallis non-parametric one way Anova test).

and 58 ug/l respectively (P< 0.1). The mean PSA three months after treatment was 15 and 4.1 µg/l for patients in Groups A and B respectively compared to 159 ug/l for patients in Group C (p< 0.001). After 6 months of treatment, the mean PSA was 61 ug/l and 4.0 ug/l for patients in Groups A and B respectively, compared to 181 ug/l for patients in group C as shown in Fig 56 (P < 0.001).

There was a steady rise in mean serum Suramin from 39 ug/ml (range 27-52) on day 7 of treatment to 130 ug/ml(range 11-149) on day 42 of treatment as shown in Table 45 and Fig 57. The mean value of serum Suramin 3 and 6 months post-commencement of treatment was 65 ug/ml and less than 1 ug/ml respectively.

8.3.1 Side effects

The side effects encountered in this study are shown in Table 46. EMP produced significant side effects necessitating discontinuation of therapy with EMP in 4/6 and 1/6 in groups A and B patients respectively before the third month of treatment.

One patient in each group had proven deep venous thrombosis. There was one death in group B three months after treatment; the cause of death was not established.

8.3.2 Response to treatment

8.3.2.1 Changes in PSA

As Table 44 and Fig 56 show, there was a statistically significant fall in the mean value of PSA in group B patients (4.1 ug/l) compared to group A patients (15 ug/l) at the end of the third month of treatment (P<0.01). At the end of six months of treatment the differences in the mean PSA between group A (61 ug/l) and group B (4.0 ug/l) became more obvious (P < 0.001). The PSA started to rise again in group A patients before the end of six months of treatment.

8.3.2.2 Changes in analgesic requirement

Requirements for analgesia in the three groups of patients before commencement of the study and 3 months after treatment with EMP or EMP + Suramin is as shown in Table 47. There

Table 45

Trough serum Suramin levels of patients with hormone resistant prostate cancer receiving weekly i.v. Suramin 1 gm. for 6 weeks (Group B patients)

Days after Rx started	Trough Serum Suramin ug/ml n = 6	Mean Trough Suramin ± SEM (ug/ml)
0	0	0
7	27, 40, 50, 32, 33, 52	39 ± 5.9
14	74, 76, 51, 67,88, 94	75 ± 8.9
21	103, 68, 74, 100, 85, 56	81 ± 10.7
28	95, 115, 116, 88, 97, 89	100 ± 7.2
35	120, 115, 130, 118, 150, 117	125 ± 7.9
42	111, 140, 145, 120, 115, 149	130 ± 9.6
120	46, 35, 71, 87, 85, 66	65 ± 12.1
180	0, 3, 0, 1, 0.6 *	0.9 ± 0.7

* 1 patient died before the end of the 6th month of treatment.

Fig 57

Trough Suramin levels of patients with hormone resistant prostate cancer receiving weekly i.v Suramin 1 gm for 6 weeks.

Serum Suramin Level (ug/ml)

Time after Starting Treatment in Days
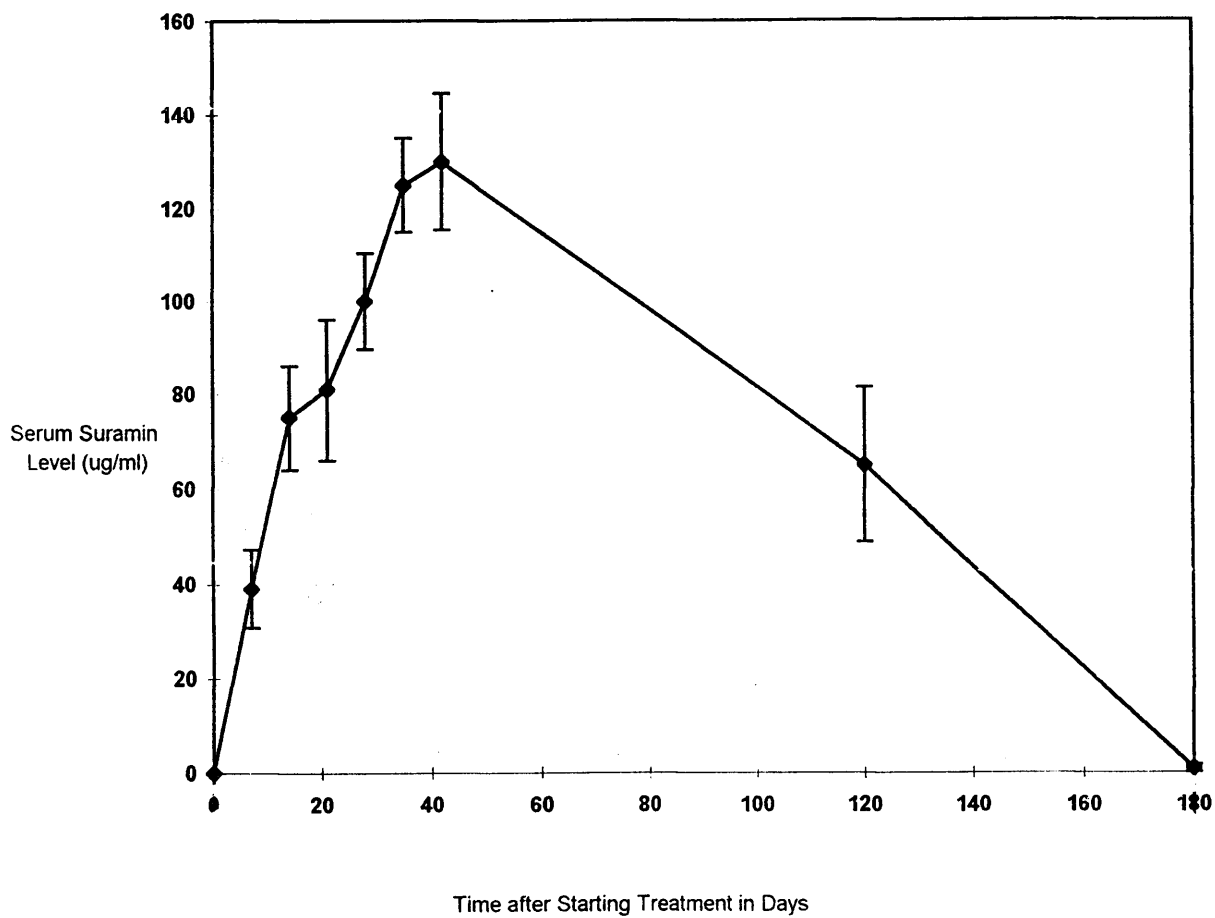
Our regimen maintained serum Suramin levels well below the toxic level of 270 ug/ml during induction of treatment with Suramin

At serum Suramin concentrations of > 270 ug/ml, Suramin produced significant toxicity.

was worsening of bone pain and requirement for more potent analgesics for patients in groups A and C compared to group B patients. There was a marked reduction in bone pain usually reported by patients within a week of treatment with the EMP and Suramin combination in all group B patients. Two out of 5 surviving patients at the end of 3 months of treatment with EMP and Suramin stopped taking analgesics altogether. One patient in group A who did not require analgesia at the beginning of treatment required regular narcotic analgesics by the third month of treatment. In contrast, one patient in group B who did not have bone pain at the commencement of treatment remained pain-free 3 months after treatment with the EMP and Suramin combination.

8.3.2.3 Changes in bone scan

A bone scan six months after treatment revealed no change in bony metastases in 2 and 4 patients in groups A and B respectively whereas in 4 and 1 patients in groups A and B respectively there was progression of the disease. One patient in group B had died by the sixth month of treatment before a bone scan could be repeated. There were no bidimensionally measurable metastases in the 12 patients.

Table 46

Side effects of treatment in patients with hormone resistant prostate cancer receiving EMP alone (Group A) or EMP and Suramin (Group B)

Side Effects		Group A n = 6	Group B n = 6
Nausea/Vomiting		4	0
CVS	Clinical DVT	1	1
Fluid retention		5	4
Abnormal LFT		0	0
Albuminuria		0	0
Gynaecomastia		2	0
Mortality		0	+1
Discontinuation of EMP before 3/12		4	1

+   Died suddenly at home. ? myocardial infarction.
       Responding to treatment at the time of death.

CVS = cardiovascular

DVT = deep vein thrombosis

LFT = liver function test

Table 47

Analgesic requirements for bone pain for patients with hormone resistant prostate cancer undergoing treatment with EMP alone (Group A) or EMP + Suramin (Group B) or Control (Group c)

Treatment Group → Severity of Pain ↓		Before treatment			3 months post-treatment		
		A n=6	B n=6	C n=6	A n=6	B n=5	C n=6
None	0	1	1	0	0	2	0
Mild	1	1	2	2	0	2	1
Moderate	2	2	2	1	3	1	1
Severe	3	1	0	1	2	0	2
Intractable	4	1	0	2	1	0	2

- worsening of bone pain in group A and C patients and requirement for stronger analgesic.

- marked reduction in bone pain in group B patients. None required stronger analgesic.

Analgesic requirements (bone pain)

None   0 = no bone pain. No analgesic

Mild   1 = non-narcotic analgesic occasionally required

Moderate   2 = non-narcotic analgesic regularly required

Severe   3 = narcotic analgesics occasionally required

Intractable 4 = narcotic analgesics regularly required ± DXT.

8.4 Discussion

The results of this pilot study suggest that the combination of EMP and Suramin seems to be better tolerated than EMP alone. Furthermore, the response to treatment as judged by the fall in PSA, the reduction in bone pain and the reduced requirement for analgesics was much better with the combination of EMP and Suramin than with EMP alone. The difference in the changes in PSA at the end of 6 months of treatment is statistically significant ($P < 0.001$ Kruskal-Wallis non-parametric one way Anova test) between group B and A or C patients.

Furthermore, EMP and low-dose Suramin produced fewer side-effects than Suramin and Mitomycin as reported by Rapoport et al, 1993, and far fewer side-effects than high-dose Suramin alone (La Rocca et al. 1991). The side effects produced by the combination of EMP and Suramin were probably due to the EMP component. It is tempting to speculate that, since Suramin and EMP show synergistic activity in vitro, a further reduction in the EMP dose to, say, 280 or 140 mg daily, plus low-dose Suramin might produce the same clinical response as 560 mg daily of EMP and Suramin. We intend to pursue this line of investigation in a Phase III clinical trial. One main advantage of our regimen is that EMP is given orally and can also be given to patients who have had previous irradiation for bony metastasis. This is in contradistinction to Mitomycin C, which is given intravenously, and is not advisable for patients with compromised bone marrow reserve. None of the patients in group B developed the albuminuria or dermatological lesions usually seen with high-dose Suramin (Rapoport et al. 1993) The present regimen also obviates the need to use hydrocortisone for adrenal replacement therapy as is necessary with high-dose Suramin. This study has also confirmed that the reduction in bone pains observed in patients given high-dose Suramin (> 300 ug/ml) + hydrocortisone is not due to the hydrocortisone component of the treatment as some researchers have argued (Eisenberger et al. 1992, Harland and Duschensne, 1993). Our study indicates that Suramin in its own right, and probably in low doses (< 200 ug/ml), most likely has analgesic properties.

The conclusions from this limited preliminary study are that in reasonably matched groups of 6 patients, EMP and Suramin significantly reduced PSA at 6 months compared to continued

maximal androgen blockade (P <0.001) and second-line EMP alone (P<0.001). Side-effects from low dose Suramin (<200 ug/ml) seemed minor, and most side effects in group B patients were due to EMP. There was a significant reduction in bone pain and no progression of disease on bone scan was observed in group B patients. There was also a significant reduction in requirements for analgesics in group B patients, compared to groups A and C patients. Survival figures analysis were not available at the time of this report, because all the patients had been followed up for less than 1 year.

Further studies are warranted to determine whether other drug schedules involving the use of Suramin in combinations can produce clinically acceptable results with less toxicities than high dose Suramin monotherapy for patients with hormone resistant prostate cancer.

CHAPTER 9   ***SUMMARY AND CONCLUSIONS***

Section A   **Summary**

Section B   **Conclusions**
                       Key words

SUMMARY AND CONCLUSIONS

Section A

9.1   Summary

9.1.1   The effects of Suramin on established human prostate cancer cell lines - in vitro studies

In vitro experiments carried out in this study confirmed that Suramin at a concentration of 0 - 270 ug/ml produced dose-dependent growth inhibition on the following established prostate cancer cell lines: LNCaP (androgen-dependent), PC3 and DU145 (androgen independent cell lines). At a concentration of 270 ug/ml, Suramin produced 50%, 49%, and 68% inhibition of growth of LNCaP, PC3 and DU145 CaP cell lines respectively on day 6 (Kehinde et al. 1992). These results are similar to those reported previously (Stein et al. 1989, La Rocca et al. 1991, Kim et al. 1991). However, these effects are cytostatic as the withdrawal of Suramin on day 6 after exposing the cells to 270 ug/ml led to continuation of cell growth. The effects of Suramin can also be reversed if the cells are grown in the presence of excess growth factors such as EGF, or TGF alpha, indicating that a possible mechanism of action of Suramin is by interference with growth factor induced cell proliferation of CaP cells. This is confirmed by the effect of anti-epidermal growth factor receptor which inhibits prostate cancer cell growth.

In an attempt to improve the therapeutic profile of Suramin we carried out a series of in vitro experiments using combinations of growth factor inhibitors, to see whether we could produce synergy with Suramin and render its action cytocidal. We used the following combinations :

(a)   Suramin and Somatostatin (SMS 201-995)

(b)   Suramin and EMP

(c)   EMP and SMS 201-995

The only combination which produced a synergistic effect was EMP and Suramin. Suramin at a concentration of 10 ug/ml produced 14% growth inhibition on the PC3 cell line. The addition of EMP 12.5 ug/0.5 ml to Suramin 10 ug/0.5 ml increased the percentage growth inhibition from 14% to 64% - (P = 0.001), a difference that is statistically significant. A possible explanation for the synergistic activity between EMP and Suramin is that the 2 drugs act at different points in the growth pathway of prostate cancer cells. The addition of SMS 201-995 to Suramin did not improve the growth inhibitory effect over either agent used alone even though independently, the 2 drugs produce dose dependent growth inhibition. This is presumably because Suramin and SMS 201-995 are displacing the same types of growth factors from receptors on CaP cell membrane.

9.1.2   The effects of Suramin on prostate cancer cells obtained by primary culture

Repeatedly passaged established cell lines have limitations. Consequently, the above experiments were repeated using cells obtained by primary culture. In primary epithelial growth medium (WAJC 404 medium-based) Suramin inhibited the clonal growth of CaP cells obtained by primary culture in a dose-dependent fashion. However, the effect of Suramin on CaP cells obtained by primary culture was cytostatic and reversible more than 24 hours after the cells had been exposed to Suramin. These findings agree with those of Peehl et al. (1991). The combination of Suramin and EMP produced more growth inhibition on cells obtained by primary culture than Suramin alone - an effect which proved to be additive rather than synergistic unlike the effect of the same combination on established prostate cancer cell lines. The effect of Suramin and EMP is also cytostatic and reversible - even though to a lesser extent than Suramin alone - 24 hours after exposure to the two drugs. A SMS 201-995 and Suramin combination failed to induce a greater percentage of growth inhibition than either agent alone, similar to the effect of this combination on established prostate cancer cell lines.

9.1.3 **Results of clinical trials using Suramin as monotherapy in patients with metastatic hormone resistant prostate cancer**

La Rocca et al. (1991), first established that for Suramin to be used as monotherapy for metastatic CaP, a serum Suramin concentration of between 250 and 300 ug/ml was necessary. To achieve this, Suramin was given as a continuous infusion and the serum Suramin concentration was monitored daily. Using this regimen Manyak et al. (1992) and Eisenberger et al, 1992, reported a response rate of over 50% in patients with measurable lesions, more than a 75% decrease in PSA, major symptomatic improvement and an overall 53% response rate. However, at a serum concentration of 300 ug/ml Suramin produced many side-effects, which included abnormal reversible coagulopathy (73%), rashes (42%), malaise (34%), reversible peripheral neuropathy (30%) reversible vortex keratopathy (8%) and suppression of adrenal activity necessitating replacement doses of hydrocortisone (10-30 mg per day) (La Rocca et al. 1991). The use of high dose Suramin in the USA was said not to be associated with any mortality in the first 95 patients treated. However, Van Oosterom et al. (1990), in Belgium reported 7 out of 9 patients dying of malignancy within 9 weeks of starting Suramin infusion, but they noted partial responses in 4 out of 9 patients and reported that the side-effects of Suramin were dose-dependent. Hence, it could be argued that Suramin as a monotherapy in metastatic hormone-resistant CaP produces modest short-term responses, but at the cost of significant toxicity when a therapeutic serum level of 250 - 300 ug/ml is used.

9.1.4 **Results of clinical trials using Suramin in combination with other chemotherapeutic agents in metastatic hormone resistant prostate cancer**

Despite Suramin's obvious activity in patients with hormone-resistant metastatic CaP, its clinical use is limited by its toxicity and short duration of response. One therapeutic strategy to overcome this is to combine Suramin with other drugs that are effective against CaP. This allows the use of lower doses of Suramin without losing anti-tumour activity. Rapoport et al. (1993) reported their experience on Suramin and Mitomycin C combination.

Suramin at 350 mg/m² daily was given for 5 days, followed by 350 mg/m² weekly starting on day 4. Mitomycin C 12 ug/m² was given every 5 weeks starting on day 14. During the induction of treatment, the median serum Suramin level was 140 ug/ml (range 93-270 ug/ml). In 32 patients, one complete and six partial responses were observed and in 15 patients the disease stabilized. The median time to treatment failure (rising PSA, reduced performance status) was 103 days and the median survival was 209 days. Significant side-effects noted included haematological toxicity (59%), fatigue (34%), neurotoxicity (31%), proteinuria (28%), and skin rashes (25%). Rapoport et al. (1993) concluded that the combination of Suramin and Mitomycin C has therapeutic activity but causes significant toxicity in patients with hormone-resistant CaP. However, the level of toxicity they found using the combination of drugs and lower levels of Suramin, was less than that reported for high doses of Suramin alone. Significant bone marrow depression produced by Mitomycin C is bound to limit the usefulness of this regimen especially in patients with CaP with bone metastasis and reduced bone marrow reserve due to the disease process or previous radiotherapy for bone pain.

9.1.5 Suramin and Estramustine phosphate (EMP) combination therapy in hormone resistant prostate cancer - the Leicester regimen: Phase II clinical trial: (preliminary report)

In vitro studies on the established human CaP cell lines LNCaP, PC3 and DU145, and on prostate cancer cells obtained by primary culture in Leicester confirmed that Suramin and EMP produce synergistic activity against cell growth. Consequently, we carried out a preliminary and limited clinical trial on patients with metastatic hormone resistant prostate cancer (Stage D₃) comparing the effect of EMP 280 mg bd alone with that of EMP 280 mg b.d. and low dose Suramin, that is, maintaining a mean serum concentration of 140 (range 90-180) ug/ml during the induction of Suramin therapy. Patients who received the EMP and Suramin combination had a statistically significant reduction in PSA (P < 0.001), a reduction of bone pain, and requirement for analgesics (P < 0.01) compared to patients who had EMP only or the 'control' group after 6 months of treatment. Toxicities encountered in this limited preliminary study were seen with EMP only. No side effects of Suramin were seen in patients who received low dose Suramin. Similarly, hydrocortisone replacement therapy was not required with this regimen unlike in high dose Suramin monotherapy. EMP is administered orally and, more

importantly, it does not cause significant bone marrow depression unlike Mitomycin C. These factors combine to make an EMP and Suramin combination the best combination treatment for hormone-resistant CaP up to date (Kehinde et al. 1995).

Section B

9.2   Conclusions

New insights regarding the biology of CaP have shown that there are major growth factors other than testosterone responsible for cellular proliferation of androgen-resistant prostate cancer cells. In vitro studies have confirmed the efficacy of growth factor inhibitors such as Suramin and SMS 201-995 in reducing cellular proliferation of androgen dependent LNCaP and independent PC3 prostate cancer cell lines as well as prostate cancer cells obtained by primary culture. Initial clinical trials using high dose Suramin (peak serum concentration 250-300 ug/ml) as monotherapy in patients with hormone-resistant prostate cancer have shown some promise, but the period of response to therapy has been short-lived and Suramin toxicity is a problem. To minimise Suramin's toxicity without reducing anti-tumour activity, studies evaluating its use in combination with other chemotherapeutic agents are attractive in CaP.

Suramin and Doxorubicin, tumour necrosis factor and Estramustine Phosphate have shown in vitro synergy. The preliminary clinical trial carried out in Leicester on patients with hormone-resistant CaP has shown that the combination of EMP 280 mg b.d. and Suramin 1 gm weekly infusions for 6 weeks, compared to EMP 280 mg bd alone showed a statistically significant difference in the rate of depression of PSA levels after 6 months of treatment (P < 0.001), and a statistically significant reduction of bone pain and analgesic requirement in patients on combination therapy (100% compared to 0% for patients on EMP alone). A key benefit of our regimen is immediate bone pain relief. This regimen produces the least toxicity up to date.

Further studies to determine whether other drug schedules involving the use of Suramin in combinations can produce acceptable results with less toxicities than high dose Suramin monotherapy for patients with hormone resistant prostate cancer are warranted. A possible option to explore will include low dose Suramin in combination with a further reduction of the dose of EMP to about 280 mg once daily to further reduce the side-effects of EMP without compromising therapeutic efficacy.

It is no hyperbole to state that there has not been any major contribution to the management of metastatic prostate cancer since the landmark observations of Huggins and Hodges in 1941 regarding the efficacy of androgen ablative therapy in the treatment of metastatic CaP. Fortunately, in recent years, we are now witnessing a resurgence of interest in the problem of metastatic hormone-resistant prostate cancer, which almost invariably kills most patients with the disease. With new insights regarding the role of growth factors in regulating the growth of hormone-resistant metastatic prostate cancer, novel treatment strategies should be forthcoming during the next few years.

9.2.1   Key Words:

Growth Factors, Hormone-Resistant Prostate Cancer, Suramin Monotherapy, Combination Therapy, Estramustine Phosphate.

Appendix A

Preparation of media used for experiments on established prostate cancer cell lines LNCaP, DU145 and PC3.

A.1   Foetal calf serum (FCS) (10%, 2.5% and 1.25%)

Foetal calf serum (FCS)

- 10% FCS was used to culture all cell lines during passaging. 12 ml added to cells in 200 ml tissue culture flask.

- 200 ml of 10% FCS prepared as follows:

    - add 22 ml FCS +

    - 2 ml L - Glutamine +

    - 2 ml Penicillin and Streptomycin 100 ug/ml +   174 ml of RPMI 1640 (GIBCO).

For all experiments, assessing the inhibitory effects of drugs on LNCaP cells, 2.5% FCS was used. 200 mls of 2.5% FCS was prepared as follows:

- 5.5 mls of FCS + 2 ml L - Glutamine + 2 mls Penicillin and Streptomycin 100 ug/ml + 190.5mls of RPMI

For growth stimulatory tests on LNCaP cells, 1.25% FCS was used in all experiments, 200 mls of 1.25% FCS prepared as follows:

- 2.75 mls FCS + 2 ml L - Glutamine + 2 ml Penicillin and Streptomycin 100 ug/ml and 193.25 ml RPMI.

A.2   **Preparation of RPMI and ITS for experiments on PC3 and DU145 cells.**

For all growth stimulatory and inhibitory tests on androgen independent prostate cell line, the medium used consisted of RPMI and ITS 200 mls was prepared as follows:

-         2 mls of sterile ITS (Insulin Transferrin Sodium Selenite) + 2 mls of Penicillin + Streptomycin 100 ug/ml + 196 mls of RPMI.

A.3   **Preparation of nutrient broth/yeast extract**

This was used to test for sterility of tissues or culture medium.

Dissolve the following in 1 litre of distilled water:

-         5 gm yeast extract +
-         2 gm nutrient broth +
-         8 gm NaCL

The solution was split into 500 ml and autoclaved.

A.4   **Cell culture techniques**

A.4.1   **Preparation of freezing medium - used to preserve cells that were not needed:**

-         45 mls RPMI 1640 and
-         5 mls DMSO
-         Add to 50 mls of 50% FCS
-         Sterilise by filtration
-         Divide into 20 mls amounts
-         Freeze at -20^0 C.

        Check for sterility using liquid nutrient broth before use.

A.4.2   **Cell culture techniques testing solutions for sterility**

        This was done each time a new batch of medium was prepared:

-         add 1 ml of test solution to a bijoux
-         add approximately 2 mls of nutrient broth

- incubate at 37^0 (for 72 hours)

- a slight turbidity indicates contamination

A.4.3   **Removing attached cells from the flask - sub-cultures or passaging cells**

- Pour off culture medium from each flask aseptically. Wash twice with calcium and magnesium free Hanks Balanced Salt Solution (HBSS).

- Add 5 mls of Trypsin/EDTA solution, swirl solution around in flask and put flask into an incubator for 3-5 minutes.

- Check that the cells have detached from flask.

- Stop action of Trypsin by adding 5 mls of 10% FCS.

- Centrifuge at 1000 rpm for 5 minutes.

- Wash pellets twice with 10% FCS.

- Make up cells in the medium at concentration as required.

- Label flasks with date, passage number, and cell type.

- Return to incubator.

- Check for cell growth/confluence in flask every 2-3 days.

A.4.4   **Feeding attached cells in cultures**

All the cells used in our experiments were attached to tissue culture flasks.

- Remove old culture medium leaving about 2 mls behind.

- Add fresh culture medium about 10-12 mls for 200 mls tissue culture flasks.

A.4.5   **Freezing cells (liquid nitrogen storage)**

- Transfer cells to a sterile universal container.

- Centrifuge at 1000 rpm for 5 minutes.

- Decant supernatant.

- Add 0.5 mls of freezing medium and mix gently. You need about 1×10^6 cells.

- Transfer to freezing vial and place in ice immediately.

- Transfer to -70^0C freezer and leave overnight (packed in polystyrene).

- Transfer to liquid nitrogen.

A.4.6   **Setting up cultures from frozen cells**

- Thaw cells (from liquid nitrogen) into 37^0C water bath - as quickly as possible.

- Add 8-10 mls of complete medium (10% FCS) to a universal bottle and add the cells.

- Spin at 1000 rpm for 5 minutes.

- Decant supernatant.

- Add 15 mls of culture medium to the cell pellet and mix slowly.

- Transfer to a 75 ml incubation flask.

A.4.7   **Suppliers of reagents and drugs**

Most of the reagents used in this work were obtained from Sigma Cell Culture Reagents, Sigma Chemical Co Ltd, Dorset, England, except where otherwise stated.

Worthington Collagenase I was obtained from Lorne Lab Ltd, Reading, England.

- ^{3}H-thymidine stock was obtained from Amersham International Plc, Little Chalfont Buckinghamshire, England

- Suramin was supplied free of charge by Bayer, UK Ltd, Newbury, England.

- Somatostatin SMS 201-995 was supplied free by Sandoz Pharmaceuticals, Research Division, Leeds, England.

- Estramustine Phosphate was supplied free of charge by Kabi-Pharmacia, Milton Keynes, England.

A.5   **Preparation of drugs used in various experiments**

A.5.1	Suramin (MW 1429.2)
A.5.2	Somatostatin 201-995 (MW 1079.4)
A.5.3	Estramustine phosphate (MW 564.4)
A.5.4	Epidermal growth factor

Stock solution of 4 mg/ml made by dissolving 4 mg Suramin in 1 ml of distilled water. Solution filtered using 0.2 u filters.

Subsequent strengths of Suramin obtained as follows:

(a)   1 ml stock and 3.938 mls medium to give 810 ug/ml solution.

(b)   1 ml of 810 ug/ml solution and 2 ml medium to give 270ug/ml solution.

(c)   1 ml of 270 ug/ml solution and 2 ml medium to give 90 ug/ml solution.

(d)   1 ml of 90 ug/ml solution and 2 ml medium to give 30 ug/ml solution.

(e)   1 ml of 30 ug/ml solution and 2 ml medium to give 10 ug/ml solution.

810 ug/ml   ≡ 3.02 mM Solution

270 ug/ml   ≡ 1.006 mM Solution

90 ug/ml   ≡ 0.335 mM Solution

30 ug/ml   ≡ 0.111 mM Solution

10 ug/ml   ≡ 0.0372 mM Solution

Half strength solutions of Suramin prepared as follows:

-     2 ml stock of 4 mg/ml and 2.938 ml medium = 810 ug/0.5ml solution

-     1 ml of 810 ug/0.5 ml solution and 2 ml medium = 270 ug/0.5ml solution, ie. 540 ug/ml

-     1 ml of 270 ug/ml/0.5 ml solution and 2 ml medium = 90 ug/0.5ml solution, ie. 180ug/ml

-     1 ml of 90 ug/0.5ml solution and 2 ml medium = 30ug/0.5ml solution, ie. 60 ug/ml

-     1 ml of 30 ug/0.5ml solution and 2 ml medium = 10ug/0.5ml solution, ie. 20 ug/ml.

A.5.2   Somatostatin SMS 201-995 M.W. 1079.4

Stock solution 1 mg/ml, that is dissolve 1 mg in 1 ml of distilled water and then sterilise by filtering with 0.2 um filter.

-     1 mg/ml stock solution and 2400 ul of medium = 20 ug/ml solution

-     300 ul of 200 ug/ml and 2700 ul of medium   = 2 ug/ml solution

-     300 ul of 20 ug/ml and 2700 ul of medium   = 0.2 ug/ml solution

-     300 ul of 2 ug/ml and 2700 ul of medium   = 0.02 ug/ml

-     300 ul of 0.2 ug/ml and 1700 ul of medium   = 0.002 ug/ml

A.5.3   Estramustine phosphate M.W. 564.4

-     Stock solution 4 mg/ml = 4000 ug/ml.

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- Diluted with sterile water.

Working solutions prepared as follows:

- 100 ul of working solution + 7.9 mls of medium = 50 ug/ml solution

- 4 ml of 50 ug/ml solution + 4 mls of medium = 25 ug/ml solution.

- 4 ml of 25 ug/ml solution + 4 mls of medium = 12.5 ug/ml solution.

- 4 ml of 12.5 ug/ml solution + 4 mls of medium = 6.25 ug/ml solution

- 4 ml of 6.25 ug/ml solution + 4 mls of medium = 3.1 ug/ml solution.

A.5.4   Epidermal growth factors

- Stock solution 100 ug/10 ml = 10 ug/ml = 10,000 ng/ml

- Diluted with HBSS

Working solutions prepared as follows:

- 1 ml of stock (10,000 ng/ml) + 15 mls medium = 625 ng/ml solution

- 1 ml of 625 ng/ml solution + 4 mls of medium = 125 ng/ml solution

- 1 ml of 125 ng/ml solution + 4 mls of medium = 25 ng/ml solution

- 1 ml of 125 ng/ml solution + 9 mls of medium = 12.5ng/ml solution

- 1 ml of 25 ng/ml solution + 4 mls of medium = 5 ng/ml solution

- 1 ml of 5 ng/ml solution + 4 mls of medium = 1 ng/ml solution

A.5.5   Hanks balance salt solution (HBSS)

A washing solution which was prepared in bulk.

A 250 ml solution contained the following ingredients:

1. NaCl 1.7675 gm

2. KCL 0.0375 gm

3. Glucose 0.18 gm

4. KH_2PO_4 0.034 gm

5. HEPES 1.7875 gm

6. Dissolve in 100 ml of distilled water

7. Add phenol red 0.25 ml

8. 4N NaOH is added while stirring to bring pH up to 7.6

9. H_2O is added to a final volume of 250 ml

Mix ingredients, filter solution and refrigerate at 4°C.

A.6   Measurement of serum Suramin levels

Plasma Suramin levels were assayed by the method of Klecker and Collins (1985). Briefly, Suramin was extracted from $100\mu l$ of plasma by adding 40 μl of 0.5 M tetrabutyl ammonium phosphate (TBAP, HPLC grade from Sigma, # T1531) followed by 400 μl HPLC grade methanol. This was vortexed vigorously and centrifuged at 2,000 rpm for 5 minutes. The supernatant was decanted into a separate tube and the extraction procedure was repeated on the pellet. This was repeated a third time and the pooled supernatants were centrifuged at 2,000 rpm for 5 minutes. Fifty microliters of the supernatant was injected directly onto the HPLC column. Separation was performed on a Whatman partisil ods-3 reverse phase C18 column with 10 μl particle size. Peak detection was performed with a Waters model 450 variable wavelength detector at 313 mm. A two-solvent system was employed. Solvent A contained 5 mM TBAP, 10 mM ammonium acetate (ACS grade from Sigma # A8920), and 30% HPLC methanol (v/v). Solvent B contained 5 mM TBAP, 10 mM ammonium acetate, and 90% HPLC methanol (v/v). Initial conditions were 40% solvent B at 2ml/min. Analytical runs progressed to 80% solvent B over 10 minutes; this was performed in linear fashion with a Waters model 660 solvent programmer. Plasma spiked with known concentrations of Suramin was extracted and served as standards. Peaks were analysed on a Hewlett Packard 3390A integrator (Fig 58). Mr N Mistry performed all the Suramin assays described in this work.

Hawkins, 1978, has shown that using Suramin 1 gm weekly for 6 weeks maintains a peak serum Suramin concentration of under 200 ug/ml and a trough level of about 10-15 ug/ml 3-6 months after discontinuation of Suramin injections. In Leicester the HPLC (Figs 58 and 59) was used to determine trough Suramin levels weekly for the first 7 weeks and at 2 monthly intervals subsequently. The next dose of Suramin was omitted if the previous week's Suramin trough level was more than 300 ug/ml. The aim of this regimen was to maintain a peak serum Suramin concentration of less than 200 ug/ml during induction of treatment and a trough concentration of over 15 ug/ml at 3 or 6 months. The patients were observed in hospital overnight following the first dose of Suramin. Urinalysis was performed during each visit before Suramin injection was given. If albuminuria was detected, and serum Suramin was more than 200 ug/ml, the next dose of Suramin was not given.

Fig 58 High pressure liquid chromatography machine used to measure serum Suramin.
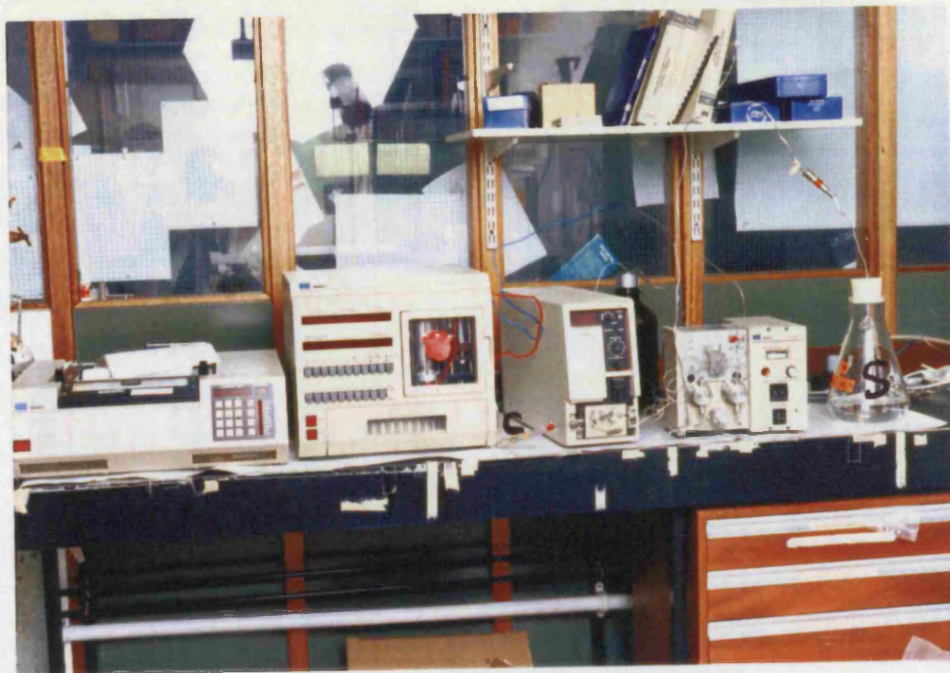
Fig 58   Schematic diagram of the HPLC equipment used for the serum Suramin Assay
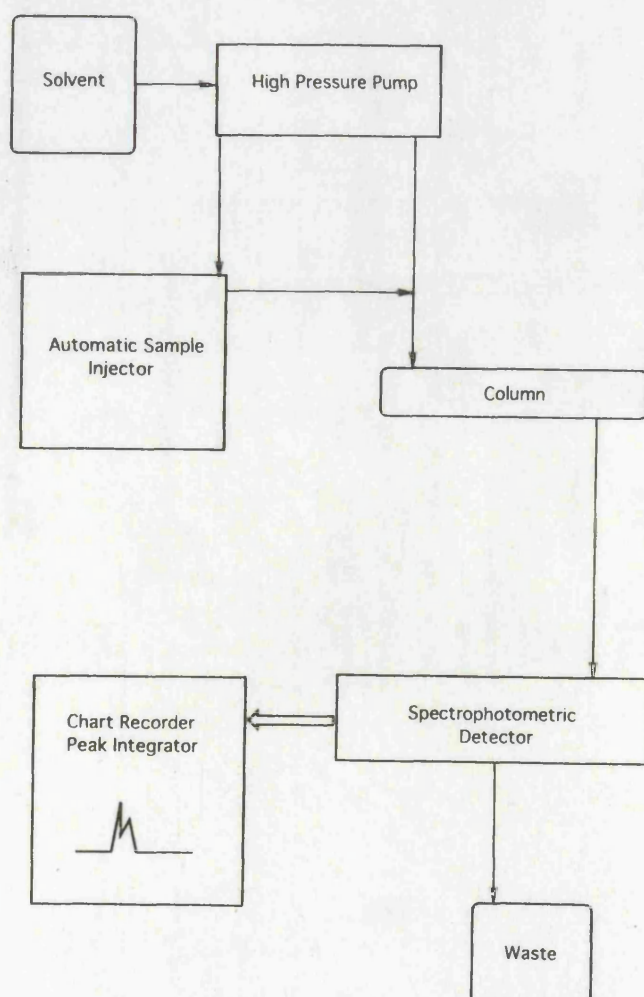
Fig 59

Chromatograms of fresh Suramin measurements using H.P.L.C. method

Figure 59 (b₁) Chromatogram of fresh suramin concentration equivalent to 50 ug/ml

INJECT

SAMPLE POSITION   01   SINGLE MODE   WISP REPORT

INJECTION VOLUME   0025
NUMBER OF INJECTIONS   1   INJECTIONS REMAINING   0
RUN TIME   00:06   EQUILIBRATION DELAY   00:00
NON-DEFAULT SYS MSG'S: 6500-0030 PSI.7048
WISP CODES GENERATED:

FEB. 2, 1994   07:06:05   CHART 0.50 CM/MIN   FLOW   0.90 ML/MIN
                                 RUN #34   CALC #0
COLUMN   SOLVENT   OPR ID: 6

CALIBRATION

PEAK#	AMOUNT	RT	EXP RT	AREA		RF
UNK		1.32		4173981	F	
UNK		1.86		39892	L	
1	1250.00000	2.58		28720282	S	0.435200E-1
UNK		4.00		635173	T	

PEAK#	RT	AMOUNT	RF
1	2.58	1250.00000	0.435200E-1

60 μg/ml = 50 mg/ml.

Inj vol = 25 μl

= 25×50

= 1250 mg/ml 25 μl

00	02/02/94	DATE
01	07:13:44	TIME
02	0.50	CHART SPEED. CM/MIN
03	OFF	PLOT
04	OFF	PEN 2
05	10	PEN 1 ZERO
06	90	PEN 2 ZERO
07	OFF	AUTO ZERO
08	LC	GPC LC
09	OFF	CALIB
20	OFF	AUTO PARAMETER
21	12	PEAK WIDTH
22	75.000	NOISE REJECTION

μl = 125/x × 1000

= 50 μg/ml (suramin)/ml

INJECTION VOLUME
NUMBER OF INJECT
RUN TIME
NON-DEFAULT SYS
WISP CODES GENER

Figure 59 (b₁₁) Chromatogram of AB suramin spiked with suramin and extracted as per protocol, concentration equivalent to 50 ug/ml

FEB. 2, 1994   07:28:27   CHART 0.50 CM/MIN   FLOW   0.90 ML/MIN
                                   RUN #36   CALC #0
COLUMN   SOLVENT   OPR ID: 6

EXTERNAL STANDARD QUANTITATION

PEAK#	AMOUNT	RT	EXP RT	AREA		RF
UNK		1.32		3831788	F	
UNK		1.86		112291	L	
1	2430.11000	2.56		56988222	S	0.435200E-1
UNK		3.98		722242	T	
TOTAL	2480.11000					
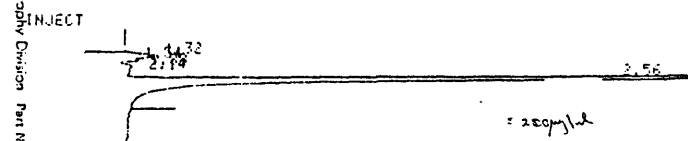
INJECT

SAMPLE POSITION   01   SINGLE MODE   WISP REPORT

INJECTION VOLUME   0025
NUMBER OF INJECTIONS   1   INJECTIONS REMAINING   0
RUN TIME   00:06   EQUILIBRATION DELAY   00:00
NON-DEFAULT SYS MSG'S: 6500-0030 PSI.7048
WISP CODES GENERATED:

FEB. 2, 1994   07:35:59   CHART 0.50 CM/MIN   FLOW   0.90 ML/MIN
                                   RUN #37   CALC #0
COLUMN   SOLVENT   OPR ID: 6

EXTERNAL STANDARD QUANTITATION

PEAK#	AMOUNT	RT	EXP RT	AREA		RF
UNK		1.32		3878184	F	
UNK		1.88		266482	F	
UNK		2.14		194586	F	
1	4406.84000	2.56		101260969	L	0.435200E-1
TOTAL	4406.84000					

INJECT
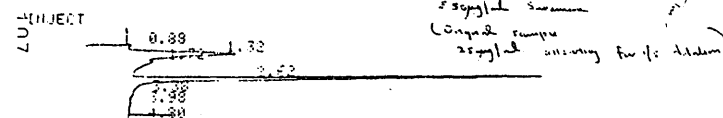
Statistical Methods

For ease of presentation, the data collected in this thesis have been summarised as means and their standard deviations or errors - as appropriate. Figures have been used to illustrate important differences more visually. The results presented represent the mean of at least 3 experiments, performed in most cases in 3 separate weeks.

Analysis of variance was used to determine differences in groups of surviving cells after exposure to different drugs, Fisher's exact test was used in suitable instances.

Correlation square (r^2) or Pearson's Correlation Coefficient $r = (\sqrt{r^2} = $ Square root of $r^2)$ was used to determine correlation between corresponding observations. A perfect correlation gave $r^2 = 1$, while a good correlation gave a value of 0.5 - 1.0 and no correlation was taken as values $\angle 0.50$

For comparison of the biochemical indices especially PSA in patients with prostate cancer in our clinical trial, Kruskal-Wallis non-parametric one-way Anova test was used.

The statistical methods used in the analysis of any set of results was decided after consultation with Dr. Charles Barkhit, biostatician, Department of Mathematics, College of Science, Sultan Qaboos University, Muscat, Oman.

Raw Data

Raw data for Table 13

The inhibition of growth of LNCaP and PC3 cell lines by Suramin

LNCaP								
	1st Experiment		2nd Experiment		3rd Experiment		MEAN ± SEM	
Dose of Suramin ug/ml	No of cells (DPM) control	No of cells (DPM) Expt	No of cells (DPM) control	No of cells (DPM) Expt	No of cells (DPM) control	No of cells (DPM) Expt	Cont.	Expt
0 (contr)	6581	6581	7156	7156	6439	6439	6725 ± 219	6725 ± 219
10	6581	6289	7156	6798	6439	5988	6725	6358 ± 237
30	6581	5989	7156	6123	6439	5576	6725	5896 ± 165
90	6581	3435	7156	4029	6439	3239	6725	3568 ± 238
270	6581	3356	7156	3499	6439	3071	6725	3309 ± 126
810	6581	2764	7156	2719	6439	2511	6725	2665 ± 78
PC3								
0 (contr)	7875	7875	8510	8510	10300	10300	8895 ± 727	8895 ± 729
10	7875	5591	8510	7659	10300	8652	8895	7301 ± 903
30	7875	4883	8510	7114	10300	6994	8895	6330 ± 725
90	7875	4181	8510	6305	10300	5644	8895	5377 ± 628
270	7875	4040	8510	5532	10300	5119	8895	4897 ± 445
810	7875	2433	8510	7650	10300	2565	8895	1921 ± 580

contr = control

Figures represent the number of cells per well as determined by ^{3}H thymidine incorporation assay (DPM).

Raw data for Table 14

The inhibition of growth of LNCaP and PC3 cell lines by SMS 201-995.

	1st Experiment		2nd Experiment		3rd Experiment		Mean	
Dose of SMS ug/ml	DPM control	DPM Expt Well	DPM Control	DPM Expt Well	DPM Control	DPM Expt Well	Contr	Expt Well
0	6581	7875	7156	7156	6439	6439	6725 ± 219	6725 ± 219
0.002	6581	5923	7156	7013	6439	6053	6725	6230 ± 344
0.02	6581	5660	7156	6941	6439	6053	6725	6218 ± 379
0.2	6581	2764	7156	4723	6439	3863	6725	3783 ± 568
2.0	6581	1448	7156	644	6439	837.1	6725	976.3 ± 243

(LNCaP)

0	7875	7875	8510	8510	10300	10300	8895 ± 727	8895 ± 727
0.002	7875	7009	8510	6127	10300	8443	8895	7193 ± 676
0.02	7875	6458	8510	5061	10300	8034	8895	6517 ± 860
0.2	7875	5434	8510	4425	10300	7004	8895	5621 ± 751
2	7875	4253	8510	3489	10300	5562	8895	4435 ± 606

(PC3)

Appendix C

Raw data for Table 15

The inhibition of growth of LNCaP and PC3 cell lines by EMP.

Cell line	LNCaP				PC3			
EMP ug/ml	1st Expt	2nd Expt	3rd Expt	Mean ± SEM	1st Expt	2nd Expt	3rd Expt	Mean ± SEM
0	12669	16215	29559	19481 ± 5148	15895	18609	21324	18609 ± 1569
3.1	10583	14651	20897	15379 ± 3003	10550	15454	20358	15454 ± 2385
6.25	7348	14791	14545	12228 ± 2444	7854	11191	14527	11191 ± 1929
12.5	2101	7929	3112	4381 ± 1800	6066	7857	9647	7857 ± 1035
25.0	188	4629	938	1918 ± 1374	5471	5581	5691	5581 ± 64
50.0	127	1121	700	649 ± 288	3997	4074	4150	4074 ± 44

Figures represent the number of cells per well as determined by ^{3}H thymidine incorporation assay (DPM).

Raw data for Table 16

The effect of Suramin (0-810 ug/ml) or Suramin (0-405 ug/0.5 ml) and SMS 201-995 (0.01 ug/0.5 ml) combination on cellular proliferation of prostate cancer cell line LNCaP.

Suramin ug/ml	LNCaP						
	Suramin Only				Suramin + SMS 0.01 ug/ml		
	DPM	well	DPM Mean ± SEM	Mean % Surviving cells	DPM/well	DPM Mean ± SEM	Mean % Surviving cells
0	Week 1 Week 2 Week 3	43891 16636 42428	34318 ± 11792	100%	43891 16636 42428	34318 ± 8862	100%
10	Week 1 Week 2 Week 3	41491 11615 35256	29454 ± 9110	86%	38611 10309 28239	25720 ± 8276	75%
30	Week 1 Week 2 Week 3	38063 76800 13015	19586 ± 9377	57%	29882 66995 10666	15749 ± 7167	46%
90	Week 1 Week 2 Week 3	23503 56798 12148	14443 ± 5843	42%	17799 55351 93161	10883 ± 3630	32%
270	Week 1 Week 2 Week 3	6899 2389 6135	5141 ± 1395	15%	9938 3988 65418	68223 ± 2985	19%
810	Week 1 Week 2 Week 3	4978 922 2689	2863 ± 2034	8%	9809 1726 2175	45705 ± 4543	13%

Raw data for Table 17

The effect of Suramin (0-810 ug/ml) or Suramin (0-405 ug/ 0.5ml) and SMS 201-995 (0.01 ug/ 0.5 ml) combination on cellular proliferation of prostate cancer cell line PC3.

PC3							
Suramin ug/ml	Suramin Only				Suramin + SMS 0.01 ug/0.5 ml		
	DPM	well	DPM Mean ± SEM	Mean % Surviving cells	DPM/well	DPM Mean ± SEM	Mean % Surviving cells
0	Week 1 Week 2 Week 3	22908 35849 36106	31621 ± 4362	100%	22842 35849 36372	31681 ± 4431	100%
10	Week 1 Week 2 Week 3	10706 33481 22977	22388 ± 6589	71%	12227 36216 22499	23647 ± 6957	75%
30	Week 1 Week 2 Week 3	9136 33040 15710	19295 ± 7138	61%	10527 36875 17939	21780 ± 5592	69%
90	Week 1 Week 2 Week 3	8040 30438 11828	16769 ± 6939	53%	7346 35931 35931	19238 ± 7651	61%
270	Week 1 Week 2 Week 3	6818 30055 7103	14659 ± 6231	46%	8566 33956 10348	17623 ± 8192	56%
810	Week 1 Week 2 Week 3	6318 16888 4159	9122 ± 4638	29%	6720 20309 5055	10695 ± 4837	33%

Raw data for Table 19(a)

The effect of Suramin (0-810) ug/ml and EMP (3.1 ug/ml) on prostate cancer cell line LNCaP.

Surami ug/ml	Suramin only							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Survivin g cells/well 1st Expt	% Survivin g cells/well 1st Expt	% Survivin g cells/well 1st Expt	Mean % Survivin g cells
0	32296	34908	37812	39730 ± 1594	100	100	100	100
10	22521	32405	24931	26619 ± 2856	56	83	62	67
30	14796	17238	35904	22646 ± 1156	48	72	50	57
90	13574	16513	18780	16289 ± 1508	24	66	32	41
270	2445	14901	11253	9535 ± 3701	22	28	24	24
810	2135	1199	5009	2781 ± 1147	8	3	9	7

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

% inhibition = 100 - % Surviving cells.

Raw data for Table 19(b)

The effect of Suramin (0-810 ug/ml) or Suramin (0-405 ug/0.5 ml) and EMP (1.5 ug/ 0.5 ml) combination on prostate cancer cell line LNCaP.

Suramin ug/0.5 ml	Suramin (0-405 ug/0.5 ml) and EMP (1.5 ug/ 0.5 ml)							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	32296	49082	37812	39730 ± 1594	100	100	100	100
10	20024	36812	28359	27811 ± 4852	62	75	73	70
30	16471	17670	21175	21852 ± 1413	51	48	56	55
90	15502	20127	11722	15892 ± 2433	48	41	31	40
270	4521	5399	7184	7151 ± 784	14	11	19	18
810	1938	4417	3403	3178 ± 120	6	9	9	8

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 20(a)

The effect of Suramin (0-810 ug/ml) and EMP (12.5 ug/ml) on prostate cancer cell line PC3.

Suramin ug/ml	Suramin only							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	88048	38505	76198	67584 ± 14954	100	100	100	100
10	66917	32344	74674	58122 ± 13026	76	84	98	86
30	62514	26183	51815	46633 ± 10791	71	68	68	69
90	45785	18482	35813	33116 ± 7986	52	48	47	49
270	26414	15787	31241	25006 ± 4570	30	41	40	37
810	7044	5776	1448	4756 ± 1696	8	15	19	14

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Appendix C

Raw data for Table 20b

The effect of Suramin (0-405 ug/0.5 ml) and EMP (6.25 ug/0.5 ml) combination on prostate cancer cell line PC3.

Suramin ug/0.5 ml	Suramin + EMP 6.25 ug/0.5 ml							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	88048	38505	76198	67584 ± 2025	100	100	100	100
10	49307	23488	41147	37981 ± 7628	56	61	54	57
30	42263	20408	37337	33792 ± 6624	48	53	49	50
90	29056	13092	15239	19599 ± 5008	33	34	20	29
270	14968	3851	13716	10138 ± 3520	17	10	18	15
810	8805	1926	9144	6625 ± 2354	10	5	12	9

DPM = disintegration per minute - measurement of surviving cells in culture using ^3H thymidine incorporation assay.

Raw data for Table 21a(i)

The effect of Suramin (0-810 ug/ml) and EMP (12.5 ug/ 0.5 ml) combination on prostate cancer cell line LNCaP

Surami ug/ml	Suramin only							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Survivin cells
0	32296	49082	37812	39730 ± 1594	100	100	100	100
10	22521	32405	24931	26619 ± 2979	56	83	62	67
30	14796	17238	25904	22646 ± 3374	48	72	50	57
90	13574	16513	18780	16289 ± 1508	24	66	32	41
270	2445	14901	11253	9535 ± 37801	28	24	24	24
810	2135	119	5009	2781 ± 1148	3	9	7	7

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 21a(ii)

The effect of Suramin (0-405 ug/0.5 ml) and (EMP 12.5 ug/0.5ml) combination on prostate cancer cell line LNCaP.

Suramin ug/0.5 ml	Suramin and EMP 12.5 ug/ 0.5ml							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	32296	49082	37812	39730 ± 1594	100	100	100	100
10	6661	2298	10288	9933 ± 1798	24	42	9	25
30	3764	20945	9867	12355 ± 7022	18	39	8	22
90	2606	7768	6897	5197 ± 2110	11	15	7	11
270	3546	3922	3524	3734 ± 154	9	7	6	7
810	947	4298	2612	2619 ± 791	3	6	5	5

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 21b(i)

The effect of Suramin (0-810 ug/ml) and (EMP 12.5 ug/ml) on prostate cancer cell line PC3.

Suramin ug/ml	Suramin only							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	25295	20808	29287	25130 ± 2452	100	100	100	100
10	18261	13112	26528	19300 ± 3912	93	84	80	86
30	14560	8436	26762	11498 ± 2503	74	54	69	66
90	11174	6297	11478	87358 ± 1993	57	40	51	49
270	7141	5127	9372	6134 ± 823	36	33	32	34
810	3628	3599	6342	4523 ± 911	12	23	18	18

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 21b(ii)

The effect of Suramin (0-405 ug/0.5 ml) and (EMP 6.25 ug/ 0.5 ml) combination on prostate cancer cell line PC3.

Suramin ug/0.5 ml	Suramin and EMP 6.25 ug/0.5 ml							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	18974	21720	23664	20347 ± 1122	100	100	100	100
10	3511	3794	15865	7723 ± 4076	18	26	65	36
30	2878	2900	9850	5363	15	19	40	25
90	1179	1532	6672	3123 ± 1777	6	10	27	14
270	581	1558	3094	1746 ± 1221	3	9	13	8
810	865	708	188	587 ± 205	1	5	1	2

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Appendix C

Raw data for Table 21c(i)

The effect of Suramin (0-405ug/0.5 ml) and (EMP 12.5 ug/0.5 ml) combination on prostate cancer cell line PC3.

Suramin ug/0.5 ml	Suramin and EMP (12.5 ug/0.5 ml)							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	5945	8151	6199	6765 ± 698	100	100	100	100
10	1486	2445	1239	1788 ± 368	24	22	20	22
30	951	1956	1239	1383 ± 299	16	24	20	20
90	654	734	578	659 ± 45	11	9	7	9
270	595	652	372	541 ± 21	10	8	6	8
810	418	326	558	435 ± 68	3	4	9	6

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 21c(ii)

The effect of Suramin (0-405 ug/0.5ml) and (EMP (12.5 ug/0.5ml) combination on prostate cancer cell line PC3.

Suramin ug/0.5 ml	Suramin and EMP 12.5 ug/0.5ml							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	23655	38505	76198	46119 ± 1565	100	100	100	100
10	7681	7786	41239	18902 ± 1118	32	20	54	35
30	5029	5312	29349	13230 ± 8070	21	14	39	25
90	3089	2808	13022	6306 ± 3363	13	7	17	12
270	2809	2163	6586	3853 ± 1381	12	6	9	9
810	2584	1824	3815	2741 ± 581	11	5	5	7

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 22

The effect of SMS 201-995 (0-20 ug/ml) or Somatostatin (0-10 ug/0.5 ml) and EMP (1.5 ug/0.5 ml) combination on prostate cancer cell line LNCaP.

SMS 201-995 ug/ml	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
			SMS + EMP 1.1 ug/ml					
0	13128	10074	14894	12699 ± 1409	100	100	100	100
0.02	11290	9470	14299	11683 ± 1410	86	94	96	92
0.2	6827	6548	8043	7138 ± 460	52	65	54	59
2	3807	4312	5809	4649 ± 602	29	43	39	37
20	2101	2519	3724	2781 ± 487	16	25	25	25

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 23

The effect of SMS 2091-995 (0-20 ug/ml) or Somatostatin (0-10 ug/0.5 ml) and EMP (12.5 ug/0.5 ml) combination on prostate cancer cell line PC3.

SMS 201-995 ug/0.5 ml	SMS + EMP 12.5 ug/ml							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	16231	19761	7643	14545 ± 3603	100	100	100	100
0.02	13959	12054	5274	10472 ± 2639	86	61	69	72
0.2	9576	11264	5350	8730 ± 1761	59	57	70	62
2	7953	10473	2752	7091 ± 2276	49	53	36	46
20	8765	8497	2904	6722 ± 1821	54	43	38	45

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 24

The effect of combining drug concentrations producing less than 5% on growth inhibition on prostate cancer cell line PC3 and DU145.

DRUGS	cells/well (DPM) 1st Expt Control 0	cells/well (DPM) 2nd Expt EMP 1.5 ug/ml	cells/well (DPM) 3rd Expt Sur 3.3 ug/ml	Mean ± SEM cells/well EMP 0.8 ug/0.5 + Sur 1.7 ug/0.5 ml	% Surviving cells/well 1st Expt Control 0	% Surviving cells/well 1st Expt EMP 1.5 ug/ml	% Surviving cells/well 1st Expt Sur 3.3 ug/ml	Mean % Surviving cells EMP 0.8 + Sur 1.7 ug/0.5 ml
PC3 Experiment								
a	7583	7356	7431	6597	100	97	98	87
b	7851	7380	7459	6438	100	94	95	82
c	8804	8012	8364	8393	100	91	95	86
Mean ± SEM	8079 ± 371	7582 ± 215	7756 ± 307	7143 ± 628	100	93	96	85
DU145 Experiment								
a	11637	11172	11288	10008	100	96	97	86
b	11391	10594	10594	10480	100	93	93	92
c	15867	15074	15074	14122	100	93	95	89
Mean ± SEM	12965 ± 1454	12187 ± 1409	12317 ± 1381	11539 ± 1301	100	94	95	89

% growth inhibition = 100 - % surviving cells in well

Raw data for Table 25

The effect of combining drug concentrations producing less than 5% growth inhibition on prostate cancer cell line LNCaP.

	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
DRUGS	Control 0	EMP 1.5 ug/ml	Sur 3.3 ug/ml	EMP 0.8 ug/0.5 + Sur 1.7 ug/0.5 ml	Control 0	EMP 1.5 ug/ml	Sur 3.3 ug/ml	EMP 0.8 + Sur 1.7 ug/0.5 ml

Experiment								
a	5791	5560	5444	4981	100	96	94	86
b	6742	6540	6540	5433	100	97	97	88
c	5503	5283	5229	4898	100	96	95	89
Mean ± SEM	6012 ± 375	5794 ± 370	5738 ± 406	5271 ± 166	100	96	95	88

Raw data for Table 31a

The effect of anti-epidermal growth factor receptor on growth of prostate cancer cell lines LNCaP and PC3.

Anti-EGFR ug/ml	LNCaP							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	8391	9482	7628	8500 ± 539	100	100	100	100
0.1	7560	8012	6889	7480 ± 326	90.1	84.5	90.3	88.2
1	6537	7510	6415	6834 ± 346	77.9	79.2	84.1	80.4
10	6167	8069	6492	6909 ± 588	73.5	85.1	79.3	79.3
100	6671	7311	5744	6574 ± 455	79.5	77.1	75.3	77.3

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 31b

The effect of anti-epidermal growth factor receptor on growth of LNCaP and PC3 prostate cancer cell line.

| | PC3 | | | | | | |
Anti-EGFR ug/ml	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	14174	12370	15751	14098 ± 978	100	100	100	100
0.1	10673	9661	11892	10757 ± 646	75.3	78.1	75.5	76.3
1	8278	8560	10096	9009 ± 566	58.4	69.2	64.1	63.9
10	8788	8299	9451	8883 ± 334	62	67	60	63.0
100	9156	7150	10490	8882 ± 271	64.6	57.8	66.6	63.0

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 31c

The effect of anti-EGF-Receptor on growth of DU 145 prostate cancer cell line.

Anti-EGFR ug/ml	cells/well (DPM) 1st Expt.	cells/well (DPM) 2nd Expt.	cells/well (DPM) 3rd Expt.	Mean ± SEM cells/well	% Surviving cells/well 1st Expt.	% Surviving cells/well 1st Expt.	% Surviving cells/well 1st Expt.	Mean % Surviving cells
0	63944	75810	85211	74998 ± 6160	100	100	100	100
0.1	4834	5193	5845	5317 ± 296	75.6	68.5	68.6	70.9
1	3210	4473	4601	4080 ± 444	50.2	59.0	54.0	54.4
10	3146	4162	3645	3817 ± 294	49.2	54.9	48.6	50.9
100	3203	3722	4823	3892 ± 478	50.1	49.0	56.6	51.9

Anti-EGFR ug/ml	% growth inhibiton
0	0
0.1	29.1
1	45.6
10	49.1
100	48.1

% growth inhibition = 100 - % surviving cells.

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 35a

Prostatic acid phosphatase levels in the supernatant of culture mediums of cells derived from BPH, NDCaP and HRCaP specimens.

Days in Culture	PAP IU/L			
	BPH			
	1st Expt	2nd Expt	3rd Expt	Mean ± SEM
2	0.1	0.1	0.1	0.1 ± 0.00
4	0.16	0.15	0.15	0.15 ± 0.10
6	0.22	0.19	0.17	0.19 ± 0.06
8	0.19	0.21	0.15	0.18 ± 0.07

BPH   =   benign prostatic hyperplasia

NDCaP   =   newly diagnosed carcinoma of the prostate

HRCaP   =   hormone resistant cancer of the prostate

Raw data for Table 35b

Prostatic acid phosphatase levels in the supernatant of culture medium of cells derived from BPH, NDCaP and HRCaP specimens.

Days in Culture	PAP IU/L			
	NDCaP			
	1st Expt	*2nd Expt*	*3rd Expt*	*Mean ± SEM*
2	2.5	2.1	1.7	21. ± 0.4
4	2.9	4.4	3.8	3.7 ± 0.4
6	4.6	3.4	4.3	4.1 ± 0.7
8	5.1	5.6	4.9	5.2 ± 0.6

BPH   = benign prostatic hyperplasia

NDCaP = newly diagnosed carcinoma of the prostate

HRCaP = hormone resistant cancer of the prostate

Appendix C

Raw data for Table 35c

Prostatic acid phosphatase levels in the supernatant of culture mediums of cells derived from BPH, NDCaP and HRCaP specimens.

Days in Culture	PAP IU/L			
	HRCaP			
	1st Expt	2nd Expt	3rd Expt	Mean ± SEM
2	2.5	2.4	2.9	2.6 ± 0.7
4	3.1	2.3	3.6	3.0 ± 0.8
6	5.1	5.1	4.8	5.0 ± 0.1
8	5.7	4.9	5.3	5.3 ± 0.9

BPH = benign prostatic hyperplasia

NDCaP = newly diagnosed carcinoma of the prostate

HRCaP = hormone resistant cancer of the prostate

Raw data for Table 38a

The effect of Suramin (0-270 ug/ml) or Suramin (0-135 ug/0.5 ml) and EMP (12.5 ug/0.5 ml) combination on prostate cancer cells obtained by primary culture.

	Suramin							
Suramin ug/ml	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	2144	3710	3038	2964 ± 454	100	100	100	100
10	1951	3265	1944	2386 ± 440	91	88	64	81
90	1244	1707	1489	1482 ± 134	58	46	49	51
270	858	1288	1367	1176 ± 158	40	35	45	40

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 38b

The effect of Suramin (0-135 ug/0.5 ml) and (EMP 12.5 ug/0.5 ml) combination on prostate cancer cells obtained by primary culture.

Suramin ug/0.5 ml	Suramin + EMP 12.5 ug/0.5 ml							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	2144	3710	3038	2964 ± 454	100	100	100	100
10	772	1447	1094	1097 ± 195	36	39	36	37
90	686	1076	881	889 ± 113	32	29	29	30
270	600	742	729	690 ± 45	28	20	24	24

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 38c

The effect of Suramin (0-135 ug/0.5 ml) and (EMP 25 ug/0.5 ml) combination on prostate cancer cells obtained by primary culture.

Suramin 0.5 ug/ml	Suramin + EMP25 ug/0.5ml							
	cells/well (DPM) 1st Expt	cells/well (DPM) 2nd Expt	cells/well (DPM) 3rd Expt	Mean ± SEM cells/well	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	% Surviving cells/well 1st Expt	Mean % Surviving cells
0	2144	3710	3038	2964 ± 454	100	100	100	100
10	793	1187	1003	994 ± 114	37	32	33	34
90	536	742	911	741 ± 109	25	20	30	25
270	515	631	668	604 ± 46	24	17	22	21

DPM = disintegration per minute - measurement of surviving cells in culture using ^{3}H thymidine incorporation assay.

Raw data for Table 40

Reversibility of the effects of Suramin 270 ug/ml, EMP 25 ug/ml, SMS 2 ug/ml, or EMP 12.5 ug/0.5 ml and Suramin 135 ug/0.5 ml. combination on the proliferation of prostate cancer cells obtained by primary culture.

Hours of continued exposure		0	4	8	16	24	144
Drug concentration		% Surviving Cells					

Suramin 270 ug/ml	Expt a	100	94	86	59	47	11
	Expt b	100	88	85	57	55	9
	Expt c	100	88	84	64	57	7
MEAN ± SEM		100	90 ± 5	85 ± 5	60 ± 6	53 ± 3	9 ± 2

EMP 25 ug/ml	Expt a	100	66	59	37	34	10
	Expt b	100	62	45	45	36	6
	Expt c	100	73	55	44	32	8
MEAN ± SEM		100	67 ± 5.6	50 ± 5	42 ± 2	34 ± 2	8 ± 5

EMP 12.5 ug/0.5 ml + Suramin 135 ug/0.5 ml	Expt a	100	51	33	27	20	2
	Expt b	100	53	39	31	17	5
	Expt c	100	37	33	31	17	8
MEAN ± SEM		100	47 ± 4	35 ± 3	29 ± 2	18 ± 1.1	5 ± 3

SMS 201-995 2 ug/ml	Expt a	100	97	92	91	78	19
	Expt b	100	99	96	85	82	14
	Expt c	100	89	94	88	86	15
MEAN ± SEM		100	95 ± 3.1	94 ± 1.2	88 ± 1.7	82 ± 2.3	16 ± 1.52

% surviving cells/well calculated from:

$$\frac{\textbf{\textit{No of cells in experimental well}}}{\textbf{\textit{No of cells in control well}}} \quad \textbf{\textit{X} \ 100\%}$$

Raw data for Table 42

The effect of EGF on Suramin induced growth inhibition of prostate cancer cells obtained by primary culture.

Days		2	4	6	8
Drugs		No of cell per well (DPM)			

Control No drug	Expt a	7784	25000	21530	34721
	Expt b	9500	13031	21465	29731
	Expt c	9274	10723	19780	34731
MEAN ± SEM		8852 ± 539	16251 ± 4430	20925 ± 573	33061 ± 1667

EGF 5 ng/ml	Expt a	7784	9705	16231	31310
	Expt b	9500	16400	20961	22479
	Expt c	9274	13072	18971	39211
MEAN ± SEM		8852 ± 539	13059 ± 1935	18721 ± 1346	31000 ± 4838

Suramin 270 ug/ml	Expt a	7784	7271	6871	7737
	Expt b	9500	6644	5900	9602
	Expt c	9274	5798	10101	8824
MEAN ± SEM		8852 ± 539	6571 ± 427	7624 ± 1271	8721 ± 542

EGF 2.5 ng/0.5 ml + Suramin 135 ug/0.5 ml	Expt a	7784	4746	9763	10151
	Expt b	9500	6159	8124	9046
	Expt c	9274	6321	8342	8751
MEAN ± SEM		8852 ± 539	5742 ± 501	8743 ± 515	9316 ± 427

Photocopy letters of approval from

- Medicines Control Agency, London
- Leicestershire Ethics Committee Approval
- Bayer UK Limited

Protocol for Clinical Trial

- Quality of Life assessment questionnaire
- Patient information sheet.

Department of Health

MEDICINES CONTROL AGENCY

Market Towers   1 Nine Elms Lane   London SW8 5NQ

Facsimile 071-273 0493 *Telephone* 071-273 0327/0328

MCA

Professor P R F Bell   Our Ref: MF 8000/5254
Leicester Infirmary   Product: Suramine
Department of Surgery
Leicester General Hospital
Gwendolen Road
Leicester LE5 4PW   11th June 1993

Dear Professor Bell

I am writing in connection with your notification under the
Medicines (Exemption from Licences) (Special Cases and
Miscellaneous Provisions) Order 1972 which relates to a
proposed trial using Suramine
supplied by Bayer PLC.

The Licensing Authority does not propose to issue a
direction under Article 4(2)(v) of the above order in this
case. The above named supplier may therefore supply the
product for the purpose outlined in your notification
without a product licence or clinical trial certificate
being required.

This exemption is conditional on your agreement that all
serious or unexpected adverse reactions occuring during the
course of the trial will be notified to the Licensing
Authority at the earliest opportunity.

Your attention is drawn to the fact that your notification
to the Licensing Authority under the above Order
(i.e.1972/1200) was not accompanied by the detailed and
extensive data which is required to support a product
licence or a clinical trial certificate. It must therefore
be emphasised that the Licensing Authority has not
positively assessed the product for safety, quality or
efficacy.

You may wish to ensure that the hospital, or other
appropriate, Ethics Committee is aware of the limitations of
the Licensing Authority's approval of this trial.

We shall be pleased to see a copy of any report which is
produced as a result of this trial.

Yours sincerely

Trupti Sangani

Miss Trupti Sangani.

Our Ref: AEC/MS

27 August, 1993

Dear Mr. Kehinde,

Suramin Therapy in Advanced Prostate Cancer

Further to your application dated 8th July, 1993, you will be pleased to know that the Ethics Committee at its meeting held on the 6th August, 1993 approved your request to undertake the above-mentioned research conditional upon a patient information sheet being produced and submitted for the Committee's formal approval. Also the Committee would welcome clarification on the study design since the application form refers to randomisation into three treatments whilst the protocol study design refers to randomisation into Groups A and B. The Committee requested an early report on toxicity after a small number of patients had been treated.

I would remind you, however, that your research project has been given approval only in relation to its acceptability from an ethical point of view. If, subsequently, departure from the methodology outlined in your protocol is contemplated, the Ethics Committee must be advised in order that the proposed changes may be approved. Also a report should be made to the Ethics Committee if any significant adverse reactions are noted during the course of the study.

In addition, any NHS resource implications of your project must be discussed with the appropriate Trust Chief Executive/Unit General Manager. Similarly, it may be that the research project has implications for other disciplines and, if so, you are advised to discuss them with the appropriate departmental manager. Researchers should also be able to assure the Ethics Committee that satisfactory arrangements have been made for the labelling, safe storage and dispensation of drugs and pharmaceutical staff are always willing to provide advice on this.

Researchers' attention is also drawn to correspondence from the Regional Director of Public Health dated 28th January, 1991 relating to Clinical Trials which sets out revision of the procedures to be followed, and the Clinical Trials Indemnity Letter and Deed of Guarantee. Researchers should ensure that these indemnity arrangements have been complied with.

Researchers intending to study selective groups of patients in the community are reminded that their first approach should be to the individual patient's general practitioner to ascertain whether the particular patient was suitable for inclusion in the study. Equally, when the researcher contacts the patient it should be emphasised that the approach is made with the knowledge of the General Practitioner, with whom the patient may discuss this research, if the patient so wished.

Yours sincerely,

G. M. Morgan

Director of Public Health. PPMS

Mr. E. O. Kehinde,
Research Fellow, Department of Surgery,
Leicester General Hospital.

Bayer

Bayer plc
Pharmaceutical Business Group
Bayer House
Strawberry Hill
Newbury
Berkshire RG13 1JA

Telephone: 0635 39000
Fax: 0635 563393
Telex: 847205 Baynew G

Mr.E.O.Kehinde,
Department of Surgery,
School of Medicine,
Leicester General Hospital,
Gwendolen Road,
Leicester   LE5 4PW

Your ref	Our ref	Direct dialling	Direct fax	Date
	GM/sjh45mar	0635 5613	3616 56	24th March 1993

Dear Mr.Kehinde,

Thank you for your telephone call yesterday. As I indicated, I phoned several times following your call to my secretary some time after my letter of the 25th November, and in the interim assumed there were too many snags to conduct the study you originally proposed. I understand that you have obtained a method of assaying suramin but that the dose levels employed are not likely to cause toxicity.

As promised, I have completed form MLA163, duly signed, and this is enclosed together with two copies of form MLA162, lest one of them becomes spoilt in completion. Professor Bell's signature is required in section 3 of the first page, as the form has to be signed by the consultant in charge of the patients. If an alternative consultant assumes responsibility, I will need to alter form MLA163. You will see that they request typed completion or the use of block capitals using black ink on all three pages.

In terms of certain of the boxes to be completed on page 3 - the chemical name of the drug is obviously suramin and against structure of drug, I suggest you use the term "polysulfonated naphthylurea, a reverse transcriptase inhibitor". In box 10 the entry should be "1g IV vials". The answers to questions 11a and 11b are "no", so there is no PL number to complete. Please insert my name and address in box 12, and Bayer plc as supplier in box 13, with the same address as in box 12.

Please forward form MLA163 with your forms MLA162, noting the different room numbers in Market Towers. When you receive their decision to proceed, please advise accordingly and I will arrange for the suramin to be supplied to you promptly.

I hope the study will prove informative.

Yours sincerely,

Dr.G.Macdonald
Medical Director

SURAMIN THERAPY IN ADVANCED CANCER OF PROSTATE

Patients with hormonal resistant Cancer of prostate:
e. satge D2 with 2 successive rises in PSA

Study Design

Group A - EMP 280 mg t.d.s. x 3/12 orally.

Group B - EMP 280 mg t.d.s. x 3/12.
    + Suramin 1 g weekly i.v. x 6/52.
      Test dose of 200 mg to start.
    + i.v. chlorpheniramine 10 mg start.

Group C - Control - hydrocortisone 10 mg t.d.s. x 6/52.
    Stop all hormone manipulation x 3/12.

PROTOCOL FLOW CHART: Suramin therapy in advanced prostate cancer.

WEEK

	1	2	3	4	5	6	7	8	9	10	11	12
Visit WEEK												
Growth hormone profile	X					X						X
Prolactin	X					X						X
Testosterone	X					X						X
U & E's	X					X						X
LFT	X					X						X
PSA	X					X						X
FBC	X					X						X
SAP	X											
Urinalysis (Grp. B patients)	X	X	X	X	X	X						X
Lymph Node Mets.	X											X
USS Pros. + Pelvic nodes	X											X
Karnofsky Score	X											X
Analogue Pain Score	X											X
Analgesic Requirements	X											X
Clinical Evaluation	X											X
Serum Suramin level (Grp. B Pts.)		X	X	X	X	X						X

<u>QUESTIONNAIRE FOR PATIENTS</u>

(To be completed on Days 1, 90, 180, 270, 365 of Commencement of
Treatment)

CLINICAL TRIALS
TREATMENT OF PATIENTS WITH HORMONE RESISTANT METASTATIC CARCINOMA
OF PROSTATE (STAGE D3) WITH ESTRAMUSINE PHOSPHATE OR ESTRAMUSINE
<u>PHOSPHATE + SURAMIN</u>

ASSESSMENT of QUALITY of LIFE

Name: .... Date of Birth: ....

Address: .... Hospital No: ....

....

....

....

We are interested in some things about you and your health. There are no
'right' or 'wrong' answers. Please answer all the questions yourself by
circling the number that best applies to you. The information that you
provide will remain strictly confidential.

Please fill in today's date: Day .... Month ..... Year .....

<u>Because of your present condition:</u>	<u>Yes</u>	<u>No</u>
1. Do you need help with eating, dressing, bathing or using the toilet?	1	2
2. Do you have to stay in bed or a chair for most of the day?	1	2
3. Do you have to stay indoors most or all of the day?	1	2
4. Do you have any trouble either taking a walk or climbing a few flights of stairs?	1	2
5. Do you have any trouble bending, lifting or stooping?	1	2
6. Are you limited in any way in doing your work or household jobs?	1	2

During the past week:	Not At All	A Little	Quite A Bit	Very Much
7. Did you have to urinate more frequently than is normal for you?	1	2	3	4
8. Did you have difficulty controlling your urination?	1	2	3	4
9. Did you pass blood when you urinated?	1	2	3	4
10. Did you have pain when you urinated?	1	2	3	4
11. Did you have pain in other parts of your body?	1	2	3	4
12. Did you have any treatment for pain?	1	2	3	4
How much did it help?	1	2	3	4
13. Were you physically well?	1	2	3	4
14. Did your condition limit your interest in sex?	1	2	3	4
15. Did your condition interfere with your enjoyment of sex?	1	2	3	4
16. Has your condition interfered with your family or social life?	1	2	3	4
17. Has your medical treatment interfered with your family or social life?	1	2	3	4

18. Date questionnaire was completed .... 199...

Please check to make sure that you have answered all the questions.

PATIENT INFORMATION SHEET

SURAMIN THERAPY IN CANCER OF PROSTATE

Dear Patient

The purpose of this write up is to inform you about the clinical trials in which you have agreed to participate.

You have been diagnosed to have prostate cancer. Prostate cancer feeds on the male sex hormone called testosterone which is produced by the testis. To suppress the growth of prostate cancer it is important to counteract the production of testosterone. This can be done by tablets (eg cyproterone acetate, flutamide, stilboesterol) injection therapy (eg Zoladex) or by surgery to remove the testis. You must have been on one of these forms of therapy for sometime.

One of the problems is that with time - the prostate cancer can get used to this type of treatment and escape control. At present, therapeutic options are very limited for patients whose cancer no longer respond to hormones. However, research efforts in Leicester and some centres in the USA have indicated that Estramustine phosphate and Suramin may be useful under such circumstances. Estramustine phosphate is taken by mouth at a dosage of 280 mg three times daily. It must not be taken with milk or milk products as this affects its potency. It may cause minor enlargement of your breasts and it may also make you feel sick.

Suramin is administered intravenously weekly for 6 weeks. You will have a test dose to make sure that you will not react to the drug. It may also make you feel sick or may make your vision blurred, these side effects are temporary and will resolve once drug administration is discontinued. Suramin tends to depress the function of your adrenal gland. You will therefore receive hydrocortisone 10 mg TDS to counteract this effect. Hydrocortisone also makes some patients feel good within themselves.

As with any new treatment, extra care has to be taken to monitor side effects, which are not always apparent. You will be asked at each visit if you have experienced any side effects. This study has been reviewed and approved by the local Ethics Committee of this hospital.

In the unlikely event that serious problems arise that are due to treatment with the study drug, you will receive appropriate treatment and compensation as laid down by government regulations.

If for any reason, you cannot tolerate the drugs, obviously we will have to withdraw you from this study.

If you notice any untoward side effects while on these drugs, please inform the following researchers immediately:

1.   Mr E O Kehinde - Telephone Leicester 490490, Bleep 4075, 736179

2.   SN Barbara Peacock - Telephone Leicester 490490 Ext 4601, 777180

3.   Mrs Janice White, Mr Terry's Secretary - Telephone Leicester 584450

Participation in this study is entirely voluntary and you will be free to withdraw from the study at anytime.

We hope you will be one of the patients who will benefit from the results of our research efforts.

Thank you for your co-operation

Cancer of Prostate Research Group
c/o Department of Urology
Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

- **Published abstracts of thesis**

- **Published articles from thesis**

LIST OF ABSTRACTS AND PUBLICATIONS IN JOURNALS

Kehinde, E.O., Horsburgh, T., Mistry, N., Terry, T., Donnelly, P.K. Growth factor inhibition and carcinoma of prostate. Br J Cancer 1992; (XVII): 1.

Kehinde, E.O., Horsburgh, T., Mistry, N., Terry, T., Donnelly, P.K. Inhibitors of growth factors in the therapy of hormone resistant prostate carcinoma. Br J Surg 1992; 79 (11): 1240.

Kehinde, E.O., Terry, T.R., Mistry, N., Horsburgh, T., Iqbal, S.J., O'Reilley, K., Bell, P.R.F. Primary Culture of Cancer of Prostate Epithelial Cells: The Leicester Experience. Urol Res. 1993: 21(6): 447.

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PRESENTATIONS TO LEARNED SOCIETIES

Kehinde, E.O., Horsburgh, T., Mistry, N., Terry, T., Donnelly, P.K. Inhibitors of growth factors in the therapy of hormone resistant prostate carcinoma. Surgical Research Society of Great Britain, Summer Meeting 1992 (Abstract): 98.

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Kehinde, E.O., Horsburgh, T., Donnelly, P.K., Terry T., Mistry, N., Edwards, C. The effects of combination therapy using Suramin and Somatostatin on cellular proliferation of cancer of prostate cell lines PC3 and LNCaP. Edinburgh Urological Festival 1992 (Abstract): 36.

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Horsburgh, T., Kehinde, E.O., Lycett, A., Bell, P.R.F., Donnelly, P.K., Terry, T. Histocompatability antigen expression in prostate cancer biopsies and in prostate cancer cell iines. British Prostate Group Autumn Meeting, Stirling, Scotland - 13 November 1992.

Kehinde, E.O., Liu, S., Donnelly, P.K., Sandhu, S.D., Terry, T.R. A combination chemo-hormonal therapy for metastatic hormone resistant carcinoma of the prostate: results of in vitro studies adapted for clinical trials. Urological Research Society of Great Britain Annual Scientific Meeting, London - 8 January 1993 (Abstract): 24.

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Kehinde, E.O., Terry, T.R., Mistry, N., Horsburgh, T., Iqbal, S.J., O'Reilley, K., Bell, P.R.F. Primary culture of cancer of prostate epithelial cells: the Leicester Experience. 10th Congress of European Society for Urological Oncology and Endocrinology, Berne, Switzerland. 10-13 March 1994 (Abstract): 24.

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