

**COMBINATORIAL CHEMOPREVENTION OF PROSTATE
CANCER**

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ABSTRACT

Chemoprevention of prostate cancer is a relatively new concept which is attracting a great deal of interest from clinicians and scientists alike as it offers real hope of reducing the burden of this disease on society in the future. The ultimate goal is to develop safe and efficacious agents which, when administered over a long period of time, will prevent or delay prostatic carcinogenesis. Improved understanding of the molecular biology of prostate cancer has led to the identification and development of a number of agents which may be well suited to the task of chemoprevention. In the work described here, two novel agents, resveratrol and gefitinib, have been evaluated for their anticancer effects using *in vitro* and *in vivo* models of prostate cancer. Specifically, the hypothesis that these two agents can act synergistically to achieve a greater antineoplastic effect has been tested. Resveratrol is a diet-derived naturally-occurring polyphenol which displays anti-oxidant and anti-inflammatory properties. Gefitinib (ZD1839. "Iressa", AstraZeneca Pharmaceuticals) is a small molecule inhibitor of the epidermal growth factor (EGFR) tyrosine kinase. EGFR is increasingly implicated in prostatic carcinogenesis. The results presented here demonstrate that both resveratrol and gefitinib inhibit the proliferation of hormone-sensitive and hormone-resistant cells *in vitro*, although there was no synergy between these two compounds. Both compounds modulate cell cycle kinetics causing arrest in various phases of the cycle. Gefitinib was shown to effectively abrogate EGFR phosphorylation in prostate cancer cell lines. No effect of resveratrol on oxidative DNA damage in prostate cancer cells was observed and its effects on COX-2 expression could not be evaluated. Gefitinib potently inhibited the development of DU145 xenografts in a nude mouse model of prostate cancer. No significant effect of resveratrol could be seen in the same mouse model. These results suggest that gefitinib may well have a role to play in the

chemotherapy and chemoprevention of prostate cancer. Despite its potent *in vitro* antiproliferative effects, further *in vivo* evaluation of resveratrol is required prior to its use alone or in combination with other agents can be recommended in prostate cancer.

DEDICATION

This thesis is dedicated to my parents and to my wife, Joanna.

Thanks for experiencing the rollercoaster ride of research with me.

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Chapter 1: Introduction

“Is a cure possible for those whom it is necessary? Is it necessary for those in whom it is possible?”

With these two statements, Whitmore summarised the conundrum facing urologists treating men with prostate cancer. Although it is the most frequently diagnosed solid tumour in men in the Western world, the natural history of the disease runs a variable course, being aggressive and leading to the death of some patients whilst remaining relatively indolent in others. Curative options are available for early prostate cancer in the form of radical prostatectomy, radical radiotherapy, as well as newer approaches such as brachytherapy and cryotherapy, but they are associated with a high risk of urinary incontinence and/or impotence. With increasing public awareness of prostate cancer and PSA testing, greater numbers of early tumours are being identified on prostatic biopsy. There is as yet no way of determining which of these biopsy-detected cancers will go on to cause clinical disease, although there is a lot of research currently taking place to address this important question. Thus, it is inevitable that a proportion of patients with early prostate cancer will be over-treated with the attendant operative risks and post-operative complications. They may in fact, be a group in whom a cure is possible but undesirable.

Metastatic prostate cancer may initially be responsive to androgen ablation by surgical or pharmacological means. Eventually, however, a proportion of these tumours progress to a phase of hormone-independent growth, at which point the only therapeutic options are palliative in nature. Thus, for this group of patients in whom a cure is highly desirable, we are unable to provide one.

It can thus be appreciated that current management of established prostate cancer remains far from ideal. New strategies are being developed both to prevent the disease and slow its progression by the administration of either naturally occurring or synthetic agents. This is known as chemoprevention and it is one of the most exciting areas of cancer research at present. If the promise shown by early *in vitro* and *in vivo* studies is borne out by large clinical trials, chemoprevention offers a real hope of reducing the burden of prostate cancer on subsequent generations of men.

In this thesis introduction, the topics of prostate cancer and chemoprevention will be reviewed and the evidence for the use of two novel agents, resveratrol and ZD1839 as prostate chemopreventive agents will be considered.

1.1.1 Epidemiology

Prostate cancer is one of the most common diseases afflicting men in the Western world, being the most frequently diagnosed tumour and accounting for the second highest number of cancer deaths in the United States. In the United Kingdom, it accounts for 20,000 new cases and approximately 9,000 deaths annually (Quinn, 1999). Its incidence increases with age, an important fact which has implications for our demographically ageing society. The incidence of prostate cancer in the UK increased by 179% between 1971 and 1993 from 6174 cases to 17210 (Majeed, 2000), a rise of 104% following age standardisation. The age-standardised mortality rate of prostate cancer in the UK has also increased by 38% between 1971 and 1998, although this trend disguises a fall of 8% between 1995 and 1998 (Majeed, 2000).

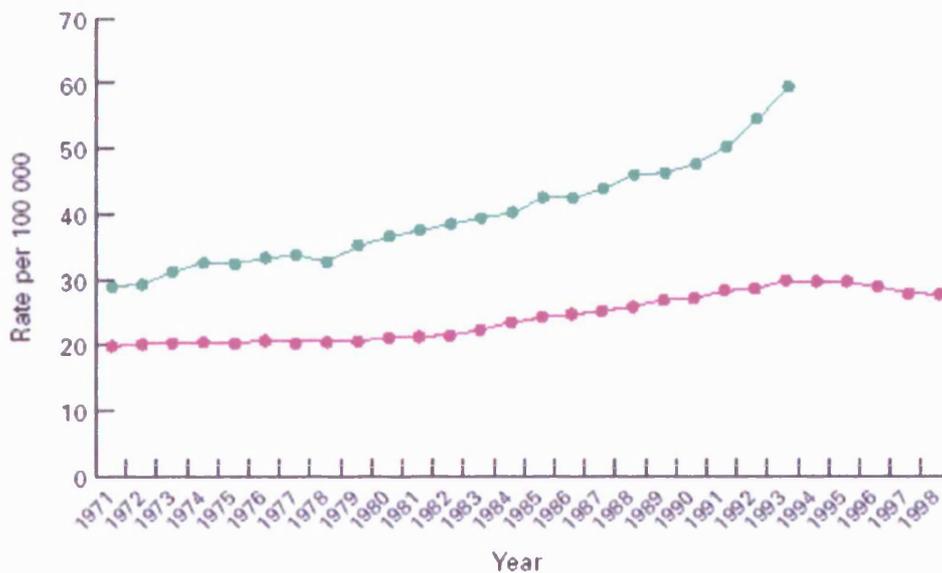


Figure 1. Prostate cancer incidence and death rates in the UK. Taken from (Majeed, 2000).

The true prevalence of prostate cancer is difficult to quantify, as a proportion of men will die of other medical conditions prior to their prostate cancer becoming clinically evident. Autopsy studies have revealed that the prevalence of histologically diagnosed prostate cancer increases with age, and that approximately 30% of healthy men over the age of 50 will have microscopic evidence of prostate cancer (Sakr, 1994).

Although the prevalence of occult prostate cancer is found to be similar around the world, the incidence of clinically evident disease varies greatly. Prostate cancer is primarily a disease of the Western World. African American men have the highest worldwide incidence of prostate cancer, and are more likely to present with advanced prostate cancer and subsequently die of their disease than their white American counterparts (Morton, 1994). China and other Oriental countries have the fewest cases of prostate cancer. Within Europe, the highest incidence is seen in Scandinavian countries (figure 2). Interestingly, migrants tend to take on the risk of their adopted country (Meikle and Smith, 1990).

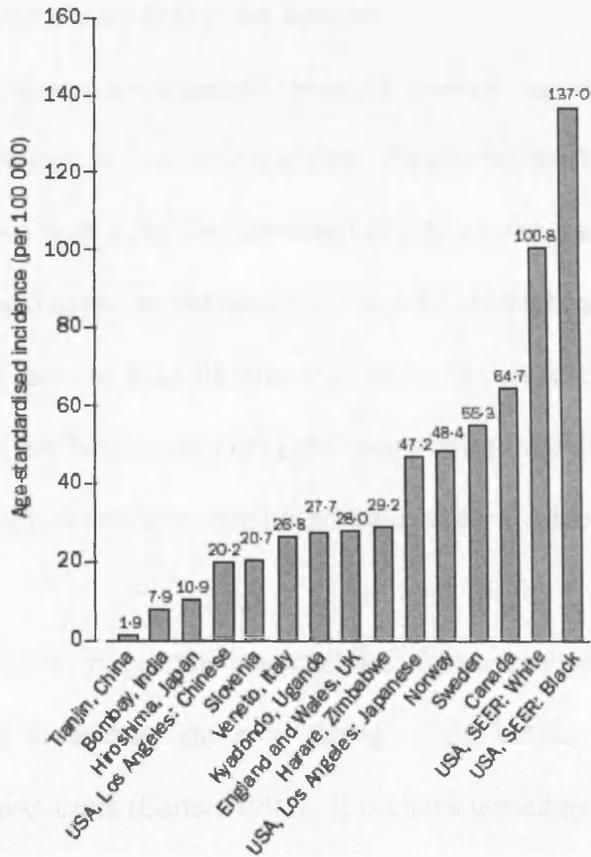


Figure 2. Age standardised incidence of prostate cancer worldwide. Taken from (Gronberg, 2003).

1.1.2 Risk factors

As with most malignancies, prostate cancer is thought to arise through an interaction between environmental and genetic factors which lead to a stepwise progression to malignancy. Much remains unknown regarding prostatic carcinogenesis but intensive research is leading to the identification of new genetic and environmental risk factors. Increasing age remains the most important risk factor for prostate cancer, as has already been discussed. Other risk factors can generally be divided into those possessing a genetic or environmental basis.

1.1.2.1 Hereditary risk factors

Evidence for a genetic basis of prostate cancer arises from epidemiological studies on families with several members affected by prostate cancer. It has been demonstrated that men with a first degree-relative with prostate cancer are around twice as likely to develop the disease themselves (Keetch, 1995),(Steinberg, 1990),(Carter, 1993) and as the number of cases in a family increase, there is a concurrent increase in individual risk to a member of that family. Monozygotic twin studies from the USA and Scandinavian countries show a high concordance rate for prostate cancer (Lichtenstein, 2000),(Gronberg, 2003).

Hereditary prostate cancer (HPC) forms a subset of the disease accounting for around 9% of all prostate cancers by the age of 85, but may account for a greater proportion of early-onset cases (Carter, 1992). It is characterised by three generations of men with the disease, early onset of malignancy (<55years old) or three or more first degree relatives affected. It is believed to be transmitted in an autosomal dominant fashion with high penetrance. The genes responsible for HPC were first mapped to the *HPC1* locus on chromosome 1 in 1996 (Smith, 1996), but this remains controversial (Eeles, 1998). There is some evidence that mutations in the tumour-suppressor *RNASEL* gene in the *HPC1* region may be responsible for a number of cases of HPC (Gronberg, 2003).

Single inherited genetic traits such as those discussed above, which are transmitted in a Mendelian fashion, and lead to cancer development with high penetrance tend to be uncommon and their overall effect on a population's risk of malignancy is likely to be low. A more common genetic contribution to prostate cancer susceptibility may be in the form of a number of polymorphisms in genes regulating prostate growth and homeostasis,

particularly genes coding for steroid hormones or androgen receptor (AR)-mediated signalling proteins (Makridakis and Reichardt, 2001). Indeed, androgenic stimulation is thought to be essential to prostate carcinogenesis as it is rare in men who have never been exposed to testosterone. Some studies have shown higher circulating levels of androgens in African American men compared to whites, which may explain their elevated prostate cancer risk (Ross, 1998). Some investigators have shown that reduced CAG repeats in the ELAC/HPC2 region of the AR gene are associated with an increased risk of prostate cancer (Stanford, 1997).

Another important polymorphism may involve *SRD5A2*, which codes for the enzyme 5- α reductase, the enzyme which metabolises testosterone to its more active form, dihydrotestosterone (DHT). It has been suggested that *SRD5A2* polymorphisms which result in a greater degree of enzymatic activity might be associated with an increased risk of prostate cancer (Henderson and Feigelson, 2000).

1.1.2.2 Environmental risk factors

As was previously mentioned, migrants tend to take on the prostate cancer risk of their host populations. This suggests that either environmental factors which are present in the host country can increase risk of the developing the disease, or conversely that some protective factors are present in the migrant's country of origin. There has been a great deal of research into the nature of these harmful or protective factors, most of which has focused on dietary habits. The Western diet is high in animal fat and meat intake, containing high quantities of n-6 polyunsaturated fatty acids. There is some experimental evidence in

animal models that these fatty acids exert promotional effects on prostatic carcinogenesis (Connolly, 1997). The mechanisms by which a high-fat diet may mediate prostate carcinogenesis are unknown, but one theory centres on the role of insulin-like growth factor-1 (IGF-1). This peptide growth factor regulates proliferation, apoptosis and differentiation, and its plasma level may be consistently increased with a high fat diet. Several studies demonstrated a link between high levels of IGF-1 and prostate cancer risk (Harman, 2000),(Stattin, 2000).

A number of protective dietary factors have also been identified including vitamin E, selenium, phytoestrogens, vitamin D and lycopene. These nutrients will all be discussed in detail under the topic of chemoprevention. Other environmental factors such as smoking, vasectomy, and occupational exposure have all been suggested to increase prostate cancer risk, but there is little evidence to support these claims.

1.1.2.3 Molecular genetics of prostate cancer

It is dogmatically held that malignancy is the end result of an accumulation of genetic damage, which in turn is mediated by hereditary and environmental factors such as those discussed above. This multi-step model of carcinogenesis is well characterised in colorectal cancer, where the stages of malignant transformation have been correlated with particular genetic events. Unfortunately, such a cascade of genetic instability remains poorly characterised in prostate cancer. In general, genetic defects may occur on the nucleotide or chromosomal levels. Nucleotide defects may take the form of point

mutations, deletions or substitutions. Chromosomal abnormalities include loss or gain of whole or parts of chromosomes or chromosome translocation.

Fluorescent in situ hybridisation (FISH) and comparative genomic hybridisation (CGH) are two techniques used to study chromosomal anomalies in prostate cancer. Chromosomal losses are believed to be more important than gains, in particular, losses on chromosomes 8p and 13q (Qian, 1995; Cher, 1996; Alers, 2000). Nevertheless, some investigators have shown gains on several chromosomal regions, including 2p, 11p, 1q, 3q, 4q, 7q, 8q, 11q, 12q and Xq (Bova and Isaacs, 1996).

CGH and FISH provide an overview of chromosomal changes, but do not specify which alleles may be involved. Microsatellite marker and loss of heterozygosity (LOH) studies seek to address this question, and have shown allelic losses in the 16q, 17q, 13q and 8p regions in particular. Allelic losses increase with disease progression (Karan, 2003).

Whatever the manner by which genomic damage is brought about, it ultimately results in the expression of oncogenes or inhibition of tumour-suppressor genes (TSGs). The latter are a group of genes vital to the negative regulation of cell growth. *p53* is one such gene, popularly known as the “guardian of the genome”. Located on chromosome 17p, it has a plethora of actions, principal among them being induction of apoptosis in cells following irreparable DNA damage. A number of studies have revealed the presence of mutated *p53* in 42% of cases of advanced prostate cancer (Bookstein, 1993) and only 10-20% of cases of earlier stage disease (Voeller, 1994). Another important TSG, the retinoblastoma gene (*Rb*), and its protein product have been found to be mutated or absent in both early and late

stage prostate cancers (Phillips, 1994; Ittmann and Wiczorek, 1996). Inactivation of the *glutathione-S-transferase pi (GSTP)* gene is the most common genetic anomaly in prostate cancer and is brought about by hypermethylation of the promoter sequence in 96% of primary prostatic carcinomas (Lee, 1994). This important gene codes for an enzyme which is important for detoxification of potential carcinogens. Proto-oncogenes are generally positive regulators of proliferation, which in their native form, are essential for cell survival. However, following genetic damage, their unregulated expression or function may lead to unchecked cell growth and proliferation, and they are then known as oncogenes. Several oncogenes which may be relevant to prostatic carcinogenesis have been identified. These include *c-erbB-1* and *c-erbB-2*, which code for growth factor receptors and will be discussed later, and *c-myc*, which codes for a transcription factor.

Other genes, whilst not directly influencing cell proliferation, have a crucial role in determining metastatic potential of a cell. These include genes such as *CDH1* which codes for the cell-cell adhesion molecule, E-cadherin. This molecule has been found to be down-regulated in prostate cancer (Bussemakers, 1992; Girolodi and Schalken, 1993). *KAI1*, which codes for another cell-cell interaction molecule has been shown by some studies to be lost in the progression from normal prostatic epithelium to localised prostatic cancer to metastatic disease (Dong, 1996).

Based on these findings, Karan et al (Karan, 2003) have constructed a putative genetic sequence of events underlying the various stages of prostatic carcinogenesis (figure 3).

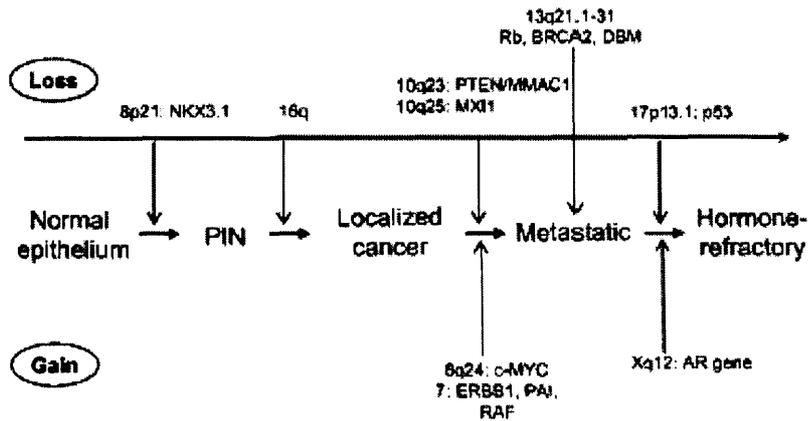


Figure 3. A possible molecular genetic model of prostate cancer. Taken from (Karan, 2003).

1.1.3 Pathology

1.1.3.1 Macroscopic and microscopic pathology

The vast majority (95%) of prostatic cancers are adenocarcinomas, the remainder comprising transitional cell carcinomas, neuroendocrine carcinomas and sarcomas. Macroscopically, 60-70% of tumours occur in the peripheral zone of the prostate, and may be multifocal. Microscopically, a proliferation of small acini with multiple patterns is usually seen. Diagnosis relies on a combination of cytological and architectural features. Cellular features of malignancy include hyperchromatic, enlarged nuclei with large, eccentric nucleoli. The basal cell layer, an important component of normal prostatic tissue, is conspicuously absent in prostate cancer. Perineural invasion is common in prostate cancer, but is not pathognomonic of malignancy.

1.1.3.2 Precursor lesions

High-grade prostatic intraepithelial neoplasia has been identified as a precursor lesion for prostate cancer. It frequently develops in the peripheral zone of the prostate, the same site as carcinoma (Haggman, 1997). In addition, a number of biomarkers that are found to be abnormal in prostate cancer including changes in oncogene and tumour suppressor gene expression, growth factors and receptor expression are also found in HGPIN (Bostwick, 1996). HGPIN has similar cytological characteristics to frank adenocarcinoma, the key difference being presence of a basement membrane in HGPIN. However, although HGPIN is considered to be a precursor to prostate cancer, it is not a prerequisite step. Some early, low grade cancers do not seem to be associated with areas of HGPIN (DeMarzo, 2003). The frequency of HGPIN on prostatic biopsy has been reported as between 1.5-16.5% (Wills, 1997; Hu, 1998; Orozco, 1998; Novis, 1999). Some investigators have shown that the presence of HGPIN in prostatic biopsy specimens is a significant predictor of concurrent prostate cancer (Prange, 2001). If HGPIN is detected on an initial biopsy, the risk of cancer on subsequent biopsies is 23-35% (Davidson, 1995; O'Dowd G, 2000; Kronz, 2001).

1.1.3.3 Grading and Staging

The degree of differentiation of the glandular architecture in adenocarcinoma specimens provides important prognostic information, and is graded according to a system developed by Gleason *et al* (Gleason and Mellinger, 1974). For any particular specimen, a pathologist will assign two individual Gleason grades from 1-5 corresponding to the two most prevalent grades of cancer in the specimen. These two values are added together to give the Gleason sum or score. Well-differentiated cancers typically have a Gleason sum of 4 or

less, whilst poorly differentiated cancers have Gleason sums of 8 or over, the remainder being moderately differentiated. There is good inter-operator correlation of Gleason scores when the system is used by uro pathologists (Allsbrook, 2001). Gleason scores have been strongly correlated with malignant behaviour and prognosis (Sogani, 1985).

Prostate cancer is staged according to the TNM classification (1997), illustrated in table 1

Table 1 TNM staging of prostate cancer

Stage	Description
T0	No evidence of tumour
T1a	Malignancy in <5% of TURP chips
T1b	Malignancy in >5% of TURP chips
T1c	Tumour detected on needle biopsy
T2a	Tumour palpable in less than half of one lobe
T2b	Tumour palpable in more than half of one lobe
T2c	Tumour palpable in both lobes
T3a	Unilateral extracapsular extension
T3b	Bilateral extracapsular extension
T3c	Seminal vesicle invasion
T4a	Fixed to bladder neck, external sphincter or rectum
T4b	Invasion of levator muscle or pelvic floor

N0- no nodal metastases N1-pelvic nodal metastases

M0-no distant metastases

M1a-distant nodal metastases M1b-bone metastases M1c-Other distant metastases

1.1.4 Clinical manifestations of prostate cancer and their management

1.1.4.1 Organ-confined prostate cancer

The current treatment options for men with this type of prostate cancer on diagnosis are “watchful waiting” (active monitoring), radical radiotherapy, radical prostatectomy, brachytherapy or cryotherapy, although the latter two treatments are newer approaches. The choice of appropriate treatment is influenced by tumour characteristics, patient characteristics and patient choice.

Patients with small, low grade tumours who may be unfit for radical surgery or radiotherapy are ideally suited to the watchful waiting regimen, which involves regular monitoring of disease by clinical and biochemical means. Indeed, a recently reported randomised controlled trial demonstrated no difference in overall survival (Holmberg, 2002) or quality of life (Steineck, 2002) between patients treated with radical surgery and those monitored in a watchful waiting regime, although disease-specific survival rate was much higher in the group who underwent surgery. Watchful waiting may eventually permit disease progression, at which point treatment with hormone ablation therapy may be initiated.

Radical prostatectomy is an option for younger, fitter patients with organ-confined tumours. This operation may be performed via either perineal or retropubic routes, and improved understanding of pelvic anatomy has led to the development of nerve-sparing techniques. The procedure may also be performed laparoscopically (Matin, 2003). Following radical prostatectomy, PSA levels should fall to undetectable values, allowing PSA monitoring to be an accurate means of detecting disease progression (Pound, 1999). The reported 5-year

progression-free rates from several studies give figures of between 77-80% (Catalona and Smith, 1994; Trapasso, 1994; Zincke, 1994; Pound, 1997). The pathological stage of the cancer is the single most important predictor of recurrence, with organ-confined disease having recurrence free rates of greater than 90% at 5 years (Epstein, 1993). Morbidity associated with radical prostatectomy can be significant, and is related to the experience of the surgeon. Total urinary incontinence tends to be rare, but more minor degrees of stress incontinence may be present in up to 20% of patients (Zincke, 1994; Catalona, 1999). Rates of post-operative impotence, another major complication of the procedure, vary a great deal between reported studies (Catalona, 1999; Rabbani, 2000; Stanford, 2000), and may be 60% or higher.

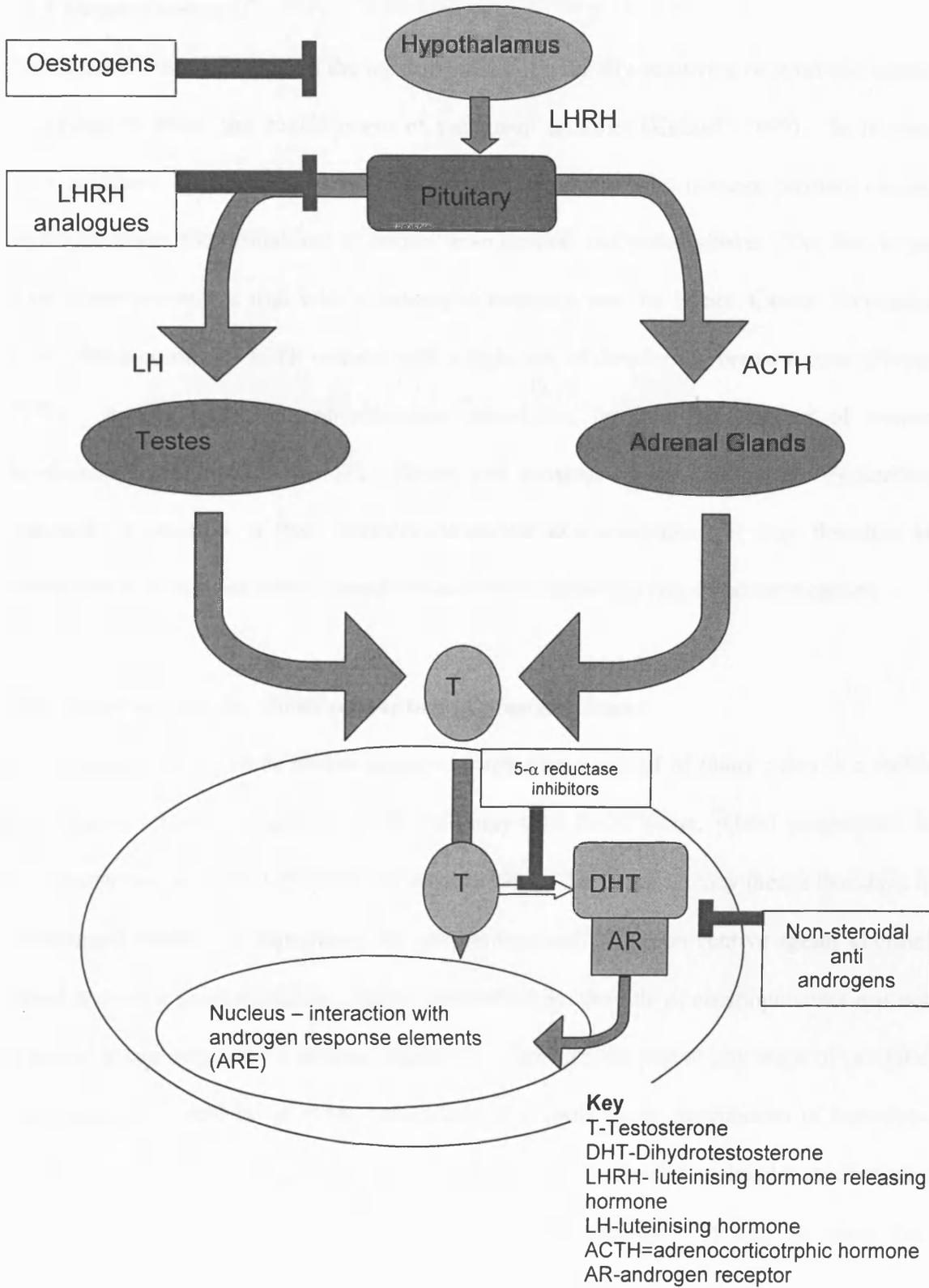
External beam radiotherapy (EBRT) provides an alternative to surgery for the treatment of localised disease. Improved imaging and planning software has led to the development of conformal radiotherapy (Fraass, 1995), enabling a high dose to be delivered in an anatomically accurate fashion. Results from EBRT compare favourably with radical surgery (Lu-Yao and Yao, 1997).

1.1.4.2 Metastatic prostate cancer

Huggins and Hodges (Huggins and Hodges, 1941) first described the androgen responsiveness of prostate cancer over 50 years ago. Androgen ablation therapy remains the mainstay of treatment of metastatic disease. The hypothalamo-pituitary-gonadal axis is shown in figure 4, and hormonal manipulation can be effected at any level of this axis. After this initial period of encouraging biochemical and clinical response, prostate cancer invariably becomes resistant to hormone ablation, the median times to progression and

survival being 16-18 months and 2-3 years respectively (Group, 1984; Eisenberger, 1986; Denis, 1993; Eisenberger, 1998). Mean survival following the onset of hormone-refractory disease is 40-60 weeks. Current initial management involves cessation of androgen blockade therapy and this results in a PSA response in up to 30% of patients. Chemotherapeutic regimens involving mitoxantrone and estramustine may be employed, but with little impact on overall survival. Hormone-refractory prostate cancer remains incurable.

Figure 4. Hormonal manipulation of prostate cancer



1.2 Chemoprevention

1.2.1 General concepts

Chemoprevention is defined as the administration of naturally occurring or synthetic agents to prevent or delay the development of malignant tumours (Kelloff, 1999). In its pre-emptive nature, this concept is very attractive to clinicians who manage prostate cancer, especially given the limitations of current management discussed above. The first large-scale chemopreventive trial with a successful outcome was the Breast Cancer Prevention Trial, which enrolled 13,388 women with a high risk of developing breast cancer (Fisher, 1998). In this trial, the anti-oestrogen, tamoxifen, reduced the number of women developing breast cancer by 45%. Breast and prostate cancer share some similarities, especially in relation to their hormone-dependent characteristics. It may therefore be reasonable to expect hormonal manipulation to help reduce the risk of prostate cancer.

1.2.2 Opportunities for chemoprevention in prostate cancer

It is accepted that prostatic carcinogenesis occurs over a period of many years in a multi-step fashion. The development of HGPIN may take 20-30 years, whilst progression to cancer may take another 3-15 years (Bostwick, 1992). This long latency means that there is a prolonged window of opportunity for intervention with chemopreventive agents to either retard or reverse carcinogenesis. Unlike chemotherapy, the role of chemoprevention is not confined to one stage of the disease (figure 5). Chemoprevention at any stage of prostatic carcinogenesis would be of value particularly if it could delay progression of hormone-sensitive to -insensitive disease, and the identification of potential molecular mechanisms by which this transformation occurs is a research priority. It can be seen that

chemopreventive and chemotherapeutic agents share a degree of overlap, and the boundaries of what constitutes a chemopreventive agent as opposed to a chemotherapeutic agent are necessarily blurred.

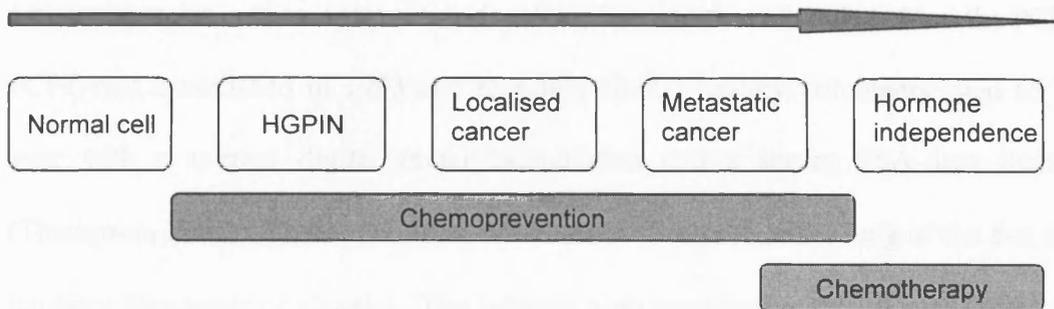


Figure 5. The scope of chemoprevention

1.2.3 Chemopreventive agents

A number of chemopreventive agents are undergoing rigorous pre-clinical and clinical evaluation at present. Their acceptance will only come about through evidence obtained from large randomised, controlled trials (RCTs). Considerations for any potential chemopreventive agent are:

- Biological rationale for action
- Low (or no) toxicity on chronic administration
- Patient acceptability
- Financial viability

The most promising agents under development will be summarised below:

1.2.3.1. Finasteride

The interest of both the urological and scientific communities in prostate cancer chemoprevention has recently been heightened by the publication of results from the first prospective, randomised controlled Prostate Cancer Prevention Trial (PCPT). The vital role of androgens in prostatic carcinogenesis led to the supposition that blocking their action may serve a chemopreventive function, forming the rationale behind the PCPT. The PCPT was established in 1993 and recruited 18,000 healthy volunteers aged 55 years or over with a normal digital rectal examination and a serum PSA less than 3ng/ml (Thompson, 2001). These men were randomised to receive either 5mg of the 5- α reductase inhibitor finasteride or placebo. The subjects were examined annually with DRE and serum PSA. Suspicious findings resulted in biopsy. After 7 years, all subjects were scheduled to undergo end of study prostatic biopsies. The primary endpoint was the period prevalence of biopsy-detected prostate cancer, and the study was powered to reveal a 25% reduction in prostate cancer incidence in the presence of finasteride (Feigl, 1995). Secondary endpoints included health-related quality of life (HRQOL), cancer stage at diagnosis, as well as overall survival. The first results were expected to be available in October 2004, but the study was terminated prematurely and the results reported in 2003 (Thompson, 2003). The results showed that finasteride administration resulted in a 24.8% reduction in prostate cancer incidence over the study period. This is the first prospective study to demonstrate such a large clinically-relevant reduction in prostate cancer. However, the study also revealed that the finasteride group developed a significantly higher proportion of high-grade tumours of Gleason sum 7 and above (6.4%) than the placebo group (5.1%). Sexual side-effects were also more problematic in the finasteride group. These results have

sparked much controversy and discussion. The authors of the study argue that the higher incidence of high-grade tumours may be an artefactual consequence of finasteride altering prostate cancer histology (Thompson, 2003), although the possibility of a causative link between finasteride-induced androgen deprivation and high grade prostate neoplasia must not be dismissed. On the basis of the results of the PCPT, finasteride chemoprevention cannot be recommended unreservedly; a careful risk-benefit analysis with the patient must be undertaken (Marberger, 2003). Nevertheless, despite the obvious *caveats*, the study provided a valuable insight into the design and conduct of a large scale chemoprevention trial and certainly provides a rationale for further such trials.

1.2.3.2 Selenium and Vitamin E

Both selenium and vitamin E are important intracellular antioxidants. The evidence which initially led to the consideration of both selenium and vitamin E as chemopreventive agents in prostate cancer arose from studies involving entirely different cancers. In the case of vitamin E, this was the Scandinavian α -tocopherol, β -carotene (ATBC) study (ATBC Study Group, 1994). This was a double-blind, randomised placebo-controlled trial with 4 study arms, one receiving two placebo tablets, the next receiving one placebo and one 50mg vitamin E tablet, the third receiving one placebo and one 20mg beta carotene tablet and the final group receiving both vitamin E and beta carotene. 29, 133 male smokers were recruited and the aim of the study was to assess whether there was any reduction in the incidence of lung cancer following administration of these agents. The authors concluded that dietary supplementation with α -tocopherol had no effect on the incidence of lung cancer in this group and that the group who received beta carotene had a significantly

higher risk of contracting lung cancer. However, a secondary analysis showed that patients who received α -tocopherol had a significantly lower incidence of prostate cancer (Heinonen, 1998).

Similarly, the chemopreventive action of 200mcg daily selenium in the form of selenized yeast on skin cancer in 1,312 patients with a history of basal or squamous cell carcinoma of the skin was investigated. Patients were randomised to receive either selenized or non-selenized yeast (Clark and Combs, 1996). They retrospectively tested stored blood for PSA levels before and after treatment, and performed a secondary analysis of the incidence of prostate cancer in the study groups. Again, there was no effect on the primary endpoint of skin cancer, but the secondary analysis revealed a 63% reduction in the incidence of prostate cancer (Clark, 1998).

The prostate-related findings of these studies must be treated with caution, as both studies set out to answer another question and used highly selected groups. However, on the basis of this evidence, a large randomized controlled prostate cancer chemopreventive trial known as the selenium and vitamin E chemoprevention trial (SELECT) has recently been established (Klein, 2001) and will eventually recruit 32,400 participants. Participants will be randomized into four groups, to receive placebo, selenium, α -tocopherol, or a combination of both drugs. Recruitment will finish in 2006 and results are expected in 2013.

1.2.3.3. Phytoestrogens

Native Japanese men have a high intake of soya-bean derived foodstuffs, a dietary habit which is thought to contribute to this population's low incidence of prostate cancer (Adlercreutz, 1991). These foodstuffs are rich sources of compounds known as phyto-oestrogens, non-steroidal compounds with a weak oestrogenic activity (Eldridge and Kwolek, 1983). Three main classes are recognised; isoflavonoids, flavonoids and lignans. Soya beans are rich in conjugates of the isoflavonoids genistein and daidzein, and other plant sources such as cereals, grains and fruits are rich in the lignans enterolactone and equol. Flavonoids such as quercetin and resveratrol are found in the outer epidermis of leaves and fruits (Knowles, 2000).

Isoflavonoids, lignans and flavonoids all have weak oestrogenic activity (Miksicek, 1993) and are thought to compete with oestrogen for binding to the oestrogen receptor (ER). Thus, their chemopreventive potential in breast cancer is clear. Whether this oestrogenic effect has any bearing on prostate cancer is unknown. Phytoestrogens stimulate the liver to produce greater quantities of sex hormone binding globulin (SHBG) (Adlercreutz, 1987) which binds 98% of circulating testosterone in the male. Only the remaining 2% is biologically active. If more SHBG is produced, perhaps this would leave less biologically active free testosterone. Genistein is also known to inhibit tyrosine kinase enzymes, which may be important in prostatic carcinogenesis (Burvall, 2002).

1.2.3.4. Lycopene

Lycopene is a member of the carotenoid family of compounds, a group of complex hydrocarbons found as plant pigments. Lycopene is commonly found in tomatoes, and is the most abundant carotenoid in human serum (Clinton, 1998). It has potent antioxidant properties which are thought to be relevant to its chemopreventive effect (Hwang and Bowen, 2002). Data from the health professionals follow-up study (HPFS) using dietary questionnaires revealed that high intake of tomato-based products was associated with a lower risk of prostate cancer (Giovannucci and Clinton, 1998). Lycopene certainly has been shown to have an antiproliferative effect on prostate cells *in vitro* (Obermuller-Jevic, 2003). Recently a study showed that men given tomato sauce-based pasta dishes prior to their radical prostatectomy showed lower levels of oxidative DNA damage in peripheral leucocytes and in resected prostatic tissue (Chen, 2001).

1.2.3.5. Vitamin D

The role of vitamin D in calcium homeostasis is well known, but its possible role in the chemoprevention of prostate cancer is perhaps more surprising. An association was first suggested by a study which correlated higher rates of prostate cancer death with a lack of exposure to sunlight (Schwartz and Hulka, 1990). The authors of this study argued that population groups with a high risk of prostate cancer were also vitamin D deficient. Subsequent *in vivo* and *in vitro* work has confirmed the inhibitory effect of vitamin D on prostate cancer cells (Schwartz, 1997). The mechanism of action may be related to modulation of the cell cycle, causing the cancer cells to arrest in G1 phase (Wang, 1996), and induction of apoptosis (Blutt, 2000).

1.2.3.6. Non-steroidal anti-inflammatory drugs (NSAIDs)

Inflammatory mediators such as prostaglandins (PG) stimulate the growth and spread of tumours (Xu, 2002). The enzyme responsible for the production of these prostaglandins is cyclo-oxygenase (COX), which exists in two forms, COX-1 and COX-2. COX-1 is the constitutive form of COX, whereas COX-2 is the inducible form, which is expressed in inflamed tissues and in many malignancies. In addition to metabolising arachidonic acid, COX-1 and COX-2 can catalyse the transformation of harmless pro-carcinogens into carcinogens, and may be involved in tumour angiogenesis. There is evidence of COX-2 over-expression in prostate cancer (Gupta, 2000; Uotila, 2001) and high levels of PGE₂ have been found in prostate cancer specimens compared with surrounding normal prostate tissue (Chaudry, 1994). Celecoxib is a COX-2 selective inhibitor which is being used to reduce polyp load in familial adenomatous polyposis (FAP), and also has powerful anti-angiogenic effects. Sulindac sulfone is another agent which induces apoptosis in cell lines *in vitro* (Lim, 1999) and in prostate cancer xenograft models (Goluboff, 1999). Another prostate cancer chemoprevention trial using the COX-2 selective inhibitor, rofecoxib is currently recruiting (Basler and Piazza, 2004).

1.2.4 Biomarkers

The phase III chemopreventive studies which are currently taking place are large, expensive and time-consuming. This is, in part due to the long latency of the primary endpoint, ie prostate cancer. It would be useful to identify events which occur earlier the carcinogenetic pathway, and whose detectable presence correlates strongly with eventual development of prostate cancer. These events are known as surrogate endpoint biomarkers (Kelloff, 2001; Montironi, 2003). Any potential biomarker has to be validated

mechanistically using *in vitro* and *in vivo* models in order to prove that it is indicative of malignant potential before being tested in small clinical trials. Successful biomarker development will expedite the progression of chemoprevention clinical trials by identifying any beneficial effect earlier than is currently possible. A number of biomarkers of prostatic carcinogenesis have been identified and are summarised in table 2. Surrogate endpoint biomarkers are distinct from tumour markers, which are used to diagnose and monitor established malignancy, and risk biomarkers, which indicate a genetic or environmental predisposition to disease in a healthy individual.

Class of biomarker	Examples
Histological	HGPIN
Markers of increased cell proliferation	Ki67 expression, angiogenesis
Markers of differentiation	Loss of high-molecular weight cytokeratins Altered antigen profile
Genetic changes	Chromosomal loss or gain Loss of heterozygosity <i>GTSP</i> promoter hypermethylation

Table 2. Possible surrogate endpoint biomarkers in prostate cancer, adapted from (Kelloff, 2001)

1.3 Resveratrol as a chemopreventive agent

Resveratrol (3,4,5 trihydroxystilbene) is a naturally-occurring polyphenol found in red grapes and also in a variety of other plants (Gusman, 2001). Its chemical structure is shown in figure 6.

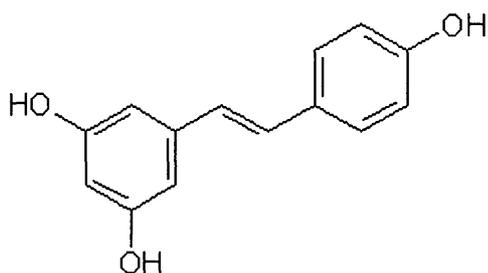


Figure 6. The chemical structure of resveratrol

It is synthesised in leaf epidermis and grape skin, probably as a response to environmental stress such as that which occurs in fungal infection. Its concentration in grape skins varies, and it was first detected in wine in 1992 (Mattivi, 1993). It is also found in non-alcoholic red grape juice. Given its status as a naturally occurring substance, which is found in common foodstuffs, one would expect it to have low toxicity on chronic administration, a key feature of any chemopreventive agent. Resveratrol came to prominence during the course of investigation of the so-called “French paradox” – i.e. the realisation that the population of France have a relatively high fat diet accompanied by a steady albeit moderate intake of alcohol in the form of red wine whilst their incidence of heart disease is lower than would be expected (Kopp, 1998). Resveratrol has been implicated in this effect due to its actions on the modulation of lipid metabolism, vasorelaxant activity, and inhibition of platelet aggregation amongst others. Its cancer chemopreventive effects were

discovered in 1997 when it was shown to inhibit the carcinogenic process at the initiation, promotion and progression stages (Jang, 1997). These studies were performed using a panel of cells originating from human lymphocytic leukaemia and murine mammary and skin tumours.

1.3.1 Experimental evidence of antineoplastic effect of resveratrol

Many *in vitro* studies have addressed the antiproliferative or pro-apoptotic effects of resveratrol in human prostate cancer cells, and its mechanism of action. The cell lines used include both hormone-sensitive and hormone-resistant cells in order to mimic the heterogeneous state of clinical prostate cancer. LNCaP cells are hormone-sensitive, PC-3 is a hormone-independent line possessing dysfunctional androgen receptors (ARs), and DU145 are androgen-independent cells, devoid of ARs. Resveratrol was found to inhibit the growth of LNCaP cells in a concentration-dependent manner (Hsieh and Wu, 1999; Mitchell, 1999) and was most potent at a concentration of 2.5×10^{-6} M. The growth of the DU-145 and PC-3 cells was inhibited as well, but it required a 10-fold higher concentration (Hsieh and Wu, 1999).

The potential *in vivo* efficacy of a putative chemopreventive agent can be tested in rodent models, especially those which bear a genetic defect of relevance to the genesis of the human disease which is to be prevented. In the light of the paucity of knowledge of genetic defects underlying human prostate cancer, the topic of rodent models and their relevance to human prostate cancer is currently the subject of considerable debate (Sharma and Schreiber-Agus, 1999). Nevertheless there have recently been impressive reports of the prostate cancer chemopreventive efficacy of genistein (Mentor-Marcell, 2001) and a

standardised polyphenol-containing green tea extract (Gupta, 2001) in the so-called TRAMP (“transgenic adenocarcinoma of the mouse prostate”) mouse. This rodent model expresses SV40 early genes, probably abrogating the function of the *p53* and retinoblastoma genes, which in turn engenders the formation of prostate carcinoma in all mice. Convincing evidence for the notion that resveratrol prevents prostate cancer also in models of prostate cancer *in vivo* has yet to be presented.

1.3.2 Mechanisms of action of resveratrol

Potential mechanisms of action of resveratrol have been studied extensively, and as yet there is no clear consensus on the matter. It should be noted that many of these mechanistic studies summarised below have been carried out on cell lines other than those derived from prostate cancer cells, and their relevance to the prostate have to be interpreted with caution.

1.3.2.1 Anti-oxidant and anti-inflammatory effects

Oxidative stress is thought to be an important cause of damage to DNA, with subsequent mutation. The interaction of free radicals with DNA results in the formation of a number of adducts which can be detected and quantified (DeWeese, 2001). One study showed that treating LNCaP cells with physiological levels of androgens seemed to increase oxidative stress within these cells (Ripple, 1997). Thus, due to the presence of high levels of androgens, prostate cells may be particularly vulnerable to oxidative stress. Hydroxyl radicals can attack DNA directly, or can initiate autocatalytic lipid peroxidation, which results in the formation of genotoxic products. Resveratrol has been shown to exert a strong inhibitory effect on the formation of free radicals in human macrophages (Martinez and Moreno, 2000), and may thus act to reduce oxidative stress within pre-malignant cells.

It is known that inflammatory mediators such as prostaglandins (PG) and nitric oxide (NO) stimulate the growth and spread of tumours by a number of mechanisms, including stimulation of cellular proliferation, reduction in immune surveillance and the induction of angiogenesis (Gusman, 2001). Large concentrations of these substances may be found in inflamed prostatic tissues. Resveratrol has been shown to inhibit the activity of enzymes responsible for the production of some of these inflammatory mediators, namely COX-1 and COX-2 and the nitric oxide synthase (NOS) enzymes [endothelial NOS (eNOS) or inducible NOS (iNOS)]. Resveratrol treatment decreased the production of NO significantly in PC-3 and DU-145 cells, but not in LNCaP cells (Kampa, 2000). Resveratrol has also been shown, in non-prostate inflammatory models, to reduce the production of iNOS in response to stimuli such as lipopolysaccharide (LPS) (Jang and Pezzuto, 1999). Interference with intracellular signalling mechanisms responsible for regulation of NOS production is thought to be important. In particular, resveratrol exerts an inhibitory effect on actions mediated by the transcription factor NF- κ B. NF- κ B regulates the transcription of both NOS and COX genes, and it is involved in the control of the cell cycle (Holmes-McNary and Baldwin, 2001). As well as metabolising arachidonic acid, COX-1 and COX-2 also have hydroperoxidase activity, and thus can catalyse the transformation of harmless pro-carcinogens into carcinogens (Gusman, 2001). COX-2 may be important in tumour angiogenesis, and its activation may suppress apoptosis (Sawaoka, 1999). Resveratrol has been shown to have inhibitory effects on both the arachidonic acid metabolising and hydroperoxidase activities of both COX-1 and COX-2. In one study the inhibitory effect of resveratrol on cyclooxygenase activity of COX-1 was greater than that

mediated by aspirin (Jang, 1997). There is some evidence *in vitro* which suggests that resveratrol inhibits COX-2 induction in response to substances such as phorbol esters (Subbaramaiah and Dannenberg, 2001). As with the iNOS enzyme, this effect may be mediated by interference with intracellular signalling mechanisms, which lead to COX-2 expression, among them the protein kinase C system and NF- κ B.

1.3.2.2 Modulation of cytochrome P450 system

Many carcinogens require metabolism in the body to acquire their detrimental activity. One of the most important enzymes in the formation of carcinogens is the cytochrome P450 isoenzyme 1A1 (CYP 1A1). This enzyme is responsible for a pivotal step in the biological activation of many aryl hydrocarbon (AH) carcinogens, such as benzo(*a*)pyrene (B(*a*)P). AHs bind to the cytosolic AH receptor which then translocates to the nucleus, and in conjunction with the AH nuclear translocator, binds to the *CYP 1A1* promoter, resulting in increased transcription of *CYP 1A1*. Resveratrol has been shown to inhibit the activity of CYP 1A1 and also to inhibit the transcription of *CYP 1A1* in hepatocytes (Ciolino and Yeh, 1999), by preventing the binding of the AH receptor to the promoter region of the gene. CYP 1A1 has been demonstrated in human prostatic tissue [Williams, 2000], but the extent of its involvement in prostatic carcinogenesis remains unknown. Nevertheless it is conceivable that resveratrol inhibits CYP1A1 expression in prostate cells and that such inhibition is related to chemoprevention.

1.3.2.3 Modulation of cell cycle and apoptosis

The loss of control of the cell cycle and lack of normal apoptosis are two of the hallmarks of malignant lesions. The cell cycle is normally controlled by a number of proteins, including p53, p21^{WAF1}, the cyclin-dependent kinases (cdks), and their activators, the cyclins. Apoptosis is particularly linked with the bax and bcl-2 proteins. The progress of a cell through its cycle is mediated by specific activation of complexes between cdks and cyclins. Once activated, these complexes shepherd a cell through the transition between phases in the cell cycle. Other molecules such as p21^{WAF1} can have an inhibitory effect on these cyclin-cdk complexes, i.e. they are cdk inhibitors. Resveratrol has been shown to interfere with the cell cycle and act on many of these biomolecules. It significantly increased the proportion of LNCaP cells undergoing apoptosis (Hsieh and Wu, 1999). Another study showed that it up-regulates p53 expression and induces apoptosis in a mouse epidermal cell line. This pro-apoptotic effect of resveratrol was absent in p53-deficient fibroblast cells (Huang, 1999), which suggests that resveratrol acts through a p53-dependent mechanism. In another study in epidermoid carcinoma lacking wild-type p53 resveratrol was pro-apoptotic, probably mediated *via* increased expression of p21^{WAF1} (Ahmad, 2001). The expression of p21^{WAF1} resulted in cell cycle arrest between the G₁ and S phases. This arrest is thought to be irreversible, the inert cell then undergoing apoptosis. Although this model is appealing, contradictory evidence as to the role of p21^{WAF1} has been presented in LNCaP cells, in which p21^{WAF1} levels were markedly decreased following resveratrol treatment, concomitant with an increase in apoptotic cell number (Mitchell, 1999). Resveratrol can have other effects on the mitotic process, exemplified by inhibition of ribonucleotide reductase (Fontecave, 1998) and DNA polymerase (Sun, 1998).

1.3.2.4 Effects on the androgen receptor

As has already been discussed, androgen receptor (AR) mediated signalling is of paramount importance in the development of prostatic carcinoma. Following ligand binding, the AR-ligand complex initiates gene transcription by cofactor-mediated binding to androgen response elements (ARE). Thus androgen binding is the most potent stimulus to AR activity. Furthermore, AR-regulated gene activity is important even in the “androgen-independent” stage of prostate cancer. For example, serum levels of PSA, the most widely studied AR-regulated gene product, continue to rise in hormone-refractory metastatic disease. AR is still expressed in hormone-refractory disease, and it is possible that they are activated by ligand-independent pathways, such as by interaction with peptide hormone signalling molecules, as will be discussed later. Given the central role of AR, it is possible that resveratrol exerts its chemopreventive action in part by modulating the expression or function of this receptor.

Several studies have focussed on the effect of resveratrol on the expression of ARs in LNCaP cells, and they have produced conflicting results. In one study, resveratrol treatment of LNCaP cells failed to reduce the expression of AR, although it resulted in a marked decrease in secreted and cytosolic PSA (Hsieh and Wu, 2000). The authors suggested that resveratrol reduces PSA secretion in an androgen receptor-independent manner. In contrast, in another study in the same cell type, AR levels were decreased in a time- and dose-dependent manner by resveratrol, and the AR-binding site in androgen inducible genes was thought to be a target for resveratrol (Mitchell, 1999). There is evidence to suggest that the p21^{WAF1} gene is upregulated by androgens, thus giving androgens direct control over the cell cycle (Lu, 1999).

1.4 Epidermal growth factor receptor (EGFR) and prostate cancer

Several families of genes are implicated in the process of prostatic carcinogenesis, including the group of *erbB* genes which codes for the four transmembrane receptors epidermal growth factor receptor (EGFR or *erbB-1*), HER-2 (*neu*, *erbB-2*), HER-3 (*erbB-3*) and HER-4 (*erbB-4*) (Coussens, 1985; Kraus, 1989; Plowman, 1993; Sedlacek, 2000). These receptors have been strongly implicated in many human cancers (Wells, 2000). The *erbB* subfamily is one of around twenty groups of receptors known as receptor tyrosine kinases (RTKs), all of which are functionally related. Numerous strategies are being developed to inhibit the function of these receptors and their downstream signalling pathways, and these agents have chemopreventive as well as chemotherapeutic potential. One such agent, the monoclonal antibody trastuzumab (Herceptin, Genentech), has entered clinical practice in the form of therapy for ER-negative HER-2 positive breast cancer.

Gefitinib (ZD1839, Iressa), one of the agents used in our experiments, is a molecule targeted to the EGFR tyrosine kinase enzyme. These agents, including gefitinib, have attracted much attention from the scientific and medical community. Their potent activity *in vitro* and *in vivo* models of cancer and low toxicity make them theoretically well suited to the purpose of chemoprevention.

In order to understand the putative role for gefitinib in prostate cancer chemoprevention, it is necessary to review the biology of EGFR-mediated signalling and consider the evidence linking this molecule to prostatic carcinogenesis.

1.4.1 EGFR structure and ligands

The structural similarity of EGFR to the viral oncogene *v-erbB*, encoded by the avian erythroblastosis retrovirus, is striking (Downward, 1984). Its expression and functional role in numerous human tumours, including prostate cancer has been studied. Although the other three receptors in the *erbB* family are related to EGFR, there are some important structural and functional differences between them. EGFR is a 170kD tyrosine kinase transmembrane glycoprotein which has high affinity for a number of ligands, principally epidermal growth factor (EGF) and transforming growth factor- α (TGF- α), as well as amphiregulin, betacellulin and heparin-binding epidermal growth factor (HB-EGF) (Kim, 1999; Klapper, 2000). *ErbB-2* shares some homology with EGFR, but the key difference is that no ligand has been identified for this receptor, and indeed, it may not possess one. It has thus become known as an “orphan” receptor. The other two receptors, *erbB-3* and *erbB-4*, bind a number of ligands including the neuregulins and epiregulin (Aldkofer and Lai, 2000).

1.4.2 EGFR activation

Binding of a ligand to EGFR results in its dimerisation, either with another EGFR molecule (homodimerisation) or with other receptors of the *erbB* family (heterodimerisation). The precise mechanism of this pivotal step in EGFR activation is still not fully understood. One theory (Greenfield, 1989) suggests that ligand binding induces a conformational change in the receptor which results in ability to dimerise, whereas other investigators have suggested that ligands may be bivalent and thus bind two receptors together (Gullick, 1994). It has been recently demonstrated by X-ray crystallography that there is direct receptor-receptor

interaction, mediated by the extracellular region of the EGFR molecule (Ogiso, 2002). Whatever the mechanism, heterodimerisation is hierarchical with HER2 being the favoured partner of all other *erbB* receptors (Graus-Porta, 1997). Heterodimers are generally more active than homodimers and can result in prolonged activation of downstream signalling pathways (Moghal and Sternberg, 1999). The stimulatory ligand plays a key role in determining which components of the diverse EGFR signalling network are activated.

Following dimerisation, phosphorylation of intracellular tyrosine residues on the intracellular portion of the receptor takes place, and this is catalysed by the receptor tyrosine kinase domain (Olivier, 1993). Tyrosine phosphorylation is the key step in EGFR activation. Various intracellular molecules containing specific domains known as *src* sequence homology (SH2) or phosphotyrosine binding sites (PTB) can then recognise and complex with phosphotyrosine residues on EGFR and activate intracellular signalling pathways (figure 7). The principal signalling pathways involved are the mitogen activated protein kinase (MAPK) pathway, the phosphatidyl inositol 3-Kinase (PI3-K) pathway and the phospholipase-C γ (PLC- γ) pathway. These pathways are illustrated in figure 8.

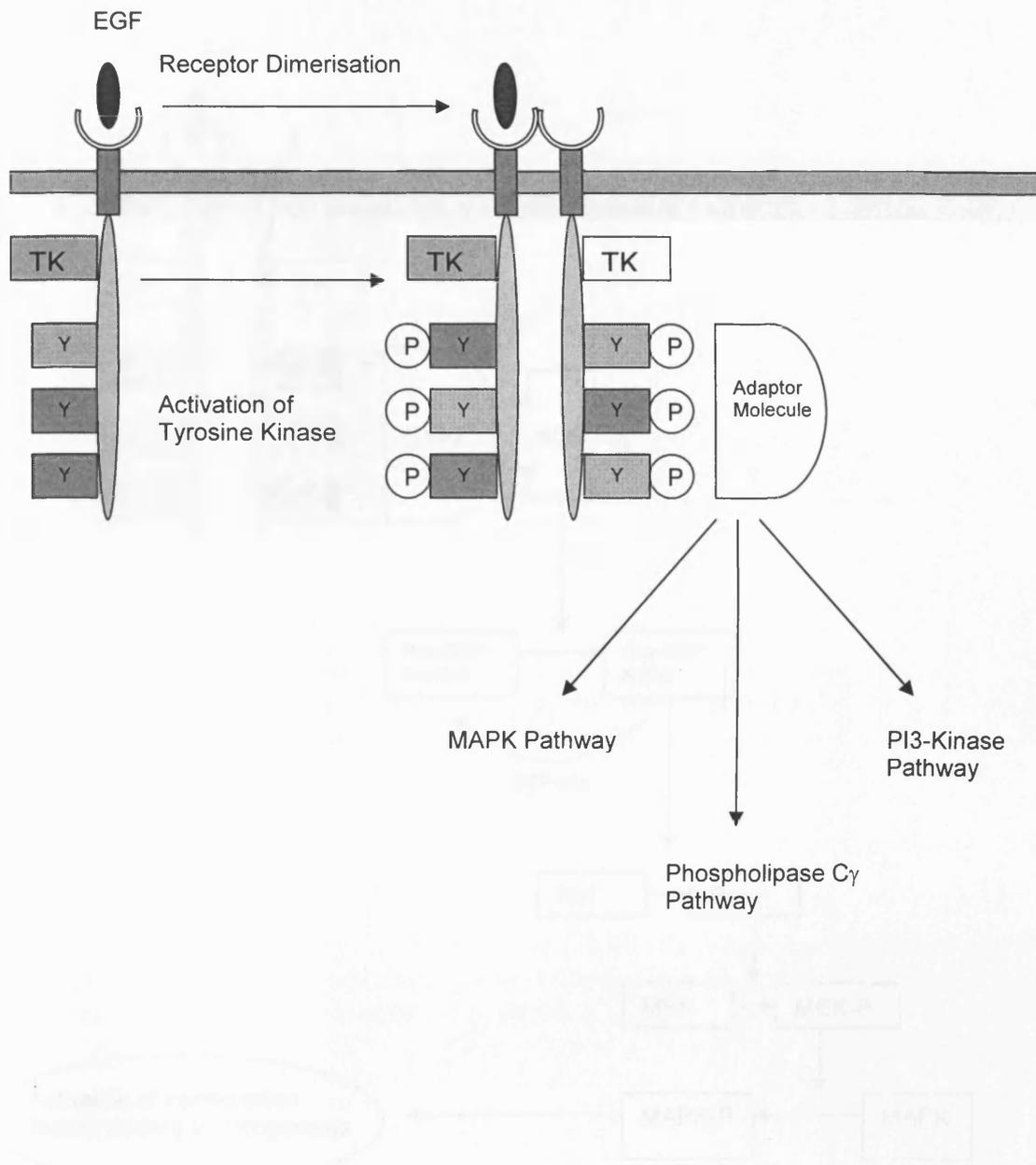


Figure 7. Following binding of a ligand to EGFR, the receptor dimerizes with another receptor of the same family, activating the tyrosine kinase (TK). This results in phosphorylation of the tyrosine (Y) residues. The phosphotyrosine residues are recognized by a number of adaptor molecules possessing *src* sequence homology (SH2/ SH3). These molecules subsequently activate other intracellular signalling pathways.

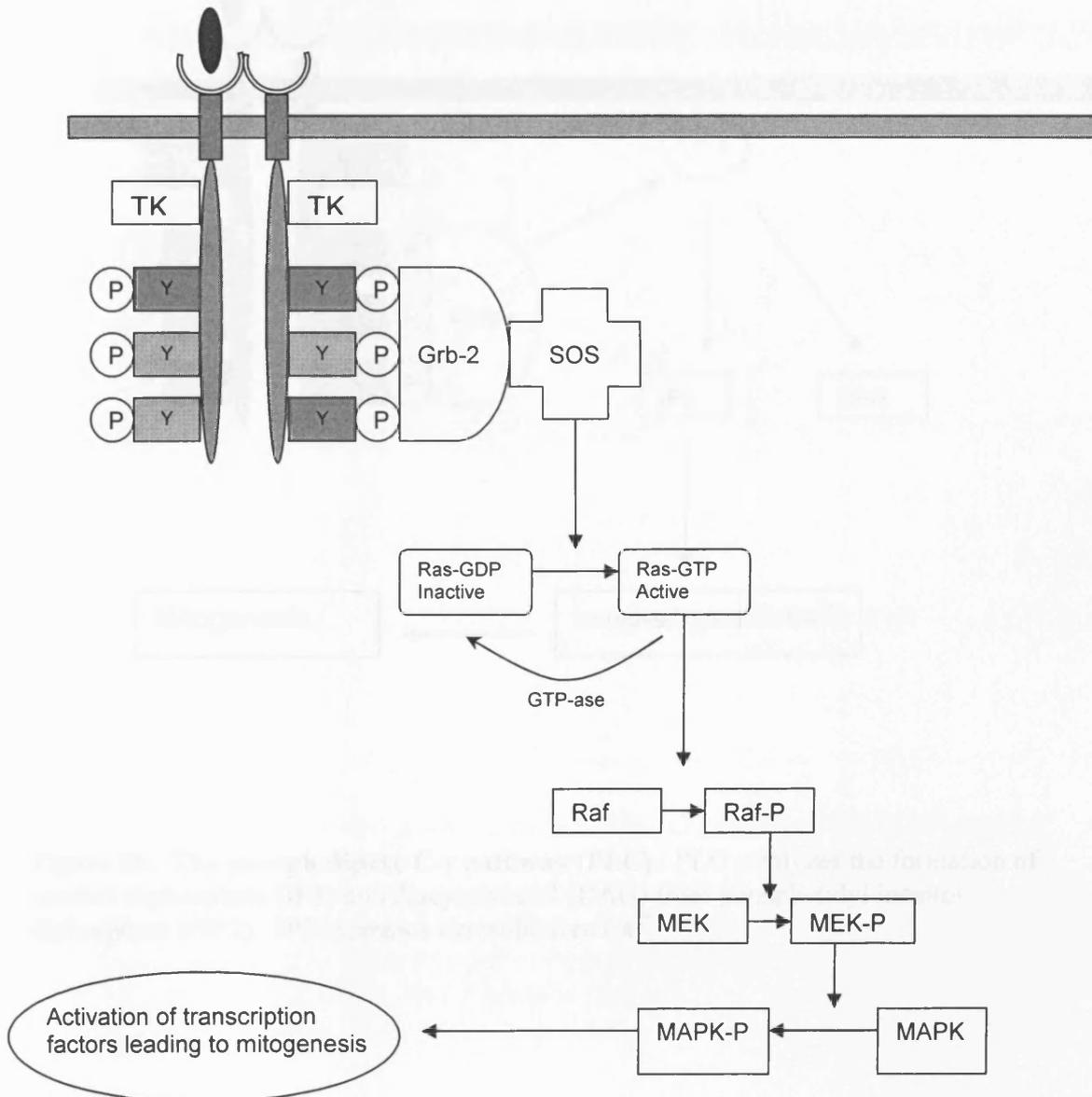


Figure 8a. The mitogen activated protein kinase pathway. Here, the adaptor molecule is Grb-2. Following interaction of Grb-2 with SOS (son of sevenless) protein, the linked ras-GDP is phosphorylated to ras-GTP which activates it. Subsequently, a cascade involving raf and other protein kinases is triggered, leading ultimately to the activation of nuclear transcription factors and mitogenesis.

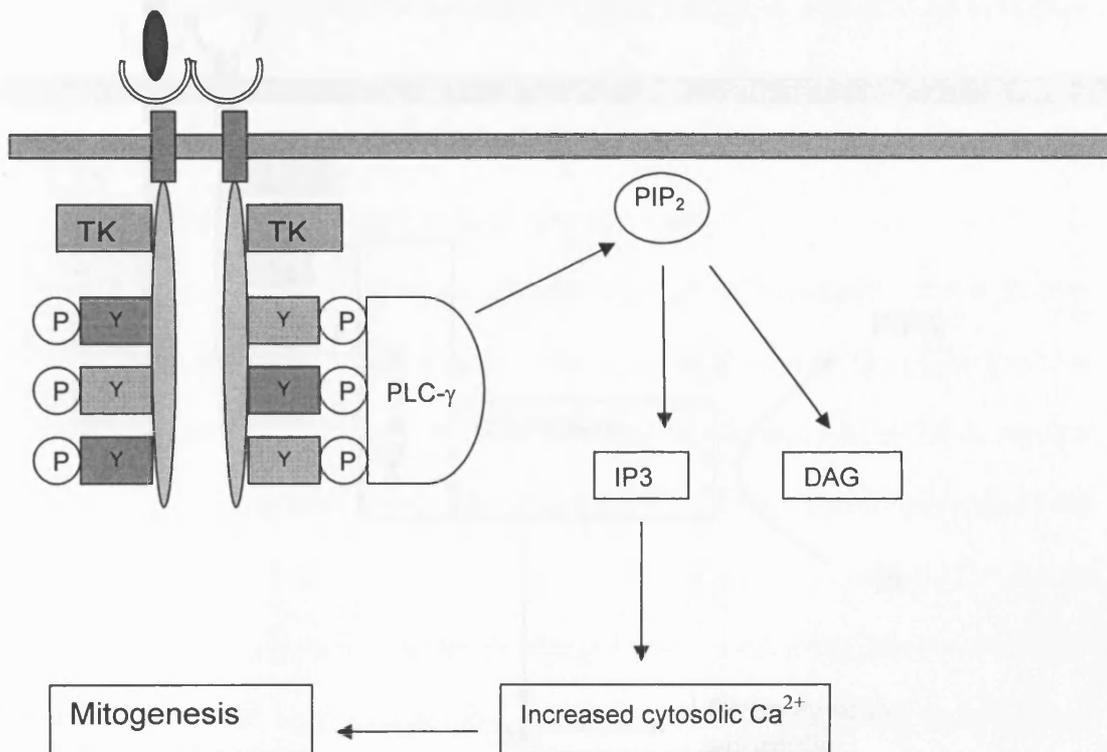


Figure 8b. The phospholipase C- γ pathway (PLC). PLC catalyzes the formation of inositol triphosphate (IP₃) and diacylglycerol (DAG) from phosphatidylinositol diphosphate (PIP₂). IP₃ increases cytosolic free Ca²⁺.

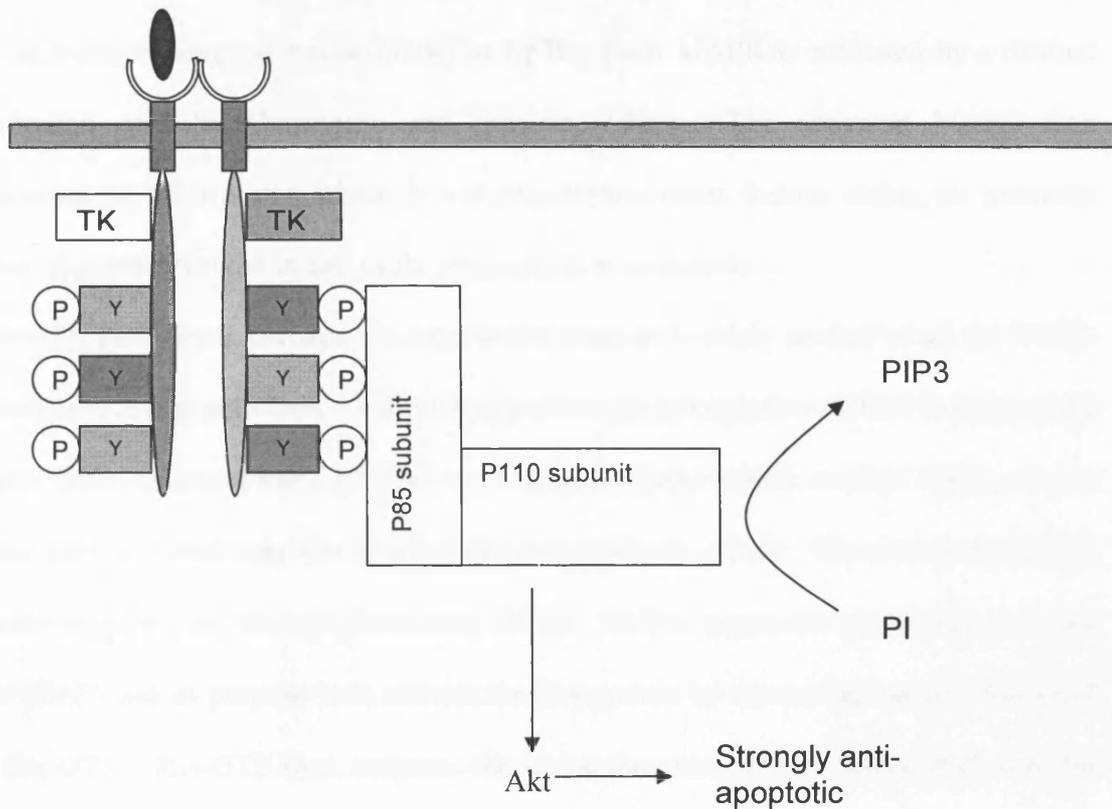


Figure 8c. The phosphatidylinositol 3-kinase (PI3-K) pathway. The PI3-K enzyme comprises two subunits, the p85 and p110 components. The p85 subunit recognises the phosphotyrosine residues on activated EGFR, whilst the p110 subunit catalyses the formation of phosphatidylinositol triphosphate (PIP3) from phosphatidylinositol and PIP2. The enzyme also controls downstream activation of the anti-apoptotic protein kinase B or “Akt” (it is the analogue of the viral *v-akt* gene product).

1.4.2.1 The mitogen activated protein kinase (MAPK) pathway

The MAPK family comprises three members known as extracellular-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK) and p38. Each MAPK is activated by a distinct multiprotein cascade (Garrington and Johnson, 1999). The activated MAPK then translocates to the nucleus where it activates transcription factors acting on promoter regions of genes involved in cell cycle progression or metastasis.

The MAPK pathway is perhaps the most well-known and widely studied of all the EGFR-mediated signalling pathways. Following tyrosine phosphorylation of EGFR intracellular domain, there is recruitment of Shc, an adaptor protein, which recruits Grb2, another adaptor protein. Grb2 may also be recruited independently of Shc. The next protein in the pathway is known as 'Son Of Sevenless' (SOS). This is a guanine nucleotide exchange factor (GEF) and its purpose is to activate the Ras protein by converting inactive Ras-GDP into Ras-GTP. Ras-GTP then activates the serine-threonine protein kinase Raf, and Raf subsequently activates MAPK Erk1/2 via the dual specificity kinase MEK1. There is some evidence to suggest that if Shc is recruited following EGFR activation, the JNK pathway is preferentially activated, whereas if Grb2 interacts with EGFR directly, the ERK pathway is activated (Hashimoto, 1999). The activation of JNK pathway by EGFR may involve small GTPases, Rac1 and Cdc42, either downstream of Ras (Minden, 1995), or independently of Ras (Coso, 1995). The adaptor protein Crk is critical for Ras-independent activation of Rac-JNK pathway (Dolfi, 1998).

1.4.2.2 The PI3-K pathway

The PI3-kinases are a family of lipid-kinase enzymes found in mammalian cells, all of which catalyse the phosphorylation of inositol lipids. There are three classes of these enzymes of which class I is activated by EGFR (Leevers, 1999). The enzyme comprises two subunits, the adaptor protein, p85, which contains SH2 domains and binds to EGFR, and the catalytic p110 component. Following activation, phosphatidylinositol 3-phosphate (PIP), phosphatidylinositol 3,4-bisphosphate (PIP₂) and phosphatidylinositol 3,4,5-trisphosphate (PIP₃) are formed. PIP₂ and PIP₃ bind to molecules containing pleckstrin homology (PH) domains (Lemmon, 1997). Of these, protein kinase B (PKB or Akt) has stimulated a lot of interest. Binding of PIP₂ and PIP₃ to the PH2 domain of Akt results in its translocation to the cell membrane (Downward, 1998), where it is activated by another enzyme, PIP₃-dependent kinase 1 (PDK1). As its name suggests, PDK1 is also reliant on PIP₃ activation. Activated Akt protects cells from apoptosis, perhaps through inactivation of the pro-apoptotic member of the Bcl-2 family, BAD (del Peso, 1997), or by activation of the transcription factor, NFκB (Datta, 1999). Recently, the EGFR-mediated stimulation of NFκB via PI3K and PKC has been shown to be important for cell cycle progression in oestrogen receptor-negative mammary adenocarcinoma cell lines (Biswas, 2001). Other proteins containing PH2 domains include several guanine nucleotide exchange factors (GEFs), which catalyse the activation of small GTPases, including those of the Rho family. These GTPases are involved in the regulation of the cell cytoskeleton and may be important in cell motility and adhesion (Hall, 1998).

1.4.2.3 The PLC γ pathway

PLC γ is an enzyme which hydrolyses membrane-bound phosphatidylinositol-4,5 bisphosphate (PIP₂) into inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). PLC γ contains SH2 domains, which bind to phosphotyrosine residues on the intracellular domain of activated EGFR. This association brings PLC γ into contact with its substrate, PIP₂, on the cell membrane and results in the phosphorylation and activation of the enzyme. The two products of this enzyme act as second messengers for further signal transduction. IP₃ mobilizes calcium stores in the endoplasmic reticulum to increase free cytosolic calcium, an important cofactor for many enzymes. DAG in conjunction with calcium activates the enzyme protein kinase C (PKC). Protein kinase C is implicated in a variety of responses, including activation of cell cycle progression, activation of the MAPK pathway and inhibition of apoptosis (Musashi, 2000).

1.4.2.4 The fate of EGFR

Following signal transduction, EGFR complexes undergo endocytosis in clathrin-coated pits. They may then be subsequently ubiquitinated and degraded. This process is facilitated by the recruitment of an ubiquitin ligase known as Cbl to the complex, which targets receptors for ubiquitination and lysosomal degradation. In the absence of Cbl, EGFR is recycled to the cell membrane (Levkowitz, 1998). EGFR homodimers form more stable complexes with Cbl than EGFR-HER2 heterodimers, which tend to dissociate in endosomes and are recycled to the cell membrane where they can participate in further signalling (Lenferink, 1998).

1.4.3 The function of EGFR in the normal prostate and prostate cancer

Immunohistochemical staining of well-preserved histological specimens has revealed the presence of EGFR in normal prostatic epithelium, in tissue undergoing benign hyperplasia, as well as in malignant prostatic cells (Cohen, 1994). The role of EGFR in the normal adult prostate is still not clear, although, as it is found in basal epithelial cells, is thought to help maintain epithelial integrity. It plays a key role during embryonic prostatic morphogenesis, prominently influencing folding of prostatic lobules (Kim, 1999).

In contrast to carefully regulated physiological EGFR-mediated signalling which occurs in health, there is increasing evidence that abnormal, prolonged EGFR signalling plays a significant role in prostatic carcinogenesis. This aberrant signalling may occur as a result of EGFR over-expression, expression of mutant EGFR, a switch in production of predominant stimulatory ligand from EGF to TGF- α , establishment of an autocrine stimulatory loop, or interaction of EGFR with other signalling pathways, particularly the androgen receptor (AR)-mediated pathway. These mechanisms will now be discussed in greater detail.

1.4.3.1 The expression of wild-type (WT-) and mutant EGFR in prostate cancer

The status of WT-EGFR and mutant EGFR expression in prostate cancer remains unclear. It has been shown that EGFR mRNA expression in cancerous tissue is higher than in normal prostatic tissue or even BPH tissue (Kumar, 1998). In addition, *in vitro* studies have demonstrated the presence of EGFR in both hormone-sensitive and -insensitive prostate cancer cell lines by Western blotting (Sherwood, 1998). However, investigations

into comparative EGFR protein expression in benign and malignant human prostatic tissue have proven contradictory, with some groups finding smaller amounts of the receptor in malignant epithelial cells compared to normal tissue (Mellon, 1992) and other investigators finding the opposite (Glynne-Jones, 1996). These differences have been attributed to variations in antibodies used for detection of the protein and assay techniques.

Recent work from one group of investigators has revealed the presence of a mutated form of EGFR known as EGFRvIII in prostate cancer cells (Olapade-Olaopa, 2000). This variant form of the receptor lacks 267 amino acids from its extracellular domain, resulting in loss of a large proportion of the ligand-binding pocket (Pedersen, 2001). It is constitutively active in the absence of a ligand. Using EGFRvIII-specific antibodies, it was shown that this variant receptor was over-expressed in human prostate cancer tissue, and that this was related to a concomitant decrease in expression of WT-EGFR (Olapade-Olaopa, 2000). As EGFRvIII is not detected in assays for normal or wild-type EGFR (WT-EGFR), it may contribute to reported discrepancies in EGFR expression in prostate cancer specimens. It must be emphasised, however, that the importance of EGFRvIII in prostate cancer remains to be confirmed by further studies.

It is clear that no consensus exists on the question of whether EGFR is over-expressed in prostate cancer. It is possible that the other mechanisms leading to aberrant EGFR-mediated signalling described below are of more importance than protein overexpression.

1.4.3.2 TGF- α as the dominant ligand in cancer

Although both EGF and TGF- α are mitogenic, they have been shown to have differential effects on the expression of EGFR in prostate cancer cell lines. TGF- α has been shown to preferentially stabilise EGFR mRNA, a mechanism thought to be of pivotal importance in post-transcriptional regulation of EGFR (Seth, 1999). EGFR-ligand complexes undergo endocytosis in clathrin-coated pits prior to their degradation (see 1.4.2.4). EGFR-EGF complexes remain stable in endosomes and are rapidly targeted by lysosomes for degradation. However, TGF- α -EGFR complexes rapidly dissociate in endosomes leading to recycling of the receptor rather than degradation. Thus, for the same initial number of EGF receptors, a switch in ligand production from EGF to TGF- α would result in prolonged signalling, as may occur in cancer (Seth, 1999). This hypothesis was borne out by a study which demonstrated that TGF- α is found in negligible quantities in normal prostate tissue, in small amounts in BPH and in much larger quantities in prostatic carcinoma (Harper, 1993).

1.4.3.3 Establishment of an autocrine loop in cancer

Prostatic tissue is a composite of epithelial and stromal elements. Although epithelial cells undergo malignant transformation, stromal cells are important in regulating proliferation by way of secreting growth factors. One study looked at the distribution of EGFR and its ligands in histological specimens of normal prostate and prostate cancer (Leav, 1998). In normal and benign glands, epithelial cells express EGFR, whilst EGF and TGF- α are primarily found in the stromal compartment. This suggests modulation of epithelial cell growth by stromal cells (paracrine control). In cancerous tissue, however, epithelial cells

co-express both ligand (predominantly TGF- α) and EGFR. This is very important as it implies epithelial autocrine stimulation, and loss of stromal modulation of EGFR function.

Stromal regulation of epithelial cell growth is mediated by a number of other growth factors, including transforming growth factor-beta (TGF- β), which acts on TGF- β receptors, T β R-I, T β R-II and T β R-III. In the normal gland, TGF- β is a negative regulator of epithelial proliferation and induces apoptosis (Ilio, 1995). It has been shown that TGF- β is over-expressed in prostate cancer specimens and that there is a loss of TGF- β receptors in malignant cells (Lee, 1999). It is thought that loss of T β R-I receptors by malignant prostatic cells confers a survival advantage by reducing their sensitivity to the growth inhibitory properties of TGF- β . Over-expression of TGF- β in prostate cancer seems paradoxical, given its inhibitory properties. However, it can also stimulate extracellular matrix production and promote angiogenesis, thereby making the surrounding environment more favourable for cancer cell growth and metastasis.

Neuroendocrine cells are found in the prostate and are thought to contribute to the control of growth and differentiation of the prostate gland. Over-expression of EGFR and HER-2 by these cells has been reported, and may be another mechanism by which *erbB*-mediated pathways are involved in carcinogenesis (Iwamura, 1998).

1.4.3.4 Interaction between EGFR and androgens

There is strong *in vitro* evidence of an interaction between androgens and growth factors. Androgen stimulation has been shown to up-regulate expression of EGFR (Schuurmans, 1988b). Another study showed that a combination of dihydrotestosterone (DHT) and EGF stimulated the growth of LNCaP cells synergistically (Schuurmans, 1988a).

The mechanisms underlying the development of androgen-independent prostate cancer remain unclear, but one hypothesis is that in the absence of androgens, activation of peptide growth factor receptors can lead to phosphorylation and subsequent activation of AR via the MAPK or PI3-K pathways (Feldman and Feldman, 2001). Indeed, EGF and other peptide growth factors can induce the transcription of AR-regulated genes, which is a possible explanation of why levels of prostate specific antigen, the product of an AR-regulated gene continue to rise in these later stages of prostate cancer. A recent immunohistochemical study (Di Lorenzo, 2002) showed that EGFR expression was higher in hormone-refractory metastatic prostate cancer than in radical prostatectomy and LHRH-treated specimens. Additionally, AR activation in the presence of low levels of androgens is dramatically increased by the presence of peptide growth factors (Jenster, 2000). Following androgen ablation therapy, a patient with advanced prostate cancer is likely to have very low levels of circulating androgens, which, when facilitated by *erbB*-mediated signalling, would be enough to activate AR-mediated cell proliferation.

Other studies, however, dispute any interaction between the two systems. It was shown that hormone-sensitive LNCaP cells stimulated to grow by the administration of DHT were

not inhibited by blockade of EGFR with anti-EGFR antibodies (Sherwood, 1998) or EGFR-specific tyrosine kinase inhibitors (Jones, 2001).

1.4.4 HER-2 in Prostate Cancer

HER-2 has been studied extensively in breast cancer and is over-expressed in a subset of 20-30% of these tumours (Slamon, 1989). High levels of HER-2 have been correlated with a poorer response to hormonal therapy in breast cancer (Slamon, 1987; Slamon, 1989). The role of HER-2 in prostate cancer is under intense scrutiny. It was shown that LNCaP cells, which are normally hormone-dependent, underwent hormone-independent growth following the forced over-expression of HER-2 (Craft, 1999). In a mouse prostate cancer xenograft model, androgen-independent xenografts expressed higher levels of HER-2 than their androgen-dependent equivalents (Craft, 1999).

There have been conflicting results relating to *c-erbB-2* gene amplification in human prostate cancer specimens. The same inconsistencies arise when studying HER-2 protein expression, which has been reported in 0-100% of cases (Ross, 1997). The reasons underlying these inconsistencies are possibly related to differences in specimen preparation, of the reagents used and in strict definitions of “positive” results. Two recent papers have aimed to clarify the situation, and have investigated HER-2 expression in different clinical stages of prostate cancer (Signoretti, 2000; Shi, 2001). Both groups of investigators detected HER-2 expression in prostate cancer and found that the level of expression was higher in prostate cancer. In addition they found that the level of expression was higher in prostatic tissue which had been exposed to androgen ablation therapy. The highest levels of expression were found in androgen-resistant metastatic specimens.

Thus, HER-2 over-expression appears to be associated with androgen-independent growth, and studies to determine the reason for this are underway. In common with EGFR signalling, HER-2 stimulation has been shown to activate AR in a ligand-independent fashion. HER-2 may also potentiate EGFR signalling, as HER-2/EGFR heterodimers dissociate rapidly in endosomes, with resultant recycling of EGFR and HER-2 and prolongation of signalling.

1.4.5 *ErbB* receptors as therapeutic targets in prostate cancer

Given the likely importance of EGFR and HER-2 mediated signalling in prostatic carcinogenesis, therapeutic abrogation of their function has become a research priority (Barton, 2001). This has proved fruitful in breast cancer, where a monoclonal antibody directed against HER-2, trastuzumab (“Herceptin”, Genentech), has been shown to be effective in women whose tumours express high levels of HER-2 (Agus, 2000). Agents directed against the *erbB* family of receptors in prostate cancer may prove particularly useful in the androgen-independent stage of the disease, and may delay the onset of this phase. At present, profiling of prostate cancer specimens for EGFR or HER-2 expression is not routinely carried out, but this will assume importance if those patients most likely to respond to highly targeted anti-EGFR therapy are to be identified. Numerous such therapeutic approaches may be used to inhibit the action of EGFR and HER-2, most of which are still in preclinical or early phase of clinical investigation. The most promising agents under development are summarised below.

1.4.5.1 Monoclonal antibodies

Cetuximab or C225 (ImClone) is a chimeric monoclonal antibody which has been developed to target the ligand-binding domain of the EGFR. It binds to the EGFR with higher affinity than its naturally occurring ligands. It has been shown to be effective in preclinical *in vivo* models of prostate cancer, both on its own and in combination with other agents. Clinical trials are in progress to assess its usefulness in prostate cancer and preliminary data from a phase I/II study in androgen-independent prostate cancer has shown that C225 combined with doxorubicin resulted in stabilization of disease in 38% of patients after 4 courses of treatment (Slovin, 1997). Trastuzumab, a monoclonal antibody directed against HER-2 may prove valuable in hormone refractory prostate cancer. *In vivo* studies using androgen-dependent and independent prostate cancer xenografts showed that trastuzumab had an anti-tumour activity when combined with paclitaxel (Agus, 1999). EGFRvIII presents another therapeutic target and a highly potent antibody-toxin conjugate, scFv(14E1)-ETA, showed marked activity against the mutant receptor as opposed to WT-EGFR (Schmidt, 1998). ABX-EGF (Abgenix/ Immunex) is a monoclonal antibody directed against EGF and has entered phase II clinical evaluation in hormone-refractory prostate cancer patients in the USA.

1.4.5.2 Inhibition of tyrosine kinase activity

Inhibition of ligand-induced EGFR tyrosine kinase activity is an attractive therapeutic target and a number of agents are being developed for this purpose. They tend to be small molecules which interact with the adenosine triphosphate (ATP) binding site on the intracellular domain of EGFR. Gefitinib (ZD1839, "Iressa" AstraZeneca Pharmaceuticals), is a highly-selective EGFR tyrosine kinase inhibitor, which shows good activity against

various cancer cells *in vivo* (Sirotnak, 2002), alone, and in combination with other cytotoxic agents (Ciardiello, 2000). A phase I clinical trial on 19 patients with advanced prostate cancer showed promising results in terms of symptom improvement of variable duration (Barton, 2001). Reported side effects of gefitinib include an acneiform skin rash as well as gastrointestinal symptoms including diarrhoea. Similar tyrosine kinase inhibitor compounds such as erlotinib (OSI-774, “Tarceva” OSI pharmaceuticals) have been developed. PKI 166 (Novartis pharmaceuticals) is a dual EGFR/HER-2 tyrosine kinase inhibitor and shows good activity against both hormone-sensitive and -insensitive prostate tumours (Mellinghoff, 2002). The constitutively active EGFRvIII can also be targeted by tyrosine kinase inhibitors, and a small synthetic compound, tyrphostin AG1478, has been developed for this purpose (Han, 1996).

1.4.5.3 Other approaches

Antisense oligonucleotides may prove useful as highly specific agents, but are at a very early stage of development. They are artificial nucleotide base sequences which can be synthesised so that they are complementary to specific regions of mRNA coding for a particular protein such as EGFR. By binding to these mRNA strands, they can reduce protein synthesis (Barton, 2001). One preclinical study (Rubenstein, 1996) investigated the antitumour properties of antisense oligonucleotides directed against EGFR and TGF- α mRNA in a xenograft model using PC3 cells. Haemorrhagic necrosis of these tumours was seen following this treatment, which was generally well tolerated (Rubenstein, 1996; Rubenstein, 1997; Rubenstein, 2003).

1.4.6 EGFR as a target for chemoprevention

It can be seen that aberrant EGFR and HER-2 mediated signalling is strongly implicated in prostatic carcinogenesis. Small-molecule tyrosine kinase inhibitors such as gefitinib have been extensively evaluated *in vitro* and *in vivo* and are effective in abrogating EGFR function. The low toxicity of gefitinib, even when administered at chemotherapeutic doses fulfils one of the most important requirements of any putative chemopreventive agent. What remains to be resolved is the optimum timing for targeting EGFR in a chemopreventive sense. The accumulating evidence suggests that both EGFR and HER2 are most relevant to the development of hormone-independent disease, and administering EGFR-targeted therapeutic agents to patients with hormonally treated metastatic prostate cancer may delay the onset of hormone-independent malignancy.

1.5 Specific aims of this project

As discussed, in section 1.1.3, cancer is the end result of a multi-step process, with a large number of intracellular processes becoming dysregulated. It is unreasonable, therefore to expect a single agent to be entirely successful in preventing carcinogenesis. It may, however, be possible to identify the most important changes which lead to cancer, and use a combination of agents to target these pathways. This rationale led to the seminal work by Torrance et al (Torrance, 2000) who used two agents, sulindac and EKI-569, to prevent development of intestinal adenomatous polyps in the murine APC^{Min/+} model of familial adenomatous polyposis. Min mice lack the tumour-suppressor gene APC and always develop numerous adenomatous intestinal polyps. COX-2 and EGFR overactivity have both been linked with colonic carcinogenesis. In Torrance's study, sulindac was chosen as a COX-1 and -2 inhibitor, and EKI-569 as an inhibitor of the EGFR tyrosine kinase. The investigators demonstrated that the mice which were given a combination of these two agents developed far fewer tumours than the control group or the two groups which received one or the other of the two agents. Even more impressively, giving EKI-569 allowed the dose of sulindac to be reduced by 75%, thus reducing the GI toxicity of the NSAID. One explanation for this synergistic effect is that there is cross-talk between the EGFR and COX-2 regulatory mechanisms. Indeed, it has been shown that EGFR-mediated MAPK-induced transcription factor activation can up-regulate COX-2 expression (figure 9).

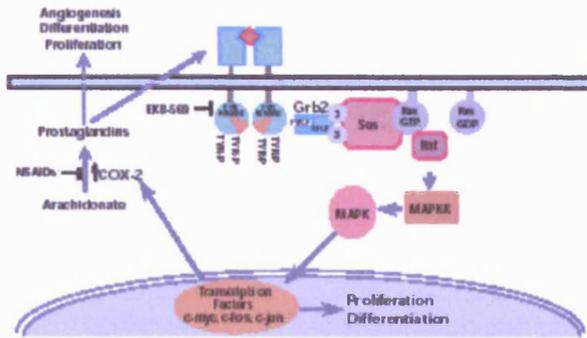


Figure 9. EGFR-mediated MAPK pathways can up-regulate COX-2 expression through induction of transcription factors. Taken from (Gupta, 2000)

Based on the discussion of the importance of COX-2 and EGFR (sections 1.3 and 1.4) to prostatic carcinogenesis, it would seem logical to apply this paradigm to the combination of resveratrol and gefitinib. Resveratrol is a potent inhibitor of COX-2 (1.3.2.1), and gefitinib specifically inhibits EGFR tyrosine kinase (1.4.5.2). Resveratrol, being diet-derived, would be expected to have low or no toxicity, and so far, phase I and II studies have shown gefitinib to be well-tolerated, even in high chemotherapeutic doses. Thus, both agents satisfy the first few criteria for any chemopreventive agent; they have low or no toxicity, there is a biological rationale for their action, and are both are likely to be tolerated by an individual. The aim of the work described in this thesis was to answer the question of whether they can both be shown to have antineoplastic effect in *in vitro* and *in vivo* models of human prostate cancer. Such preclinical evaluation is essential prior to the consideration of clinical trials involving agents of this type.

The specific aims of the project were to test the following hypotheses:

1. Resveratrol and gefitinib inhibit the growth of prostate cancer cell lines *in vitro*.
2. A combination of gefitinib and resveratrol result in an increased growth inhibitory effect.
3. The mechanisms by which resveratrol acts include cell cycle arrest, down-regulation of COX-2 expression and prevention of oxidative DNA damage.
4. The mechanisms by which gefitinib acts include cell cycle arrest mediated by inhibition of EGFR autophosphorylation.
5. Resveratrol and gefitinib inhibit the growth of prostate cancer xenografts in a nude mouse model.

Chapter 2: Materials and Methods

2.1 Suppliers

General reagents

All general laboratory chemicals and reagents including resveratrol were obtained from Sigma-Aldrich Company (Poole, U.K.), unless stated otherwise. All solvents were obtained from Fischer Scientific (Loughborough, UK). Gefitinib was a gift from AstraZenca Pharmaceuticals (Macclesfield, UK).

Cell culture

LNCaP, DU145 and PC3 were all obtained from ECACC (London, UK). U87MG- Δ EGFRvIII cells were a gift from Dr. Webster Cavenee (University of California, San Diego). Cell culture flasks, dishes, and 24 well-plates and all pipettes were all obtained from Nunclon. Growth media RPMI1640 and MEM, non essential amino acids were from Gibco. Foetal calf serum was obtained from Gibco after suitable batch testing. Isoton was obtained from Beckman-Coulter.

Western Blotting

Bradford Protein Assay Reagent and all Western blotting plastic equipment including broad-range molecular weight marker ladders were obtained from Biorad (Hemel Hempstead, UK). Nitrocellulose membranes, chemiluminescence kit and detection film were obtained from Amersham (Little Chalfont, Bucks). Total EGFR (polyclonal sc-03) and phospho-EGFR (monoclonal sc-1684) antibodies were from Santa Cruz biotechnology (Santacruz,, CA, USA). The corresponding anti-goat and anti-rabbit secondary antibodies were from the same company. EGFRvIII (monoclonal) antibody was from Zymed

Laboratories. α -tubulin antibody was obtained from Oncogene Research Products (Beeston, Notts). Polyclonal goat anti-COX-2 antibody and secondary donkey anti-goat antibody was obtained from Santa Cruz Biotechnology.

M₁G Assay

Murine M₁G monoclonal antibody D10A1 was prepared as has already been described (Sevilla, 1997) and obtained from Prof L. Marnett, Vanderbilt University, USA. Anti-murine horseradish peroxidase antibody was purchased from Dako (Ely, UK). M₁G standards were synthesised and characterised by Dr Raj Singh, University of Leicester. SuperSignal West Dura Extended Duration Substrate was from Pierce, (Rockford, USA). Immunoslot-blot apparatus, nitrocellulose filter paper and gel-blotting paper were from Schleicher and Schuell (Keene, Germany). DNA extraction kit was obtained from Qiagen.

In vitro experiments

Outbred ICRF and inbred MF1 nude mice were obtained from Harlan Biotechnology (Bicester, UK). RM1 diet was obtained from Special Dietary Services (Witham, UK). Liebowitz medium was obtained from Sigma Chemicals (Poole, Dorset).

2.2 Stock solutions

The following stock solutions will be referred to throughout the rest of this chapter.

Cell culture media

For DU145:

500ml	Minimum Essential Medium (MEM)
1ml	1mM non-essential amino acids (NEAA)
5mls	1mM Na pyruvate
50mls	fetal calf serum

For LNCaP and PC3:

500ml	RPMI 1640
5mls	sodium pyruvate
5mls	glutamax
50mls	fetal calf serum

Cell storage (freezing) medium

50%	FCS
40%	base medium (serum-free)
10%	DMSO

Cell lysis buffer

120mM	NaCl
50mM	Tris base
0.5%	Triton X-100
1mM	EDTA
1mM	EGTA
1mM	Na ₃ VO ₄
1mM	Sodium pyrophosphate
1mM	β-glycerophosphate
1%	Protease cocktail inhibitor

Cell lysis buffer for COX-2 determination

150mM	NaCl
1%	Tris ph8
50mM	Diethyldithiocarbamic acid
1mM	EDTA
1%	Tween 20
1x	Protease cocktail inhibitor

4x Resolving gel buffer (pH 8.8)

1.5M	Tris base
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4x Stacking gel buffer (pH 6.8)

0.5M	Tris base
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Tank buffer

0.025M	Tris base
0.192M	Glycine
0.1%	SDS

Transfer buffer 5L

29g	Tris base
14.5g	Glycerine
1L	Methanol
9.25ml	20% SDS

Tris buffered saline with tween (TBS-T) 10L

500ml	1M Tris base pH7.5
87.8g	NaCl
10ml	Tween 20

Stripping buffer

2%	SDS
7.8%(w/v)	Tris base
0.8%	β -mercaptoethanol

7.5% Resolving gel (x2 1.5mm thickness gels)

5ml	Acrylamide solution
5ml	4x resolving gel buffer
200 μ l	10% SDS solution
9.7ml	ddH ₂ O
200 μ l	10% APS solution
15 μ l	TEMED

Stacking gel (x2 1.5mm thickness gels)

0.88ml	Acrylamide solution
1.66ml	4x stacking gel buffer
66 μ l	10% SDS solution
4.06ml	ddH ₂ O
200 μ l	10% APS solution
15 μ l	TEMED

2.3 Methods

2.3.1 Establishment of cell cultures from frozen cells

Frozen cells were thawed in a water bath and resuspended in 10mls of fresh medium. This cell suspension was then centrifuged at 4°C for 5 min at 350g. The supernatant was poured off and the cells resuspended in fresh medium (10ml) and transferred into 25cm² tissue culture flasks. Cells were examined daily under the light microscope. Once 80-90% confluent, cells were passaged as described below.

2.3.2 Routine passage of cells

Flasks were removed from the incubator and the old waste medium pipetted out and discarded. Cells were washed with PBS to remove non-adherent dead cells and any traces of waste medium. An aliquot of 5ml of trypsin was added to each of the flasks and left in the incubator for 5 minutes. Following this, the flasks were gently tapped to detach any remaining adherent cells before 5 ml of fresh medium was added to neutralise the trypsin. The cell suspension was then centrifuged at 350g for 5 min to form a cell pellet. After pouring away the supernatant, this pellet was resuspended in 5ml of fresh medium. An aliquot of 50µl of this suspension was then mixed with 50µl of trypan blue solution and the cells counted in a haemocytometer using a light microscope. Cell viability as assessed by trypan blue exclusion was never less than 99%. Cells were then seeded into a fresh 75cm² flask at a density of 1×10^6 in 25ml of fresh medium and the flasks returned to the incubator at 37.5°C, 5%CO₂.

2.3.3 Preparation of cells for storage

The procedures detailed above were followed to obtain a cell suspension in fresh medium from a flask of adherent cells. Following cell counting using a haemocytometer, the suspension was centrifuged again, this time at 200g for 10 min. The resultant pellet was resuspended in an appropriate amount of freezing medium in order to give a concentration of $1-6 \times 10^6$ cells/ml. This suspension was transferred into freezing vials (cryotubes) and frozen in the -20°C freezer for 24 hrs prior to transfer to the -80°C freezer or liquid nitrogen for long-term storage.

2.3.4 The effect of resveratrol and gefitinib on prostate cancer cell proliferation

Cells were passaged as described above to obtain a suspension of cells in fresh medium, which were counted using a haemocytometer. Cells were seeded at a density of 10^4 in each well of 24 well plates in 1ml of medium. These plates were incubated for 24 hrs to allow cell adhesion. An aliquot of 1ml of medium containing the desired final concentration of resveratrol or gefitinib was added to each well, using an equivalent volume of DMSO-containing medium as control. The four concentrations of resveratrol and gefitinib chosen were $1\mu\text{M}$, $5\mu\text{M}$, $10\mu\text{M}$ and $25\mu\text{M}$, and $0.1\mu\text{M}$, $0.5\mu\text{M}$, $1\mu\text{M}$ and $5\mu\text{M}$ respectively.

Plates were replaced in the incubator. At 72 hrs, one plate was removed from the incubator and the medium from each well was carefully aspirated. Cells were gently washed with PBS. Aliquots of 0.5mls trypsin solution were added to each well and incubated for 5 min at 37°C . After this time, 0.5ml of fresh medium was added to neutralise the trypsin. 200 μl of the resultant cell suspension was removed and mixed with 9.8ml of isoton. Cells

were counted using a Coulter Z2 particle counter. This procedure was repeated with a new plate every 24 hrs until 168 hrs (DU145 and PC3 cells) or every 48 hours until 240 hrs (LNCaP cells) to obtain cell growth curves.

IC₅₀ values calculated during the exponential growth phase as determined from the growth curve (144 hrs for DU145 and PC3 cells, 192 hrs for LNCaP cells). The statistical model used to derive the IC₅₀ value was a non-linear regression sigmoidal dose-response curve as calculated by GraphPad Prism 3 software.

2.3.5 The effect of a combination of gefitinib and resveratrol on cell proliferation

After the initial experiments, the two agents were used in combination. Cells were seeded at a density of 1×10^4 in 24 well plates and left to adhere for 24 hours as above. A further 1ml of medium containing various combinations of resveratrol, gefitinib and control (DMSO) added to each well. The resveratrol concentration was kept constant at $5 \mu\text{M}$, and the gefitinib concentrations used were 0.5, 1 and $5 \mu\text{M}$. The cells were counted using a Coulter Z2 at 120 hrs (DU145 and PC3) or 168 hrs (LNCaP) as these timepoints corresponded to the exponential growth phase determined by the previous experiments.

These experiments yielded useful information on the effect of combining the two agents, (see 3.2.2) but it was not possible to determine whether any combined effect was additive or synergistic. To clarify this relationship a further set of combination experiments was designed in conjunction with a biostatistician. In this experiment, three concentrations of

resveratrol and three concentrations of gefitinib were combined with each other to give a 4x4 design as shown below:

	Gefitinib concentration (μM)			
Resveratrol concentration (μM)	0	0	0	0
	0	0.1	0.5	1
	2	2	2	2
	0	0.1	0.5	1
	4	4	4	4
	0	0.1	0.5	1
	6	6	6	6
	0	0.1	0.5	1

Table 3: Design of combination experiment. Resveratrol concentration shown in blue, gefitinib in red.

2.3.6 The effect of resveratrol and gefitinib on cell cycle kinetics

Cells were grown and passaged as described previously. Cells were seeded at a density of 1×10^6 in 10ml of medium in 10cm Petri dishes and incubated for 24 hr. Fresh medium (10ml) containing various concentrations of gefitinib, resveratrol or DMSO control were then added to the dishes. Cells were incubated for a further 72 hr. At this time, cells were washed with ice cold PBS and detached by incubation with 2ml trypsin solution. Following centrifugation at 350g for 5 minutes, the pellets obtained were resuspended in 200 μl PBS. Aliquots of 2ml 70% ethanol were then added whilst vigorously agitating the tube, in order to permeabilise the cell membrane. This cell suspension was then incubated at 4 $^{\circ}\text{C}$ for at least 2 hr. The suspension was centrifuged at 600g for 10 min. The supernatant was carefully discarded and the pellet was resuspended in 800 μl PBS. Aliquots of 100 μl RNase solution (1mg/ml) and 100 μl propidium iodide solution (50 $\mu\text{g}/\text{ml}$) were

added, and this suspension was incubated for 3-4 hr. The cell suspension was then analysed in a Beckton-Dickinson flow cytometer set up to detect the fluorescence of propidium iodide, and thus the relative quantities of DNA content. The flow cytometry readings obtained were subsequently analysed using Modfit software running on an Apple Macintosh PowerPC. This enabled the determination of the proportion of cells in the G0/G1, S and G2/M fractions of the cell cycle. The experiments were performed in triplicate and repeated once. The mean values obtained from the two experiments were very similar.

2.3.7 Determination of EGFR content of prostate cancer cells and the effect of gefitinib on EGFR autophosphorylation

2.3.7.1 Preparation of cell lysates

Cells were passaged, seeded at a density of 1×10^6 per 10cm plate and treated with gefitinib as described previously (see 2.3.6). For determination of mutant EGFRvIII content, lysates of the U87MG Δ EGFRvIII cell line, which over-expresses this receptor, were used as a control. The concentrations of resveratrol and gefitinib used ranged between 1 and 50 μ M, and 0.01 to 10 μ M respectively. Following 72hr incubation, plates were removed from the incubator and waste medium removed. Ice-cold PBS was used to wash the cells. 200 μ l of ice-cold lysis buffer was then added to each plate, and the cell debris scraped off and transferred to 1.5ml Eppendorf tubes. These tubes were then centrifuged at 13,000g for 15min. The resulting supernatant, containing the cytosolic proteins was then removed and transferred to fresh Eppendorf tubes for protein quantification by Bradford analysis.

2.3.7.2 Bradford protein assay

The Bradford protein assay was performed in order to ensure equal loading of samples prior to gel electrophoresis. The principle of the test relies on the ability of protein to effect a colour change of the Bradford reagent, the degree of which can be quantified using a spectrophotometer.

In order to construct a standard curve to which samples could be compared, a range of volumes of bovine serum albumin solution (BSA, 1mg/ml) from 1 μ l to 20 μ l were dissolved in 1ml of HPLC-grade water. An aliquot of 800 μ l of each sample was then mixed with 200 μ l of Bradford reagent and transferred to a cuvette. The sample was analysed in a spectrophotometer at a wavelength of 595nm using Cary WinUV 50 software.

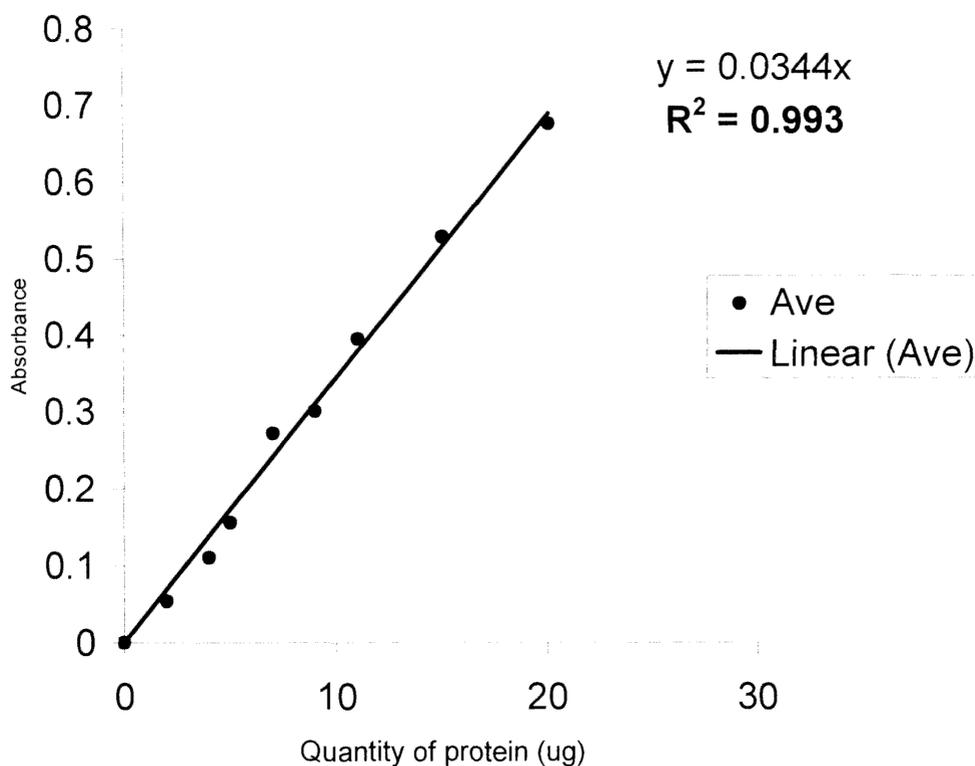


Figure 10. A typical Bradford protein assay standard curve

To determine the protein concentration in each sample, between 1 and 5 μ l of sample was dissolved in 1ml of water. Aliquots of 800 μ l of this solution were then mixed with 200 μ l of Bradford reagent and analysed using the spectrophotometer. Protein concentration was calculated from the standard curve.

2.3.7.3 Electrophoresis and transfer

Gels were constituted between 1.5mm glass plates as described above. Once the stacking gel had set, the apparatus was transferred to a gel tank filled with running buffer. Volumes of cell lysate containing 100 μ g of protein were loaded into each of 9 wells along with 10 μ l of Biorad broad-range molecular weight marker in the 10th well. Gels were run at constant potential difference of 100V for approximately 2-3 hr. After this time, the gel was carefully transferred to a blotting cassette in contact with a strip of nitrocellulose membrane. The cassette was transferred into a tank containing transfer buffer and 130V was applied for 2hr.

2.3.7.4 Development of gel

Following transfer, the nitrocellulose membrane was removed and placed in TBS-T containing 0.5% milk powder (TBS-T/ milk solution) overnight. The following day, the relevant primary antibody (Table 6) was constituted in TBS-T/ milk solution, added to the nitrocellulose membrane and constantly agitated on rocking apparatus for two hours. The antibody-containing solution was then discarded and the membrane washed 5 times with TBS-T solution for 5 minutes at a time. The secondary antibody was then constituted in TBS-T/ milk solution and incubated with the membrane for 1 hr. The membrane was

washed again with TBS-T as described above. Chemiluminescence reagents were mixed and poured over the surface of the membrane. A minute later, the excess liquid was discarded and the gels were placed in acetate holders before being transferred to an x-ray cassette. The gels were exposed to detection film for between 5 and 15 min. The films were developed in a dark room using an AGFA photo developer.

Primary antibody (concentration)	Secondary antibody (concentration)
Total EGFR (1:1000) polyclonal	Anti-rabbit HRP conjugate (1:1000)
EGFRvIII (1:1000) monoclonal	Anti-mouse HRP conjugate (1:1000)
Phospho-EGFR (1:1000) polyclonal	Anti-goat HRP conjugate (1:2000)

Table 4: Concentrations of primary and corresponding secondary antibodies used.

2.3.7.5 Determination of α -tubulin content

In order to ensure equal loading of protein for all samples, the membranes obtained were stripped of antibody by incubating them with stripping buffer at 60°C for 45 min. Following overnight incubation with TBS-T/milk solution, the membrane was incubated with α -tubulin antibody for 2 hr followed by 5x5 min TBS-T washes and 1 hr incubation with anti-mouse secondary antibody. The membrane was developed as described above.

2.3.8 Effect of resveratrol on COX-2 expression

Cells were prepared and incubated with concentrations of resveratrol varying between 1 and 25 μ M of resveratrol as described in section 2.3.7. After 72hr incubation, cells were washed and lysed with COX-2 lysis buffer. Protein content was quantified with the Bradford protein assay (2.3.7.2) and gels were run and developed as described in 2.3.7.3-

2.3.7.5. Primary antibody was 1:1000 goat anti-COX-2 antibody, and the corresponding secondary antibody was donkey anti-goat immunoglobulin. 100l of purified COX-2 enzyme was used as a positive control. Following these initial experiments, no constitutive expression of COX-2 was demonstrated, so cells were then stimulated by 1hr pre-treatment with 10nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Lysates were prepared and analysed in the same manner.

2.3.9 Effect of resveratrol on malondialdehyde-deoxyguanosine (M₁G) levels in DU145 cells

2.3.9.1 Cell preparation

1x10⁶ DU145 cells were seeded into 175cm² flasks containing 50ml fresh medium. After 24hr, resveratrol-containing material at various concentrations ranging from 1-25µM was added and flasks were incubated for a further 72hr.

2.3.9.2 DNA extraction from cells for M₁G analysis (Qiagen Method)

DNA extraction from human prostate cancer cells was performed for M₁G analysis using the Qiagen method by Mr. Sanjeev Pathak at the University of Leicester. Cells were harvested from large flasks with 10 ml of trypsin (1%). Cell suspensions were then centrifuged in 50 ml falcon tubes containing 40 ml of growth medium at 500g for 5 minutes at 4°C. The supernatant was discarded and the cell pellet was re-suspended initially in 2 ml of cell media followed by further dilution in 5-8 ml of cell media. Cell counts were performed using trypan blue and the haemocytometer. Ice cold Phosphate Buffered Saline (PBS) was added to the cell suspension to make up to a final volume of 50 ml. The suspension was centrifuged at 1500 x g for 10 minutes at 4°C. The supernatant was

discarded and the cell pellet was again re-suspended in 2 ml of PBS. 2 ml of ice cold Qiagen C1 lysis buffer and 6 ml of ice cold water (HPLC grade) was added. The suspension was inverted several times and then incubated on ice for 10 minutes before centrifugation at 1300g for 15 min at 4°C. The supernatant was discarded and the pellet re-suspended in 1 ml of Qiagen C1 lysis buffer and 3 ml water, following gentle vortexing, then centrifugation at 1300 x g for 15 minutes at 4°C was repeated. The supernatant was again discarded and the nuclear pellet completely re-suspended in 5 ml G2 buffer and vortexed for up to 30 seconds. Aliquots of 95 µls of Proteinase K and 162 µls of RNAase A were added and the suspension incubated for 2 hours at 37°C in water bath. The 100/G Qiagen genomic tips were equilibrated with 4 mls of QBT buffer, and the samples were then run through the genomic tips, and the tips were washed twice with 7.5ml QC buffer.

DNA was eluted from the genomic-tips with 5mls of QF elution buffer (50°C) into 15 ml falcon tubes containing 3.5 ml of 100% isopropanol, and precipitated by gently inverting the tubes 10-20 times, followed by centrifugation at 4000g for 20 minutes at 4°C. The resultant supernatant was discarded and the pellet washed with 0.5-1.5 ml of ethanol (70%), then transferred into a 2 ml eppendorf tube. These were then centrifuged at 5000g for 10 min at 4°C. Finally, the supernatant was discarded and the pellet allowed to dry for a few minutes at room temperature, prior to being resuspended in 200µl of water and stored at -80°C for subsequent analysis.

2.3.9.3 Estimation of sample DNA content by spectrophotometry

Following DNA extraction and re-suspension in 200 μ l, each DNA sample concentration was determined by uv spectrophotometry. The samples were taken and diluted by a factor of 200 in HPLC grade water. The samples were placed in a quartz cuvette and analysed using the Christian-Warburg parameter. The concentration and purity of DNA was then used to calculate the volume of DNA sample needed to give 50 μ g of DNA for 8-oxo-dG determination.

$$[\text{DNA sample- } \mu\text{g}/\mu\text{l}] = \text{wavelength (260 nM)} \times \text{dilution factor (200)} \times C (0.05)$$

2.3.9.4 Quantification of the oxidative DNA adduct, M₁G by immunoslot blot

Calibration curve determined by obtaining a series of standards containing 5 μ g of calf thymus DNA in a volume of 50 μ ls. Standards were developed by diluting control calf thymus DNA with MDA treated calf thymus DNA as below.

Sample	control calf thymus DNA (μ ls)	MDA treated calf thymus DNA ((μ ls)	M ₁ G (fmol)
1	50	0	0.2
2	48	2	0.4
3	45	5	1.0
4	40	10	2.0
5	35	15	3.0
6	25	25	5.0
7	20	30	6.0
8	10	40	8.0
9	0	50	10.0

Following gentle vortexing of the above standards, 35 μ l (3.5 μ g) of each standard was transferred into a 1 ml eppendorf containing 65 μ ls of KP buffer, 150 μ l of PBS was added and the samples gently vortexed. Each specimen was transferred into a 1 ml eppendorf, the volume transferred containing 3.5 μ g of DNA. KP buffer was added to make a final volume of 100 μ l. Finally, 150 μ l of PBS was added and the samples gently vortexed. The standards and DU145 DNA specimens were centrifuged at 14,000 rpm for 1 minute.

DNA was sonicated for 20 minutes, thus allowing the DNA strands to be broken down into 100 base pair lengths, and heated to 100^oC for 5minutes to break double strands into single strands. All samples were then cooled on ice for 10min. Aliquots of 250 μ l of ammonium acetate (1M) added, vortex mixed and centrifuged at 14,000 rpm for 2min.

Immunoslot blot apparatus was set up, and aspiration was commenced. A volume of 142 μ l of standards and specimen was added to the wells in triplicate and the wells were then allowed to run dry. To each well 200 μ l of 1M ammonium acetate was added and again allowed to run dry. The aspirator was left on for a further 15 minutes. The nitrocellulose filter paper was removed and baked in a pre-heated vacuum oven at 80^oC for 1.5 hr. The filter paper was then washed in 100 ml of PBS-T containing 5% milk powder for 1 hour at room temperature. The filter paper was then washed twice in 50 mls PBS-T for 5 minutes. The filter paper was then incubated in 40 ml of PBS-T containing 0.5% milk powder and 5 μ l of primary antibody (M₁G) and gently rocked for 2 hours at room temperature, followed by gentle rocking overnight in the cold room (4^oC). The filter paper was then washed with 50 mls of PBS-T for 1 min, followed by two further 5 min washes. The filter paper was

then incubated in 32 ml of PBS-T containing 0.5% milk powder and 8 μ l of secondary antibody (goat anti-mouse) and rocked for 2 hours at room temperature. The filter paper was then washed again in 50 ml PBS-T for 15 minutes followed by two further 5 minute washes. The supersignal ultra chemiluminescent reagent working solution was prepared by mixing equal volumes (4 ml) of the ultra enhancer solution with the ultra stable peroxide solution. The filter paper was bathed in 8 ml of chemiluminescent reagent for 5 minutes at room temperature and blotted onto a paper towel prior to ECL analysis in the GenegnomeTM Bioimaging system.

In order to determine equal loading of samples, the immunoblot was re-stained with propidium iodide. Following PBS washing, the filter paper was bathed in 50 ml of PBS containing 50 μ l of propidium iodide and gently rocked for 3 hours at room temperature. (Protected from sunlight by placing container in black box). This was followed by washing in 50 mls of PBS for 1 hr, followed by a fresh wash for 30 minutes. The image was then captured on the GenegeniusTM Bioimaging system, and M₁G adduct levels were corrected on the base of PI staining of each band, and were expressed as adducts/10⁷ nucleotides.

2.3.10 The effect of resveratrol and gefitinib on growth of DU145 xenografts in nude mice

2.3.10.1 Care of Mice

The first set of experiments involving ICRF nude mice were carried out at the MRC Toxicology Unit Division of Biomedical Sciences. The second and third experiments involving MF1 and ICRF mice respectively were carried out at the Robert Kilpatrick Clinical Sciences Building. All these animals were kept in numbered cages in sterile

isolators. RM1 standard diet and water was administered in bottles and the animals were weighed weekly.

2.3.10.2 Pilot Experiment

In order to determine whether prostate cancer cell lines would form xenografts in nude mice, a pilot experiment was designed involving 12 ICRF nude mice. These mice were randomised to two groups of 6 mice, and marked with identifying ear punches. The two cell lines used were LNCaP and DU145. These two cell lines were chosen as they represent hormone-dependent and hormone-independent models respectively, and they are both sensitive to the growth inhibitory effects of both gefitinib and resveratrol. Prior to *in vivo* experiments commencing, twelve 175cm² flasks of both cell lines were grown. The cells were passaged and counted using a haemocytometer. Cells were then resuspended in Liebowitz medium at a concentration of 2×10^7 /ml. The mice were anaesthetised using gaseous isoflurane and 100µl of cell suspension (containing 2×10^6 cells) was injected subcutaneously into the right flank of each mouse, 6 mice receiving DU145 cells and the other 6 receiving LNCaP cells. All tumour injections were performed by Dr. Richard Verschoyle. Following this, the mice were returned to their isolators and normal diet was administered. Mice were weighed weekly and any tumours were measured using callipers.

DU145 cells were found to proliferate well in nude mice, forming well-defined subcutaneous tumours after a latency period of 2-3 weeks. However, LNCaP cells did not form tumours even 8 weeks following injection of the cells. It was thus decided to proceed with a feeding experiment using DU145 xenografts.

2.3.10.3 Feeding study using DU145 xenografts in ICRF nude mice

The first feeding study was designed to test the hypothesis that gefitinib and resveratrol would inhibit the growth of DU145 xenografts, when used individually and in combination. To this end, 24 ICRF nude mice were obtained, randomly allocated to four groups of 6 mice, and marked with ear punches. Group 1 were to receive control RM1 diet for the duration of the study. Group 2 would receive resveratrol at a dose of 240mg/kg mixed with diet following tumour injection. This dose of resveratrol was chosen as another member of our group in the Department of Oncology (Mr S. Sale) used the same dose in murine models of carcinogenesis and found it to be effective (unpublished data – Sale 2003). Group 3 initially received gefitinib at a dose of 100mg/kg mixed with diet following tumour injection. The dose of gefitinib was decided upon after reviewing the literature regarding *in vivo* experiments using this compound. The dose of 100mg/kg seemed the most commonly used effective dose. Group 4 received both 240mg/kg resveratrol and 100mg/kg gefitinib in the diet following tumour injection.

The mice were all 2 weeks of age upon arrival, and tumour injections were performed by Dr. Richard Verschoyle 3 weeks later. All mice received 3×10^6 DU145 cells subcutaneously in the right flank. Up until the point of tumour injection, all mice were fed control diet, and thereafter the diet allocated to them. The mice were weighed weekly and the size of any tumours were measured with callipers. After 5 weeks, it was noted that some mice in the 2 groups which received gefitinib were losing weight, mainly through what appeared to be an appetite suppressant effect of the drug. It was thus decided to halve the dose of gefitinib in these mice. The study was terminated after 10 weeks. Two mice in Group 2 (receiving resveratrol) grew large tumours which ulcerated prior to the conclusion

of the study, and were culled immediately in accordance with the animal licence requirements.

Mice were culled by terminal exsanguination under anaesthesia by Dr. Richard Verschoyle, and the plasma and tumour tissue preserved for subsequent biochemical analysis. The experiment was repeated with another group of 24 ICRF nude mice under the same conditions, including the drop in gefitinib dose from 100mg/kg to 50mg/kg after 5 weeks.

2.3.10.4 Further experiments to investigate the effect of resveratrol on tumour growth

To determine whether the findings regarding the tumour growth rate response to dietary resveratrol were valid, two further experiments were conducted. The first of these two experiments was designed to investigate whether there was a strain-specific effect, and employed 24 MF1 mice. These mice were randomised to receive either dietary resveratrol (240mg/kg) or control diet following the injection of 3×10^6 DU145 cells in the right flank. The experiment was terminated after 7 weeks.

The second additional experiment was designed to investigate the dose-response effect of resveratrol. A group of 36 ICRF nude mice (the same strain as initially used) were randomised into three groups of 12. Following injection of 3×10^6 DU145 cells in the right flank, group 1 received control diet, group 2 received resveratrol at a dose of 120mg/kg and group three received a dose of 480mg/kg. The experiment was terminated after 8 weeks, and the mice culled by terminal exsanguinations under anaesthesia by Dr. Verschoyle.

2.3.10.5 Calculation of tumour volume

Callipers were used to measure tumour size in two dimensions. From these values, tumour volume was calculated using the formula:

$$V = ((A+B)/2)^3/2$$

Where A and B are the two measured dimensions in mm, and V is the volume in mm³.

Thus, if A and B were 12mm and 8mm respectively, tumour volume would be 500mm³.

2.3.11 Mass spectrometry analysis of plasma from MF1 mice fed 120mg/kg and 480 mg/kg resveratrol

Plasma containing resveratrol and any metabolites was obtained from mice fed resveratrol as described in 2.3.10.3. Plasma samples were stored at -80C following extraction. The samples were subsequently thawed at room temperature whilst being kept in the dark. An aliquot of 250 µl of the plasma was pipetted into a clean 1.5 ml eppendorf tube and 250 µl of room temperature acetonitrile was added. The sample was then vortex mixed for 1 minute and returned to the freezer for 5-10 min. Following this, the sample was centrifuged for 15min at 13000 rpm, the resultant supernatant being pipetted into a fresh eppendorf tube. The supernatant was dried down under a stream of gaseous nitrogen. Once dry, the sample was reconstituted in 150 µl of mobile phase solvent (a 50:50 mix of methanol with 2% propan-2-ol: 5mM Ammonium acetate with 2 % propan-2ol). Samples were analysed with an API 2000 mass spectrometer with inline liquid chromatography (Agilent 1100). Samples were injected at 1.5ml/minute and passed through a Zorbax SB C18 3.5u 4.6x150mm column. Retention time was 22 min. Resveratrol and its metabolites

were identified on mass spectroscopy by specifying monitoring of molecular weight of the compounds as follows:

Resveratrol 227g

Resveratrol glucuronide 403g

Resveratrol sulfite 307g

Resveratrol disulfite 387g

Resveratrol glucuronide sulfite 483g

Chapter 3: Results

3.1 The effect of resveratrol and gefitinib on the proliferation of prostate cancer cell lines

Figures 11 to 13 show representative growth curves obtained by treating cell lines with gefitinib, along with graphs showing the growth inhibition at the timepoint at which IC_{50} values were calculated. Gefitinib was extremely active against DU145 cells and demonstrated activity against LNCaP cells, albeit with a higher IC_{50} . This compound displayed relatively less activity against PC3 cells.

Figures 14 to 16 show representative growth curves obtained by treating cell lines with resveratrol, along with graphs showing the growth inhibition at the timepoint at which IC_{50} values were calculated. Resveratrol inhibited the growth of all three cell lines, the IC_{50} values being similar. Table 5 summarises the IC_{50} values obtained by treating the three cells with resveratrol and gefitinib.

Table 5: IC_{50} values (μM) with standard deviation in brackets

	Resveratrol	Gefitinib
LNCaP	6.88 (3.57)	3.83 (4.59)
DU145	4.87 (2.31)	0.14 (0.02)
PC3	6.57 (2.57)	8.20 (0.16)

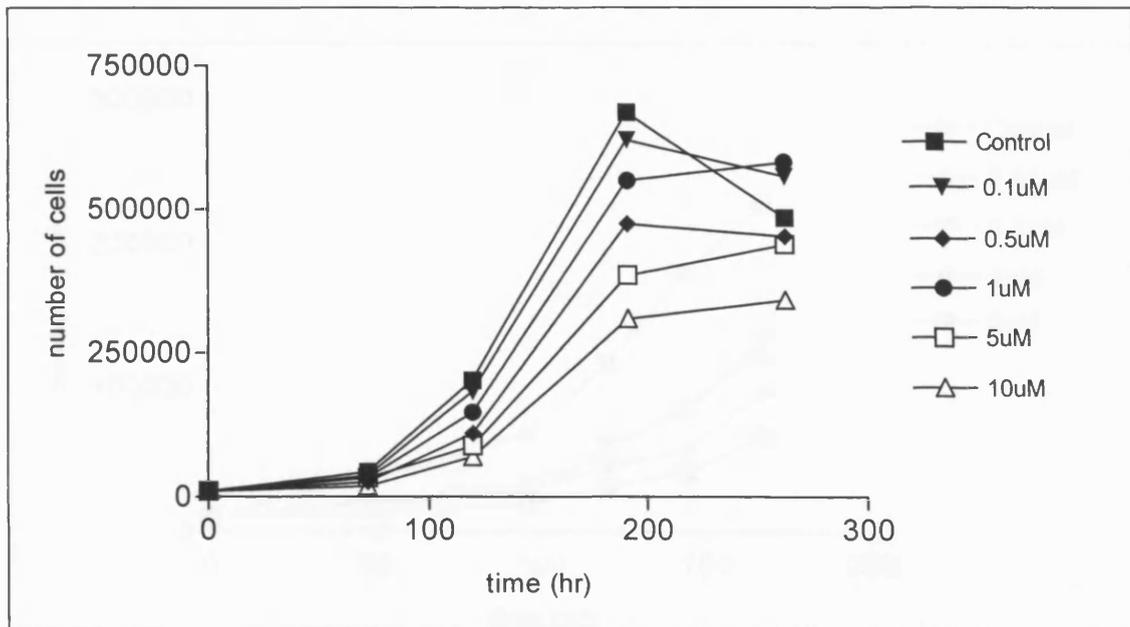


Figure 11a: the effect of gefitinib on the proliferation of LNCaP cells over 268 hours. Figures represent mean of 2 values, and the graph is representative of an experiment performed in triplicate.

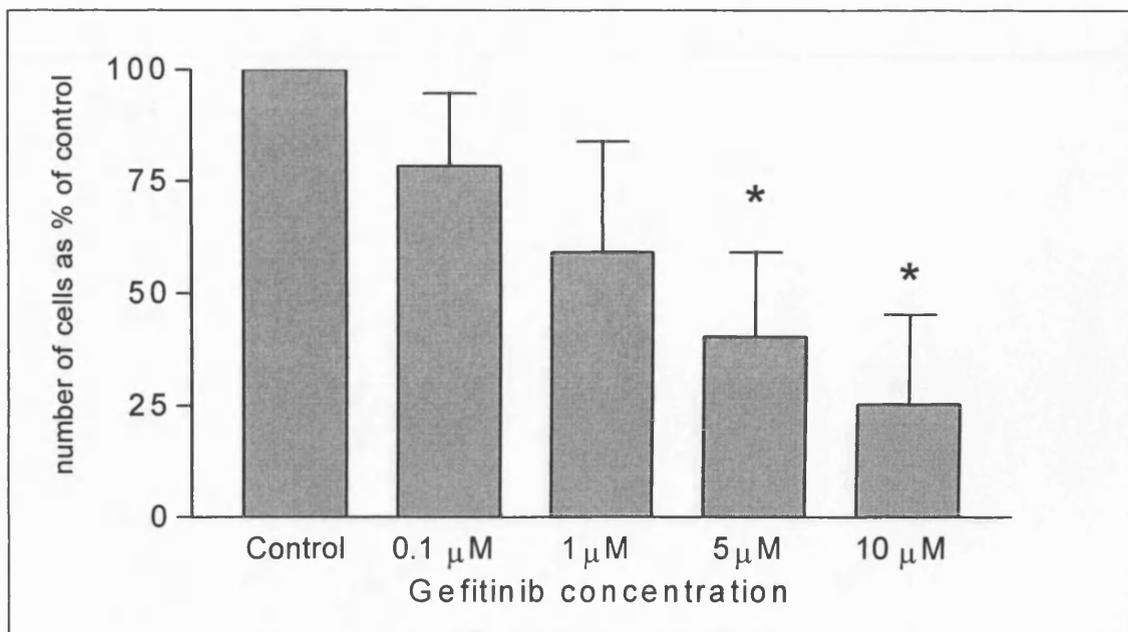


Fig 11b: the number of LNCaP cells as a proportion of control following 192 hrs treatment with various concentrations of gefitinib. All values represent the mean of 3 experiments, with error bars representing SD. * represent values significantly less than control at $p < 0.05$

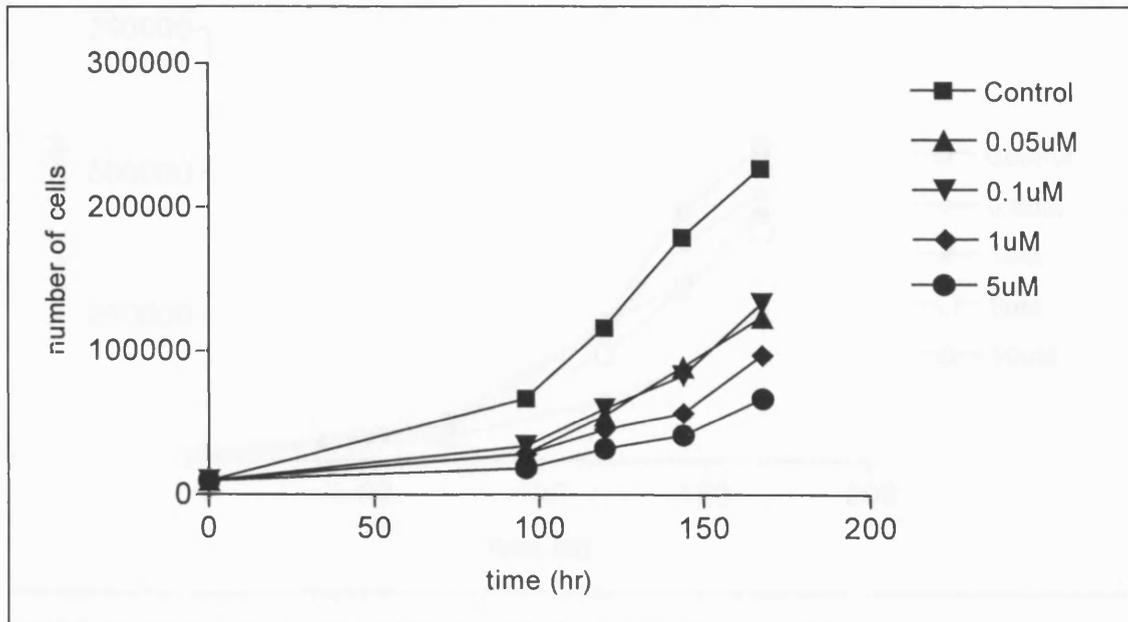


Figure 12a: the effect of gefitinib on the proliferation of DU145 cells over 168 hours. Figures represent mean of 2 values, and the graph is representative of an experiment performed in triplicate.

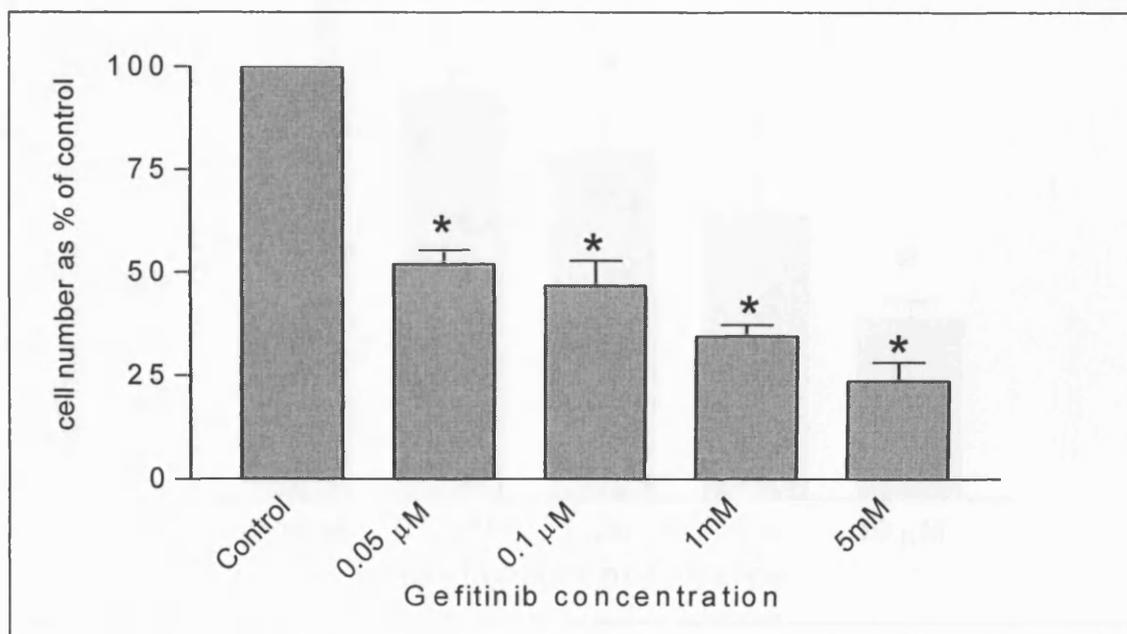


Figure 12b: the number of DU145 cells as a proportion of control following 144 hrs treatment with various concentrations of gefitinib. All values represent the mean of 3 experiments, with error bars representing SD. * represent values significantly less than control at $p < 0.05$

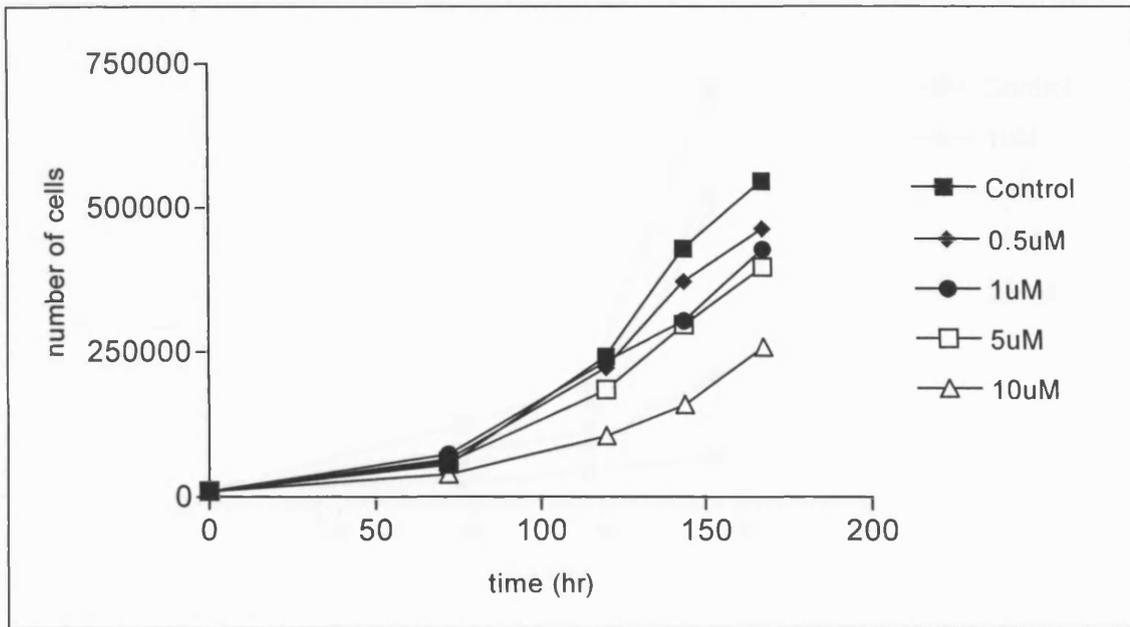


Figure 13a: the effect of gefitinib on the proliferation of PC3 cells over 168 hours. Figures represent mean of 2 values, and the graph is representative of an experiment performed in triplicate.

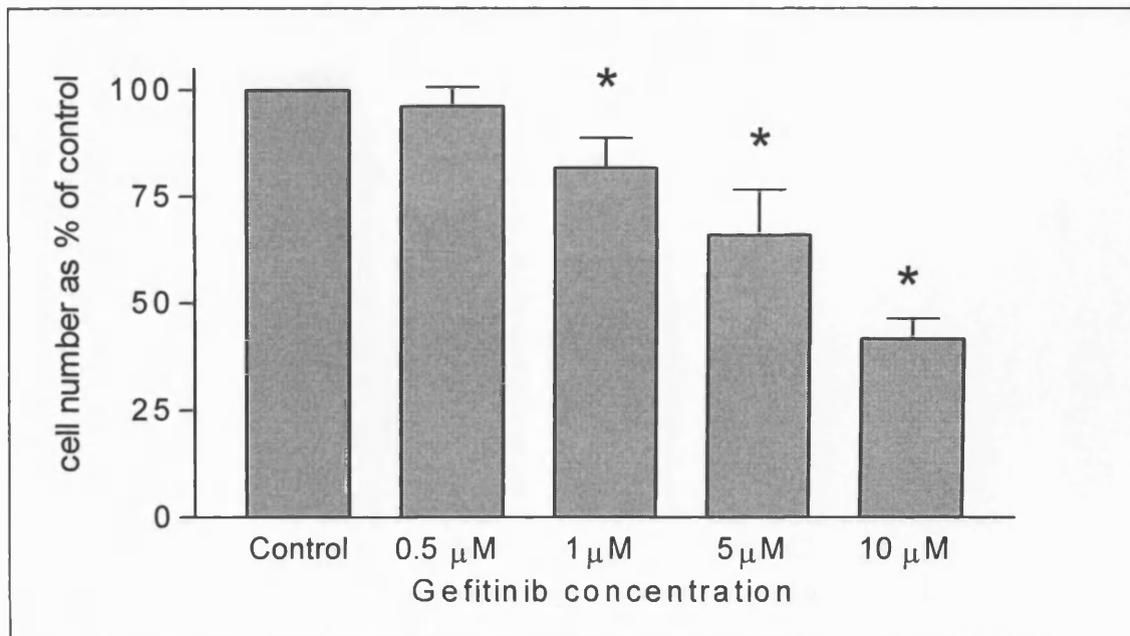


Figure 13b: the number of PC3 cells as a proliferation of control following 144 hr treatment with various concentrations of gefitinib. All values represent the mean of 3 experiments, with error bars representing SD. * represent values significantly less than control at $p < 0.05$

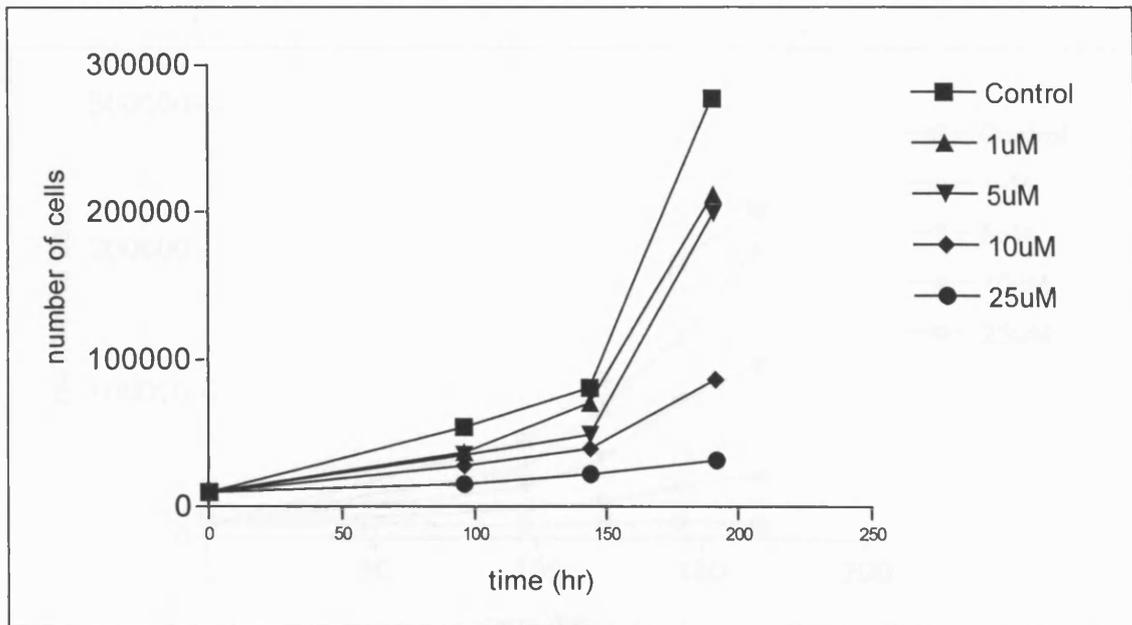


Figure 14a: the effect of resveratrol on the proliferation of LNCaP cells over 192 hours. Figures represent mean of 2 values, and the graph is representative of an experiment performed in triplicate.

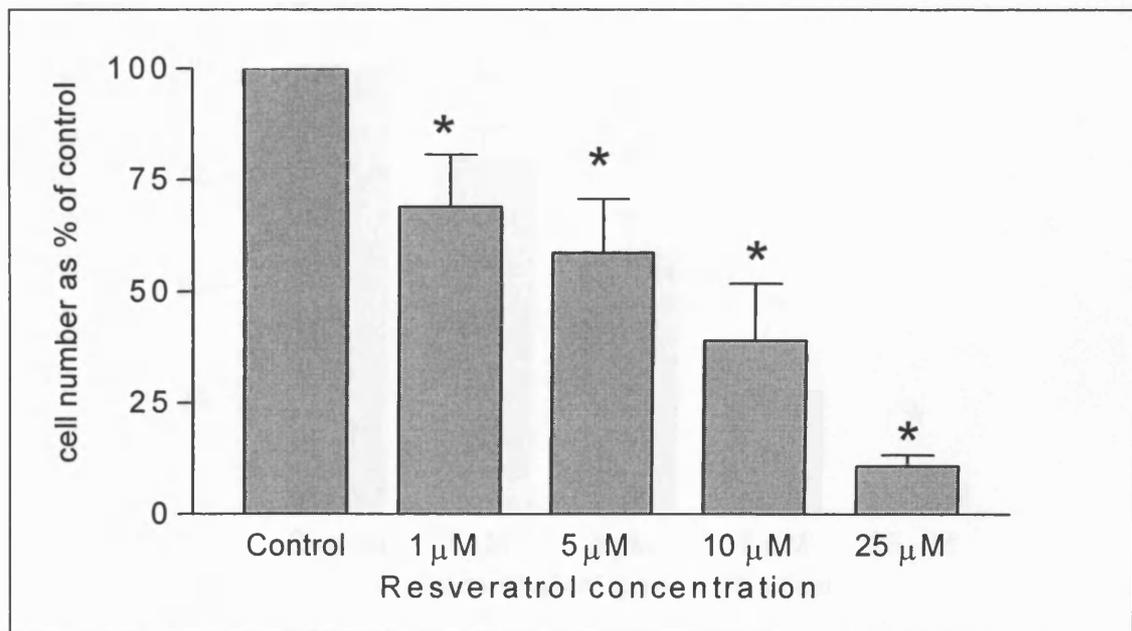


Figure 14b: the number of LNCaP cells as a proportion of control following 192 hrs treatment with various concentrations of resveratrol. All values represent the mean of 3 experiments, with error bars representing SEM. * represents values significantly less than control at $p < 0.05$

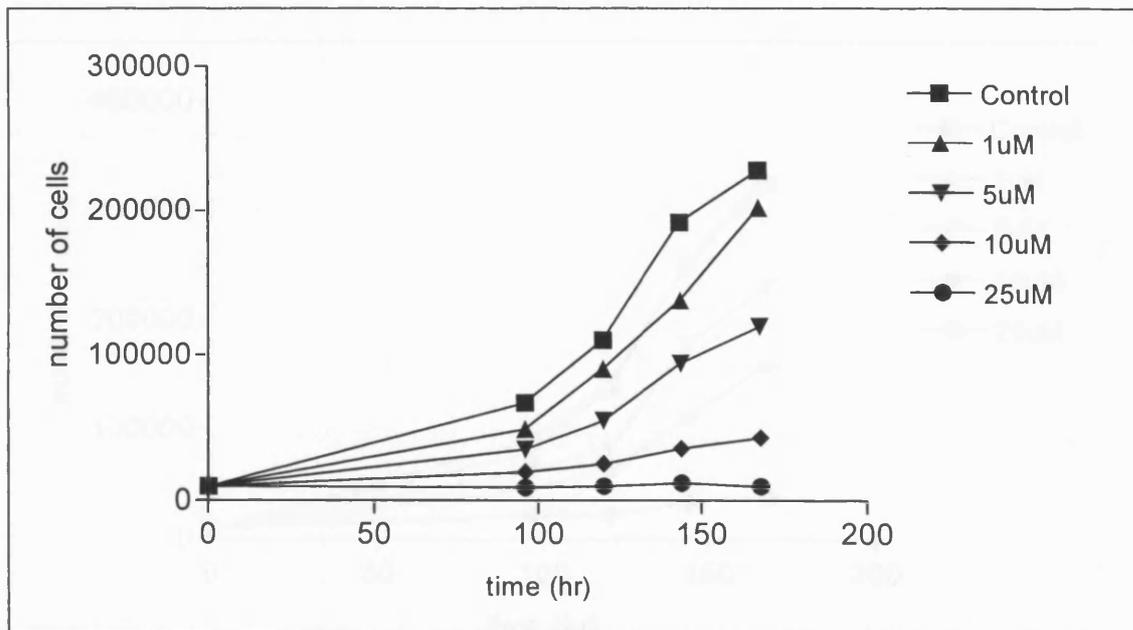


Figure 15a: the effect of resveratrol on the proliferation of DU145 cells over 168 hours. Figures represent mean of 2 values, and the graph is representative of an experiment performed in triplicate.

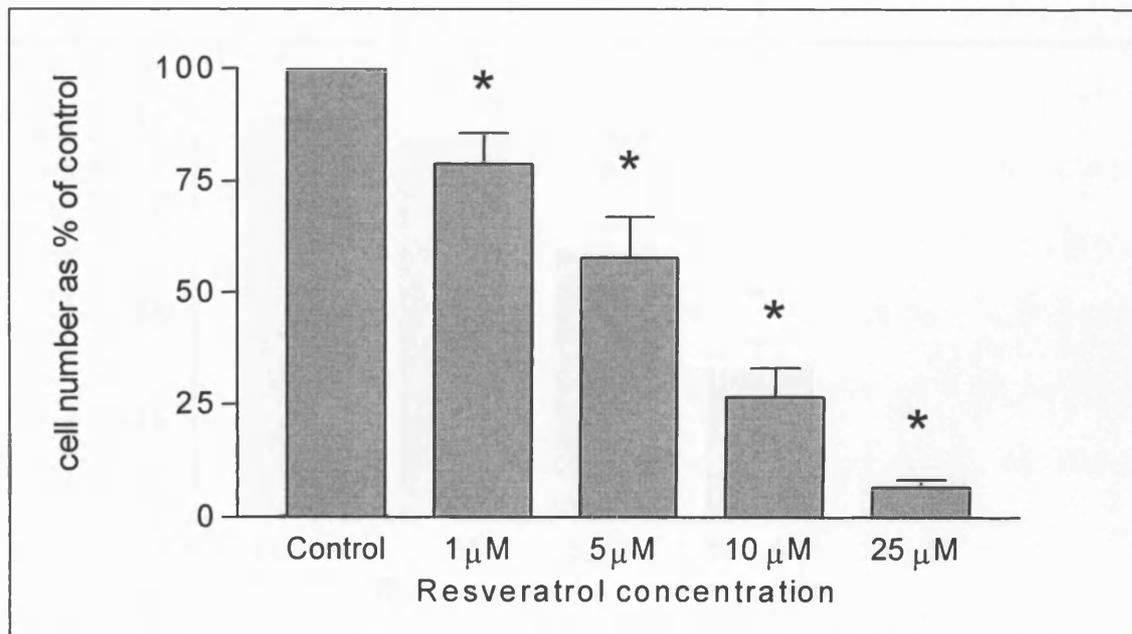


Figure 15b: the number of DU145 cells as a proportion of control following 144 hrs treatment with various concentrations of resveratrol. All values represent the mean of 3 experiments, with error bars representing SD. * represents values significantly less than control at $p < 0.05$

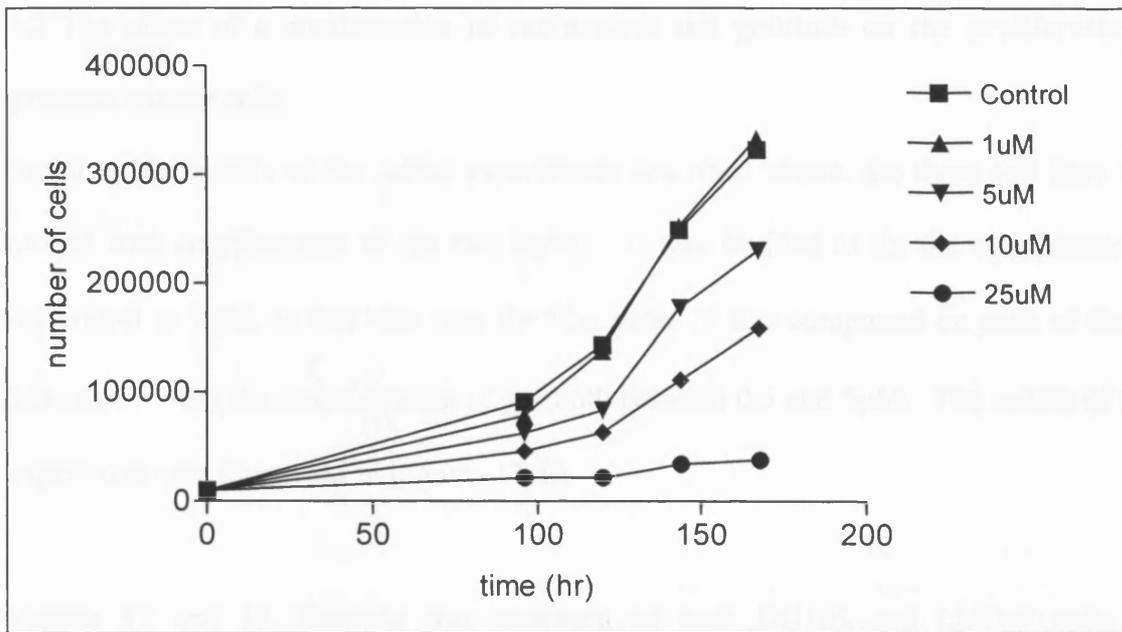


Figure 16a: the effect of resveratrol on the proliferation of PC3 cells over 168 hours. Figures represent mean of 2 values, and the graph is representative of an experiment performed in triplicate

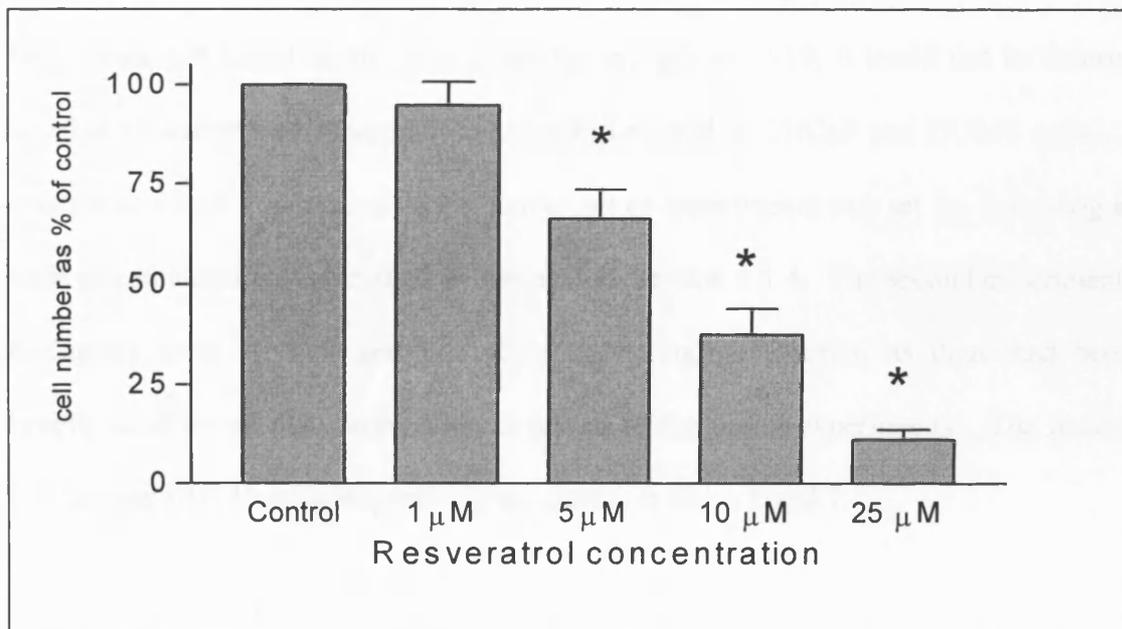


Figure 16b: the number of cells as a proportion of control following 144 hrs treatment with various concentrations of resveratrol. All values represent the mean of 3 experiments, with error bars representing SD. * represents values significantly less than control at $p < 0.05$.

3.2 The effect of a combination of resveratrol and gefitinib on the proliferation of prostate cancer cells.

Based on the results of the initial experiments described above, the three cell lines were treated with combinations of the two agents. It was decided to fix the concentration of resveratrol at 5 μ M, as this was near the IC₅₀ value of this compound on each of the cell lines and to vary the concentration of gefitinib between 0.5 and 5 μ M. The results of these experiments are illustrated in Figures 17-19.

Figures 17 and 18 illustrate that treatment of both DU145 and LNCaP cells with combinations of the two agents resulted in significantly greater inhibition of proliferation of these cell lines than either agent used alone. However, this is not the case in PC3 cells (figure 19). This set of experiments provided an initial insight into the interaction of these two agents, but based on the data presented in figures 17-19, it could not be determined whether an additive or synergistic relationship existed in LNCaP and DU145 cells. After consultation with a biostatistician, a further set of experiments was set up, involving a 4x4 table of combinations as detailed in the methods section 1.1.4. The second experiment was performed using LNCaP and DU145 cells, but not PC3 cells, as there had been no beneficial effect of the combination observed in the initial experiments. The results for LNCaP and DU145 cells respectively are shown in tables 6 and 7.

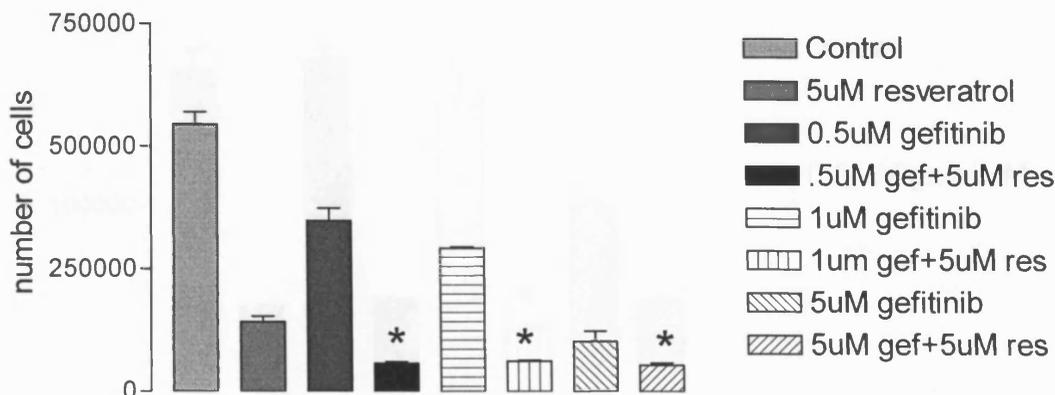


Figure 17: The effect of combined resveratrol (5 μ M) and various concentrations of gefitinib on the proliferation of LNCaP cells. * represents a significant difference in number between the cells treated by the combination of agents and the cells treated by either 5 μ M resveratrol or the corresponding gefitinib dose (0.5, 1 or 5 μ M) at $p < 0.05$.

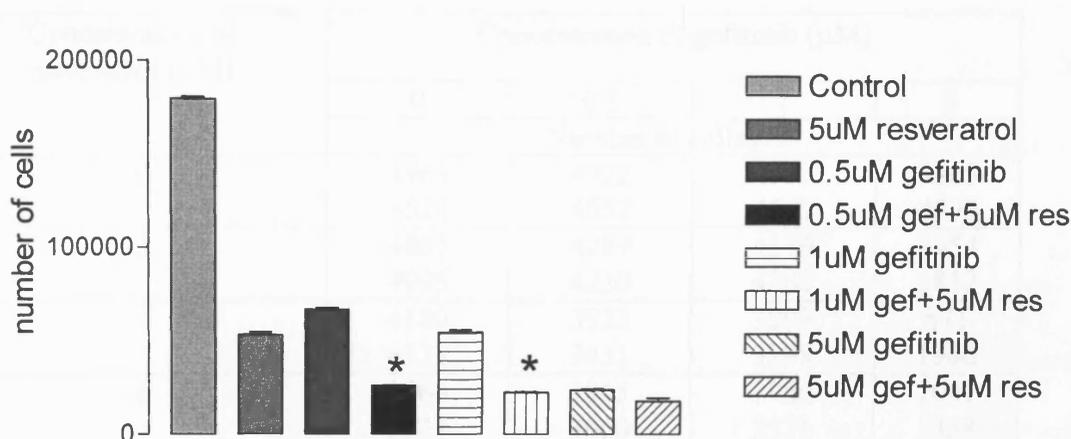


Figure 18: The effect of combined resveratrol (5 μ M) and various concentrations of gefitinib on the proliferation of DU145 cells. * represents a significant difference in number between the cells treated by the combination of agents and the cells treated by either 5 μ M resveratrol or the corresponding gefitinib dose (0.5, 1 or 5 μ M) at $p < 0.05$.

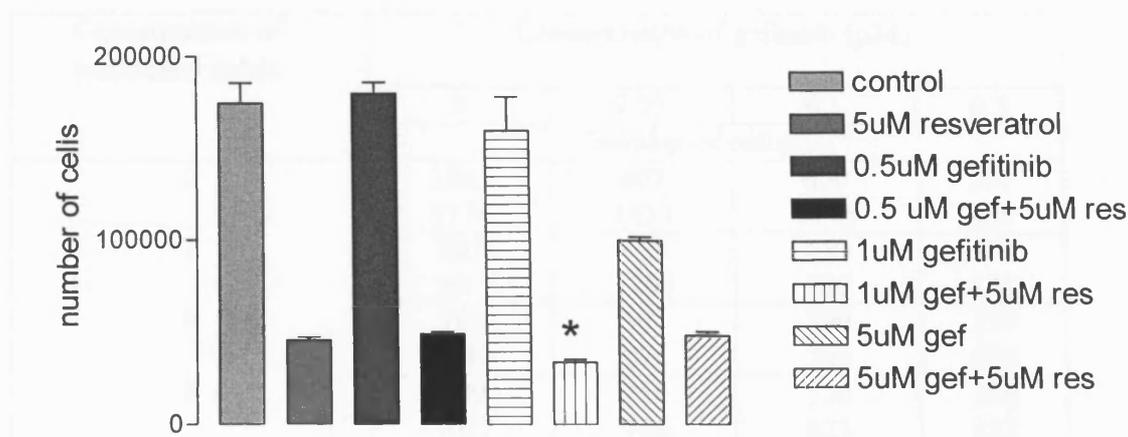


Figure 19: The effect of combined resveratrol (5 μ M) and various concentrations of gefitinib on the proliferation of PC3 cells. * represents a significant difference in number between the cells treated by the combination of agents and the cells treated by either 5 μ M resveratrol or the corresponding gefitinib dose (0.5, 1 or 5 μ M) at $p < 0.05$.

Concentration of resveratrol (μ M)	Concentration of gefitinib (μ M)			
	0	0.5	1	5
	Number of cells $\times 10^2$			
0	4963	4722	4799	3263
	5520	4552	4562	4999
2	4805	4289	4176	2454
	4995	4230	4303	2812
4	4180	3922	3293	871
	4333	3431	3298	1980
6	2460	2845	1986	1041
	3327	3060	2571	1358

Table 6: Effect of a combination of gefitinib and resveratrol on LNCaP cell proliferation (the numbers represent number of cells $\times 10^2$, results are in duplicate).

Concentration of resveratrol (μM)	Concentration of gefitinib (μM)			
	0	0.05	0.1	0.5
	Number of cells $\times 10^2$			
0	1445	607	650	507
	1776	1434	1176	809
2	802	465	450	363
	1617	1209	930	648
4	765	307	390	399
	1680	974	703	603
6	589	258	370	308
	1027	786	623	412

Table 7: Effect of a combination of gefitinib and resveratrol on DU145 cell proliferation (the numbers represent number of cells $\times 10^2$, results are in duplicate).

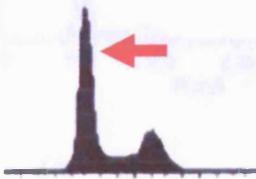
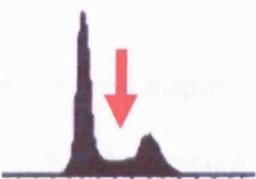
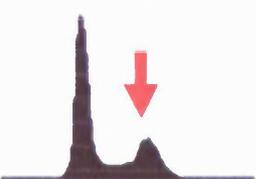
These results were statistically analysed with MINTAB 13 using a general linear model one-way ANOVA with analysis of interaction. These results confirm the findings of the previous experiments by showing that resveratrol and gefitinib independently reduce cell proliferation of both LNCaP and DU145 cells significantly ($p < 0.0001$). However difference in cell number attributable to the interaction of the two agents was not statistically significant ($p = 0.143$ for LNCaP cells, $p = 0.736$ for DU145 cells). In other words, the behaviour of one agent was not influenced by the presence of the other agent, which means that the observed increased inhibitory effect of using the two agents together is purely additive rather than synergistic.

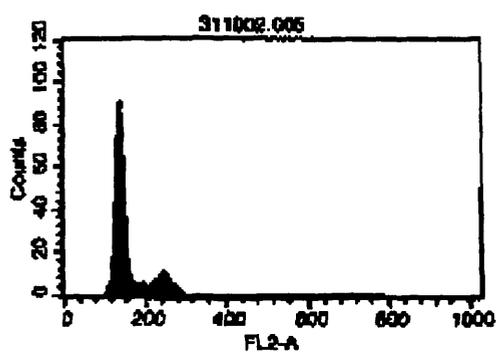
3.3 The effect of resveratrol and gefitinib on cell cycle kinetics of prostate cancer cells

The flow cytometry traces take the form of frequency histograms, showing the number of cells over a range of fluorescent intensity (FL2-A on the x-axis of the graphs).

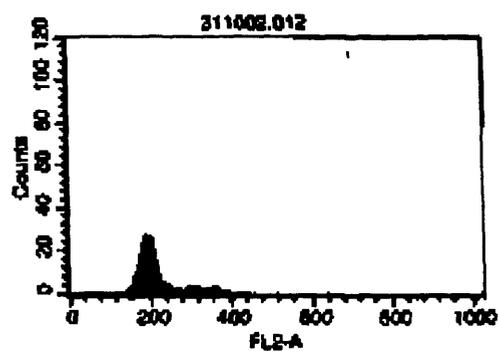
Fluorescence is directly related to cellular content of propidium iodide, which in turn, binds directly to DNA. Thus, replicating cells, which contain more DNA, will appear more fluorescent to the flow cytometer. From this quantitative estimation of DNA content, it is possible to determine the proportion of cells in each phase of the cell cycle. This concept is illustrated in table 8. Figures 20 and 21 illustrate the traces obtained following the treatment of LNCaP cells with resveratrol and gefitinib respectively, and Figures 22 and 23 represent DU145 cells treated with the same compounds. Figure 24 shows the effect of resveratrol on PC3 cells. These flow cytometry histograms were quantified using Modfit software and the mean of triplicate experiments are presented in figures 25-29.

Table 8: Flow cytometric representation of cell cycle

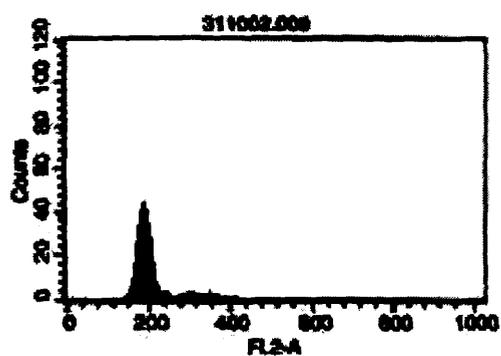
Phase of cell cycle	DNA content	Representation on graph
G1/ G0 (Resting)	2n	
S (synthetic)	Between 2n and 4n	
G2/ M (mitotic)	4n	



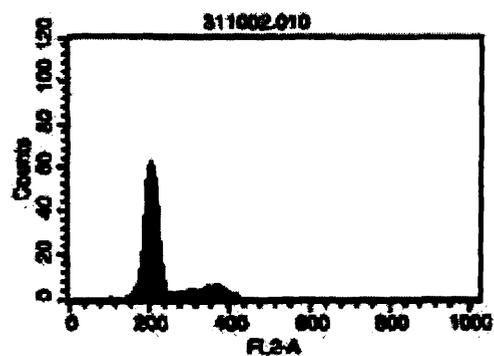
(a)



(b)



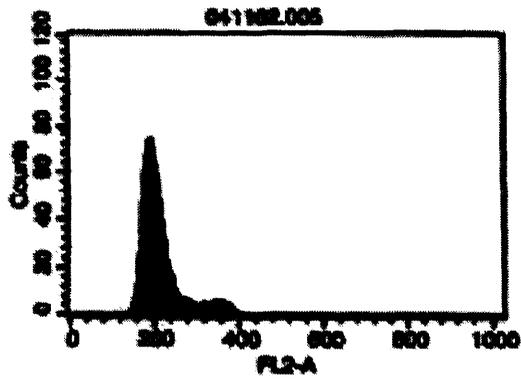
(c)



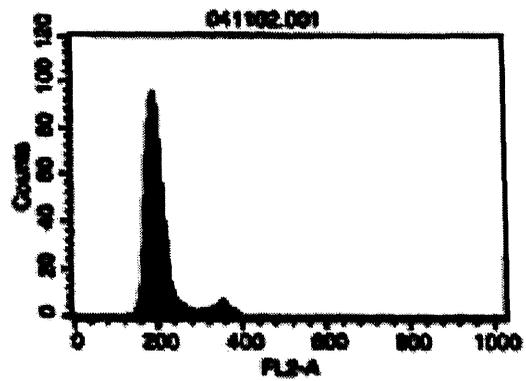
(d)

Figure 20: The effect of 72hr resveratrol treatment on cell cycle distribution of LNCaP cells.

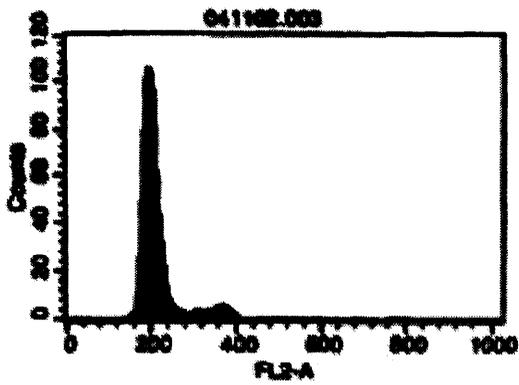
(a) control (b) 1 μ M resveratrol (c) 25 μ M resveratrol (d) 50 μ M resveratrol.



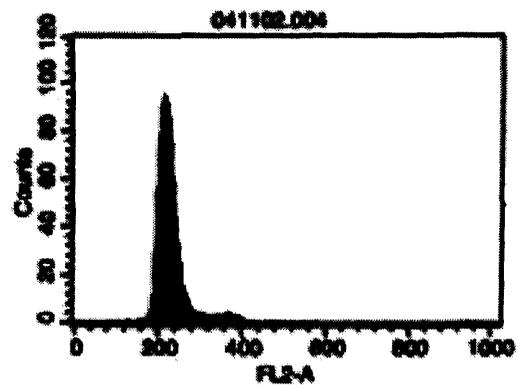
(a)



(b)



(c)



(d)

Figure 21: The effect of 72hr gefitinib treatment on cell cycle distribution of LNCaP cells. (a) control (b) 0.5 μ M gefitinib (c) 1 μ M gefitinib (d) 5 μ M gefitinib

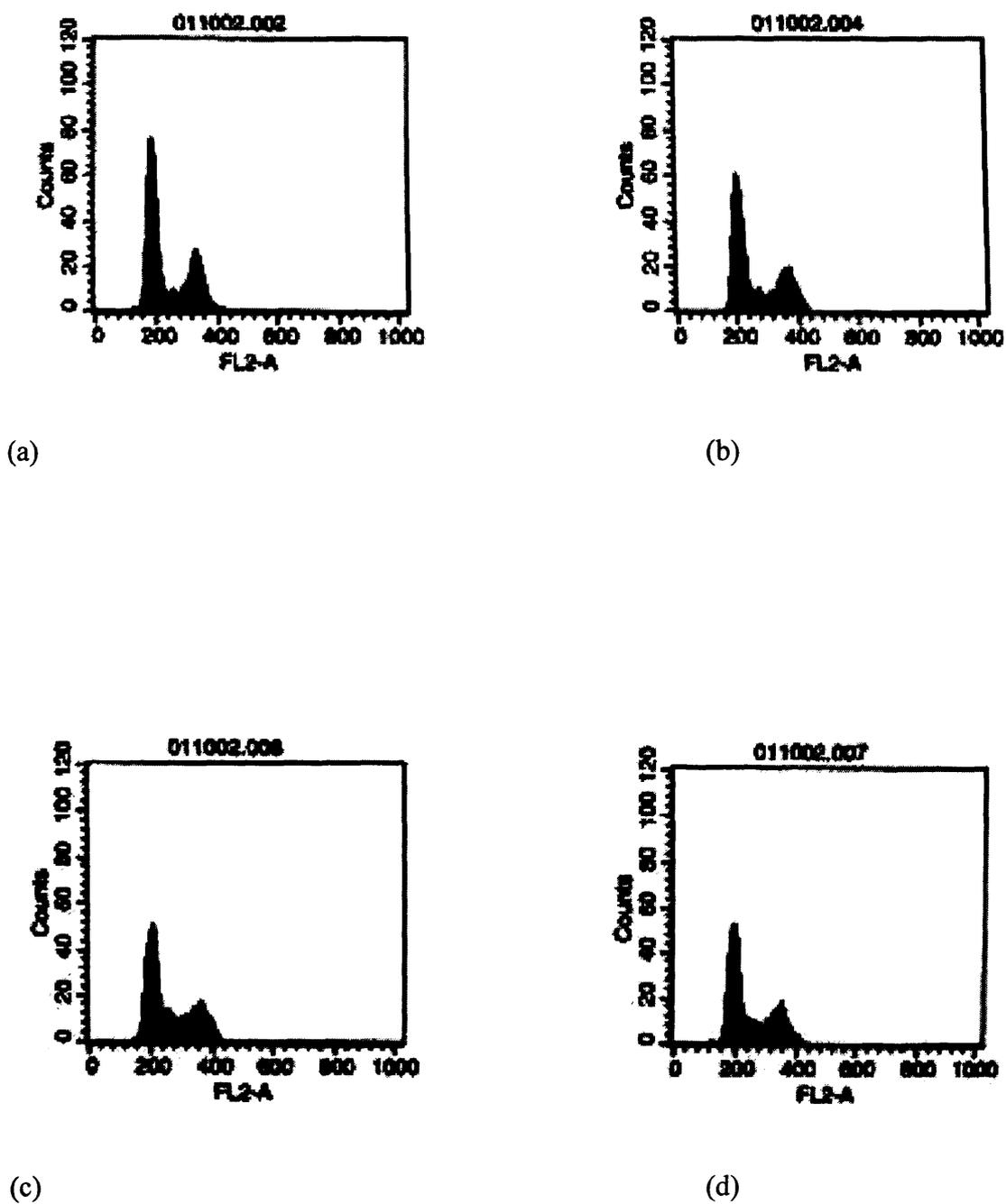
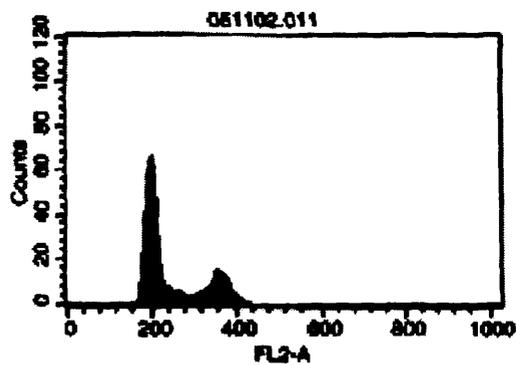
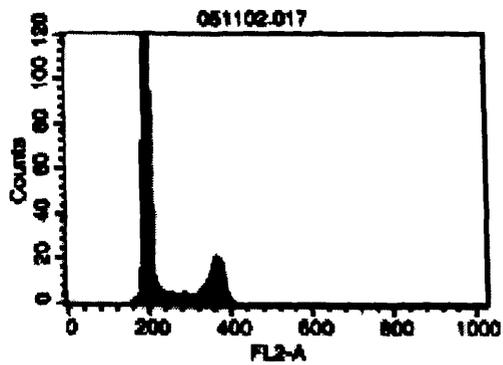


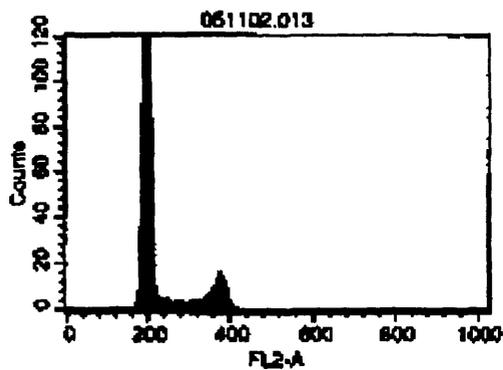
Figure 22: The effect of 72hr resveratrol treatment on cell cycle distribution of DU145 cells. (a) control (b) 1 μ M resveratrol (c) 25 μ M resveratrol (d) 50 μ M resveratrol.



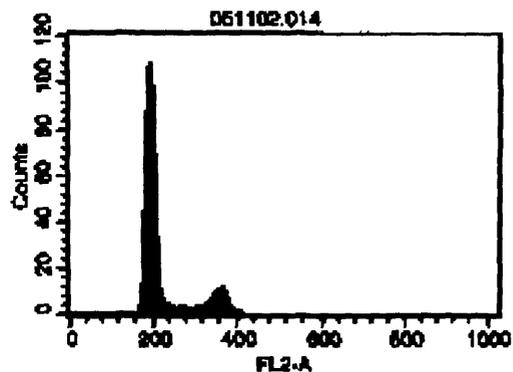
(a)



(b)



(c)



(d)

Figure 23: The effect of 72hr gefitinib treatment on cell cycle distribution of DU145 cells. (a) control (b) 0.01 μ M gefitinib (c) 0.05 μ M gefitinib (d) 0.1 μ M gefitinib

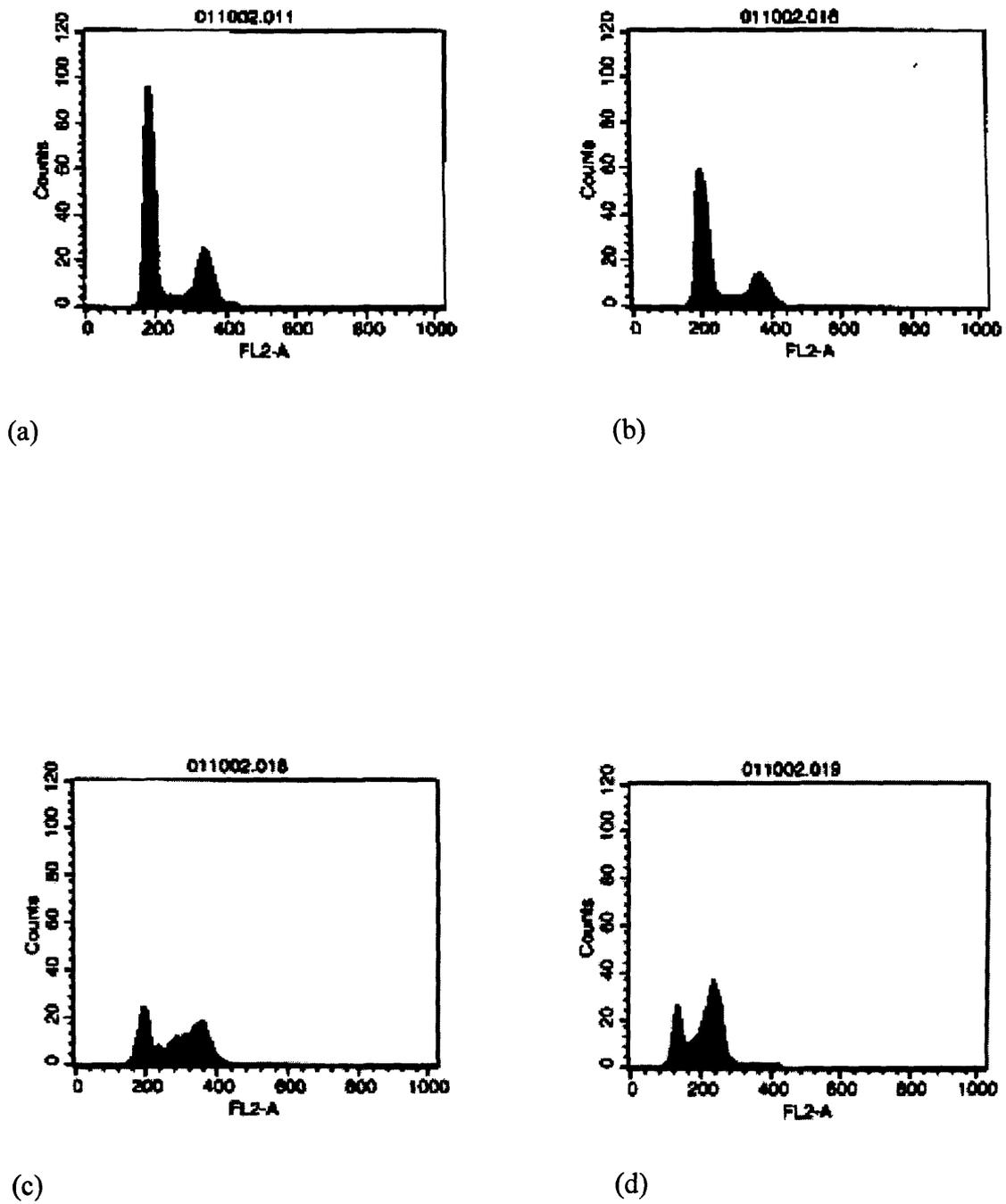


Figure 24: The effect of 72hr resveratrol treatment on cell cycle distribution of PC3 cells. (a) control (b) 10 μ M resveratrol (c) 25 μ M resveratrol (d) 50 μ M resveratrol.

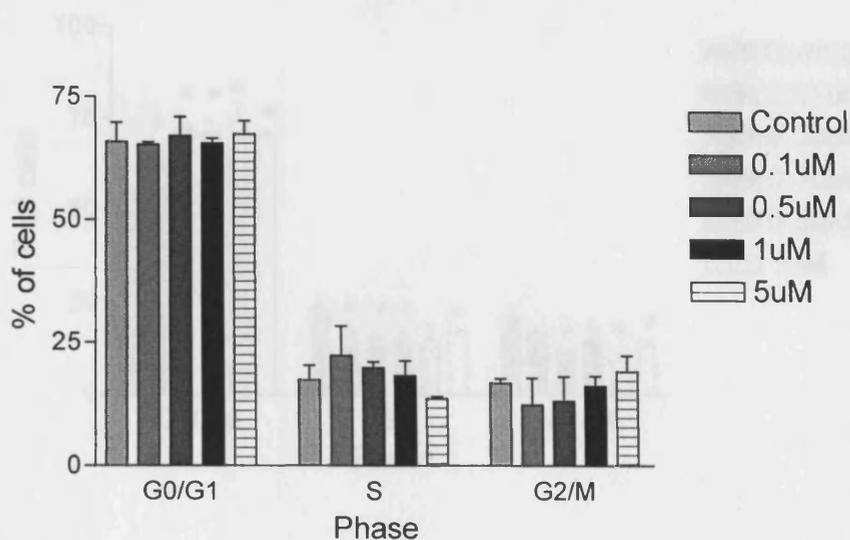


Figure 25: The effect of 72hrs gefitinib treatment on the cell cycle distribution of LNCaP cells. Figures represent the mean and SD of three values.

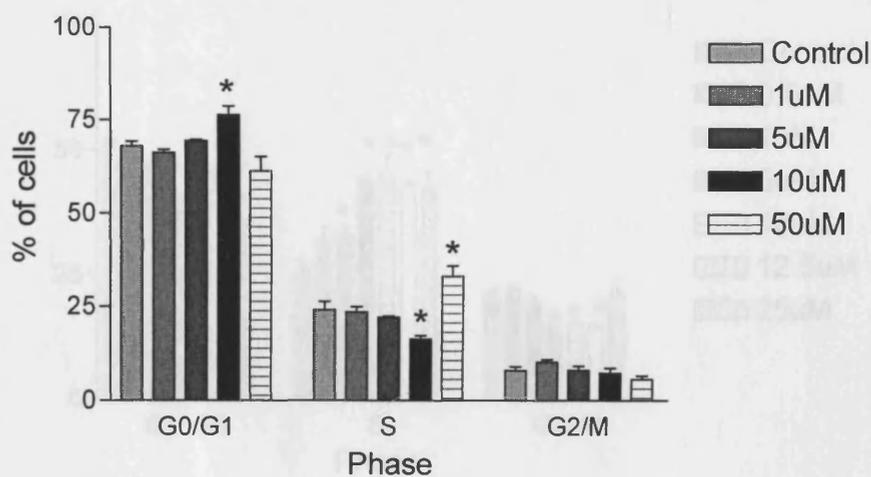


Figure 26: The effect of 72hrs resveratrol treatment on the cell cycle distribution of LNCaP cells. Figures represent the mean and SD of three values. * represent a significant difference between the proportion of cells in a particular phase of the cell cycle between the treatment group and control, $p < 0.05$.

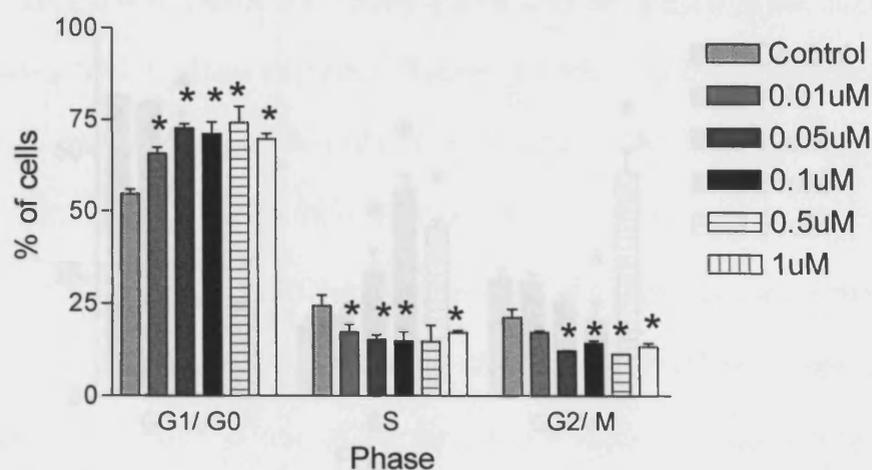


Figure 27: The effect of 72hrs gefitinib treatment on the cell cycle distribution of DU145 cells. Figures represent the mean and SD of three values. * represent a significant difference between the proportion of cells in a particular phase of the cell cycle between the treatment group and control, $p < 0.05$.

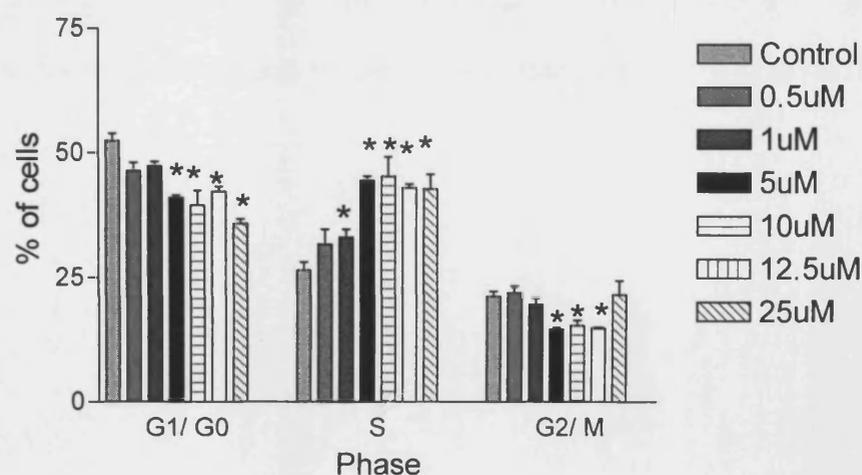


Figure 28: The effect of 72hrs resveratrol treatment on the cell cycle distribution of DU145 cells. Figures represent the mean and SD of three values. * represent a significant difference between the proportion of cells in a particular phase of the cell cycle between the treatment group and control, $p < 0.05$.

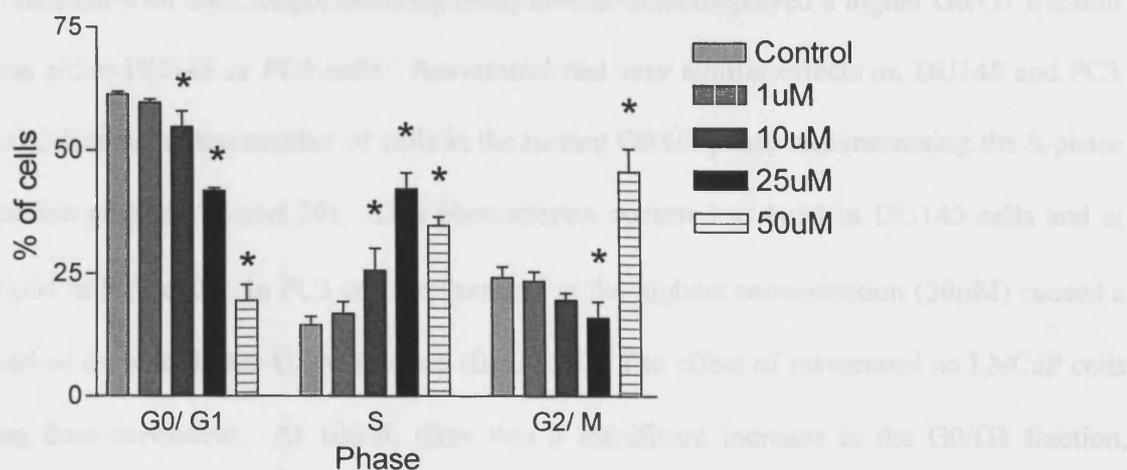


Figure 29: The effect of 72hrs resveratrol treatment on the cell cycle distribution of PC3 cells. Figures represent the mean and SD of three values. * represent a significant difference between the proportion of cells in a particular phase of the cell cycle between the treatment group and control, $p < 0.05$.

Consistent with their longer doubling time, LNCaP cells displayed a higher G0/G1 fraction than either DU145 or PC3 cells. Resveratrol had very similar effects on DU145 and PC3 cells, decreasing the number of cells in the resting G0/G1 phase and increasing the S-phase fraction (figures 28 and 29). This phenomenon occurred at 1 μ M in DU145 cells and at 10 μ M in PC3 cells. In PC3 cells, resveratrol at the highest concentration (50 μ M) caused a marked increase in the G2/M fraction (figure 28). The effect of resveratrol on LNCaP cells was dose-dependent. At 10 μ M, there was a significant increase in the G0/G1 fraction, representing a cell cycle arrest at this stage. However, at 50 μ M, there was a significant S-phase accumulation (figure 26).

Gefitinib caused a G1/G0 arrest in DU145 cells, even at the lowest concentration used (0.01 μ M). This arrest was sustained at higher concentrations up to 1 μ M (figure 27). There was no appreciable effect of gefitinib on cell cycle progression in LNCaP cells (figure 25).

3.4 The determination of total EGFR and EGFRvIII expression

Figures 30 and 31 are Western blots of total (phosphorylated and unphosphorylated) EGFR and EGFRvIII expression of the three prostate cancer cell lines under basal growth conditions in 10%FCS. The U87MG Δ EGFRvIII cell line was used as a positive control for EGFRvIII.

3.5 Effect of gefitinib on total EGFR and phospho-EGFR levels

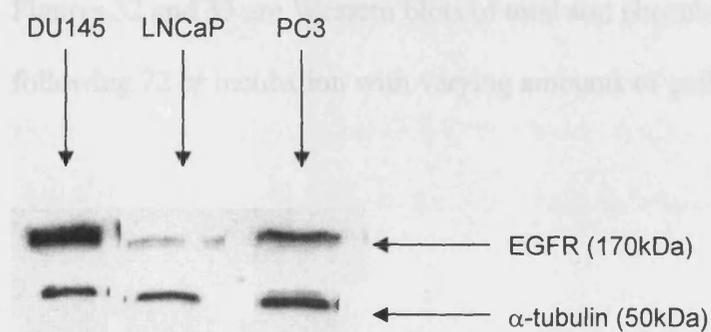


Figure 30 EGFR expression in prostate cancer cell lines

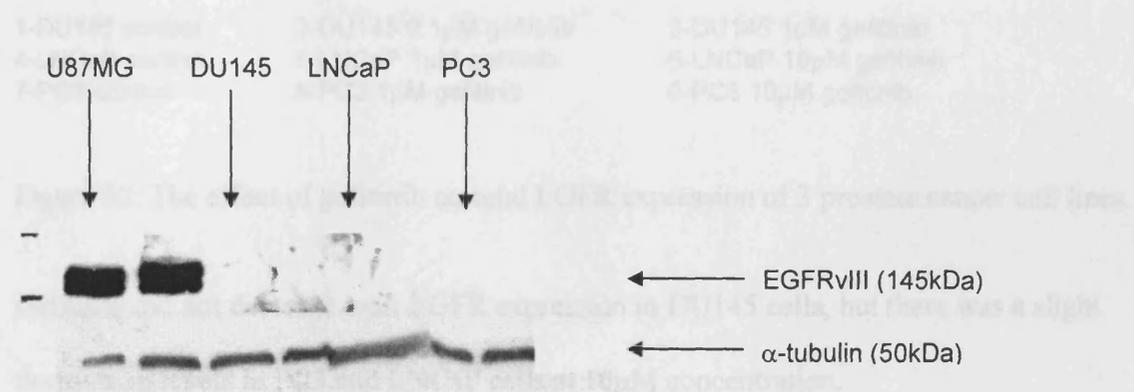


Figure 31 EGFRvIII expression in prostate cancer cell lines (two samples for each cell line)

It can be seen that DU145 cells expressed the highest quantities of total EGFR, followed by PC3 cells, whilst LNCaP cells expressed very low levels of the receptor. This finding may

explain why DU145 cell proliferation was inhibited by gefitinib at a much lower concentration than the other two cell lines. There was no detectable expression of EGFRvIII in the 3 prostate cancer cell lines, but the control cells expressed very high levels as would be expected.

3.5 Effect of gefitinib on total EGFR and phospho-EGFR levels

Figures 32 and 33 are Western blots of total and phospho-EGFR expression respectively following 72 hr incubation with varying amounts of gefitinib.

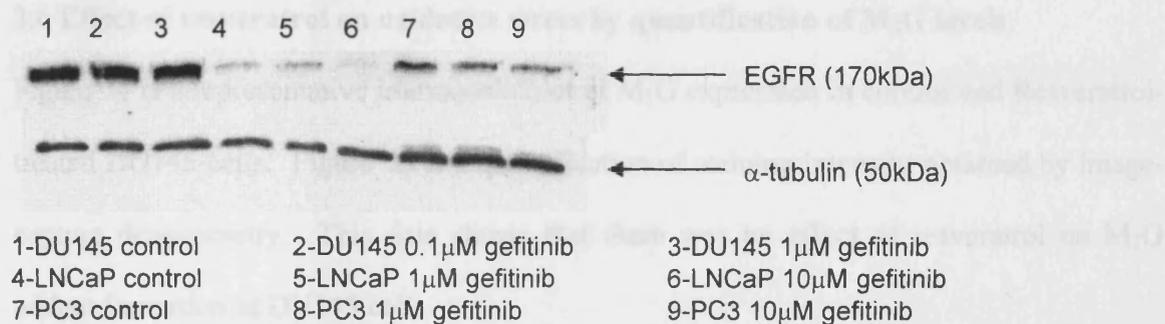


Figure 32: The effect of gefitinib on total EGFR expression of 3 prostate cancer cell lines.

Gefitinib did not decrease total EGFR expression in DU145 cells, but there was a slight decrease in levels in PC3 and LNCaP cells at 10 μM concentration.

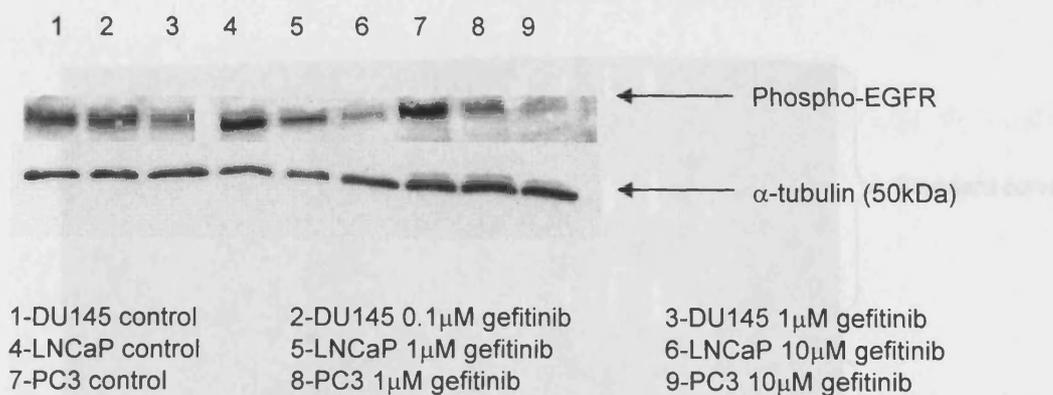


Figure 33: Effect of gefitinib on phospho-EGFR expression in 3 prostate cancer cell lines.

Gefitinib decreased phospho-EGFR expression in all 3 cell lines. Interestingly, levels of phospho EGFR were similar in the 3 cell lines although total EGFR levels vary markedly.

3.6 Effect of resveratrol on oxidative stress by quantification of M₁G levels

Figure 34 is a representative immunoslotblot of M₁G expression in control and Resveratrol-treated DU145 cells. Figure 35 is a quantification of staining intensity obtained by image-capture densitometry. This data shows that there was no effect of resveratrol on M₁G adduct formation in DU145 cells.

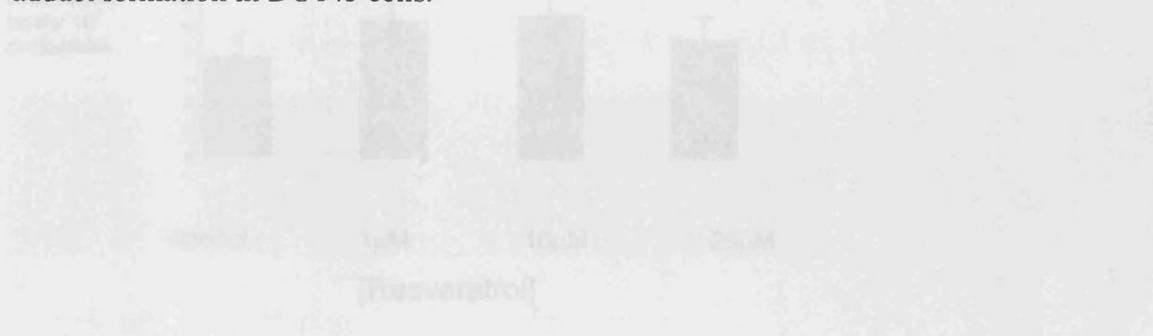


Figure 35: The effect of resveratrol on DNA M₁G adduct levels. Error bars represent SD.

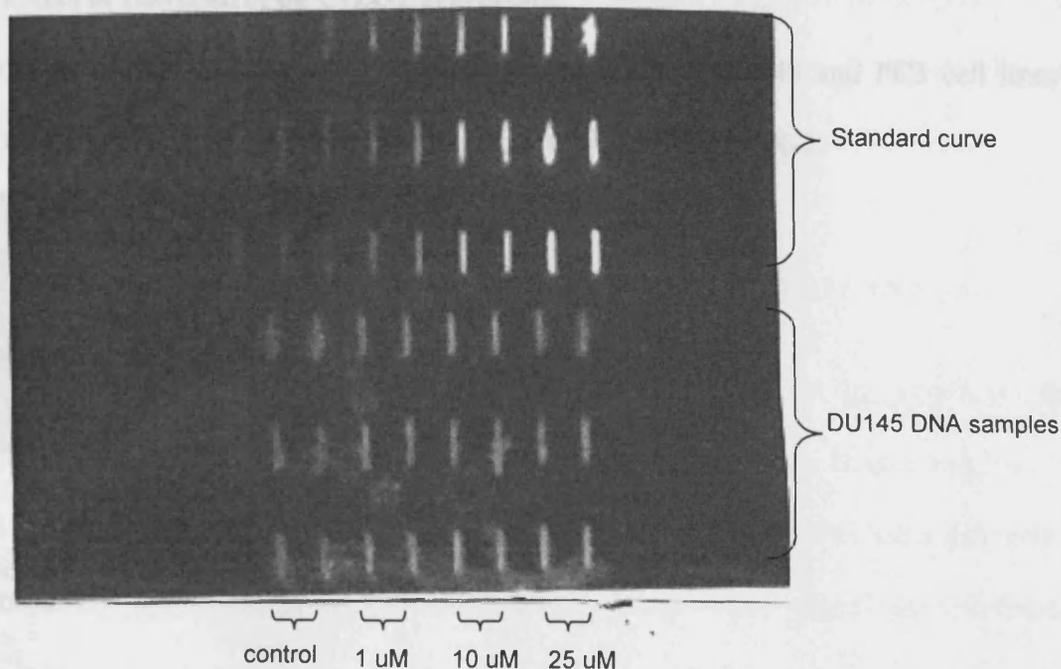


Figure 34: The basal expression of COX-2 in LNCaP, PC3 and DU145 cell lines (Western blot representative of 3 experiments).

Figure 34: An immunoslotblot showing the effect of Resveratrol on M₁G adduct levels in DU145 cells

Following stimulation with 100nM IGF1, it was possible to induce COX-2 expression in PC3 cells, and possibly in LNCaP cells, but not in DU145 cells (Figure 37).

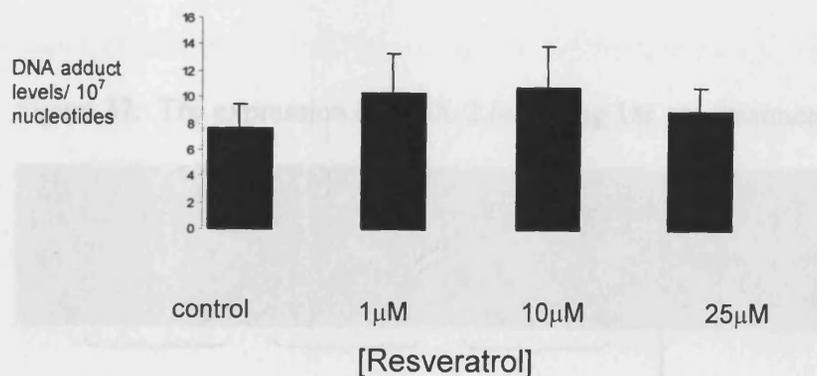


Figure 35: The effect of resveratrol on DNA M₁G adduct levels. Error bars represent SD.

3.7 Effect of resveratrol on COX-2 expression

The Western blot of basal COX-2 expression in LNCaP, DU145 and PC3 cell lines is shown in Figure 36. These cells do not constitutively express COX-2.

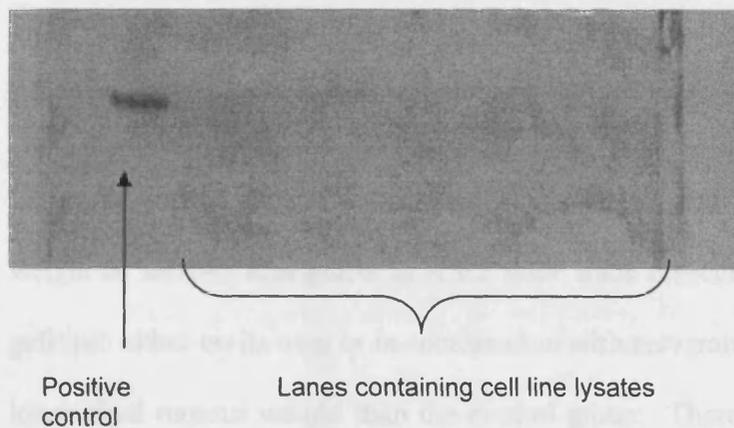
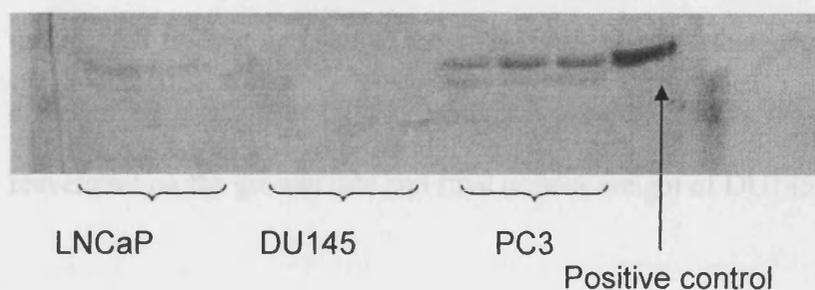


Figure 36: The basal expression of COX-2 in LNCaP, PC3 and DU145 cell lines (Western blot representative of 3 experiments).

Following stimulation with 10nM TPA, it was possible to induce COX-2 expression in PC3 cells, and possibly in LNCaP cells, but not in DU145 cells (Figure 37).

Figure 37: The expression of COX-2 following 1hr pre-treatment with 10nm TPA



It was felt that the level of expression of COX-2 even following TPA stimulation was too weak to assess whether resveratrol prevented it. Thus, no evidence to support the anti-COX-2 activity of resveratrol in prostate cancer cell lines was found.

3.8 Effect of gefitinib and resveratrol on DU145 xenograft growth in nude mice

Figure 38 and 39 illustrate the effect of resveratrol and gefitinib on the growth and final weight of DU145 xenografts in ICRF nude mice respectively. The mice which received gefitinib either on its own or in combination with resveratrol in their diet had a significantly lower final tumour weight than the control group. There was no significant difference in final tumour weight between the group which received gefitinib on its own and that which received a mixture of the two agents.

The group which received resveratrol had a larger mean final tumour weight, but this did not reach statistical significance at $p < 0.05$ using one-way ANOVA analysis. Figure 38 shows that the mean final tumour volume of the resveratrol-fed mice was greater, and that these mice tended to develop tumours faster than the other three groups. This was an unexpected finding and led to the establishment of further experiments to investigate the effect of resveratrol on DU145 xenograft growth. Figure 40 and 41 show the effect of resveratrol on the growth rate and final tumour weight of DU145 in MF1 nude mice.

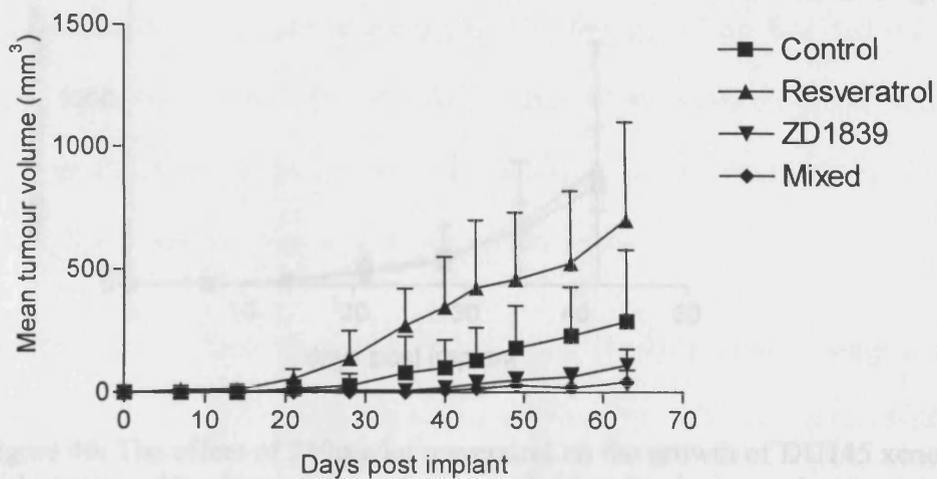


Figure 38: The effect of resveratrol, gefitinib or a combination of both agents on the growth of DU145 xenografts in ICRF nude mice. Numbers represent mean of 6 animals in each group with error bars representing SD. The dose of resveratrol was 240mg/kg, the dose of gefitinib was 100mg/kg for 35 days followed by 50mg/kg for the rest of the study period.

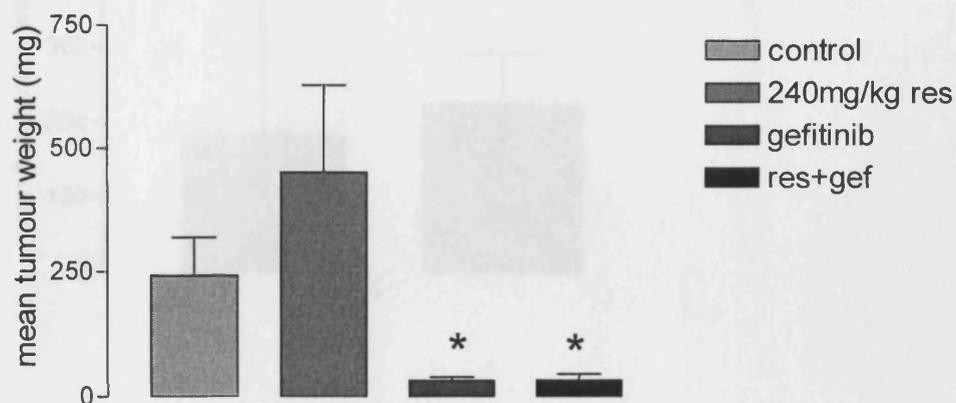


Figure 39: Effect of resveratrol, gefitinib or a combination of both agents on the mean final weight of DU145 xenografts in ICRF nude mice. Numbers represent mean of 12 animals in each group except the resveratrol only group (10 animals) with error bars representing SD. * represent significant difference between tumour weight of treated group and control $p < 0.05$.

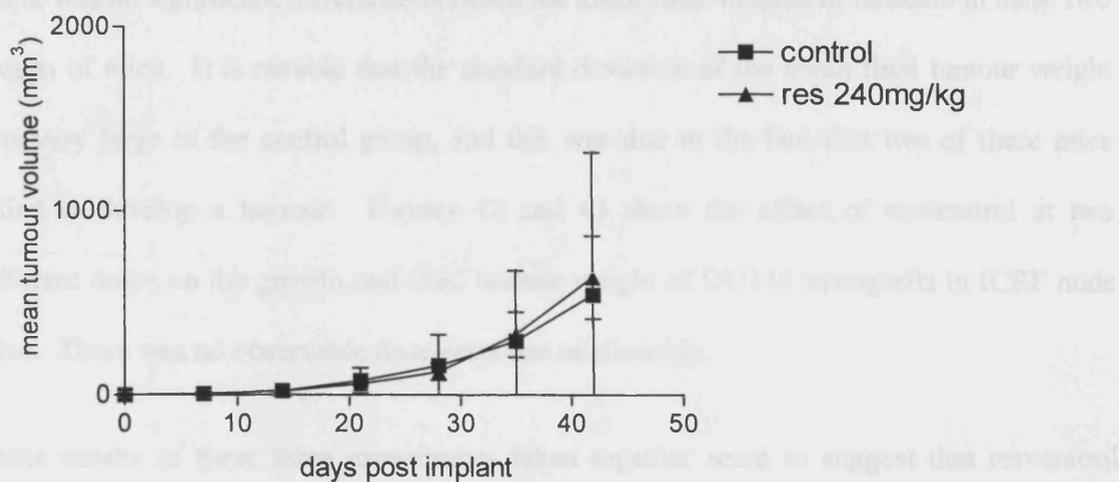


Figure 40: The effect of 240mg/kg resveratrol on the growth of DU145 xenografts in MF-1 nude mice. Numbers represent mean of 12 animals in each group with error bars representing SD.

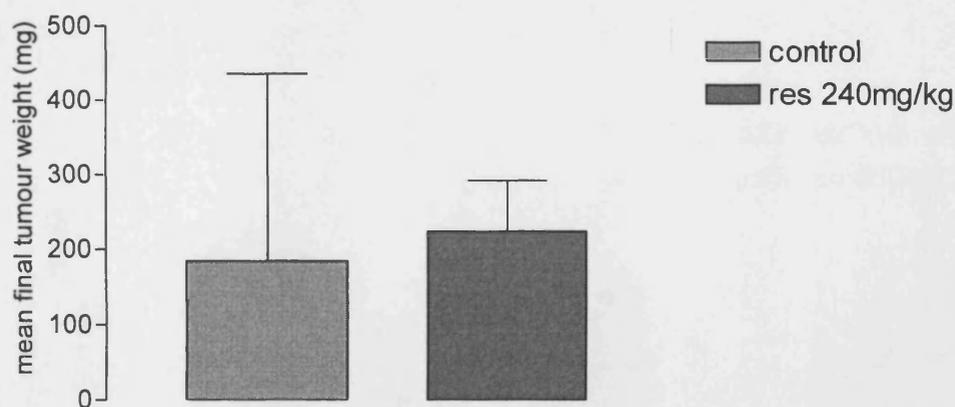


Figure 41: The effect of 240mg/kg resveratrol on mean final weight of DU145 xenografts in MF-1 nude mice. Numbers represent mean of 12 animals in each group with error bars representing SD.

There was no significant difference between the mean final weights of tumours in these two groups of mice. It is notable that the standard deviation of the mean final tumour weight was very large in the control group, and this was due to the fact that two of these mice failed to develop a tumour. Figures 42 and 43 show the effect of resveratrol at two different doses on the growth and final tumour weight of DU145 xenografts in ICRF nude mice. There was no observable dose-response relationship.

These results of these three experiments taken together seem to suggest that resveratrol does not have a significant effect on the growth of DU145 xenografts in either ICRF mice or MF-1 mice.

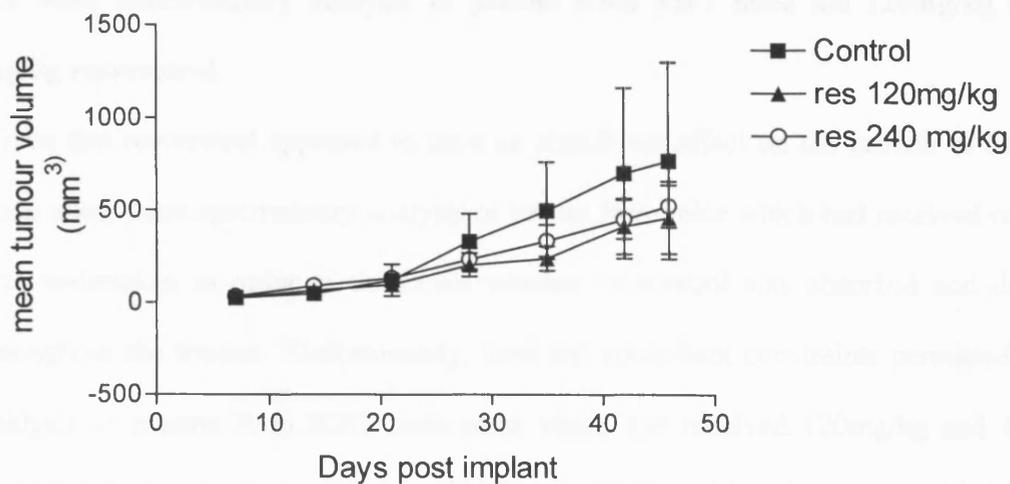


Figure 42: The effect of 120 mg/kg and 480mg/kg resveratrol on the growth of DU145 xenografts in ICRFnude mice. Numbers represent mean of 12 animals in each group with error bars representing SD.

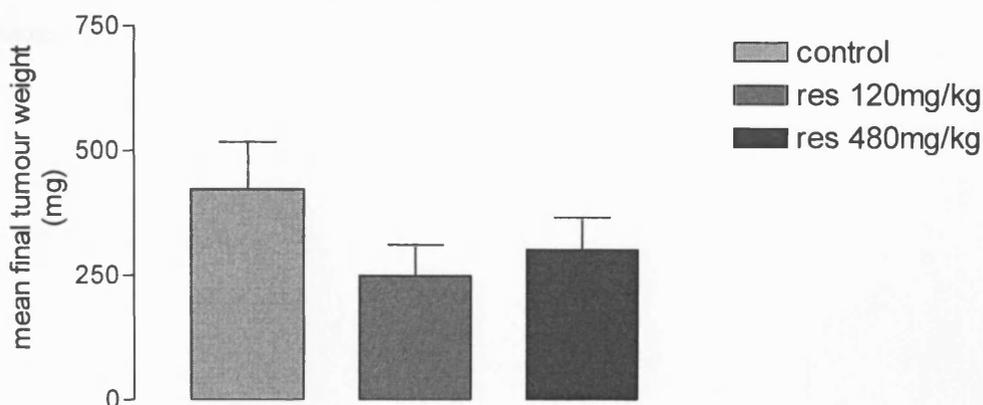


Figure 43: The effect of 240mg/kg resveratrol on mean final weight of DU145 xenografts in MF-1 nude mice. Numbers represent mean of 12 animals in each group with error bars representing SD. There was no significant difference between these values at $p < 0.05$.

3.9 Mass spectrometry analysis of plasma from MF1 mice fed 120mg/kg and 480 mg/kg resveratrol

Given that resveratrol appeared to have no significant effect on the growth of tumours in nude mice, mass spectrometry analysis of tissues from mice which had received resveratrol was undertaken in order to determine whether resveratrol was absorbed and distributed throughout the tissues. Unfortunately, time and equipment constraints permitted only the analysis of plasma from ICRF nude mice which had received 120mg/kg and 480mg/kg resveratrol. The results are presented in figures 44-46. Although resveratrol itself was not seen in the plasma of the treated groups, its glucuronide conjugate was present in both groups and its sulfite conjugates was seen in the plasma of the 480mg/kg group. Unfortunately, equipment constraints did not permit the analysis of tumour tissue, so it is not known whether the resveratrol metabolites seen in the plasma were also present in this tissue.

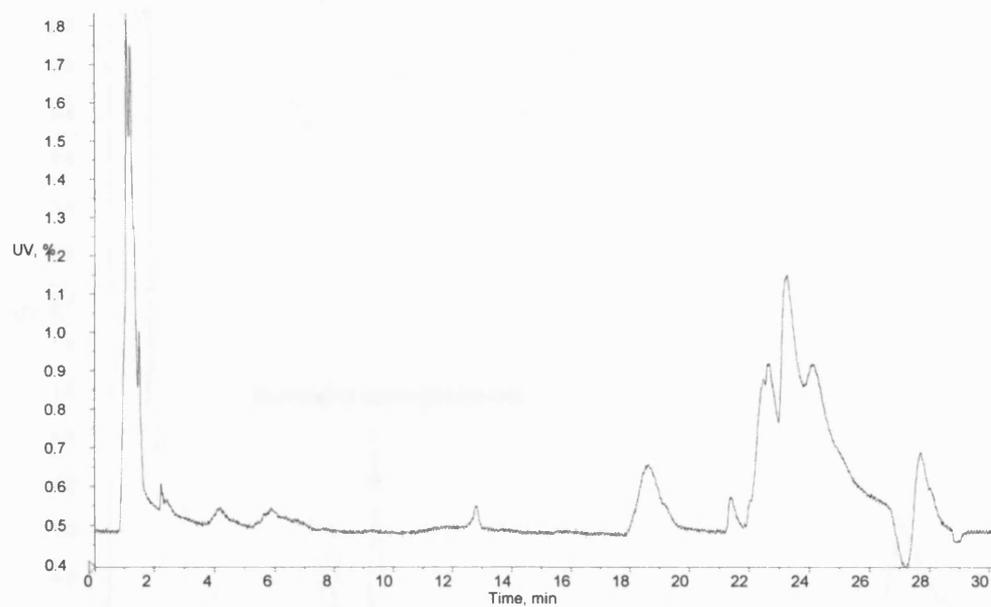


Figure 44: HPLC analysis of plasma from an MF-1 mouse receiving control diet. No peaks were identified on mass spectroscopy.

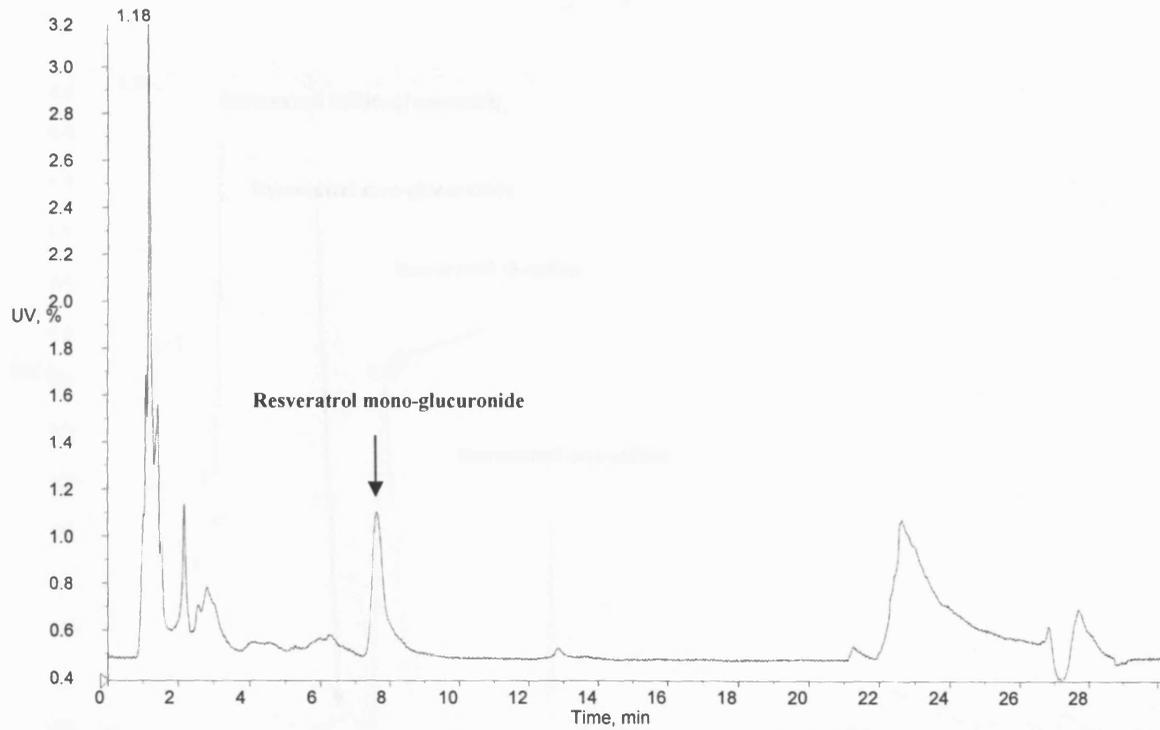


Figure 45: HPLC analysis of plasma from a mouse receiving 120mg/kg of dietary resveratrol. The only peak identified on mass spectroscopy has been labelled.

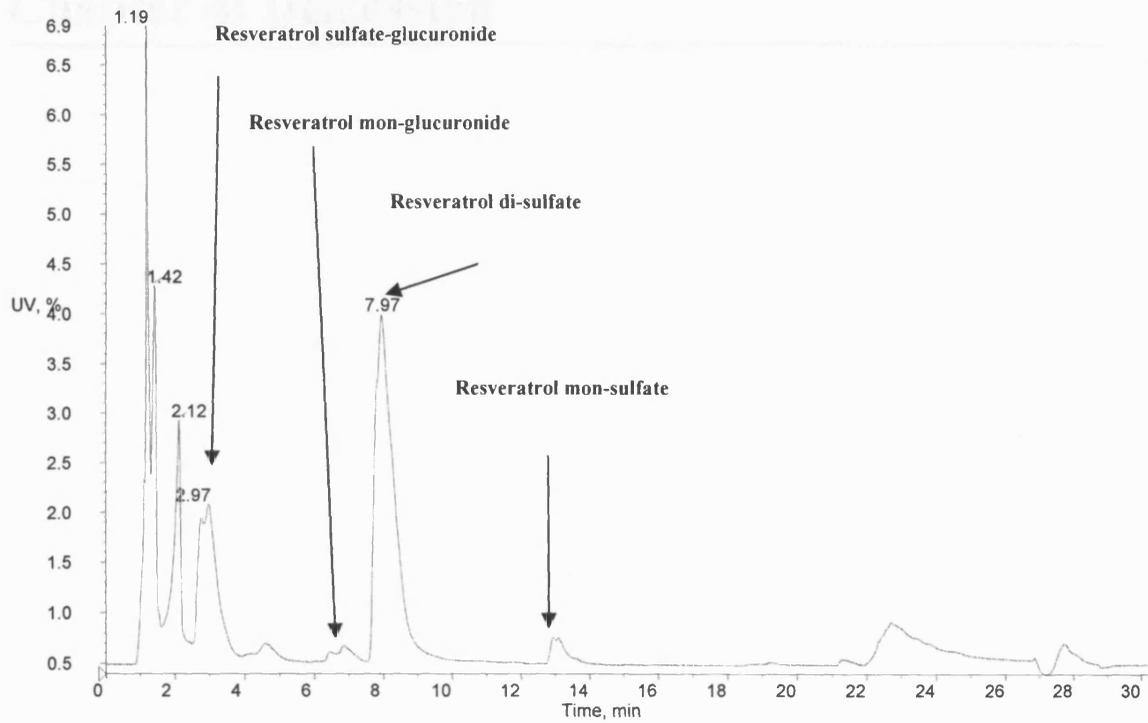


Figure 46: HPLC analysis of plasma from a mouse receiving 480mg/kg of dietary resveratrol, showing peaks which were confirmed on mass spectroscopy.

Chapter 4: Discussion

The work described in this thesis was designed to investigate whether resveratrol and gefitinib would be suitable agents for prostate cancer chemoprevention and if so, to determine a mechanistic rationale for their use. Specifically, the following hypotheses have been addressed:

1. Resveratrol and gefitinib inhibit the growth of prostate cancer cell lines *in vitro*.
2. A combination of gefitinib and resveratrol result in increased growth inhibition.
3. The mechanisms by which resveratrol acts include cell cycle arrest, down-regulation of COX-2 expression and prevention of oxidative DNA damage.
4. The mechanisms by which gefitinib acts include cell cycle arrest mediated by inhibition of EGFR autophosphorylation.
5. Resveratrol and gefitinib inhibit the growth of prostate cancer xenografts in a nude mouse model.

The following discussion will address each of these hypotheses in turn, whilst relating the findings of the experiments to other work in the area of prostate cancer chemoprevention.

4.1 The effect of resveratrol and gefitinib on the proliferation of prostate cancer cells *in vitro*

The hormone-dependent cell line LNCaP and hormone-independent cell lines PC3 and DU145 were chosen to reflect the biological diversity of prostate cancer encountered in clinical practice. LNCaP cell line was derived from a lymph node metastasis (Horoszewicz, 1983), and several subclones have subsequently been developed (van Steenbrugge, 1989), some of which are androgen independent. The parent cell line was

used in the investigations described here. DU145 cell line was established from a brain metastasis in 1977 (Stone, 1978), whilst PC3 cells were derived from a vertebral metastasis the following year (Kaighn, 1979). Phenotypic characterisation of these three cell lines has been extensively carried out, and LNCaP cells are found to express AR protein whilst the other two cell lines do not (Mitchell, 2000). It would have been ideal to complement these three cell lines with a cell line representing benign prostatic tissue or HGPIN in order to study the earlier stages of prostatic carcinogenesis during which chemopreventive agents may act. However, there is a paucity of such commercially-available cell lines. PNT1A is a cell line derived from benign prostatic epithelium and immortalised with the *T-SV40* gene (Cussenot, 1991). However it displays a number of highly abnormal features, including polyploidy and failure of expression of AR (Mitchell, 2000), and thus its similarity to normal prostatic epithelium is debatable.

The effect of resveratrol and gefitinib proliferation of LNCaP, PC3 and DU145 cells was investigated as described in 2.3.4. The IC_{50} values showed that resveratrol had a similar antiproliferative effect on all 3 cell lines studied, with an IC_{50} of around $5\mu\text{M}$ (Table 5). This is similar to IC_{50} values reported by other authors using the same cell lines (Hsieh and Wu, 2000),(Mitchell, 1999) and lower than yet other reported studies (Hsieh and Wu, 1999), bearing in mind differences in techniques used to determine IC_{50} values in all these studies. The fact that the efficacy of resveratrol was similar in the three cell lines regardless of androgen receptor status is perhaps an indication that resveratrol acts in an AR-independent manner *in vitro*.

The effect of resveratrol on the proliferation of a number of human cancer cell lines has been investigated intensively. It has been found to inhibit the growth of cell lines derived from colon cancer (Delmas, 2002), pancreatic cancer (Ding and Adrian, 2002), epidermoid cancer (Ahmad, 2001), hepatic cancer (Delmas, 2000) and leukaemia (Gao, 2002) amongst others. The IC_{50} values of resveratrol in all these cell lines vary widely, but our values of approximately $5\mu\text{M}$ are towards the lower end of the spectrum.

The hormone insensitive DU145 cell line expressed the highest levels of total EGFR followed by PC3 and then LNCaP cells. All three cell lines expressed similar levels of phospho-EGFR, and EGFR autophosphorylation was markedly reduced by gefitinib. Contrary to previous findings (Olapade-Olaopa, 2000), no EGFRvIII expression was detected in any of the three cell lines. The effect of gefitinib on basal cell growth did not correlate with level of total EGFR expression in the cell lines. As might be expected, DU145 cells were the most sensitive to gefitinib, with an IC_{50} of $0.14\mu\text{M}$. However, LNCaP cells which express less total EGFR than the PC3 cells were more sensitive to gefitinib, with a significantly lower IC_{50} . The level of p-EGFR was similar in all three cell lines, and it was shown that gefitinib successfully reduced EGFR phosphorylation in all 3 cell lines, demonstrating that gefitinib effectively abrogates EGFR-mediated signalling in all three cell lines. Perhaps the insensitivity of PC3 cells to gefitinib is a reflection of the relative unimportance of the EGFR pathway in PC3 cells.

4.2 The effect of a combination of gefitinib and resveratrol on prostate cancer cell proliferation *in vitro*

The sensitivity of LNCaP and DU145 cells to gefitinib and resveratrol when employed alone lead to the supposition that there may be a synergistic reaction when a combination of the two agents was used. Little combined effect was expected on PC3 cells, which were relatively insensitive to gefitinib. Analysis of the initial results of experiments investigating the combined effect of gefitinib and resveratrol on the proliferation of LNCaP and DU145 did indeed seem to reveal an additive or synergistic effect. The experiments were repeated in order to determine the nature of this interaction, and statistical analysis clearly revealed the combined effect of the two agents to be additive rather than synergistic. The results of the combination experiments would seem to suggest that there is little mechanistic interaction between EGFR-mediated signalling pathways abrogated by gefitinib treatment and any pathways which are affected by resveratrol. However, an additive effect of using two agents together is still noteworthy, as if it could be translated to the clinical environment, it would result in greater disease response than using either agent alone.

4.3 The effect of gefitinib and resveratrol on cell cycle progression in prostate cancer cells

Cell cycle modulation has been suggested as a possible mechanism of action of both resveratrol and gefitinib (Sgambato, 2001; Yano, 2003). In our experiments, the effects of resveratrol on the cell cycle varied between cell lines and were dependent on the concentration of resveratrol used. The most striking effect of resveratrol on DU145 cells was an accumulation of cells in S-phase. This effect was also seen in PC3 cells, although at

the highest concentration of resveratrol, there was a G2/M phase arrest. The effect on LNCaP cells was entirely different; at low concentrations, resveratrol caused a G1/G0 arrest, and at higher concentrations a G2/M arrest. Variable effects of resveratrol on the cell cycle *in vitro* have been documented by a number of other investigators using different concentrations of resveratrol. These are summarised in Table 9.

Gefitinib caused a marked G1/G0 arrest in DU145 cells, but had little effect on cell cycle progression in LNCaP cells. Few other studies have investigated the effect of gefitinib on cell cycle progression *in vitro*. G1/G0 arrest was observed in CAL33 head and neck cancer cells 24 hours following treatment with gefitinib (Magne, 2003). The mechanism by which this arrest is mediated is thought to be via activation of the inhibitory proteins p21 and p27, both being negative regulators of the cell cycle.

Investigators	Cell line	[Resveratrol]	Apoptosis	Cell cycle distribution	p21	p27	Cyclin D	Cyclin E	Cyclin B	Cdk4	
(Kuwajerwala, 2002)	LNCaP	5-30 μ M	N/A	\uparrow S \downarrow G2/M	\downarrow		\downarrow	N/A	\uparrow	N/A	N/A
(Volter, 2001)	Caco-2 HCT-16	12.5-200 μ M	N/A	\uparrow S (both)	N/A		\downarrow	\uparrow	N/A	\downarrow cdk-4	
(Pozo-Guisado, 2002)	Mcf-7	10-200 μ M	+++	\uparrow S (50-150 μ M)	\uparrow		-	\uparrow	\uparrow	\uparrow	\downarrow cdk4 (both)
	MDA-231		None	No effect	-	-	\downarrow	\downarrow	\downarrow		
(Joe, 2002)	Seg1, SW480, MCF7, HL60	300 μ M	+++ (all)	\uparrow S	N/A		N/A	\downarrow	N/A	\downarrow	N/A
(Park, 2001)	U937	0-120 μ M	N/A	30-60 μ M - \uparrow S 90 μ M+ No effect	N/A		\downarrow	\uparrow	\uparrow	N/A	N/A
(Hsieh and Wu, 1999)	LNCaP	0.25-25 μ M	+	No effect	N/A		N/A	N/A	N/A	N/A	N/A
	DU145, PC3 and JCA1		No effect	\uparrow S							
(Hsieh, 2002)	Mitogenically-stimulated lymphocytes	0-100 μ m	\downarrow	\uparrow G1/G0, \downarrow S	N/A		N/A	\downarrow (50 μ M)	$\downarrow\downarrow\downarrow$	N/A	N/A
(Ahmad, 2001)	A431	1-50 μ M	+++	\uparrow G0/G1	\uparrow		N/A	\downarrow	\downarrow	N/A	N/A

Table 9: A summary of investigations into the effect of resveratrol on cell cycle kinetics

4.4 The effect of resveratrol on COX-2 expression and oxidative DNA damage

There is an increasing amount of evidence linking over-expression of COX-2 with a number of human malignancies, including prostate cancer, as discussed in section 1.3.3.3. However, in the experiments described here, there was no basal expression of COX-2 in any of the three cell lines. Following treatment with TPA, it was possible to demonstrate faint COX-2 expression in PC3 cells, but not convincingly in the other two cell lines. These results are in agreement with another study (Zha, 2001), in which basal COX-2 expression was not detected in PC-3, DU15 and LNCaP cells. Other investigators have, however, found immunohistochemical evidence of COX-2 overexpression in prostate cancer (Gupta, 2000; Kirschenbaum, 2000; Tanji, 2000; Uotila, 2001; Fujita, 2002). While the role of COX-2 in established prostate cancer remains controversial, there is stronger evidence for its role as an inflammatory mediator in the earlier stages of prostatic carcinogenesis. Chronic inflammation is associated with carcinogenesis in a number of cancers including bladder, skin and gastric cancers (Weitzman and Gordon, 1990). Inflammation is a common finding on prostatic biopsy, and focal prostatic atrophy, which is associated with chronic inflammation, is highly proliferative (De Marzo, 1999). These areas of so-called proliferative inflammatory atrophy (PIA) have low apoptotic indices which may be associated with bcl-2 upregulation (De Marzo, 2003), and may ultimately be precursors of HGPIN. COX-2 has been found to be upregulated in areas of PIA (Zha, 2001). Thus, the ultimate role of COX-2 inhibitors in prostate cancer may be in the early stages of prostatic carcinogenesis, preventing PIA development.

Increased oxidative stress is thought to be important to carcinogenesis and has been found to correlate with androgen exposure in prostate cancer cells (Ripple, 1997). One consequence of oxidative stress in cells may be the formation of mutagenic DNA adducts (Zhang, 2002).

Peroxidation of lipids by reactive oxygen species, generates mutagenic carbonyl compounds such as malondialdehyde (MDA) (Chaudhary, 1994), which can form adducts with DNA. These MDA-DNA adducts can be measured, and one such assay measures the levels of malondialdehyde-deoxyguanosine (M₁G), which may represent a useful biomarker of oxidative stress in chemoprevention studies. It has been used previously to demonstrate a reduction in M₁G levels in colonic mucosa following treatment with curcumin, another putative chemopreventive dietary agent (Sharma, 2001). Resveratrol at the highest dose of 25µM was not found to decrease M₁G levels in prostate cancer cells (Figure 20). Further experiments using different measures of oxidative stress are needed to clarify the antioxidant effect of resveratrol in prostate cancer cells. Our experimental evidence does not support the hypothesis that the mechanisms by which resveratrol acts include down-regulation of COX-2 expression and prevention of oxidative DNA damage.

4.5 The effect of resveratrol and gefitinib on the growth of DU145 xenografts in nude mice

The well-established ICRF nude mouse xenograft model was chosen as a model of *in vivo* tumour development. Pilot experiments (data not shown) revealed the rapid establishment and growth of DU145 xenografts. Attempts to grow LNCaP xenografts in these mice were unsuccessful, and thus it was decided to proceed only with DU145 xenografts

Figures 38 and 39 show a reduction in tumour growth rate and final weight in the mice which received gefitinib. There was no difference between the groups which received gefitinib on its own or in combination with resveratrol. Other studies have also demonstrated the efficacy of gefitinib in xenograft models. The activity of gefitinib administered as a single daily dose of

12.5mg/kg or above was shown to significantly inhibit the growth of DU145 xenografts in nude mice (Barton, 2001). Another group of investigators recently reported that gefitinib inhibited the further growth of both androgen-dependent and -independent CWR22 prostate cancer xenografts in nude mice by 54% and 76% respectively (Sirotnak, 2002). In the same study, co-administration of gefitinib improved the therapeutic activity of carboplatin in this nude mouse model. The same group of investigators showed the benefit of combining gefitinib with a number of cytotoxic chemotherapeutic drugs in other solid tumour xenograft models with varying degrees of EGFR expression (Sirotnak, 2000). Our study differed from previous investigations in two ways. Firstly, gefitinib was administered continuously in the diet as opposed to by gavage. Secondly, the administration of gefitinib commenced at the time the tumours were implanted as opposed to after they had become established. These modifications were made in order to expand the nude mouse xenograft model to provide an insight into the chemoprevention of tumour development rather than the treatment of an established solid tumour.

In our experiments, the group of mice which received 240mg/kg resveratrol developed larger tumours than the control group, although the values did not reach statistical significance at $p < 0.05$. Two further experiments failed to reproduce any apparent stimulatory effect of resveratrol on tumorigenesis by using a different strain of nude mice or by using different resveratrol doses. The effect of resveratrol on DU145 xenograft growth and development remains uncertain following these experiments. It has been shown by using ¹⁴C-radiolabelled resveratrol that this compound does indeed penetrate mouse tissues following oral administration, being concentrated in the liver and kidneys (Vitrac, 2003). What remains unknown is whether any resveratrol is able to penetrate into tumour tissue. Mass spectrometry

analysis of plasma from our animals which received both 120mg/kg and 480mg/kg revealed the presence of resveratrol metabolites in the plasma, and attempts are currently ongoing to analyse tumour tissue from these mice using mass spectrometry.

The nude mouse model has limitations as a model of chemoprevention. It does not model the transition from benign to malignant cells, and is perhaps more useful as a model of chemotherapy of established tumours. Other mouse models of prostatic carcinogenesis have been developed, including the TRAMP model that expresses the oncogene SV40 T antigen specifically in the epithelium of the prostate. These mice initially have benign prostatic tissue and then all mice develop malignancy. This model thus serves as a better paradigm of chemoprevention, and especially of early malignant transformation (Foster, 1998).

How do the results of the investigations described in this thesis relate to the clinical objective of combinatorial chemoprevention of prostate cancer? It would be difficult from the results obtained, to comment on the use of either of these compounds in the early phases of carcinogenesis, as the models used in these investigations were derived from cells which were already neoplastic. However, it has certainly been shown within the limitations of our models that gefitinib is very active against DU145 cells *in vitro* and *in vivo*. This cell line also expresses the highest levels of EGFR. Thus, gefitinib may be able to prevent the development of hormone-resistant tumours where aberrant EGFR-mediated signalling is important in progression to androgen independence. This paradigm is somewhat similar to the use of herceptin in HER-2 positive breast cancer and may require profiling of prostatic tumour tissue for EGFR expression.

Although resveratrol showed promising activity *in vitro*, the *in vivo* activity of this compound in our experiments was inconsistent, and further experiments need to be performed prior to recommendation of the use of this compound in the chemoprevention or chemotherapy of prostate cancer. The same is thus true of a combination of gefitinib and resveratrol. However, combinatorial administration of chemopreventive agents is likely to supersede the use of single agents (for example as in the SELECT trial-see section 1.2.3.2), in the same way as chemotherapeutic regimens now commonly employ a plethora of therapeutic combinations. Thus, the future of cancer prevention may well lie in identifying suitable combinations of agents.

4.6 The current status of clinical chemoprevention trials

The publication of the PCPT has sparked a great deal of interest in the field of chemoprevention, and there are a number of NCI-sponsored trials currently recruiting and taking place in the USA. Table 10 summarises these Phase I, II and III trials.

Table 10. Clinical chemoprevention trials in prostate cancer

Agent	Study population	Phase	Endpoints	Duration
Selenium	Men with HGPIN	III	Incidence of prostate cancer	3 years
Selenium and Vitamin E (SELECT)	Healthy population age >55 (50 if African American)	III	Incidence of clinical prostate cancer	7-12 years
Soy	Men with PSA 5-10ng/L, biopsy negative for prostate cancer	II	Effect on proliferative biomarkers	1 year
Eflornithine (DFMO)	Healthy relatives of prostate cancer patient <70 yrs old	II	Effect on progression genes and polyamines in prostatic tissue	1 year
Doxecalciferol	Pre-prostatectomy patients with prostate cancer	II	Effect on intermediate endpoint biomarkers	28 days
Toremifene	Pre-prostatectomy patients with prostate cancer	II	Effect on levels of HGPIN	3-6 weeks
Low fat, high fibre diet	Prostate cancer patients being treated by watchful waiting	II	Effect of patient serum on prostate cancer cell growth in vitro	3 weeks
Low fat and/or high flaxseed diet	Prostate cancer patients awaiting radical prostatectomy	II	Effect on histological and serum biomarkers	At least 3 weeks
Soy isoflavones	Prostate cancer patients awaiting radical prostatectomy	II	Biomarkers of oxidative stress	3-4 weeks
Vitamin E, Selenium, Soy isolate	Patients with biopsy-proven HGPIN	II	Histological prostate cancer	3 years
Lycopene	Healthy volunteers	I	Dose-escalation study; pharmacokinetics	28 days
Celecoxib	Patients with prostate cancer awaiting prostatectomy	I	Effect on tissue biomarkers	4 weeks

It is clear that phase III chemopreventive trials in prostate cancer are large, expensive, and given the long latency of prostatic carcinogenesis, extremely time-consuming. Financial constraints would prevent the screening of every putative chemopreventive agent in the manner of the PCPT or SELECT trials. Successful surrogate endpoint biomarker development will expedite the progression of chemoprevention clinical trials by identifying any beneficial effect of an agent earlier than is currently possible. The search for suitable biomarkers is thus a research priority.

With our understanding of the molecular pathology of prostate cancer improving, and the development of better preclinical models of the disease, the future holds much promise for both the identification of new molecular targets as well as efficient evaluation of new agents. Results from pre-clinical studies assessing the chemopreventive activity of a range of different agents have already given us cause for cautious optimism. The first large-scale prostate cancer chemoprevention trial, the PCPT has given us a tantalising vision of the potential of chemoprevention as a viable therapeutic option. The reduction in biopsy-detected prostate cancer found in the PCPT is tempered only by the relatively higher incidence of high-grade tumours, although the reasons for this observed phenomenon remain unknown at present. The results from this trial and other large trials such as SELECT over the next 10-20 years will establish whether there is a role for the widespread use of chemopreventive agents to reduce the impact of prostate cancer on society.

4.7 Suggestions for further research

There are a number of issues arising from the work described in this thesis which could be investigated in the future. Firstly, further *in vitro* work would be needed to clarify the mechanisms by which resveratrol and gefitinib act on prostate cancer cells. The only mechanism of action which was found for resveratrol was cell cycle inhibition, and further analysis of cell cycle protein expression following resveratrol treatment by way of Western blotting would yield useful information. Gefitinib was shown to potently inhibit EGFR phosphorylation, but the downstream pathways which are affected were not identified. Again, Western analysis of proteins involved in the MAPK, PI3-K and PLC pathways could be performed.

Investigations into the effect of resveratrol on xenograft tumour growth *in vivo* yielded conflicting results, and further larger experiments using ICRF nude mice could be performed to clarify the situation. However, the nude mouse model has severe limitations as a model of chemoprevention, as discussed above, and it would perhaps be more useful to perform the same feeding studies on a different model. At the present time, the TRAMP model of prostatic carcinogenesis seems like the best choice for further studies. Additionally, it would be interesting to determine at what dose gefitinib remained effective in preventing tumour growth in nude mice. The lowest dose used in the work described here was 50mg/kg, but if this agent is being considered for its chemopreventive potential, the lowest possible dose which remained effective would need to be determined.

The pharmacokinetics of gefitinib in the mouse xenograft model were not analysed as a method of detecting gefitinib and its metabolites by HPLC or other mass spectrometry was not

available. It would be useful to establish the pharmacokinetic profile to assist in determining the optimal dosing regimen of gefitinib.

Finally, from the results presented here, there is a rationale for a small clinical trial involving the administration of gefitinib to patients with hormone-responsive advanced prostate cancer in order to determine whether their progression to hormone-independent disease could be delayed.

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Review article

Resveratrol—A prostate cancer chemopreventive agent?

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Abstract

The incidence of prostate cancer in Western countries continues to rise. Whilst opinion remains divided on the best treatment for localized disease, intervention for metastatic, hormone-independent cancer remains extremely limited. The concept of chemoprevention is gaining popularity as an effective means of reducing the burden of prostate cancer on the population, and many compounds with putative chemopreventive activity are currently under investigation. Resveratrol is a plant-derived polyphenolic compound which has a wide spectrum of biological activity. It has anti-oxidant and anti-inflammatory properties, and may induce apoptosis as well as modulate the function of the androgen receptor in prostate cancer cell lines. Further studies to evaluate the use of this compound as a chemopreventive agent in prostate cancer are warranted. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Prostatic neoplasms; Chemoprevention; Resveratrol

1. Introduction

Prostate cancer is one of the biggest threats to men's health in the western world. It accounts for the second largest number of male cancer deaths in the United States, and is also the most frequently diagnosed cancer in North American men [1]. Although the treatment of localized disease remains controversial, there are potentially curative options such as radical prostatectomy or radiotherapy. Once the disease is metastatic, however, the outlook is poor. These tumors may initially be responsive to hormone ablation therapy, but they often progress to a stage of hormone-independent growth, with little scope for further intervention. Thus new strategies to combat prostate cancer are being developed, one of the most exciting of which is the use of chemopreventive agents.

2. Materials and methods

2. Prostate cancer and chemoprevention

Chemoprevention can be defined as the prevention or reduction of cancer risk by the ingestion of natural or synthetic compounds with low toxicity that are able to sup-

press, delay or reverse carcinogenesis [2]. A well-known example of chemoprevention is the use of tamoxifen in breast cancer. Commonly administered for five years following surgery for primary breast cancer, it has been found to significantly reduce the risk of tumor recurrence [3]. Tamoxifen engenders the essential qualities of a chemopreventive agent; it has a well-defined biological action, is well-tolerated by patients, has a low toxicity, and is an economically viable therapy. There are now several agents, especially those derived from the diet, for which there is some evidence of their ability to prevent prostate cancer. In this review, attention is focussed on one such agent, the red grape constituent resveratrol. The aim of the article is to alert the urology community to the intriguing biological properties of resveratrol germane to its potential anti-carcinogenic activity.

An individual's susceptibility to prostate cancer is affected by both genetic and environmental factors. For example, in prostate cancer, first-degree relatives of an affected male are 1.5 to 3 times more likely than men with unaffected relatives to experience the disease [4]. Unlike adenocarcinoma of the colon, in which a number of genetic markers of risk have been identified, the role of genetics in prostate cancer remains unclear. There is compelling evidence for an environmental component in the aetiology of prostate cancer. The incidence of the disease in the United

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States is thirty times that in Japan [5]. However, the incidence amongst Japanese migrants to the USA rises to approximately half that of the indigenous, white North Americans [5]. One theory which may explain this observation is that the diet of native Japanese men contains substances, which, when consumed over many years, may reduce the risk of developing prostate cancer i.e. the Japanese diet may be rich in naturally occurring chemopreventive agents. It must be stressed that a single chemopreventive agent is unlikely to act as a panacea in all cases of cancer, as borne out by the observation that the Japanese have a high susceptibility to develop gastric adenocarcinoma. Much effort has centered around the identification of cancer chemopreventive agents in the Japanese diet, and possible candidates are compounds contained in the soya bean, a major constituent of the Japanese diet [6]. These agents are known as soy isoflavones.

The multistep theory of carcinogenesis stipulates that cancer ultimately results from damage to DNA, which causes mutations in proto-oncogenes and/or tumor-suppressor genes. The damage may be caused by genotoxic reactive oxygen species, carcinogenic metabolites, or by physical processes, such as ionizing radiation. Proto-oncogenes and tumor-suppressor genes usually control vital cellular regulatory mechanisms and signaling pathways. Following damage, these fastidiously regulated systems start to function aberrantly, leading to unrestricted cellular proliferation and ultimately, metastasis. High-grade prostatic intraepithelial neoplasia, which may represent the histological precursor of invasive carcinoma [7], develops over a period of around 20 years, and the progression to clinically significant carcinoma may take another 13 to 25 years [8]. This long latency period, in conjunction with the extremely high incidence of the disease makes prostate cancer an attractive target for chemoprevention.

The evaluation of chemopreventive agents in prostate cancer is in its infancy, and several compounds are under clinical evaluation. The antiandrogenic 5- α reductase inhibitor finasteride is at the center of a large prospective chemopreventive trial in the USA, the "Prostate Cancer Prevention Trial" (PCPT) [9]. Another large-scale clinical trial with the acronym SELECT, which has just been activated, aims at investigating the chemopreventive properties of selenium and vitamin E alone and in combination [10]. Putative prostate cancer preventive agents currently at various stages of preclinical or early clinical evaluation include the tomato ingredient, lycopene [11], non-steroidal anti-inflammatory agents, tyrosine kinase inhibitors [12], flavinoids such as genistein and quercetin, polyphenols such as epigallocatechin gallate and trihydroxystilbenes such as resveratrol.

2.2. Resveratrol

Resveratrol (3,4,5 trihydroxystilbene) is not only found in red grapes but also in a variety of other plants [13]. Its chemical structure is shown in Fig. 1. It is synthesized in the leaf epidermis and grape skin, probably as a response to en-

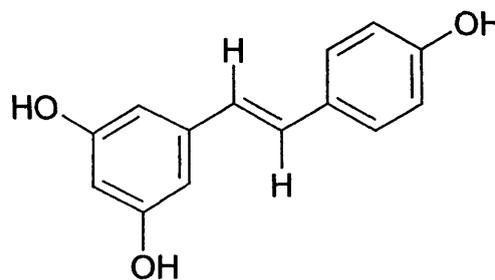


Fig. 1. The structure of resveratrol.

vironmental stress such as that which occurs in fungal infection. Its concentration in grape skins varies, and it was first detected in wine in 1992. It is also found in non-alcoholic red grape juice. Given its status as a naturally occurring substance, which is found in common foodstuffs, one would expect it to have low toxicity on chronic administration, a key feature of any chemopreventive agent. Resveratrol came to prominence during the course of investigation of the so-called "French paradox" – i.e. the realization that the population of France have a relatively high fat diet accompanied by a steady albeit moderate intake of alcohol in the form of red wine whilst their incidence of heart disease is lower than would be expected. Resveratrol has been implicated in this effect due to its actions on the modulation of lipid metabolism, vasorelaxant activity, and inhibition of platelet aggregation among others. Its cancer chemopreventive effects were discovered in 1997 when it was shown to inhibit the carcinogenic process at the initiation, promotion and progression stages [14]. These studies were performed using a panel of cells originating from human lymphocytic leukemia and murine mammary and skin tumors.

Many *in vitro* studies have addressed the antiproliferative or pro-apoptotic effects of resveratrol in human prostate cancer cells, and its mechanism of action. The cell lines used include both hormone-sensitive and hormone-resistant cells in order to mimic the heterogeneous state of clinical prostate cancer. LNCaP cells are hormone-sensitive, PC-3 is a hormone-independent line possessing dysfunctional androgen receptors (ARs), and DU145 are androgen-independent cells, devoid of ARs. Resveratrol was found to inhibit the growth of LNCaP cells in a concentration dependent manner [15,16] and was most potent at a concentration of 2.5×10^{-6} M [15]. The growth of the DU-145 and PC-3 cells was inhibited as well, but it required a 10-fold higher concentration [15].

The potential *in vivo* efficacy of a putative chemopreventive agent can be tested in rodent models, especially those that bear a genetic defect of relevance to the genesis of the human disease that is to be prevented. In the light of the paucity of knowledge of genetic defects underlying human prostate cancer (*vide supra*), the topic of rodent models and their relevance to human prostate cancer is currently the subject of considerable debate [17]. Nevertheless there have

recently been impressive reports of the prostate cancer chemopreventive efficacy of the soya constituent genistein [18] and a standardized polyphenol-containing green tea extract [19] in the so-called TRAMP (“transgenic adenocarcinoma of the mouse prostate”) mouse. This rodent model expresses SV40 early genes, probably abrogating the function of the p53 and retinoblastoma genes, which in turn engenders the formation of prostate carcinoma in all mice. Convincing evidence for the notion that resveratrol prevents prostate cancer also in models of prostate cancer *in vivo* has yet to be presented.

Potential mechanisms of action of resveratrol have been studied extensively, and there is no clear consensus on the matter. It should be noted that many of these mechanistic studies summarized below have been carried out on cell lines other than those derived from prostate cancer cells, and their relevance to the prostate have to be interpreted with caution.

2.3. Anti-oxidation/ anti-inflammation

Oxidative stress is thought to be an important cause of damage to DNA, with subsequent mutation. The interaction of free radicals with DNA results in the formation of a number of adducts which can be detected and quantified [20]. One study showed that treating LNCaP cells with physiological levels of androgens seemed to increase oxidative stress within the cells [21]. Thus, due to the presence of high levels of androgens, prostate cells may be particularly vulnerable to oxidative stress. Hydroxyl radicals can attack DNA directly, or can initiate autocatalytic lipid peroxidation, which results in the formation of genotoxic products. Such products may also be formed as a result of prostaglandin biosynthesis by cyclooxygenase (COX) enzymes. Resveratrol has been shown to exert a strong inhibitory effect on the formation of free radicals in human macrophages [22], and may thus act to reduce oxidative stress within pre-malignant cells.

It is known that inflammatory mediators such as prostaglandins (PG) and nitric oxide (NO) stimulate the growth and spread of tumors by a number of mechanisms, including stimulation of cellular proliferation, reduction in immune surveillance and the induction of angiogenesis [13]. Large concentrations of these substances may be found in inflamed prostatic tissues. Resveratrol has been shown to inhibit the activity of enzymes responsible for the production of some of these inflammatory mediators, namely the enzymes COX-1 and COX-2 and the nitric oxide synthase (NOS) enzymes [endothelial NOS (eNOS) or inducible NOS (iNOS)]. Resveratrol treatment decreased the production of NO significantly in PC-3 and DU-145 cells, but not in LNCaP cells [23]. Resveratrol has also been shown, in non-prostate inflammatory models, to reduce the production of iNOS in response to stimuli such as lipopolysaccharide [24]. Interference with intracellular signaling mechanisms responsible for regulation of NOS production is thought to be important. In particular, resveratrol exerts an inhibitory

effect on actions mediated by the transcription factor NF- κ B. NF- κ B regulates the transcription of both NOS and COX genes, and it is involved in the control of the cell cycle [25]. COX-1 is the constitutive form of COX, whereas COX-2 is the inducible form, which is expressed in inflamed tissues and in many malignancies. As well as metabolizing arachidonic acid, COX-1 and COX-2 also have hydroperoxidase activity, and thus can catalyse the transformation of harmless pro-carcinogens into carcinogens [13]. There is evidence of increased COX activity and COX-2 over-expression in prostate cancer [26], and high levels of PGE₂ have been found in prostate cancer specimens as compared to surrounding normal prostate tissue [27]. COX-2 may be important in tumor angiogenesis, and its activation may suppress apoptosis [28]. Resveratrol has been shown to have inhibitory effects on both the arachidonic acid metabolizing and hydroperoxidase activities of both COX-1 and COX-2. In one study the inhibitory effect of resveratrol on cyclooxygenase activity of COX-1 was greater than that mediated by aspirin [14]. The effect on COX-2 activity is slightly more controversial; in the above study resveratrol had no discernible effect on this enzyme. However, there is some evidence *in vitro* which suggests that resveratrol inhibits COX-2 induction in response to substances such as phorbol esters [29]. As with the iNOS enzyme, this effect may be mediated by interference with intracellular signaling mechanisms, which lead to COX-2 expression, among them the protein kinase C system and NF- κ B.

2.4. Modulation of cytochrome P450

Many carcinogens require metabolism in the body to acquire their detrimental activity. One of the most important enzymes in the formation of carcinogens is the cytochrome P450 isoenzyme 1A1 (CYP 1A1). This enzyme is responsible for a pivotal step in the biological activation of many aryl hydrocarbon (AH) carcinogens, such as benzo[*a*]pyrene (B[*a*]P). AHs bind to the cytosolic AH receptor which then translocates to the nucleus, and in conjunction with the AH nuclear translocator, binds to the CYP 1A1 promoter, resulting in increased transcription of CYP 1A1. Resveratrol has been shown to inhibit the activity of CYP 1A1 and also to inhibit the transcription of CYP 1A1 in hepatocytes [30], by preventing the binding of the AH receptor to the promoter region of the gene. CYP 1A1 has been demonstrated in human prostatic tissue [31], but the extent of its involvement in prostatic carcinogenesis remains unknown. Nevertheless it is conceivable that resveratrol inhibits CYP1A1 expression in prostate cells and that such inhibition is related to chemoprevention.

2.5. Modulation of cell cycle and survival

The loss of control of the cell cycle and lack of normal apoptosis are two of the hallmarks of malignant lesions. The cell cycle is normally controlled by a number of proteins, including p53, p21^{WAF1}, the cyclin-dependent kinases (cdks),

and their activators, the cyclins. Apoptosis is particularly linked with the bax and bcl-2 proteins. The progress of a cell through its cycle is mediated by specific activation of complexes between cdks and cyclins. Once activated, these complexes shepherd a cell through the transition between phases in the cell cycle. Other molecules such as p21^{WAF1} can have an inhibitory effect on these cyclin-cdk complexes, i.e. they are cdk inhibitors. Resveratrol has been shown to interfere with the cell cycle and act on many of these biomolecules. It significantly increased the proportion of LNCaP cells undergoing apoptosis [15]. Another study showed that it up-regulates p53 expression and induces apoptosis in a mouse epidermal cell line. This pro-apoptotic effect of resveratrol was absent in p53-deficient fibroblast cells [32], which suggests that resveratrol acts through a p53-dependent mechanism. In another study in epidermoid carcinoma lacking wild-type p53 resveratrol was pro-apoptotic, probably mediated via increased expression of p21^{WAF1} [33]. The expression of p21^{WAF1} resulted in cell cycle arrest between the G₁ and S phases. This arrest is thought to be irreversible, the inert cell then undergoing apoptosis. Although this model is appealing, contradictory evidence as to the role of p21^{WAF1} has been presented in LNCaP cells, in which p21^{WAF1} levels were markedly decreased following resveratrol treatment, concomitant with an increase in apoptotic cell number [16]. Resveratrol can have other effects on the mitotic process, exemplified by inhibition of ribonucleotide reductase [34] and DNA polymerase [35].

2.6. Effects on the androgen receptor

Androgen receptor (AR) mediated signaling is of paramount importance in the development of prostatic carcinoma. This notion is supported by the fact that prostate cancer does not develop in those castrated prior to the onset of puberty. In the early stages of prostate cancer the AR gene is amplified [36]. Following ligand binding, the AR-ligand complex initiates gene transcription by cofactor-mediated binding to androgen response elements (ARE). Thus androgen binding is the most potent stimulus to AR activity. Furthermore, androgen-regulated gene activity is important even in the “androgen-independent” stage of the disease. For example, serum levels of PSA, the most widely studied androgen-regulated gene product, continue to rise in hormone-refractory metastatic disease. ARs are still expressed in hormone-refractory disease, and it is possible that they are activated by ligand-independent pathways, such as by interaction with peptide hormone signaling molecules. Given the central role of AR, it is possible that resveratrol exerts its chemopreventive action in part by modulating the expression or function of this receptor. Several studies have focussed on the effect of resveratrol on the expression of ARs in LNCaP cells, and they have produced conflicting results. In one study, resveratrol treatment of LNCaP cells failed to reduce the expression of AR, although it resulted in a marked decrease in secreted and cytosolic PSA [37]. The authors suggested that

resveratrol reduces PSA secretion in an androgen receptor-independent manner. In contrast, in another study in the same cell type, AR levels were decreased in a time- and dose-dependent manner by resveratrol, and the AR-binding site in androgen inducible genes was thought to be a target for resveratrol [16]. There is evidence to suggest that the p21^{WAF1} gene is upregulated by androgens, thus giving androgens direct control over the cell cycle [38].

3. Conclusions

This short review summarizes the evidence which suggests that resveratrol is a chemopreventive agent of potential promise in prostate cancer. This supposition is based on the fact that resveratrol interferes with a plethora of processes pivotal for the prostatic malignant phenotype, and that it thus may compromise progression of prostatic carcinogenesis at a number of biochemical levels. By its anti-oxidant and anti-inflammatory effects, it prevents the formation of free radicals, and of arachidonic acid metabolites. It interferes with the activation of aryl hydrocarbon pro-carcinogens by inhibiting the activity and expression of CYP 1A1. It modulates the control of the cell cycle and androgen-mediated signaling pathways. Resveratrol is present in common dietary constituents, and thus on chronic administration it probably lacks adverse effects. More mechanistic work needs to be performed in prostate-derived cells in order to pinpoint its exact mechanisms of action. The elucidation of its potential efficacy in rodent models relevant to human prostate carcinogenesis is imperative for the further development of resveratrol as a potential prostate cancer preventive agent. In the light of the depressing scarcity of therapeutic options open to urologists who treat advanced prostate cancer, diet-derived chemopreventive agents—exemplified by resveratrol—may constitute a ray of hope. Their preclinical evaluation should be vigorously pursued, because it may furnish leads which ultimately convert this hope into tangible clinical efficacy.

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ErbB receptors: possible therapeutic targets in prostate cancer?

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INTRODUCTION

Prostate cancer is one of the greatest threats to men's health in the Western world, accounting for $\approx 20\,000$ new cases and 9000 deaths annually in England and Wales. Although there is controversy about the optimum treatment of localized prostate cancer, there are potentially curative options. However, once the disease has disseminated, the prognosis is poor, with the principal treatment options being palliative. It is therefore important to investigate the mechanisms underlying prostate carcinogenesis, particularly the transformation from androgen-dependent to androgen-independent disease, to develop new targets for chemotherapeutic and chemopreventive drugs

Carcinogenesis is a multistep process which occurs as a consequence of damage to proto-oncogenes and tumour-suppressor genes. After DNA damage the normally fastidious cellular control mechanisms fail, resulting in proliferation, invasion and metastasis. Several families of genes are implicated in these processes, including the group of erbB genes which code for the four transmembrane receptors, epidermal growth factor receptor (EGFR or erbB-1), HER-2 (neu, erbB-2), HER-3 (erbB-3) and HER-4 (erbB-4). The link between EGFR and cancer was first established when the structural similarity of this receptor to the viral oncogene v-erbB, encoded by the avian erythroblastosis retrovirus, was noted. Since then, its expression and functional role in numerous human tumours, including prostate cancer, has been studied. Although the other three receptors are related to EGFR, there are some important structural and functional differences between them. EGFR is a 170-kDa

tyrosine kinase transmembrane glycoprotein (Fig. 1) which has high affinity for several ligands, principally EGF and TGF- α , as well as amphiregulin, betacellulin and heparin-binding epidermal growth factor-like factor (HB-EGF). ErbB-2 shares some homology with EGFR, but the key difference is that no ligand has been identified for this receptor, and indeed, it may not possess one. It has thus become known as an 'orphan' receptor. The other two receptors, erbB-3 and erbB-4, bind several ligands including the neuregulins and epiregulin (Fig. 2). Although all four receptors should be regarded as an integrated family which modulate the function of each other, this review will concentrate on EGFR and HER-2, as there are few data about HER-3 and -4 in prostate cancer.

EGFR ACTIVATION

The binding of a ligand to EGFR results in its dimerization, either with another EGFR molecule (homodimerization) or with other receptors of the erbB family (heterodimerization). HER-2 is the favoured dimerization partner for all other erbB receptors. After dimerization, intracellular tyrosine residues on the intracellular portion of the receptor are phosphorylated and this is catalysed by the receptor tyrosine kinase domain. Tyrosine phosphorylation is the key step in EGFR activation. Various intracellular molecules containing specific domains known as src sequence homology (SH2) or phosphotyrosine binding sites can then recognize and complex with phosphotyrosine residues on EGFR and activate intracellular signalling pathways (Fig. 3). The principal signalling pathways involved are the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3-K) pathway and the phospholipase-C γ (PLC- γ) pathway; these pathways are illustrated in Fig. 4.

THE FUNCTION OF EGFR IN THE NORMAL PROSTATE AND IN PROSTATE CANCER

Immunohistochemical staining of well-preserved histological specimens shows the presence of EGFR in normal prostatic epithelium, in tissue undergoing benign hyperplasia, and in malignant prostatic cells [1]. The role of EGFR in the normal adult prostate is still unclear, although, as it is found in basal epithelial cells, it is thought to help maintain epithelial integrity. It is important during embryonic prostatic morphogenesis, prominently influencing the folding of prostatic lobules [2].

In contrast to carefully regulated physiological EGFR-mediated signalling which occurs in health, there is increasing evidence that abnormal, prolonged EGFR signalling is significant in prostatic carcinogenesis. This aberrant signalling may occur as a result of EGFR over-expression, expression of mutant EGFR, a switch in production of predominant stimulatory ligand from EGF to TGF- α , establishment of an autocrine stimulatory loop, or interaction of EGFR with other signalling pathways, particularly the androgen receptor (AR)-mediated pathway. These mechanisms are discussed in greater detail.

THE EXPRESSION OF WILD-TYPE (WT) AND MUTANT EGFR IN PROSTATE CANCER

The status of WT-EGFR and mutant EGFR expression in prostate cancer remains unclear. It has been shown that EGFR mRNA expression in cancerous tissue is higher than in normal prostatic tissue or even BPH tissue [3]. In addition, *in vitro* studies show the presence of EGFR in both hormone-sensitive and -insensitive prostate cancer cell lines by Western blotting. However, investigations into comparative EGFR protein expression in benign and malignant human prostatic tissue

have been contradictory, with some groups finding less of the receptor in malignant epithelial cells than in normal tissue [4] and others finding the opposite [5]. These differences have been attributed to variations in the antibodies used to detect the protein, and to assay techniques.

Recent work from one group shows the presence of a mutated form of EGFR, known as EGFRvIII, in prostate cancer cells [6]. This variant form of the receptor lacks 267 amino acids from its extracellular domain, resulting in the loss of a large proportion of the ligand-binding pocket. It is constitutively active in the absence of a ligand. Using EGFRvIII-

specific antibodies, this variant receptor was over-expressed in human prostate cancer tissue, and this was related to a concomitant decrease in expression of WT-EGFR [6]. As EGFRvIII is not detected in assays for normal or WT-EGFR it may contribute to reported discrepancies in EGFR expression in prostate cancer specimens. However, the importance of EGFRvIII in prostate cancer remains to be confirmed by further studies.

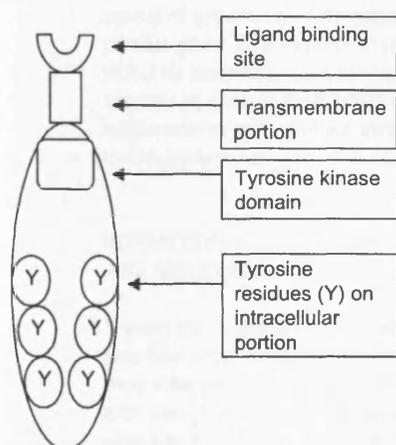
It is clear that there is no consensus on the question of whether EGFR is over-expressed in prostate cancer. It is possible that the other mechanisms leading to aberrant EGFR-mediated signalling, described below, are more important than protein overexpression.

receptor rather than degradation. Thus, for the same initial number of EGFRs, a switch in ligand production from EGF to TGF- α would result in prolonged signalling, as may occur in cancer [7]. This hypothesis was supported by a study which showed that TGF- α is found in negligible quantities in normal prostate tissue, in small amounts in BPH and in much larger quantities in prostatic carcinoma [8].

ESTABLISHMENT OF AN AUTOCRINE LOOP IN CANCER

Prostatic tissue is a composite of epithelial and stromal elements. Although epithelial cells undergo malignant transformation, stromal cells are important in regulating proliferation, by secreting growth factors. One study evaluated the distribution of EGFR and its ligands in histological specimens of normal prostate and prostate cancer [9]. In normal and benign glands, epithelial cells express EGFR, whilst EGF and TGF- α are primarily found in the stromal compartment. This suggests modulation of epithelial cell growth by stromal cells (paracrine control). However, in cancerous tissue epithelial cells co-express both ligands (predominantly TGF- α) and EGFR (Fig. 5). This is very important as it implies epithelial autocrine stimulation, and loss of stromal modulation of EGFR function. Stromal regulation of epithelial cell growth is mediated by several other growth factors, including TGF- β , which acts on TGF- β

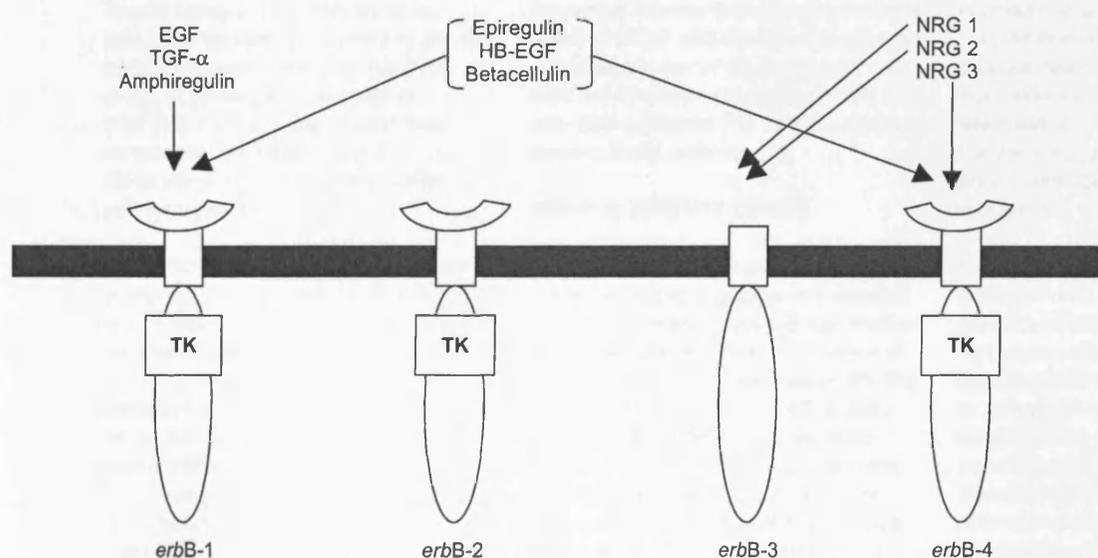
FIG. 1. Schematic representation of the EGFR.



TGF- α AS THE DOMINANT LIGAND IN CANCER

Although both EGF and TGF- α are mitogenic, they have been shown to have different effects on the expression of EGFR in prostate cancer cell lines. TGF- α preferentially stabilizes EGFR mRNA, a mechanism thought to be of pivotal importance in the post-transcriptional regulation of EGFR. EGFR-ligand complexes undergo endocytosis in clathrin-coated pits before their degradation. EGFR-EGF complexes remain stable in endosomes and are rapidly targeted by lysosomes for degradation. However, TGF- α -EGFR complexes rapidly dissociate in endosomes, leading to recycling of the

FIG. 2. ErbB receptors and their ligands.



receptors, T β R-I, -II and -III. In the normal gland, TGF- β is a negative regulator of epithelial proliferation and induces apoptosis [10]. TGF- β is over-expressed in prostate cancer specimens and there is a loss of TGF- β receptors in malignant cells [11]. It is thought that loss of T β R-I receptors by malignant prostatic cells confers a survival advantage by reducing their sensitivity to the growth inhibitory properties of TGF- β . Over-expression of TGF- β in prostate cancer seems paradoxical, given its inhibitory properties. However, it can also stimulate extracellular matrix production and promote angiogenesis, thereby making the surrounding environment more favourable for cancer cell growth and metastasis.

Neuroendocrine cells are found in the prostate and are thought to contribute to the control of growth and differentiation of the prostate gland. Over-expression of EGFR and HER-2 by these cells has been reported, and may be another mechanism by which erbB-mediated pathways are involved in carcinogenesis [12].

INTERACTION BETWEEN EGFR AND ANDROGENS

Prostate carcinoma does not develop in men who have never produced testosterone or in those who are resistant to its effects. Thus, even though peptide growth factors can stimulate mitogenesis, their actions must be modulated by androgens, at least during the androgen-dependent stage of the disease.

There is strong *in vitro* evidence of an interaction between androgens and growth factors. Androgen stimulation has been shown to up-regulate expression of EGFR [13]. Another study showed that a combination of dihydrotestosterone and TGF- α stimulated the growth of LNCaP cells synergistically [14].

The mechanisms underlying the development of androgen-independent prostate cancer remain unclear, but one hypothesis is that in the absence of androgens, activation of peptide growth factor receptors can lead to phosphorylation and subsequent activation of AR via the MAPK or PI3-K pathways [15]. Indeed, EGF and other peptide growth factors can induce the transcription of AR-regulated genes, which is a possible explanation of why levels of PSA, the product of an AR-regulated

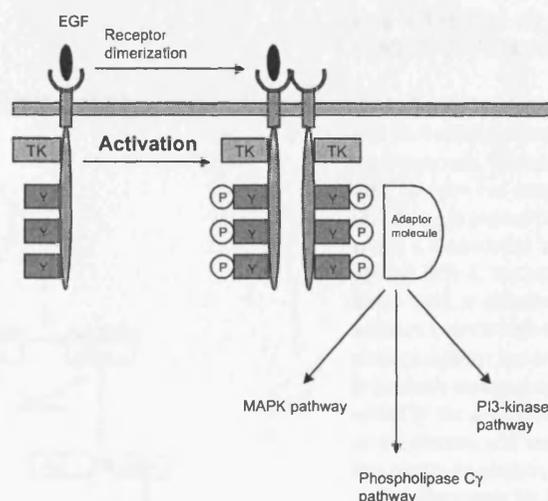


FIG. 3. After binding of a ligand to EGFR, the receptor dimerizes with another receptor of the same family, activating the tyrosine kinase (TK). This results in phosphorylation of the tyrosine (Y) residues. The phosphotyrosine residues are recognized by several adaptor molecules possessing src sequence homology (SH2/SH3). These molecules subsequently activate other intracellular signalling pathways.

gene, continue to increase in these later stages of prostate cancer. A recent immunohistochemical study [16] showed that EGFR expression was higher in hormone-refractory metastatic prostate cancer than in specimens obtained after radical prostatectomy or LHRH treatment. Additionally, AR activation in the presence of low levels of androgens is dramatically increased by the presence of peptide growth factors [17]. After androgen-ablation therapy, a patient with advanced prostate cancer is likely to have very low levels of circulating androgens, which, when facilitated by erbB-mediated signalling, would be enough to activate AR-mediated cell proliferation.

However, other studies dispute any interaction between these systems; hormone-sensitive LNCaP cells stimulated to grow by the administration of dihydrotestosterone were not inhibited by blockade of EGFR with anti-EGFR antibodies [18] or EGFR-specific tyrosine kinase inhibitors [19].

HER-2 IN PROSTATE CANCER

HER-2 has been studied extensively in breast cancer and is over-expressed in a subset of 20–30% of these tumours, and high levels of HER-2 correlate with a poorer response to hormonal therapy in breast cancer [20]. The role of HER-2 in prostate cancer is under intense scrutiny. LNCaP cells, which are normally hormone-dependent, underwent hormone-independent growth after the forced over-expression of HER-2 [21]. In a mouse prostate cancer xenograft model,

androgen-independent xenografts expressed higher levels of HER-2 than their androgen-dependent equivalents [21].

There have been conflicting results relating to c-erbB-2 gene amplification in human prostate cancer specimens. The same inconsistencies arise when studying HER-2 protein expression, which has been reported in 0–100% of cases [22]. The reasons underlying these inconsistencies are possibly related to differences in specimen preparation, of the reagents used and in strict definitions of 'positive' results. Two recent papers aimed to clarify the situation, and investigated HER-2 expression in different clinical stages of prostate cancer [23,24]. Both groups of investigators detected HER-2 expression in prostate cancer and found that the level of expression was higher in prostate cancer. In addition, the level of expression was higher in prostatic tissue which had been exposed to androgen ablation therapy. The highest levels of expression were in androgen-resistant metastatic specimens.

Thus, HER-2 over-expression appears to be associated with androgen-independent growth, and studies to determine the reason for this are underway. In common with EGFR signalling, HER-2 stimulation has been shown to activate AR in a ligand-independent fashion. HER-2 may also potentiate EGFR signalling, as HER-2/EGFR heterodimers dissociate rapidly in endosomes, with resultant recycling of EGFR and HER-2 and prolongation of signalling.

FIG. 4.

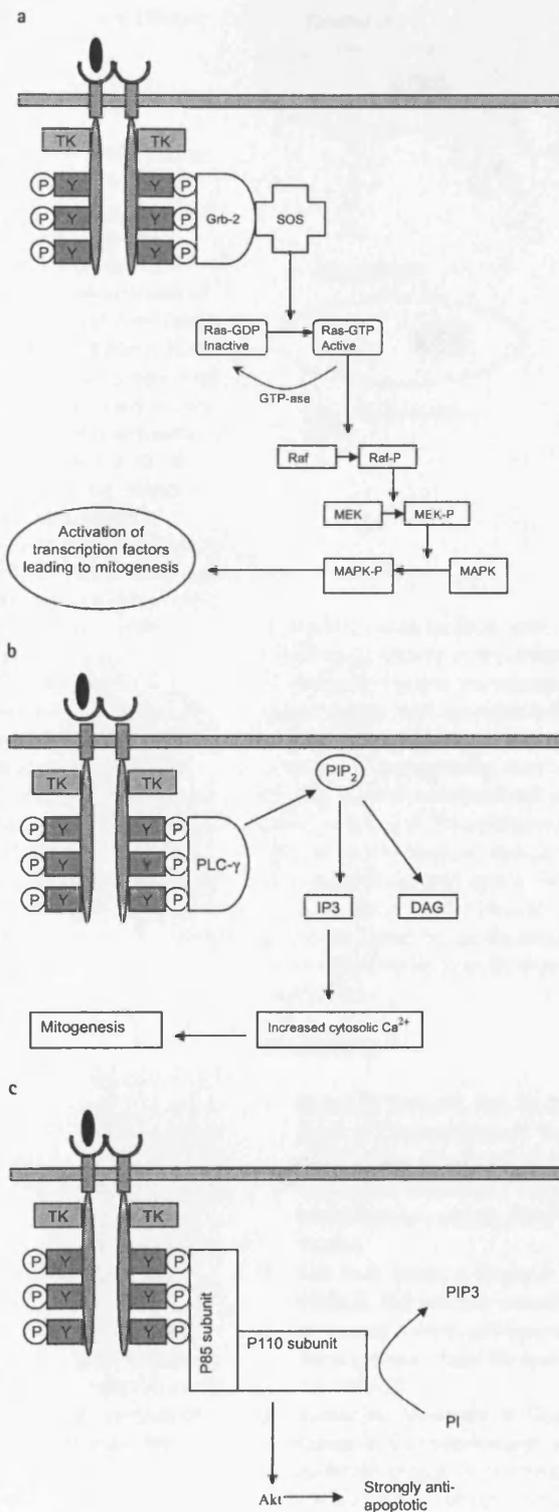
(a) The MAPK pathway; here, the adapter molecule is Grb-2. After interaction of Grb-2 with SOS (son of sevenless) protein, the linked ras-GDP is phosphorylated to ras-GTP which activates it.

Subsequently, a cascade involving raf and other protein kinases is triggered, leading ultimately to the activation of nuclear transcription factors and mitogenesis.

(b) The PLC pathway; PLC catalyses the formation of inositol triphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol diphosphate (PIP2).

IP3 increases cytosolic free Ca²⁺ + 0. (c) The PI3-K pathway; the PI3-K enzyme comprises two subunits, the p85 and p110 components. The p85 subunit recognises the phosphotyrosine residues on activated EGFR, whilst the p110 subunit catalyses the formation of phosphatidylinositol triphosphate (PIP3) from phosphatidylinositol and PIP2.

The enzyme also controls downstream activation of the anti-apoptotic protein kinase B or 'Akt' (the analogue of the viral v-akt gene product).



ErbB RECEPTORS AS THERAPEUTIC TARGETS IN PROSTATE CANCER

Given the likely importance of EGFR- and HER-2-mediated signalling in prostatic carcinogenesis, therapeutic abrogation of their function has become a research priority [25]. This has proved fruitful in breast cancer, where a monoclonal antibody directed against HER-2, trastuzumab ('Herceptin', Genentech), is effective in women whose tumours express high levels of HER-2. Agents directed against the erbB family of receptors in prostate cancer may prove particularly useful in the androgen-independent stage of the disease, and may delay the onset of this phase. At present, profiling of prostate cancer specimens for EGFR or HER-2 expression is not routine, but this will assume importance if those patients most likely to respond to highly targeted anti-EGFR therapy are to be identified. Numerous such therapeutic approaches might be used to inhibit the action of EGFR and HER-2, most of which are still in preclinical or early phases of clinical investigation. The most promising agents under development are summarized below.

MONOCLONAL ANTIBODIES

Cetuximab or C225 (ImClone) is a chimeric monoclonal antibody developed to target the ligand-binding domain of the EGFR. It binds to the EGFR with higher affinity than its naturally occurring ligands. It was effective in preclinical *in vivo* models of prostate cancer, both alone and combined with other agents. Clinical trials are in progress to assess its usefulness in prostate cancer, and preliminary data from a phase I/II study in androgen-independent prostate cancer has shown that C225 combined with doxorubicin resulted in stabilization of disease in 38% of patients after four courses of treatment [26]. Trastuzumab, a monoclonal antibody directed against HER-2, may be valuable in hormone-refractory prostate cancer. *In vivo* studies using androgen-dependent and -independent prostate cancer xenografts showed that trastuzumab had an antitumour activity when combined with paclitaxel [27]. EGFRvIII presents another therapeutic target and a highly potent antibody-toxin conjugate, scFv(14E1)-ETA, showed marked activity against the mutant receptor, as opposed to WT-EGFR [28]. ABX-EGF (Abgenix/ImmuneX) is a monoclonal antibody directed against EGF and has entered phase II clinical evaluation in

patients with hormone-refractory prostate cancer in the USA.

INHIBITION OF TYROSINE KINASE ACTIVITY

Inhibition of ligand-induced EGFR tyrosine kinase activity is an attractive therapeutic target and several agents are being developed for this purpose. They tend to be small molecules which interact with the ATP-binding site on the intracellular domain of EGFR. Gefitinib (ZD1839, 'Iressa' AstraZeneca Pharmaceuticals) is a highly selective EGFR tyrosine kinase inhibitor, which shows good activity against various cancer cells *in vivo*, alone and combined with other cytotoxic agents [29]. A phase I clinical trial in 19 patients with advanced prostate cancer showed promising results in terms of symptom improvement of variable duration [25]. Reported side-effects of gefitinib include an acneiform skin rash and gastrointestinal symptoms including diarrhoea. Similar tyrosine kinase inhibitor compounds such as erlotinib (OSI-774, 'Tarceva' OSI Pharmaceuticals) have been developed. PKI 166 (Novartis Pharmaceuticals) is a dual EGFR/HER-2 tyrosine kinase inhibitor and shows good activity against both hormone-sensitive and -insensitive prostate tumours [30]. The constitutively active EGFRvIII can also be targeted by tyrosine kinase inhibitors, and a small synthetic compound, tyrphostin AG1478, has been developed for this purpose [31].

OTHER APPROACHES

Antisense oligonucleotides may prove useful as highly specific agents, but are at a very early stage of development. They are artificial nucleotide base sequences which can be synthesized so that they are complementary to specific regions of mRNA coding for a particular protein such as EGFR. By binding to these mRNA strands, they can reduce protein synthesis. One preclinical study investigated the antitumour properties of antisense oligonucleotides directed against EGFR and TGF- α mRNA in a xenograft model using PC3 cells. Haemorrhagic necrosis of these tumours was detected after this treatment [32].

CONCLUSIONS

Aberrant signalling mediated by the related receptors EGFR and HER-2 is important

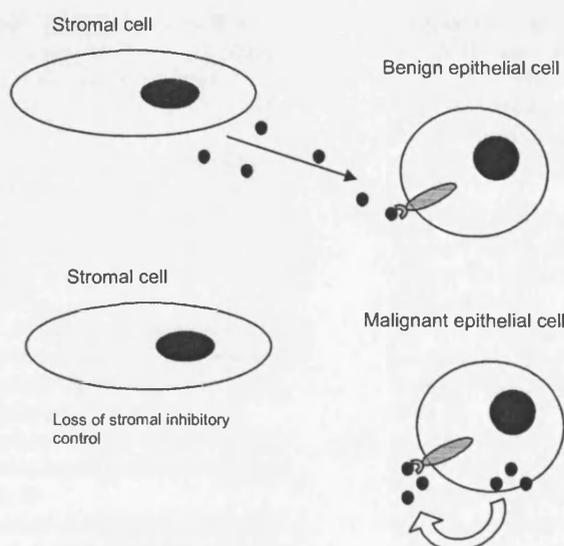


FIG. 5.

The switch from paracrine to autocrine control in prostate cancer. In normal prostatic tissue, growth factors are produced by stromal cells, and act upon EGFR on epithelial cells. This is an example of paracrine control. In malignant prostatic tissue, epithelial cells gain the ability to produce their own supply of growth factors which leads to the establishment of an autocrine loop. In addition, malignant cells lose Tbr-1 receptors, which renders them less susceptible to the antiproliferative action of TGF- β .

in prostatic carcinogenesis, both in the initial stages leading to the development of malignancy and in the subsequent transformation into androgen-independent tumours. Improved understanding of the subtleties of the signalling mechanisms involved in these erbB-mediated pathways in cancer will help in the development of mechanistically targeted chemopreventive and chemotherapeutic agents. Pre-clinical and early phase I and II trials of some such agents are promising, but the need for further well-designed trials must be strongly emphasized.

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Abbreviations: EGF(R), epidermal growth factor (receptor); HB-EGF, heparin-like binding epidermal growth factor-like factor; SH2, src sequence homology; MAPK, mitogen-activated protein kinase; PI3-K, phosphatidylinositol 3-kinase; PLC- γ , phospholipase-C γ ; AR, androgen receptor; WT, wild type.