STUDIES ON THE MODE OF ACTION OF COUMARINS

(COUMARIN, 6-HYDROXYCOUMARIN,
7-HYDROXYCOUMARIN & ESCULETIN)

AT A CELLULAR LEVEL.

A thesis submitted for the degree of Ph.D.

by

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Under the supervision of Professor Richard O'Kennedy

For my parents, Patrick & Annette

Declaration:

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy, is entirely my own work, and has not been taken from the work of others, save and to the extent that such work is cited and acknowledged within the text of my work.

Signed Deirdre Roske

Date: 25/2/99

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Abbreviations

Ab	Antibody
AIDS	Acquired immune deficiency syndrome
AP	Alkaline phosphatase
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
AUC	Area under curve
BCA	Bichonic agent
BCG	Bacille Calmette-Guerin
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
BRTK	Binary receptor tyrosine kinase
BSA	Bovine serum albumin
C	Carbon
CL	Chemiluminescence
DAG	Diacylglycerol
DMEM	Dulbeccos minimum essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECAR	Extracellular acidification rate
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
Esc	Esculetin
FCS	Foetal calf serum
FDA	Food and Drug Administration (U.S.)
FGF	Fibroblastic growth factor
GAP	GTPase activating protein
GCR	G-protein coupled receptor
GDP	Guanosine diphosphate
Gen	Genistein
GM-CSF	Granulocyte-macrophage colony stimulating factor

Grb	Growth factor receptor binding protein
GRF	Guanine nucleotide releasing factor
GTP	Guanosine triphosphate
HBSS	Hanks balanced salts solution
6-HC	6-hydroxycoumarin
7-HC	7-hydroxycoumarin
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HMP	Hexose monophosphate
HPLC	High performance liquid chromatography
HPO	High protein oedema
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMEM	Iscoves minimum essential medium
IR	Infra-red
kDa	kilodalton
LAL	Limulus ameobocyte lysate
LAPS	Light-addressable potentiometric sensor
LED	Light-emitting diode
LDH	Lactate dehydrogenase
LPS	Lipopolysaccaride
IC_{50}	50% Inhibitory Concentration *i.e.* Drug concentration causing 50% growth inhibition
i.v.	intravenous
Mab	Monoclonal antibody
MAF	Macrophage activating factor
MAP	Mitogen-activated protein
MB/E	Methylene blue/Eosin
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MPS	Mononuclear phagocyte system
mRNA	messenger RNA
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NBT	Nitroblue tetrazolium

NGF	Nerve growth factor
NK	Natural killer cells
NMR	Nuclear magnetic resonance
NSE	Non-specific esterase
o-HPAA	o-hydroxyphenylacetic acid
o-HPLA	o-hydroxyphenyllactic acid
OZ	Opsonified zymosan
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PMSF	Phenyl methyl sulphonylfluoride
p.o.	peroral
PSA	Prostate specific antigen
PTyr	Phosphorylated tyrosine
RCC	Renal cell carcinoma
RLU	Relative light units
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
s.d.	standard deviation
SDS	Sodium dodecyl sulphate
SHP	Src-homology phosphatase
TAM	Tumour-associated macrophage
TBS	Tris-buffered saline
TGF	Transforming growth factor
T_H	Helper T-cells
TMB	3,3′,5,5′-tetramethylbenzidine
TNF	Tumour necrosis factor

Tris	Tris(hydroxymethyl)aminomethane
T_S	Suppressor T-cells
TYR	Tyrphostin
UV	Ultraviolet
V_d	Volume of distribution
WGA	Wheat-germ agglutinin

Publications and Presentations

Cooke D., Fitzpatrick B., O'Kennedy R., McCormack T. & Egan D. (1997) "Coumarins – Multifaceted molecules with many analytical and other applications" In: *Coumarins: Biology, Applications and Mode of Action,* (Eds: R O'Kennedy & R.D. Thornes), John Wiley & Sons, Chichester, pp 303-332.

Egan D., James P., **Cooke D.** & O'Kennedy R. (1997) "Studies on the cytostatic and cytotoxic effects and mode of action of 8-nitro-7-hydroxycoumarin" *Cancer Letts.*, **118**, 201-211.

Bogan D., **Cooke D.**, Duffy C., Byrden T. & O'Kennedy R. (1996) "Studies on the analysis, metabolism and mode of action of coumarins" *The Biochemist*, **S59**, 53.

Cooke D. & O'Kennedy R. (1997) "The effect of coumarin compounds on tyrosine phosphorylation in growth factor-stimulated cells". IACR Annual Conference, April 1997.

Cooke D. & O'Kennedy R. (1997) "The effect of coumarin compounds on growth factor signalling in cancer cells". AACR Special Conference on "Cell Signalling and Cancer Treatment", Feb. 1997.

Cooke D. & O'Kennedy R. (1996) "A novel biosensor system for examining tyrosine kinase inhibition". St, Vincents ERC Annual Biomedical Research Symposium, Nov. 1996.

Cooke D. & O'Kennedy R. (1996) "Replacement of animal experiments with sensor-based toxicity studies". Irish Medical Devices Seminar, April 1996.

Abstract

Coumarin, a member of the benzopyrone family of compounds, is a natural substance that has shown anti-tumour activity *in vivo*, with this effect believed to be due to its metabolites. However, no definitive mode of action has been identified, and this thesis aimed at gaining further insight into the precise target of coumarin molecules at a cellular level. A novel biosensing instrument, the Cytosensor Microphysiometer, which detects cellular metabolism, was used throughout, to aid this investigation.

The effect of four coumarin compounds (coumarin, 6-hydroxycoumarin, 7-hydroxycoumarin and esculetin) on the growth, metabolism and metastatic potential of a range of tumour cell lines was investigated. The toxicity of these four compounds was examined using a variety of *in vitro* tests (medium-term growth assays, Lactate dehydrogenase (LDH) assay and a tetrazolium salt-based (MTT) assay). A superior method to the MTT assay for examining the effect of compounds on metabolism was achieved using the Cytosensor Microphysiometer. The effect of coumarins on the metastatic potential of tumour cells (in terms of their protease secretion) was also explored.

The effect of 7-hydroxycoumarin and esculetin on growth signalling pathways within tumour cells was probed. Using the A431 cell line (which over-expresses the EGF-Receptor) and EGF as a model growth factor signalling mechanism, the effect of the two coumarins on tyrosine phosphorylation events in cells was explored. Direct *in vitro* tyrosine kinase assays with purified EGF-receptor, and ELISA, Western Blotting and Cytosensor studies in intact cells, were used to achieve this. The involvement of coumarin compounds in protein kinase C signalling was also examined.

A "model" monocyte system was developed and used in a preliminary assessment of the immunomodulatory role of coumarins. The activation of two "monocytic" cell lines was assessed using the Cytosensor Microphysiometer. Subsequently, the effect of coumarins on the release of reactive oxygen species, reactive nitrogen intermediates and proteases from activated immune cells, was accomplished using luminometric, colourimetric and substrate gel analyses, respectively.

Chapter 1

Introduction to the Coumarin Family of Compounds

1.1 INTRODUCTION

Coumarin, which derives its name from the plant *Coumarouna odorata,* is a naturally occurring component of many plants and essential oils. This introduction describes the chemistry, metabolism and pharmacology of coumarin and many of its derivatives. The uses of various coumarins in both industrial and clinical settings are reviewed. Finally, the utilisation of coumarin in cancer therapy is discussed.

Coumarin is classified as a member of the benzopyrone family of compounds. The benzopyrones, all members of which consist of a fused benzene and pyrone ring (Figure 1.1), can be sub-classified on the basis of the position of the oxygen atom within the pyrone ring - the benzo-α-pyrones, to which the coumarins belong, and the benzo-γ-pyrones, of which the flavonoids are the principal members.

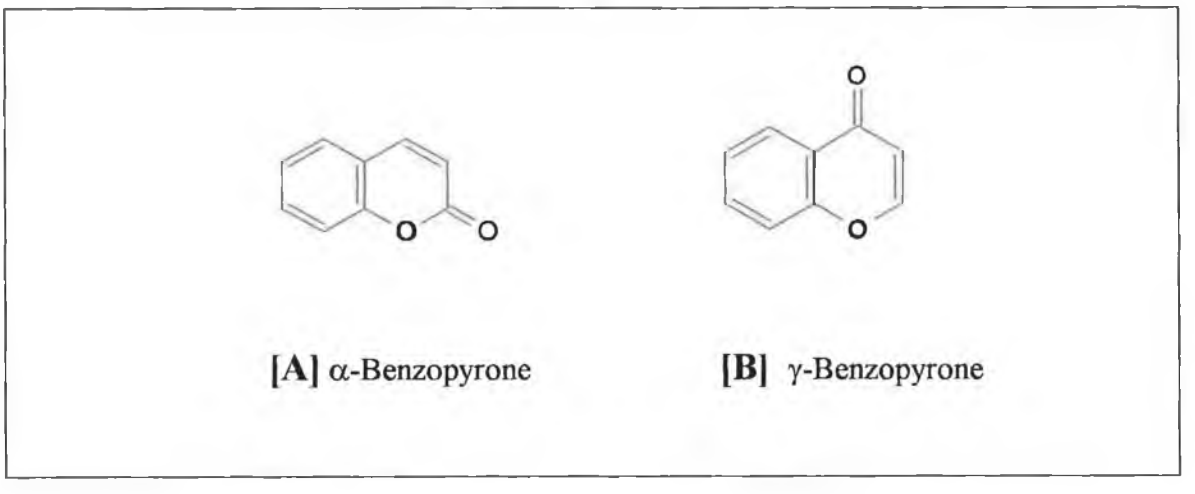


Figure 1.1: Chemical structures of the benzopyrone sub-classes, with the basic coumarin structure (benzo-α-pyrone) [A], and flavonoid (benzo-γ-pyrone) structure [B].

Dietary exposure to benzopyrones is quite significant, as these compounds are found in fruit, vegetables, nuts, seeds, tea, coffee and wine, and it is estimated that the average western diet contains approximately 1g/day of mixed benzopyrones (Pierpoint, 1986). Compounds belonging to both sub-classifications have been studied intensively in research and clinical settings, and both groups have been shown to possess a wide variety of useful pharmacological and physiological activities (Table 1.1). The lead for the discovery of many such properties was taken from traditional medicines, and benzopyrone compounds are now known to be the active agents in many folk remedies.

Pharmacological Activity	*α-Benzopyrones*	*γ-Benzopyrones*
Analgesic	Soine (1964)	Formica & Regelson (1995)
Anti-allergic		Middleton & Drzewiecki (1982)
Anti-coagulant	Link (1959); Feuer (1979)	
Anti-inflammatory	Fontaine *et al.*(1967); Lee *et al.* (1981)	Moroney *et al.*(1988)
Anti-microbial/anti-viral	Soine (1964); Feuer (1979)	Kaul *et al.* (1985)
Anti-oxidant	Paya *et al.* (1992); Chang *et al.* (1996)	Das & Ray (1988); Paya *et al.* (1992)
Anti-pyretic	Ritschel *et al.* (1984)	Middleton (1984)
Sedatory	Ritschel & Hardt (1983b)	
Vasodilatory	Soine (1964)	Duarte *et al.* (1993)

Table 1.1: **This table summarises the important pharmacological and physiological activities of both coumarin and flavonoid molecules, and includes relevant references.**

1.2. OCCURRENCE

By virtue of its structural simplicity coumarin has been assigned as head of the benzo-α-pyrones, although it is generally accepted that 7-hydroxycoumarin (umbelliferone) be regarded as the parent compound (see below). Since 1820, when coumarin was first isolated from the tonka bean by Vogel, over one thousand coumarin derivatives have been described. These have been mainly isolated from natural sources (higher plants and micro-organisms), although the organic synthesis of many "unnatural" derivatives has also been accomplished. The derivatives range from simple coumarins with hydroxyl, alkoxyl and alkyl side chains, to more complex forms containing furanoyl, pyranoyl and benzoyl functions (Table 1.2) (Murray *et al.*, 1982). All but 35 of these are oxygenated at C-7, and as a result 7-hydroxycoumarin is often regarded, both structurally, and as shall be seen later, biochemically, as the parent of the more complex coumarins.

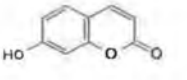
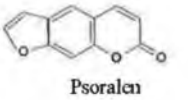
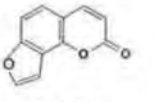
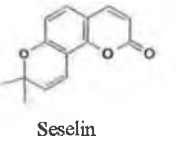
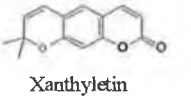
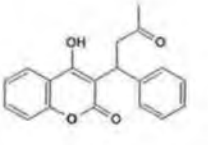
Classification	*Features*	*Examples*
Simple coumarins	Hydroxylated, alkoxylated or alkylated on benzene ring	 7-hydroxycoumarin
Furanocoumarins	5-membered furan ring attached to benzene ring. Linear or Angular	 Psoralen  Angelicin
Pyranocoumarins	6-membered pyran ring attached to benzene ring. Linear or Angular	 Seselin  Xanthyletin
Pyrone-substituted coumarins	Substitution on pyrone ring, often at 3-C or 4-C position	 Warfarin

Table 1.2: **The four main coumarin sub-types are outlined in this table. The main structural features and examples of each are also given.**

Both Murray *et al.* (1982), and more recently, Keating and O'Kennedy (1997) have reviewed the botanical source of all naturally isolated coumarin compounds. Coumarins are usually found free, or in combination with sugars as glycosides, in many higher plants, especially those of the *Umbelliferae, Rosaceae* and *Rutaceae* families. They are found distributed throughout the roots, leaves, stems and fruits, occurring at highest levels in the fruits, but the levels tend to vary with seasonal and environmental changes (Feuer, 1979). Generally a number of different coumarins are found within one plant. Although known to be secondary plant metabolites (see section 1.3) the role of coumarins in plants is still obscure, although their distribution appears to correlate with an ability to protect against disease or infection (Feuer, 1979). It has also been suggested that their role may be as plant growth regulators (Riordan & Daly, 1954).

Some important coumarin members have been isolated from microbial sources *e.g.* novobiocin and coumermycin from *Streptomyces,* and aflatoxins from *Aspergillus* species, but again their role in these organisms is unclear.

1.3. BIOSYNTHESIS

The coumarins are secondary metabolites in plant metabolism and are synthesised via the shikimate-chorismate pathway. This pathway is a central biosynthetic route in plants and micro-organisms, and shikimate and chorismate are key intermediates in the biosynthesis of the aromatic acids L-phenylalanine, L-tyrosine and L-tryptophan. This pathway follows the route shown in Figure 1.2, and the reader is referred to any plant biochemistry text for full details of the pathway. Briefly, shikimate is converted to chorismate through sequential phosphorylation, condensation and elimination reactions (not shown in Figure 1.2), involving 3-enolpyruvyl-shikimate-5-phosphate as an intermediate. The conversion of chorismate to prephenate is achieved enzymatically, after which aromatisation of prephenate yields phenylpyruvate, which is transaminated to become phenylalanine. Enzymatic elimination of ammonia from phenylalanine produces *trans*-cinnamic acid, which is the precursor for the production of all coumarin species (Murray *et al.*, 1982; Keating & O'Kennedy, 1997).

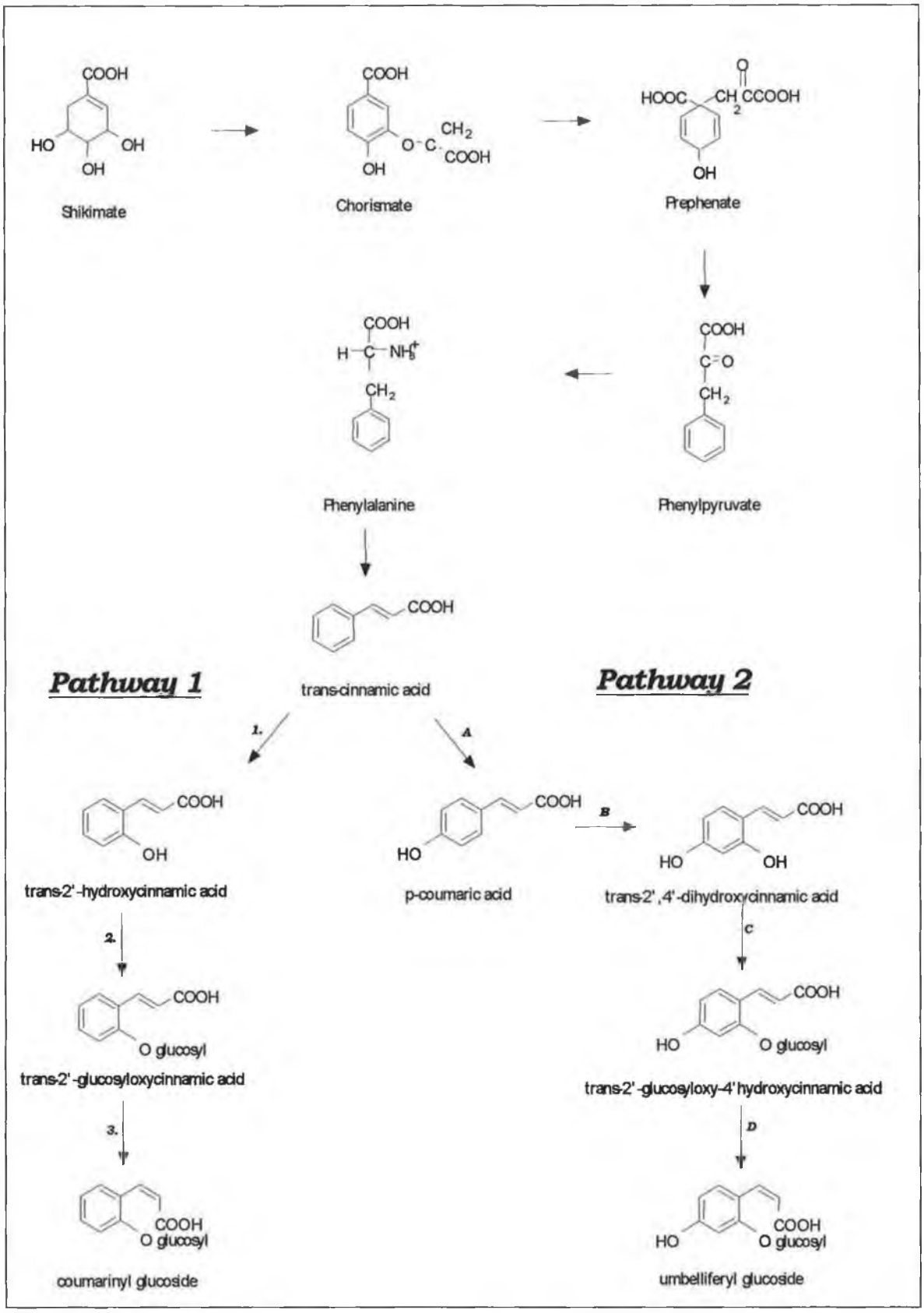


Figure 1.2: Biosynthetic routes for the natural production of coumarin and 7-hydroxycoumarin. Details of the pathways are included in the text.

At this stage the biosynthetic pathway diverges (pathways 1 and 2 in Figure 1.2), giving rise to two distinct coumarin types – those which are oxygenated at carbon-7 (most natural coumarins) and those that are not. In the latter case (pathway 1, Figure 1.2), *trans*-cinnamic acid undergoes *ortho*-hydroxylation (1) to yield *trans*-2'-hydroxycinnamic acid, which becomes glucosylated (2) to give *trans*-2'-glucosyloxycinnamic acid. This is finally converted to the *cis*-isomer in an U.V.-mediated reaction (3) to yield *cis*-2'-glucosyloxycinnamic acid (or coumarinyl glucoside) which is the primary form in which coumarin exists in plants.

The simple 7-oxygenated coumarins follow a very similar biosynthetic scheme (pathway 2, Figure 1.2), the only difference being an initial, additional step where *trans*-cinnamic acid is converted to *p*-coumaric acid by a *para*-hydroxylation (A). *p*-Coumaric acid then becomes *ortho*-hydroxylated (B), glucosylated (C) and undergoes an U.V.-dependent *trans-cis*-isomerisation (D) to form umbelliferyl glucoside. As mentioned previously the coumarin derivatives exist naturally in plants primarily as glycosides, and free compounds are released through physical or chemical disruption to cells, an example being the release of odouriferous coumarin when grass is cut.

1.4. PHARMACOKINETICS

Following administration the therapeutic action of any drug requires satisfactory concentration of the active compound into the area surrounding the target tissue. The movement of drug molecules from the site of administration to the target site {absorption and distribution} leads to this concentration effect, but its length and intensity is offset by negative factors such as the chemical modification of the active compound into non-active forms {metabolism} and their subsequent removal {elimination} (Rang & Dale, 1987).

1.4.1. Absorption and Distribution

The ability of a compound to be absorbed well in the gastro-intestinal tract following oral administration relies heavily on its physico-chemical characteristics. Ritschel *et al.* (1981) studied the biopharmaceutical properties of coumarin and its principal metabolite 7-hydroxycoumarin, in order to gain some insight into their possible behaviour in the body. Coumarin and 7-hydroxycoumarin are both poorly soluble in water (0.22% and 0.031%, respectively). This characteristic is cause for concern with respect to their bioavailability *in vivo*, as 0.3% solubility in water is considered the critical value at which the dissolution of a compound limits its rate of absorption (Ritschel *et al.*, 1981). However, both compounds have high partition coefficients

(21.5% for coumarin and 10.4% for 7-hydroxycoumarin), which is considered favourable for the rapid absorption of the compounds once they are in aqueous solution. This coupled with the fact that coumarin is non-polar, suggests that in theory coumarin should cross lipid bilayers easily by passive diffusion.

In reality absorption from the gastro-intestinal tract has been shown to be quite high, with most of the dose absorbed within 1 hour of administration (Ritschel *et al.*, 1977). However, coumarin undergoes an extreme first pass effect *i.e.* metabolism to 7-hydroxycoumarin and its glucuronide, on passage from the gut, via the liver, to the systemic circulation, and only 2-6 % is available systemically unchanged following absorption (Ritschel *et al.*, 1979). Ritschel and Hoffman (1981) managed to dramatically improve this low availability by using an oral sustained-release formulation, and succeeded in making 35% of coumarin available to the systemic circulation unchanged. The low bioavailability of coumarin, in addition to its short half-life (see below) has brought into question its importance *in vivo* and it is now accepted that coumarin is actually a pro-drug, with 7-hydroxycoumarin being the compound of therapeutic relevance.

Once absorbed any active compound must be distributed via the plasma to its target site (Rang & Dale, 1987). Most drugs bind reversibly to plasma proteins, particularly albumins, and this can prove crucial in terms of the availability of drug at its target site. Ritschel *et al.* (1981) have shown that 35% of coumarin and 47% of 7-hydroxycoumarin bind to plasma proteins. They decided that because these proportions were well below the accepted critical value of 80% binding, that availability of the compounds at their target tissues should not prove problematic. They also showed that 27% of coumarin and 21% of 7-hydroxycoumarin is bound to erythrocytes, and suggested that this might provide the drugs with temporary protection from biotransformations during transport.

Distribution of coumarin and its metabolites in the body is widespread as demonstrated by pharmacokinetic studies in humans (Ritschel *et al.*, 1977, 1979). An open two-compartment model was found to best fit the experimentally-derived data. Ritschel *et al.* (1977) showed that coumarin has a short half-life *in vivo,* which is independent of the route of administration (1.02 hrs *p.o* vs 0.8 hrs *i.v.*). As mentioned previously, coumarin is rapidly metabolised to form 7-hydroxycoumarin, which quickly becomes conjugated as the glucuronide, such that the concentrations of 7-hydroxycoumarin are always low and rarely exceed 2.2% of the levels of 7-hydroxycoumarin-glucuronide (Ritschel *et al.*, 1977).

The distribution of 7-hydroxycoumarin-glucuronide has also been shown to fit an open two-compartment model and the glucuronide has been shown to have a very large volume of distribution (V_d). This indicates the possibility of the glucuronide partitioning into fat stores in the body during its distribution. It has been suggested that this may act as a "reservoir", with cellular glucuronidases reconverting it to the active 7-hydroxycoumarin form, but to date this effect has not been observed *in vivo*. The large clearance value of the glucuronide (greater than ten times the glomerular filtration rate of 120ml/min) suggests that it is excreted by active renal tubular processes (Ritschel *et al.*, 1977).

The pharmacokinetic profiles of coumarin in various species have been obtained (Ritschel & Grummich, 1981; Waller & Chasseud, 1981; Hardt & Ritschel, 1983; Ritschel & Hardt, 1983a, 1983b). Of the species tested only baboons, gerbils and certain mouse strains (DBA/2J) have similar profiles to humans. Tests in animals, using radiolabelled coumarin, have demonstrated its distribution throughout the body to nearly all organs and tissues, and highlighted its accumulation in the liver and kidney. This information has proven useful in illustrating which species are appropriate animal models for studies on the toxicity and therapeutic relevance of coumarin.

1.4.2. Metabolism

1.4.2.1. Introduction to Coumarin Metabolism

Traditionally coumarin has been viewed by pharmacologists as an ideal model for studying the complex metabolism of a structurally simple organic molecule, and as such, its metabolic fate has been extensively researched (Kaighen & Williams, 1961; Shilling *et al.*, 1969; Pelkonen *et al.*, 1985; Moran *et al.*, 1987; Fentem & Fry, 1992; van Iersel *et al.* 1994).

Like many lipophilic compounds, coumarin is oxidatively metabolised in the liver by microsomal cytochrome P450 enzymes. Metabolism occurs principally via hydroxylation, can take place at all available carbon atoms (Figure 1.3) and is required for phase II conjugation reactions by UDP-glucuronosyl transferases and sulfotransferases. The most common routes of hydroxylation are at positions 7 and 3 to yield 7-hydroxycoumarin and 3-hydroxycoumarin respectively. Hydroxylation at carbon 3 results in further metabolism via ring-opening, yielding two further products, o-hydroxyphenyllactic acid and o-hydroxyphenylacetic acid. The metabolism is notable for its display of large inter-species (Fentem & Fry, 1992) and inter-individual variability (Rautio *et al.*, 1992; Iscan *et al.*, 1994) which has proven significant with

regard to its toxicity and therapeutic relevance. The metabolism of coumarin has been recently reviewed (Pelkonen *et al.* 1997), and a few salient points will be discussed below.

1.4.2.2. Metabolism in man

Human metabolic studies usually involve oral dosage followed by urine collection with or without timed fractionation (Moran *et al.*, 1987; Egan & O'Kennedy, 1992; Bogan *et al.*, 1995), although more recent studies have concentrated on the *in vitro* study of metabolism using human liver microsomes or precision cut liver slices (van Iersel *et al.*, 1994; Steensma *et al.*, 1994). Analysis is by one of a number of techniques including spectrofluorimetry, HPLC and more recently, capillary electrophoresis (see section 1.6).

In humans, as in primates, the major metabolic route is via 7-hydroxylation, catalysed by the specific cytochrome P450 enzyme CYP2A6. Various studies have shown that the conversion of coumarin to 7-hydroxycoumarin is affected by the dose administered: with a dosage range of 5-200mg p.o. about 60-70% on average is excreted into the urine as 7-hydroxycoumarin (Moran *et al.*, 1987; Rautio *et al.*, 1992), while a peroral dose of 1-2g results in only 20-40% being recovered in the urine as the 7-hydroxy-metabolite (Sharifi *et al.*, 1991).

Substantial interindividual differences in the metabolism of xenobiotics are common (Conney and Kappas, 1985), these differences often being a result of genetic or environmental factors. Recently it has been shown that some individuals can metabolise a considerable proportion of coumarin through pathways other than 7-hydroxylation. In particular, van Iersel *et al.* (1994), have shown using human liver microsomes *in vitro*, that the contribution of the 3-hydroxylation pathway can equal, or in some cases exceed, that of 7-hydroxylation. The existence of 3-, 5-, 6- and 4-hydroxylations has also been shown (Fentem & Fry, 1992; Steensma *et al.*, 1994). Early metabolic studies detected the presence of esculetin (6,7-dihydroxycoumarin), as well as 7-hydroxycoumarin in the urine of normal and pathological human urines (van Sumere *et al.*, 1959). It would appear, therefore, that although 7-hydroxycoumarin is the main human metabolite, other hydroxylation pathways are important in humans, and as such the therapeutic relevance of non-7-hydroxymetabolites should be examined rather than disregarded.

1.4.2.3 Metabolism in other species

Species differences in the metabolism of coumarin are quite significant and have been well-reported. Kaighen and Williams (1961) found that in the rat the major route of metabolic

conversion is via 3-hydroxylation, with several end-products including o-HPAA and o-HPLA (see Figure 1.3). The major urinary metabolites in rabbit are o-HPAA and 3-hydroxycoumarin, with approximately 10% excreted as 7-hydroxycoumarin, and trace amounts of 4-, 5-, 6- and 8-hydroxycoumarin also formed (Kaighen & Williams, 1961). As is mentioned in the next section, the observed hepatotoxic nature of coumarin appears to be due to metabolites of the 3-hydroxy-pathway. Hence it has proven important to discover effective animal 7-hydroxylators of coumarin, in order to effectively mirror the toxicity effects (if any) of coumarin in humans.

Pelkonen *et al.* (1997) in their recent review included a table outlining the metabolites of coumarin in 13 different species. From this it is obvious that baboons and certain mouse strains are effective 7-hydroxylators, but most other species included in the table appear to prefer other routes for metabolism. As mentioned, the activity of coumarin-7-hydroxylase in mouse is very strain-dependent, with DBA/2J mice proving the most effective (Wood & Conney, 1974). In addition to those listed by Pelkonen *et al.* (1997), two other species, gerbils and chickens, have been shown to possess high coumarin 7-hydroxylase activity (Dominguez *et al.*, 1990; Cacini & Ritschel, 1980), and as such are probably also appropriate animal models in any future testing involving coumarin.

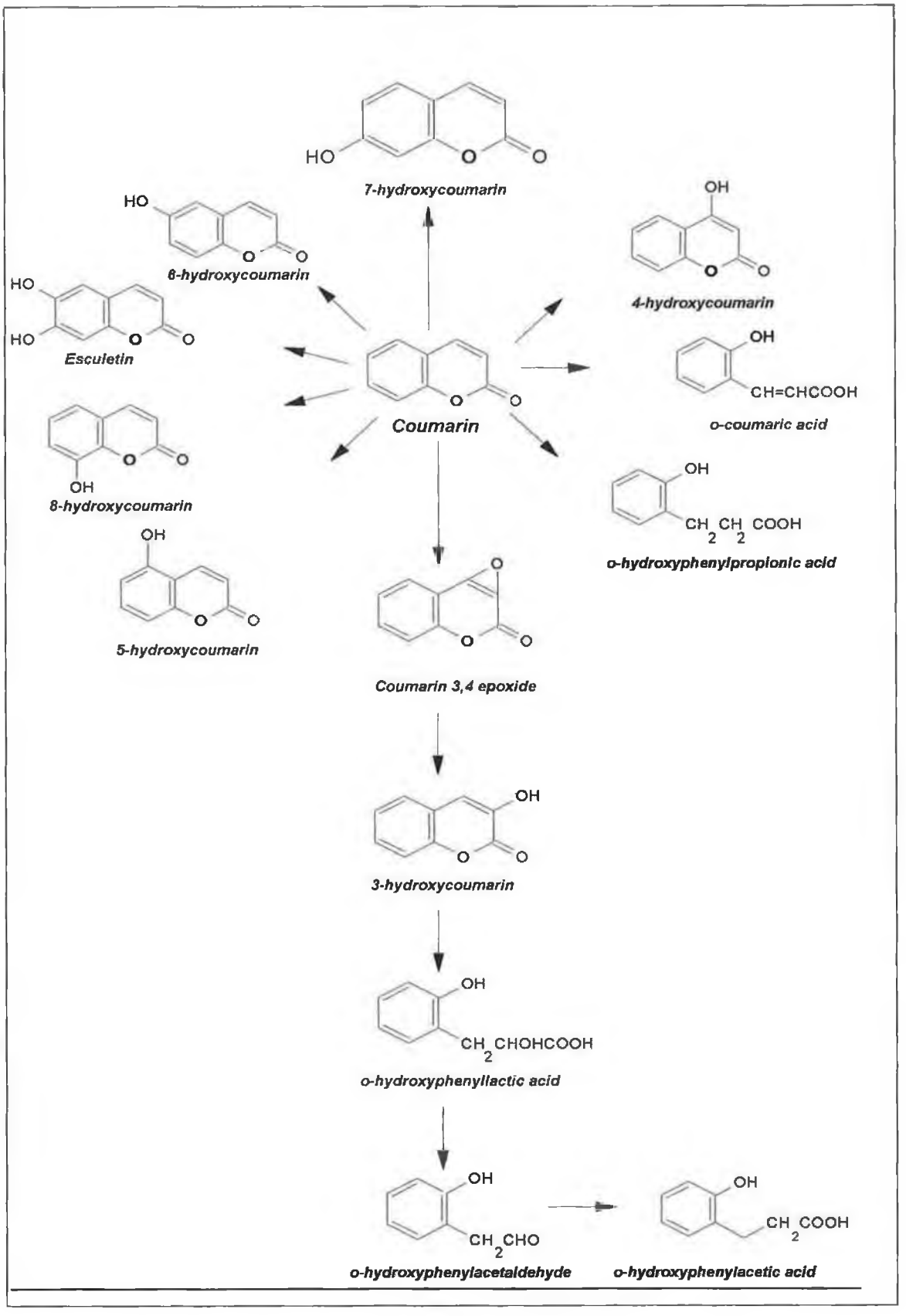


Figure 1.3: **Metabolism of coumarin. All biotransformations are possible, although the metabolism is species-specific and the reader is referred to the text for details.**

1.5. TOXICOLOGY

In 1954 the U.S. Food and Drug Administration (FDA) classified coumarin as a toxic substance and banned its use, labelling as adulterated all foods containing coumarin (Casley-Smith & Casley-Smith, 1986). This move was not copied by their European counterparts until 20 years later. The FDA action was taken based on results from routine toxicity tests, which showed that coumarin initiated toxic liver damage in rats. No pathological effects were observed in any other organs. Hazelton *et al.* (1956) subsequently obtained similar results in their studies on rats and dogs, and to date numerous investigators have reported on the toxicity of coumarins (Jenner *et al.*, 1964; Bar & Griepentrog, 1967; Endell & Seidel, 1978; Evans *et al.*, 1979). The information reported has often been contradictory, which has created confusion in this whole area. It is now believed that the evaluation of the toxicity of coumarin in man on the basis of data obtained in rats and rabbits is unjustified and inherently flawed (see below).

With respect to acute toxicity studies, coumarin has been shown to produce narcosis, loss of reflexes, coma and death, in rats, mice and guinea pigs, when administered in large doses. Hepatotoxicity was implicated by the presence of hepatic enzymes in the serum, and on death, liver degeneration with necrosis was observed. Unusually, LD_{50} values were found to differ within species *e.g.* LD_{50} values in rat ranged from 290-680 mg/kg (Hazelton *et al.*, 1956; Jenner *et al.*, 1964), an anomaly explained by investigators to result from differences in carriers! LD_{50} values in mice were also wide-ranging, but this trait appeared to be strain-dependent (Endell & Seidel, 1978; Seidel & Kreuser, 1979). It is important to note here that in all cases the LD_{50} figures obtained were extremely large compared to the actual relative doses given to humans.

Chronic toxicity studies are often perceived to be more useful than acute trials in assessing toxicities of compounds and in many chronic studies coumarin has been shown to have no or little effect. In baboons given 22.5 mg/kg /day for two years, no evidence of hepatotoxicity was observed (Evans *et al.*, 1979). Baboons fed 67.5mg/kg/day in the same study did show slight hepatotoxic signs (slightly enlarged liver). Rats fed coumarin at a dose of 100mg/kg/day for two years and golden hamsters fed up to 500mg/kg/day for the same period showed no adverse effects from this treatment (Bar & Griepentrog, 1967; Ueno & Hirono, 1981).

The reason for conflicting evidence from these studies appears now to be clarified, due to the wealth of information gained from metabolic studies in various species. The toxicity of coumarin in certain species can be related to the species-dependent metabolism of the compound. Therefore, animals which hydroxylate coumarin via 7-hydroxylation (*e.g.* baboons, gerbils, DBA

mice) are not affected in toxicity studies as adversely by coumarin, as are common 3-hydroxylators (*e.g.* rats, rabbits) [c.f. section 1.4]. As stated in a recent report from the National Toxicology Program "Organ-specific toxicity occurs in species and strains only, that metabolise coumarin qualitatively and quantitatively different from man" (Weinmann, 1997). This would suggest that some metabolite of the 3-hydroxylation pathway is causing the observed toxic effects, and as the principal metabolic pathway in humans is 7-hydroxylation, this toxicity may not be relevant in humans. Therefore, extrapolation of data from animal studies requires a cautious approach, and in reality, only data derived from appropriate animal models, or *in vitro* approaches, should be considered in an assessment of coumarins toxicity.

Clinically, studies carried out on humans have shown little evidence of liver dysfunction. An idiosyncratic-type hepatotoxicity was observed in a clinical trial of 2163 patients, but with only a 0.37% incidence (Cox *et al.*, 1989). In addition, cessation of coumarin therapy returned the elevated liver enzymes to normal levels, in patients with this response.

Coumarin has been cited as a chemical carcinogen by NIOSH [National Institute for Occupational Safety and Health] (Egan *et al.*, 1990), and has been reported as a probable carcinogen in rodent carcinogenicity bioassays (Jones & Easterley, 1991). However, as before caution needs to be taken in extrapolating this information to human situations as these tests were carried out in rodents. Various tests (Ames, micronucleus) have shown that coumarin and its metabolites are non-mutagenic (Egan *et al.*, 1990). Although coumarin can cause chromosome and DNA breaks in plant cells (Grigg, 1977), it offers anti-mutagenic protection in *E. coli* cells exposed to U.V. radiation or 4-nitro-quinoline-1-oxide (Ohta *et al.*, 1983). In cultured Chinese hamster ovary cells, neither coumarin nor 7-hydroxycoumarin induces sister chromatid exchange (Sasaki *et al.*, 1987). Coumarin is non-teratogenic (Preuss-Uberschar *et al.*, 1984) and non-phototoxic (Prosser *et al.*, 1990).

1.6. ANALYSIS

The previous two sections have highlighted the wealth of information that has been collected on the pharmacology and toxicology of coumarin and its derivatives. This work could not have been achieved, without parallel development in the area of analysis, to provide the most sensitive and selective methods for detecting coumarin derivatives. The significant dietary, clinical, and now industrial exposure of humans to coumarins (section 1.7), with questions still

unanswered about their precise action *in vivo*, also prompted significant development of state-of-the-art procedures for the detection of these compounds.

Although numerous analyses of coumarins in plant extracts and foodstuffs have been achieved (Thompson & Hoffmann, 1988; Gamache *et al.*, 1993; Nykolov *et al.*, 1993), most analytical techniques have focussed on the determination of coumarin and its metabolites in various biological fluids (Tan *et al.*, 1976; Egan & O'Kennedy, 1992; Bogan *et al.*, 1995). The techniques have been applied to the qualitative and quantitative detection of coumarin and its derivatives in a variety of samples, including urine, plasma, serum, whole blood and tissue homogenates. Through these studies much insight has been gained into the pharmacokinetic character of the coumarin molecule, in both humans and other species (see section 1.4). This information has demonstrated the inter-species variability in coumarin metabolism, and is invaluable for choosing appropriate animal models in future clinical studies.

Currently the analytical methods available include both traditional and modern chromatographic, spectrophotometric and immunoanalytical approaches. Bogan *et al.* (1997) have reviewed this area comprehensively, but a few important procedures will be outlined below.

1.6.1. Chromatographic Methods

To date High Performance Liquid Chromatography (HPLC) has proven the most popular and versatile analytical technique for the analysis of coumarin compounds. It has been employed in the analysis of coumarin flavourings and plant extracts, as well as in *in vivo* and *in vitro* metabolic studies. Separation has been achieved with a range of stationary (C-8 and C-18) and mobile phases, and with a variety of detection systems (UV, fluorescence, electrochemical and radiochemical). Two reviews have effectively summarised this area with thorough reference to separating conditions and detection limits (Shkarenda and Kuznetsov, 1993; Bogan *et al.*, 1997).

As mentioned earlier, most analytical procedures have concentrated on the area of coumarin metabolism, and HPLC studies have yielded extensive information on the pharmacological fate of coumarin, in particular orally administered coumarin. Walters *et al.* described the first method for the separation and quantification of coumarin and its metabolites in 1980. The procedure employed was quite successful with excellent separation of a range of coumarin derivatives (o-hydroxyphenylacetic acid, 8-hydroxycoumarin, 7-hydroxycoumarin, 6-hydroxycoumarin, 5-hydroxycoumarin, 4-hydroxycoumarin, 3-hydroxycoumarin, coumarin and o-coumaric acid). However the analysis time taken was long at 65 minutes in addition to sample

pre-treatment, and in subsequent years groups have concentrated on diminishing sample preparation and analysis times. They have achieved this goal, albeit by concentrating on the principal metabolites of interest *i.e.* 7-hydroxycoumarin and its glucuronide. Various groups have developed sensitive and accurate methods for the detection of coumarin and these two metabolites in plasma and urine (Egan and O'Kennedy, 1992; Sharifi *et al.*, 1993; Bogan and O'Kennedy, 1996b). In addition to decreasing processing times, increases in detection limits have also been attained. Urinary excretion profiles in man following oral administration of coumarin have been obtained (Moran *et al.*, 1987). Inter-individual variabilities in the extent and rate of 7-hydroxycoumarin formation has also been shown using HPLC methods (Rautio *et al.*, 1992).

HPLC has also been applied in following the *in vitro* metabolism of coumarin by hepatic microsomes and liver slices from a range of species (Fentem & Fry, 1992; Lake *et al.*, 1992; van Iersel *et al.*, 1994). *In vitro* phase II glucuronidation reactions using uridine diphosphate glucuronyl transferase (UDPGT) have also been followed using HPLC separation (Killard *et al.*, 1996).

Capillary electrophoresis is the most recent addition to the array of techniques available to the analyst. Despite the advantage of rapid analysis times, it has as yet enjoyed only limited use in the coumarin analysis field. The area of concentration has again been the analysis of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide. The separation of 7-hydroxycoumarin from coumarin in urine or serum can be accomplished in less than a minute (Bogan *et al.*, 1995). Separation of 7-hydroxycoumarin from its glucuronide in urine without sample clean-up has also been achieved (Bogan *et al.*, 1996c), and the *in vitro* metabolism of coumarin has been followed (Bogan *et al.*, 1996a).

1.6.2. Spectroscopic methods

The principal spectroscopic method of interest in the analysis of coumarin is spectrofluorimetry. The original method developed by Tan *et al.* (1976), which allowed the quantitative analysis of coumarin and 7-hydroxycoumarin mixtures in whole blood, was extremely sensitive and interference-free, but did suffer from long assay times due to the intensive sample preparation involved. Even so, several groups have modified this technique and used it in metabolic studies following both the *in vivo* and *in vitro* fate of coumarin in humans (Ritschel *et al.* 1977, 1981; Rautio *et al.*, 1992; Egan and O'Kennedy, 1993a; Iscan *et al.*, 1994) and other species (Ritschel and Hardt, 1983b; Kaipainen *et al.*, 1985).

1.6.3. Immunoanalytical methods

The specific recognition of an antibody for its antigen is the basis for very selective and sensitive analytical methods, and has been exploited in many formats for the determination of coumarin and its derivatives. Immunoanalytical approaches have included ELISA-based methods for the detection of coumarin and 7-hydroxycoumarin in urine (Egan & O'Kennedy, 1993b; Reinartz *et al.*, 1996). Antibody biosensors have also been employed, with either electrochemistry (Dempsey *et al.*, 1993), or surface plasmon resonance [BIAcore®] (Keating, 1998) facilitating label-free detection of coumarin compounds in various matrices.

1.7. USES

The coumarins are well-known in terms of their use in clinical settings. However, the versatility of this family of compounds is only appreciated, when one considers the major expansion of their usage in non-clinical settings. This expansion has re-addressed concerns over the toxicity of coumarins, and prompted calls for research to probe the precise mode of action of these compounds within the body. This will establish if benefits from their widespread usage outweigh the toxicity concerns.

1.7.1. Industrial Uses of Coumarins

Coumarin, with its strong, pleasant fragrance, is one of the most extensively used synthetic aroma chemicals (Taylor, 1987). It is applied in a variety of industrial settings, the most important being the perfumery industry. It is widely used by perfumists to enhance the fragrance of many essential oils such as lavender, rosemary and citrus, in addition to a more limited use in other cosmetics such as lotions, talcum powders, *etc.* 6-methylcoumarin, with a more subtle odour, is also a popular choice.

Coumarin has enjoyed only limited use as a food additive, as a result of the 1954 FDA ban and the subsequent confusion over its toxicity, as discussed in section 1.5. A coumarin derivative, 3,4-dihydrocoumarin, has GRAS (generally regarded as safe) status, and with its sweet, caramel-like taste, is often used to fortify flavours such as vanilla, rum and caramel. Coumarin itself has been included as an odour enhancer in tobacco products and is commonly used as an odour-masker, with applications in the paints, plastics and synthetic rubber industries.

Coumarin addition to electroplating baths has been shown to cause metals (especially nickel, zinc and cadmium) to deposit with increased brightness and decreased porosity, which has resulted in frequent application of coumarin in the electroplating industries (Meuly, 1987).

The final industrial application of coumarin and its derivatives finds root in the natural U.V. absorbance and resulting fluorescence displayed by many of these compounds. 7-hydroxycoumarin, 7-hydroxy-4-methylcoumarin and 7-amino-4-methylcoumarin have all been added to soaps and detergents to act as textile fluorescent brighteners. 7-hydroxycoumarin has also been used in sunscreen lotions and creams (Meuly, 1987).

1.7.2. Analytical Uses of Coumarins

The coumarin family of compounds has also found widespread use in many analytical scenarios. In many cases this application arises from their inherent fluorescence properties, which offer increased sensitivity in detection, compared to absorbance/colourimetric measurements. This area has been extensively reviewed recently by Cooke *et al.* (1997), but the most important applications will be briefly outlined.

1.7.2.1. Use of coumarins in enzyme assays

The most prevalent use of coumarin-based compounds is their application in research and diagnostic enzymology, as moieties coupled to enzyme substrates. The initial use, developed in the 1950s and 1960s, utilised the 4-methylumbelliferone (4-MU) moiety coupled to various derivatives (glucuronide, glycoside, acetate) to assay for levels of enzyme activity (glucuronidase, glucosidase, esterase) in biological samples (Mead *et al.*, 1955; Leaback, 1961). On enzymatic hydrolysis of the appropriate substrate, *e.g.* 4-MU-Glucuronide by β-glucuronidase, the liberation of 4-MU could be followed fluorimetrically {λ_{ex} = 364nm, λ_{em} = 448nm}. The basic principle outlined here has also been applied to other coumarins, and has lead to a proliferation of sensitive peptide substrates with attached coumarin groups *e.g.* the peptide amides of 7-amino-4-methylcoumarin (AMC) and 7-amino-4-trifluoro-methylcoumarin (AFC). These commercially available substrates have been applied extensively in research and diagnostic enzymology as reviewed by Cooke *et al.* (1997).

1.7.2.2. Use of coumarins as macromolecule labelling agents

The fluorescent labelling of various molecules has been achieved with coumarin derivatives, with 7-amino-4-methylcoumarin acetic acid (AMCA) a popular choice for protein labelling (Aubry *et al.*, 1990).

Coumarin itself has been used to label oligonucleotides for use in *in situ* hybridisation experiments. These blue fluorescing probes have proved particularly useful in experiments requiring simultaneous multiple probing of sequences, and have been frequently used in conjunction with rhodamine- and fluorescein-labelled DNA [red and green fluorescing, respectively] (Wiegant *et al.*, 1993).

1.7.2.3. Use of coumarins in chemical analyses

Coumarins have found widespread use in a variety of chemical/analytical scenarios. The pH sensitivity of their absorbance/fluorescence spectra means they can be exploited as pH indicators (Dement, 1995)

Coumarin compounds are also well-established as analytical reagents in the determination of metals. Both photometric and gravimetric determination of a variety of metals has been accomplished using a range of hydroxycoumarin derivatives [*e.g.* 6,7-dihydroxycoumarin, 6,7-dihydroxy-4-methylcoumarin and 3-acetyl-4-hydroxy-coumarin] (Jain and Singh, 1967; Katyal and Singh, 1968).

Coumarin-based compounds have also been used as ion indicators, with ion complexation causing the absorbance and emission spectra to undergo extreme shifts allowing determination. Calcein Blue (4-methylumbelliferone-6-methyleneimino-diacetic acid), is commonly used as an indicator in metal ion titrations for the determination of Ba, Ca, Co, Cu, Fe(III), Ni and Sr (Wilkins, 1960; Huitnik *et al.*, 1974). Similarly, 7-hydroxycoumarin, 4-methylcoumarin and 7-hydroxy-8-amino-methylcoumarin diacetic acid can be used for the detection of Ca and Cu (Huitnik and Diehl, 1974).

The fluorescent coumarin entity is the basis for four new fluorescent probes for calcium described by Iatridou and co-workers (1994). The detection of aluminium and alkali earth metals using a crown ether-linked coumarin has also been outlined (Bourson *et al.*, 1992). A simple and

sensitive method for the detection of nitrite ion using 4-hydroxycoumarin has been accomplished (Ohta *et al.*, 1986).

Aside from their uses as metal and ion indicators, the coumarins are also valuable for the determination of other biologically and chemically important compounds. 4-Bromomethyl-7-hydroxycoumarin (BrMmC) reacts with carboxylic acids to produce ester derivatives of marked blue fluorescence (Dunges, 1977; Wolf and Korf, 1992). This reaction is useful for pre-column derivatisation of compounds prior to their separation by chromatography, leading to enhanced sensitivity in the detection of the separated compounds. An analogous reagent is BrMaC (4-Bromomethyl-7-acetoxycoumarin), and both have been used for the pre-column derivatisation of various biological samples including prostaglandins, bile acids and gibberellins in a variety of biological matrices (Crozier *et al.*, 1982; Tsuchiya *et al.*, 1982; Guldutuna *et al.*, 1993). BrMmC has also been used for the derivatisation of compounds containing imidic nitrogens of an acidic nature, and has been used for the pre- and post-column derivatisation of thiouracil and thiouridine (Iwamoto *et al.*, 1984).

Coumarin derivatives have also been employed for the derivatisation of hydroxyl moieties in biological molecules, with coumarin-3-carbonylchloride and 7-[(chlorocarbonyl)methoxy]-4-methylcoumarin (CMMC), two such examples. CMMC has been used to derivatise various steroid compounds (Karlsson *et al.*, 1985), while coumarin-3-carbonylchloride was applied to the fluorescent detection of mycotoxins (Cohen and Boutin-Muma, 1992).

Coumarins have been used extensively to tune lasers to desired wavelengths and there are a number of coumarin dyes available for this purpose. The use of these dyes is based on their broad absorption and fluorescent spectra, which allow the possibility of producing tunable lasers at a relatively low cost. Coumarin 153 has been used by Spanner and Niessner (1993) to tune a laser between 520 and 600nm, for the non-invasive determination of blood components. Coumarin 440 has been used in conjunction with other dyes in the determination of polynuclear aromatic compounds using laser-excited synchronous fluorescence (Stevenson and Tuan, 1995). Nilsson and co-workers (1995) have used the coumarin 500 dye to set their laser at 488nm for use in the fluorescence imaging of capillary electrophoretic separation of DNA samples in "real time".

1.7.3. Clinical Uses of Coumarins

The diverse pharmacological and physiological effects of the coumarin family of compounds have been referred to previously (see Table 1.1). In the clinical arena, it is the activation of the immune system by coumarin, which has led to its use in a variety of disease states, including High Protein Oedemas, chronic infections and immune system disorders. Other coumarin derivatives have proven important in anti-coagulation therapy, and more recently as potential HIV therapies.

1.7.3.1. High Protein Oedema (HPO)

The lymph system is responsible for drainage of interstitial fluid within our tissues. If excess fluid is present, as a result of injury or lymph vessel blockage, the transport capacity of the lymph system is compromised, and oedema (swelling of tissue) develops. There are numerous detrimental effects of oedemas to the patient - apart from the burden of pain and swelling, oedemas can cause severe damage to the involved tissue. Often there is extensive injury to blood vessels, with fibrin deposition and fibrosis common effects. The oedema interferes with the metabolism of the tissue cells and reduces oxygen transport, resulting in problematic wound healing (Casley-Smith and Casley-Smith, 1986).

In the case of high protein oedemas (HPO), there is an accumulation of protein in the tissue following trauma or inflammation, with resulting permeability of the capillaries causing water leakage in the tissue spaces. If HPOs continue for some weeks they cause chronic inflammation with many associated problems: increased numbers of small blood vessels and lymphatics with damaged vessel walls cause increased leakage, exacerbating the problem; excess fibroblasts and protein in the tissues provide an excellent incubation medium for microbial infections to fester; and excess macrophage infiltration, though initially advantageous, eventually cause problems as they become inactive and filled with lipid. Many disease states are associated with high protein oedemas, ranging from extremely severe and chronic (*e.g.* lymphoedema and elephantiasis) through more common and acute forms (*e.g.* burns, accidental and surgical traumas). All forms have been shown to benefit from benzopyrone treatment (Casley-Smith and Casley-Smith, 1997).

Coumarin and numerous other benzopyrones have been tested in high protein oedema, and all have been shown to successfully reduce the swelling. Casley-Smith and co-workers have carried out most of the laboratory work in this area, and they have demonstrated that the

benzopyrones act by increasing normal proteolysis in the oedematous tissue. In a succession of experiments, they demonstrated that this proteolytic increase arises from an increase in both the numbers and activity of macrophages in the tissue (Piller and Casley-Smith, 1975; Piller 1977a, 1977b, 1977c, 1978). Proteolysis by macrophages results in a rapid decrease in oedema, as the high molecular weight proteins responsible for water retention are broken down into fragments and removed by capillaries. Unlike diuretic therapy, which acts only symptomatically to eliminate oedema fluid, coumarin treatment acts to eliminate the source of the oedema (accumulated plasma proteins) thus providing permanent reduction of swelling. Casley-Smith and colleagues further substantiated the crucial role of the benzopyrone/macrophage interaction by demonstrating that selective killing of the macrophages using silica prevented the oedema reduction observed with coumarin (Casley-Smith *et al.*, 1978).

There have been over 300 clinical trials investigating the effects of coumarin and benzopyrones in oedematous presentations (Casley-Smith and Casley-Smith, 1986). In addition to laboratory-based work, Casley-Smiths team has been very closely involved with many of these clinical investigations. Full details of these trials have been recently reviewed by John and Judith Casley-Smith (1997), with respect to lymphoedema treatment with either coumarin alone, or a combined coumarin/troxerutin treatment (commercially available as Venalot®). All trials have shown that coumarin either alone, or in combination with other benzopyrones, leads to both objective (circumference and volume), and subjective (tension, heaviness, fullness, pain) improvement, and has lead to the introduction of coumarin as a lymphoedema treatment in a number of western and developing countries. A further potential area of clinical application is that of thermal wounding treatment, and a colleague of Casley-Smiths, Neil Piller, has shown that coumarin can consistently reduce the duration and damage of thermal wounding (Piller 1997). To date however, no clinical trials have been completed in this area.

1.7.3.2. Chronic Infections

In addition to its stimulatory effect on macrophages, coumarin has been shown to activate other cells of the immune system. This has lead to its classification pharmacologically as a biological response modifier. Coumarin is known to enhance the response of peripheral blood lymphocytes to phytohemagglutinin, but not to pokeweed mitogen or concanavalin A *in vitro* (Marshall and Hollingsworth, 1987). It also increases the ratio of T_H : T_S cells and stimulates natural killer cells *in vitro* (Thornes & Lynch, 1983; Thornes, 1997). More recently coumarin has been shown to potentiate the release of cytokines from monocytes in response to lipopolysaccharide stimulation (Stuhlmeier *et al.*, 1991; Zlabinger *et al.*, 1993).

As a result of these effects coumarin has been used clinically to treat chronic infections such as brucellosis (Thornes, 1983). In chronic brucellosis *Brucella abortis* infects macrophages, thus eluding the immune response. When immunostimulatory drugs such as coumarin are administered, the symptoms of chronic brucellosis (chronic tiredness, intermittent fever and night sweats, depression, anxiety) disappear. Coumarin was found by Thornes (1983) to be the most effective and least toxic immunostimulant. It was also effective at augmenting and maintaining IgA levels (normally suppressed in chronic brucellosis) and restoring delayed hypersensitivity reactions (Thornes, 1983). These results have encouraged the use of coumarin in other chronic infections such as mononucleosis, mycoplasmosis, toxoplasmosis and Q fever.

A combined coumarin/troxerutin preparation has been tested on patients with chronic tuberculosis, in an open trial in conjunction with normal therapies. The trial was established to determine if the benzopyrone preparation could decrease the intensity of fibrosis surrounding the tubercles in patients, thus allowing better antibiotic penetration into the lesions. In a nine-year follow-up it was found that scar formation was reduced in coumarin-treated patients (Seeliger, 1988)

1.7.3.3. Anti-Coagulant Therapy.

The recognition of the anti-coagulant activity of certain coumarin derivatives dates back to a report by Butt *et al.* (1941), which concerned studies on the toxic agent of spoiled sweet clover hay which causes serious hemorrhagic conditions in cattle. The responsible agent was identified as a coumarin derivative, dicoumarol (3,3'-methylenebis-4-hydroxycoumarin). Subsequent studies by Link and other groups (Chmielewska and Cieslak, 1958; Link, 1959) concluded that the minimum structural requirements for anti-coagulant activity were an intact 4-hydroxycoumarin with a substituent at position 3. In 1944 Ikawa and colleagues synthesised a very potent agent, warfarin (3-(α-acetonylbenzyl-4-hydroxy-coumarin), which quickly underwent trials and was recommended for human use.

In vivo, the coumarin anti-coagulants act by blocking the synthesis of four blood factor proteins essential to the blood clotting process. The factors (Factor II, VII, IX and X) are necessary components of the prothrombin complex, and vitamin K is essential for their correct post-translational modification. It is known that warfarin and other coumarin anti-coagulants interfere with this vitamin K-dependent process, by inhibiting the formation of vitamin K from its precursor (Vitamin K epoxide). This interference causes abnormal processing of blood factors,

leading to accumulation of abnormal prothrombin proteins called PIVKAs, with a resultant depressed clotting activity.

The coumarin anti-coagulants are thus employed therapeutically to depress blood clotting. They have been applied in the treatment of venous thromboembolism, acute myocardial infarction and threathened stroke. Warfarin (also known as coumadin) is particularly extensively used in the clinical treatment of all thromboembolic disorders.

1.7.3.4. AIDS Therapies

Recently, studies have been published highlighting the potential for using coumarin derivatives in AIDS treatment regimes. A single dose of coumarin derivatives (warfarin, 4-hydroxycoumarin and 7-hydroxycoumarin) exhibited a specific inhibition of HIV replication in lymphocytes (Bourinbaiar *et al.*, 1993a). The investigating group examined the effect of these three coumarins on the *in vitro* infectivity of the HIV virus, in MOLT-4 lymphocytes, and found that all decreased infectivity dose-dependently. In addition, the transmission of the virus via cell-cell interactions was decreased by the presence of coumarins. This occurred at low drug dosages and was observable even five days post-infection. In further experiments, lymphocytes chronically infected with the HIV virus, were exposed to coumarins *in vitro*, and a marked decline in *gag* p24 release and reverse transcriptase activity was observed, with a 50% reduction occuring at 10^{-9}M and 10^{-6}M, respectively (Bourinbaiar *et al.*, 1993a, 1993b). The observed activities were almost identical for all three compounds tested, suggesting that a basic hydroxycoumarin structure was essential for the observed anti-viral effect.

Tummino *et al.* (1994) showed that warfarin inhibits the HIV retroviral protease in a competitive manner in the micromolar concentration range. Screening of further active 4-hydroxycoumarin derivatives is ongoing (Thaisrivongs *et al.*, 1994; Zhao *et al.*, 1997). Two advantages of warfarin derivatives over other promising HIV therapy candidates are their non-toxic effects on cells and their well-documented pharmacology, both of which facilitate clinical trials.

1.8. COUMARINS IN CANCER

1.8.1. Current Cancer Therapies

Over the last twenty-five years scientists have waged "war on cancer", with many victories – they have advanced the public understanding of the disease, battled to discover early

diagnostic markers and invented a weaponry of effective anti-cancer drugs. Despite these successes, cancer is still one of the most common and vicious causes of death globally – this year, hundreds of thousands of otherwise healthy people will die from some form of cancer.

Anti-cancer drugs have traditionally been targeted to damage the aberrantly dividing cell by interrupting the cell division process (Carter *et al.*, 1989). Reagents used include DNA intercalating agents (*e.g.* adriamycin), DNA cross-linking agents (*e.g.* cis-platin), topoisomerase inhibitors (*e.g.* campothecins), cytoskeleton-disrupting agents (*e.g.* vinblastin) and anti-metabolites (*e.g.* mercaptopurine). These drugs though effective, are cytotoxic, and thus exhibit severe side-effects, particularly on normal proliferating tissues such as the hematopoietic system. Often combination therapies, whereby several cytotoxic agents are combined in the treatment regime, offer better results with fewer toxic side-effects, as they are carefully regulated to allow recovery of normal, but not malignant cells, from drug exposure (Carter *et al.*, 1989).

Currently, chemotherapy, radiotherapy and surgery combined, offer the best outcomes for cancer patients, and treatment combinations have been successfully applied to particular cancer types, for example, Hodgkins lymphoma, testicular cancer and various leukemias. Otherwise success has been limited, and the majority of cancers remain refractory to treatment. As existing therapies are unable to extend the life expectancy of patients, the search for new, effective approaches, to supplement current ones has proved relentless.

The interest in coumarin and 7-hydroxycoumarin as anti-cancer agents, arose from reports (discussed below) that these agents had achieved objective responses in some patients with advanced malignancies. To date, the particular mode of action of these agents in cancer cells is obscure, although various possibilities have been hypothesised, and these are also outlined below.

1.8.2. Coumarin in Malignant Melanoma

Early diagnosis of malignant melanoma facilitates surgical removal of the primary lesion and achieves a good prognosis. However, if the lesion progresses, the risk of recurrence becomes serious and represents a major challenge to the oncologist, as no satisfactory treatment for recurrent malignant melanoma currently exists. In the past malignant melanoma has been shown to respond well to immune cell manipulation *e.g.* macrophage stimulation *in vivo* with B.C.G. has been used as an adjuvant therapy, and recently, immunotherapies such as IL-2 and interferon have been proposed as treatment strategies.

Original work with coumarin derivatives in the treatment of melanoma focussed on the use of warfarin as a maintenance therapy. This compound was known to inhibit tumour spread, and to stimulate granulocytes, lymphocytes and macrophages (Thornes *et al.,* 1968). In 1980, Maat illustrated that the decrease in tumour metastases due to warfarin was macrophage-dependent. This data prompted Thornes to assess the potential application of coumarin, the parent compound of warfarin, as an adjuvant therapy in melanoma. His rationale was manifold – like warfarin, the *in vivo* actions of coumarin were also known to be macrophage-derived (Piller, 1978); coumarin was non-toxic and conveniently administered; unlike warfarin, coumarin had no anti-coagulant activity, and previous administration by Riordan had resulted in subjective improvement in cancer patients (Thornes, 1997).

Coumarin was first compared to warfarin in a small-scale trial on patients with stage IB [lesions > 1.70mm thickness] and stage II melanomas after primary resection. Five patients were anti-coagulated with warfarin and six were given 25mg coumarin daily. During the first year, coumarin matched warfarin in its therapeutic effectiveness, with one patient in each group recurring (Thornes, 1993).

In a small-scale study, Thornes added cimetidine (to reduce T-suppressor cells) to the coumarin regime, in melanoma patients with stable disease. Addition of cimetidine resulted in 5 objective regressions in 10 patients, but was only of benefit to patients who had remained stable for greater than two years on coumarin therapy alone (Thornes, 1982). In a similar study by Marshall *et al.* (1989a), 3/22 responses were obtained for a combined coumarin/cimetidine regime. All responses were correlated with low tumour burdens (Marshall *et al.,* 1989a).

In 1984, a placebo-controlled, randomised, double-blind trial (50mg coumarin alone, daily for 2 years) was established, with twenty-seven patients admitted. All patients were treated with standard surgical excision of the melanoma lesions prior to coumarin treatment. In 1987 the trial results were published, and showed 10 recurrences in the control (N=14), but only 2 in the treated group (N=13). Since the trial termination, two more patients in the treated group have recurred, at two and four years (Thornes, 1997).

Only one further trial in melanoma patients has yielded results: an open trial in Australia found a dosage of 300mg of coumarin daily to be of no benefit to treated patients (Thornes, 1997). Thornes has continuously stated the importance of optimal dosing in the establishment of trials, believing that high doses of coumarin may inhibit rather than stimulate the immune system.

Such a hypothesis may explain these conflicting results, but it is evident that further dosing trials are required to determine the real benefit of coumarin in malignant melanoma therapy.

1.8.3. Coumarin in Renal Cell Carcinoma

The clinical course of renal cell carcinoma (RCC) has been well-documented, with long-lasting stable periods and rapid tumour growth its principal features. Surgery remains the standard patient care, however lesions usually recur, with the lungs, liver and bones the common sites of secondary occurrence. Prognostically, long intervals between nephrectomy and metastasis development, lung metastases only and no bone involvement are favourable factors (Stahl *et al.*, 1992). Currently there is no curative therapy for renal cell carcinoma, and in the past twenty years neither hormonal therapy, nor chemotherapy have improved survival rates for patients with metastatic lesions. Spontaneous tumour regressions have been reported at a rate of 1-7%, which has aroused interest in the potential use of immunotherapies as treatment modalities. In particular, IFN-α and IL-2 have received most attention, and they have demonstrated objective responses of 11-17.5% [IFN-α only], 0-33% [IL-2 only] and 0-50% [IFN-α/IL-2 combined] in RCC patients (Stahl *et al.*, 1992). Combination of IFN-α with cytotoxic drugs such as vinblastine has also yielded good results [remission range 10-45%, and tumour stabilisation in 30% of patients] (Stahl *et al.*, 1992). However, further drugs, which alone, or in combination with other agents might exhibit higher response rates and longer response durations, are constantly being sought.

Interest in the coumarin family of compounds as such an alternative, stemmed from reports by Thornes, of the immunomodulatory activity of coumarin and its utility in malignant melanoma (Thornes, 1982). With the clear response of RCC to immunotherapy regimes, Marshall *et al.* (1987b) set about investigating the clinical activity of coumarin in renal cell carcinoma patients. They did so initially by applying a treatment regime courtesy of Thornes [coumarin at 100mg/day oral dosage, with the addition of cimetidine 4 X 300mgs/day from day 15] to RCC patients. This preliminary study yielded some interesting results, with 14 objective results among 45 patients with metastatic RCC, and almost no toxic side effects. Validation of this anti-tumour activity was further demonstrated by other investigators (Dexeus *et al.*, 1990; Kokron *et al.*, 1991).

Following this success, it became obvious that additional information regarding doses and toxicities was required, and Marshall and colleagues implemented a phase I trial to define the

maximally tolerated dose, and dose-limiting toxicities of coumarin and cimetidine. 54 patients with a variety of advanced malignancies were admitted to this trial, with 3 patients each, given one coumarin dose in the range 400mg-7000mg. Coumarin was administered as a single, oral dose continuously during the trial, with cimetidine [4 doses of 300mg daily] added to the dosage regime on day 15. All coumarin doses were well-tolerated, with the most common side-effect of nausea attributable to the intense aroma of coumarin. Objective responses were observed in 7 patients, all with renal cell carcinoma, these responses being observed across a range of coumarin doses [600-5000mgs] (Marshall *et al.,* 1991a).

Kokron *et al.* (1993) questioned the significance of cimetidine in the observed responses in a 1989-1993 study. They showed similar response rates [3/19 for coumarin, and 3/25 for coumarin/cimetidine combined], suggesting the dispensability of cimetidine. However, patients in the cimetidine group did have longer survival times (294 days vs 191 days) compared to coumarin-only recipients (Kokron *et al.,* 1993).

Since completion of their 1991 phase I trial, Marshall and colleagues have proceeded to conduct a trial of coumarin alone [1g daily] in the treatment of RCC patients. A double-blind randomised trial was implemented with patients receiving daily oral doses of either coumarin [1g daily; 10 tablets] or placebo [10 tablets]. Patients were evaluated monthly for anti-tumour response. Dosage was continued until progression of the disease was evidenced, after which placebo patients were crossed-over to receive coumarin, while patients on coumarin were removed from the study and followed for survival data. Although complete results from this trial remain to be published, it is known that objective responses were seen in patients receiving coumarin, while no responses were observed in placebo patients (Ebbinghaus *et al.*, 1997).

Only one trial has been completed which compares the efficacy of coumarin with existing therapies. Sagaster *et al.* (1995) compared the efficacy of a combined coumarin/cimetidine/IFN-α combination therapy to IFN-α monotherapy in RCC patients. The results showed no improvement in response rates or survival by combining coumarin with IFN-α.

Ebbinghaus *et al.* (1997) have critically evaluated the current status of coumarin trials in renal cell carcinoma, and highlighted future trial requirements. It is quite evident from their summary that a mass of work remains to be achieved, before any clear decision on the effectiveness of coumarin in renal cell carcinoma can be reached.

1.8.4. Coumarin in Prostate Cancer

Prostate cancer is characterised by a very slow growth rate and a wide biological variability, especially with regard to hormonal sensitivity (Bosland, 1991). These two traits have curbed attempts at curative treatments for patients, as most effective chemotherapeutic drugs rely on fast growth kinetics in the tumour mass, and due to differential hormonal dependencies hormonal therapy (androgen deprivation) is not effective in all presented cases. At present, early detection, and removal of clinically significant tumours by surgery or radiation, has been the focus of clinical strategies: this can lead to "cure", as evidenced by undetectable prostate specific antigen (PSA) levels and organ confinement. However, patient survival is dependent on metastases [principal sites are regional lymph nodes and bone], and advancement often occurs, with palliative care the only therapeutic option. Approximately 80% of patients with metastatic symptomatic prostate cancer respond to androgen deprivation [average life expectancy = 43 months], the other 20% have hormone-independent disease and survive an average of 7-9 months (Mohler *et al.*, 1997). Eventually, however, almost every prostate carcinoma that initially regressed on androgen deprivation will relapse into a hormonal-insensitive state and grow in the absence of androgen. Evidently, better therapeutic approaches to control both metastases and hormone-insensitive prostate carcinomas are required.

One suggested approach has been augmentation of normal immune cell functions, in an attempt to curb the spread of micrometastatic disease. As coumarin had previously appeared to exert immunomodulating effects in other cancers, a small-scale study to test the efficacy of coumarin in prostate cancer was set up (Marshall *et al.*, 1990). 14 patients received a single oral dose of 100mg coumarin daily continuously during the trial, with 300mg of cimetidine 4 times daily added to the regime from day 15 onwards. Both drugs were continued until disease progression was observed. No objective responses were observed, but a significant subjective improvement in bone pain was noted in patients, which prompted further studies.

A phase I trial involving 40 patients with metastatic, hormone-naïve or hormone-refractory prostatic cancer was conducted (Mohler *et al.*, 1992). Participants were administered 3g of coumarin daily, and evaluated for toxicity and anti-tumour responses. 3 partial responses occurred, all in patients with low tumour loads. One responder remained with 3 responsive bone metastases and stable PSA levels for 7 years following the trial.

On the basis of these results a US FDA-supervised, multi-centre study has been undertaken, to examine the effect of coumarin in metastatic (stage M1) prostate cancer. The

study will investigate the effect of 1g coumarin daily on 80 patients with either hormone-naïve or hormone-independent metastatic disease [Group A]. Additionally, 80 further patients with positive signs of micrometastases following prostatectomy, will partake in a randomised trial, receiving either placebo or 1g coumarin daily [Group B]. In the most up-to-date report, 24 patients have enrolled in group A, and only 1 has not progressed after 54 months. In Group B, with 40 patients participating to date, 8 and 9 patients in each group [coumarin and placebo, respectively] have progressed [PSA evaluations]. To date no patient on this trial has developed symptoms, local clinical progression or bone metastases (Mohler *et al.*, 1997). Final trial results will prove very important in assessing the value of coumarin as a future therapy for prostate cancer patients.

1.8.5. Mode of Action

The precise mode of action of coumarin *in vivo,* which accounts for the described clinical responses in patients with advanced malignancies, remains obscure. Direct anti-tumour properties, immunomodulatory properties and chemopreventive properties have all been cited as possible explanations. Due to the pleiotropic effects of coumarins and other polyphenols (*c.f.* Table 1.1) *in vivo*, it is probable that all are valid proposals. However, in order to understand and exploit the multiple effects of any drug correctly, it is essential to locate its precise cellular or molecular target(s).

This thesis aimed at achieving this with respect to coumarin and its derivatives. This was implemented through further examination of direct and immunological effects of coumarin derivatives in human *in vitro* models, in Chapters 5 and 6, respectively. Detailed accounts of the direct anti-tumour effects, and immunological effects, of coumarin are therefore included in these chapters, and only a brief discussion of these will follow below.

1.8.5.1. Direct Anti-tumour Effects

In recent years interest in studying the direct effects of coumarin has stemmed from clinical observations of its efficacy in cancer patients. Coumarin and its principal human metabolite, 7-hydroxycoumarin, have been shown to reversibly inhibit the *in vitro* growth of a range of cell lines, in a dose- and time-dependent manner (Moran *et al.*, 1993; Siegers & Bostelmann, 1993; Marshall *et al.*, 1994). Some cell lines *e.g.* renal, breast, prostate, glioblastoma and leukemic, have been shown to be more sensitive than others, to these effects.

This direct effect is cytostatic in nature, with treated cells illustrating decreased synthesis of DNA, RNA and protein (Marshall *et al.*, 1994). In all cases, 7-hydroxycoumarin has been reported to be more potent in its effects than coumarin at equal concentrations, which correlates well with the belief that coumarin is a pro-drug for the active agent 7-hydroxycoumarin.

The mechanisms underlying the growth-inhibitory effects remain unclear, although various hypotheses have been presented. One proposal is that coumarin may exert its effects by down-regulating autocrine loops in growing cells. Some indirect evidence for this lies in the fact that both coumarin and 7-hydroxycoumarin inhibit the growth of glioblastoma cell lines that are heavily dependent on a PDGF autocrine loop (Seliger, 1993). More direct evidence backing this theory, is clear from the observation that 7-hydroxycoumarin reversibly and selectively downregulates the expression of PDGF mRNA in glioblastomas (Seliger & Pettersson, 1994a).

Another suggestion is that coumarin modulates the progression of the cell cycle through accumulation of coumarin-treated cells in G_0/G_1 phase, as shown by Conley and Marshall (1987), using flow cytometry. It is thought that coumarin may exert this effect by affecting the expression of the *ras* gene, which is important for the $G_1 \rightarrow S$ transition. Zanker (1993) showed that 7-hydroxycoumarin could alter the level of Ha-*ras* expression in the RBA cell line. Following this Kahn *et al.* (1994) showed that coumarin could decrease ras expression, and simultaneously decrease the number of cells entering S phase, in steroid-inducible cells transformed with the *ras* gene.

One final theory is that coumarin acts by inducing cell differentiation, as evidenced by timed kinetic analysis of glioblastoma cells which had been exposed to 7-hydroxycoumarin. 7-hydroxycoumarin induced increases in I-CAM and GFAP expression, with a maximum reached after 10 days exposure to the drug (Seliger and Pettersson, 1994b).

1.8.5.2. Immunomodulatory Effects

The immunomodulatory effects of coumarin and its derivatives *in vivo* have been mentioned previously with regard to the clinical application of coumarin in oedemas and chronic infections (sections 1.7.3.1 and 1.7.3.2.). Briefly, coumarin activates macrophages and natural killer cells (Piller, 1978; Thornes, 1997). It also increases the ratio of T_H : T_S cells *in vitro* (Thornes & Lynch, 1983). Coumarin has been shown to enhance the expression of DR and DQ antigens on peripheral blood monocytes both *in vitro* and *in vivo*, a situation which is consistent with an activated cell state (Marshall *et al.*, 1989a, 1991b). Coumarin also increases the release

of inflammatory cytokines from monocytes in response to lipopolysaccharide stimulation (Stuhlmeier *et al.*, 1991). It is believed therefore, that coumarin augments the activation level in the cells of the immune system, particularly those of the monocyte/macrophage lineage, which may cause them to become tumouricidal *in vivo*.

1.8.5.3. Chemopreventive Effects

Chemoprevention of cancer involves controlling the onset and progression of cancer, through administration of compounds which curb the effects of carcinogenic agent exposure. At present 30 classes of chemicals with cancer chemopreventive properties have been described, and include phenolic anti-oxidants, flavonoids, indoles, cinnamates and coumarins. In animal cells, it is believed that their anti-carcinogenic properties arise due to their ability to enzymatically induce the inactivation of reactive electrophilic carcinogen forms. This is achieved by promoting increased activities in phase II xenobiotic-metabolising enzymes *e.g.* UDP-glucuronidases, glutathione-S-tranferases (GST), quinone reductases.

Coumarin protects against hydrocarbon-mediated carcinogenesis (Feuer *et al.*,1976; Wattenberg *et al.*, 1979), and causes GST elevation in rodent tissues (Sparnins *et al.*,1982). It also raises quinone reductase levels in a murine hepatoma cell line (DeLong *et al.*, 1986). However, the importance of these chemopreventive effects in human situations is debatable, when inter-species metabolism differences are considered.

1.9. CHAPTER SUMMARY

In this chapter the coumarin family of compounds were reviewed, as a prelude to the work described in this thesis. The occurrence, sub-classification and biosynthesis of coumarins were described. Particular focus was given to the metabolism and toxicology of the parent compound, coumarin, with the importance of coumarin metabolites addressed. Many of these metabolites, and other coumarin derivatives are very important, both clinically and industrially, and the uses of coumarins in these settings were summarised. Finally, a review of the use of coumarin itself, in cancer therapy, the focus of this thesis, was given.

1.10. AIMS OF THIS THESIS

The aim of this thesis was the examination of the effects of coumarin compounds on human cells, with determination of the precise anti-tumour mode of action of coumarin, a primary goal. This aim was addressed specifically as follows:

1. Determination of the effect of coumarin and hydroxycoumarins on the growth, metabolism and metastatic potential of human tumour cells. This investigation examined the properties of a number of hydroxycoumarins, with relevance to various anti-tumour mechanisms (anti-proliferative (cytostatic vs cytotoxic), anti-metastatic *etc.*).

2. Examination of the effect of two hydroxycoumarins, 7-hydroxycoumarin and esculetin on signalling pathways within human tumour cells. This aimed at identifying a precise cellular target/mechanism by which coumarin achieves its anti-proliferative effect.

3. Examination of the effect of coumarin compounds on monocyte function, using a model monocyte system. Through this investigation, the immunomodulatory effect of coumarins, believed to be important as an anti-cancer mechanism, was addressed.

Chapter 2

Materials & Methods

2.1. MATERIALS

2.1.1. SOURCE OF COUMARIN COMPOUNDS.

Coumarin was kindly donated by Schaper and Brummer, Salzgitter, Germany. 7-hydroxycoumarin and esculetin were purchased from Sigma Chemical Co., Poole, Dorset, England. 6-hydroxycoumarin was synthesised by Dr. Oliver Egan, BEST Centre, Dublin City University, Dublin 9, Ireland, as outlined in section 2.2. with chemicals purchased from Aldrich Chemical Co. Ltd., The Old Brickyard, New Road, Gillingham, Dorset, England.

2.1.2. REAGENTS AND CHEMICALS

All chemicals used were analytical grade unless otherwise specified. All reagents, unless otherwise specified, were purchased from Sigma Chemical Co., Poole, Dorset, England. Calbiochem-Novabiochem, Boulevard Industrial Estate, Beeston, Nottingham, England supplied Tyrphostin A25, suramin and H-7.

Cell culture media and supplements were obtained from Sigma Chemical Co., and sera were purchased from Life Technologies, 3 Fountain Drive, Inchinnan Business Park, Paisley, Scotland. Cell lines were purchased from American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia, U.S.A. (A431, 28SC), European Collection of Animal Cell Cultures, Porton Down, Salisbury, U.K. (MCF-7, PC-3, T-24, A-549, HL-60), or obtained from DCU stocks (SW480, SW620, NIH3T3).

Anti-phosphotyrosine antibodies were purchased from Sigma Chemical Co. (address above), and Boehringer Mannheim U.K., Bell Lane, Lewes, East Sussex, U.K. Alkaline phosphatase-labelled antibodies were purchased from Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, U.S.A. Bromo-chloro-indolyl-phosphate and nitroblue tetrazolium, for Western blotting, were also supplied by Promega Corporation. Nitrocellulose was purchased from BDH Chemicals Ltd., Poole, Dorset, England.

Interferon-γ, luminol and LDH Assay kit were supplied by Boehringer Mannheim, (address above). Greiss reagents were purchased from Molecular Probes Europe, PoortGebouw, Rijnsburgerweg 10, 2333AA, Leiden, The Netherlands. Bichonic agent (BCA) reagent for protein

determination, and the non-radioactive tyrosine kinase kit were purchased from Pierce Chemicals, 3747 North Meridian Road, PO Box 117, Rockford, IL 61105, U.S.A.

2.1.3. PLASTIC CONSUMABLES

Sterile plastic disposables for cell culture (flasks, plates. pipettes etc.) were purchased from Corning-Costar Corporation, 45 Nagog Park, Acton, MA 01720, U.S.A. Eppendorf tubes and micropipette tips were purchased from Sarstedt Ltd., Sinnottstown Lane, Drinagh, Co Wexford, Ireland. 15- and 50-ml polypropylene centrifuge tubes were purchased from Greiner Labortechnik, Greiner GmBH, Maybachstrasse, PO Box 1162, D-7443 Frickenhausen, Germany.

96-well ELISA plates were purchased from NUNC, Postbox 280, Kamstrup DK, Roskilde, Denmark. White cliniplates for luminometric assays were supplied by Labsystems Affinity Sensors Ltd., Saxon Way, Bar Hill, Cambridge, U.K.

All consumables for the Cytosensor Microphysiometer were supplied by Molecular Devices Ltd., Unit 6, Raleigh Court, Rutherford Way, Crawley, West Sussex, England.

2.1.4. EQUIPMENT

All pH measurements were performed on a Sentek pH Electrode (p56/BNC) from Sentek, Unit 6-7 Crittall Court, Crittall Drive, Braintree, Essex, England. I.R. analysis was performed on a Nicolet I.R. spectrometer supplied by the Nicolet Instrument Corporation, 5225-1 Verona Rd., Madison, WI 53711, U.S.A. NMR analysis was performed on a 400MHz AC NMR Spectrometer from Brucker, Banner Lane, Coventry, England.

Cell culture was performed in a Holten 2448K laminar air-flow cabinet supplied by Holten Laminair A/S, Gydevang 17, DK 3450 Allerod, Denmark. Incubations were carried out in a Heraeus CO_2 Incubator from Heraeus Instuments Inc., 111-A Corporate Blvd., South Plainfield, N.J. 07080, U.S.A. Cells were viewed using a Nikon Diaphot phase contrast microscope from Nikon Corporation, 2-3 Marunouchi 3-chome, Chiyoda-ku, Tokyo, Japan.

Centrifugation was performed on the centrifuges from Heraeus Instruments Inc., 111-A Corporate Blvd., South Plainfield, N.J. 07080, U.S.A (appropriate models are specified in the methods sections). Ultracentrifugation was performed on a Beckman L8-70M Ultracentrifuge, supplied by Beckman Instruments, High Wycombe, Bucks HP11 1JV, U.K.

The Cytosensor Microphysiometer was purchased from Molecular Devices Ltd., Unit 6, Raleigh Court, Rutherford Way, Crawley, West Sussex, England.

Absorbances from MTT, LDH, BCA, ELISA and Greiss assays were measured using a Titertek Multiscan plate reader produced by Flow Laboratories Ltd., Woodcock Hill, Harefield Rd., Richmansworth, Hertsfordshire, England. Luminometric measurements were performed on a Luminoskan RS luminometer from Labsystems Affinity Sensors, Saxon Way, Bar Hill, Cambridge, England. Spectrophotometric measurements were made on a UV-160A spectrophotometer from the Shimadzu Corporation, 1 Nishinokyo-Kuwabaracho, Nakagyo-ku, Kyoto, Japan.

Protein Electrophoresis was performed on apparatus supplied by the ATTO Corporation, 2-3 Hongo 7-chome, Bunkyo-Kui, Tokyo, Japan. Western blotting was accomplished using a Transblot Electrophoretic Transfer Cell (170-3910), from Bio-Rad Laboratories, 3300 Regatta Boulevard, Richmond, CA 94804, U.S.A. Gel Analysis and photography was performed on a Ultraviolet Products Gel Documentation System Image Store 7500 version 7.01, supplied by Medical Supply Company, Damastown, Dublin 15, Ireland. Densitometric analysis was achieved with a Biorad GS670 densitometer with Biorad Software Molecular Analyst, version 1.7.

2.2. METHODS

2.2.1. PREPARATION OF 6-HYDROXYCOUMARIN

6-hydroxycoumarin (6-HC) was synthesised by Dr. Oliver Egan, BEST Centre, Dublin City University, Ireland, using the outlined method supplied by Dr J.R. Fry, University of Nottingham, U.K. (Personal Communication). The method was a modification from that reported by Murayama *et al.* (1972) for the synthesis of suberosin and osthol. The reaction scheme is outlined in Figure 2.1.

2.5g of 2,5-Dihydroxybenzaldehyde was dissolved in 20mls of ethanol. Following the addition of 5.5mls of diethylmalonate and 5 drops of piperidine, the mixture was refluxed for 6 hours. The mixture was allowed to cool slightly, an equal volume of water was added and the solution allowed to stand at 4 °C overnight. The resulting yellow crystals (putative 3-carboethoxy-6-HC) were collected by vacuum filtration.

The putative 3-carboethoxy-6-HC was dissolved in 50 mls of hot ethanol and the solution maintained at 65 °C (just below boiling), prior to the addition of 30mls of 10%(w/v) sodium hydroxide. Heating with stirring was continued for 10 mins, after which the mixture was cooled on ice and acidified to pH 3 with concentrated HCl.

The precipitate (approximately 5g of putative 3-carboxy-6-HC) was collected by vacuum filtration, and refluxed with 40mls of 30% (w/v) aqueous sodium bisulphite solution, until no further CO_2 evolved. 30mls of concentrated sulphuric acid was added dropwise and refluxing continued for 1 hour. After cooling, the mixture was extracted three times with diethylether, the pooled organic layers were dried and the solvent evaporated to give crude 6-hydroxycoumarin. This was decolourised by refluxing with activated charcoal for 1 hour. This solution was filtered hot and, on cooling, crystals of 6-hydroxycoumarin (m.p. 242-245 °C) precipitated. The purity and identity were confirmed by I.R. and NMR analysis.

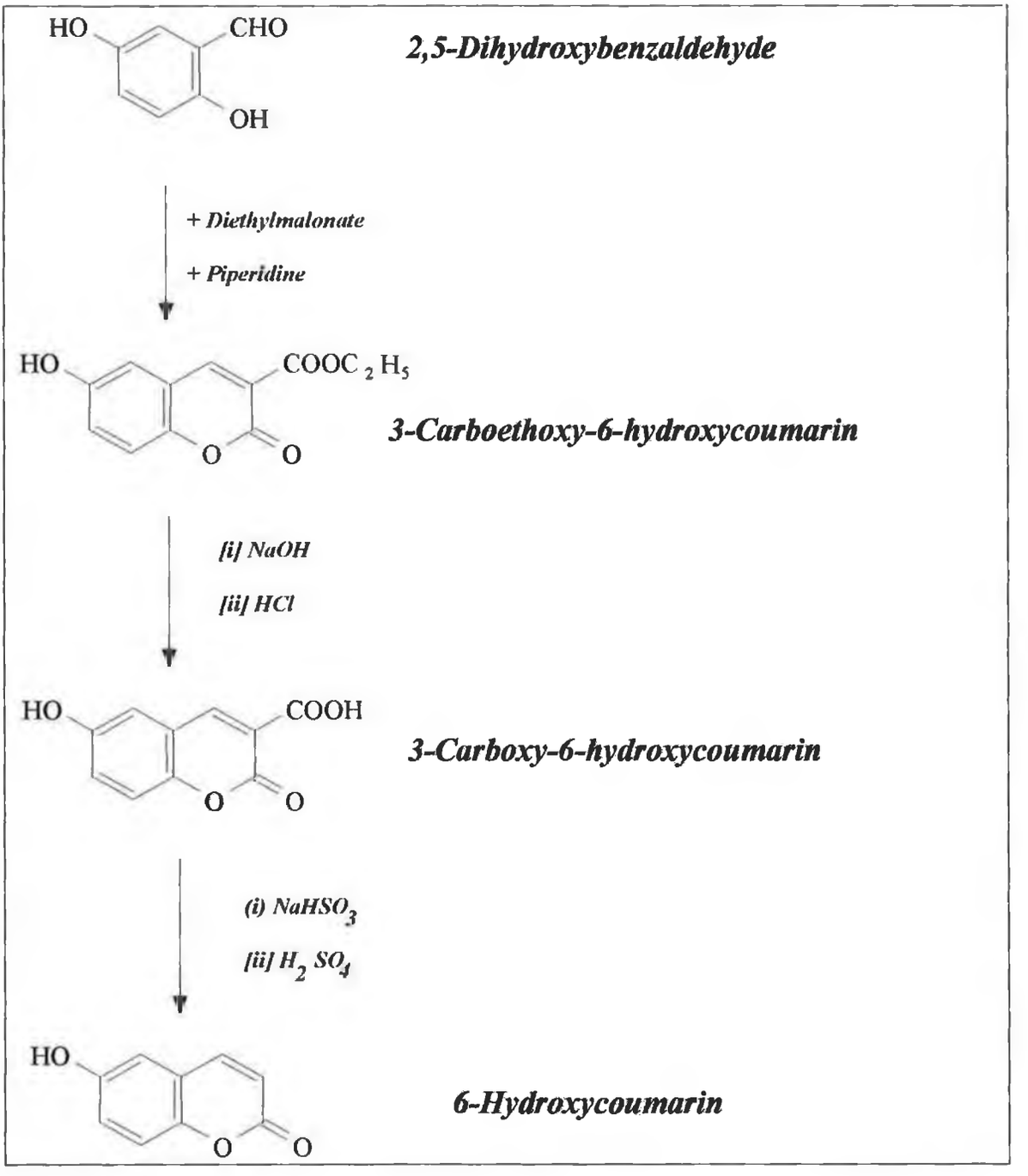


Figure 2.1: **Reaction scheme for the synthesis of 6-hydroxycoumarin from 2,5-dihydroxybenzaldehyde.**

2.2.2. PROTEIN TECHNIQUES

2.2.2.1. BCA Protein Assay

The BCA assay was used to detect protein quantitatively and was obtained commercially as a kit from Pierce, Rockford IL. In this assay, Cu^{2+} reacts with protein under alkaline conditions to give Cu^{+}, which reacts with BCA to give a coloured product. Two separate reagents are supplied as part of the kit: Reagent A, an alkaline bicarbonate buffer, and Reagent B, a 4% (w/v) copper sulphate solution. The working solution was prepared fresh each time by combining 50 parts of Reagent A to 1 part Reagent B. Protein standards in the range 0-1mg/ml were prepared in 0.1M PBS, pH 7.4.

For the assay 10µl of sample or standard was placed in the well of a 96-well plate. 200µl of the BCA working solution was added and the plate was gently swirled to ensure mixing of the solutions. The plate was then incubated at 37°C for 30 mins, before the absorbance of each well at 560nm was determined on a Titertek Twinplus plate-reader. All standards and samples were determined in triplicate. The absorbances of the standard solutions were used to construct a standard curve from which the protein concentration of the samples could be determined.

2.2.2.2. Protein Electrophoresis

2.2.2.2.1. Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) was carried out using the discontinuous system, in the presence of sodium dodecyl sulphate (SDS), as described by Laemmli (1970). 8% (w/v) resolving and 4% (w/v) stacking gels were normally used and prepared from the following stock solutions as outlined in Table 2.1.

Stock Solutions

(A) 30% (w/v) acrylamide containing 0.8% (w/v) bis-acrylamide.
(B) 1.5M Tris-HCl, pH 8.8, containing 0.4% (w/v) SDS.
(C) 0.5M Tris-HCl, pH 6.8, containing 0.4% (w/v) SDS.
(D) 10% (w/v) ammonium persulphate.

Solution	*Resolving Gel*	*Stacking Gel*
Acrylamide (A)	5.33 mls	1.2 mls
Distilled Water	9.6 mls	5.46 mls
Separating Gel Buffer (B)	5 mls	-------
Stacking Gel Buffer (C)	--------	5 mls
Ammonium Persulphate (D)	100µl	75µl
TEMED	10µl	10µl

Table 2.1 **This table outlines the quantities of stock solutions required for an 8% resolving and 4% stacking polyacrylamide gel.**

Samples were dissolved in solubilisation buffer (0.06M Tris-HCl, pH 6.8, 2% (w/v) SDS, 25% (v/v) glycerol, 0.014M 2-mercaptoethanol, 0.1%(w/v) bromophenol blue) and boiled for 8 mins before loading onto the gel. The gel was electrophoresed in electrode buffer, pH 8.3, containing Tris (0.025M), glycine (0.192M) and 0.1% (w/v) SDS, at 15mA (stacking) and 25mA (resolving) using an ATTO vertical mini electrophoresis system until the blue dye reached the bottom of the gel.

2.2.2.2.2. Staining with Coomassie Brilliant Blue

Gels were stained for 1 hour in 0.2% (w/v) Coomassie Brilliant Blue in methanol:acetic acid:distilled water (3:1:6), and destained overnight in the same solvent system.

2.2.2.3. Western Blotting

Following electrophoresis, as outlined above (Section 2.2.2.2.), the gel was soaked for 30 mins in cold (4°C) transfer buffer (0.25M Tris-HCl, pH 8.3, 0.192M glycine, 20% (v/v)

methanol). A sheet of nitrocellulose and six sheets of Whatman filter paper cut to the same size as the gel, were also soaked in transfer buffer. The proteins were transferred from the gel to the nitrocellulose using a Bio-Rad Wet Transfer system for 16 hours at 30V.

After transfer, the blot (nitrocellulose) was washed twice in Tris-Buffered Saline (10mM Tris-HCl, pH 8.0, 150mM NaCl), prior to incubation in Blocking Buffer (2% (w/v) BSA, 0.5% (v/v) Tween-20, in TBS) for 2 hours at room temperature. Following blocking, the blot was washed once with TBS and incubated with primary antibody (at the appropriate dilution in TBS containing 1% (w/v) BSA, 0.1% (v/v) Tween-20) for 2½ hours at room temperature. The blot was then washed extensively in TBS, prior to incubation with anti-mouse IgG, alkaline phosphatase conjugate (Promega), [Diluted 1/5000 in TBS containing 1% (w/v) BSA, 0.1%(v/v) Tween-20] for 2½ hours at room temperature. The nitrocellulose membrane was washed five times in TBS and once in alkaline phosphatase buffer (100mM Tris-HCl, pH 9.5, 100mM NaCl, 5mM $MgCl_2$), before incubation in substrate solution (175µg/ml BCIP, 500µg/ml NBT in alkaline phosphatase buffer). Colour development was allowed to proceed at room temperature without shaking, until the desired bands were apparent, at which time the reaction was stopped by washing the membrane in PBS containing 20mM EDTA.

2.2.3. CELL CULTURE TECHNIQUES

2.2.3.1. Cell Lines and Media Preparation.

The cell lines used in the experimental work and their appropriate culture media are outlined in Table 2.2. In all cases, the culture medium was supplemented with FCS (at either 5 or 10% (v/v) as outlined in Table 2.2.), L-glutamine (2mM), HEPES (1mM) and gentamycin (5µg/ml). Sodium pyruvate (1mM) and non-essential amino acids (1% (v/v)) were also used to supplement the MCF-7 and PC-3 media, respectively. The 28SC culture medium was additionally supplemented with 0.03mM thymidine, 0.1mM hypoxanthine and 0.05mM 2-mercaptoethanol.

Cell Line	*ATCC No.*	*Description*	*Culture Medium*
A431	CRL 1555	Human Epidermoid Carcinoma	DMEM.S_{10}
MCF-7	HTB 22	Human Caucasian Breast Carcinoma	DMEM.S_5
PC-3	CRL 1435	Human Caucasian Prostate Adenocarcinoma	Hams F12. S_{10}
T-24	HTB 4	Human Caucasian Bladder Carcinoma	DMEM.S_{10}
A549	CCL 185	Human Caucasian Lung Carcinoma	DMEM.S_{10}
SW480	CCL 228	Human Caucasian Colon Adenocarcinoma	DMEM.S_{10}
SW620	CCL 227	Human Caucasian Colon Adenocarcinoma: Lung Metastasis	DMEM.S_{10}
HL-60	CCL 240	Human Caucasian Promyelocytic Leukemia	RPMI 1640. S_{10}
NIH3T3	CRL 1658	NIH Swiss Mouse Embryo	DMEM.S_5
28SC	CRL 9855	Human Peripheral Blood Monocyte	IMEM.S_{10}

Table 2.2: List of Cell Lines and their culture media used throughout this work.

2.2.3.2. Recovery of Frozen Cells.

Cells were recovered from liquid nitrogen by thawing rapidly at 37°C and transferring to a sterile universal tube containing 10mls of DMEM.S_{10} [DMEM containing 10% (v/v) Foetal Calf Serum (FCS)]. The cells were centrifuged at 2000 rpm for 10 mins, resuspended in fresh medium, transferred to culture flasks, and incubated at 37°C in a humid 5% CO_2 atmosphere. Prior to incubation a 100µl sample was removed and tested for viability using Trypan Blue (see section 2.2.3.5). Only cultures with >90% viability on recovery from long term storage were further cultured and used in experiments.

2.2.3.3. Culture of cells in suspension

HL-60 cells and 28SC cells were maintained in DMEM.S_{10} and IMEM.S_{10} (see section 2.2.3.1), respectively, seeded at cell densities of 2 X 10^5 cells/ml, using 15mls of medium per 75cm^2 culture flasks. Cells were incubated in a humid, 5% CO_2 environment at 37°C, ensuring that at all times their densities did not exceed 1 X 10^6 cells/ml. They were harvested by flushing from the bottom surface with medium from the culture flask using a sterile pasteur pipette. These cells were either used for experiments (see sections 2.2.4. and 2.2.7.), or used for subculturing as follows: the cells were centrifuged in a sterile universal tube at 2000 rpm for 10 mins and resuspended in medium at the density outlined above.

2.2.3.4. Culture of adherent cells

The eight other cell lines used in the experiments were adherent cell lines, and were maintained in the appropriate growth medium (see section 2.2.3.1.) at 37°C in a humid, 5% CO_2 environment. All cells were strongly adherent and required trypsinisation for harvesting prior to subculturing or experimental usage.

For trypsinisation the medium was decanted and 3 mls of trypsinising solution (Gibco 0.025% (w/v) trypsin with 0.02% (w/v) EDTA in 0.1M PBS, pH 7.4) was used to rinse the flask, thus removing residual FCS which contains a trypsin-inhibitory activity. After this volume was decanted a further 5mls of the trypsinising solution was added to the flask and the flask was incubated at 37°C until all the cells had detached from the flask surface. 5mls of DMEM.S_{10} was added to this cell suspension, which was then transferred to a sterile universal tube and centrifuged at 2000 rpm for 10 mins. The cells were resuspended in culture medium and seeded at 2 X 10^5 - 1 X 10^6 cells/ml, using 15mls of medium per 75cm^2 culture flask.

2.2.3.5. Cell counts and viability testing

Trypan Blue was used routinely to determine cell numbers and viabilities. 20µl of Trypan Blue stain (Sigma, 0.25% (w/v)) was mixed with 100µl of cell suspension and allowed to incubate for 3 mins. A sample of this mixture was loaded onto the counting chamber of an improved Neubauer Haemocytometer slide, and cell numbers and viabilities were determined.

Viable cells excluded the dye and remained white, while dead cells stained blue. In all cases the minimum number of cells counted was 200.

2.2.3.6. Long-term storage of cells

Cells required for long-term storage were frozen in liquid nitrogen. Harvested cells were pelleted and resuspended in freezing medium (90% FCS, 10% DMSO, (v/v)) to a concentration of 1 X 10^6 cells/ml. 1ml aliquots were then transferred to sterile cryotubes, and lowered slowly (over a 3 hour period) into the gas phase, before being eventually immersed in liquid nitrogen.

2.2.4. TOXICITY TESTING

2.2.4.1. Drug Preparations

Drug solutions (coumarins) were prepared fresh for each experiment. Stock solutions of all drugs were made in DMSO, from which all required drug dilutions were made into culture medium, in such a way that the final concentration of solvent exposed to cells was always less than 0.1% (v/v). In all experiments, control cells were exposed to 0.1% (v/v) DMSO in culture medium.

2.2.4.2. In Vitro Proliferation Assays

Cells were seeded into 25cm^2 flasks at the appropriate density (see Table 2.3) and allowed to adhere for 6 hours, prior to addition of the drug of interest (coumarin, 6-hydroxycoumarin, 7-hydroxycoumarin and esculetin) at the desired concentration. Incubation of cells with drugs was continued for 96 hours, after which the cell number and viability was determined using Trypan Blue as outlined in section 2.2.3.5. All experiments were carried out in duplicate on at least three separate occasions. The increase in cell number for drug-treated cells was expressed as a percentage of the increase for untreated control cells, and growth curves were constructed from this data.

Cell Lines	*Seeding Density*
MCF-7, PC-3, A549, T-24, SW480, SW620,NIH3T3	1 $X10^5$ cells/flask
A431, HL-60	2 $X10^5$ cells/flask

Table 2.3: Seeding Densities for *In Vitro* Proliferation Assays.

2.2.4.3. Cytotoxicity Detection: Lactate Dehydrogenase (LDH) Assay

This assay is used to examine cell toxicity by quantifying plasma membrane damage. When the plasma membrane of any cell is damaged due to exposure to a toxic insult, LDH, a ubiquitous cytoplasmic enzyme, is rapidly released into the cells environment. The LDH levels in cell culture supernatants can be measured by the 2-step enzyme reaction outlined in Figure 2.2. A commercially available kit (Boehringer Mannheim) was used to carry out this assay.

Cells were seeded at 5 X 10^4 cells/well in sterile 96 well plates, allowed to adhere and exposed to drug concentrations (range 0-500μg/ml) for 24 hours at 37°C. Each drug concentration was tested in 7 separate wells in a randomised manner, with each experiment repeated on at least three separate occasions.

Immediately prior to testing supernatants, the LDH reaction mix was prepared from the kit components as follows: 250μl of catalyst solution (Diaphorase/NAD^+ mixture) was mixed with 11.25mls dye solution (iodotetrazolium chloride (INT) and sodium lactate). Test supernatants were prepared by centrifugation of the cells in their 96 well plates at 250g for 10 mins. From each well, 100μl of supernatant was transferred to a second 96 well plate, 100μl of LDH reaction mix was added, and the plates were incubated for 30 minutes at room temperature. Absorbances at 492nm were determined on a Titertek Twinplus plate-reader, with a reference wavelength of 620nm. The absorbances obtained were compared to that of cells exposed to a 1% (v/v) Triton X-100 solution (maximal LDH release).

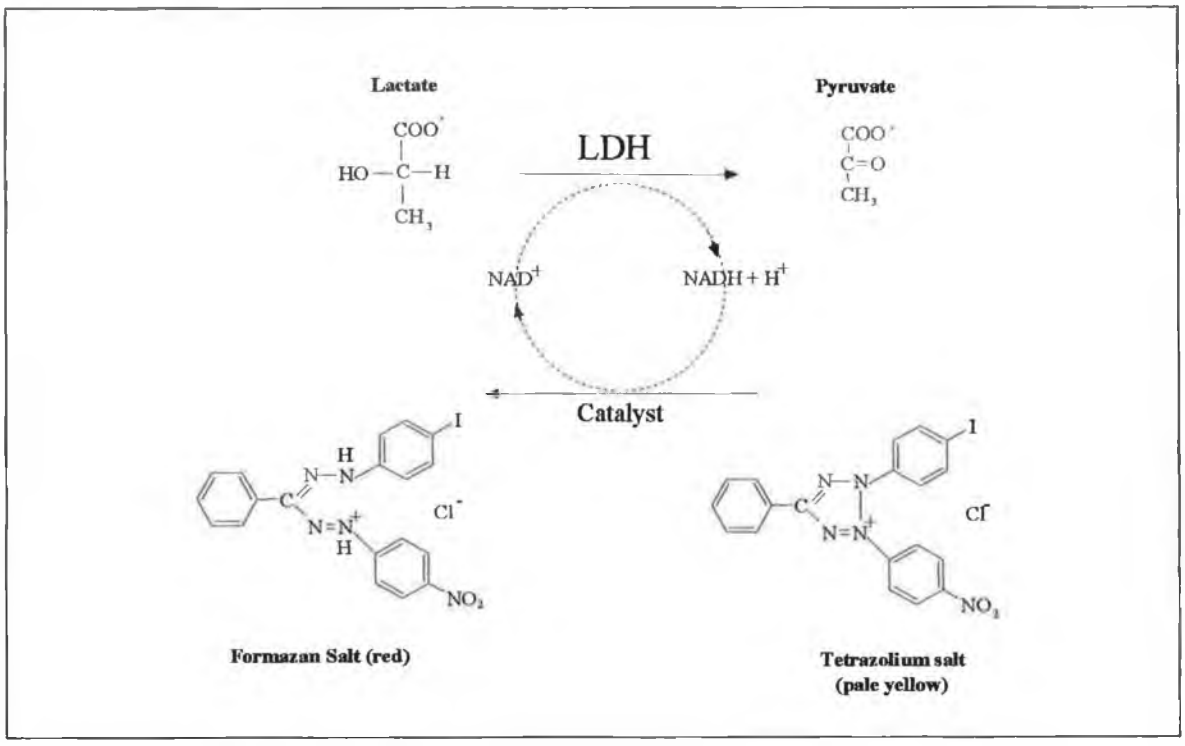


Figure 2.2: Principle of the LDH Assay.

2.2.4.4. MTT Assay

This non-radioactive colourimetric assay system is a modification of that first described by Mosmann *et al.*(1983), and involves the use of a tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). In metabolically active cells, this yellow MTT salt is cleaved to form purple formazan crystals, and as such can be used to assess the cellular metabolism and viability.

Cells were seeded at 5 X 10^4 cells/well into sterile 96 well plates, and allowed to adhere prior to the addition of 100µl of the appropriate drug solution (concentration range: 0-500µg/ml). Incubation with drug was continued for 24 or 96 hours. Following this time period, 10µl of MTT [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide] labelling reagent (5mg/ml in 0.1M PBS) was added to each well, and the plates were incubated at 37°C for 4 hours to allow for formazan crystal formation. 100µl of solubilisation buffer (10% SDS in 0.01M HCl) was added to dissolve the crystals overnight. The following morning the absorbance at 560nm was read on a Titertek Twinplus plate-reader. Drug-treated cells were compared to untreated control

cells. Each drug concentration was tested in 7 separate wells in a randomised manner and each experiment was repeated on at least three separate occasions.

2.2.4.5. Cytosensor Microphysiometer Studies on Toxicity

2.2.4.5.1. Pre-experimental Preparations.

On the day prior to the experiment A431 cells were seeded into transwell cell capsules at a density of 2.5 X 10^5 cells/capsule in DMEM.S_{10} and allowed to adhere overnight.

On the day of the experiment, running media was prepared from stock liquid 10X DMEM, as a 1X DMEM solution with a low buffering capacity i.e. without HEPES or sodium bicarbonate. Sodium chloride was added at 0.044M to compensate for the osmolarity as a result of the absence of sodium bicarbonate. Other supplements included L-glutamine (2mM), gentamycin (5μg/ml) and FCS (0.5% (v/v)). After all these additions, the pH was adjusted to 7.35 with 1M NaOH, and the media was filter-sterilised (0.22μm) using a Millipore apparatus. This media was heated to 37 °C prior to use.

2.2.4.5.2. Toxicity Studies

On the day of the experiment the cells were assembled onto the Cytosensor Microphysiometer, and running medium (37°C) was passed over them at a 50% flow-rate (~ 100μl/min). The cells were allowed to stabilise on the instrument for 3-4 hours. A pump cycle of 4 minutes duration was used with the acidification rate being measured in the final 30 secs of this 4 minute cycle. Once a steady base-line acidification rate was obtained the cells were exposed to drugs (6-hydroxycoumarin, 7-hydroxycoumarin, esculetin) in the concentration range 0-100μg/ml (final concentrations in running medium) for 4, 12 or 24 hours. The acidification rate was measured and recorded every 4 mins during this exposure period.

2.2.4.5.3. Reversibility Studies

To assess the reversibility of the toxic insult, the cells were re-exposed to drug-free running medium on cessation of the drug exposure period. The acidification rate was again monitored and recorded every 4 mins. The recovery of the cells was followed for 4, 12 or 24 hours.

2.2.5. METASTASES STUDIES

2.2.5.1. Collection of Conditioned Media.

A431 cells were seeded at a density of 1 X 10^6 cells/flask in 25cm^2 flasks and maintained in DMEM.S_{10} for 6 hours, after which this medium was decanted and the cells rinsed with sterile 0.1M PBS, pH 7.4. 5mls of fresh serum-free medium was then added to each flask, with or without the appropriate drug concentration, and the cells were returned to the incubator for 24 hours. After this time period, the medium ("conditioned medium") was collected from each flask. This conditioned medium was clarified by centrifugation at 3000rpm for 10 mins, and stored at –80°C until further analysis was performed. Prior to storage, aliquots were removed from each sample for protein determination using the BCA Assay (section 2.2.2.1.).

2.2.5.2. Substrate Gel Analysis.

Conditioned medium from cells exposed to different concentrations of drug (section 2.2.5.1.) was analysed for the presence of proteases by substrate gel analysis. This involves incorporating a protein substrate (gelatin) within the polymerised matrix of an acrylamide gel, and running the samples under non-reducing conditions, in such a way that any protease activity present can be renatured following electrophoresis, and allowed to degrade the gelatin in the gel.

The gel was prepared as a 10% (w/v) acrylamide gel containing 1mg/ml gelatin, as outlined in Table 2.4. The solutions used were the same as those described in section 2.2.2.2.1. Samples were mixed with solubilisation buffer (0.06M Tris-HCl, pH 6.8, 2.5% (w/v) SDS, 1% (w/v) sucrose, 0.02% (w/v) bromophenol blue), ensuring that equal quantities of protein were loaded onto lanes of the gelatin-containing gel. The gel was electrophoresed in electrode buffer, pH 8.3, containing Tris (0.025M), glycine (0.192M) and 0.1% (w/v) SDS, at 15mA (stacking gel) and 20mA (resolving gel), using an ATTO vertical mini electrophoresis system until the blue dye reached the bottom of the gel.

Following electrophoresis the gel was soaked in 2.5% (v/v) Triton X-100, with gentle shaking, for 30 minutes (with one change of solution) at room temperature, in order to renature the proteins by removal of SDS. The gel was then rinsed in substrate buffer (50mM Tris-HCl, pH 8.0, 5mM $CaCl_2$) and incubated overnight in the same solution at 37°C. Following this incubation the gel was stained with Coomassie Blue (0.5% (w/v) in isopropanol:water:acetic acid,

3:6:1, v/v/v) for 30 mins with shaking. Destaining in distilled water was continued until clear bands (representing protease bands) were visible.

Solution	*Resolving Gel*	*Stacking Gel*
Acrylamide (A)	6.67 mls	1.5 mls
Distilled Water	3.33 mls	6.0 mls
Separating Gel Buffer (B)	5 mls	-------
3mg/ml Gelatin Stock	5 mls	-------
Stacking Gel Buffer (C)	--------	2.5 mls
Ammonium Persulphate (D)	100µl	100µl
TEMED	15µl	15µl

Table 2.4: **Quantities of stock solutions (Section 2.2.2.2.1.) required for Gelatin gels. These gelatin gels were used for the analysis of proteases in conditioned medium collected from A431 cells (Sections 2.2.5.1)**

2.2.6. CELL SIGNALLING STUDIES

2.2.6.1 Purification of Active Epidermal Growth Factor Receptor Tyrosine Kinase (EGF-RTK)

A431 cells overexpress the Epidermal Growth Factor Receptor (EGF-R) and its associated tyrosine kinase activity, and were therefore used as the source of the enzyme in the purification process. Lectin column chromatography can easily purify the receptor to adequate levels for tyrosine kinase studies, while maintaining the tyrosine kinase in an unactivated, but activatable, form.

A431 cells were grown to confluency in a $75cm^2$ flask. The culture medium was decanted and the cells were washed twice with 0.1M PBS, pH 7.4. The cells were harvested by incubation with 0.1M PBS containing 1mM EDTA at 37°C for 1hour. The detached cells were pelleted at 600g for 10mins. 2 X 10^7 cells were resuspended in 1 ml of cold solubilisation buffer (50mM HEPES, pH 7.6, 1% (v/v) Triton X-100, 150mM NaCl, 5mM EGTA, 1mM PMSF, 25mM benzamidine, 50µg/ml aprotinin and 5µg/ml leupeptin), for 20mins at 4°C. The lysed cells were ultra-centrifuged at 100,000g for 30 mins and the supernatant loaded onto a WGA (Wheat germ agglutinin)-Agarose column (500µl of resin). The column was shaken gently for 2hrs at 4°C, after which unabsorbed material was removed and the resin washed sequentially twice with HTN buffer (50mM HEPES, pH 7.6, 0.1% (v/v) Triton X-100, 150mM NaCl), twice with HTN buffer containing 1M NaCl, and twice with HTNG buffer (HTN with 10% (v/v) glycerol). The bound EGF-R was eluted with HTNG containing 0.5M N-acetyl-D-glucosamine. The concentration and purity of the purified EGF-R were determined using the BCA Assay (Section 2.2.2.1) and Western Blotting (Section 2.2.2.3) respectively. The purified EGF-RTK was used for studies on tyrosine kinase inhibition as outlined in Section 2.2.6.2.

2.2.6.2. EGF-RTK Inhibition Studies

A commercially available non-radioactive tyrosine kinase kit (Pierce) was used to assess the tyrosine kinase activity of the purified EGF-RTK (Section 2.2.6.3.) and its inhibition by a variety of known and putative inhibitors. The basis of the method utilised in the kit is outlined in Figure 2.3. Briefly a biotinylated TK peptide (Biotin-EGPWLEEEEEAYGWMDF-Amide), is added to a Neutravidin-coated well, where it binds. A tyrosine kinase sample is added, resulting

in the phosphorylation of the tyrosine residue present in the bound peptide. An anti-phosphotyrosine antibody (PY20), conjugated with Horseradish Peroxidase (HRP), is added for specific detection of the phosphotyrosine residues, via the HRP-TMB colourimetric reaction. Kinase activity can be quantitated by comparison with a phosphopeptide curve, and the tyrosine kinase inhibitory activity of compounds can also be ascertained, by comparison of samples incubated with the putative inhibitor, with control kinase samples.

Experimentally, this was achieved as follows: 50µl of Biotinylated peptide was added per well of the microtitre plate and allowed to incubate at 37°C for 30 mins. During this incubation time, EGF-RTK (60µg of protein) was incubated with EGF (100ng) for 30mins at room temperature to allow activation of the receptor tyrosine kinase activity. Following the 30 minute peptide binding period, the wells of the microtitre plates were emptied and washed three times with Tris Buffered Saline (25mM Tris-HCl, pH 7.2, 150mM NaCl). To each experimental well, 10µl reaction buffer (100mM HEPES, pH 7.4, 500µM sodium orthovanadate, 1mM DTT), 10µl of distilled water, 20µl ATP solution (5mM ATP, 50mM $MgCl_2$ in TBS) and 10µl of activated EGF-RTK was added, and the microtiter plate was incubated at 37°C for 20 mins. Appropriate controls, and phosphopeptide standards were run concurrently. Following this incubation step, all wells were emptied and washed three times with TBS. 75µl of anti-phosphotyrosine (1/500µl in 1% BSA) was added to each well and the plate incubated at 37°C for 1 hour. Following extensive washing with TBS, 100µl of TMB (3,3′,5,5′-tetramethylbenzidine) substrate was added to all wells. Colour development was allowed to continue for 15 mins, after which time the reaction was stopped with 100µl of 1N H_2SO_4. The absorbances of the wells were determined at 450nm.

To test the tyrosine kinase inhibitory activity of various compounds, the above procedure was adopted with the inclusion of 10µl of the test compound instead of distilled water at the reaction mix step. At the conclusion of the experiment, absorbances from wells containing putative inhibitors were compared to those from control wells.

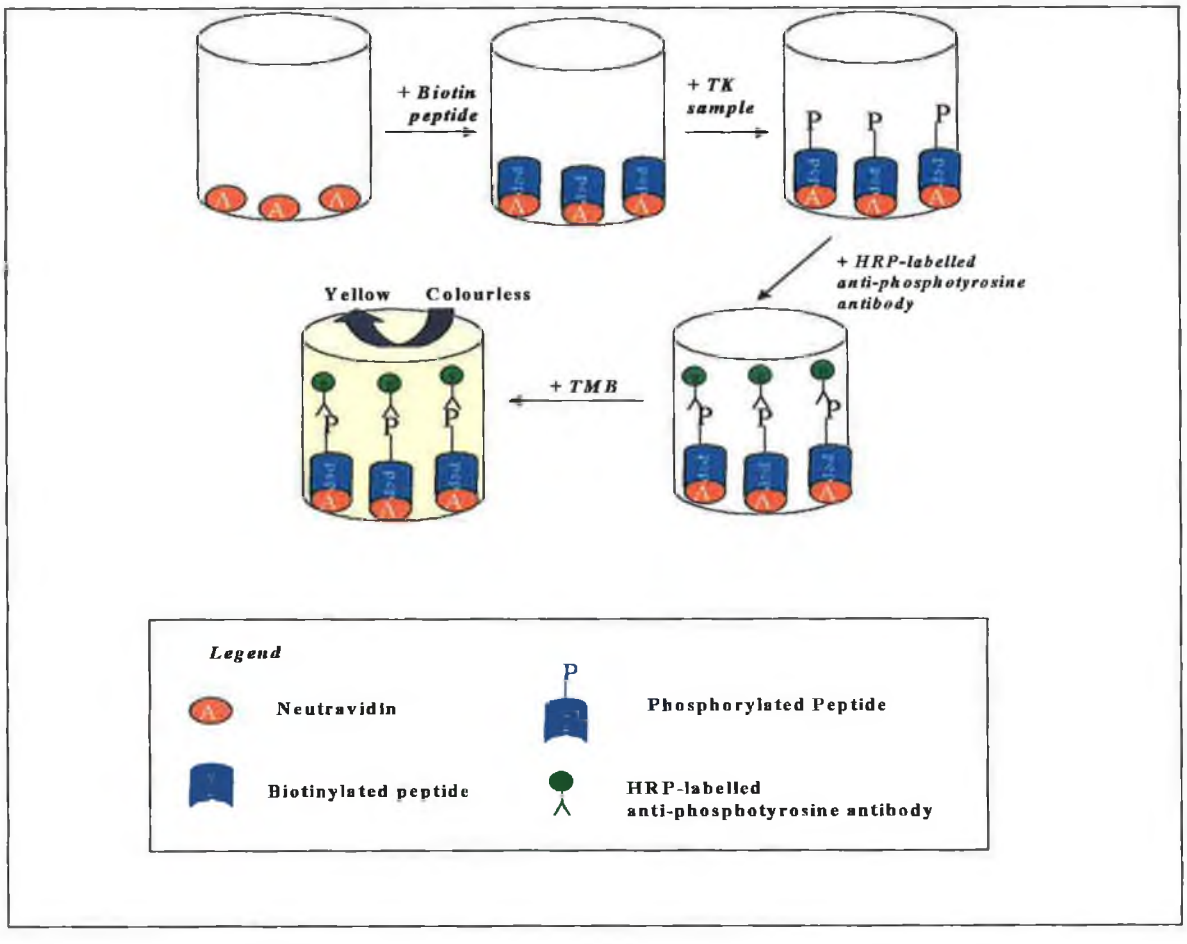


Figure 2.3: **Diagram outlining the basic protocol involved in the tyrosine kinase assay used in Section 2.2.6.2. The accompanying text details the above outlined steps.**

2.2.6.3. ELISA for Detecting Tyrosine Kinase Activity in Whole Cells

This ELISA was developed as a modification of a method published by Trinks *et al.* (1994). Sterile 96 well plates were seeded with A431 cells at a density of 5 X 10^4 cells/well and allowed to adhere for 6 hours in DMEM.S_{10}, afterwhich the cells were subjected to a 16hr period of serum-depletion. Following this serum-free period, each well of cells was stimulated with 100ng/ml EGF for 15 minutes at 37°C. The media was immediately removed and the wells were washed with ice-cold PBS containing 100μM sodium orthovanadate, after which the cells were fixed with methanol for 10 minutes at –20°C.

200μl of Blocking solution (10% (v/v) FCS, 0.2% (v/v) Tween-20, 100μM sodium orthovanadate in Tris Buffered Saline, TBS [10mM Tris-HCl, pH 8.0, 150mM NaCl]) was

added per well and the plates were incubated at 37°C for 1½ hours. After incubation, the blocking solution was removed and the plate was washed once with TBS containing 0.1% (v/v) Tween-20. 100µl of primary antibody (Anti-phosphotyrosine, dilution of 1/750 in TBS containing 10% (v/v) FCS, 0.1% (v/v) Tween-20) was added per well. The plates were incubated for 1 hour at 37°C.

Following incubation the plates were washed five times with TBS containing 0.1% (v/v) Tween-20, and twice with TBS. Anti-mouse IgG-alkaline phosphatase conjugate (Promega) was diluted 1/2000 in TBS containing 10% (v/v) FCS, 0.1% (v/v) Tween-20. 100µl of this solution was added per well and incubation was achieved at 37°C for 1 hour. Following incubation the plates were washed five times with TBS containing 0.1% (v/v) Tween-20 and twice with TBS. 100µl of pNPP was added per well and colour development achieved for 45 minutes before the absorbance at 405nm was measured.

To determine the effect of the coumarin compounds on tyrosine phosphorylation, the ELISA was performed as outlined above, with cells being pre-exposed to drug solutions (in the concentration range 0-100µg/ml), for 1, 6 or 12 hours (as appropriate) prior to EGF stimulation. Controls were used which were positive tyrosine kinase inhibitors (Genistein, 1 hour exposure, and Tyrphostin A25, 6 hour exposure). In all experiments, increases in absorbances (405nm) in stimulated cells compared to unstimulated cells were determined. Absorbance increases for drug-treated cells were normalised versus untreated control cells. Inhibition of tyrosine kinase activity was demonstrated if the absorbance increase for the drug-treated cells was diminished compared to the absorbance increase for control cells.

2.2.6.4. Cytosensor Studies for Detecting Tyrosine Kinase Activity

2.2.6.4.1. Pre-experimental Preparations.

On the day prior to the experiment, A431 cells were seeded into transwell cell capsules at a density of 2.5 X 10^5 cells/capsule in DMEM.S_{10} and allowed to adhere for 6 hrs. Serum-free medium was then used to replace the culture medium and the cells were serum-starved for 16 hours. Running media was prepared from stock liquid 10X DMEM, as a 1X DMEM solution with a low buffering capacity i.e. without HEPES or sodium bicarbonate. Sodium chloride was added at 0.044M to compensate for the osmolarity as a result of the absence of sodium bicarbonate. Other supplements included L-glutamine (2mM), gentamycin (5µg/ml) and BSA (1mg/ml). After all these additions the pH was adjusted to 7.35 with 1M NaOH, and the media

was filter-sterilised (0.22μm) using a Millipore apparatus. The media was heated to 37 °C prior to use.

2.2.6.4.2 Optimisation of EGF stimulation

On the day of the experiment the cells were assembled into the Cytosensor Microphysiometer and running medium (37°C) was passed over them at a 60% flow-rate (~ 120μl/min). The cells were allowed to stabilise on the instrument for 3-4 hours. A pump cycle of 2 minute duration was used, with the acidification rate being measured in the final 30 secs of this 2 minute cycle. Once a steady base-line acidification rate was obtained, the cells were exposed to various concentrations of EGF in the range (0-100ng/ml). By carrying out these experiments in quadruplicate, a dose-response curve for the EGF-EGF-R response in the A431 cell line was constructed. From this it was determined that the maximal response was obtained at 100ng/ml of EGF and this was used in all subsequent experiments (section 2.2.6.4.3.)

2.2.6.4.3. EGF-Receptor Tyrosine Kinase Inhibition Studies.

The passing of EGF (100ng/ml) over A431 cells on the Cytosensor Microphysiometer was shown from experiments in Section 2.2.6.4.2. to cause a substantial (~ 170%) immediate increase in the acidification rate of the cells. This increase was mainly due to the activation of the receptor-associated tyrosine kinase (on EGF binding to the receptor), and could be blocked by pre-exposure of the cells to tyrosine kinase inhibitors such as genistein and tyrphostin.

In order to determine if the coumarin compounds could inhibit the activation of the EGF-RTK the cells were pre-exposed to 7-hydroxycoumarin (0-100ug/ml) or esculetin (0-20ug/ml) for either 1 or 6 hours prior to the EGF stimulation (100ng/ml). In all experiments genistein and tyrphostin were used as positive controls.

2.2.6.5. Western Blotting for Detecting Tyrosine-Phosphorylated Proteins

A431 cells were seeded into 10cm petri-dishes at a concentration of 1 X 10^6 cells/plate and allowed to adhere to the surface by culturing in DMEM.S_{10} media for 6 hours. The media was then replaced with DMEM.$S_{0.5}$ and the cells were serum-starved for 16 hours. The serum-starved cells were then exposed to EGF (100ng/ml) for 15 minutes at 37°C. These stimulated cells were washed twice with ice-cold PBS containing sodium orthovanadate (100μM), prior to

lysis with 100µl of lysis buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 1% (v/v) Triton X-100, 1mg/ml BSA, 1mM PMSF, 100µM sodium orthovanadate). Protein concentrations in each sample were determined using the BCA Assay (Section 2.2.2.1.). Equal concentrations were run on an 8% SDS-PAGE as outlined in section 2.2.2.2.1. This gel was then immunoblotted and probed with an anti-phosphotyrosine antibody (1:1000 dilution) as outlined in section 2.2.2.3.

To determine the effect of the coumarin compounds on tyrosine phosphorylation as a result of EGF stimulation, treatment with drugs (concentration range 0-100µg/ml) was carried out during the 16 hour serum-starvation period for the appropriate pre-exposure time period (1, 6 or 12 hours). Cells exposed to drugs were immunoblotted and levels of tyrosine phosphorylated proteins compared to controls. Positive tyrosine kinase inhibitors (Genistein, 1 hour exposure, and Tyrphostin A25, 6 hour exposure) were also included.

2.2.6.6. Cytosensor Studies of Protein Kinase C Activity

2.2.6.6.1. Pre-experimental Preparations

Running media and cell seeding preparations for these experiments were exactly as outlined in Section 2.2.6.4.1.

2.2.6.6.2. Activation of Protein Kinase C (PKC) Activity

On the day of the experiment the cells were assembled onto the Cytosensor Microphysiometer, and running medium (37°C) was passed over them at a 60% flow-rate (~ 120µl/min). The cells were allowed to stabilise on the instrument for 3-4 hours. A pump cycle of 2 minute duration was used, with the acidification rate being measured in the final 30 secs of this 2 minute cycle. A 20 minute exposure of cells to PMA (optimised at 0.05ng/ml) was used to assess the activation of Protein Kinase C activity within the cells. Pre-exposure of cells to H-7 (25µg/ml), a specific PKC inhibitor, blocked the observed activation.

In order to determine if the coumarin compounds could affect the activation of Protein Kinase C, the cells were pre-exposed to 7-hydroxycoumarin (0-100ug/ml) or esculetin (0-100ug/ml) for either 1 or 6 hours prior to the PMA stimulation.

2.2.7. IMMUNE CELL EXPERIMENTS

2.2.7.1. Endotoxin Prevention

Although media, media supplements and commercial solutions were purchased endotoxin-free, they were tested for endotoxin contamination using a commercial E-Toxate® kit (Sigma diagnostics). Any homemade solutions also underwent scrutiny with this Limulus Ameobocyte Lysate (LAL)-based kit.

Briefly, 100µl of sample to be tested was incubated with 100µl of the E-Toxate*(LAL) working solution, in a baked glass tube, for 1hr at 37°C. Positive and negative control samples were also included. After this incubation period, the test sample was examined for evidence of gelation, by 180 ° inversion of the tube. A positive test was the formation of a hard gel, permitting complete inversion of the tube without disturbance of the gel.

* E-Toxate is prepared from a lysate of the circulating ameobocytes of the horseshoe crab, *Limulus polyphemus*. When exposed to minute quantities of endotoxin, the lysate increases in opacity and viscosity, and gels, if the concentration of endotoxin is sufficiently high.

2.2.7.2. Preparation of Opsonified Zymosan.

Zymosan is a cell wall preparation of *Saccharomyces cerevisiae* which alone can act as a phagocytic stimulant. However, coating of this preparation with immunglobulins and complement (a process known as opsonisation) yields a much more potent stimulator of phagocytic cells.

Opsonified Zymosan (OZ) was prepared according to the method of Allen (1986) as follows: A 1ml suspension of zymosan at a concentration of 2.5mg/ml in 0.85% NaCl (w/v) was prepared, and heated in a boiling water bath for 20 mins. This suspension was cooled to 22°C, centrifuged at 500g for 10 mins and the supernatant discarded. The resultant pellet was opsonified by resuspension in 2 mls of normal rabbit serum, with 20 mins of rotation on a blood-tube rotator. Following this incubation step, the solution was centrifuged at 500g for 10 mins, and the pellet subjected to a second incubation with 2mls serum, as before. The opsonified zymosan was again pelleted, and washed three times with normal saline to remove any residual protease activity. The final pellet was resuspended in 1ml saline, aliquoted and stored at -80 °C until required.

2.2.7.3. Characterisation of Monocytic Cells

2.2.7.3.1. Cytocentrifugation

Glass slides were alcohol-washed and labelled prior to assembly into the cytospin apparatus. A cell suspension of the appropriate cells at a density of 1X 10^6 cells/ml was prepared following counting (section 2.2.3.5). A 100µl aliquot of this suspension was transferred to the plastic inserts. The cells were centrifuged onto the slides at 1000 rpm for 5 mins using the Heraeus Labofuge Ae cytocentrifuge. The resulting cytospins were fixed and stained as described in sections 2.2.7.3.2. and 2.2.7.3.3

2.2.7.3.2. Eosin/Methylene Blue Staining

Slides were prepared as outlined in section 2.2.7.3.1. prior to fixing and staining with Eosin/Methylene Blue (from a Rapi-diff II, triple stain set from Diagnostic Developments) to assess cell morphology. Subsequently, the cells were fixed with methanol for 20 seconds, stained with Eosin for 15-20 seconds and counterstained with Methylene Blue for 15-20 seconds. Excess stain was removed with distilled water and following air-drying, the cells were viewed and photographed under 40X magnification.

2.2.7.3.3. α-Naphthyl Acetate Esterase Activity

Esterase activity is routinely used to identify and characterise mononuclear phagocytes, and is generally demonstrated by the use of either α-naphthyl acetate or butyrate as a substrate. The naphthol liberated by the esterase is coupled with a diazonium salt to give an insoluble coloured azo dye. The naphthyl acetate esterase activity was determined in HL60 and 28SC cells using a specific kit purchased from Sigma Diagnostics.

Briefly, the stain was prepared fresh each time as follows: 1ml of Sodium Nitrite (0.1M) and 1ml of Fast Blue BB Base Solution (15mg/ml in 0.4M HCl) were mixed and allowed to stand for at least 2 mins before adding to 40mls of pre-warmed (37°C) distilled water. 5mls of Trizmal (Trizma-Maleate, 1M, pH 7.6) and 1ml of α-naphthyl acetate (12.5mg/ml in methanol) were subsequently added, and this stain transferred to a staining chamber.

Cells, prepared on slides as outlined in section 2.2.7.3.1., were fixed in a Citrate (18mM citric acid, 9mM sodium citrate, 12mM sodium chloride, pH 3.6): Acetone: Formaldehyde (37%

(v/v)) solution (25:65:8) for 30 seconds, rinsed for 60 seconds in distilled water, and incubated in the staining solution for 30 mins at 37°C. Following this incubation the cells were rinsed with distilled water for 3 mins, and counterstained with Hematoxylin for 2 mins. Excess stain was rinsed off with tap water and the cells allowed to air-dry, prior to viewing and photographing under 40X magnification.

2.2.7.3.4. Nitroblue Tetrazolium Reduction for detection of monocytic respiratory burst

1 X 10^6 cells in 1 ml of culture medium were incubated with 0.2% nitroblue tetrazolium and 100ng/ml PMA for 30 mins at 37°C. 100µl of cell suspension was centrifuged onto slides as outlined in section 2.2.7.3.1. and the number of cells containing blue/black intracellular deposits was determined, counting at least 200 cells in total.

2.2.7.4. Activation of Monocytic Cells: Cytosensor Studies

2.2.7.4.1. Pre-experimental preparations

Running medium was prepared as outlined previously in Section 2.2.4.5.1. As both HL60 and 28SC are non-adherent cell lines, their assembly into the transwell capsules prior to mounting on the Cytosensor Microphysiometer is different to that described in Sections 2.2.4.5.1. and 2.2.6.4.1., and involved entrapment of the cells using an agarose matrix.

To achieve this a suspension of cells at a density of 4 X 10^6 cells/ 300µl was prepared in running medium. To this was added 100µl of the supplied Agarose cell entrapment medium, which had been previously melted in a beaker of boiling water for 5 mins. 10µl of this cell/agarose mixture was then carefully pipetted into the centre of each transwell capsule cup, and the agarose was allowed to solidify for 10 mins before the capsules were assembled onto the instrument in the conventional way. This procedure yielded a final concentration of 1 X 10^5 cells in each capsule cup.

2.2.7.4.2. Activation of Monocytic Cells

The cells were assembled onto the Cytosensor Microphysiometer, and running medium (37°C) was passed over them at a 60% flowrate (~ 120µl/min). The cells were allowed to stabilise on the instrument for 3-4 hours. A pump cycle of 2 minute duration was used with the acidification rate being measured in the final 30 secs of this cycle. Once a steady baseline

acidification rate was obtained, the cells were exposed to a 20 min pulse of PMA (500ng/ml) to activate PKC within the cells. The effect of exposure to various modulators (LPS and IFN-γ) was also examined.

2.2.7.5. Determination of Reactive Oxygen Species by Luminescence

Phagocytic cells, when activated, are capable of reducing molecular oxygen to superoxide, via a process called the "respiratory burst". Conversion of superoxide to hydrogen peroxide can occur, and both these processes produce oxygen species with electronically excited states, which results in the emission of luminescence from the cells. Measurement of this light emission, can therefore be useful in examining the effect of various agents on the inflammatory response of immune cells.

The luminescence method used to monitor production of reactive oxygen species from monocytic cells was modified from that of Labro *et al.* (1989). HL60 or 28SC cells were pelleted, washed with HBSS (Hanks Balanced Salt Solution) supplemented with 0.1% (w/v) gelatin, and resuspended at a concentration of 1 X 10^6 cells/ml in the HBSS/gelatin solution. 200µl of this cell suspension was incubated at 37°C, with gentle mixing, with 25µl of luminol (0.25mM). At time zero, the cells were stimulated with the appropriate stimulant (500ng/ml PMA or 250µg/ml OZ) and following a 10 min "lag period" where light emission was not recorded, luminescence measurements were obtained for the ensuing 60 min. Under the Repeat Scan mode, light emission was recorded for 30 secs every 3 mins for this 1 hr period. Results were expressed as integral light units emitted over this 1 hr period.

In experiments determining the effect of coumarins on the generation of reactive oxygen species in monocytic cells, cells were pre-exposed to the appropriate drug concentration (0-100µg/ml) for either 1 or 24 hours pre-stimulation.

2.2.7.6. Determination of Reactive Nitrogen Intermediates

2.2.7.6.1. Collection of Nitrite-Containing Samples

HL60 or 28SC cells were seeded at a density of 1 X 10^5 cells/well in a 96-well plate, prior to exposure to combinations of a variety of modulating/stimulating agents (PMA, LPS, IFN-γ) over a 24 hour incubation period at 37 °C. Following this incubation, test supernatants

were prepared by centrifugation of the cells in their 96 well plates at 250g for 10 mins. From each well, 100µl of supernatant was collected for analysis of nitrite content as outlined in Section 2.2.7.6.2.

2.2.7.6.2. Determination of Nitrite Using Greiss Reagents

Fresh Greiss reagent was prepared for each experiment from stock solutions of N-(1-naphthyl)ethylenediamine dihydrochloride (1mg/ml in distilled water) and sulphanilic acid (10mg/ml in 5% (w/v) phosphoric acid). Equal volumes of these two components were mixed to produce the Greiss reagent. Experimental samples, collected as outlined in Section 2.2.7.6.2., and standards of sodium nitrite (0-100µM) were analysed using this Greiss reagent. In each microplate well the following components were mixed: 13.5µl of Greiss Reagent, 100µl of Nitrite containing sample (sample or standard), and 86.5µl of distilled water. The microplate was incubated for 30 mins at room temperature and the absorbances of each well at 540nm were determined on a Titertek Twinplus plate-reader. All standards and samples were determined in triplicate. The absorbances of the standard solutions were used to construct a standard curve from which the nitrite concentration of the samples was determined.

2.2.7.7. Determination of Protease Activity

2.2.7.7.1. Collection of Conditioned Medium

HL60 cells and 28SC cells were seeded at a density of 1 X 10^6 cells/well in 2mls serum-free medium in a 12-well plate. The appropriate stimulant (PMA [500ng/ml], OZ [250µg/ml]) was added and the cells incubated at 37°C for 24hours. Wells with appropriate drug concentrations (coumarin, 7-hydroxycoumarin, or esculetin [0-100µg/ml]) were also included. After this time period, the medium ("conditioned medium") was collected from each flask. This conditioned medium was clarified by centrifugation at 3000rpm for 10 mins, and stored at –80°C until further analysis by substrate gel analysis as outlined in Section 2.2.7.7.2.

2.2.7.7.2. Substrate Gel Analysis

Substrate gel analysis of the conditioned medium collected from stimulated HL60/28SC cells (Section 2.2.7.7.1.) was analysed exactly as outlined in Section 2.2.5.2.

Chapter 3

Introduction to the Cytosensor Microphysiometer

This chapter introduces the reader to the Cytosensor Microphysiometer, an instrument used extensively throughout this work, which measures extracellular acidification rates. A brief overview of cellular metabolism and methods for measuring metabolism are given, followed by an outline of the cytosensor components and capabilities. A brief summary of the application of this instrument in our work is also included.

3.1. INTRODUCTION TO CELLULAR METABOLISM

Metabolism is defined as the overall biochemical process through which living systems acquire free energy from their environment, and utilise this free energy to maintain their normal functioning. Central to this overall process is the ubiquitous cellular energy transmitter, adenosine triphosphate (ATP), the measurement of which is often used to assess the physiological state of the cell (Lehninger, 1982).

As part of their metabolic cycle, cells intake nutrients and harness the inherent energy of these (via ATP), for use in cellular processes such as growth, motion, biosynthesis of complex molecules, active transport *etc*. The primary carbon sources used are sugars, amino acids and fatty acids, with glucose and glutamine of most importance in *in vitro* cell culture. The important ATP-yielding reactions of the cell utilising these two carbon sources are outlined in Table 3.1. From this it is seen that glucose can be converted to lactic acid via glycolysis, or to CO_2 via respiration (glycolysis followed by the citric acid cycle and oxidative phosphorylation). As shown in this table and described in more detail by Owicki & Parce (1992), respiration is more proficient energetically than glycolysis, and also produces much less acid per ATP generated (compare 1 H^+/ATP and 0.167 H^+/ATP, for glycolysis and respiration, respectively). However, although respiration is the predominant ATP source of cells *in vivo*, cells cultured *in vitro* utilise glycolysis as their major ATP-generating reaction. Glutamine metabolism has also been shown to contribute a proportion of ATP to mammalian cells in culture [estimated at 30-100% depending on cell type and culture medium composition] (Owicki & Parce, 1992).

An intracellular build-up of the protons produced during metabolism is detrimental to the cell, therefore they are excreted via numerous mechanisms *e.g.* proton channels, Na^+/H^+antiport, monocarboxylate carriers. This proton excretion occurs at a rate of $\approx 1 \times 10^8$ H^+/sec/cell (known as the extracellular acidification rate), and under unchanging environmental conditions remains constant (Owicki & Parce, 1992). Any event (stimulatory or inhibitory) which causes an alteration

in the metabolic rate of a cell will be efficiently mirrored by a change in the extracellular acidification rate of the cell. Therefore, measurement of this entity can yield useful information on the metabolic state and well-being of a cell at any particular instant.

Carbon Source	*Pathway*	*Reaction*	*ATP Yield*
Glucose	Glycolysis	glucose $\rightarrow$ 2 lactate$^-$ + 2H$^+$	2
Glucose	Respiration	glucose + 6O_2 $\rightarrow$ 6HCO_3^- + 6H$^+$	36
Glucose	HMP shunt + glycolysis + oxidative phosphorylation[a]	3 glucose + 11/2 O_2 $\rightarrow$ 5 pyruvate$^-$ + 3HCO_3^- + 5H_2O + 8H$^+$	27
Glutamine	Respiration	glutamine + 9/2 O_2 + 3H_2O $\rightarrow$ 5HCO_3^- + 2NH_4^+ + 3H$^+$	27

Table 3.1: Summary of the major ATP-yielding reactions within cultured cells.
[a]HMP shunt is not itself a primary source of ATP, but it produces NADPH which can couple to oxidative phosphorylation to yield ATP.

3.2. MEASURING CELLULAR METABOLISM

Cellular metabolism has been studied by a variety of both invasive and non-invasive methods. Most of these simply involve the measurement of either the rate of uptake of reactants, such as glucose or oxygen, or the rate of production of heat or acidic products. The main methods used for these determinations are outlined in Table 3.2. The measurements obtained have been primarily used to examine the effects of various toxicants (drugs, industrial chemicals and environmental pollutants) on the physiological state of cells. During this work we have used a recent addition to these tools, the Cytosensor Microphysiometer (section 3.3), to examine the effects of coumarins on various cellular events.

Measurement of	*Technique used*	*References*
Glucose	Enzymatic (G-6-P-D)	Olson (1994)
	Microbial Biosensor	Svitel *et al.* (1998)
Oxygen	Oxygen Electrode	Li *et al.* (1988)
		Schnellmann (1994)
		Ara *et al.* (1994)
Heat	Microcalorimetry	Gebreselassie & Schon (1993)
		Schon & Wadso (1988)
Acidic Products	Enzymatic (LDH)	Olson (1994)
(Lactate, CO_2)	Cytosensor Microphysiometer	McConnell *et al.* (1992)
Adenylate	HPLC	Park *et al.* (1994)
Compounds	Enzymatic	Lundin (1984)

Table 3.2: List of techniques used to study cellular metabolism in recent years.

3.3. THE CYTOSENSOR MICROPHYSIOMETER

The Cytosensor Microphysiometer is an instrument which determines the effects of various agents on the metabolism of cells. It achieves this by detecting the acidity of the culture medium bathing a small sample of cells (Figure 3.1), using a pH-sensitive sensing chip (LAPS sensor, section 3.3.2). The measurement of this extracellular acidification rate (ECAR) is used as a measure of the catabolic rate (McConnell *et al.*, 1992).

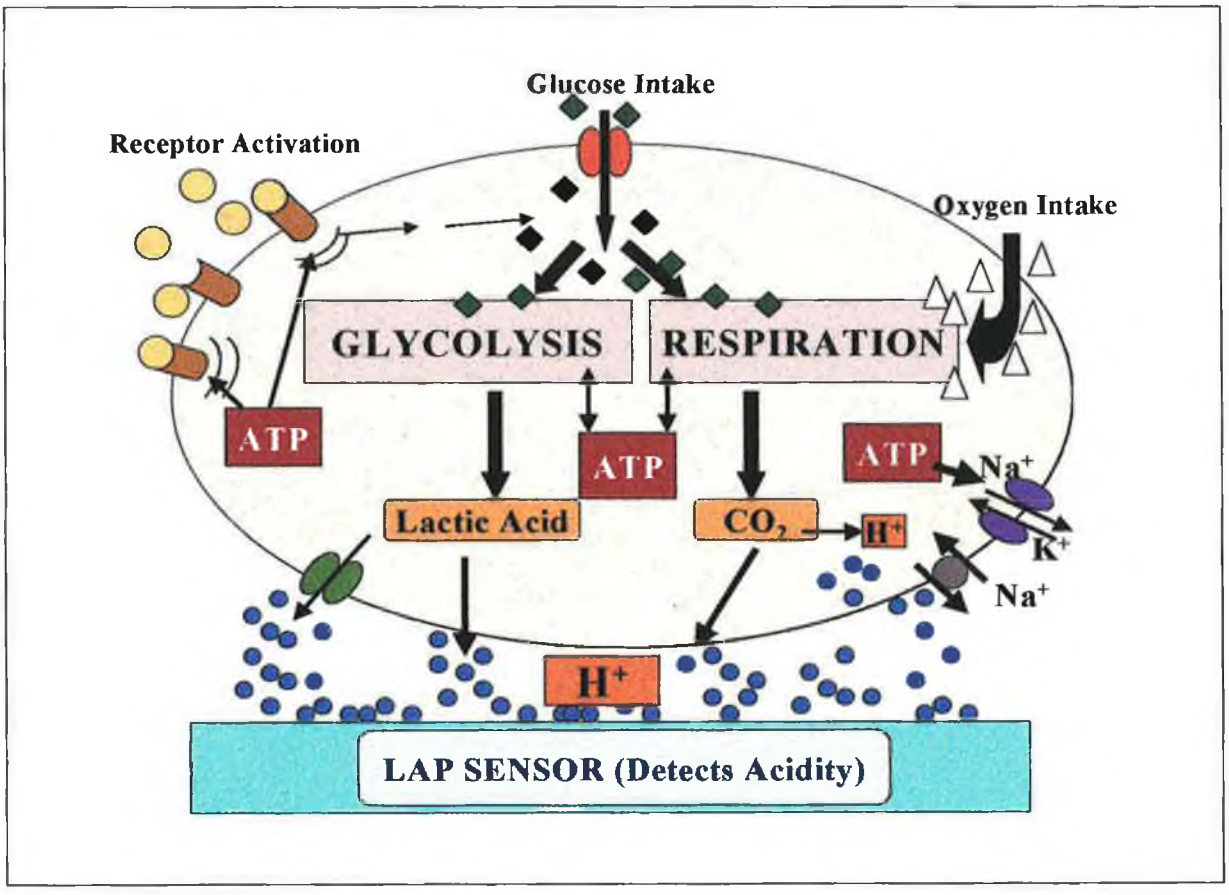


Figure 3.1: Diagrammatic representation of cellular metabolism and its relationship to cellular processes such as receptor activation. Glucose and oxygen are utilised in the production of the cellular energy transmitter ATP, with the concurrent production of acidic waste products (lactic acid and carbon dioxide). ATP is consumed by a variety of cellular processes, *e.g.* growth, movement or receptor activation (as depicted in this diagram). In the Cytosensor Microphysiometer, changes in metabolic rate due to ATP turnover as a result of such processes, can be detected by measuring changes in the extracellular levels of acidic waste products, using the pH-sensitive LAPS sensor.

3.3.1. Cytosensor Components

In the Cytosensor Microphysiometer, cells are assembled into a disposable capsule, in such a way that they are sandwiched between two porous polycarbonate membranes. This assembly is then placed in a micro-flow chamber in aqueous diffusive contact with the pH-sensitive LAPS chip, which serves as the bottom wall of the flow chamber (see Figure 3.2). As the location at which all pH readings are taken, this sensor chamber is the heart of the instrument, and with a volume of only 3μL allows for the acquisition of very precise and sensitive readings.

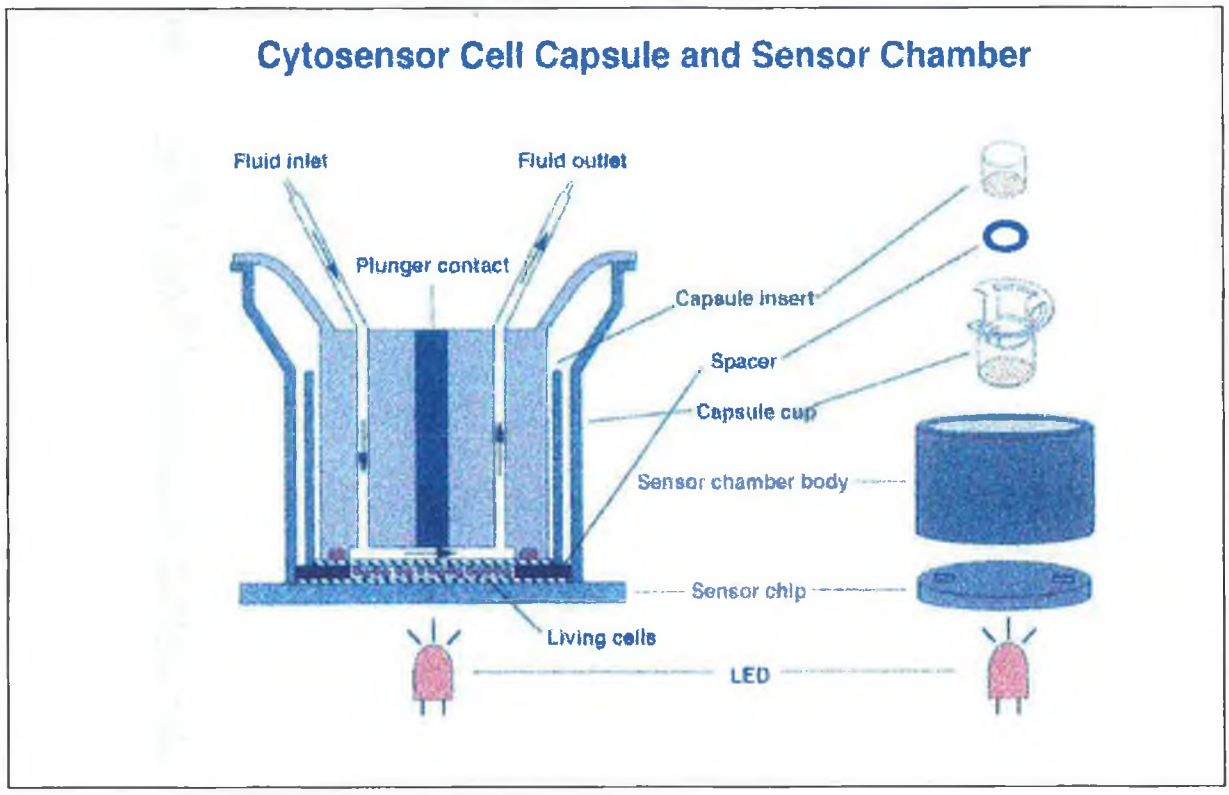


Figure 3.2: **Diagrammatic representation of the Cytosensor cell capsule and sensor chamber. This diagram was reproduced with kind permission from Molecular Devices Corporation, Menlo Park, California, U.S.A.**

As shown in Figures 3.3(a) and (b) [Photograph and Schematic diagram of the Cytosensor Microphysiometer components, respectively], a constant flow of fresh low-buffered running media is pumped through the system, and introduced into this sensor chamber, providing the encased cells with fresh nutrients and removing any waste products from their immediate environment. Other components of the system (Figure 3.3) function as follows:

- ***Debubbler*** — removes bubbles and reduces the amount of dissolved gases in the fluid.
- ***Valves*** — valve switches introduce different effector agents into the sensor chamber via the second fluid channel
- ***Reference Electrode*** — stabilises the pH readings from the sensor chamber
- ***Computer*** — controls pumps and valves during the experiment
  controls data display, acquisition, and analysis.

3.3.2. The LAPS sensor

The Cytosensor Microphysiometer uses a light-addressable-potentiometric sensor (LAPS) to measure the rate of extracellular acidification of the cells. This LAPS sensor consists of a doped silicon chip, with a thin insulating layer of silicon oxide and silicon nitride on its surface, (Figure 3.4). If a potential is applied across this insulating layer (through an external circuit connecting the aqueous layer and the uninsulated side of the silicon), an electric field is created in the insulating layer and at the silicon-insulator surface. If the silicon is simultaneously pulsed with light from a light-emitting diode (LED), electron-hole pairs will be formed due to light absorption. These electron-hole pairs move in opposite directions in the electric field, producing an alternating photocurrent detectable in the external circuit (Figure 3.4).

The applied potential (ψ) at which the photocurrent begins to flow is defined by the sum of a variety of constant potentials (*i.e.* contacts) and the surface potential of aqueous-insulator interface. This surface potential varies due to titration of the surface groups on the insulating layer [in turn dependent on the pH of the solution bathing this layer] (Figure 3.4). Therefore, changes in the magnitude of the applied potential required to produce a photocurrent are pH-sensitive. This is the

basis of the measuring capability of the LAPS system. A properly prepared Si-nitride surface has a sufficiently high density of titratable groups to make the pH response of the surface potential Nernstian [*i.e.* 61mV/pH unit at 37°C] (McConnell *et al.* 1992).

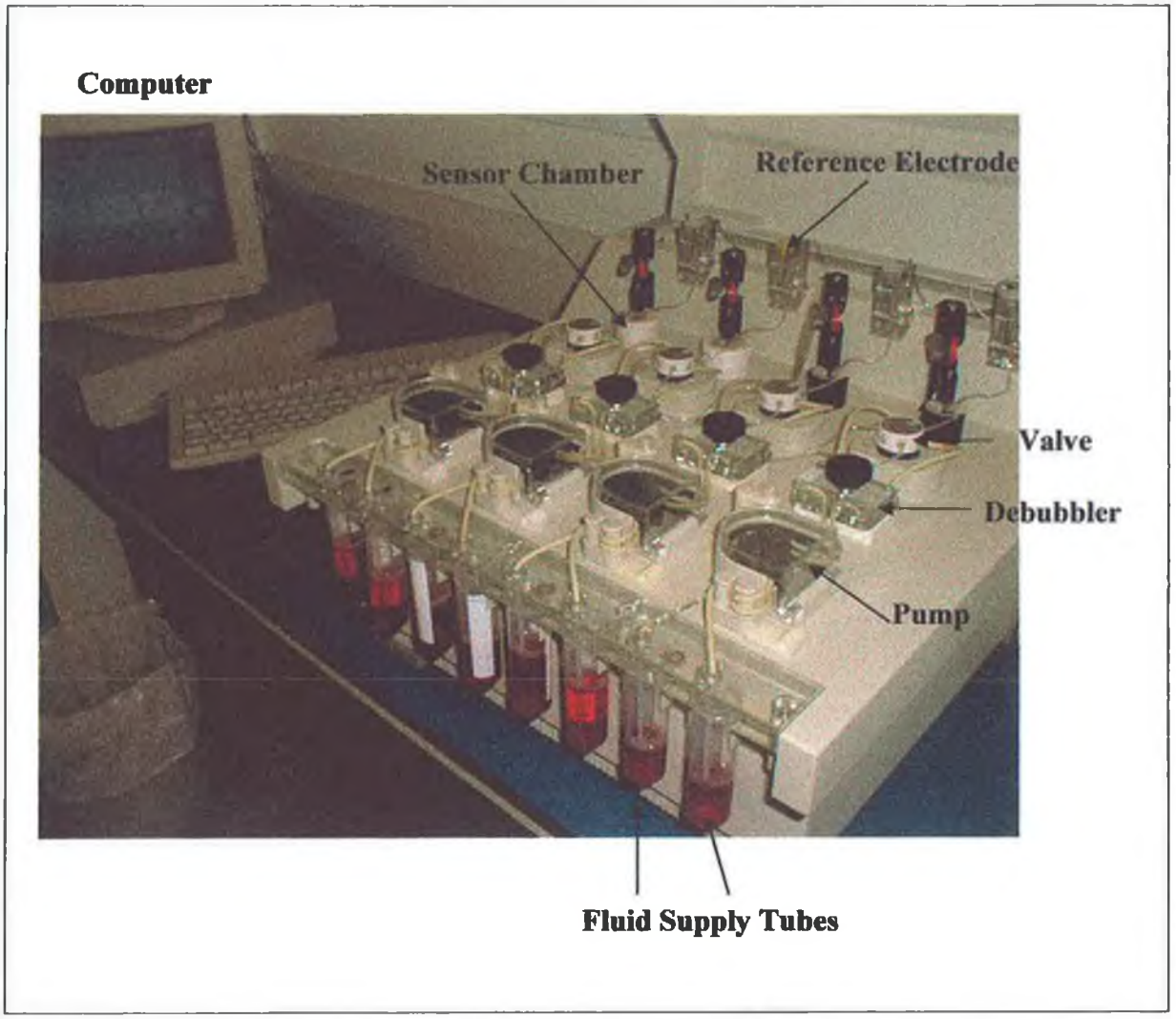


Figure 3.3a: Photograph depicting the major components of the Cytosensor Microphysiometer system. The photograph should be compared with the schematic diagram (Figure 3.3b)

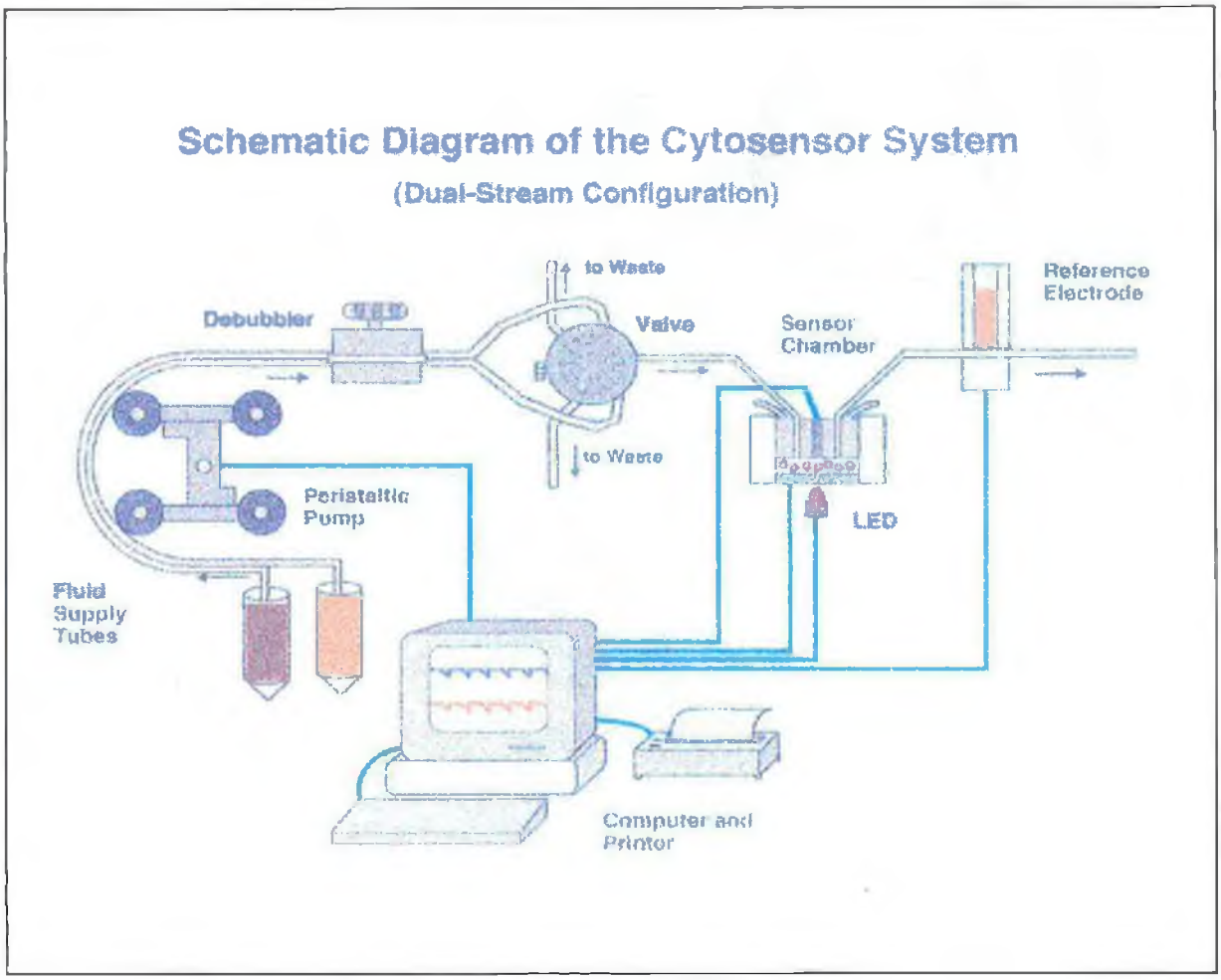


Figure 3.3b: Schematic diagram of the major components of the Cytosensor Microphysiometer system. This diagram was reproduced with kind permission from Molecular Devices Corporation, Menlo Park, California, U.S.A.

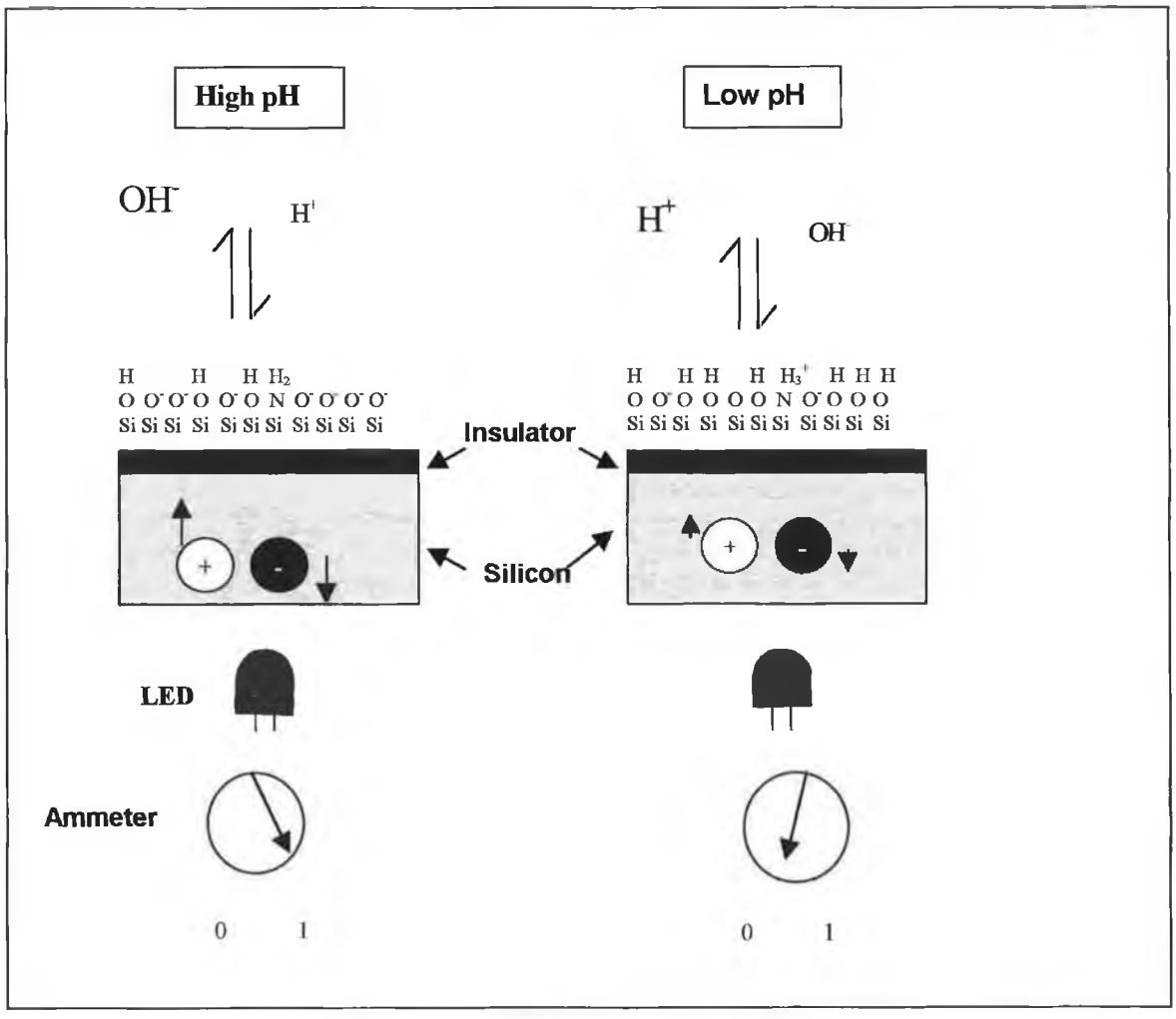


Figure 3.4.: **How the LAPS works. The left-hand side of the diagram illustrates what occurs with a high pH solution bathing the sensor: A strong negative charge is created at the chip surface, which results in the repulsion of light-generated electrons from the surface, and attraction of the holes to this negative charge. A photocurrent is generated as a result. When a low pH solution is in contact with the surface (as depicted on the right-hand side of the diagram), there is only a weak surface charge, with subsequent little repulsion/attraction of electrons/holes, therefore the photocurrent is negligible.**

3.3.3. Measuring Acidification Rates

The pH of the sensor chamber remains constant once media is allowed to flow through it, as no build-up of acidic waste products can occur. The pH remains steady at approximately the pH of the running medium (pH 7.3-7.4). If the pump is stopped, acidic metabolites (lactic and carbonic acids) begin to accumulate in the sensor chamber with a subsequent pH drop, a drop which can be detected by the LAPS sensor chip. When the pump is turned on again the flow sweeps out the acidic metabolites, returning the pH of the chamber to its original value, where it remains stabilised until the pump is stopped again. This pump on-off cycle generates data traces such as that shown in Figure 3.5. The pump off periods are quite short (10-40s) which prevents the build-up of harmful levels of metabolic products.

The data obtained during the pump-off interval (also known as the get rate period) are fitted to a straight line, the slope [-μV/sec] of which is plotted as the acidification rate (Figure 3.5). If the metabolic rate of the cells changes in response to an effector agent (either stimulatory or inhibitory), the acidification rate data will reflect this change (Figure 3.5).

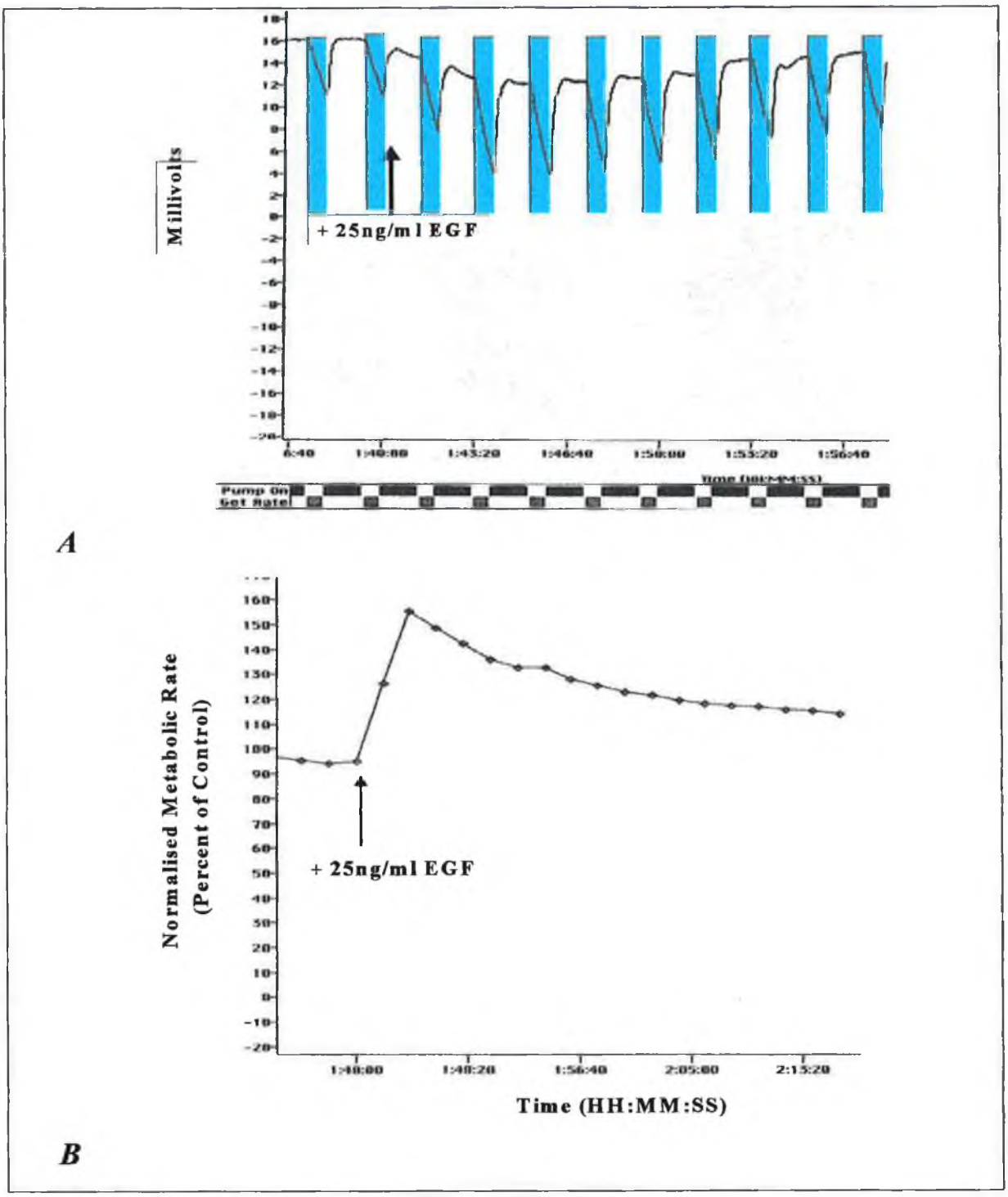


Figure 3.5: **Raw (A) and Rate (B) data derived from an experiment in which A431 cells were exposed to 25ng/ml EGF for 12 mins (Time of EGF exposure = 1:40:00). As can be seen from the raw data, a noticeable increase in the acidification rate during the pump-off cycle (highlighted in blue) denotes activation of the EGF-receptor in these cells. This change in the cells metabolic rate is reflected in the Rate data (B).**

3.4. APPLICATIONS OF THE CYTOSENSOR MICROPHYSIOMETER

A major attribute of the Cytosensor Microphysiometer is its flexibility: it accurately measures a general cellular event (extracellular acidification), and as such is applicable to a variety of biological disciplines (Table 3.3).

Discipline	*Application*	*References*
Pharmacology	Receptor characterisation	Baxter *et al.* (1994), Pitchford *et al.* (1997)
	Signal transduction	Baxter *et al.* (1992), Pitchford *et al.* (1995)
Toxicology	Ocular irritancy	Bruner *et al.* (1991)
	Neurotoxicity	Wada *et al.* (1992)
	Hepatotoxicity	Cao *et al.* (1998)
Cell Biology	Transient transfections	Chio *et al.* (1994), Skwish *et al.* (1994)
	Na^+/H^+ antiport studies	Neve *et al.* (1992), Wada *et al.* (1993)
Immunology	T-cell activation	Wada *et al.* (1994)
	B-cell activation	Renschler *et al.* (1995)
	Macrophage/monocyte studies	Romano *et al.* (1996), DeVries *et al.* (1998)
Oncology	Chemotherapeutic efficacy	Wada *et al.* (1992)
	Apoptosis	Kuo *et al.* (1994)
Microbiology	Antibiotic susceptibility assays	Libby *et al.* (1998)
	Anti-viral studies	Wada *et al.* (1992)
	Ion channel studies	Hahnenberger *et al.* (1996)

Table 3.3: List of the various research applications to-date of the Cytosensor Microphysiometer in biological studies.

As an assay system that reliably measures cell surface receptor activation, with subsequent intracellular signalling events, the cytosensor has been principally applied to pharmacological investigations, being particularly advantageous over traditional methods for determining agonist/antagonist profiles of receptors (Baxter *et al.*, 1994; Jaen *et al.*, 1995; Mierau *et al.*, 1995). However, it has also featured in toxicological evaluations of compounds and has been shown to be suitable as an *in vitro* alternative in ocular irritancy testing (Bruner *et al.*, 1991).

Although general in its method of detection, the cytosensor can be used as a specific probe of signal transduction events in cells, through the appropriate use of neutralising antibodies, antisense oligonucleotides, specific enzyme inhibitors, channel blockers *etc.* (Baxter *et al.*, 1992; Wada *et al.*, 1992). It has many advantages over traditional methods for examining signal transduction, as outlined in Table 3.4.

Advantages of the Cytosensor Microphysiometer
Non-Invasive - Internal controls; Can examine recovery from toxic insult.
"Real-time" Measurement – No need to do multiple assays for time course points
Fast Results – Quicker than traditional assays (Ca^{2+} mobilisation, IP_3 assays)
High Reproducibility
Flexibility
No Radioactivity
Reduced Animal Usage

Table 3.4 : Advantages of the Cytosensor Microphysiometer for research applications.

3.5. USE OF THE CYTOSENSOR MICROPHYSIOMETER IN THIS WORK.

The Cytosensor Microphysiometer was used extensively throughout this work to examine the effects of coumarins on cultured cells. As outlined in Chapter 4, this instrument was initially used to examine the effects of coumarin compounds on the basal metabolic rate of cultured cells. As this was part of an overall assessment of the toxicity of these compounds to tumour cells, parameters such as dose and time-dependency, as well as reversibility were examined. This system was found to be superior to the MTT assay (a well-established *in vitro* toxicity assay) with respect to toxicity prediction. As a prelude to this work, the Cytosensor Microphysiometer was evaluated for its usefulness as a toxicological tool by validating previous toxicity work carried out by different investigators (Parce *et al.*, 1989; Bruner *et al.,* 1991) in this field. This work is reported in Appendix 1.

In Chapter 5, the cytosensor was used to examine the effect of coumarins on signal transduction in human tumour cells. Two signalling events in particular were investigated: activation of Epidermal Growth Factor (EGF)-Receptor, and activation of Protein Kinase C (PKC).

In Chapter 6, the cytosensor was used to assess the activation of two monocytic cell lines (HL-60 and 28SC) in response to various immune cell modulators and stimulators.

Chapter 4

The effects of coumarins on the growth and metastasis of human tumour cells

4.1. INTRODUCTION TO CHEMOSENSITIVITY TESTING

As outlined in Section 1.8.1, current cancer therapies, even combination therapies, offer limited success for cancer patients, with many common solid tumours completely refractory to treatment. Therefore, the harsh reality for scientists and clinicians alike, is that new, more effective anti-tumour agents, and better treatment strategies are required, to supplement the current treatment modalities. As a result, a vast program of screening of compounds (both natural and synthetic) has been instigated by government and private institutions worldwide. Central to these programs, is the development and improvement of biological tests for the accurate prediction of anti-tumour properties in screened agents, also known as the field of chemosensitivity testing (Twentyman, 1985; Weisenthal & Lippman, 1985; Cramer & Woltering, 1991; Bellamy, 1992).

4.1.1. Current Chemosensitivity Tests

The introduction of chemosensitivity testing has transpired for the following three reasons:

1. The preliminary screening of novel anti-tumour agents.
2. Improvement of drug responses in patients using existing drugs. This can be achieved through the testing of new drug combinations and dose schedules, as well as from information gained about their mechanisms of action. Experiments into drug resistance in tumour cells also yield improvements in chemotherapeutic potential of existing drugs.
3. Adaptation of chemotherapy regimens to individual patients by testing for drug resistance/sensitivity in both relapsed patients or patients with a primary tumour.

For general screening procedures, as presented for the coumarin compounds in this thesis, cultured cell lines are widely utilised as the cellular source (Alley *et al.*, 1988; Kratzke & Kramer, 1996). However, the ultimate aim in the development and improvement of chemosensitivity tests is for their application in the clinical arena in the hope of eliminating the exposure of patients to harsh, non-beneficial chemotherapeutic regimens. Therefore, a substantial portion of the literature in this area involves the use of biopsied patient samples (Sargent & Taylor, 1989; Wilson *et al.*, 1990; Sevin *et al.*, 1994) and deals with the issue of correlating *in vitro* and *in vivo* observations (Bertelsen *et al.*, 1984; Cramer & Woltering, 1985; Yung, 1989)

In examining the sensitivity of tumour cells (both biopsy and cultured cell samples) to compounds, one can examine the effect of the compound on one of a number of cellular

mechanisms *e.g.* cellular proliferation, cellular metabolism, cell viability or cell morphology (Table 4.1). For many years now controversy has existed with regard to the use of clonogenic versus non-clonogenic chemosensitivity testing systems, with clonogenic assays being regarded by some as the "gold standard" against which all chemosensitivity tests should be scored. Briefly the clonogenic assays (used chiefly with biopsied samples) are similar to antibiotic sensitivity testing in bacteria – following exposure to a test compound, a single suspension of cells is plated out and following a long (weeks) incubation, the number of colonies formed is counted (Hamburger & Salmon, 1977). The proliferative cells (colony forming cells) are deemed to represent the tumour stem cells, the portion of the tumour believed to "regrow" following therapy. It is thus thought that measuring the stem cell population after drug exposure is the true measure of the therapeutic effect of a given drug. However, many investigators disagree as to the relevance of this assay to chemotherapeutic research – some believe that destruction of other tumour cells (tumour debulking) is as important as stem cell destruction, as these cells may be responsible not for growth, but for invasion/metastasis, immunosuppression and cancer-related cachexia. In addition, various papers have questioned the validity of the argument that clonogenic assays actually detect tumour stem cells (Twentyman, 1985; Weisenthal & Lippman, 1985; Yung, 1989). Weisenthal and Lippmann (1985), in particular, have addressed the theoretical problems of these assays, believing that alternative non-clonogenic methods detecting cell kill or cell damage in total tumour cell populations (Table 4.1) are as valid and more technically feasible for the prediction of chemosensitivity.

From Table 4.1 it can be seen that the available chemosensitivity assays, with the exception of xenograft implantation, all examine the effect of compounds in an *in vitro* set-up. Use of the xenograft model overcomes some of the limitations of *in vitro* testing, not least through the preservation of the histological features of the original tumour. In addition, drugs that require *in vivo* metabolic activation can be tested using xenografts, provided the mouse is an appropriate metaboliser of the test compound. However, there are disadvantages coupled to the use of the xenograft model, in particular its resource and time constraints. A full list of the advantages and disadvantages of the various chemosensitivity methods available is provided in Table 4.2.

Assay Measures	*Method*	*Reference*	*Brief Description of Assay*
CELLULAR PROLIFERATION *(In Vitro)*			
(i) STEM CELL	**Clonogenic**	Hamburger & Salmon, (1977)	Measures colony formation capability of cells (no. of stem cells)
(ii) TOTAL CELL	**Total Cell Counts**	Lickiss *et al.* (1974)	Measures total number of cells
	Radionucleotide Incorporation	Kern *et al.* (1985)	Measures rate of radioactive precursor incorporation into DNA/RNA
	Kenacid Blue	Clothier (1995)	Measures total cell protein
CELLULAR PROLIFERATION *(In Vivo)*	**Xenograft**	Tveit (1983)	Measures the change in size of human tumour cells transplanted into immune-deficient or immune-deprived mice
CELLULAR METABOLISM	**MTT Assay**	Carmichael *et al.* (1987)	Measures rate of reduction of tetrazolium salt by mitochondrial dehydrogenases in metabolically active cells
	ATP Assay	Kangas *et al.* (1984)	Measures intracellular ATP levels in cells (levels decrease as cells die)

PTO

CELL VIABILITY			
STAINING	**Dye Exclusion Assay**	Weisenthal & Kern (1991)	Measures ability of viable cells to exclude stains.
	Fluorescent Cytoprint Assay	Meitner (1988)	Measures ability of viable cells to transport, hydrolyse and retain fluorescein.
MEMBRANE DAMAGE	**LDH Assay**	Welder & Acosta (1994)	Measures release of intracellular enzyme (LDH) as a result of membrane damage
	2-Deoxy-D-Glucose	Walum & Forsby (1995)	Measures release of tritiated 2-deoxy-D-glucose from cells
CELL MORPHOLOGY		Trump & Berrezesky (1994)	Measures changes to morphological features of cell

Table 4.1: **Table outlining the current chemosensitivity tests available. These can be used for the screening of novel chemotherapeutic agents, for testing drug combinations, and for determining mechanisms of action of current and new drugs. The information gained can be used to improve the responses of cancer patients to an existing therapy or a novel one, by conducting predictive sensitivity/resistance testing for individuals.**

Table 4.2: Advantages and disadvantages of various chemosensitivity tests.

Assay	*Advantages*	*Disadvantages*
CELLULAR PROLIFERATION		
Total Cell Counts	• Simple • Rapid: Results < 1 week • Versatile *i.e.* Easily examine time- & dose-dependencies, drug combinations	• Labour-intensive • Density-dependent responses • Relevance (to *in vivo* situation)
Radionucleotide Incorporation	• Rapid: Results < 1 week • Versatile • Can be automated	• Labour intensive • Radionucleotides may be toxic to cells ⇒ cell kill over-estimation • Uncertain influence of drug on transport or pool of nucleosides
Kenacid Blue	• Simple & rapid • Semi-automated • Versatile	• Does not distinguish between live and dead cells
Clonogenic Assays	• Detects most biologically significant population • Fibroblast growth prevented	• Only subpopulation evaluated – is this truly representative? • Low cloning efficiencies • Long assay times (15 days) • Colony counting laborious • Difficult to obtain single cell suspensions without damage
Xenografts	• Tumour architecture and environment maintained • Testing of drugs requiring *in vivo* metabolic activation • Useful for optimising scheduling	• Latent period of 10-40 days before tumour appearance • Sterile handling and housing required • Maintaining athymic mice expensive

PTO

Assay	*Advantages*	*Disadvantages*
CELLULAR METABOLISM		
MTT Assay	• Rapid & Versatile • Semi-automated	• Cannot distinguish cytostatic vs cytotoxic effects • Must be optimised for each cell line • Test drug may interfere with formazan production or affect mitochondrial activity (over- or under-estimation)
ATP Assay	• Rapid & Versatile • Very sensitive assay of cell death	• Reversible ATP changes can occur without loss of viability
CELL VIABILITY		
Staining	• Simple & Rapid • Cell growth not required	• Intra-observer variability • In monolayers, dead cells detach and may be washed away ⇒ underestimation
Membrane Damage (LDH)	• Simple & Rapid • Semi-automated	• Test compound may interfere with LDH • Enzyme leakage does not always equate to irreversible injury
CELL MORPHOLOGY		
Cell morphology	• Very well-studied ⇒ excellent predictions of cell death	• Difficult to standardise • Observed changes may not mirror lethal cell damage

Table 4.2 (Continued)

There has been an increase in the use of continuous human tumour cell lines in the screening of new compounds for their anti-tumour properties (Hill, 1983; Alley *et al.*, 1988; Kratzke & Kramer, 1996). The various advantages of using these as opposed to biopsied samples for screening new agents are outlined in Table 4.3. This table also includes the limitations of using cultured cells, and some remedies that can be applied to overcome these pitfalls.

The chemosensitivity screening for novel anti-cancer compounds described above has introduced an exciting new dimension to the drug discovery field. There is little doubt this predictive screening, particularly through the use of a battery of tests, will supersede the clinical "trial and error" approach of traditional chemotherapeutic regimes, with the cancer patient ultimately reaping the benefits.

Advantages	***Limitations & Remedies***
Rapid screening capabilities – Identification of lead compounds	Artificial Environment with selection pressures causes loss of certain physiological properties and tumour characteristics ⇒ Compensate by using a wide spectrum of cell lines in the screening test.
Can study both time-and dose-dependencies	Altered drug metabolism and pharmacokinetics *in vitro* vs *in vivo* ⇒ Identify metabolite and use in assay, or include metabolising enzyme in *in vitro* test
Good supply of cellular material – large number of assays can be performed	Impossible to replicate immunological or inflammatory conditions *in vitro*.

Table 4.3: Advantages and Limitations of using cultured human tumour cells in chemosensitivity testing protocols.

4.2. OTHER ANTI-TUMOUR STRATEGIES

A new avenue currently being examined by cancer scientists is the development of anti-metastatic compounds, as opposed to anti-proliferative compounds, as tumour treatment agents (Denis & Verweij, 1997; Yu *et al.*, 1997).

4.2.1. Overview of Metastases

The ability of primary tumour cells to invade surrounding tissues and establish secondary sites of growth in areas distant from the original tumour, characterises them as a malignant, rather than benign, growth. Metastasis, the spread of a malignant tumour, is the principal cause of death in cancer patients, and has often taken place by the time a patient presents clinically. The formation of metastatic lesions entails a number of coupled, sequential steps that must be fully completed by tumour cells in order for a secondary tumour to develop (Fidler, 1990). Central to this metastatic process is the degradation of the extracellular matrix (ECM), which is accomplished through enzymatic attack of the ECM components (Duffy, 1998).

4.2.2. The Extracellular Matrix (ECM)

The ECM is a complex network of specialised glycoproteins and proteoglycans, that functions as a physical support for cells, isolating tissues and promoting attachment of cells (Haralson & Hassell, 1995). The ECM consists of the basement membrane [composed of types IV and V collagens, laminin, entactin and a number of proteoglycans], and the interstitial connective tissue [the principal component of which is collagen, with fibronectin, elastin and heparan sulphate proteoglycans, also present]. During tumour invasion and progression, disruption of the basement membrane is achieved via proteolysis, of collagen in particular, following the release of a number of enzymes (Duffy, 1998). Four main enzyme classes are implicated: serine proteinases (*e.g.* urokinase plasminogen activator or uPA), cysteine proteinases (*e.g.* cathepsin B), aspartyl proteinases (*e.g.* cathepsin D) and matrix metalloproteinases (*e.g.* MMP-2). The matrix metalloproteinases (MMPs) are believed to be most active in the advancement of this degradative process.

4.2.3. The Matrix Metalloproteinase (MMP) Family

To date this family of enzymes consists of approximately twenty members, but this list is continually growing, as new members are cloned and sequenced. All members have the following characteristics: they are endopeptidases that degrade at least one component of the extracellular matrix; they have a characteristic pattern of conserved domains, as ascertained through sequencing studies; their active site contains a zinc ion, and they require a second metal cofactor, such as calcium, for activity, they are also inhibited by chelating agents; they are secreted in a latent, proenzyme form and require activation in order to initiate proteolysis; and they are inhibited by specific tissue inhibitors of metalloproteinases [TIMPs] (Morgan, 1998).

These enzymes are involved in both normal cellular processes *e.g.* tissue remodelling and repair, and destructive pathological processes *e.g.* tumour invasion and metastasis, rheumatoid arthritis. As mentioned above the activity of these enzymes is highly controlled through secretion of latent pro-enzyme forms; the genetic expression is also intensely regulated, with transcription of genes positively regulated by a variety of biologically active agents – hormones, growth factors, cytokines, oncogenes and tumour promoters (Matrisian, 1990).

In the past this enzyme family has been sub-classified according to their substrate specificity as follows: (a) Type I collagenases, which degrade fibrillar collagenases; (b) Type IV collagenases, which degrade basement membrane collagens, and (c) Stromolysins, which have a broad substrate specificity, degrading ECM proteoglycans and glycoproteins (Matrisian, 1990). Expression of the two members of the Type IV collagenases (MMP-2 and MMP-9), is widespread and commonly elevated in human tumours (Chambers & Matrisian, 1997). In particular, enhanced *in vivo* expression of MMP-2 and MMP-9 has been shown for cancers of the prostate (Steams & Wang, 1993), breast (Monteagudo *et al.*, 1990), renal (Gohji *et al.*, 1994), skin (Karelina *et al.*, 1993) and colon. (Levy *et al.*, 1991). Regardless of tumour type both these gelatinases have been localised to the invasive front of the tumour cell mass (Chambers & Matrisian , 1997).

Due to their immense importance in cancer progression and their correlation with cancer mortality levels, inhibition of these enzymes *in vivo*, with either natural or synthetic inhibitors, is now a large focus of cancer research programs. It is hoped that suitable anti-metastatic agents, which function through inhibition of MMP expression or activity, will join current anti-proliferative agents, in future treatment strategies (Denis & Verweij, 1997; Yu *et al.*, 1997).

4.3. COUMARINS IN CANCER THERAPY

As described earlier in Section 1.8, interest in coumarins as putative anti-cancer agents arose from reports that these compounds achieved objective responses in some patients with advanced malignancies. In particular the effectiveness of coumarin *in vivo,* in malignant melanoma, renal cell and prostate cancer patients, has been described (Thomes, 1997; Ebbinghaus *et al.*, 1997; Mohler *et al.*, 1997). As a result of these observations various investigators have focussed their attention on the *in vitro* study of coumarin, and in particular its chief human metabolite, 7-hydroxycoumarin, in an attempt to determine the validity of the *in vivo* reports, and elucidate the mechanism of anti-tumour action of coumarin compounds.

4.3.1. In Vitro Testing of Coumarins

4.3.1.1. Coumarin and 7-Hydroxycoumarin

Chemosensitivity testing of coumarin and its chief human metabolite, 7-hydroxycoumarin, has been achieved by several investigators utilising *in vitro* cell proliferation-based methods. Total cell counts (Moran *et al.*, 1993; Marshall *et al.*, 1994), radionucleotide incorporation (Siegers & Bostelmann, 1993; Marshall *et al.*, 1994) and MTT growth assays (Myers *et al.*, 1994) have all been utilised. In all studies, a time- and dose-dependent inhibition of cell growth has been observed on exposure to both coumarin compounds. 7-hydroxycoumarin appears to exert a more potent inhibitory activity, which would support the widespread belief that coumarin is a pro-drug for this active agent. To date a variety of cell-lines have been tested and a pattern of cell-type sensitivity has begun to emerge, with renal, prostate, breast, glioblastoma and leukemic cell lines most susceptible to the cytostatic effects (Ebbinghaus *et al.*, 1997). Marshall *et al.* (1994) have conducted reversibility studies and found the growth–inhibitory effect of 7-hydroxycoumarin to be reversible.

Combination of coumarin and 7-hydroxycoumarin with other anti-tumour agents has shown synergistic effects *in vitro*. Zippe and co-workers (1990) showed that coumarin and 7-hydroxycoumarin in conjunction with tumour necrosis factor (TNF), caused an increased growth suppression in prostatic carcinoma cell lines. This scenario was not found for other interferon/coumarin combinations (Zippe *et al.*, 1990). In addition Myers *et al.* (1994) reported increased growth inhibition in prostatic cell lines for combined coumarin/suramin (a chemotherapeutic agent in clinical trials) dosages, compared to coumarin only exposure, illustrating the potential for coumarin usage in combination chemotherapy regimes.

4.3.1.2 Other Coumarin Derivatives

A number of natural and synthetic coumarin derivatives have also been examined *in vitro* for their growth inhibitory activity. Rosskopf and colleagues (1992) studied the anti-tumour effects of coumarin and some of its derivatives on the *in vitro* proliferation of mastocytoma (P-815) and leukaemic (P-388) cells. All compounds (7-hydroxycoumarin, 4-hydroxycoumarin, o-, m-, and p-coumaric acid) were found to be cytotoxic at concentrations greater than 100μg/ml. Egan *et al.* (1997) determined the *in vitro* activity of the synthesised derivative, 8-nitro-7-hydroxycoumarin. This compound displayed cytotoxic properties in two human cell lines tested (HL-60 and K562), inducing cell death by apoptosis. This compound exerted a cytostatic effect on the three other cell lines tested, exerted through a perturbation in their cell cycle (Egan *et al.*, 1997). Kolodziej and co-workers (1997) evaluated the cytotoxicity of 22 simple coumarins to two human cell lines (colorectal and small cell lung carcinoma) using the MTT assay. While most compounds exhibited low toxicities to these cells lines over 96hrs, they found the dihydroxy-compounds (6,7- and 6,8-dihydroxycompounds) to act as very potent anti-proliferative agents.

4.3.2. In Vivo Testing of Coumarins

The chemosensitivity testing of coumarin and its derivatives has also taken place in animal (*in vivo*) models. In various tumour models in test animals (Omarbasha *et al.*, 1989; Maucher *et al.*, 1993; von Angerer *et al.*, 1994), coumarin was shown to strongly inhibit malignant growth. Tseng (1993) also demonstrated the efficacy of coumarin in reducing tumour formation in transgenic mice, at non-toxic levels (200μM).

Raev *et al.* (1990) used xenograft models to assess the anti-tumour activity of five coumarin-pyridine derivatives. Implantation of leukaemic and lung carcinoma cells, was followed by intra-peritoneal treatment with the test compounds. Only one caused inhibition of tumour growth.

4.3.3. Further Work with Coumarin Derivatives

As part of the work described in this chapter, we chose to examine two coumarin compounds, previously unexamined by coumarin investigators for their anti-cancer properties. It

has become apparent, from the literature, that the previous belief that 7-hydroxycoumarin was the only metabolite of importance *in vivo* is flawed. As outlined in Section 1.4.2.2, the existence of other hydroxy-metabolites, has been described for humans. In particular, 3-hydroxy-, 4-hydroxy-, 6-hydroxy- and 6,7-dihydroxy-coumarin (also known as esculetin) production has been observed in humans (van Sumere *et al.*, 1959; Fentem & Fry, 1992; Steensma *et al.*, 1994). It is plausible that one of these, rather than, or in conjunction with 7-hydroxycoumarin, may be responsible for the anti-cancer properties of coumarin *in vivo*.

3-hydroxycoumarin has been well-characterised as a compound, thanks to the large number of animal studies accomplished during the controversy regarding the toxicity of coumarin to humans (Section 1.5). Being the structural backbone of the widely used anti-coagulant, warfarin, the properties of 4-hydroxycoumarin have also been well-researched, and warfarin has found use in the treatment of a variety of cancer cell types (Cooke *et al.*, 1997). However, neither 6-hydroxycoumarin nor esculetin have been investigated widely – we therefore chose to examine these two compounds, in detail in our studies.

Very little work has been reported on the biological properties of 6-hydroxycoumarin – one research group included this compound in their examination of retinal function recovery following ischemia, and the effect of various coumarins on this event. Recovery of retinal cells in the presence of 6-hydroxycoumarin was not as effective as in the presence of esculetin (Liu *et al.*, 1997). To date, no other assessment of the biological or anti-tumour activity of this compound has been reported.

The biological properties of esculetin have been investigated more thoroughly. Esculetin is a known free-radical scavenger (Paya *et al.*, 1992; Chang *et al.*, 1996), a property shared with various coumarins including 7-hydroxycoumarin. Some of its scavenging ability derives from its inhibition of the superoxide-generating xanthine-oxidase enzyme (Chang & Chiang, 1995). Esculetin has also been reported as a potent inhibitor of lipoxygenases (Sekiya *et al.*, 1982; Kimura *et al.*, 1985; Hoult *et al.*, 1994) This may be of some importance from a growth inhibitory viewpoint, as leukotrienes (formed by the action of lipoxygenases on arachidonic acid) have been shown to be involved in cell transformation, tumour growth and metastases formation (Honn & Marnett, 1984; Reich & Martin, 1996). Administration of lipoxygenase inhibitors has been shown to decrease proliferation of various cancer cell lines *in vitro* (Rose & Connolly, 1990, 1991; Hofmanova *et al.*, 1996), and some studies have examined esculetin in this capacity (Hofmanova *et al.*, 1996; Noguchi *et al.*, 1997). Esculetin could suppress growth in both the presence or absence of

linoleic acid (Noguchi *et al.*, 1997). The 6,7-dihydroxy-function on a coumarin ring was found by Kolodziej and colleagues to exert a very potent anti-proliferative effect, in their broad assessment of growth inhibition of coumarin compounds. Esculetin has also been shown to inhibit the growth of vascular smooth muscle cells (Huang *et al.*, 1993), but this effect was believed to be mediated by inhibition of cellular tyrosine kinases.

4.4. CHAPTER OUTLINE

This chapter outlines chemosensitivity studies into the effects of four coumarin compounds (coumarin, 6-hydroxycoumarin, 7-hydroxycoumarin and esculetin) on cultured human tumour cell-lines. Coumarin, 7-hydroxycoumarin and esculetin were commercially-available products, while 6-hydroxycoumarin was synthesised and characterised in DCU, by Dr. Oliver Egan (The characterisation of the synthesised compound is outlined in Appendix 2).

The effect of these four compounds on growth inhibition in nine cell lines is outlined. The effects of these compounds on cellular metabolism and protease secretion from one cell line in particular, is then described in detail.

4.5. RESULTS AND DISCUSSION

4.5.1. In Vitro Proliferation Assays

Previous investigators have used cell proliferation-based assays in their chemosensitivity assessment of coumarin compounds (Moran *et al.*, 1993; Siegers & Bostelmann, 1993; Marshall *et al.*, 1994; Myers *et al.*, 1994). For comparative purposes, we chose initially to examine the four coumarin compounds, namely coumarin, 6-hydroxycoumarin, 7-hydroxycoumarin and esculetin, under similar assay procedures, for a range of cell lines (Table 2.2). Nine cell lines were exposed to each of the four compounds for 96 hours as outlined in Section 2.2.4.2. In general, cells were exposed to drug concentrations in the range 0-200μg/ml 6-hydroxycoumarin, 0-20μg/ml esculetin, while ranges of 0-150μg/ml, were utilised for coumarin and 7-hydroxycoumarin exposure. However, variations on this dosing schedule did occur depending on observed cell line sensitivity. Following exposure, cell counts were performed, and the increase in cell number for drug-treated cells was expressed as a percentage of the increase for solvent-treated control cells, and growth curves were constructed from this data.

Figure 4.1. shows typical growth curves for cells exposed to the four coumarin compounds, for two of the nine cell lines tested – A431, an epidermal carcinoma cell line of vulval origin, and MCF-7, a breast carcinoma cell line. In the case of the A431 cell line, preliminary studies showed this cell line to be quite sensitive to the coumarin compounds and thus exposure to the four compounds is illustrated in the range 0-20μg/ml drug (Figure 4.1, top). As shown, all four compounds caused a significant cytostatic effect over the 96 hour exposure period, with 7-hydroxycoumarin illustrating the most potent effect, followed by esculetin, coumarin and 6-hydroxycoumarin, in decreasing order of potency. The growth curve shown for the MCF-7 cell line (Figure 4.1, bottom) is more typical of the curve type obtained for most of the other eight cell lines tested for two reasons. Firstly, a concentration range up to 200μg/ml was used for all compounds except esculetin, where the range 0-20μg/ml was utilised to observe cytostatic effects. Secondly, the sensitivity trends observed showed the cell line relatively insensitive to the effects of coumarin, extremely sensitive to the effects of esculetin, with an intermediate cytostatic effect observed for the two hydroxycoumarin compounds. Growth curves for the other seven cell lines tested are included in an appendix (Appendix 2).

The graphs constructed for each cell line/drug combination were utilised to obtain IC_{50} values (IC_{50} value defined as the concentration of drug that causes a 50% inhibition of growth in

drug-treated cells as compared to untreated control cells). These values are shown in Table 4.4, and together with the growth curves illustrate some important facets of the cytostatic effects of the four coumarin compounds. Firstly, the parental coumarin compound did not adversely affect the proliferation of the majority of the cell lines tested. Although a concentration-dependent decrease in proliferation was observed on exposure to coumarin, it was minimal, and seven of the cell lines tested had IC_{50} values greater than 140μg/ml. Of the two remaining cell lines, as mentioned before, the A431 cell line appeared extremely sensitive to all the coumarin compounds, and the NIH3T3 cell line is not a tumour cell line, but a normal mouse cell line, included in the assays as a representative fibroblastic, rather than epidermal-type cell. The insensitivity of most of the cell lines tested to growth inhibition by coumarin, seems to confirm the generally held belief that coumarin is not responsible for the observed *in vivo* effects, but is a pro-drug for other active metabolites.

Secondly, with regard to 6-hydroxycoumarin, addition of a hydroxyl group at the 6′-position on the parent structure did augment the growth inhibitory properties of this compound. Of seven cell lines, five showed increased growth inhibition on exposure to 6-hydroxycoumarin compared to that observed for coumarin. All growth curves showed the concentration-dependent effect of this growth inhibition. At times, a certain concentration "threshold" had to be reached before inhibition of growth was observed, *e.g.* for SW480 cells no effect on cell proliferation was observed until ~ 50μg/ml. This "threshold" effect was duly reflected in the IC_{50} value of SW480, which was large at ~149μg/ml. Of the other six cell lines, three, namely A431, MCF-7 and PC-3 (a prostate carcinoma cell line), were more sensitive to growth inhibition by 6-hydroxycoumarin, as reflected in their IC_{50} values.

Addition of a hydroxyl group at the 7′-position of the coumarin ring also introduced increased growth inhibitory properties to the core coumarin structure. This 7-hydroxycoumarin compound has been widely studied as it is the chief human metabolite, and, thus, of immense interest to investigators trying to understand the *in vivo* anti-tumour properties of coumarin. As shown here, this increased growth suppression appeared relatively cell-type specific with prostate (PC3), leukemic (HL60), bladder (T-24) and breast (MCF-7) cell lines showing increased sensitivity, but colon and lung lines insensitive to this effect. Again the cytostatic effects were dose-dependent. These results reflected well the trends outlined by previous investigators (Ebbinghaus *et al.*, 1997; Seliger, 1997)

The most evident trend from the proliferation curves and table of IC_{50} values, is the extraordinary sensitivity of all cell lines tested, to growth inhibition by esculetin (6,7-

dihydroxycoumarin). With the exception of the A431 cell line, this compound is 10-100 times more inhibitory to cell proliferation than the parent coumarin compound. This reflects well the previous observation by Kolodziej and colleagues (1997), that a dihydroxy-function in either an ortho- or meta-format, was an extremely potent chemical structure for toxicity in two human tumour cell lines, as ascertained by the MTT assay. As this potency was not evident in either of the single-hydroxycoumarin compounds, the added potency must be due to the existence of a double hydroxy-function on the coumarin ring.

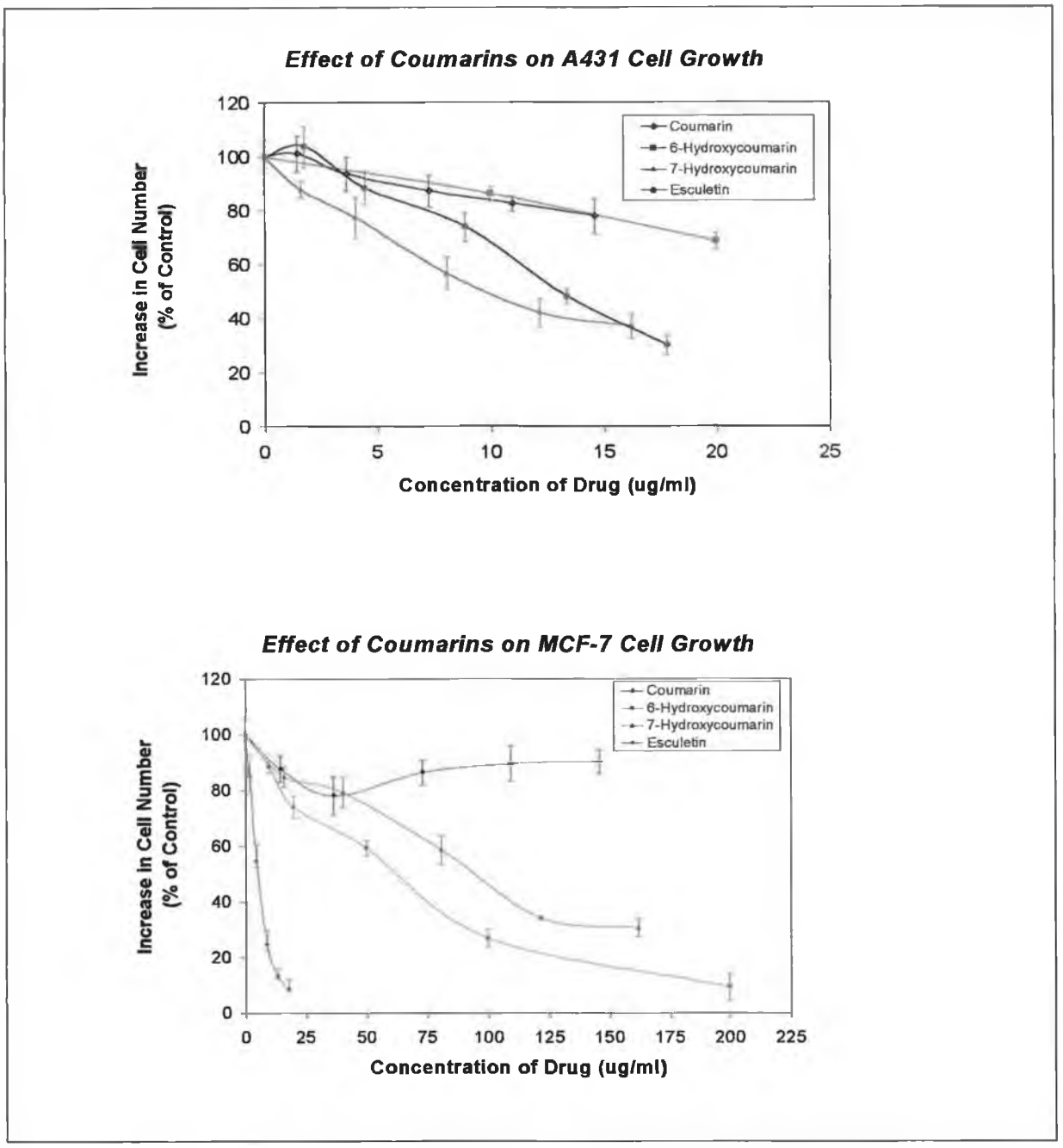


Figure 4.1: ***In Vitro*** **Cell Proliferation Assays following exposure of cells (A431, top and MCF-7, bottom) to 4 coumarin compounds for 96 hours. All experiments were conducted in duplicate on three separate occasions. The increase in cell number for drug-treated cells was expressed as a percentage of the untreated control cells and plotted versus the appropriate drug concentration.**

Cell Line	*IC_{50} Coumarin (ug/ml)*	*IC_{50} 6-HC (ug/ml)*	*IC_{50} 7-HC (ug/ml)*	*IC_{50} Esculetin (ug/ml)*
A431	40.43 ± 0.99	56.94 ± 4.17	10.31± 3.03	13.87 ± 1.17
MCF-7	> 150	57.03 ± 2.12	101.33 ± 9.36	4.67 ± 0.21
PC-3	> 150	64.44 ± 2.63	78.75 ± 1.23	8.83 ± 1.56
T-24	140.75 ± 12.02	117.96 ± 2.51	99.50 ± 6.32	3.20 ± 0.10
A549	> 150	91.19 ± 2.29	> 150	2.96 ± 0.32
HL-60	> 150	100.30 ± 7.83	69.35 ± 4.35	1.59 ± 0.09
SW480	> 150	148.82 ± 18.34	> 150	4.52 ± 0.44
SW620	> 150	N.D.	> 150	5.64 ± 0.35
NIH3T3	89.21 ± 2.11	N.D.	69.36 ± 5.10	0.73 ± 0.05

Table 4.4: **IC_{50} Values determined for the exposure of various cell lines to coumarins. The IC_{50} value is defined as the concentration that causes a 50% inhibition of growth in drug-treated cells, as compared to untreated control cells. For each cell line/drug combination, the IC_{50} value was determined from interpolation of the relevant growth curve. Each experiment was carried out in duplicate on three separate occasions (n=3), and the mean IC_{50} value (± standard deviation) for these experiments determined and tabulated above.**

4.5.2. Further Chemosensitivity Testing in A431 Cells

As outlined in Section 4.1. cell proliferation assays are only one of a number of chemosensitivity tests which can be used to examine the response of cells to chemical agents. Therefore, as part of our study into the effects of coumarins on cultured cells, we decided not to limit this investigation solely to the anti-proliferative effects of these agents, as many investigators in the past have done, but to also examine the effect of these compounds on other cellular mechanisms. We decided to limit this investigation to one cell line, A431, which as outlined in Section 4.5.1, was extremely sensitive to all four coumarins tested.

4.5.2.1. Lactate Dehydrogenase (LDH) Assay

The LDH assay is used to examine the effect of agents on cell membrane integrity and is often regarded as a measure of cytotoxicity (Welder & Acosta, 1994). When the plasma membrane of any cell is damaged as a result of toxic insult, lactate dehydrogenase (LDH), a ubiquitous cytoplasmic enzyme is rapidly released into the extracellular environment, and can be assayed as outlined in Section 2.2.4.3.

Although this assay is a rapid and simple quantification of cellular damage, one disadvantage of the assay, as detailed in Table 4.2. is the possibility that the test compound may inhibit the LDH enzyme activity, causing underestimation of membrane damage. Additionally, the test compound may also react with other assay components, *e.g.* tetrazolium salts, causing interference, which again may lead to inaccurate results. Therefore it is essential when assessing membrane damage with the LDH Assay, that these two possible interferences are excluded. Inhibition of LDH activity is tested by incubating a standard LDH solution with various drug concentrations, and determining the activity of the enzyme in the presence of these drug concentrations. The second interference can be discounted by incubating the drug in culture media with the LDH Assay reagents and detecting whether presence of the drug gives rise to an increased absorbance at 492nm (assay absorbance wavelength) compared to drug-free culture medium.

This interference testing was achieved for all four coumarin compounds under investigation (coumarin, 6-HC, 7-HC and esculetin) as shown in Figure 4.2. This diagram illustrates that none of the four compounds are inhibitors of the enzyme activity (top), or react with the assay components yielding interfering species (bottom).

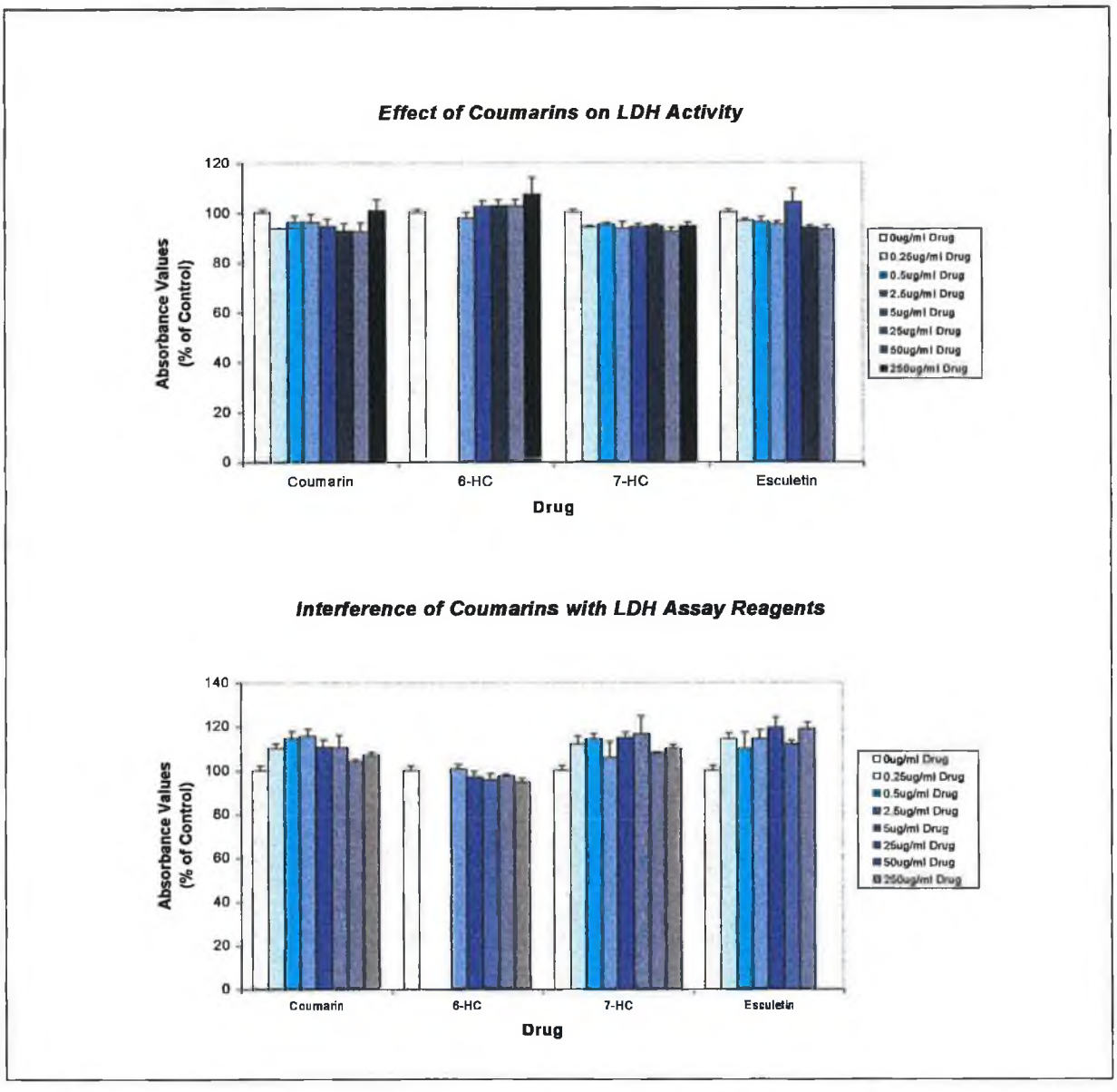


Figure 4.2: **Control tests confirming that coumarin compounds (0.25-250μg/ml) do not interfere with LDH Assay reagents. Results (A_{492nm}) for drug solutions are expressed as a percentage of the control absorbance (A_0) at 492nm. The coumarins when incubated with an LDH standard (0.1U/ml) did not inhibit this LDH activity (top). A_0 for LDH standard (0.1U/ml) = 0.594 ± 0.023. The coumarins when incubated with culture medium alone did not interfere significantly with other LDH assay reagents (bottom). A_0 for control medium = 0.181 ± 0.003**

Having excluded interferences, A431 cells were incubated with the four coumarins (range 0.25-250μg/ml) for 24 hours and the culture supernatants assessed for enzyme release using the LDH Assay, as outlined in Section 2.2.4.3. A 1% (v/v) Triton X-100 solution in culture medium was used as a positive control representing 100% membrane damage. The absorbances at 492nm for cells exposed to Triton X-100 were normalised at 100%, and absorbances of cells exposed to the various drug concentrations expressed as a percentage of this positive control (Figure 4.3). As can be seen, 24 hour exposure of A431 cells to coumarins caused no significant membrane damage when compared to untreated cells.

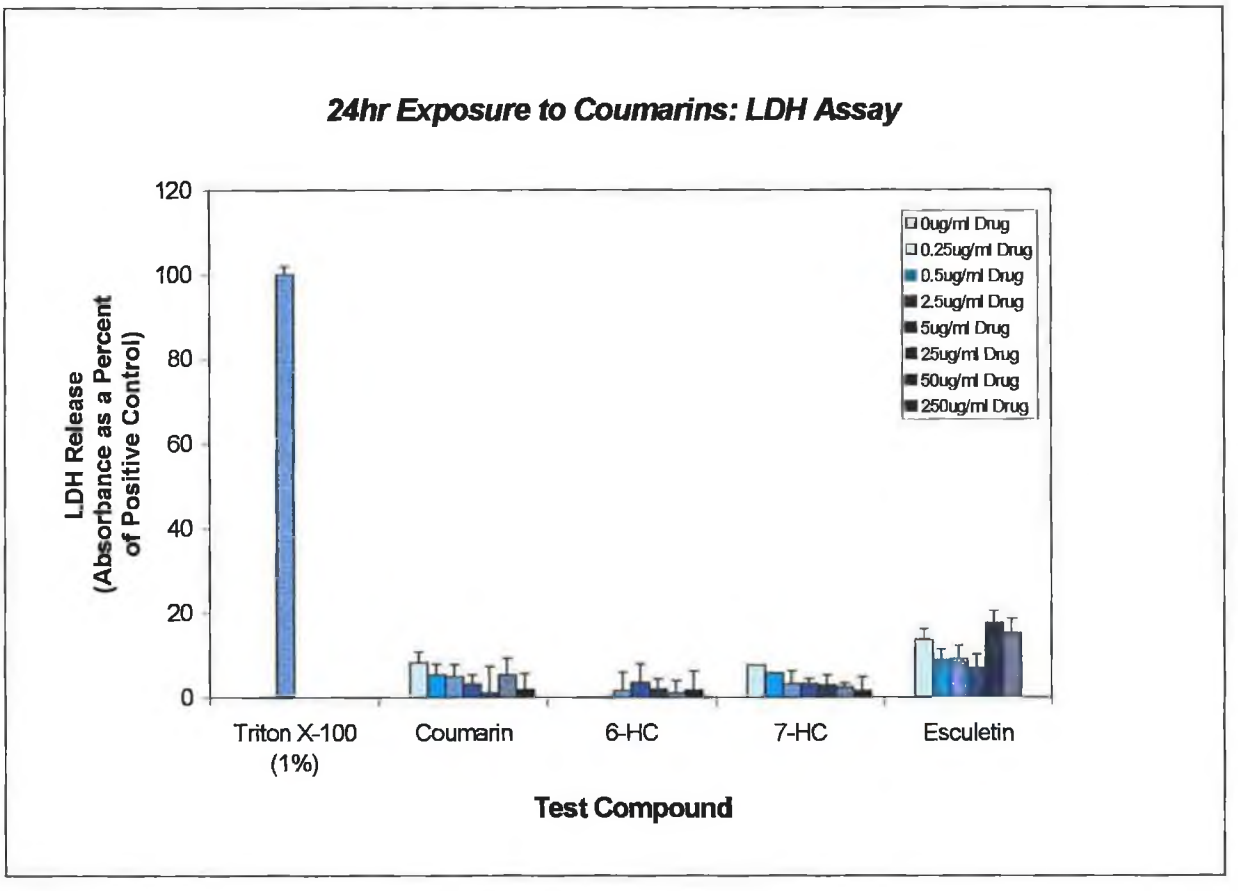


Figure 4.3: **LDH Assay of culture supernatants of A431 cells exposed to coumarins (0-250μg/ml for 24 hours. Results (A_{492nm}) for coumarin-treated cells are expressed as a percentage of A_{492nm} of positive control (Triton X-100) cells (Abs =1.417 ± 0.028). Each drug was tested in seven separate wells/plate, and the experiment was carried out on three separate occasions.**

4.5.2.2. Assays for Cellular Metabolism

We next proceeded to assess the effect of the coumarin compounds on cellular metabolism. Two methods were used in this investigation: the MTT Assay and the Cytosensor Microphysiometer. As will be outlined the latter method was found for a number of reasons to be superior for this type of assessment.

4.5.2.2.1. MTT Assay

The MTT Assay is a well-established colourimetric assay which can be used to detect the effects of agents on cellular metabolism. The assay is based on the cleavage of the yellow tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), to purple formazan crystals by mitochondrial dehydrogenases. Therefore cells must not only be alive, but also metabolically active for this reaction to occur (Mosmann, 1983). The assay can be used in either a long- (96hr) or short-term (24hr) drug-exposure format, to assess the effects of a drug on cellular growth or metabolism, respectively. Previously, the MTT assay has been used in the long-term format to assess the anti-proliferative effects of coumarins (Myers *et al.*, 1994; Kolodziej *et al.*, 1997).

The assay was carried out as outlined in Section 2.2.4.4. Prior to determining the effects of the coumarin compounds on cellular metabolism in A431 cells, optimisation of cell seeding density (Figure 4.4) and interference testing (Figure 4.5) were necessary. Optimum seeding density of A431 cells was found to be 5 X 10^5 cells/ml, and this was used in all subsequent assays. Incubating the MTT reagents with drug concentrations in the absence of cells (Figure 4.5) tested interference effects of the coumarins. As shown neither coumarin or 7-hydroxycoumarin interfered with the tetrazolium salt, while 6-hydroxycoumarin interfered very slightly with the MTT salt at 100μg/ml (ΔA_{560nm} = 0.063 ± 0.003). However, esculetin reacts mildly with the tetrazolium salt in the concentration range 10-100μg/ml, but intensely at concentrations >100μg/ml (ΔA_{560nm} = 0.431 ± 0.010 – 1.847 ± 0.044). Therefore, all subsequent MTT assays were conducted with drug concentrations ≤ 100μg/ml, and in the case of 6-hydroxycoumarin and esculetin, absorbance values were corrected for the interference effects.

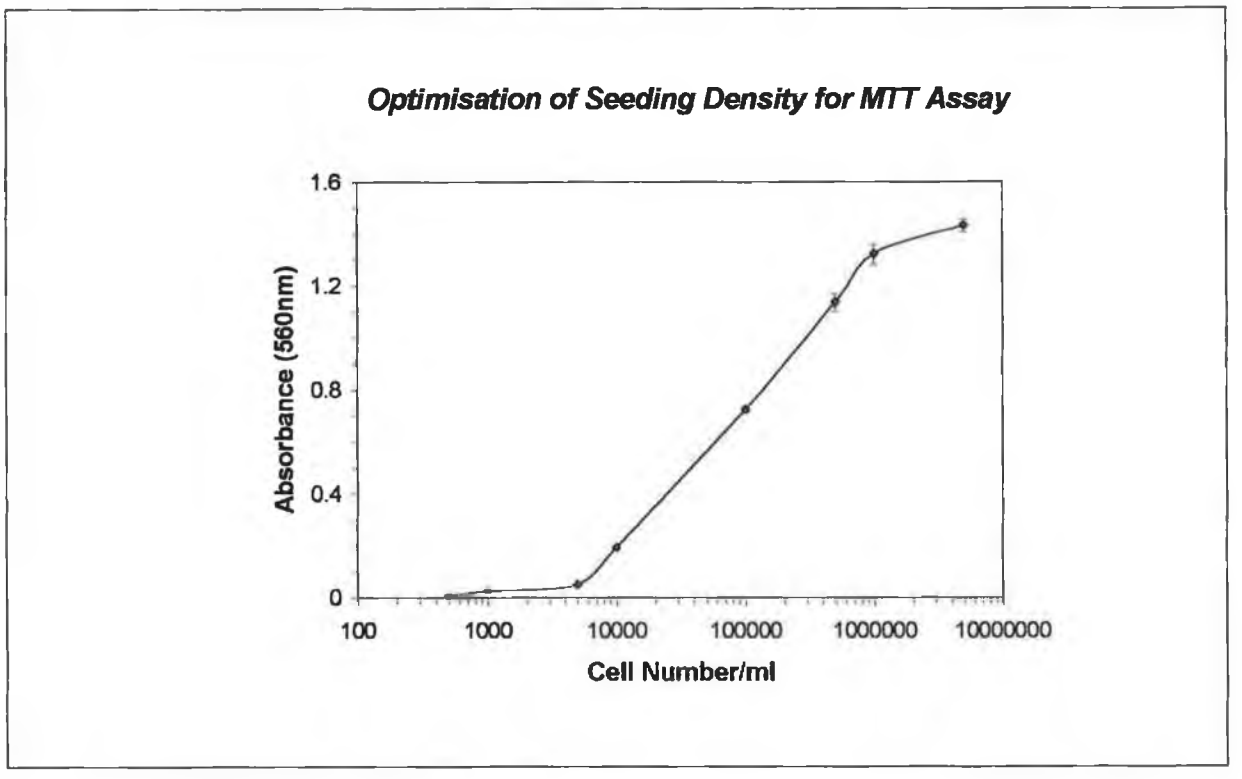


Figure 4.4: **Optimisation of Seeding Density of A431 cells for MTT Assay. 5 X 10^5 cells/ml was chosen as the optimum density as it gave a high A_{560nm} value (1.137 ± 0.033), at a pre-plateau point on the curve.**

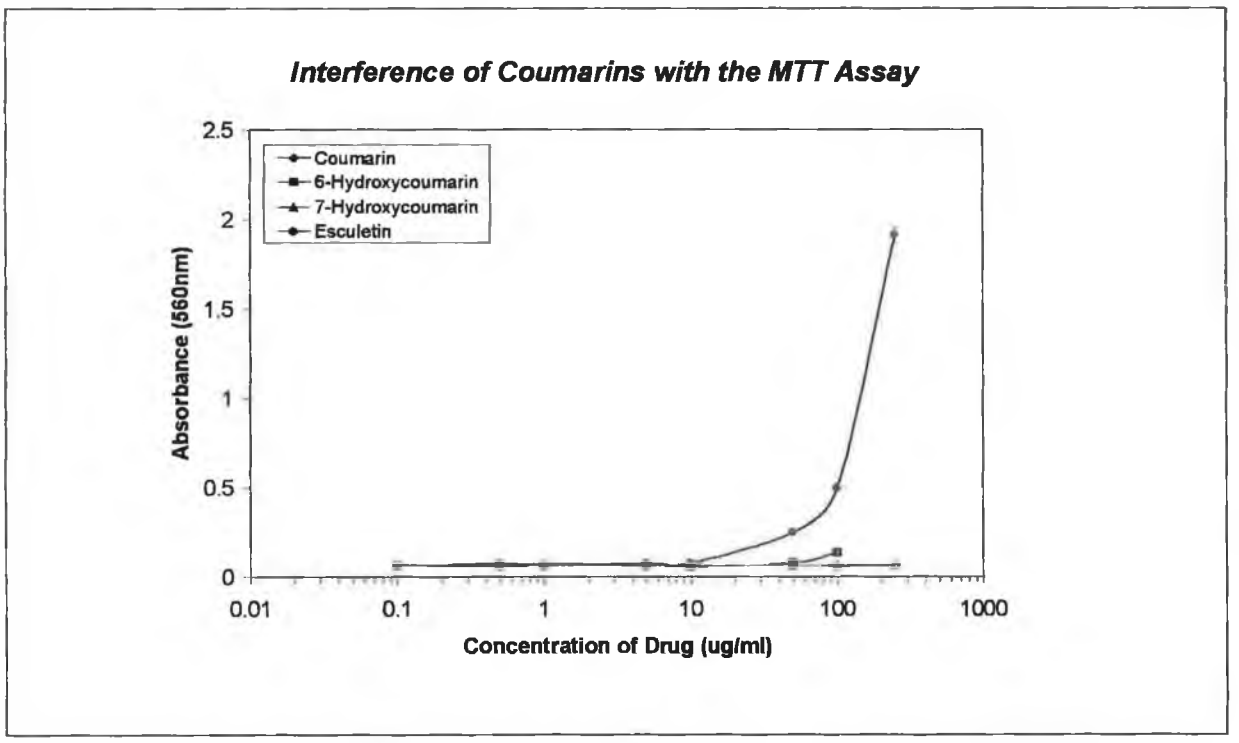


Figure 4.5: **Examination of the interference of coumarin compounds with MTT tetrazolium salt in the absence of cells. Neither coumarin nor 7-hydroxycoumarin interfere, 6-hydroxycoumarin reacts very slightly at 100μg/ml, but esculetin reacts intensely with the MTT salt especially at concentrations > 50μg/ml.**

MTT assays were conducted on A431 cells for all four coumarin compounds in the concentration range 0-100μg/ml, with 24hr drug exposure. Figures 4.6 and 4.7 show the results for these experiments. From Figure 4.6 it can be seen that neither coumarin nor 6-hydroxycoumarin exerted any detrimental effect on the metabolic activity of mitochondrial dehydrogenases in cells exposed to these drugs for 24hrs. Exposure of cells to 7-hydroxycoumarin for 24hrs caused a slight decrease in metabolic activity at concentrations greater than 10μg/ml - % decrease of 5-10% to 100μg/ml (90.37 ± 2.8%). Esculetin exerted a more serious effect on the metabolic conversion of the MTT salt compared to control cells, with decreases in activity observed at greater than 1μg/ml. An inhibition of metabolism of 28.38 ± 2.96 was observed at the highest tested concentration (100μg/ml).

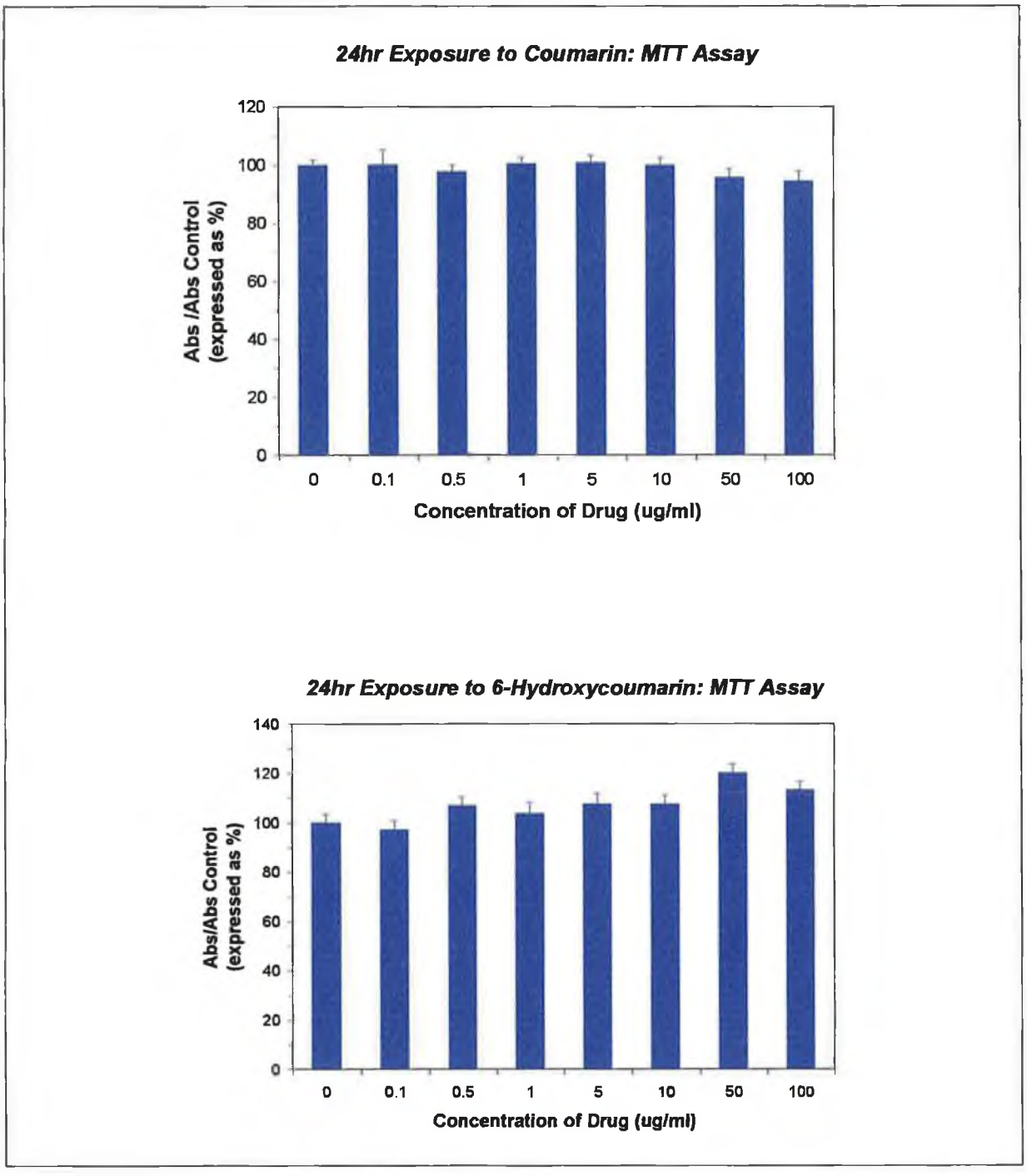


Figure 4.6: **MTT Assay following 24hr exposure to coumarin (top) and 6-hydroxycoumarin (bottom) in the concentration range 0-100μg/ml. All drug concentrations were tested in seven separate wells/plate on three separate occasions. All absorbances (560nm) of test wells were expressed as a percentage of control well absorbances (A_0). A_0 for coumarin = 1.072 ± 0.020; A_0 for 6-hydroxycoumarin = 1.185 ± 0.043**

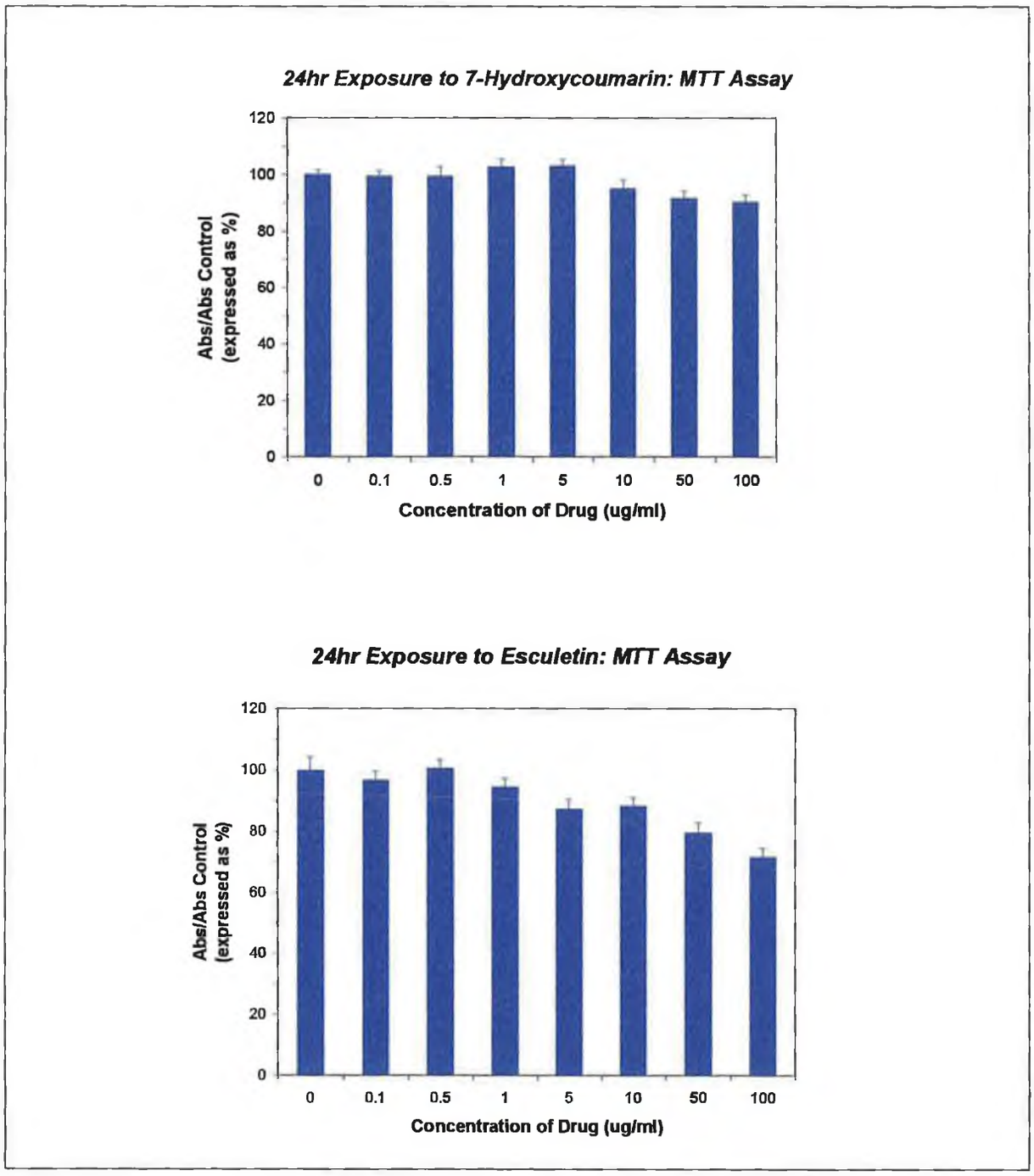


Figure 4.7: **MTT Assay following 24hr exposure to 7-hydroxycoumarin (top) and esculetin (bottom) in the concentration range 0-100μg/ml. All drug concentrations were tested in seven separate wells/plate on three separate occasions. All absorbances (560nm) of test wells were expressed as a percentage of control well absorbances (A_0). A_0 for 7-hydroxycoumarin = 0.977 ± 0.018; A_0 for esculetin = 1.012 ± 0.044.**

4.5.2.2.2. Cytosensor Microphysiometer

A second method was used to evaluate the effect of coumarins on cellular metabolism. As described in detail in Chapter 3, this biosensing instrument determines the extracellular acidification rate of cells, which is closely coupled to their basal metabolic rate. Monitoring of cellular metabolism is achieved using a pH-sensitive sensor, in "real-time", which offers distinct advantages over the end-point nature of the MTT assay, when examining the effect of chemical agents on cellular metabolism.

The effect of 24hr exposure of A431 cells to three coumarin compounds (6-hydroxycoumarin, 7-hydroxycoumarin and esculetin) was achieved using this technique (Figures 4.8-4.10). In these experiments the basal metabolic rate of the cells was determined prior to drug-exposure, and this value was normalised as 100%. This was achieved for each of the four separate sensor chambers in the Cytosensor. Following drug exposure all subsequent metabolic rates were expressed as a percentage of the basal rate of the particular sensor chamber, ensuring each chamber with its encased cells acted as its own internal control.

Figure 4.8 illustrates the dose-dependent depression of metabolism in A431 cells, on exposure of the cells to 6-hydroxycoumarin over 24hrs. Concentrations required to achieve this were high when compared to levels of 7-hydroxycoumarin and esculetin (Figures 4.9 and 4.10, respectively). Initially, during the first 4 hours of exposure, concentrations of 50 and 100μg/ml caused a slight increase in the metabolic rate of the cells, (~ 109% and 105%, respectively). This was followed by a slow suppression of metabolism over the 24hrs monitored. The metabolic rate of the cells also declined steadily after 2hrs exposure to 200μg/ml 6-hydroxycoumarin, and attained a metabolic rate ~ 65% of the basal rate after 24hrs.

Exposure of A431 cells to 7-hydroxycoumarin caused a much more immediate and damaging effect on the basal cellular metabolism (Figure 4.9). A continuous, gradual increase in metabolic inhibition was observed for all concentrations in the range 2-100μg/ml. Again a dose-dependent effect was apparent. At higher concentrations (10-100μg/ml), strong depression of the metabolic rate was evident in the first 2hrs of exposure.

Figure 4.10 illustrates the exposure of A431 cells to a range of esculetin concentrations and the consequent depression of basal cellular metabolism as a result. It is obvious from this figure that esculetin concentrations greater than 20μg/ml severely damaged the metabolic functioning of cells

over a 24hr exposure period – at these concentrations, the metabolism of cells 24hrs post-exposure was only ~ 15-20% of the basal metabolic rate. At 50 and 100μg/ml the principal damage to the cells metabolism occurred within the first ten hours of exposure. At lower concentrations, damage was dose- and time-dependent.

One detail that was impossible to ignore from the data collected, was the fact that although the trends were similar, the Cytosensor Microphysiometer was more sensitive to the effects of the coumarins on cellular metabolism than the MTT assay. For example, the MTT assay detected a decrease in cellular metabolism of ~ 28% when A431 cells were exposed to 100μg/ml esculetin for 24hrs (Figure 4.7, bottom). According to the cytosensor results, the cellular metabolism in cells exposed to 100μg/ml esculetin decreased by ~ 79% over this time period (Figure 4.10). In fact it would appear from comparing the MTT and Cytosensor results for 7-hydroxycoumarin and esculetin (Figure 4.11), that the Cytosensor Microphysiometer is a much more sensitive predictor of metabolic inhibition. The reason for this is unclear but may be coupled to the fact that the MTT assay relies on the activity of just one group of mitochondrial enzymes to predict adverse effects on metabolism, and in doing so may underestimate metabolic effects. The MTT assay has been shown to underestimate the growth inhibitory effects of interferons (IFNs) in the past (Jabbar *et al.*, 1989). This was partially attributed to a difference in the mitochondrial activity of control and treated cells, with IFN-treatment causing increased activity. Formazan production can also be induced by drugs that cause perturbations of the cell cycle (Supino, 1995). Cells have also been observed to metabolise the tetrazolium dye when lethally damaged and have lost the ability to exclude vital dyes (Maehara *et al.*,1987).

Apart from the increased sensitivity of prediction, the Cytosensor has several other advantages over the MTT assay for the detection of metabolic suppression. As mentioned before, the real-time aspect of detection with the cytosensor yields large amounts of information on the nature of the metabolic suppression (*e.g.* time of detrimental effects, *etc.*), per experiment. To attain this information with an end-point system such as the MTT Assay would require multiple, kinetic end-point assays. In addition, the exposure set-up of the Cytosensor (flow-through, perfusive) mimics the *in vivo* drug delivery/exposure more than the static exposure set-up of the MTT Assay. Finally, as described in Section 4.5.2.2.3. the Cytosensor Microphysiometer allows for reversibility studies to be carried out on drug-treated cells to assess their recovery, an experiment difficult, if not impossible to achieve with cells following the MTT Assay.

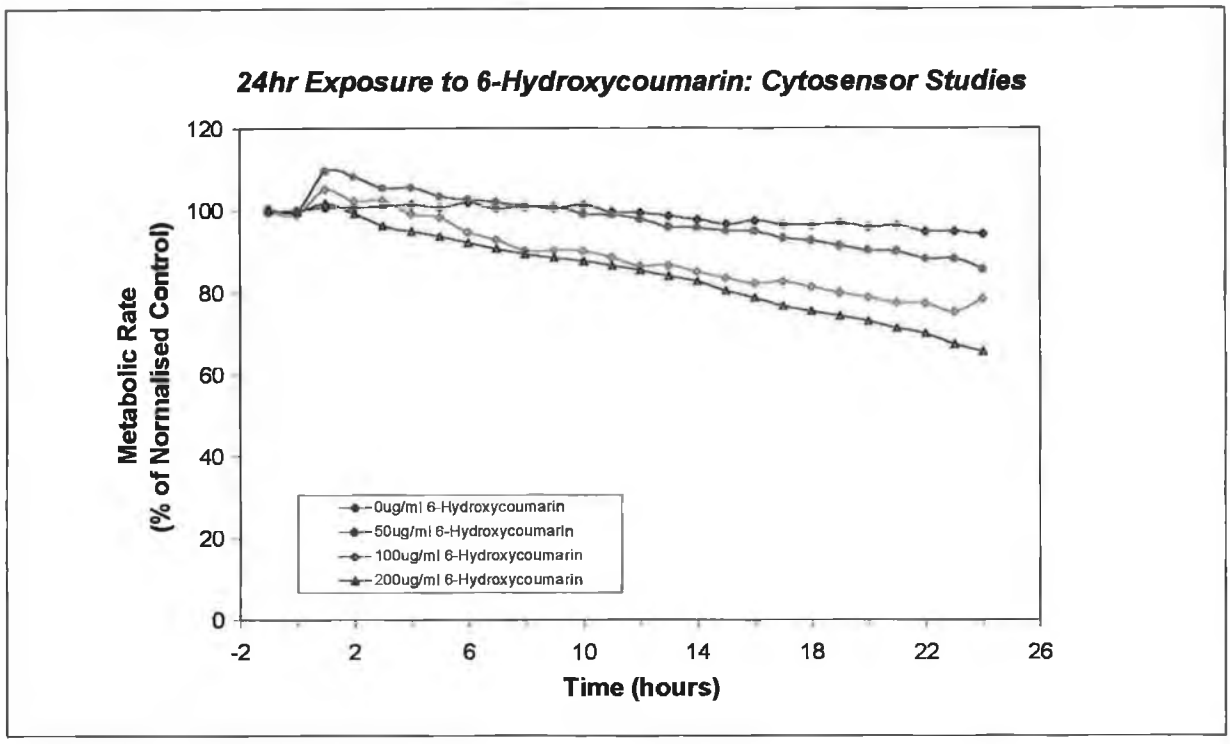


Figure 4.8: **Exposure of A431 cells to various concentrations of 6-hydroxycoumarin for 24hrs. The above plot shows mean of three experiments conducted on the Cytosensor Microphysiometer, to determine the effects of the drug on cellular metabolism (Section 2.2.4.5). The cellular acidification rate for each sensor chamber was determined prior to drug exposure, using a 4 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during drug-exposure expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates (-μvolts/sec) for the four outlined chambers from one experiment were -116.78 (Control), -120.05 (50μg/ml), -125.8 (100μg/ml) and -125.65 (200μg/ml).**

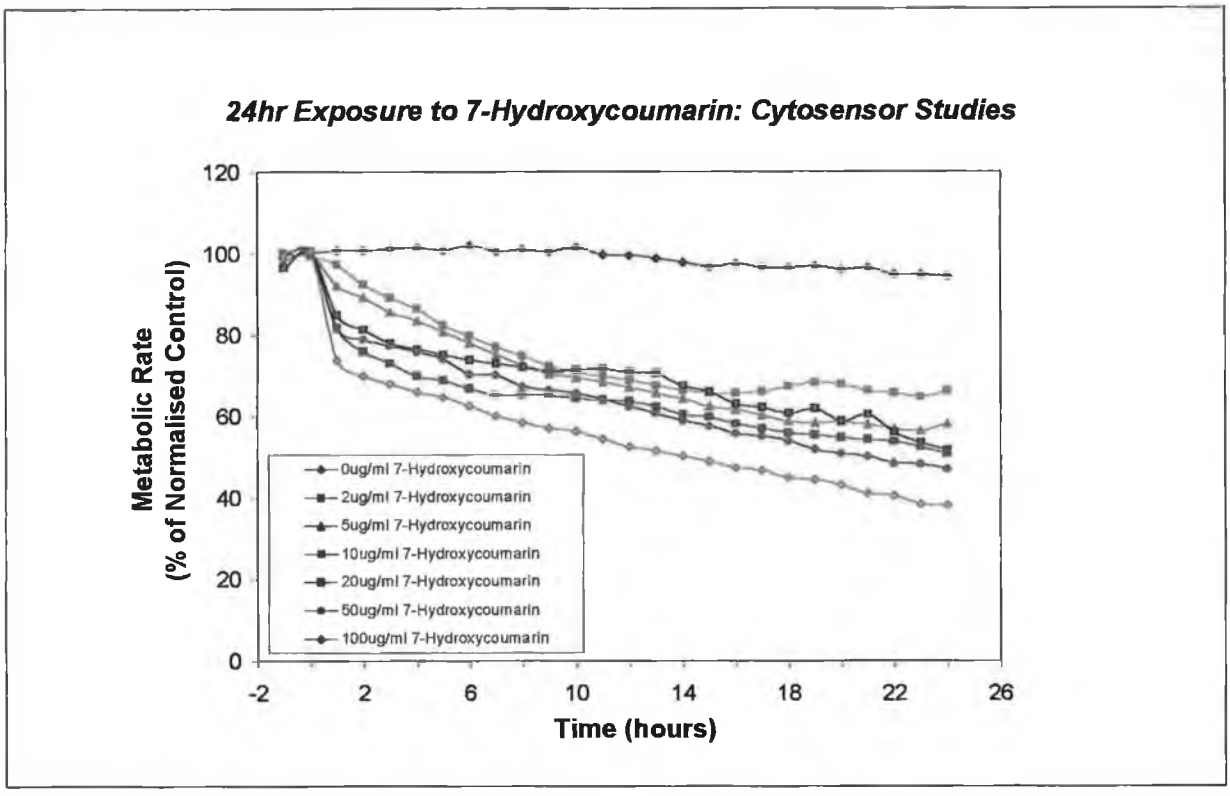


Figure 4.9: **Exposure of A431 cells to various concentrations of 7-hydroxycoumarin for 24hrs. The above plot shows the mean of four experiments conducted on the Cytosensor Microphysiometer, to determine the effects of the drug on cellular metabolism (Section 2.2.4.5). In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to drug exposure, using a 4 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during drug-exposure expressed as a percentage of this normalised value. These values of metabolic rate (mean of four experiments) were plotted vs time in the above graph. Typical basal acidification rates (-μvolts/sec) for the seven outlined chambers in one of the experiments were -139.09 (Control), -117.15 (2μg/ml), -151.22 (5μg/ml), -158.97 (10μg/ml), -116.42 (20μg/ml), -113.79 (50μg/ml), and -166.18 (100μg/ml).**

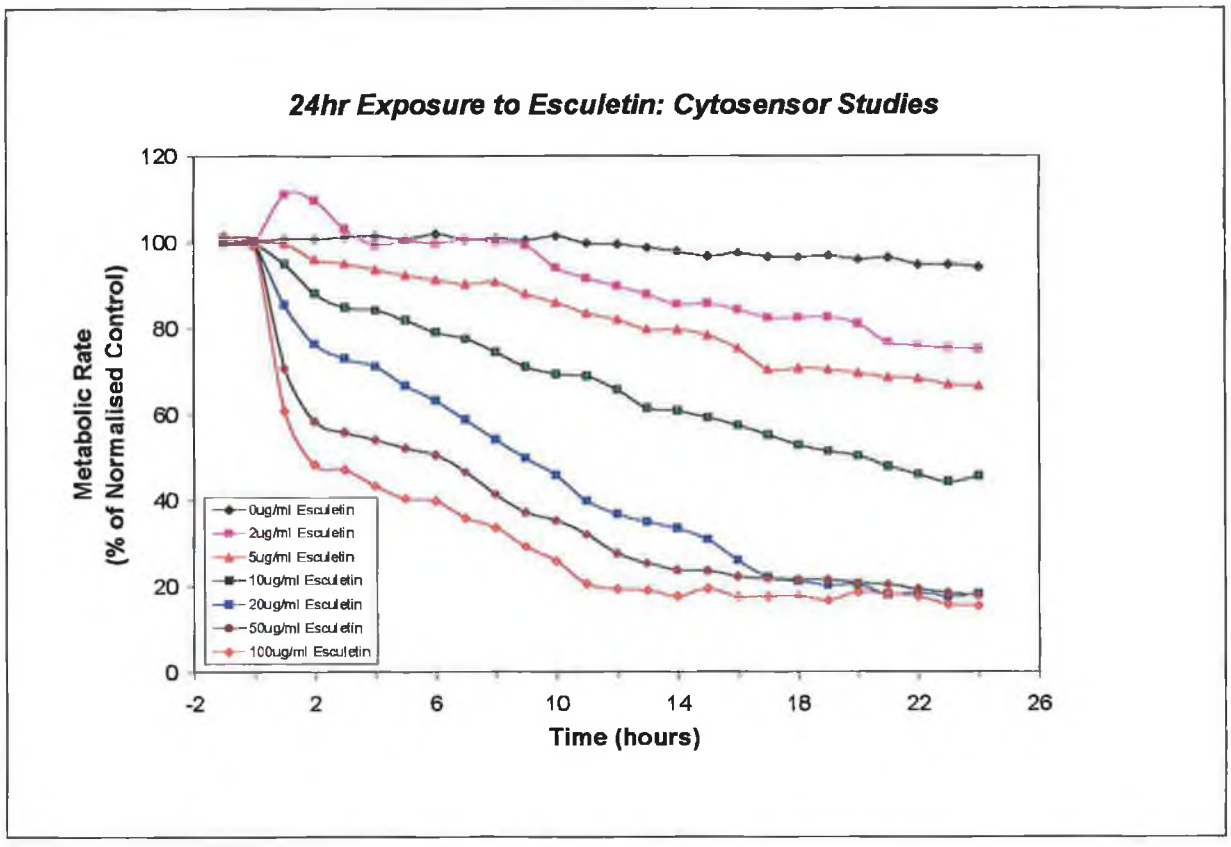


Figure 4.10: **Exposure of A431 cells to various concentrations of esculetin for 24hrs. The above plot shows the mean of four experiments conducted on the Cytosensor Microphysiometer, to determine the effects of the drug on cellular metabolism (Section 2.2.4.5). In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to drug exposure, using a 4 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during drug-exposure expressed as a percentage of this normalised value. These values of metabolic rate (mean of four experiments) were plotted vs time in the above graph. Typical basal acidification rates (-μvolts/sec) for the seven outlined chambers in one of the experiments were -124.58 (Control), -134.25 (2μg/ml), -107.59 (5μg/ml), -127.85 (10μg/ml), -145.15 (20μg/ml), -122.52 (50μg/ml), and -115.84 (100μg/ml).**

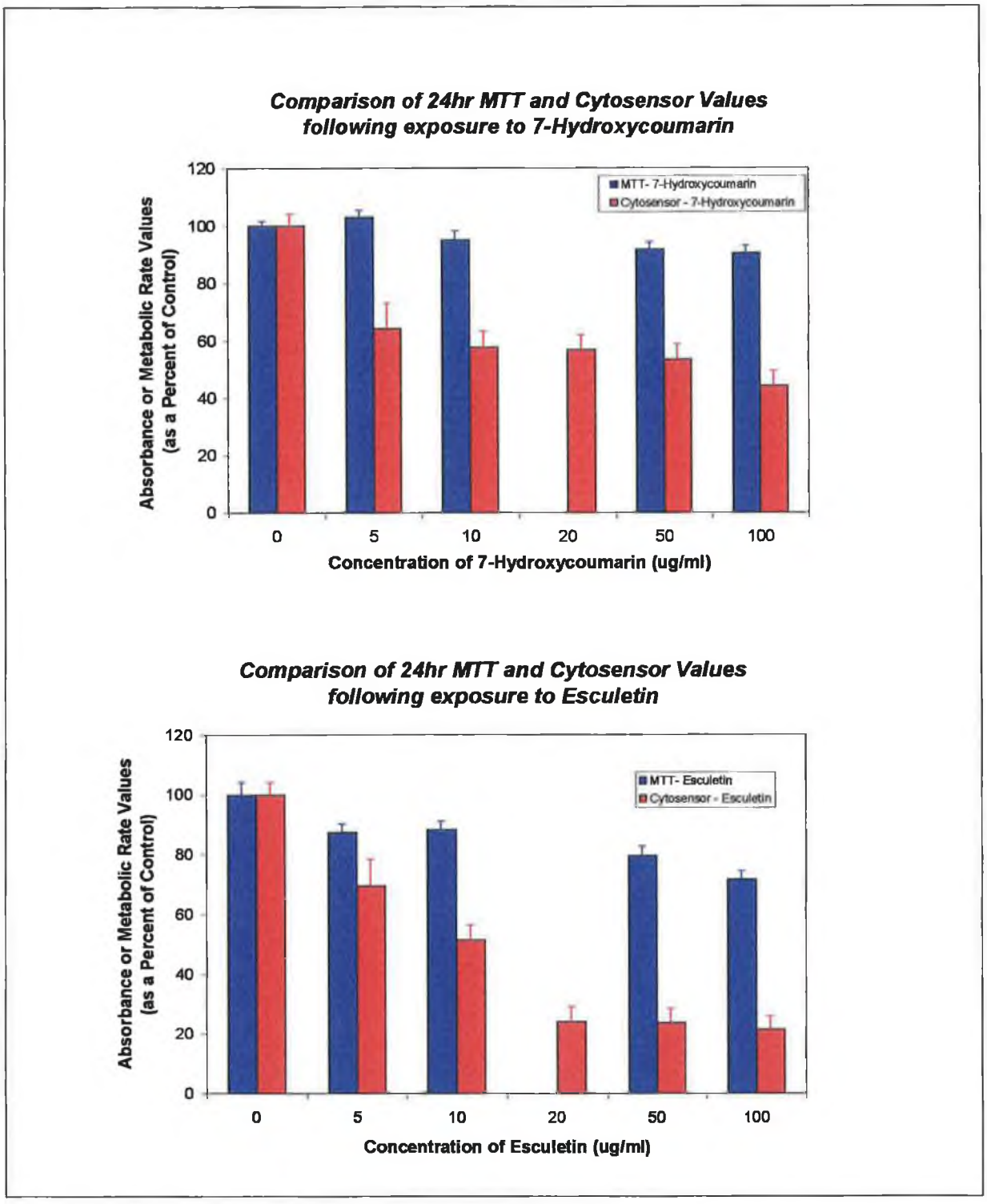


Figure 4.11: **Comparison of values obtained from MTT Assays and Cytosensor Studies on metabolism suppression in cells by 7-hydroxycoumarin (top), and esculetin (bottom) following 24hr exposures. The Cytosensor studies appear more sensitive in the prediction of metabolism perturbations.**

4.5.2.2.3. Reversibility Studies with the Cytosensor Microphysiometer

Previous reversibility studies on the effects of coumarin compounds on cultured cells have utilised proliferative-based assessments to determine if cells could recover from exposure to these compounds (Marshall *et al.*, 1994; Egan *et al.*, 1997). In most instances, this type of experiment entailed a period of drug exposure, followed by a drug-free recovery growth period for a sub-population of the original exposed cells. This introduces an element of selection into the reversibility study – outgrowth of a sub-population of drug-resistant cells could account for the appearance of a "reversible" effect.

We were, therefore, interested in assessing the recovery of cells exposed to coumarins using an alternative method, preferably internally-controlled, to avoid the above problems of sub-population selection. The Cytosensor Microphysiometer is an ideal instrument for such an experiment - each sensor chamber, with its encased cells, acts as its own internal control. During exposure of cells to a compound, the metabolic rate of the cells is compared to the metabolic rate of the same sample of cells prior to exposure; during a recovery period the same complete sample of exposed cells, not a sub-population, are allowed to recover, and again their metabolic rate during recovery is compared to the original metabolic rate. This allows for a complete and valid assessment of the reversibility of the effects of a compound on the cells metabolism.

As outlined in Section 2.2.4.5. reversibility studies were completed for 6-hydroxycoumarin, 7-hydroxycoumarin and esculetin. Various exposure/recovery periods were assessed, the results of which are shown in Figures 4.12-4.19. The recovery of A431 cells from exposure to 6-hydroxycoumarin was assessed following both 12hr (Figure 4.12) and 24hr (Figure 4.13) periods. The effects of 6-hydroxycoumarin on cellular metabolism were described previously (Section 4.5.2.2.2) as relatively slight for the high concentrations used (especially when compared to both 7-hydroxycoumarin and esculetin). However, when the exposed cells were allowed to recover from the drug-exposure, only a brief, transient increase in the metabolic rate was observed at the initiation of the recovery period. Although the metabolic rate of the cells did not continue to decrease, as during the exposure period, neither did it recover to its pre-exposure levels. Instead, it remained at a level similar to that observed immediately pre-recovery *e.g.* in Figure 4.13, we see that for cells exposed to 200μg/ml 6-hydroxycoumarin, the metabolic rate immediately pre-recovery (*i.e.* at 24hrs) was 65.5%, and excepting a slight increase in metabolism in the first five hours of recovery, the metabolic rate remained steady at ~ 61-67% over the remaining nineteen hours of recovery. Therefore it would appear that the damage to cellular metabolism inflicted by 6-

hydroxycoumarin exposure was irreversible. This trend was apparent in both the 12hr and 24hr reversibility studies (Figures 4.12 and 4.13, respectively).

Reversibility studies were also completed for A431 cells exposed to 7-hydroxycoumarin for various time periods as outlined in Section 2.2.4.5, with the results shown in Figures 4.14-4.16. In Figure 4.14, we see the effect of a 4hr exposure and 4hr recovery period, in the concentration range 20-100μg/ml. As expected exposure caused a dose-dependent decrease in the metabolic rate of the cells, but on removal of drug exposure, complete recovery of the cells to pre-exposure metabolic rates was achieved. In fact, the metabolic rate of the cells was higher on recovery than the pre-exposure rate. Figure 4.15 illustrates the data obtained for a 12hr exposure and 12hr recovery period for 7-hydroxycoumarin-exposed A431 cells. 12hrs of drug exposure again caused a dose-dependent suppression of metabolism, but this time full recovery of the cells was not achieved at any of the concentrations involved. Cells exposed to concentrations of 7-hydroxycoumarin in the range 5-20μg/ml did attain recovery of the metabolism to ~80-90% of their original values. With 50μg/ml and 100μg/ml these recovery values were ~ 68% and ~57% respectively. 24hr exposure yielded remarkably similar results – despite the increased suppression of cellular metabolism due to the increased exposure times, recovery of cellular metabolism was almost identical with recovery values of ~87%, ~78%, ~69% and ~54% for cells exposed to 10, 20, 50 and 100μg/ml 7-hydroxycoumarin, respectively (Figure 4.16).

Data obtained from reversibility studies accomplished with esculetin and A431 cells are shown in Figures 4.17-4.19. 4hr exposure to esculetin concentrations in the range 20-100μg/ml caused a suppression of cellular metabolism which was dose-dependent and reversible, as shown in Figure 4.17. Unlike 7-hydroxycoumarin, this reversibility of metabolic suppression was still achievable following 12hr exposures to the drugs (Figure 4.18), an unexpected result considering the extreme suppression of metabolism involved (at concentrations of 20-100μg/ml the cellular metabolic rate had dropped to ~ 20-30% of its original value). However, following 24hrs of esculetin exposure, this ability to fully recover from such severe metabolic suppression was lost by the cells, and only cells exposed to low (2-5μg/ml) esculetin concentrations were able to approach full recovery, with recovery reflecting exposure conditions at higher concentrations (Figure 4.19).

In many of these reversibility studies, it was observed that following drug withdrawal (0-5hrs post-withdrawal), the increase in metabolic rate was to levels above those of the control. This was observed for example in the 4hr reversibility studies of 7-hydroxycoumarin and esculetin (Figures 4.14 and 4.17). Longer temporal studies also displayed this feature *e.g.* 50μg/ml 6-

hydroxycoumarin (Figure 4.12), or 5μg/ml esculetin (Figure 4.18); in many cases cells achieved lower, more stable metabolic rates, as recovery periods continued. While we cannot explain this "overshoot", we can say it is not due to pH differences between the drug-free and drug-containing running media, as all media was adjusted to pH 7.35-7.40. An increase in the metabolic rate of cells also occurred on initial exposure to the drug [c.f. Figures 4.12 and 4.18 (2 & 5μg/ml esculetin)]. This phenomenon is not apparent for higher concentrations in the plotted graphs, but increases did occur in the first 0-10mins for all drugs tested (results not shown). This increase may represent activation of an ATP-coupled transporter protein *e.g.* the P-glycoprotein, which results in the efflux of drug from the intracellular environment. Prolonged exposure to high drug concentrations overcomes this efflux mechanism

These studies regard the reversibility of the effects of coumarins on cellular metabolism, and find that the suppression of cellular metabolism in cells exposed to coumarins is not fully reversible following prolonged (12-24hr) exposure. The extent of recovery appears to be dose-dependent. One failing of the experiment is that we do not know if all cells recovered equally *i.e.* for the sample of cells exposed to 100μg/ml esculetin for 24hrs, did 45% of the cells recover fully to their pre-exposure metabolic rate, or did 100% of the cells achieve a recovery of 45% of their metabolism, or is some other intermediate scenario responsible for the observed 45% recovery?

The results obtained disagree with previous reversibility assays carried out on coumarin compounds. Previous studies with 7-hydroxycoumarin determined their cytostatic effect on cell proliferation to be completely reversible. It is believed that ~ 40% of a cells metabolism is directed towards growth (Owicki & Parce, 1992) – one might expect then that an incomplete metabolic recovery from coumarin exposure might reflect itself in a proliferative-based reversibility study, such as that conducted by Marshall *et al.* (1994). However, Marshall and co-workers in their study of reversibility utilised an extended (9-day) recovery period, which may hide metabolic differences of cells sufficiently in terms of cell number increase.

Finally the results obtained in these cytosensor studies (exposures and recoveries) would seem to indicate a dissimilar mechanism of action of each of the three studied coumarins (6-hydroxycoumarin, 7-hydroxycoumarin and esculetin) at a sub-cellular level. This is an area that was examined in further detail in Chapters 5 and 6 of this thesis.

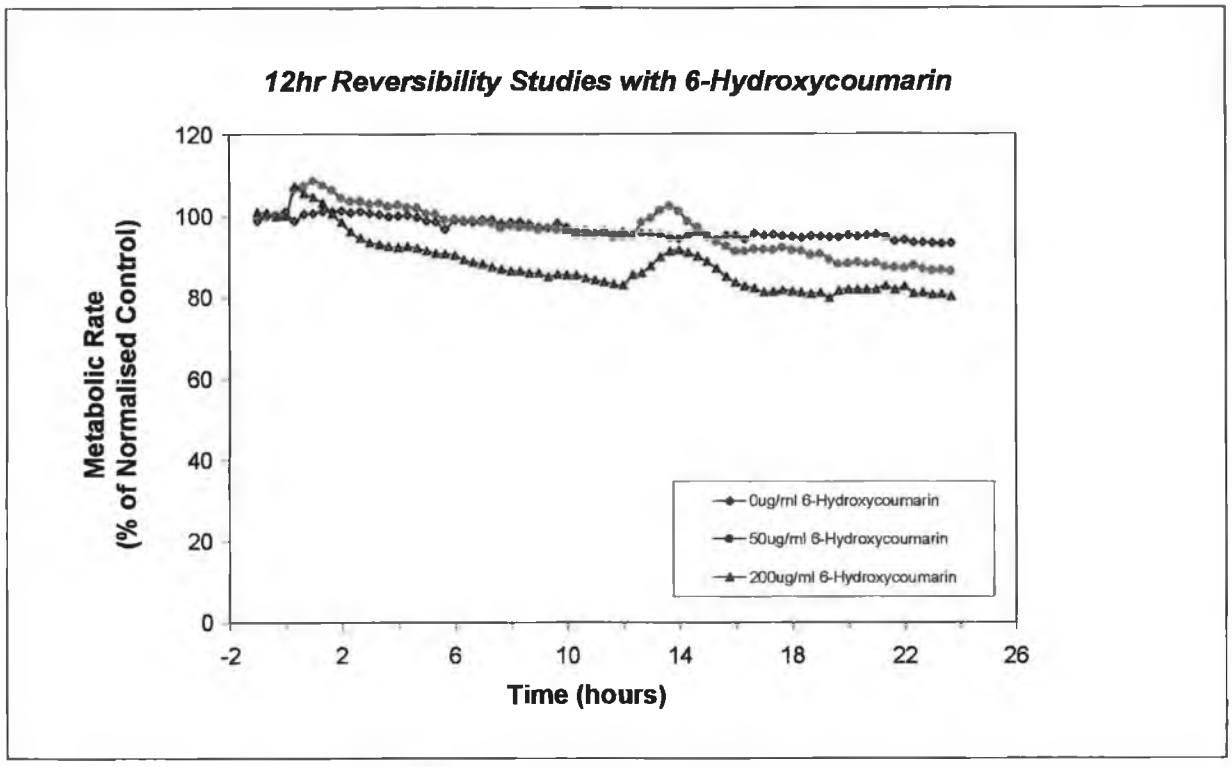


Figure 4.12: **Exposure of A431 cells to various concentrations of 6-hydroxycoumarin for 12hrs, followed by a further 12hrs period of recovery in drug-free medium. The above plot shows the mean of three experiments conducted on the Cytosensor Microphysiometer, to determine the reversibility of the effects of the drug on cellular metabolism (Section 2.2.4.5). In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to drug exposure, using a 4 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during exposure and recovery periods were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for cells in the experiments were –100 to –200 μvolts/sec.**

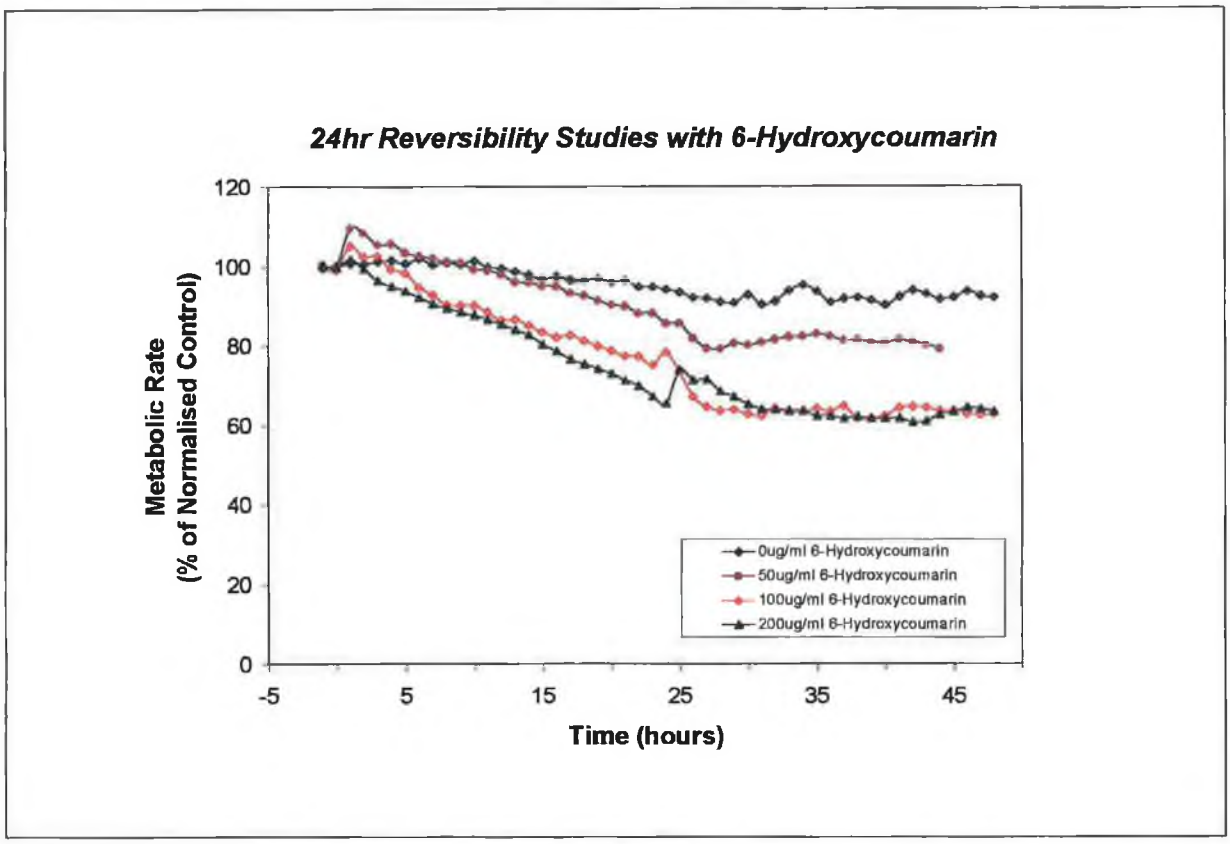


Figure 4.13: **Exposure of A431 cells to various concentrations of 6-hydroxycoumarin for 24hrs, followed by a further 24hrs period of recovery in drug-free medium. The above plot shows the mean of three experiments conducted on the Cytosensor Microphysiometer, to determine the reversibility of the effects of the drug on cellular metabolism (Section 2.2.4.5). In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to drug exposure, using a 4 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during exposure and recovery periods were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for cells in the experiments were –100 to –200 μvolts/sec.**

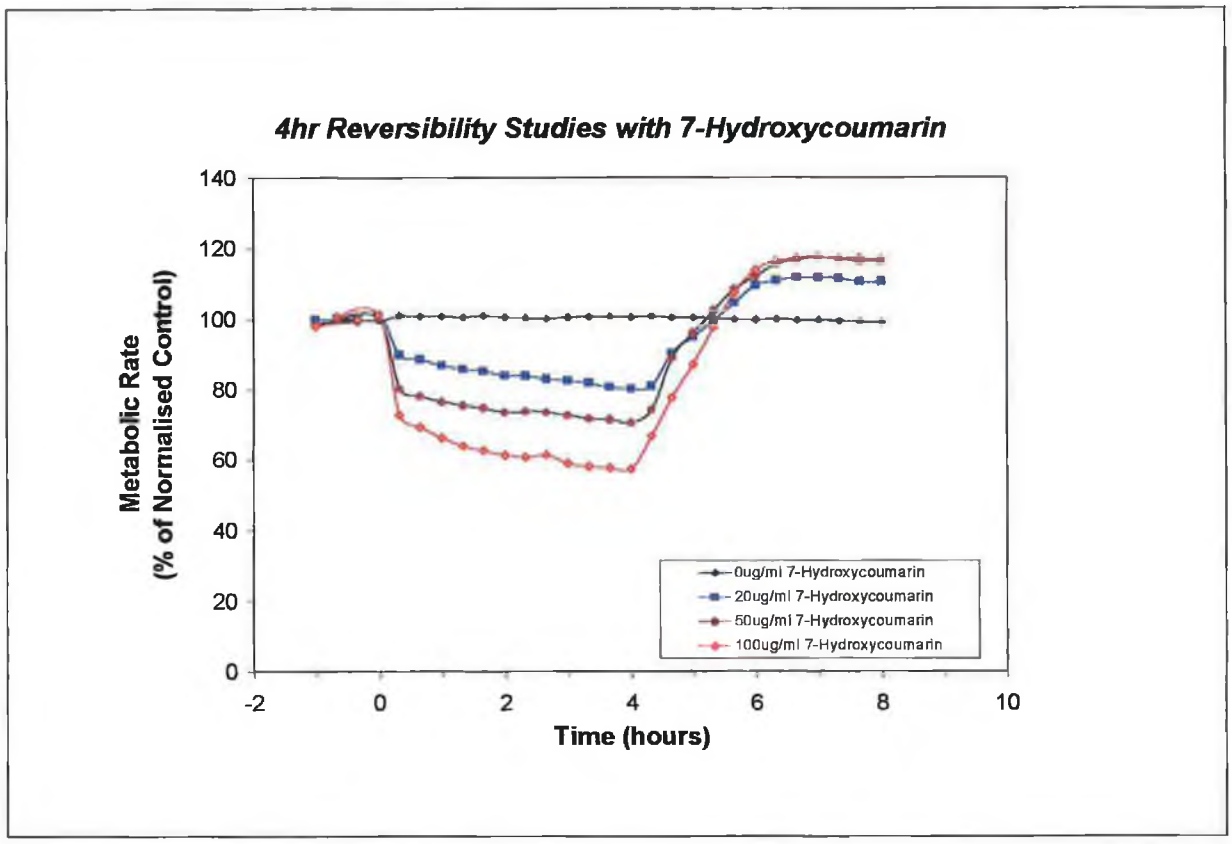


Figure 4.14: **Exposure of A431 cells to various concentrations of 7-hydroxycoumarin for 4hrs, followed by a further 4hrs period of recovery in drug-free medium. The above plot shows the mean of three experiments conducted on the Cytosensor Microphysiometer, to determine the reversibility of the effects of the drug on cellular metabolism (Section 2.2.4.5). In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to drug exposure, using a 4 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during exposure and recovery periods were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for cells in the experiments were –100 to –200 μvolts/sec.**

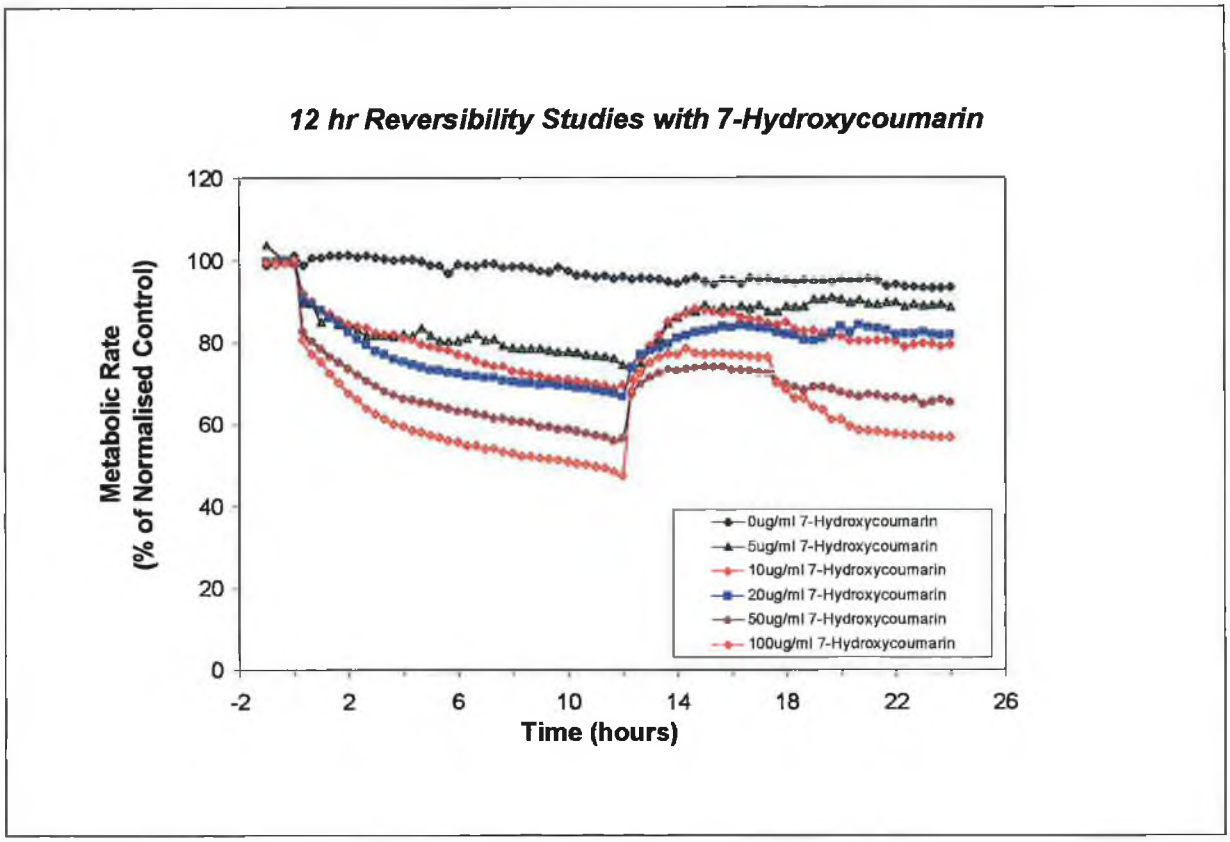


Figure 4.15: **Exposure of A431 cells to various concentrations of 7-hydroxycoumarin for 12hrs, followed by a further 12hrs period of recovery in drug-free medium. The above plot shows the mean of three experiments conducted on the Cytosensor Microphysiometer, to determine the reversibility of the effects of the drug on cellular metabolism (Section 2.2.4.5). In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to drug exposure, using a 4 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during exposure and recovery periods were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for cells in the experiments were –100 to –200 μvolts/sec.**

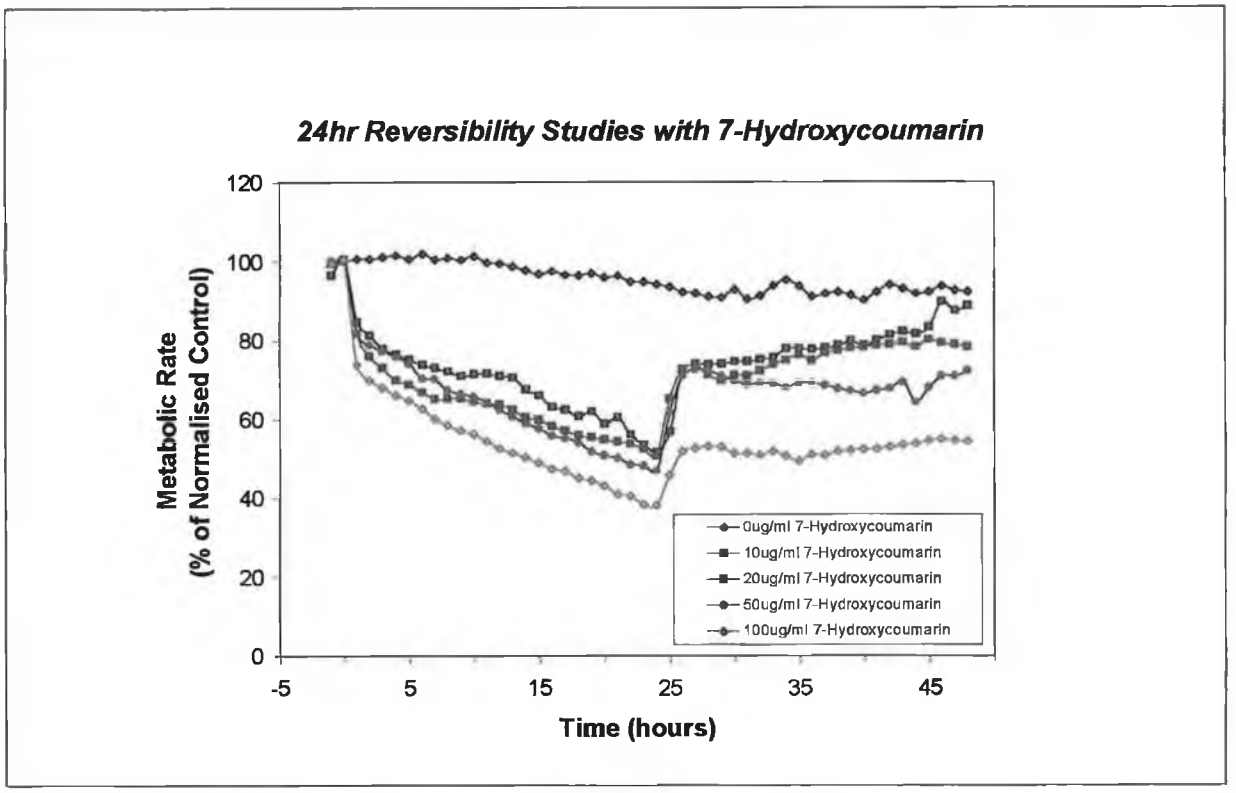


Figure 4.16: **Exposure of A431 cells to various concentrations of 7-hydroxycoumarin for 24hrs, followed by a further 24hrs period of recovery in drug-free medium. The above plot shows the mean of three experiments conducted on the Cytosensor Microphysiometer, to determine the reversibility of the effects of the drug on cellular metabolism (Section 2.2.4.5). In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to drug exposure, using a 4 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during exposure and recovery periods were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for cells in the experiments were –100 to –200 μvolts/sec.**

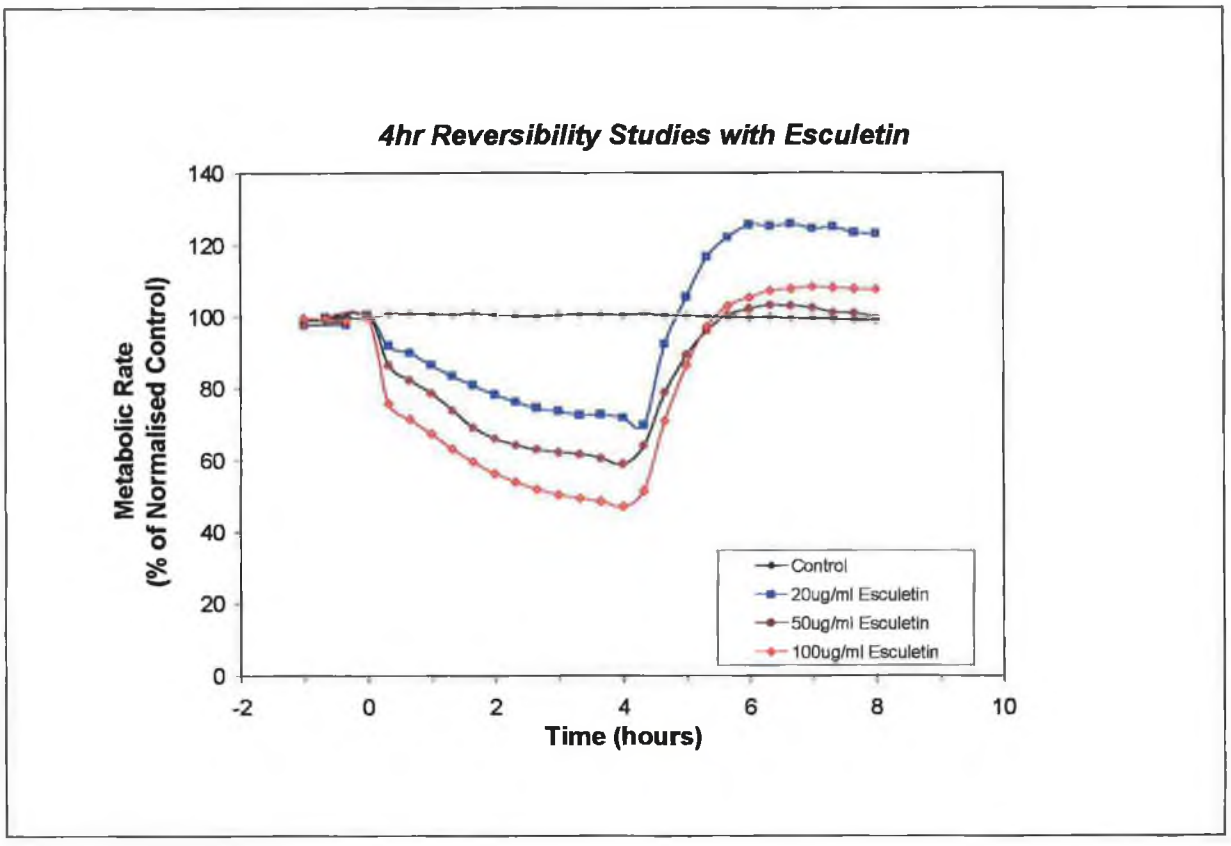


Figure 4.17: **Exposure of A431 cells to various concentrations of esculetin for 4hrs, followed by a further 4hrs period of recovery in drug-free medium. The above plot shows the mean of three experiments conducted on the Cytosensor Microphysiometer, to determine the reversibility of the effects of the drug on cellular metabolism (Section 2.2.4.5). In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to drug exposure, using a 4 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during exposure and recovery periods were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for cells in the experiments were –100 to –200 μvolts/sec.**

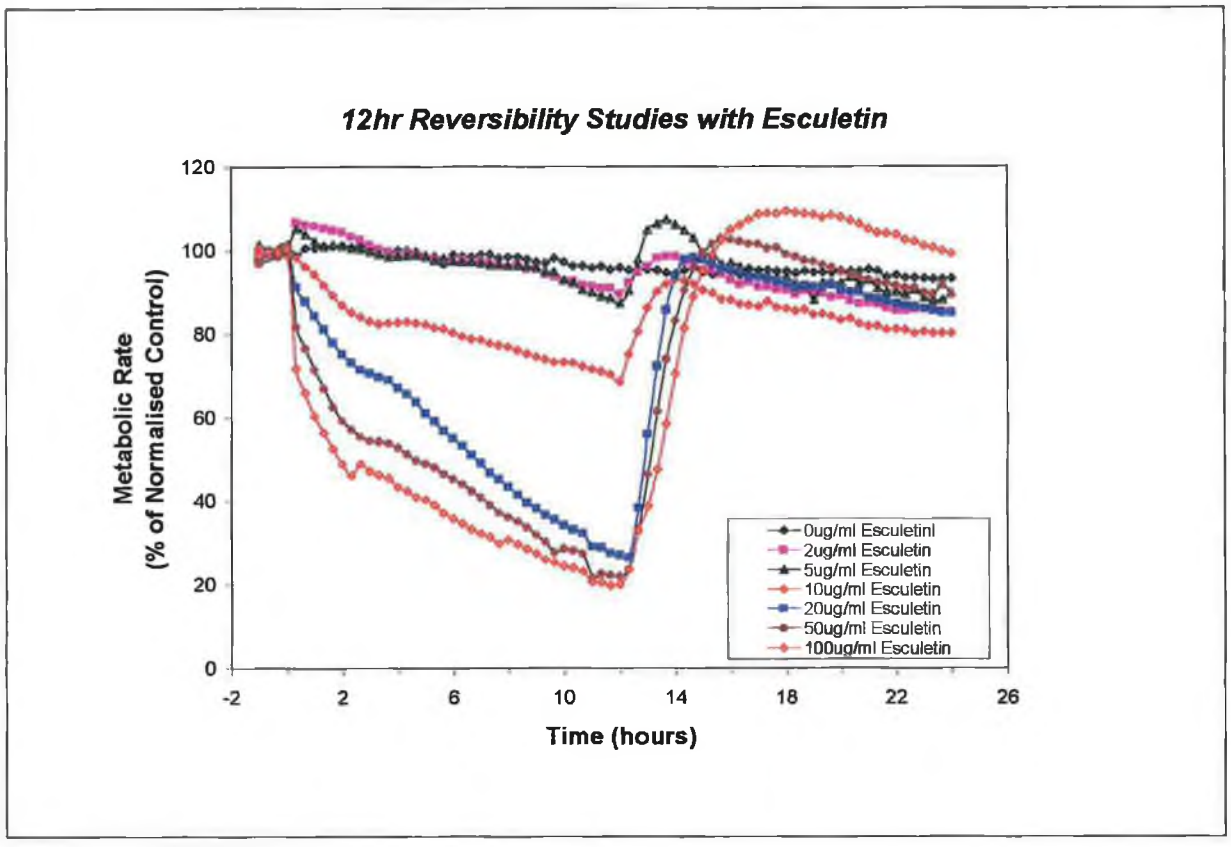


Figure 4.18: **Exposure of A431 cells to various concentrations of esculetin for 12hrs, followed by a further 12hrs period of recovery in drug-free medium. The above plot shows the mean of three experiments conducted on the Cytosensor Microphysiometer, to determine the reversibility of the effects of the drug on cellular metabolism (Section 2.2.4.5). In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to drug exposure, using a 4 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during exposure and recovery periods were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for cells in the experiments were –100 to –200 μvolts/sec.**

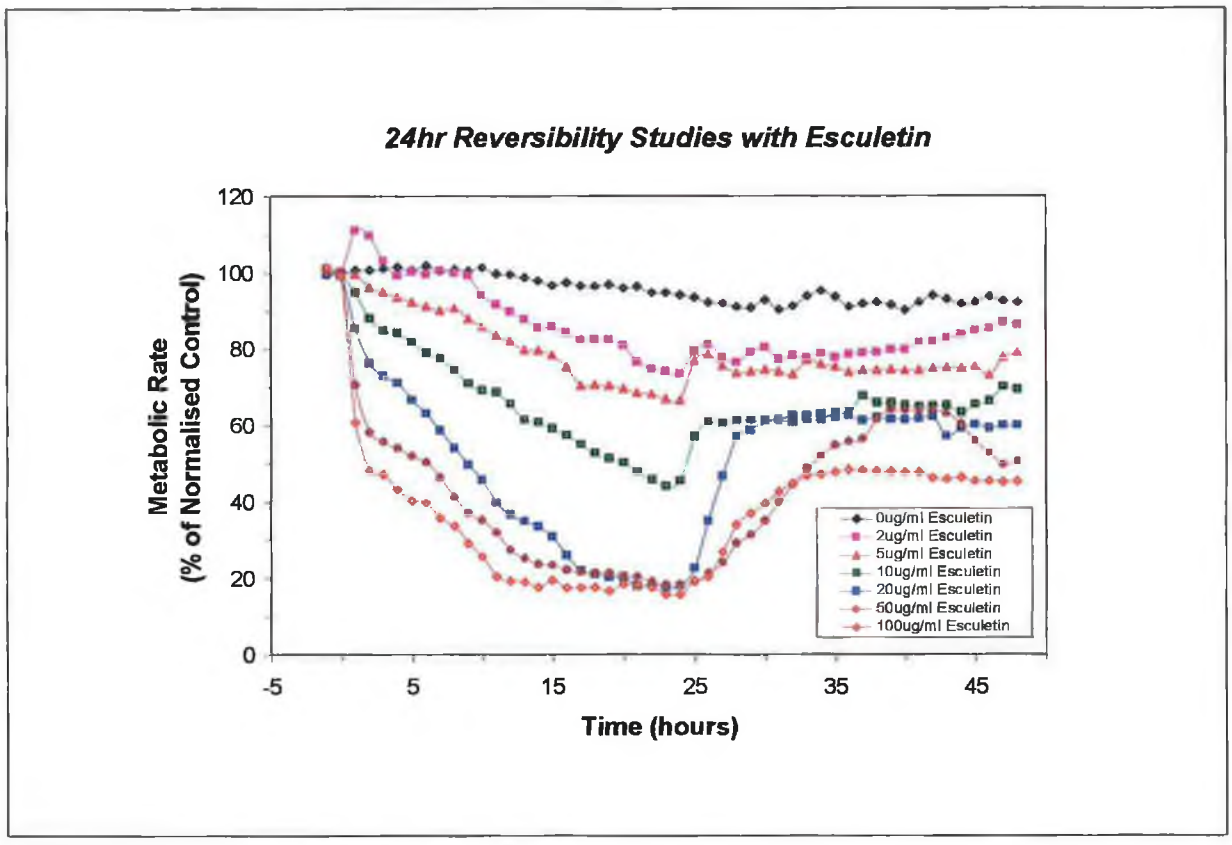


Figure 4.19: **Exposure of A431 cells to various concentrations of esculetin for 24hrs, followed by a further 24hrs period of recovery in drug-free medium. The above plot shows the mean of three experiments conducted on the Cytosensor Microphysiometer, to determine the reversibility of the effects of the drug on cellular metabolism (Section 2.2.4.5). In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to drug exposure, using a 4 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during exposure and recovery periods were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for cells in the experiments were –100 to –200 μvolts/sec.**

4.5.3. Effect of Coumarins on Protease Production by Human Tumour Cells

As outlined in Section 4.2, the expression of matrix metalloproteinases by tumour cells has correlated well with their ability to invade and metastasise *in vivo* (Chambers & Matrisian, 1997). The expression of MMP-2 and MMP-9 is enhanced *in vivo* in cancers of the skin, prostate, kidney, breast and colon during progression from the benign to malignant state (Monteagudo *et al.*, 1990; Levy *et al.*, 1991; Karelina *et al.*, 1993; Stearns & Wang, 1993; Gohji *et al.*, 1994). Coumarin compounds have been shown to be active *in vivo* against some of these malignancies. Hence, we chose to examine the effect of coumarin compounds on the activity and production of matrix metalloproteinases.

According to the method described in Section 2.2.5, A431 cells were found to constitutively secrete a protease of molecular weight ~ 70kDa (Figure 4.20). The presence of this band was inhibited by 1mM EDTA, indicating a metalloproteinase activity, which we assigned as MMP-2, a matrix metalloproteinase of molecular weight 72kDa, which has been previously showed to be expressed by A431 cells (Xie *et al.*, 1994a)

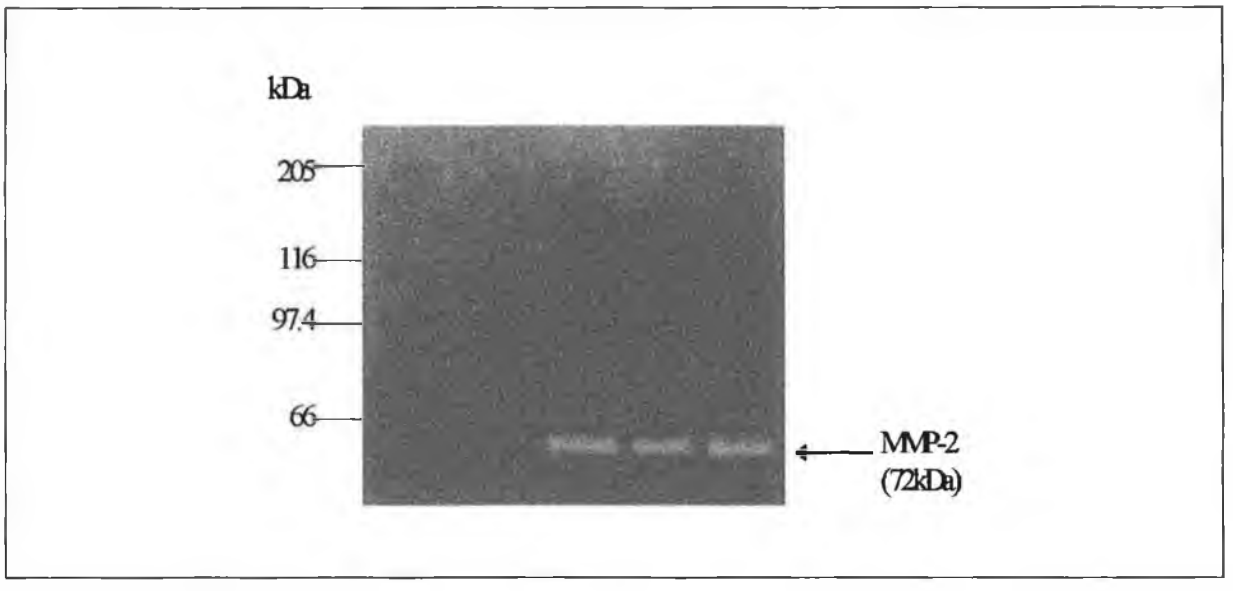


Figure 4.20: Constitutive expression of MMP-2 (72kDa) by A431 cells, as analysed by substrate gel (gelatin) analysis of their conditioned medium.

In examining the effect of coumarins on A431 MMP-2, we initially determined the effect of these compounds on the activity of this enzyme, using gelatin gel analysis. This was achieved by addition of drug to the substrate buffer post-electrophoresis. A decrease in the intensity of bands following drug exposure indicated that the coumarin was an MMP-2 inhibitor at that particular concentration. Figure 4.21 is an example of one such experiment and illustrates the inhibitory effect of 150μg/ml 6-hydroxycoumarin on the activity of the MMP-2 protease. Results of the inhibitory activity of all four drugs are summarised in Table 4.5. Briefly, coumarin only affected the catalytic activity of MMP-2 at concentrations greater than 100μg/ml, and then only mildly. 6-hydroxycoumarin was slightly more inhibitory, causing increasing enzyme inhibition from 100μg/ml upwards. 7-hydroxycoumarin displayed increasing inhibition of enzyme from 50μg/ml, while esculetin was either strongly, or totally inhibitory at the concentrations tested.

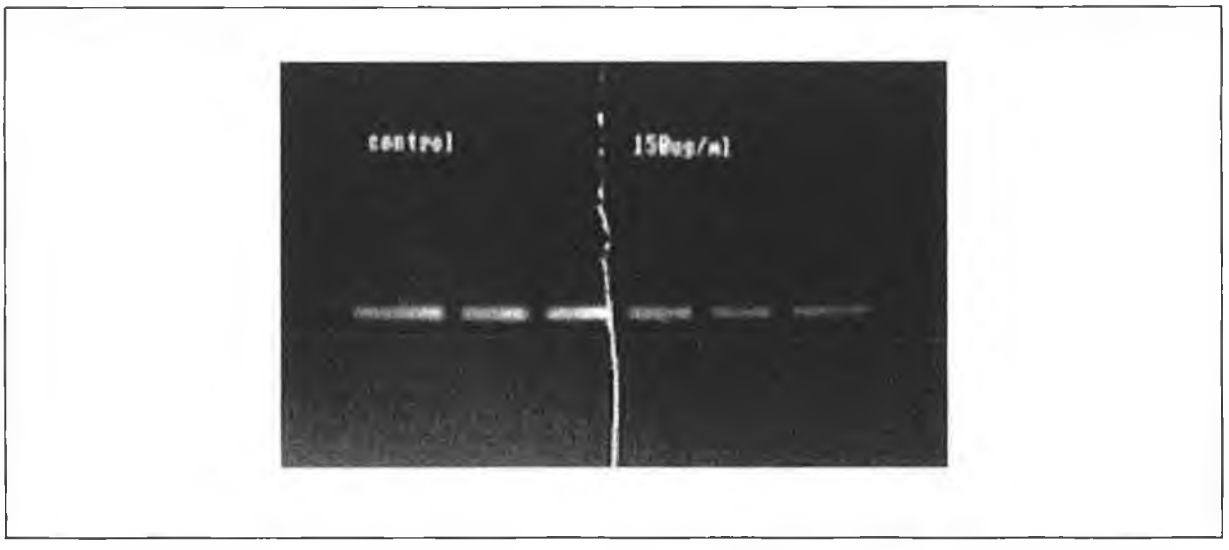


Figure 4.21: Substrate gel analysis of conditioned medium from A431 cells, to determine the effect of 150μg/ml of 6-hydroxycoumarin on the activity of the MMP-2 protease. Equal amounts of medium were electrophoresed in 6 lanes across the gel. The gel was then halved and one half placed in drug-free substrate buffer (left hand side), while the second half was incubated in substrate buffer containing 150μg/ml 6-hydroxycoumarin (right hand side). Incubation was carried out for 16hrs, after which detection of protease bands was achieved by Coomassie Blue staining and destaining of the gels. The band intensities were then compared to ascertain if the drug was inhibitory to the MMP-2 activity.

Drug	*Control*	*50μg/ml*	*100μg/ml*	*150μg/ml*
Coumarin	++++	++++	++++	+++
6-hydroxycoumarin	++++	++++	+++	++
7-hydroxycoumarin	++++	+++	++	+
Esculetin	++++	+	-	-

Table 4.5: Inhibition of MMP-2 catalytic activity by coumarin compounds as assessed using substrate gel analysis. Band intensities are described by (+) symbols, with (++++) set as control band intensity. (-) indicates no band present *i.e.* total inhibition of protease activity.

We next proceeded to examine the effect of coumarins on the production /secretion of MMP-2 from A431 cells. This was achieved by incubation of cells with the various drugs for 24 hrs, followed by collection and analysis of the conditioned medium by gelatin gel analysis. Densitometric analysis was achieved to quantify this effect, and the results of these experiments are shown in Figures 4.22 and 4.23. As is illustrated, coumarin did not affect the expression of MMP-2 except at concentrations of 100μg/ml, and the expression was only mildly affected at this concentration. The other three hydroxy-compounds displayed a dose-dependent suppression of MMP-2 expression by the cells, with the most severe effects observed with esculetin. These suppressive effects may be due to the coumarins targeting either transcription, translation or secretion of the MMP-2 enzyme. It is doubtful that these effects are due to the inhibition of enzyme activity detailed in Table 4.5, as all gels were washed intensively prior to incubation in drug-free substrate buffer. Therefore, unless the drugs had irreversibly bound to or inactivated the enzyme during drug-exposure, the suppression of MMP-2 outlined in Figures 4.22 and 4.23 should represent only negative effects on MMP production or secretion.

The inhibition of enzyme activity is not a new characteristic for coumarin compounds Coumarins have been shown to inhibit the activities of a number of enzymes in the past *e.g.*

xanthine oxidase, lipoxygenase, DNA gyrase, glyoxylase (Brandt *et al.*, 1986; Maxwell 1993; Chang & Chiang, 1995; Hoult *et al.*, 1994). The precise mechanism of inhibition is undocumented. With regard to the direct inhibition of metalloproteinase activity described above, it is possible that the coumarins may exert their inhibitory effects through one of a number of effects. Firstly, the relationship between Type IV collagenase expression and lipoxygenases has been previously documented (Reich and Martin, 1996; Liu *et al.*, 1996). The dose-dependent suppression of MMP-2 and MMP-9 expression by esculetin in human breast cells has been described (Reich & Martin, 1996), with the decrease in enzyme expression related to gene expression. The results of this study were reflected by those of Liu *et al.* (1996), where again, esculetin decreased the production of type IV collagenases in linoleic-acid stimulated human breast tumour cells. These results reflect well the outcome of our experiments with A431 cells (Figure 4.23 C& D). Coumarin and 7-hydroxycoumarin have once being reported as inhibitors of prostaglandin biosynthesis (Lee *et al.*, 1981), but no further work in this area has been achieved, and lipoxygenase inhibition is not a documented property of these compounds. Hence, it is unlikely this can explain fully their inhibition of collagenase activity/expression.

A second possibility is that chelation of metal ions may contribute to this suppressive effect: 7-hydroxycoumarin has been previously reported as a useful indicator for Ca^{2+} and Cu^{2+}in titrimetric determinations, this role being accomplished as a result of chelation of these ions (Huitnik & Diehl, 1974). The metal-chelatory properties of esculetin have also been described (Cooke *et al.*, 1997). It is possible that some of the inhibition of metalloproteinase activity and/or expression may be achieved via these effects, as chelation of the metal ions (Ca^{2+} and Mg^{2+}) essential to the correct functioning of the metalloproteinases, is detrimental to their activity. Recently it has been postulated that the therapeutic (and adverse) effects of aspirin-like drugs, are modulated through the chelation of various physiologically important metallic cations in the body (Wang, 1998)

Finally, it is intriguing to note the work of Lu and colleagues (1996) on the effect of 7-hydroxycoumarin on tumour locomotion. They described experiments whereby 7-hydroxycoumarin suppressed the motility of tumour cells in 3-D collagen matrices over a 6 day period. They correlated this anti-locomotory effect with decreases in the intracellular levels of the $p21^{ras}$ protein. The *ras* oncogene is a potent inducer of type IV collagenases (Matrisian, 1990). Thus, it is possible that the net effect of *ras* protein suppression by 7-hydroxycoumarin, was a decrease in the constitutive expression of collagenases, resulting in the prevention of tumour cell movement through the collagen matrices

Regardless of the mechanism by which the coumarins suppress the activity and expression of Type IV collagenases, the implications of these findings are important when one considers the role of matrix metalloproteinases *in vivo* in invasion and metastases (Section 4.2). The effect of coumarins on invasiveness can be determined *in vitro* using *in vitro* invasion assays with Matrigel, and such experiments should be completed to ascertain if the above suppression of metalloproteinase activity and production has real effects on tumour cell invasiveness. This may aid in an overall explanation of the observed anti-cancer effects of coumarin, *in vivo,* in patients with advanced malignancies.

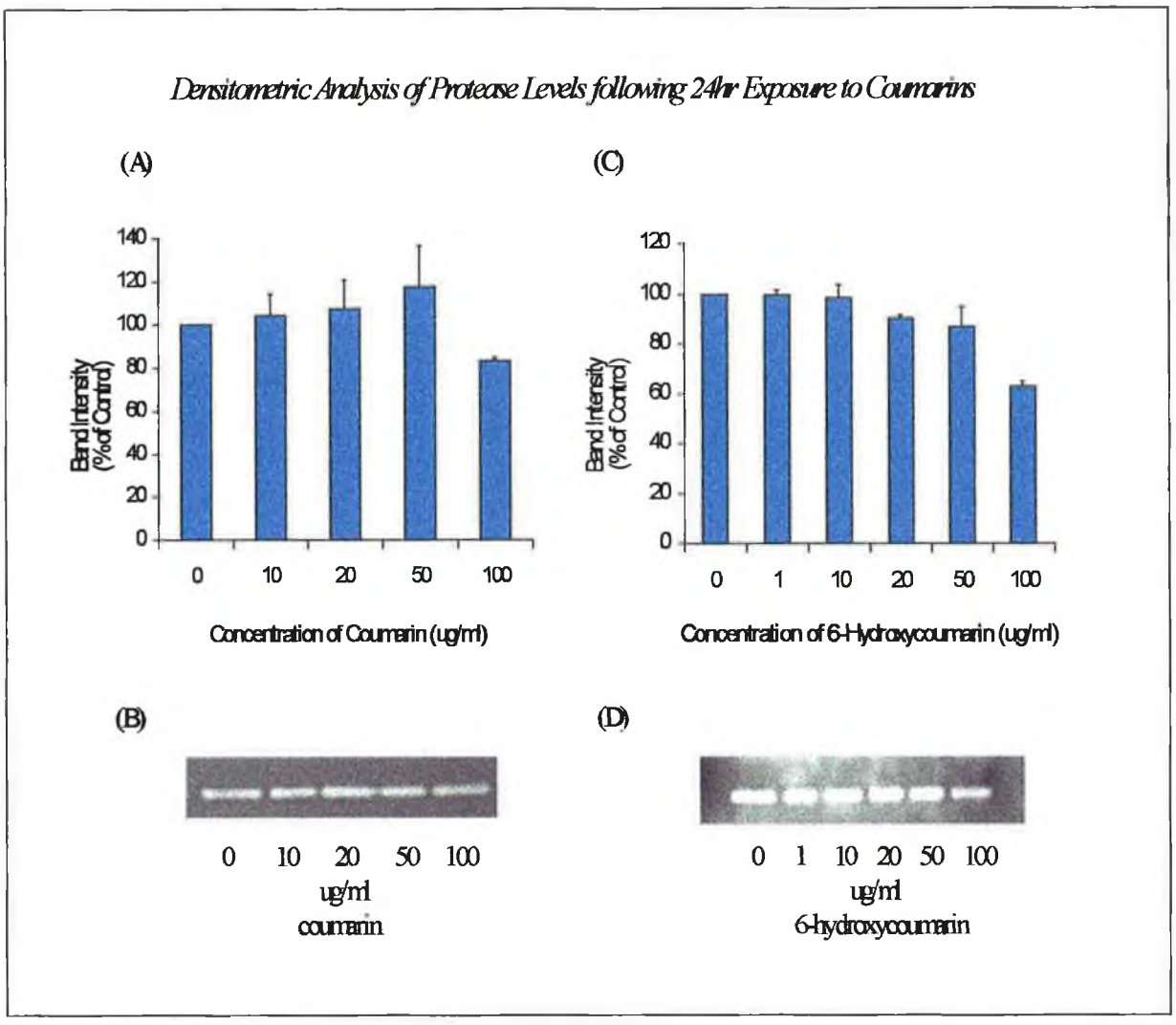


Figure 4.22: Effect of 24hr exposure to coumarins on the constitutive expression of MMP-2 from A431 cells. (A) shows the results of densitometric analysis from three different experiments (mean ± s.d.) for A431 cells exposed to coumarin in the range 0-100μg/ml. A representative coumarin gel is depicted in (B). (C) shows the densitometric analysis from three different experiments (mean ± s.d.) for A431 cells exposed to 6-hydroxycoumarin in the range 0-100μg/ml. A representative 6-hydroxycoumarin gel is depicted in (D).

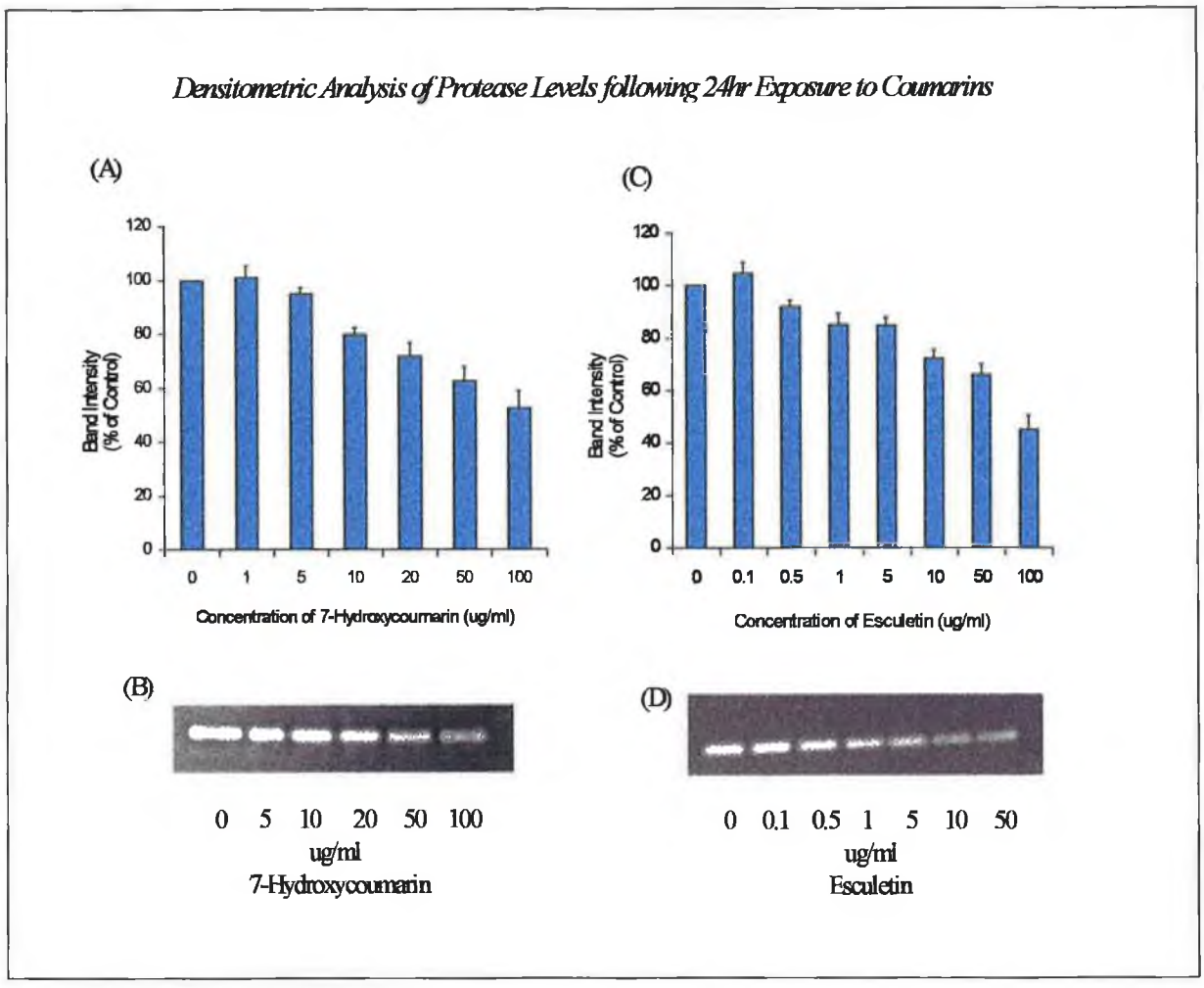


Figure 4.23: Effect of 24hr exposure to coumarins on the constitutive expression of MMP-2 from A431 cells. (A) shows the results of densitometric analysis from three different experiments (mean ± s.d.) for A431 cells exposed to 7-hydroxycoumarin in the range 0-100μg/ml. A representative 7-hydroxycoumarin gel is depicted in (B). (C) shows the densitometric analysis from three different experiments (mean ± s.d.) for A431 cells exposed to esculetin in the range 0-100μg/ml. A representative esculetin gel is depicted in (D).

4.6. CHAPTER SUMMARY

In this chapter we examined the effect of coumarin and its hydroxy-derivatives, 6-hydroxycoumarin, 7-hydroxycoumarin and esculetin, on the growth, metabolism and metastatic potential of human tumour cells.

Proliferation assays yielded some interesting information on growth-inhibition by coumarins:

1. The potency of growth inhibition by esculetin (6,7-dihydroxycoumarin)>> 7-hydroxycoumarin ≥ 6-hydroxycoumarin >> coumarin.
2. Certain cell types *e.g.* prostate, breast, bladder and leukemic cells, were particularly sensitive to growth suppression by the mono-hydroxycoumarins, while esculetin exhibited a more general growth inhibition.
3. A431 cell line was extremely sensitive to all compounds.

None of these four compounds caused significant membrane damage to A431 cells as assessed by the LDH Assay. The effects of the four coumarins on cellular metabolism was examined using both the MTT Assay and the Cytosensor Microphysiometer, although the Cytosensor method proved more useful for the following reasons:

1. "Real-time" measurement vs "end-point" MTT assay
2. Flow-through system mimics *in vivo* exposure more accurately, than static MTT conditions
3. Reversibility studies possible
4. Increased sensitivity of the Cytosensor method compared to MTT assay
5. Esculetin interfered with the MTT assay

The tested coumarin compounds all displayed a dose- and time-dependent effect on cellular metabolism, with esculetin displaying the most potent effects. The suppression of cellular metabolism in cells exposed to coumarins was not fully reversible following prolonged (12-24hr) exposure, with the extent of recovery dose-dependent.

Finally, the effect of coumarins on the metastatic potential of tumour cells was examined in terms of their effect on protease expression in tumour cells. All coumarin compounds were found to reduce the activity and expression of matrix metalloproteinases from tumour cells, although the exact mechanism by which they achieve this is unclear.

Chapter 5

Effect of coumarins on signalling pathways within cells

eventual effect of nuclear transcription. It has been determined that phosphorylation reactions are the central mechanism for the progression of these growth signals from membrane to nucleus. Protein kinases and phosphatases are enzymes that respectively, add or remove phosphate residues, on either serine/threonine or tyrosine residues of proteins, utilising ATP as a phosphate source. These enzymes are crucial to signal propagation in signalling pathways - in many cases, the phosphorylation reaction has been shown to have a profound activating effect on the enzyme activity of the targeted substrate.

The reversible phosphorylation of proteins on tyrosine residues is a particularly important event in the regulation of many eukaryotic processes. Activation of tyrosine kinases is responsible not only for control of growth signalling pathways, but is also important in cellular differentiation, cell cycle control, cell attachment, gene transcription and synaptic transmission (Hunter, 1998). However, in this work, it is the importance of tyrosine phosphorylation in growth control, and its deregulation in cancer that is addressed.

Three distinct classes of receptors exist which use tyrosine phosphorylation to propagate proliferative signals (Hunter, 1998). The details of these are summarised in Table 5.1 and Figure 5.1. As outlined, members of the Receptor Tyrosine Kinase (RTK) family of receptors have an intrinsic intracellular tyrosine kinase domain, which is activated on ligand binding. Downstream phosphorylation of proteins on tyrosine residues transduces the extracellular signal to the nucleus. The binary RTKs do not possess an intracellular tyrosine kinase domain, and can only signal by association with a cytoplasmic tyrosine kinase molecule. This event launches a cascade of phosphorylation reactions, resulting in propagation of the external signal to the nucleus. Finally, the G-protein-coupled receptors (GCRs), do not themselves use tyrosine phosphorylation to transduce the transmembrane signal. However, through GTP coupling, the GCRs cause activation of a number of signalling intermediates with the eventual downstream activation of cytoplasmic kinases. Of these three receptor types, the most important in proliferative signalling pathways is the family of Receptor Tyrosine Kinases (RTK), which are discussed in more detail in Section 5.2.2. Where necessary, the other two receptor classes will be mentioned.

Receptor Class	*Ligands*	*Activation Results*
1. **Receptor Tyrosine Kinases (RTK)**	Polypeptide Growth Factors *e.g.* Epidermal Growth Factor (EGF), Platelet Derived Growth Factor (PDGF)	Ligand binding results in activation of intracellular tyrosine kinase-domain, resulting in phosphorylation of specific tyrosine residues on intracellular targets.
2. **Binary Receptor Tyrosine Kinases (BRTK)**	Cytokines *e.g.* IFN-α, -β, -γ, IL-2, IL4.	Ligand binding results in the recruitment and activation of a cytoplasmic tyrosine kinase *e.g.* JAK activation, which propagates the signal.
3. **Seven-transmembrane/ G protein-coupled receptors (GCR)**	Mitogenic Peptides *e.g.* Bombesin, Angiotensin	Ligand binding results in activation of heterotrimeric G-proteins, with downstream activation of tyrosine kinases for mitogenic signalling

Table 5.1: **Table summarising the properties of the three classes of membrane receptors which utilise tyrosine phosphorylation as a means of propagating the external signal [c.f. Figure 5.1]. (Information derived from Hardie (1991) and Hunter (1998))**

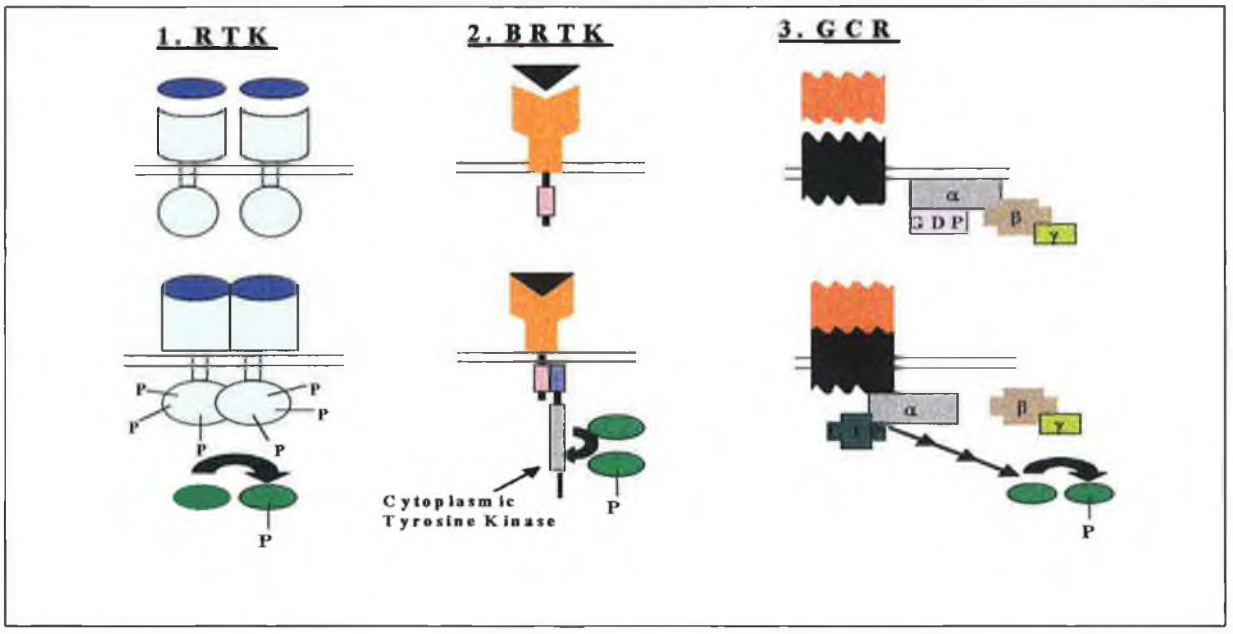


Figure 5.1: **Diagram of the three classes of membrane receptor which utilise tyrosine phosphorylation to propagate the external signal. The RTKs directly phosphorylate substrates. The BRTKs associate with a cytoplasmic kinase to induce downstream phosphorylation, and the GCRs launch signals which eventually cause downstream tyrosine phosphorylation [c.f. Table 5.1 and main text for details].**

5.2.2. Receptor Tyrosine Kinases (RTKs) and their Substrates

The Receptor Tyrosine Kinase (RTK) family of receptors consist of a group of cell-surface proteins, all members of which possess a large glycosylated extracellular ligand-binding domain, a single transmembrane domain, and an intracellular cytoplasmic domain that contains a tyrosine-kinase catalytic region (Yarden & Ullrich, 1988). Sixteen subclasses of RTKs have been described, based on sequence similarity and structural characteristics, (Hunter, 1998), and the characteristics of the five principal subclasses are represented in Figure 5.2.

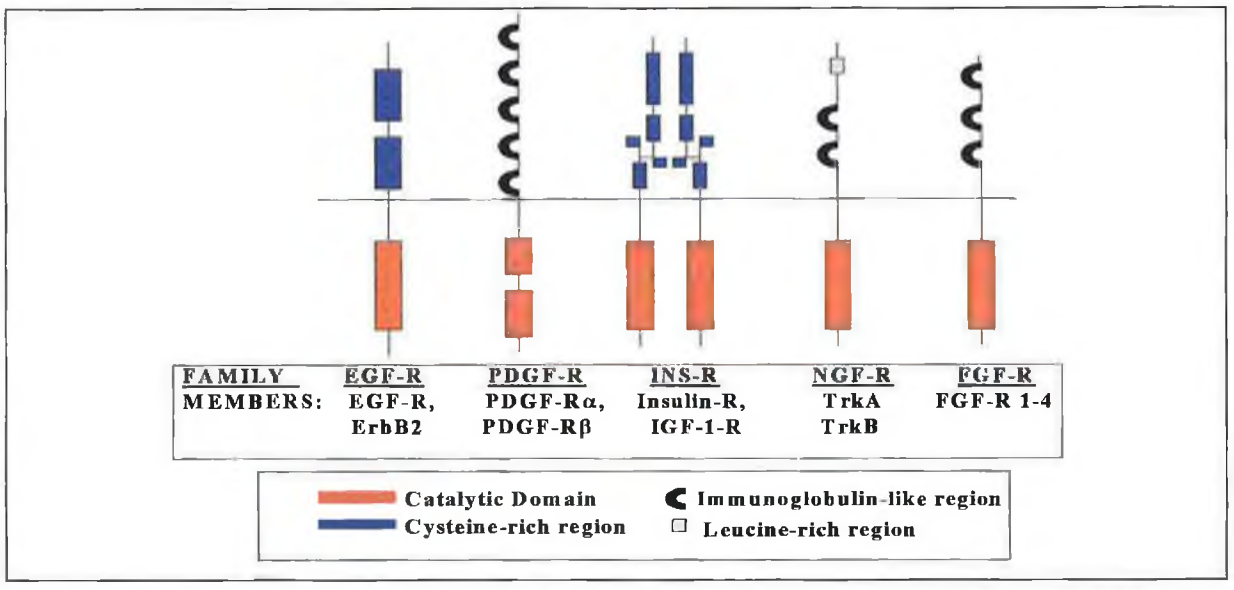


Figure 5.2: Schematic representation of the five principal Receptor Tyrosine Kinase sub-classes (Ullrich & Schlessinger, 1990; Feldkamp *et al.*, 1997; Hunter, 1998)

Many growth factors mediate their actions by binding to their cognate receptors *e.g.* the binding of epidermal growth factor (EGF) to the epidermal growth factor receptor (EGF-R), as was the focus of this work. This causes activation of the intrinsic tyrosine kinase activity, which leads to the intracellular transmission and amplification of the extracellular signal. The signal due to extracellular ligand binding must be relayed across the extracellular membrane, and this is achieved by receptor oligomerisation, usually dimerisation (Ullrich & Schlessinger, 1990; Weiss & Schlessinger, 1998). Through dimerisation, an intracellular conformational change occurs, which allows the kinase domain of one receptor molecule to phosphorylate tyrosine residues on the intracellular domain of the second receptor molecule, and *vice versa,* a process termed

autophosphorylation (Figure 5.3). Using tryptic phosphopeptide mapping and mutational analyses, the exact autophosphorylation sites for the major receptor molecules have been described (Downward *et al.*, 1984; Rosen, 1987; Malarkey *et al.*, 1995).

Receptor autophosphorylation enables the activated kinase to phosphorylate cytoplasmic molecules on tyrosine residues, thus initiating the cascade of signals downstream towards the nucleus (Figure 5.3). A number of RTK substrates have been identified to date. These include Phospholipase C-γ [PLC-γ] (Meisenhelder *et al.*, 1989; Nishibe *et al.*, 1990), p85 Phosphatidylinositol-3′ Kinase [PI3K] (Coughlin *et al.*, 1989), SHP2 [Src-Homology Phosphatase] (Lechleider *et al.*, 1993), Growth factor Receptor Binding Protein–2 [Grb2] (Arvidsson *et al.*, 1994), 120-GAP [GTPase-Activating Protein] (Kazlauskas *et al.*, 1990), and members of the non-receptor *Src* family of tyrosine kinases (Mori *et al.*, 1993). Each of these substrates recognise specific autophosphorylated residues and bind only to these, as shown for autophosphorylated PDGF-R in Figure 5.4.

Crucial to the protein-protein interactions which take place between the activated RTK and the cytoplasmic target proteins are the existence of regions known as SH2 (Src homology) domains. These are regions of ~60-100 amino acids within the target protein, which specifically recognise phosphorylated tyrosine (pTyr) residues, and, thus, in activated receptors couple strongly with autophosphorylation regions (Pawson, 1995). The amino acid sequence in which a particular pTyr lies determines which SH2 region, and thus, which target protein, binds with high affinity to that particular site, *e.g.* the SH2 domain of Src tyrosine kinases recognise the sequence pTyr-hydrophilic-hydrophilic-Ile/Pro, while the PLC-γ molecule prefers at least five hydrophobic residues following the pTyr (Malarkey *et al.*, 1995; Pawson, 1995). Thus, the SH2 domains function to recruit target substrates in a highly specific manner to the vicinity of the receptor tyrosine kinase domain (as represented in Figures 5.3 and 5.4).

Proteins with SH2 domains commonly possess another distinct sequence of ~50 amino acids, the SH3 domain, which is also critical in the protein-protein associations at the core of signal transduction. SH3 domains recognise one or more proline residues in a short (~ 10aa) peptide stretch of effector proteins, and like the SH2 domains are responsible for the accurate docking of the appropriate downstream target (Pawson, 1995). With regard to proliferative signal transduction, the SH3 domains couple with the Ras signalling pathway, the importance of which is outlined in Section 5.2.3. It has been ascertained that certain molecules, for example Grb-2 molecule, contain only SH2 and SH3 domains, with no other functional domains. It is believed that these molecules

function only as adaptor molecules to couple the activated receptor to other signalling intermediates (Malarkey *et al.*, 1995).

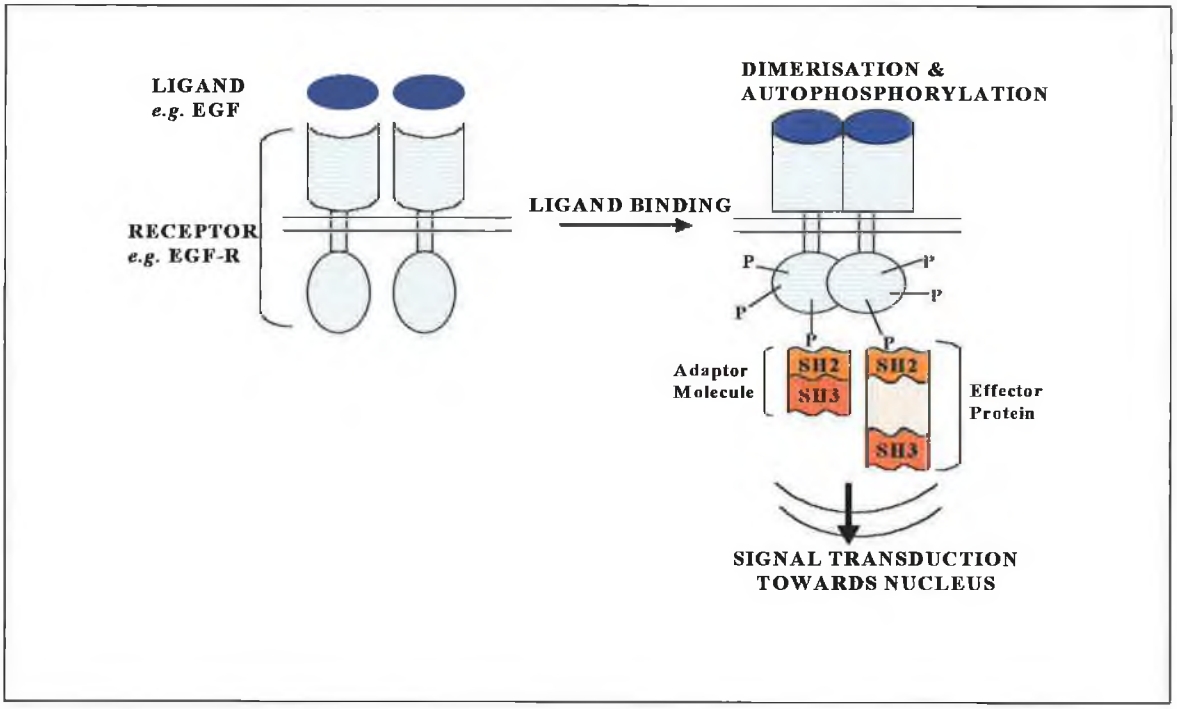


Figure 5.3: **Diagram illustrating the events which occur following ligand (*e.g.* EGF) – receptor interaction. The EGF-Receptor dimerises with a neighbouring receptor, allowing autophosphorylation of receptor tyrosine residues. Molecules containing SH2-domains (either adaptor *e.g. Grb2*, or effector proteins *e.g.* PLC-γ) can bind to tyrosine phosphorylated (P-Tyr) residues, leading to transduction of the binding signal towards the nucleus.**

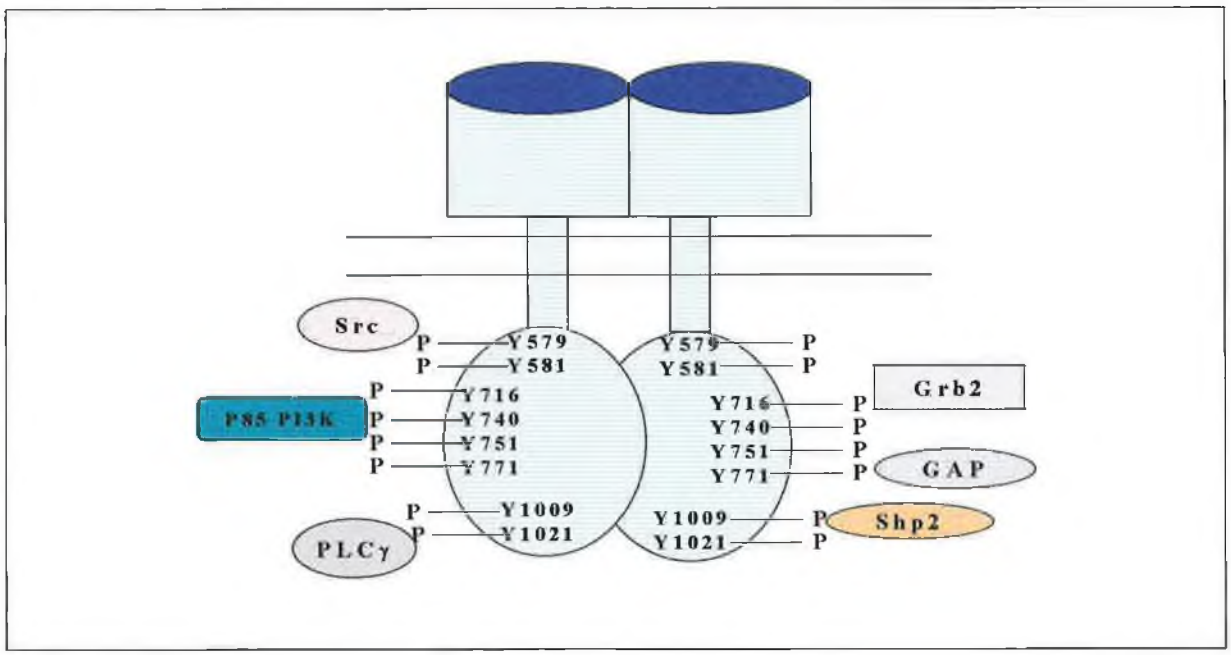


Figure 5.4: Schematic diagram illustrating the phosphotyrosine residues critical in the coupling of the PDGF-R to downstream signalling proteins.

5.2.3. The Ras Protein and its Coupled Transducers

For over a decade, the Ras protein has been assumed to act as a key regulator of cell growth and other functions. The first experiments demonstrating the importance of Ras proteins in the proliferation of normal cells, used antibodies to achieve inactivation of endogenous Ras (Mulcahy *et al.*, 1985). A major goal of research into the regulation of eukaryotic proliferation has been the identification of the critical activators and cellular targets of the Ras protein. Due to the evolutionary conservation of the signalling pathway involving Ras, much lead information on mammalian signalling has been gained from studies in lower eukaryotes, such as *Saccharomyces cerevisiae*, *Drosophilia melanogaster* and *Caenorhabditis elegans* (Egan & Weinberg, 1993).

Ras is a 21kDa guanine nucleotide-binding protein whose biological activity is determined by the bound nucleotide. Activated protein contains bound GTP, while the inactive state binds GDP. During normal signalling, activated Ras-GTP couples transiently with an effector molecule. However, the Ras protein intrinsically possesses a GTPase activity to dephosphorylate bound GTP, thus limiting the lifetime of the activated Ras-GTP species. In transformed cells, however, the Ras protein usually has diminished GTPase activity, (as a result of mutations in the amino acids

important for guanine nucleotide co-ordination), causing extended signalling periods, and the hyperproliferating cancerous cell (Downward, 1998).

Activation of Ras proteins is a key step in the signal transduction process, and is triggered by the RTKs discussed in Section 5.2.2. Central to the activation of Ras is a family of guanine nucleotide release factors (GRFs) (Feig, 1993). The GRF family are molecules responsible for the release of bound GDP from the Ras protein, thereby enabling its activation as mentioned above. Deactivation of Ras occurs by hydrolysis of GTP to GDP, a reaction dramatically hastened by GTPase activating proteins (GAPs). Therefore, the relative activities of GRFs and GAPs acting on Ras at any instant determine its activation state (Figure 5.5).

The first GRF for Ras, CDC25, was identified following genetic studies in *S. cerevisiae*. Three GRF mammalian homologues have since been cloned —2 mouse, mSos1 and mSos2, and one human, hSos. These GRF molecules function directly upstream of Ras but are coupled to the RTKs via the previously-mentioned adaptor molecule Grb2 (see Figure 5.6). This is achieved through Grb2 SH3 domain binding of a proline-rich sequence in the Sos/CDC25 molecule (Feig, 1993).

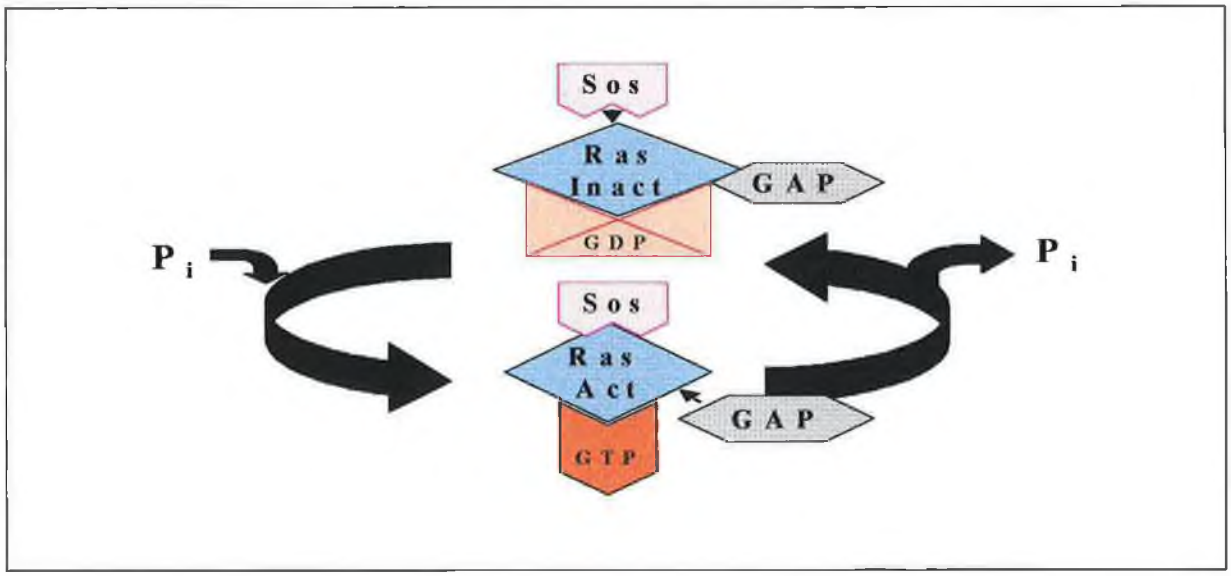


Figure 5.5: **Schematic representation of the activation/deactivation cycle of Ras. Inactive Ras binds GDP, while active Ras contains bound GTP. Two molecule types, the Guanine-nucleotide Releasing Factors (GRFs, *e.g.* Sos) and the GTPase Activating Proteins (GAPs, *e.g.* 120-GAP) control the activation state of Ras, with their relative activities at any instant, deciding its state of activation. The GRFs cause the release of bound GDP from Ras, enabling its activation. The deactivation of Ras occurs by the hydrolysis of GTP to GDP, catalysed by GAPs.**

In addition to the elucidation of upstream activators of the Ras protein, a considerable amount of research has focused on the search for downstream Ras targets (Malarkey *et al.*, 1995; Vojtek & Der, 1998). The first effectors identified were the GTPase activating proteins (GAPs), recognised to maintain normal p21 Ras in a biologically inactive GDP-bound state in *Xenopus* oocytes (Trahey & McCormick, 1987). GAPs are, therefore, negative regulators of Ras, and may be involved in a negative feedback mechanism. To date four mammalian GAPs have been identified: GAP-120, neurofibromin, $GAP1^{m}$ and $GAP1^{IP4BP}$ (Malarkey *et al.*, 1995). As mentioned in Section 5.2.2, GAP-120 is also a direct substrate of autophosphorylated RTKs.

The GAP proteins do not account for all signals emanating from Ras. A more clearly understood and crucial proliferative pathway emerging from Ras involves the activation of an evolutionarily conserved cascade of serine/threonine kinases, as outlined in Figure 5.6 (Malarkey *et al.*, 1995; Vojtek & Der, 1998). Direct interactions of the Ras protein with the amino terminal of the serine/threonine kinase, Raf-1, have been reported in both *in vitro* and yeast expression systems (Lowy & Willumsen, 1993; Van Aelst *et al.*, 1993). Raf-1, a 70-75kDa protein, has a two-domain structure, with a kinase domain occupying the carboxy-terminal half of the molecule, and a regulatory domain occupying the remainder. The Raf-1 protein preferentially binds GTP-Ras in comparison to GDP-Ras (Votjek & Der, 1998). The Ras-Raf binding reaction does not itself stimulate the inherent kinase activity of the Raf-1 protein, rather it only localises the Raf-1 protein to the plasma membrane where some unknown activation of Raf takes place (Leevers *et al.*, 1994).

Activated Raf-1 in turn phosphorylates a second kinase, MEK kinase. This enzyme controls a third family of enzymes, the Mitogen-Activated Protein Kinases (MAP kinases), again through phosphorylation on both tyrosine and threonine residues. The MAP kinases (ERK1 & ERK2) are proline-directed serine/threonine kinases, which have been implicated in the control of cell cycle progression and meiosis. Activated ERK translocates to the nucleus where it phosphorylates a number of factors (*e.g. Rsk-2*, *Elk-1*) required for the transcription of immediate early gene products *e.g. c-fos*. Thus activation of Raf-1 results in nuclear transcription (Roussel, 1998).

Other signalling intermediates can interact with Raf-1 *e.g.* Protein Kinase C (section 5.2.4) is also known to directly activate Raf-1 by direct phosphorylation (Kolch *et al.*, 1993). In addition, it appears G-protein-coupled receptors (Section 5.2.1) are capable of causing activation of the MAP Kinase signalling pathway (Malarkey *et al.*, 1995). Although the mechanism behind this recent observation is unclear, it is believed that activation of cytoplasmic kinases, including Protein Kinase C (as a result of diacylglycerol generation) couples G-protein-receptors to this pathway. Therefore,

even signals from different receptor types appear to converge and co-operate on this central proliferative pathway. Likewise, as shown in Figure 5.6, signal pathways that appear to diverge on initiation by RTK autophosphorylation, can converge later through co-operation along the MAP Kinase cascade *e.g.* Raf activation via Grb2/Sos/Ras as opposed to Raf activation via PLC-γ/DAG/PKC.

As mentioned above, the Ras/MAP Kinase pathway regulates the transcription of c-*fos*, and also c-*jun*, which together dimerise to become the AP-1 nuclear transcription factor. Another important immediate early gene is c-*myc*. This transcription factor also becomes activated following the binding of growth factor to its cognate RTK through phosphorylation of the Src family of cytoplasmic tyrosine kinases (Hunter, 1998).

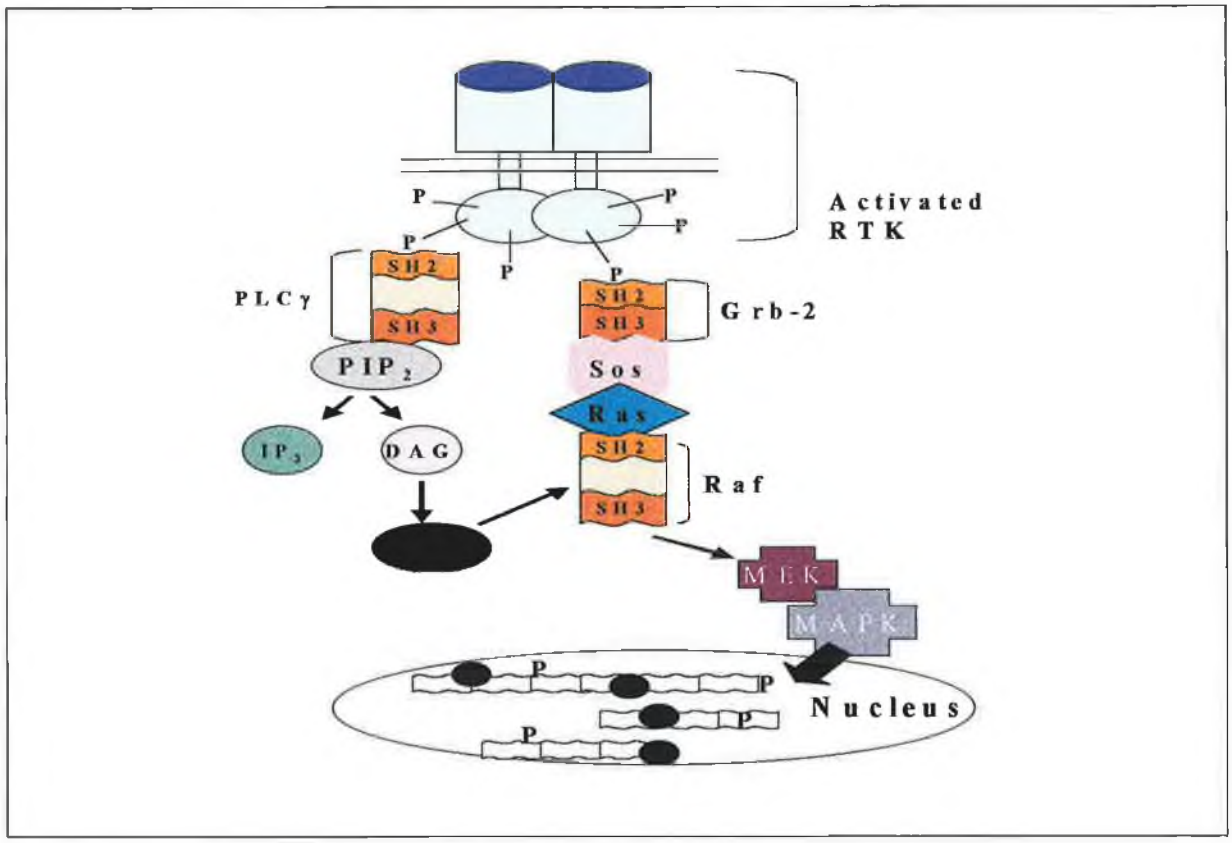


Figure 5.6: **Components of the RTK → MAP Kinase pathway. Ligand binding to its cognate receptor at the membrane surface results in signal cascades towards the nucleus via the Ras/MAP kinase pathway, with eventual nuclear phosphorylation of transcription factors (precise details in text). Divergent signals at the receptor *e.g.* activation of PLC-γ, vs Grb2 binding, can re-converge along the MAP kinase pathway as shown, for PLC-γ-mediated activation of Protein Kinase C.**

5.2.4. Protein Kinase C in Signalling Pathways

Protein Kinase C (PKC) is a large family of serine/threonine kinases involved in multiple signalling pathways. Three major sub-classes have been isolated (Table 5.2), and the members are continuously growing. The different iso-enzymes presumably activate diverse functions in the cell – secretion, ion conductance modulation, smooth muscle contraction and cell proliferation to name a few. As shown in Table 5.2, various PKC isozymes are activated by the second messengers diacylglycerol (DAG) and calcium. These are products of the phosphoinositide hydrolysis pathway, initiated through the stimulation of PLC-γ by many receptor types (Figure 5.7). The critical substrates of PKC have not been conclusively identified but include various transcription factors, Raf-1 and MEK (Kolch *et al.*, 1993; Malarkey *et al.*, 1995; Mochly-Rosen & Kauvar, 1998). Therefore, Protein Kinase C would appear to interact with proliferative pathways within the cell.

PKC Family	*Members*	*Properties*
Classical	$\alpha, \beta_1, \beta_2, \gamma$	1. Bind phospholipid, diacylglycerol (DAG) and Phorbol Myristate Acetate (PMA) 2. Ca^{2+}-dependent
Novel	$\delta, \varepsilon, \eta, \theta$	1. Bind phospholipid, DAG and PMA 2. Ca^{2+}-independent
Atypical	ζ, λ	1. Bind phospholipid; do not bind DAG and PMA 2. Ca^{2+}-independent

Table 5.2: Table summarising the properties and iso-enzymes of the various PKC families. Compiled from data in Weinstein *et al.* (1997) and Mellor & Parker (1998).

However, PKC appears to play a paradoxical role in the cellular signalling network, as it is believed to act as a negative regulator, over various steps of the cell proliferation signalling process. PKC appears to be important in decreasing the elevated Ca^{2+} levels induced by inositol triphosphates. It may achieve this by inactivating the lipid triphosphates with inositol-triphosphate-phosphatase (Connolly *et al.*, 1986). Alternatively, it may activate the Ca^{2+} transport ATPase and the Na^{+}/ Ca^{2+} exchange protein, effecting the removal of intracellular Ca^{2+} (Nishizuka, 1986). In addition, PKC acts as a negative regulator of growth factor receptors. PKC has repeatedly been shown to phosphorylate the EGF-R *in vivo* and *in vitro* on threonine residues (Cochet *et al.*, 1984;

Downward *et al.*, 1985). This action causes a rapid decrease in EGF binding, with consequent receptor downregulation.

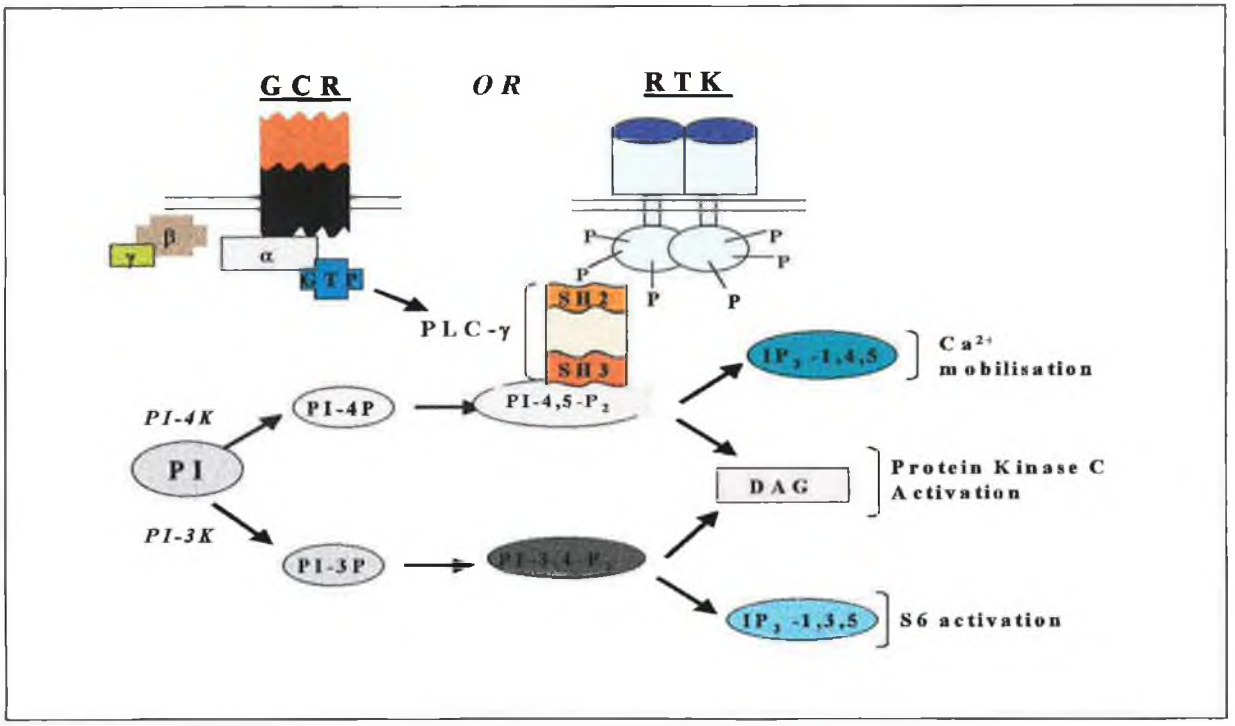


Figure 5.7: **Schematic representation of the PLC-γ -mediated Phosphoinositide hydrolysis pathway, the products of which include diacylglycerol, which can lead to Protein Kinase C activation. Either G-protein-coupled receptors or Receptor tyrosine kinases can cause activation of PLC-γ, with eventual PKC stimulation.**

5.2.5. Oncogenes and Signalling Pathways

The growth signalling pathways described in Sections 5.2.1- 5.2.4 are clearly complex, and their regulation as yet is not completely determined. While the signalling pathway from the plasma membrane to the nucleus is elucidated (the Ras/MAP kinase pathway), it is evident that other signalling intermediates infringe/converge on this pathway at each step in the cascade. Divergent pathways are also initiated from this central signalling scheme (Figure 5.8). It is apparent, therefore, that deregulation or malfunctioning at any step along this pathway has severe consequences for growth control within the cell. Oncoproteins, which are altered versions of normal growth proteins,

have been identified at all stages along the growth control pathway (Table 5.3 and Figure 5.8). In many instances the oncoprotein possesses much enhanced enzyme activity *e.g. neu* oncogenic mutant receptor has enhanced tyrosine kinase activity.

However, despite the identification of many oncoproteins with the capability to deregulate normal growth signalling pathways, only a few are commonly associated with human cancer *e.g. ras* has been associated with 30-40% of cancers. Instead, the most common cellular fault in human cancers involves autocrine activation, in association with growth factor receptor over-expression. Many tumours and cell lines co-express growth factors and receptors, including EGF/TGF-α, PDGF-A/B and FGF (Table 5.4), leading to persistent, enhanced signalling, observed as cancer.

Category	*Oncogene (Cellular Homologue)*	*Associated Cancer*
Growth Factors	*Sis* (PDGF) *Int-2* (FGF)	Simian sarcoma
Transmembrane growth factor receptors	*ErbB* (EGF-R)	Breast, ovary, prostate, renal cell, glioblastomas
Membrane-associated tyrosine kinases	*Src* *Abl*	Colorectal, renal. Leukemia
Membrane–associated guanine nucleotide binding protein	*Ras*	Colorectal, lung, prostate, pancreatic, thyroid, skin
Nuclear factors	*Myc*	Neuroblastoma, Burkitts lymphoma

Table 5.3: Selected oncogenes, their cellular homologues and cancers in which they are implicated. This table was compiled from data from a number of sources (Boutin, 1994; Carter & Kung, 1994; Levitzki, 1994; Lodish *et al.*, 1995; Kolibaba & Druker, 1997)

Growth Factor Receptor Involved	*Associated Cancer*
EGF-R Autocrine Loop / Over-expression of EGF-R & TGF-α	Glioblastomas, Prostate, Renal Cell, Breast, Bladder, Lung, Neck,
PDGF-R Autocrine Loop/ Over-expression of PDGF-R & PDGF	Glioblastomas, Breast
Over-expression of Her2/*neu*	Breast, Ovary, Non-small cell lung .

Table 5.4: **Selected growth factor autocrine loops and cancers in which they are implicated. This table was compiled from data from a number of sources (Derynck, 1988; Carter & Kung, 1994; Levitzki, 1994; Lodish *et al.*, 1995; Feldkamp *et al.*, 1997; Voldberg *et al.*, 1997)**

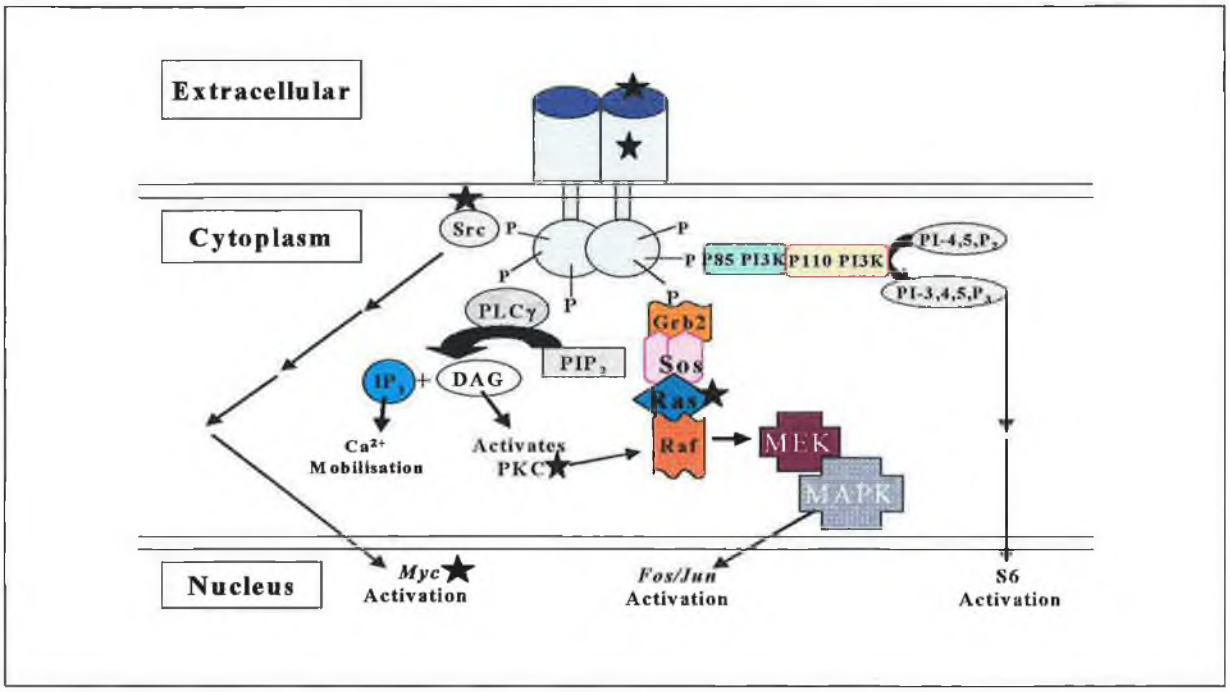


Figure 5.8: **Summary of growth factor-mediated signalling pathways within cells, illustrating the divergence and convergence of various signalling intermediates along the main MAP Kinase route, as detailed in the text. Oncoproteins at any stage along this pathway can seriously deregulate normal growth control processes, and the intermediates which are commonly associated with human cancers are highlighted with black stars.**

5.3. SIGNAL TRANSDUCTION THERAPY

As described in sections 5.1 and 5.2, cancer can arise as a result of the aberrant relay of growth signals within the cell. The persistent downstream signalling arises from either over-expression of, or mutations in, the normal signalling proteins. Signal transduction therapy involves utilising novel agents specifically designed to block signalling pathways or their components, thereby averting the uncontrolled growth of cancerous cells. Any component of the signalling pathways described in Section 5.2 can be targeted. Efforts in this approach have concentrated in two principal areas to-date: 1. Tyrosine kinase inhibition

2. Ras pathway inhibition.

5.3.1. Tyrosine Kinase Inhibition

Protein tyrosine kinases are excellent targets for the development of anti-cancer drugs, on the basis of the critical role they play in the creation of signals responsible for cell proliferation and malignancy. The transforming ability of protein tyrosine kinases is removed when recombinant DNA techniques are used to immobilise the kinase activity (Yarden & Ullrich, 1988). It would be expected if a drug could exhibit the same kinase inhibition, it would also have beneficial anti-proliferating effects. A variety of chemical structures, many based on natural products, have been determined which inhibit protein tyrosine kinases.

The first known protein tyrosine kinase inhibitor was quercetin, a member of the bioflavonoid group, which was shown to inhibit the tyrosine phosphorylation activity of p60-Src both *in vivo* and *in vitro*. However, this inhibitory activity was found to be non-specific, due to the fact that it was competitive with respect to ATP (Brunton & Workman, 1994). The isoflavone genistein proved a more efficient inhibitor of the EGF-RTK activity – it was potent to tyrosine kinases at concentrations that had no effect on serine/threonine kinases (Brunton & Workman, 1994).

Erbstatin, a natural isolate from actinomycete broth, was the first described tyrosine analogue, which had tyrosine kinase inhibitory activity. It blocks the peptide-site of the EGF-R, thus competing with the substrates for phosphorylation, in EGF-R-containing membrane fractions (Brunton & Workman, 1994). The structure of erbstatin was used as the lead for the specific and systematic design of a family of chemical tyrosine kinase inhibitors, the tyrphostins (Gazit *et al.*, 1989). Highly selective inhibitors of the EGF-RTK, PDGF-RTK and *Abl*-TK have been reported

(Gazit *et al.*, 1991; Anafi *et al.*, 1992). Other chemical families *e.g.* 4-hydroxycinnamide, dianilophthalimide and 2-phenylaminopyrimidine, have also been assessed (Shiraishi *et al.*, 1989; Trinks *et al.*, 1994; Buchdunger *et al.*, 1995). An expanding list of highly specific and potent *in vitro* inhibitors of all the major tyrosine kinases currently exists, and these are likely to enter clinical trials in the near future.

5.3.2. Inhibitors of the Ras Pathway

As a normal transducer of upstream mitogenic signals, the Ras protein plays an important role in proliferative signalling, and inhibition of Ras function is therefore an excellent target for drug discovery. Drug discovery programs have concentrated on two main areas for the inhibition of Ras function: 1. inhibition of Ras activation by Sos exchangers

2. inhibition of Ras post-translational modification.

Small molecules which interfere with the Grb2/Sos interactions and/or Sos/Ras interactions are attractive drug targets. Since Grb2 interacts with SH2 domains on phosphorylated RTKs through a small conserved sequence, in theory it should be relatively easy to screen for compounds which block Grb2 binding (Grbstatins). Active research in this area is documented (Levitzki, 1994).

Therapeutic intervention of Ras has concentrated on the area of Ras farnesylation (Gibbs *et al.*, 1994). It is well-established that membrane association is essential for Ras functioning. Localisation of Ras to the plasma membrane is mediated by the farnesyl (15-C isoprenyl) group. The post-translational addition of this farnesyl group is catalysed by a number of enzymes, the most important of which is Farnesyl-Protein-Transferase (FPTase). This enzyme is also the most important therapeutic target for the Ras molecule. Drug discovery programs have yielded successful *in vitro* FPTase inhibitors, although laboratory studies are still ongoing (Heimbrook & Oliff, 1998).

5.3.3. Other Strategies

Various other strategies for the disruption of aberrant growth signalling pathways include growth factor agonists, antibodies and anti-sense strategies. McInnes and Sykes (1997) review the current status of research in the area of peptidomimetic growth factor agonists, and highlight the various difficulties facing this approach to abolishing autocrine activation. To date, suramin is the

sole chemical agonist of growth factors, and has been used clinically in the treatment of renal and prostate cancers (Levitzki & Gazit, 1995).

Antibodies generated to block the ligand-receptor binding step, is an alternative strategy for preventing the initiation of growth signals. Murine monoclonal antibodies (Mabs) to the EGF-R were shown to inhibit proliferation of both cultured and xenograft cells. In clinical trials, successful targeting of primary lung and metastatic cells was achieved with these Mabs. A human-mouse chimeric version is currently in clinical trials. As the nature of growth inhibition with antibodies is cytostatic, combination therapies with cytotoxic chemotherapeutic agents are currently undergoing clinical trial assessment, with promising results (Fan & Mendelsohn, 1997).

Anti-sense oligonucleotides are a further intervention possibility being examined, although to date studies are in the early laboratory phases (Voldberg *et al.*, 1997).

5.4. COUMARINS IN SIGNALLING PROCESSES

Interest in the study of coumarin compounds has originated in the clinical observation of its efficacy in cancer patients, especially renal cell carcinoma, prostate cancer and melanoma. The widely-held belief, is that coumarin is the pro-drug for the active agent, 7-hydroxycoumarin, and laboratory work has concentrated on proving this belief. Much presented work has shown that both coumarin and 7-hydroxycoumarin inhibit the *in vitro* growth of a range of cell lines in a dose-and time-dependent manner, with some cell lines more sensitive than others to these effects (Chapter 4, as well as Moran *et al.*, 1993; Siegers & Bostelmann, 1993; Marshall *et al.*, 1994). In all cases 7-hydroxycoumarin was more potent than its parent compound.

To date, however, very little understanding of the growth inhibitory action has been achieved, with both direct and immunomodulatory mechanisms often cited to explain *in vivo* observations. In order to exploit the effects of any drug optimally, it is essential to locate it precise cellular or molecular target. To date this has not been achieved with the coumarins. However, it is apparent from the previous discussion on signalling pathways in normal and cancerous cells (Section 5.2), that any compound with an anti-tumour effect may infringe on these pathways, or their components, in mediating their action.

One hypothesis regarding the precise mode of action of coumarins, suggests that these compounds modulate their action by down-regulating autocrine loops in growing cells. Indirect evidence lies in the fact that both coumarin and 7-hydroxycoumarin inhibit the growth of glioblastoma cell lines (Seliger, 1993). These cell lines are heavily dependent on both PDGF and EGF autocrine loops (Feldkamp *et al.*, 1997). Direct evidence for the involvement of coumarins in down-regulating autocrine loops, stems from the report that 7-hydroxycoumarin selectively and reversibly downregulates mRNA expression of PDGF in glioblastomas. mRNA levels of PDGF-R, EGF, EGF-R and TGF-α are unaffected by 7-hydroxycoumarin treatment, and growth factor binding is also unchanged (Seliger, 1997). These results do not indicate whether the negative regulation of the PDGF loop is a cause or consequence of treatment with 7-hydroxycoumarin. Given the prevalence of autocrine loops in many cancers (Section 5.2.5), including prostate and renal cell cancers, which are responsive to coumarin *in vivo*, further work into the study of autocrine loops and their associated signalling pathways would be very beneficial.

The central role of the Ras protein in growth signalling pathways was also highlighted in Section 5.2.3. Various studies into the direct effects of coumarins have observed changes in *ras* activity and expression as a result of treatment with coumarins. Zanker (1997) reported that 7-hydroxycoumarin dramatically inhibited the growth of the RBA mammary and T-24 bladder carcinoma cell lines, which are both heavily dependent on the constitutively-activated Ha-*ras* oncogene for growth. 7-hydroxycoumarin could alter the level of Ha-*ras* expression (at the mRNA level) in the RBA cell line (Zanker, 1993). Seliger and Pettersson (1994) also observed reversible growth inhibition by 7-hydroxycoumarin in fibroblasts transformed with *ras*. Northern blot analysis revealed a decrease in the specific *ras*-mRNA levels. Kahn and colleagues (1994) showed that coumarin could decrease *ras* expression in cells transformed with the *ras* oncogene, which resulted in decreased numbers of cells entering the S-phase of the cell cycle. Therefore, coumarin compounds also appear to interact extensively with the Ras protein in transformed cells.

The effect of 7-hydroxycoumarin on the c-*myc* oncogene, an important transcription factor, has also been addressed. Zanker (1993) examined the growth inhibition and mRNA expression of c-*myc* in the DUKX cell line, which overexpresses the c-*myc* oncogene, if transiently exposed to 40°C. Concentrations of 100 and 200μg/ml caused a dose-dependent and reversible growth inhibition of this cell line. The mRNA expression was only marginally affected by 7-hydroxycoumarin treatment (Zanker, 1993). In *myc*-transformed fibroblasts, both growth and *myc*-gene expression were strongly inhibited by 7-hydroxycoumarin treatment (Seliger & Pettersson,

1994). Treatment of HL-60 leukemic cells with coumarin also caused down-regulation of c-*myc* expression (Shima *et al.*, 1989).

Finally, the effect of coumarins on Protein Kinase C pathways has been previously described in human monocytes, with regard to cytokine production (Stuhlmeier *et al.*, 1991; Zlabinger *et al.*, 1993). Coumarin, 7-hydroxycoumarin and 4-hydroxycoumarin all potentiate the release of IL-1, IL-6 and TNF-α from monocytes stimulated with LPS (Stuhlmeier *et al.*, 1991). Zlabinger and co-workers (1993) ascertained that the LPS-induced cytokine release involved both the Protein Kinase C and lipoxygenase signalling pathways, and determined that the coumarins also interacted with these pathways in displaying their augmenting effect.

It is evident from the above discussion, that the coumarin compounds exert a number of effects on important components of normal signalling pathways. Since these components are commonly deregulated in cancer, the coumarins may be exerting their anti-tumour effect by renewal of regulated signalling. The review of growth signalling pathways in Section 5.2, highlighted the importance of tyrosine phosphorylation reactions in the accurate regulation of growth control. To further the investigation of the effect of coumarin compounds on signalling components and pathways, we decided to investigate the effect of coumarins on tyrosine phosphorylation within cells.

Tyrosine phosphorylation, and in particular inhibition of tyrosine kinases, interested us for a number of reasons. Firstly, esculetin (6,7-dihydroxycoumarin) has been reported to inhibit the growth of smooth muscle cells, exerting this effect through inhibition of protein tyrosine kinases (Huang *et al.*, 1993). It also inhibited tyrosine kinase activity in T-lymphoid leukemia cells (Huang *et al.*, 1994). The origin of this activity may be derived from the structural similarity of the "phenol-moiety" of esculetin and tyrosine (Figure 5.9). In addition, many bioflavonoids, *e.g.* genistein and quercetin, are well-documented tyrosine kinase inhibitors (Boutin, 1994). Other known tyrosine kinase inhibitors (Figure 5.9) also share phenolic groups which may act as competing substrates for the tyrosine kinase activity. Lazaro *et al.* (1995) have reported on the inhibition characteristics of phenolic compounds on spleen protein tyrosine kinases. Diphenolic and triphenolic compounds were shown to display exceptional inhibitory capabilities, while monophenolic compounds were much poorer inhibitors of tyrosine kinases.

In this work, we were interested in testing the hypothesis that 7-hydroxycoumarin (a monophenolic compound) may cause slight inhibition of cellular tyrosine kinases. Such an event

would cause a dampening of persistent deregulated signalling, which may account for the observed effects of coumarins on signalling components. The fact that coumarin has shown effects *in vivo* with renal cell and prostate cancer, two cancer types with deregulated EGF/EGF-R autocrine loops, prompted us to examine this ligand-receptor system in detail. The very potent growth inhibition of the A431 cell line, which over-expresses the EGF-Receptor, by coumarin compounds observed in Chapter 4, also directed us to investigate the EGF/EGF-RTK.

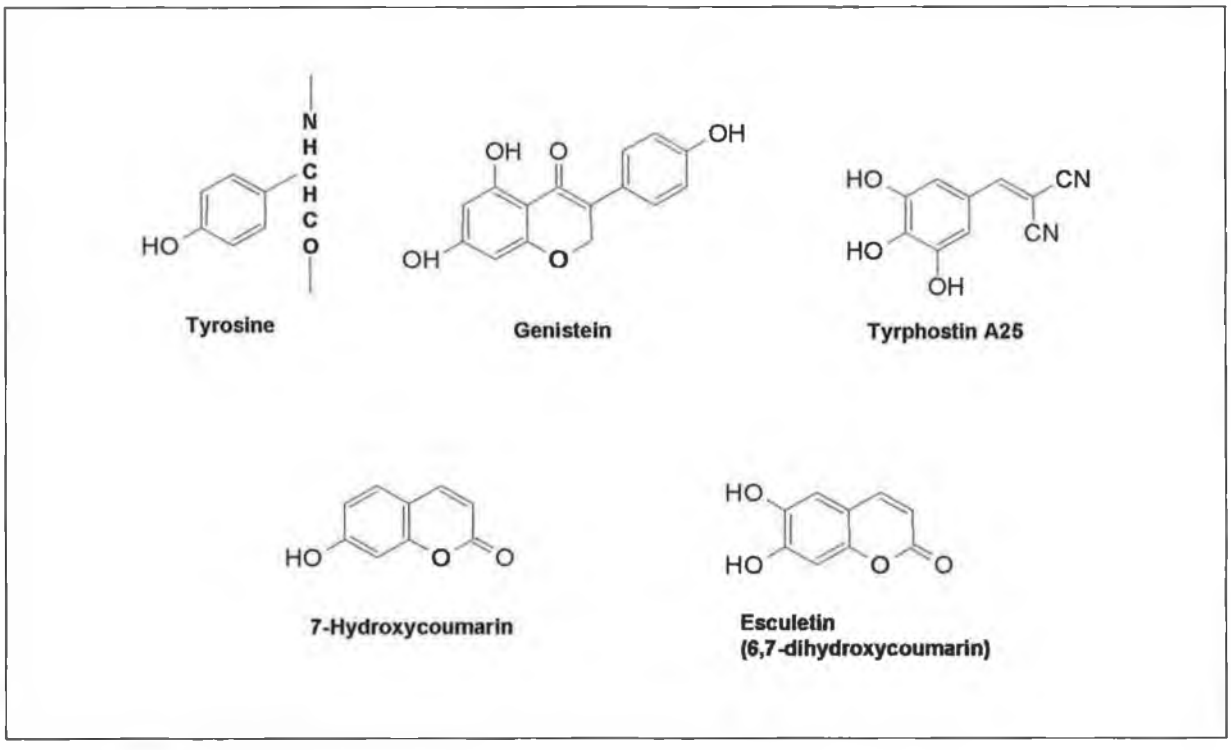


Figure 5.9: Chemical Structures of tyrosine, 2 tyrosine kinase inhibitors (tyrphostin and genistein) and coumarin compounds.

5.5. CHAPTER OUTLINE

The effect of two coumarin compounds, 7-hydroxycoumarin and esculetin, on tyrosine phosphorylation in cancer cells was examined, to determine if these compounds exhibit tyrosine kinase inhibitory properties. The EGF-Receptor tyrosine kinase, chosen as an easily-inducible TK activity, was examined in detail, using the A431 cell line as a source. The activity of this RTK was initially assessed following purification in an *in vitro* format. However, it was decided to examine this activity in intact cells, and the effect of the coumarins on tyrosine phosphorylation was examined by ELISA, Cytosensor studies and Western Blotting. The effect of the coumarins on Protein Kinase C pathways was also examined.

5.6. RESULTS AND DISCUSSION

5.6.1. Activation of the EGF-Receptor in A431 Cells:

As described in Section 5.5, this study involved examining the effect of coumarins on tyrosine phosphorylation events in cancer cells. The A431 cell line, known to over-express the EGF-Receptor was chosen in conjunction with EGF, as a model signalling pathway, with inducible tyrosine kinase activity. Preliminary studies were conducted to ensure that the A431 cells responded to EGF, using the Cytosensor Microphysiometer. As described in Chapter 3, this instrument has been used extensively in the examination of cellular responses to neurotransmitters, cytokines and growth factors. The stimulation of normal keratinocytes with EGF on the Cytosensor Microphysiometer has been reported (Parce *et al.*, 1989). Fok and colleagues (1994) also used this instrument for the detection of EGF-R ligand in the conditioned media of ovarian carcinoma cells. The metabolic response of mammary cells over-expressing the HER-2 Receptor, an EGF-R homologue, has also been reported (Chan *et al.*, 1995).

The response of A431 cells to stimulation with EGF was therefore assessed on the Cytosensor Microphysiometer, according to the method outlined in Section 2.2.6.4. As outlined in Table 5.5, various experimental conditions were optimised. Using these optimised parameters, the response of A431 cells on exposure to a range of EGF concentrations was examined. As expected a dose-dependent increase in the acidification rate of the cells was observed in the range 0-100ng/ml EGF (Figure 5.10). Through integration of the area under the curve of these response curves (Figure 5.10) a dose-response curve for the A431 cells and EGF was constructed (Figure 5.11). From this it can be seen that the maximal response was obtained at concentrations in the range 50-100ng/ml of EGF. This value corresponds well with studies using other parameters *e.g.* inositide formation (maximal response at 50-100ng/ml), to determine maximal responses to EGF in the A431 cell line (Tilly *et al.*, 1988). From this result, we concluded that our ligand-receptor model was useful for further studies, and an appropriate ligand concentration for these studies was ascertained.

Variable Examined	*Optimised Conditions*
Cell Seeding Density	2.5 X 10^5 cells/capsule
Pump Cycle	2 mins cycle @ 60% speed (~ 120µl/min)
Serum-starvation Period	16hrs
EGF Exposure Period	12mins

Table 5.5: **Optimisation of experimental conditions for the exposure of A431 cells to EGF on the Cytosensor Microphysiometer, as outlined in Section 2.2.6.4.**

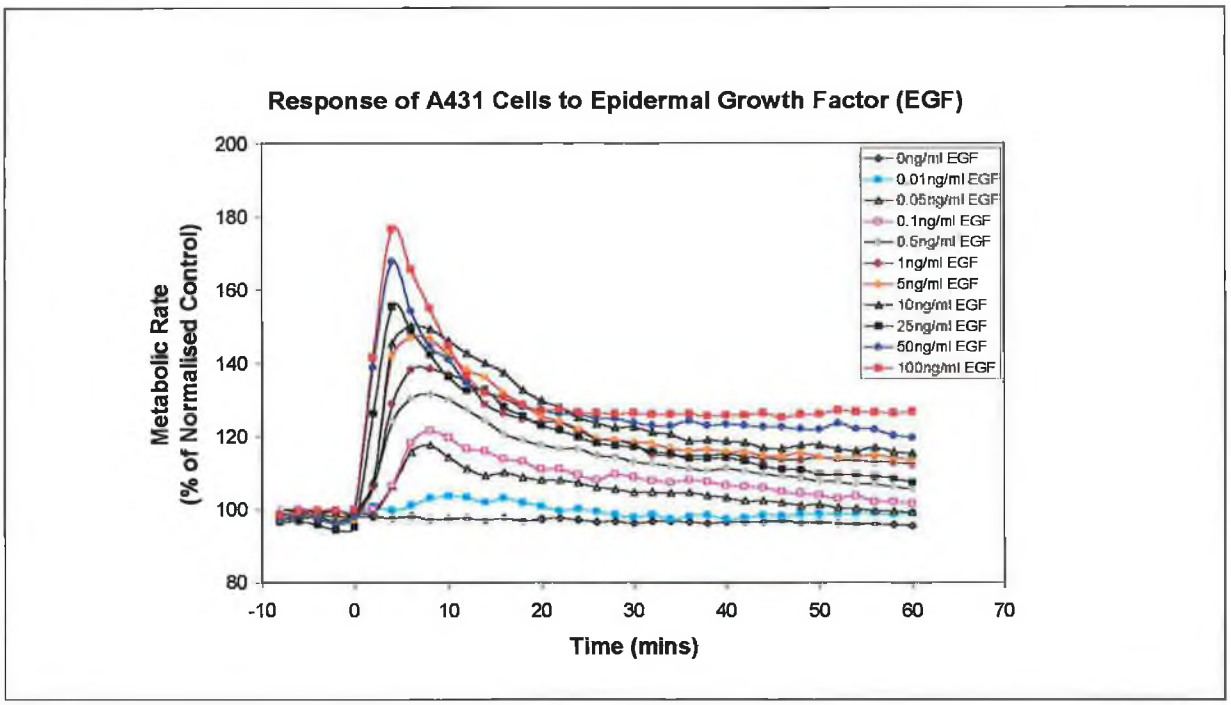


Figure 5.10: **Exposure of A431 cells to various concentrations of Epidermal Growth Factor (EGF). The above plot shows the experiment conducted on the Cytosensor Microphysiometer to determine the feasibility of using the EGF/A431 system as an appropriate ligand-receptor model. In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to EGF exposure, using a 2 min cycle, with the acidification rate measured in the final 30 secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during and following EGF exposure were expressed as a percentage of this normalised value. These values of metabolic rate (mean of four experiments) were plotted vs time in the above graph. Typical basal acidification rates for the cells in all experiments were –100 to -200µvolts/sec.**

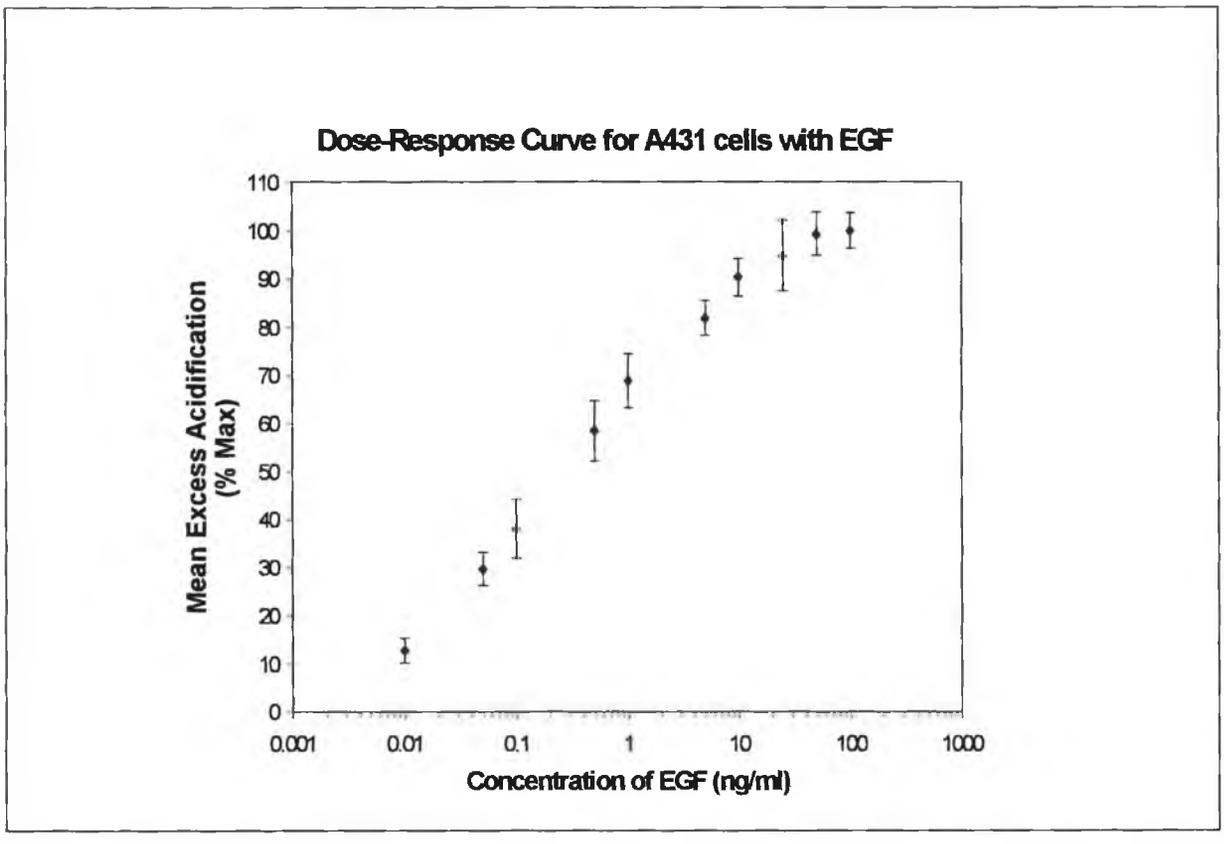


Figure 5.11: **Dose-Response curve for the interaction of EGF with the EGF-Receptor on A431 cells. This curve was constructed from the data presented in Figure 5.10 as follows: Briefly, integration of the area under each of the curves (AUC) in Figure 5.10 was achieved using the Kaleidagraph (version 3.0 – Abelbeck software) program. From this analysis, data for the AUC for zero effect, EGF concentration of maximal effect (*i.e.* 100ng/ml) and all intermediate concentrations (0.01-50ng/ml) was available. By subtracting the zero value from each concentration value, and expressing the result as a % of the maximal value a mean excess acidification value for each concentration value was obtained. The above dose response curve was obtained by plotting the mean excess acidification rate vs the concentration of interest.**

5.6.2. Studies on the Inhibition of the EGF-R Tyrosine Kinase Activity

The critical role of the EGF-Receptor at the head of signalling pathways has been discussed in Section 5.2. The prevalence of both EGF-R autocrine loops and deregulating mutations in many cancer types was also reviewed (Section 5.2.5). Due to the importance of such anomalies in prostate and renal cell cancers, two cancer types responsive to coumarin treatment, we were interested in assessing the effect of 7-hydroxycoumarin on the tyrosine kinase activity of the EGF-Receptor, and this was achieved as outlined in Sections 5.6.2.1 and 5.6.2.2.

5.6.2.1. Purification of the EGF-Receptor

In the past, purification of the EGF-Receptor has been accomplished by various methods, including use of EGF Affi-Gel affinity (Cohen *et al.*, 1980), and immunoaffinity columns with anti-EGF-Receptor (Weber *et al*, 1984). Both methods require competitive elution with EGF. However, in experiments examining kinase activity, it is preferred that the intrinsic receptor tyrosine kinase activity is not activated (through ligand binding) prior to its use. This requires the purification of active, but unactivated kinase, and was achieved in this work using lectin chromatography (using a wheat-germ agglutinin (WGA)-agarose column), as outlined in Section 2.2.6.1. This does not yield 100% pure receptor, but fractions substantially enriched for the receptor and its associated tyrosine kinase activity.

The purification process was followed using dot blots and western blots for detection of the receptor, and a typical western blot illustrating the purified EGF-Receptor (M_r = 170kDa) is outlined in Figure 5.12. The bands on the left hand side of the gel (Lanes 1 & 2) indicated the presence of EGF-R in the lysed cellular (A431) fraction, both pre- and post-ultracentrifugation – overloading of these samples was evident from the wide bands. Various washes of the column were performed to remove non-bound proteins - no EGF-receptor was lost during this procedure (Lanes 3-6). EGF-R was only present in samples obtained following specific elution of the receptor from the column (Lane 7). Some degradation of the receptor (lower bands) was apparent. The yield of total protein from the purification shown in Figure 5.12 was ~ 0.15mgs, as determined using the BCA Assay (Section 2.2.2.1). The fraction obtained was used to assess the effect of 7-hydroxycoumarin on the EGF-Receptor tyrosine kinase, as outlined in Section 5.6.2.2.

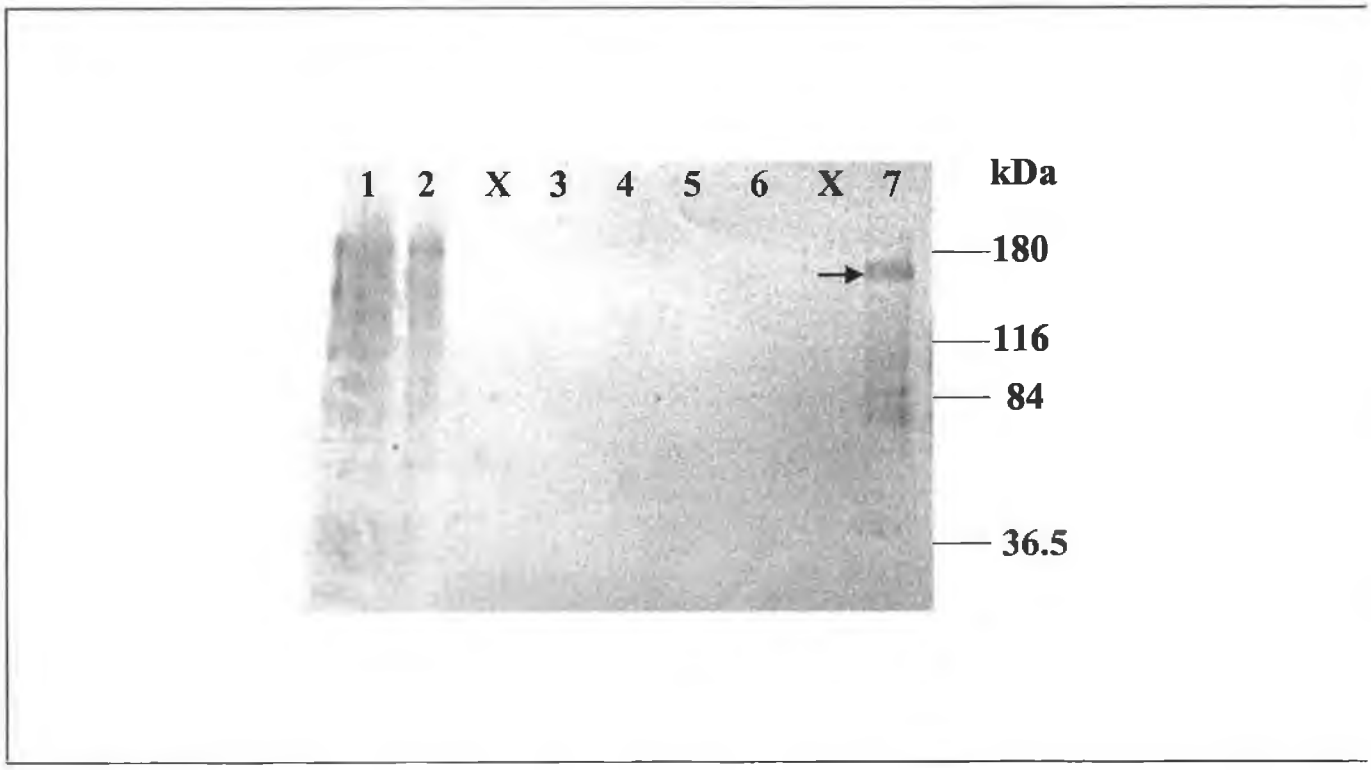


Figure 5.12: Assessment of EGF-receptor purification by Western Blot analysis with anti-EGF-Receptor antibodies. Purification was carried out as described in Section 2.2.6.1 and immunoblotting accomplished as in Section 2.2.2.3. Samples from each of the purification steps were run on an 8% SDS gel and immunoblotted, prior to probing with anti-EGF-Receptor. Samples were run as follows, with an X denoting a well where no sample was loaded: Lane 1: Solubilised cells, pre-ultracentrifugation; Lane 2: Post-ultracentrifugation supernatant; Lane 3: Unabsorbed WGA-Agarose column sample; Lanes 4-6: Column Washes; Lane 7: Eluted fraction from WGA-Agarose.

5.6.2.2. Tyrosine Kinase Assays using Purified EGF-RTK.

The phosphorylating ability of many cellular tyrosine kinases have been assessed in the past using radioactive ^{32}P-ATP (Akiyama *et al.*, 1987; Gazit *et al.*, 1989). In recent years, there has been a movement away from the use of radioactivity in experiments, which has lead to an increase in the number of non-radioactive ELISA based methods for the detection of kinase activity (Lazaro *et al.*, 1991; Farley *et al.*, 1992). In these, specific anti-phosphotyrosine antibodies are used to detect the extent of tyrosine phosphorylation following the exposure of a specific peptide to the kinase of interest. Recently, this assay format has become available commercially.

The tyrosine kinase activity of the EGF-R-containing fraction isolated in Section 5.6.2.1 was examined using one such commercial kit, as outlined in Section 2.2.6.2. Briefly, this entailed activating the EGF-Receptor Tyrosine Kinase activity, through EGF binding. The efficiency of the RTK in phosphorylating a tyrosine-containing peptide substrate, in the absence or presence of known and putative inhibitors, was then ascertained using the ELISA-based system. Tyrphostin A25, a specific inhibitor of the EGF-RTK was used as a positive control for tyrosine kinase inhibition, while 7-hydroxycoumarin was the putative inhibitor under assessment.

The results of this study are shown in Figure 5.13. It is evident that the exposure of the EGF-R to tyrphostin caused a dose-dependent decrease in the receptor-associated tyrosine kinase activity. The decrease of ~ 37% at a concentration of 100μg/ml was much lower than expected as Tyrphostin A25 has previously been stated as an extremely potent inhibitor of the EGF-RTK activity, with IC_{50} values of 3μg/ml for receptor autophosphorylation, and 0.6μg/ml using poly-GAT as an external substrate (Gazit *et al.*, 1989). Figure 5.13 also illustrated a slight decrease in the tyrosine kinase activity of the EGF-R on exposure to 7-hydroxycoumarin. This decrease appeared to be dose-dependent, but was small – at concentrations of 100μg/ml an inhibition of ~ 15% was observed. There are a number of possibilities for this: firstly, and most obviously, the 7-hydroxycoumarin molecule may not be an efficient tyrosine kinase inhibitor, or if it is, the EGF-RTK may not be its preferred/optimal target. The second reason addresses the possibility that the test system used (*i.e.* kit) may not be optimal. It has previously been shown by Songyang and colleagues (1995), using degenerate peptide libraries, that the tyrosine kinases have optimal peptide substrates *i.e.* unique sequences of peptides, that they preferentially phosphorylate. For example it has been shown that the c-Src tyrosine kinase prefers the sequence EEEIYGEFD, while the EGF-RTK will preferentially phosphorylate tyrosine in the following sequence EEEEYFELV. The number of currently available commercial tyrosine kinase kits and peptide substrates are limited,

giving rise to the possibility of sub-optimal conditions for any enzyme/substrate combination. The peptide sequence used in this kit was EGPWLEEEEEAYGWMDF, and this may account for the reduced "sub-optimal" effect of Tyrphostin. In addition, we were concerned about the stability of the EGF-R and it associated tyrosine kinase activity. Figure 5.12 had illustrated the presence of some degraded receptor in the purified fraction, and we were concerned that the purified fraction was not optimally active.

As a result of these observations it was decided to examine the effect of coumarins on tyrosine phosphorylation in intact cells, where the events observed mirror more effectively the natural situation. This approach is also more labour- and cost-efficient than the task of screening a number of pure kinases in the hope that 7-hydroxycoumarin inhibits one of them. Instead examination of the effect of this compound on total tyrosine phosphorylation, can quickly prove or disprove this research avenue as a viable one.

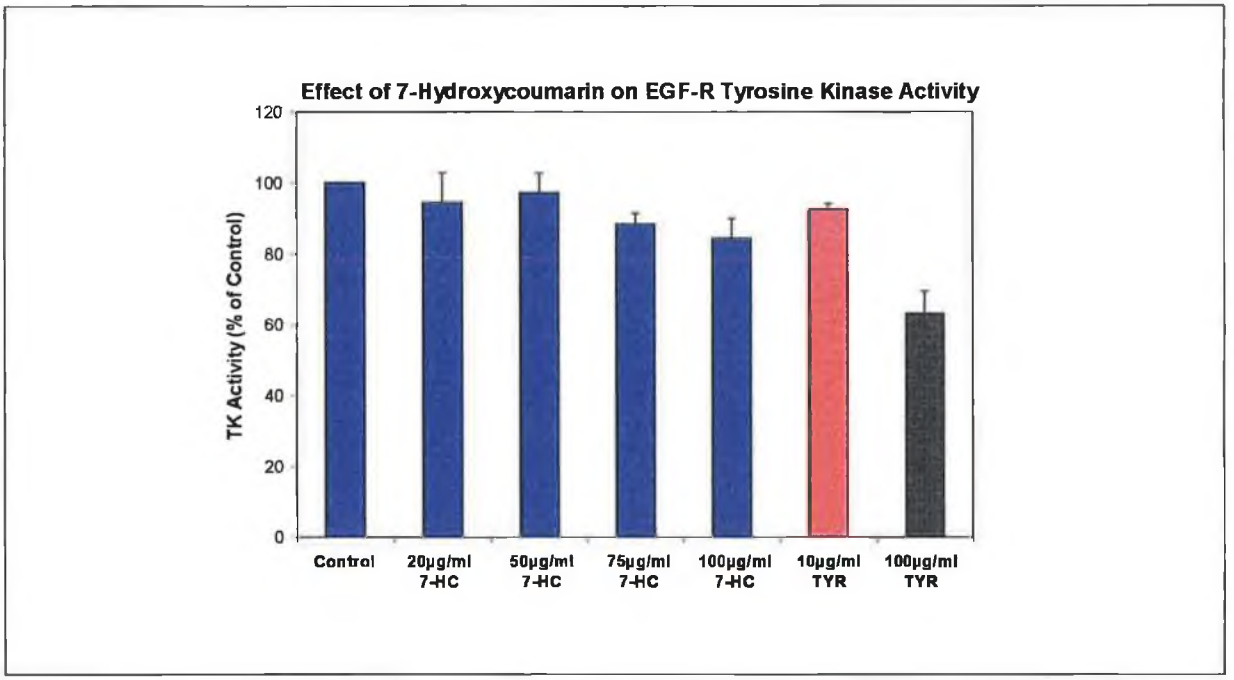


Figure 5.13: ***In Vitro*** **Tyrosine Kinase Assay to examine the effect of 7-hydroxycoumarin on the kinase activity of the EGF-RTK. The effect of 20-100µg/ml 7-hydroxycoumarin on the tyrosine kinase ability of EGF-stimulated EGF-RTK was assessed using an ELISA-based system (Section 2.2.6.3). Absorbance values for the test samples were normalised vs control wells (Abs 450nm= 1.106 ± 0.109). All experiments were carried out in duplicate on four separate occasions. Tyrphostin was included as a positive tyrosine kinase inhibitor.**

5.6.3. ELISA-Detection of Tyrosine Phosphorylation in Whole Cells

As outlined in Section 5.6.2.2, it was decided to examine the effect of coumarin compounds, namely 7-hydroxycoumarin and esculetin, on total tyrosine phosphorylation in intact cells. Initially, an ELISA-based system was used to achieve this. The ELISA used (Section 2.2.6.3) was adapted from the method of Trinks *et al.* (1994), where it had been used to screen for efficient tyrosine kinase inhibitors from a group of systematically-designed candidates. The rationale behind the assay is the following: When serum-starved A431 cells are exposed to EGF, their signalling pathways become activated, and the overall levels of phosphorylated tyrosines (P-Tyr) within the cells increase. The phosphorylated tyrosines can be detected in an ELISA-system using anti-phosphotyrosine antibodies. If cells are exposed to tyrosine kinase inhibitors, and then stimulated with EGF, the increase in P-Tyr should be diminished in inhibitor-treated cells compared to control-cells.

Initial experiments were necessary to optimise a number of parameters, and the optimised ELISA parameters are shown in Table 5.6. The effect of 7-hydroxycoumarin and esculetin on growth factor-stimulated tyrosine phosphorylation was then determined, following pre-exposure periods of 1, 6 and 12 hrs. Positive controls used in the experiments were genistein (a general tyrosine kinase inhibitor) and Tyrphostin A25 (a specific EGF-RTK inhibitor). In all experiments, increases in P-Tyr levels in drug-treated cells were compared to P-Tyr increases in control cells, with diminished increases illustrating tyrosine kinase inhibition. The results of these experiments are shown in Tables 5.7–5.9. Table 5.7 shows the results of the control compounds, genistein and tyrphostin. As shown, genistein caused a strong, dose-dependent inhibition of tyrosine phosphorylation in EGF-stimulated A431 cells, after 1hr pre-exposure, with ~ 70% inhibition at the highest concentration tested (100μg/ml). Increasing the drug pre-exposure time to 6hrs did cause a slight augmentation of this effect [~80% inhibition at 100μg/ml]. Pre-exposure of A431 cells to Tyrphostin A25 for 1hr caused increased tyrosine phosphorylation, as opposed to inhibition, at all concentrations tested. However, following 6hrs of drug pre-exposure a very potent inhibition of tyrosine kinase activity was evident, with ≥ 50% inhibition at concentrations in the range 10-100μg/ml. This delayed effect has previously been observed and is believed to result from the requirement for tyrphostin to accumulate in the cell before it potency is effected (Lyall *et al.*, 1989).

Table 5.8 shows the results obtained, following the stimulation of tyrosine phosphorylation in A431 cells pre-exposed to 7-hydroxycoumarin for various time periods. 1hr pre-exposure to 7-hydroxycoumarin lead to a slight decrease (~ 10% decrease at 100μg/ml) in the levels of cellular

tyrosine phosphorylation. 6hrs pre-exposure again saw little decrease in the levels of tyrosine phosphorylation except at the highest tested concentration (100μg/ml) where a decrease of ~21% was observed. However, following a 12hr exposure of cells to 7-hydroxycoumarin, a dose-dependent decrease in the cellular tyrosine phosphorylation was observed in the concentration range 10-100μg/ml. This decrease was not extremely potent – again the highest concentration tested caused a decrease in tyrosine phosphorylation of ~22.5%.

Table 5.9 presents the results obtained, following the stimulation of tyrosine phosphorylation in A431 cells pre-exposed to esculetin for various time periods. 1hr pre-exposure of A431 cells to esculetin followed by stimulation with EGF did not affect cellular tyrosine phosphorylation levels except at concentrations of 100μg/ml, where a decrease of ~12% was observed. However, increasing the drug pre-exposure time to 6hrs, caused an dose-dependent decrease in the P-Tyr levels observed in the cells, with a maximum inhibition of ~39%, at the highest concentration tested (100μg/ml). Incubation of cells with drug for longer time periods (*i.e.* 12hrs) maintained this level of inhibition.

These results indicate that the two coumarin compounds under investigation do inhibit growth factor-stimulated tyrosine phosphorylation by 30-40%, in a dose- and time-dependent manner. This inhibition was not as effective for 7-hydroxycoumarin as for esculetin, requiring longer time periods to exert this effect. Neither compound prevented phosphorylation with the efficiency of the positive controls, genistein (maximal effect of ~70% inhibition at 100μg/ml, following 1hr exposure) or tyrphostin (maximal effect of ~80% inhibition at 100μg/ml, following 6hr pre-exposure). This ELISA-based system yielded no information as to which kinases/substrates are affected by different compounds, therefore we examined this effect using two distinct methods: the Cytosensor Microphysiometer (Section 5.6.4) and Western Blotting (5.6.5).

Variable Examined	*Optimal Conditions*
Cell Seeding Density	5 X 10^4 cells/well
EGF Concentration	100ng/ml, 15mins.
ELISA Blocking Conditions	1½ hrs @ 37°C 10% FCS, 0.2% Tween-20 in Tris-Buffered Saline.
Primary Ab (Anti-Phosphotyrosine) Exposure	1/750 Dilution 1hr @ 37°C
Secondary Ab (AP-labelled Anti-Mouse IgG) Exposure	1/1500 Dilution 1hr @ 37°C

Table 5.6: **Optimised parameters for the ELISA-based assessment of tyrosine phosphorylation in EGF-stimulated A431 cells.**

	Alteration In Tyrosine Phosphorylation (% of Control)			
Concentration of Drug (μg/ml)	*1hr pre-exposure to Genistein*	*6hr pre-exposure to Genistein*	*1hr pre-exposure to Tyrphostin 25*	*6hr pre-exposure to Tyrphostin 25*
0	100.00 ± 1.98	100.00 ± 3.27	100.00 ± 5.12	100.00 ± 5.28
2	69.55 ± 1.88	73.62 ± 1.66	103.50 ± 4.33	102.10 ± 2.47
10	50.66 ± 1.09	47.82 ± 1.84	118.20 ± 4.89	51.57 ± 1.88
20	41.67 ± 0.89	45.70 ± 1.69	122.56 ± 3.33	37.60 ± 2.24
50	36.12 ± 1.57	35.13 ± 1.71	118.96 ± 2.87	27.57 ± 2.69
100	30.57 ± 2.43	20.74 ± 2.63	109.19 ± 2.86	21.58 ± 3.85

Table 5.7: **ELISA Results obtained for the inhibition of tyrosine phosphorylation in EGF-stimulated A431 cells, by genistein and tyrphostin. All experiments were replicated six times, and carried out on three separate occasions (Section 2.2.6.3), and absorbances for drug-treated cells was normalised vs absorbances of untreated control cells. Control absorbances were as follows: 1.279 ± 0.060 (Gen 1hr); 1.467 ± 0.053 (Gen 6hrs); 1.055 ± 0.062 (Tyr 1hr) and 1.171 ± 0.027 (Tyr 6hrs).**

	Alteration In Tyrosine Phosphorylation (% of Control)		
Concentration of 7-Hydroxycoumarin (μg/ml)	***1hr pre-exposure***	***6hr pre-exposure***	***12hr pre-exposure***
0	100.00 ± 2.82	100.00 ± 6.27	100.00 ± 5.79
2	107.38 ± 2.62	95.65 ± 1.45	N.D.
10	90.96 ± 5.34	101.34 ± 3.82	86.44 ± 7.66
20	93.00 ± 2.53	96.21 ± 4.27	85.53 ± 4.80
50	96.31 ± 5.15	92.49 ± 5.51	79.86 ± 4.33
100	89.60 ± 3.50	78.74 ± 8.41	77.53 ± 6.68

Table 5.8: **ELISA Results obtained for the time-dependent inhibition of tyrosine phosphorylation in EGF-stimulated A431 cells, by 7-hydroxycoumarin. All experiments were replicated six times, and carried out on three separate occasions (Section 2.2.6.3), and absorbances for drug-treated cells was normalised vs absorbances of untreated control cells. Control absorbances were as follows: 1.030 ± 0.031 (1hr); 1.344 ± 0.039 (6hrs); and 0.988 ± 0.057 (12hrs).**

	Alteration In Tyrosine Phosphorylation (% of Control)		
Concentration of Esculetin (μg/ml)	***1hr pre-exposure***	***6hr pre-exposure***	***12hr pre-exposure***
0	100.00 ± 3.51	100.00 ± 4.42	100.00 ± 4.00
2	100.10 ± 2.74	102.10 ± 2.47	N.D.
10	99.30 ± 3.60	92.87 ± 3.61	79.85 ± 0.78
20	98.97 ± 5.14	83.67 ± 3.49	75.04 ± 2.01
50	94.34 ± 4.80	71.39 ± 3.18	68.96 ± 1.26
100	88.17 ± 8.74	61.41 ± 2.60	63.25 ± 5.34

Table 5.8: **ELISA Results obtained for the time-dependent inhibition of tyrosine phosphorylation in EGF-stimulated A431 cells, by esculetin. All experiments were replicated six times, and carried out on three separate occasions (Section 2.2.6.3), and absorbances for drug-treated cells was normalised vs absorbances of untreated control cells. Control absorbances were as follows: 1.167 ± 0.041 (1hr); 1.178 ± 0.060 (6hrs); and 0.996 ± 0.040 (12hrs).**

5.6.4. Cytosensor Microphysiometer Studies into Tyrosine Kinase Inhibition

The Cytosensor Microphysiometer has been used in the past to dissect various cellular signalling pathways *e.g.* Nerve Growth Factor (NGF) pathway in PC-12 cells (Pitchford *et al.*, 1995), GM-CSF pathway in TF-1 cells (Wada *et al.*, 1993), *etc.* Use of chemical enzyme inhibitors, antibodies and oligonucleotide probes can all aid in the determination of the components of a specific pathway, and inhibitors of tyrosine kinase activity have been previously used (Wada *et al.*, 1993; Pitchford *et al.*, 1995). Therefore, we chose to use microphysiometry to examine the effect of 7-hydroxycoumarin and esculetin on EGF-stimulated pathways in A431 cells.

Although we had previously determined (Section 5.6.2) that 7-hydroxycoumarin appeared to slightly inhibit the RTK activity of the EGF-Receptor *in vitro*, we were unsure that the test system used to obtain these results was optimal. In addition to the question of sub-optimal peptide/enzyme recognition, there was the question of the RTK stability – the existence of degraded receptor had been shown by Western blotting (Figure 5.12). The Cytosensor Microphysiometer offered an attractive alternative for examining the inhibition of the EGF-RTK in A431 cells for two reasons:

1. The study was carried out in intact cells, thereby eliminating worries of receptor stability and suboptimal substrates (endogenous substrates are present at natural concentrations).
2. Downstream signalling events could be observed concurrently, thereby yielding more information per experiment.

The EGF-stimulation of EGF-RTK in A431 cells had been previously optimised on the cytosensor system as outlined in Section 5.6.1. Maximal stimulation of the cells was with 100ng/ml EGF (Figure 5.10) and this concentration was used in all subsequent experiments. As shown in Figure 5.14 exposure of A431 cells to 100ng/ml EGF for 12 mins caused a rapid increase in the acidification rate of the cells, to a peak of ~80% above baseline values. This peak in acidification rate was transient, but did not return immediately to baseline levels. Instead, the cells maintained a sustained higher acidification rate of ~30% above baseline for hours, following the growth factor stimulation, representing the downstream signalling and eventual nuclear transcription and translation, initiated by EGF addition. The cellular metabolism eventually returned to baseline levels ~ 8hrs post-EGF treatment (results not shown). The initial rise in acidification rate is principally due to the activation of the EGF-Receptor-associated tyrosine kinase activity, as it can be blocked by pre-exposure of cells to the tyrosine kinase inhibitors genistein or tyrphostin, for 1hr or 6hrs, respectively (Figure 5.14).

Figure 5.14 portrays the activation of EGF-RTK in cells pre-exposed to 7-hydroxycoumarin for 1 or 6hrs. The activation of EGF-RTK was unaffected by pre-exposure to 20μg/ml 7-hydroxycoumarin for either 1 or 6hrs. However, a modest inhibition of the RTK activation (~60% increase in metabolic rate vs ~ 80% in control cells) was observed in cells pre-exposed to 100μg/ml 7-hydroxycoumarin. Experiments with cells pre-exposed to 7-hydroxycoumarin for 12hrs were not carried out, as the basal metabolic rates of these cells were at levels below the rates recommended for use on the instrument (*i.e.* <-60μvolts/sec).

As shown in Figure 5.15, pre-exposure of A431 cells to 20μg/ml esculetin did not block the activation of the EGF-RTK. However, as shown, the shape of the metabolic rate curve of the esculetin-treated cells differed from that of the control cells - it did not maintain the sustained increase of ~ 30% of the control cells, instead the metabolic rate returned to baseline levels within 2hrs. This may indicate a block in the signalling pathway downstream of the EGF-RTK. 6hrs pre-exposure of the cells to 20μg/ml esculetin prior to EGF-stimulation, caused a slight decrease (~12%) in the peak of activation of the EGF-RTK (Figure 5.15). Again, the esculetin-treated cells return to their baseline acidification rates within 1hr, suggesting a downstream block of EGF-stimulated signalling pathway.

The observed results are in good accordance with the ELISA-TK results of Section 5.6.3, if one considers that the ELISA examined the tyrosine phosphorylation events in the first 15mins following EGF-stimulation. Decreases in TK activity observed for 7-hydroxycoumarin after 6hrs pre-exposure of 100μg/ml and esculetin following 6hrs exposure to 20μg/ml, reflect well the decreases in P-Tyr levels observed in Section 5.6.3. Unfortunately, 12hr experiments could not be carried out. The EGF-RTK appears modestly inhibited by both compounds, and esculetin also appears to exhibit a blockade on downstream events. To further clarify these observations, Western Blotting was carried out as reported in Section 5.6.5.

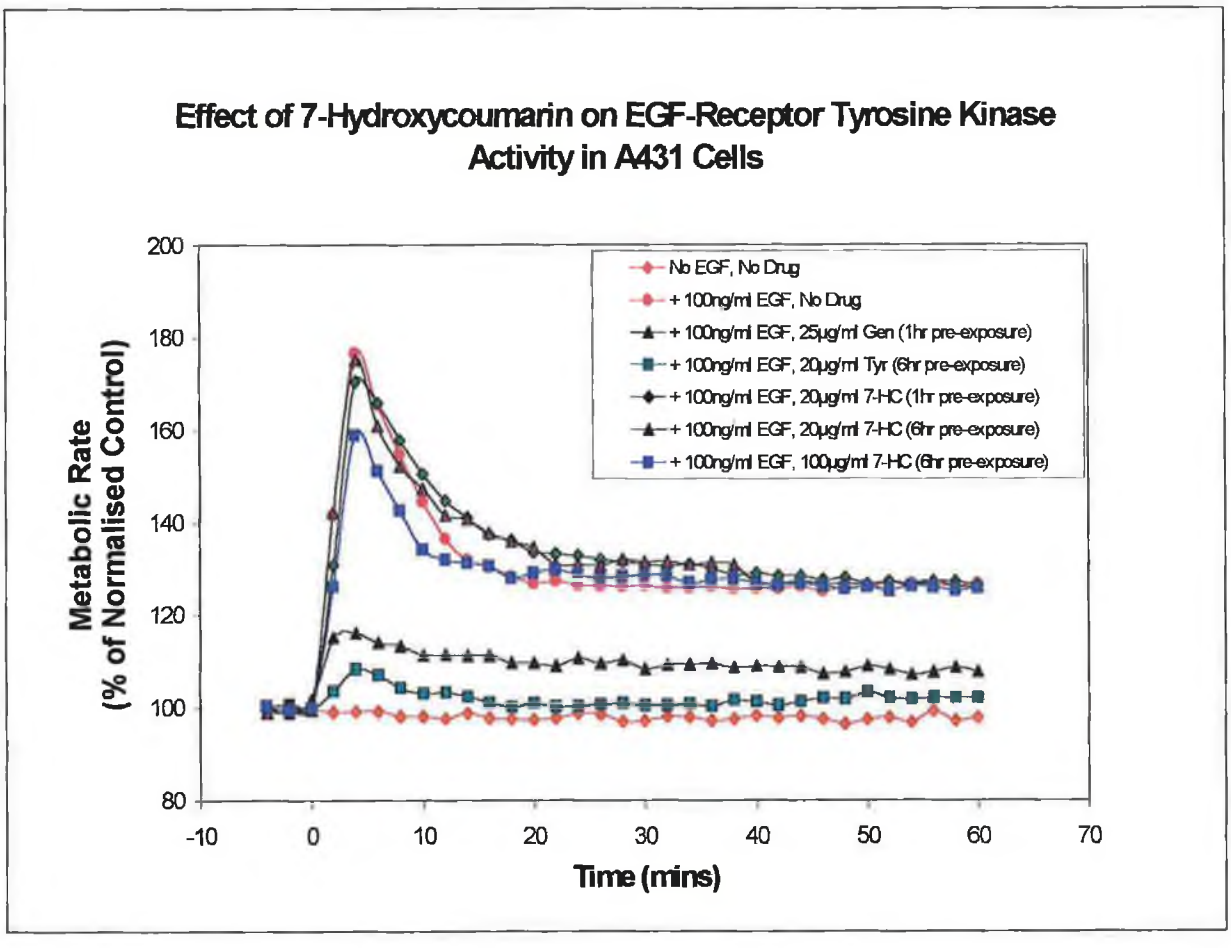


Figure 5.14: **Exposure of A431 cells to various concentrations of 7-hydroxycoumarin, prior to stimulation with 100ng/ml EGF. The above plot shows the experiment conducted on the Cytosensor Microphysiometer to determine the effect of 7-hydroxycoumarin on the activation of the EGF-RTK in A431 cells. Genistein and Tyrphostin were included as positive tyrosine kinase inhibitors controls. In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to EGF exposure, using a 2 min cycle, with the acidification rate measured in the final 30 secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during and following EGF exposure were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for the cells in all experiments were –100 to -200µvolts/sec.**

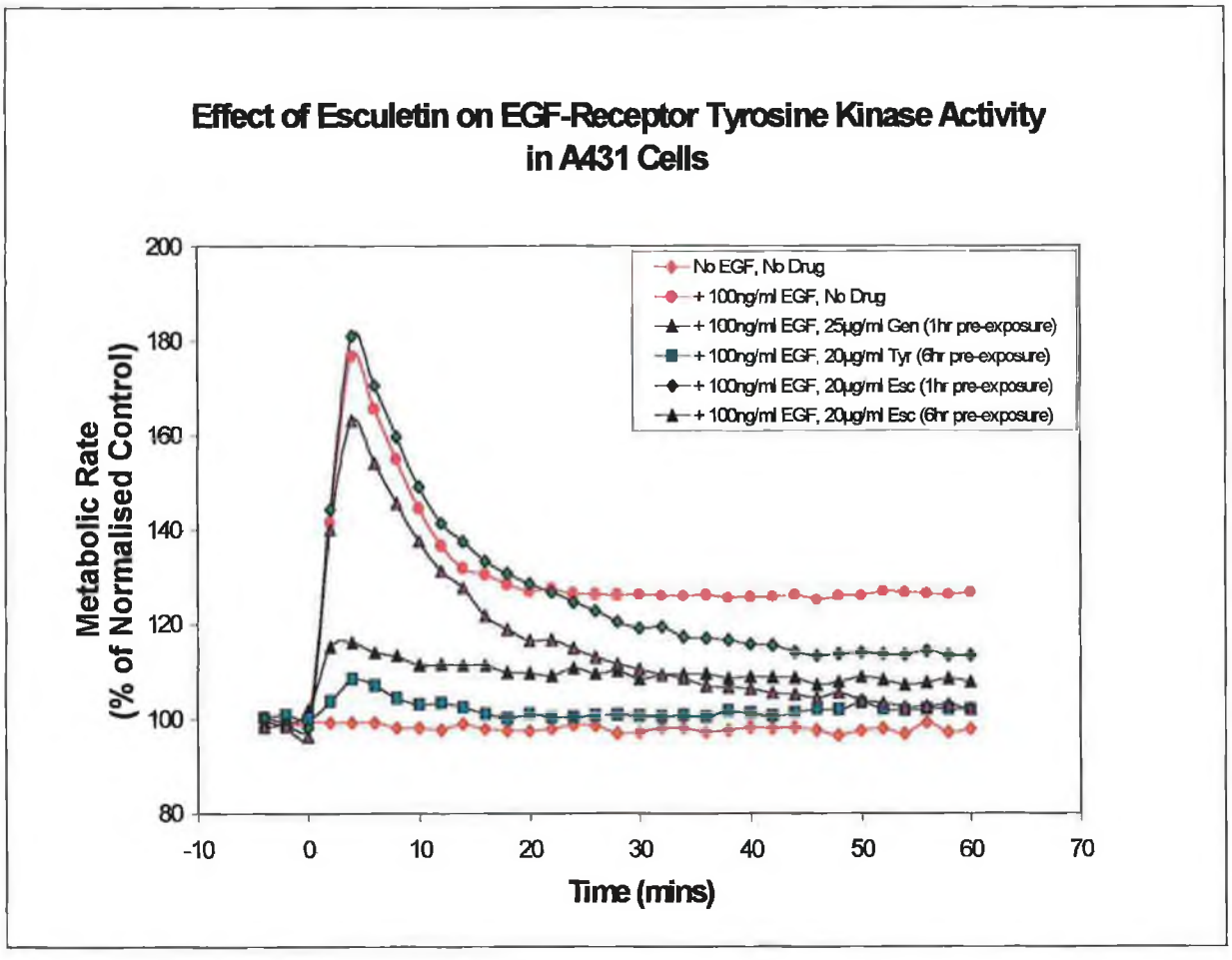


Figure 5.14: **Exposure of A431 cells to various esculetin for different time periods, prior to stimulation with 100ng/ml EGF. The above plot shows the experiment conducted on the Cytosensor Microphysiometer to determine the effect of esculetin exposure on the activation of the EGF-RTK in A431 cells. Genistein and Tyrphostin were included as positive tyrosine kinase inhibitors controls. In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to EGF exposure, using a 2 min cycle, with the acidification rate measured in the final 30 secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during and following EGF exposure were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for the cells in all experiments were –100 to -200µvolts/sec.**

5.6.5. Western Blotting Studies of Tyrosine Phosphorylation

Zippel *et al.* (1986), first described a non-radioactive immunoblotting technique using anti-phosphotyrosine antibodies to detect proteins phosphorylated on tyrosine residues in PDGF-stimulated cells. This technique is often used to dissect cellular growth signalling pathways (Druker *et al.*, 1989; Meisenhelder *et al.*, 1989) and, to a lesser extent, to examine the effect of known or putative tyrosine kinase inhibitors on tyrosine phosphorylation events in cells.

Western blotting experiments were carried out as outlined in Section 2.2.6.5, and the results are shown in Figures 5.16 – 5.20. In normal cells, approximately 0.05% of proteins are phosphorylated on tyrosine residues (Hunter, 1998) and Figure 5.16 illustrates the optimised set-up for immunoblotting these P-Tyr residues. In quiescent cells, few proteins are phosphorylated on tyrosine residues (Druker *et al.*, 1989), as shown in Figure 5.16 (Lanes 2-6). However, as shown, EGF-treatment of A431 cells, caused a number of cellular proteins, including the EGF-receptor, to become phosphorylated on tyrosine residues (arrows). The predominant band (lanes 7-11) across the top of the gel was that of the EGF-receptor (~170kDa), which becomes autophosphorylated on tyrosine residues on binding its ligand. Fainter bands of P-Tyr proteins were observed at ~110-120kDa, ~85-90kDa and ~60-70kDa, which may correspond to the GAP-120, p85 PI-3K, and c-Src/SH-PTP proteins, respectively. A faint band at ~35kDa was also apparent – a similar molecular weight band has been observed in PDGF-treated fibroblasts (Zippel *et al.*, 1986), although its identity is as yet unknown. All drug-treated samples were pre-exposed to drugs for 1hr prior to EGF-stimulation. As shown only genistein (25μg/ml) affected tyrosine phosphorylation activity, with P-Tyr levels of both the EGF-Receptor and lower molecular weight proteins affected.

Figure 5.17 illustrates the effect of 6hr pre-exposure of 7-hydroxycoumarin on EGF-stimulated tyrosine phosphorylation. A slight decrease in the density of the EGF-R band was apparent for 100μg/ml sample, but none of the other exposures affected the cellular phosphorylation. This result reflects well the ELISA and Cytosensor results of Sections 5.6.3 and 5.6.4, respectively. Figure 5.18 shows the effect of 12hr 7-hydroxycoumarin treatment on the tyrosine phosphorylation of cellular proteins. This treatment caused a dose-dependent reduction in the levels of protein tyrosine phosphorylation in EGF-stimulated A431 cells. A decrease in the density of the EGF-Receptor band indicated that receptor autophosphorylation was diminished. Corresponding decreases in the phosphorylation of lower molecular weight proteins were observed, and were probably due to decreased EGF-RTK activity. Therefore, it would appear from these

results that treatment of cells for 12hrs with 7-hydroxycoumarin caused inhibition of tyrosine kinase activity in A431 cells.

The effect of esculetin on tyrosine phosphorylation in EGF-stimulated A431 cells was also examined and the results are outlined in Figures 5.19 and 5.20. 6hr esculetin exposure (Figure 5.19) did not decrease tyrosine phosphorylation levels significantly. A slight decrease in the phosphorylation of both the EGF-R and lower molecular weight proteins was apparent, at 100μg/ml. However, this effect was significantly enhanced for cells exposed to esculetin for 12hrs (Figure 5.20). In this figure we see that 12hr exposure to esculetin caused a dose-dependent decrease in tyrosine phosphorylation for all concentrations tested. Almost complete abolition of tyrosine kinase activity was evident at concentrations of 50-100μg/ml esculetin.

Hence, these immunoblotting results indicate that both 7-hydroxycoumarin and esculetin are capable of inhibiting tyrosine phosphorylation in EGF-stimulated A431 cells. This inhibition is both dose- and time-dependent, and esculetin is more potent than 7-hydroxycoumarin in the manifestation of these effects. Inhibition of the EGF-Receptor associated tyrosine kinase activity is evident, with decreased autophosphorylation and phosphorylation of downstream targets.

The results from sections 5.6.4, 5.6.5 and 5.6.6, clearly indicate that both 7-hydroxycoumarin and esculetin cause a dose- and time-dependent decrease in tyrosine phosphorylation in growth factor-stimulated cells. Although direct inhibition of tyrosine kinases is the most evident explanation, other events may be important in the manifested result. For example, decreased growth factor binding to receptor would account for decreased RTK activation, with consequent reduction of tyrosine phosphorylation. Seliger (1997) has previously discounted this as an effect of the coumarin compounds. Another possibility is the activation of a negative regulatory mechanism to decrease tyrosine phosphorylation, examples being activation of cellular phosphatases, which would dephosphorylate tyrosine residues (Hunter, 1998), or the negative regulation of the EGF-Receptor by Protein Kinase C (Cochet *et al.*, 1984; Downward *et al.*, 1985). Given the previous reports of the involvement of coumarins in PKC signalling, activation of PKC was examined in more detail (Section 5.6.7).

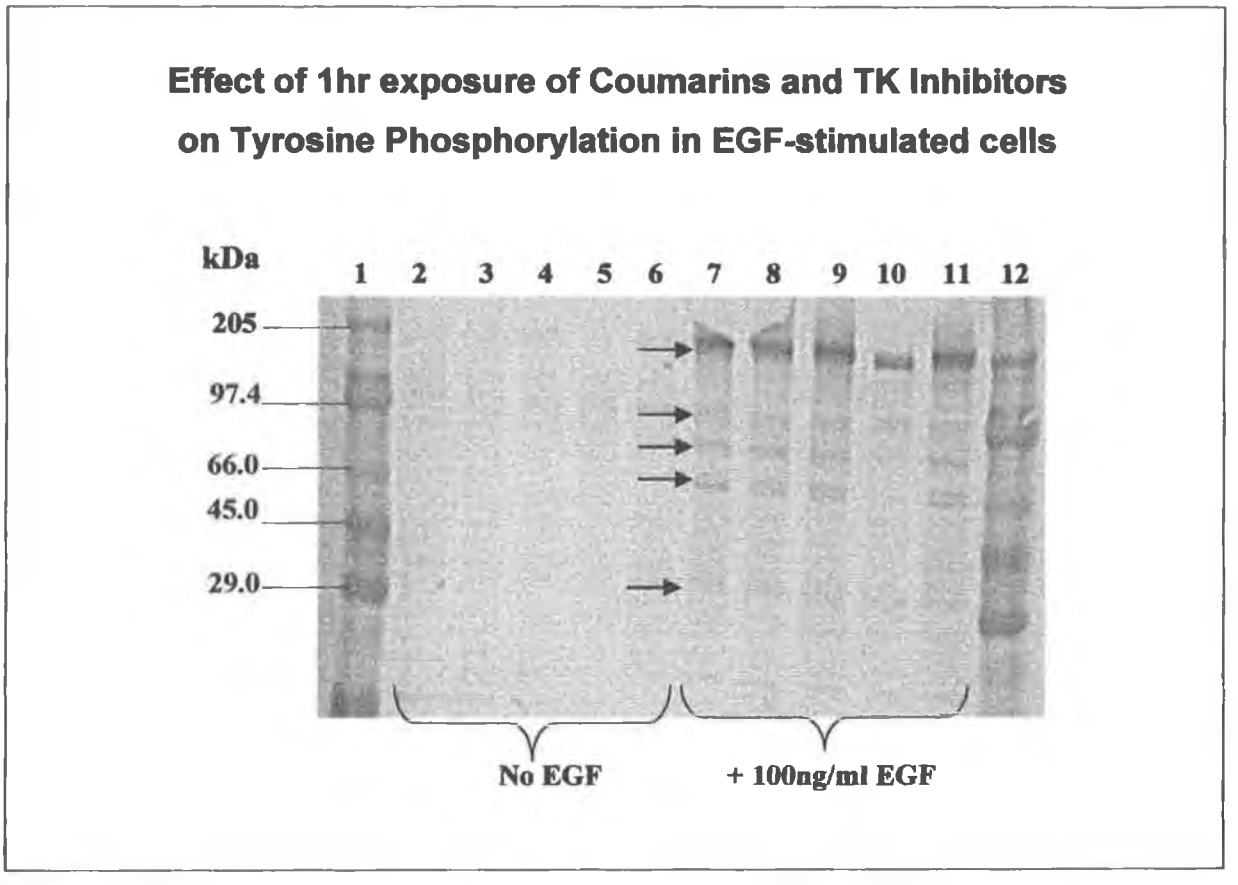


Figure 5.16: Effect of drug pre-exposure (1hr) on tyrosine phosphorylation in A431 cells. The above photograph is one of three experiments carried out to determine, by Western blotting, the levels of tyrosine phosphorylated proteins in EGF-stimulated A431 cells following exposure to various known and putative tyrosine kinase inhibitors for 1hr (Section 2.2.6.5). Cell extracts (equal protein concentrations) were run on 8% SDS gels, immunoblotted and probed with anti-phosphotyrosine antibody. As shown, samples on the right hand side of the gel were stimulated with 100ng/ml EGF for 15 mins prior to extraction, while those on the left hand side were left unstimulated The following samples were run: Lanes 1 & 12: Molecular weight markers; Lanes 2 & 7: 20µg/ml 7-hydroxycoumarin; Lanes 3 & 8: 20µg/ml esculetin; Lanes 4 & 9: 20µg/ml tyrphostin; Lanes 5 & 10: 25µg/ml genistein; Lanes 6 & 11: No drug treatment.

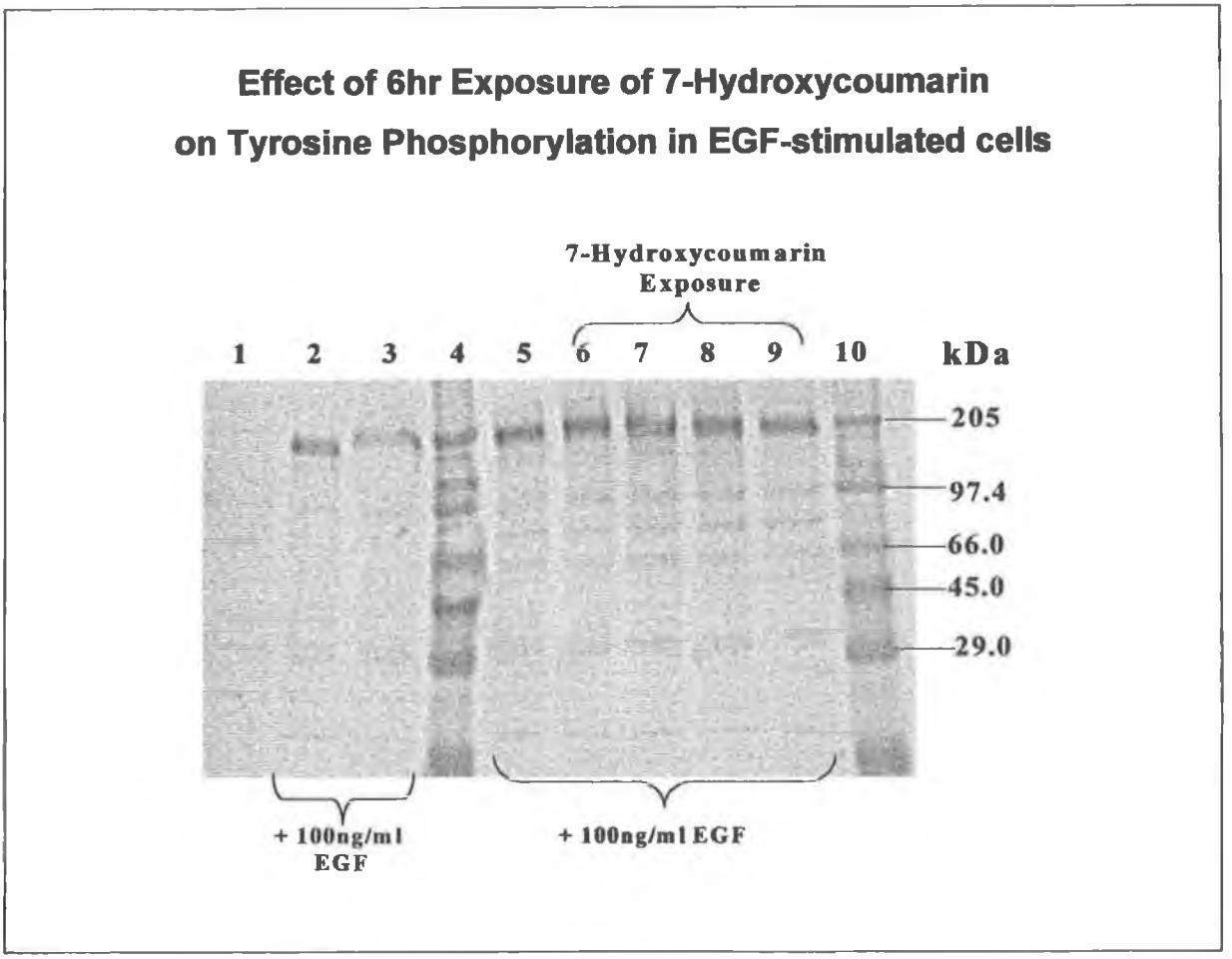


Figure 5.17: Effect of 7-hydroxycoumarin pre-exposure (6hrs) on tyrosine phosphorylation in A431 cells. The above photograph is one of three experiments carried out to determine, by Western blotting, the levels of tyrosine phosphorylated proteins in EGF-stimulated A431 cells following exposure to various concentrations of 7-hydroxycoumarin (Section 2.2.6.5). Following EGF-stimulation (100ng/ml), cell extracts (equal protein concentrations) were run on SDS gels, immunoblotted and probed with anti-phosphotyrosine antibody. The following samples were run: Lane 1: unstimulated control; Lane 2: 25µg/ml genistein; Lane 3: 20µg/ml tyrphostin; Lanes 4 &10, Molecular weight markers; Lane 5: No drug (positive phosphorylation); Lane 6: 10µg/ml 7-hydroxycoumarin; Lane 7: 20µg/ml 7-hydroxycoumarin; Lane 8: 50µg/ml 7-hydroxycoumarin; Lane 9: 100µg/ml 7-hydroxycoumarin. As illustrated samples in all lanes except Lanes 1, 4 and 10 had been stimulated with 100ng/ml EGF for 15 mins, prior to preparation.

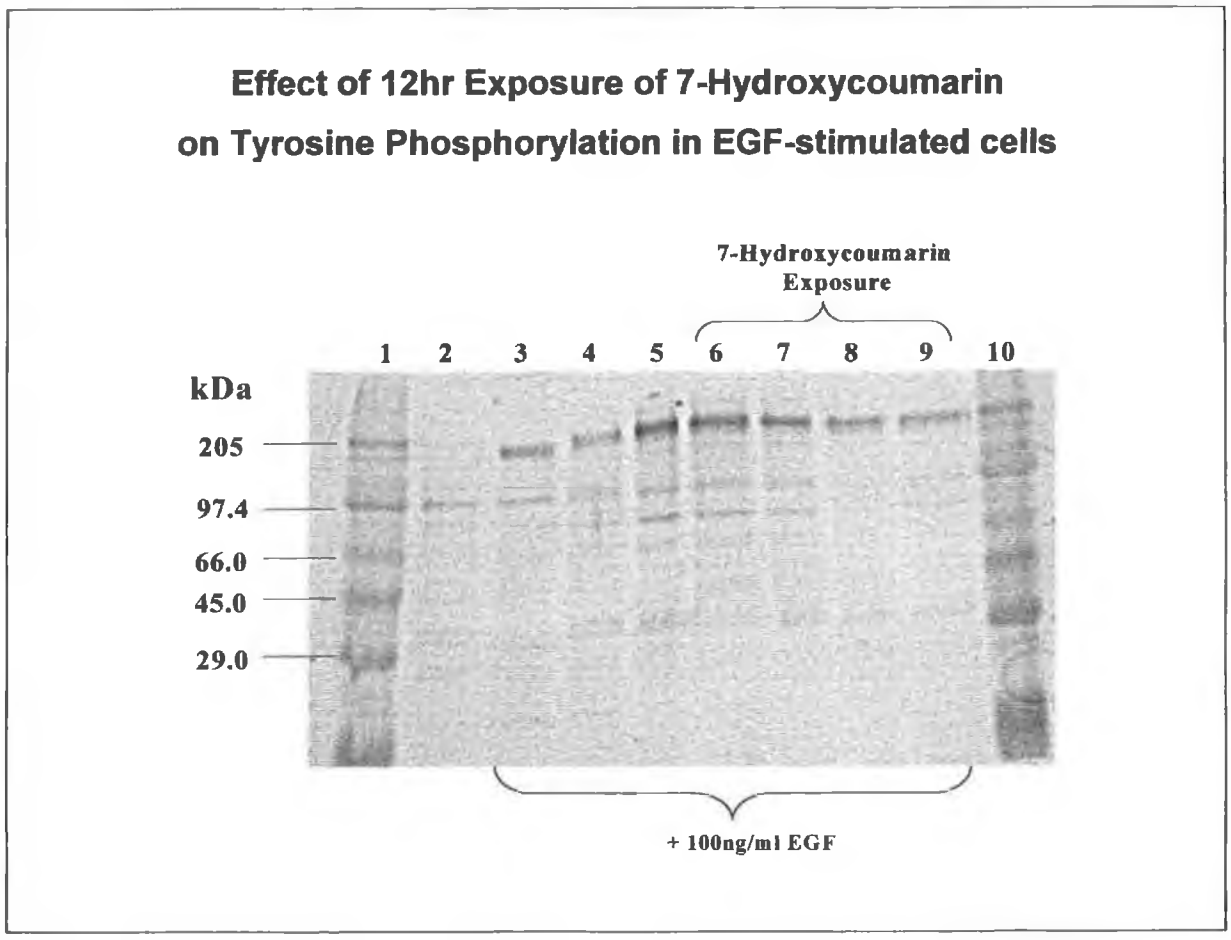


Figure 5.18: Effect of 7-hydroxycoumarin pre-exposure (12hrs) on tyrosine phosphorylation in A431 cells. The above photograph is one of three experiments carried out to determine, by Western blotting, the levels of tyrosine phosphorylated proteins in EGF-stimulated A431 cells following exposure to various concentrations of 7-hydroxycoumarin (Section 2.2.6.5). Following EGF-stimulation (100ng/ml), cell extracts (equal protein concentrations) were run on SDS gels, immunoblotted and probed with anti-phosphotyrosine antibody. The following samples were run: Lanes 1 & 10: Molecular weight markers; Lane 2: unstimulated control; Lane 3: 25µg/ml genistein; Lane 4: 20µg/ml tyrphostin; Lane 5: No drug (positive phosphorylation); Lane 6: 10µg/ml 7-hydroxycoumarin; Lane 7: 20µg/ml 7-hydroxycoumarin; Lane 8: 50µg/ml 7-hydroxycoumarin; Lane 9: 100µg/ml 7-hydroxycoumarin. As illustrated, samples in all lanes except Lanes 1, 2 and 10, had been stimulated with 100ng/ml EGF for 15 mins, prior to preparation.

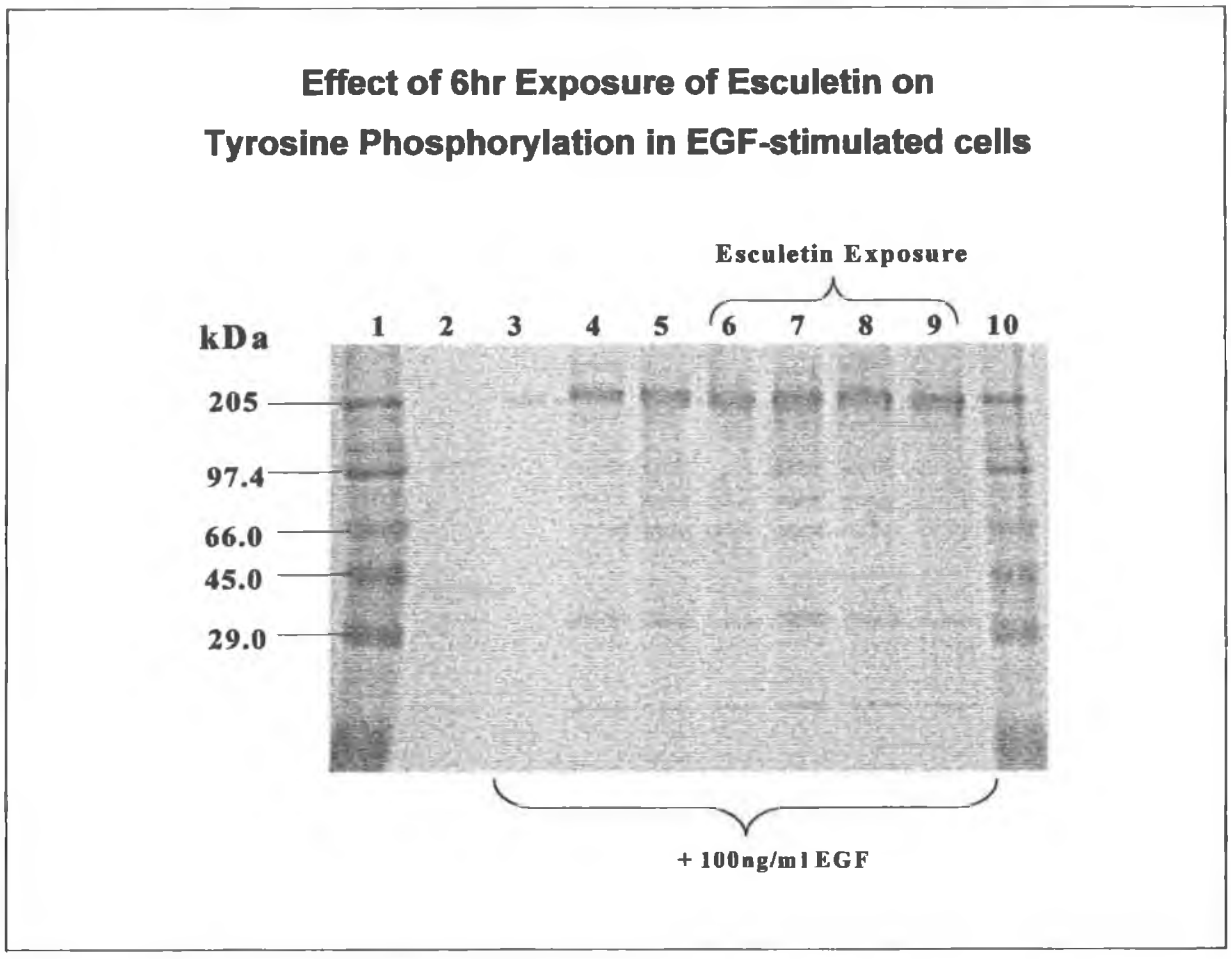


Figure 5.19: Effect of esculetin pre-exposure (6hrs) on tyrosine phosphorylation in A431 cells. The above photograph is one of three experiments carried out to determine, by Western blotting, the levels of tyrosine phosphorylated proteins in EGF-stimulated A431 cells following exposure to various concentrations of esculetin (Section 2.2.6.5). Following EGF-stimulation (100ng/ml), cell extracts (equal protein concentrations) were run on SDS gels, immunoblotted and probed with anti-phosphotyrosine antibody. The following samples were run: Lanes 1 & 10: Molecular weight markers; Lane 2: unstimulated control; Lane 3: 50µg/ml genistein; Lane 4: 20µg/ml tyrphostin; Lane 5: No drug (positive phosphorylation); Lane 6: 10µg/ml esculetin; Lane 7: 20µg/ml 7-esculetin; Lane 8: 50µg/ml esculetin; Lane 9: 100µg/ml esculetin. As illustrated, samples in all lanes except Lanes 1, 2 and 10, had been stimulated with 100ng/ml EGF for 15 mins, prior to preparation.

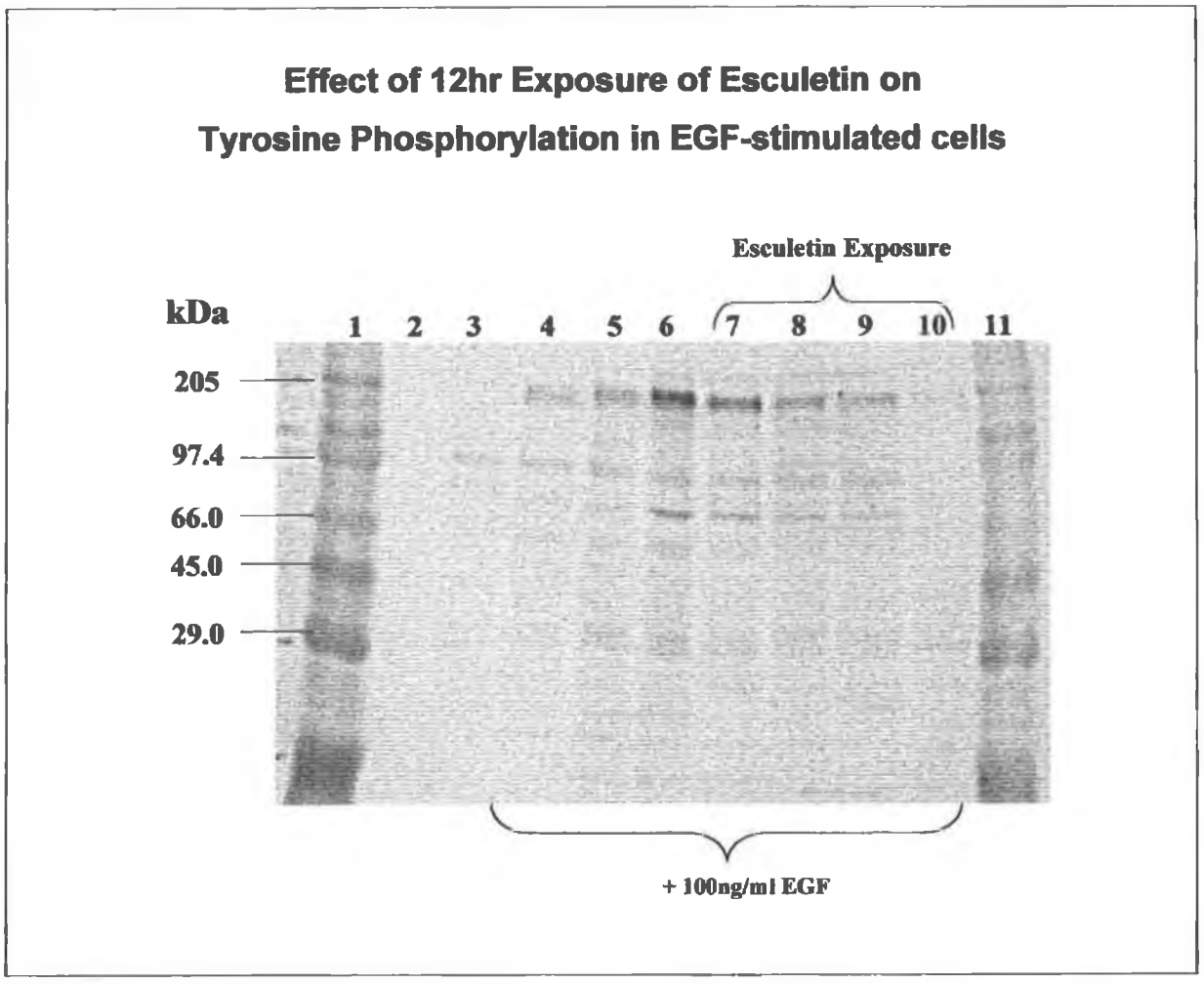


Figure 5.19: Effect of esculetin pre-exposure (12hrs) on tyrosine phosphorylation in A431 cells. The above photograph is one of three experiments carried out to determine, by Western blotting, the levels of tyrosine phosphorylated proteins in EGF-stimulated A431 cells following exposure to various concentrations of esculetin (Section 2.2.6.5). Following EGF-stimulation (100ng/ml), cell extracts (equal protein concentrations) were run on SDS gels, immunoblotted and probed with anti-phosphotyrosine antibody. The following samples were run: Lanes 1 & 11: Molecular weight markers; Lane 2: unloaded lane; Lane 3: unstimulated control; Lane 4: 25µg/ml genistein; Lane 5: 20µg/ml tyrphostin; Lane 6: No drug (positive phosphorylation); Lane 7: 10µg/ml esculetin; Lane 8: 20µg/ml 7-esculetin; Lane 9: 50µg/ml esculetin; Lane 10: 100µg/ml esculetin. As illustrated, samples in all lanes except Lanes 1, 2, 3 and 11, had been stimulated with 100ng/ml EGF for 15 mins, prior to preparation.

5.6.6. Effect of Coumarins on Protein Kinase C Activity.

Protein Kinase C (PKC) is an important intermediate in cellular signalling, which infringes on many of the important growth-regulating pathways in cells. It maintains a paradoxical role, both activating and negatively regulating, many critical signalling substrates (Section 5.2.4). PKC has been shown to reduce the EGF-R-associated tyrosine kinase activity, via phosphorylation, an event which causes both immediate decreased tyrosine phosphorylation on ligand binding, and long-term downregulation of receptor (Cochet *et al.*, 1984; Downward *et al.*, 1985). 7-hydroxycoumarin has previously been shown to augment the function of PKC in other cellular events [LPS-induced cytokine production (Zlabinger *et al.*, 1993)]. Therefore, we were interested in ascertaining whether 7-hydroxycoumarin and esculetin affected PKC signalling in A431 cells.

This was achieved with studies of A431 responses to the PKC activator PMA, in the absence or presence of coumarin compounds, using the Cytosensor Microphysiometer. The exposure of A431 cells to PMA on this instrument was optimised, and the optimised experimental parameters are shown in Table 5.10. Figure 5.21 illustrates the optimised stimulation of PKC activity in A431 cells, with exposure to 0.05ng/ml PMA for 20mins causing an ~ 12-15% increase in the metabolic rate of the cells, a response which was sustained over the monitoring period of 60mins. The activation of PKC could be very efficiently blocked by pre-exposure of A431 cells to 25μg/ml H-7 (a known PKC inhibitor) for 1hr prior to PMA activation (Figure 5.21)

Experiments were then performed in which A431 cells were pre-exposed to either 7-hydroxycoumarin or esculetin, for either 1 or 6hrs, prior to PKC activation with 0.05ng/ml PMA, and these results are shown in Figures 5.21-5.24. As shown in Figure 5.21, A431 cells pre-exposed to 7-hydroxycoumarin (20-100μg/ml) for 1hr prior to PMA stimulation, clearly exhibited a much increased response to PMA stimulation. At all concentrations tested, the metabolic rate increase in 7-hydroxycoumarin-treated cells was ~ 35-39% 1hr post-PMA, compared to ~15% for untreated cells. Increasing the drug pre-exposure time to 6hrs, maintained this augmentation of PKC activity over control cells (Figure 5.22), although the increase was not as pronounced as the drug-concentrations increased (compare ~40% increase in metabolic rate for 20μg/ml 7-hydroxycoumarin, and ~ 26% increase in metabolic rate for 100μg/ml).

Pre-exposure to esculetin for 1hr prior to PMA stimulation, yielded similar results to those obtained with 7-hydroxycoumarin, with increases due to PKC activation much higher in drug-treated cells, compared to untreated cells (Figure 5.23). As shown, the augmented activation of PKC

in esculetin-treated cells was dose-dependent – metabolic rates on cell stimulation with PMA increased to levels of ~115% (0μg/ml), ~122% (20μg/ml) and ~134-136% (50 and 100μg/ml), 1hr post-stimulation (Figure 5.23). Increasing the pre-exposure time of esculetin to 6hrs maintained this dose-dependent augmentation of metabolism in response to PKC activation (Figure 5.24). As shown in this figure, cells exposed to concentrations of 50 and 100μg/ml for 6hrs prior to stimulation with PMA, immediately became activated at levels twice that of control cells, and maintained this level of activation for the 60mins monitoring period.

Therefore, in these experiments it was demonstrated that pre-exposure of A431 cells to a range of concentrations of either 7-hydroxycoumarin and esculetin, caused augmentation in the activation of Protein Kinase C by PMA. This effect occurred at all concentrations and exposure times tested, although to varying degrees (metabolic increases in range ~1.5-3 times control increases). These two coumarin compounds, therefore, do display synergism with this enzyme, as has been previously proposed by Zlabinger and colleagues (Stuhlmeier *et al.*, 1991; Zlabinger *et al.*, 1993). Given the extensive networking role of PKC in growth signalling pathways, it is probable that this effect impacts on signalling pathways in cells. PKC has been previously shown to negatively downregulate the activity of the EGF-RTK through phosphorylation (Cochet *et al.*, 1984). It is possible that this role may be augmented by the presence of coumarins, an event which may explain the decreased tyrosine phosphorylation of both the EGF-RTK and its downstream substrates in EGF-stimulated A431 cells, observed in sections 5.6.3-5.6.5.

Variable Examined	*Optimised Conditions*
Cell Seeding Density	2.5 X 10^5 cells/capsule
Pump Cycle	2 mins cycle @ 60% speed (~ 120μl/min)
Serum-starvation Period	16hrs
PMA Concentration	0.05ng/ml
PMA Exposure Period	20mins

Table 5.10: Optimisation of experimental conditions for the exposure of A431 cells to PMA on the Cytosensor Microphysiometer, as outlined in Section2.2.6.6.

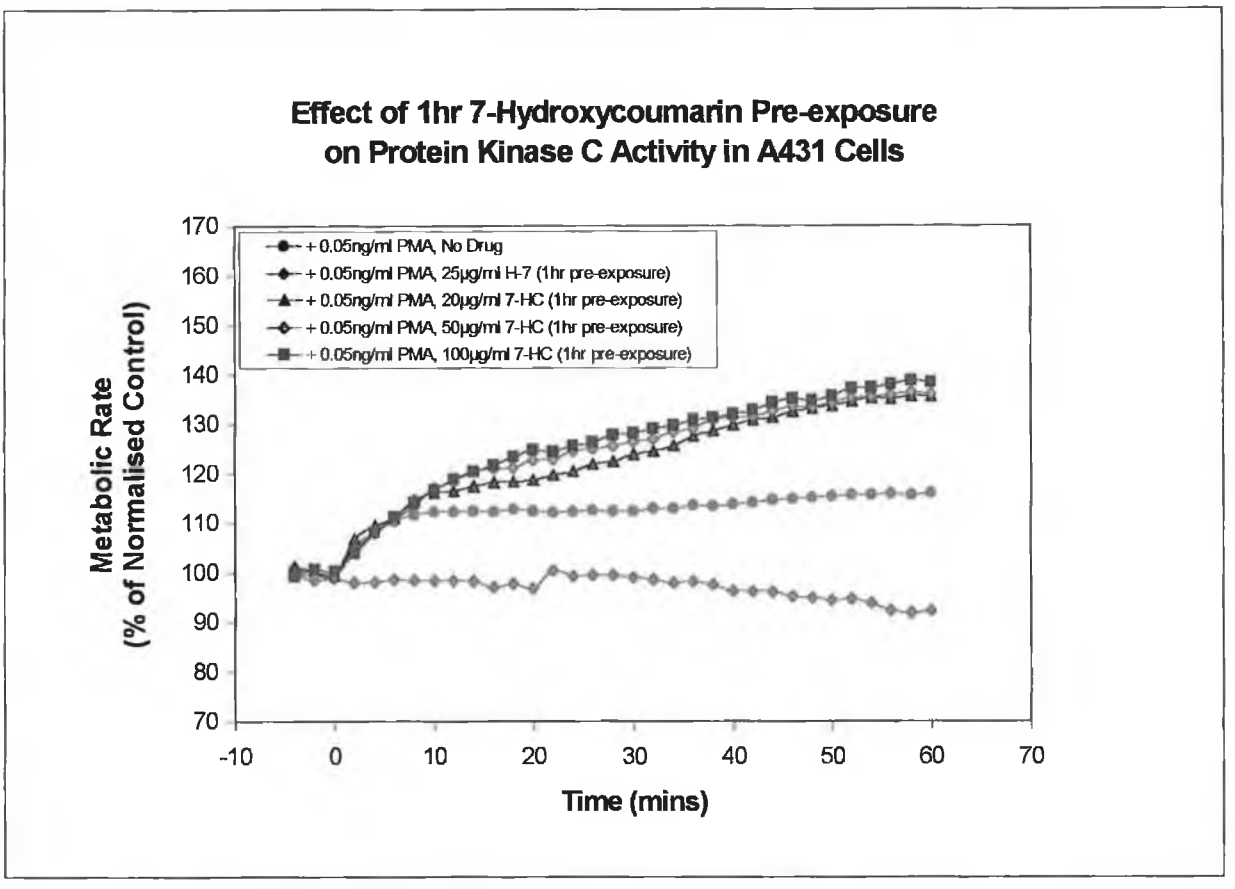


Figure 5.21: **Effect of 7-hydroxycoumarin on Protein Kinase C Activity in A431 cells. The above plot shows the experiment conducted on the Cytosensor Microphysiometer to determine the effect of 1hr 7-hydroxycoumarin exposure on the activation of the Protein Kinase C, with 0.05ng/ml PMA, in A431 cells. H-7 was used as a positive PKC inhibitor. In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to EGF exposure, using a 2 min cycle, with the acidification rate measured in the final 30 secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during and following PMA exposure were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for the cells in all experiments were –100 to –200 µvolts/sec.**

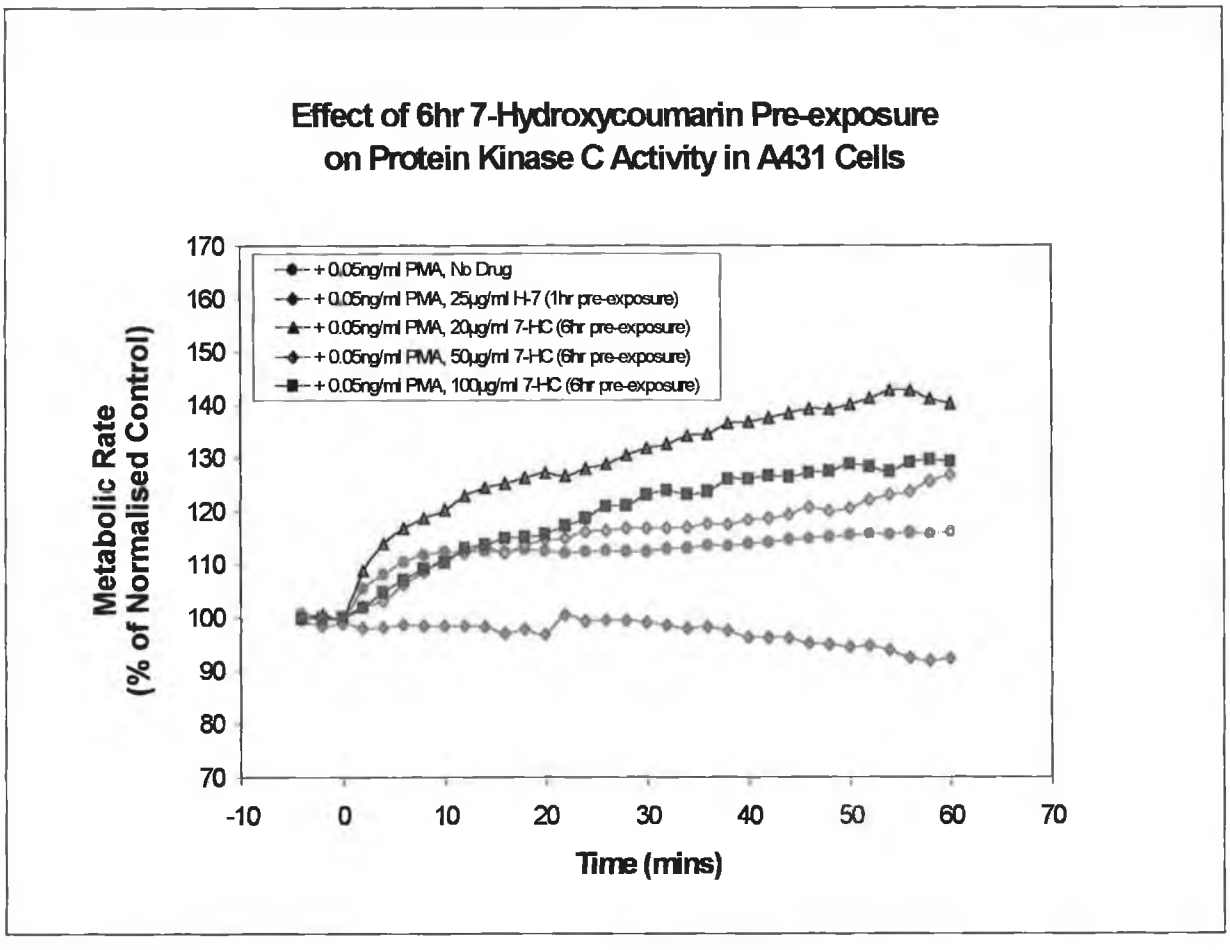


Figure 5.22: **Effect of 7-hydroxycoumarin on Protein Kinase C Activity in A431 cells. The above plot shows the experiment conducted on the Cytosensor Microphysiometer to determine the effect of 6hr 7-hydroxycoumarin exposure on the activation of the Protein Kinase C, with 0.05ng/ml PMA, in A431 cells. H-7 was used as a positive PKC inhibitor. In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to EGF exposure, using a 2 min cycle, with the acidification rate measured in the final 30 secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during and following PMA exposure were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for the cells in all experiments were –100 to –200 µvolts/sec.**

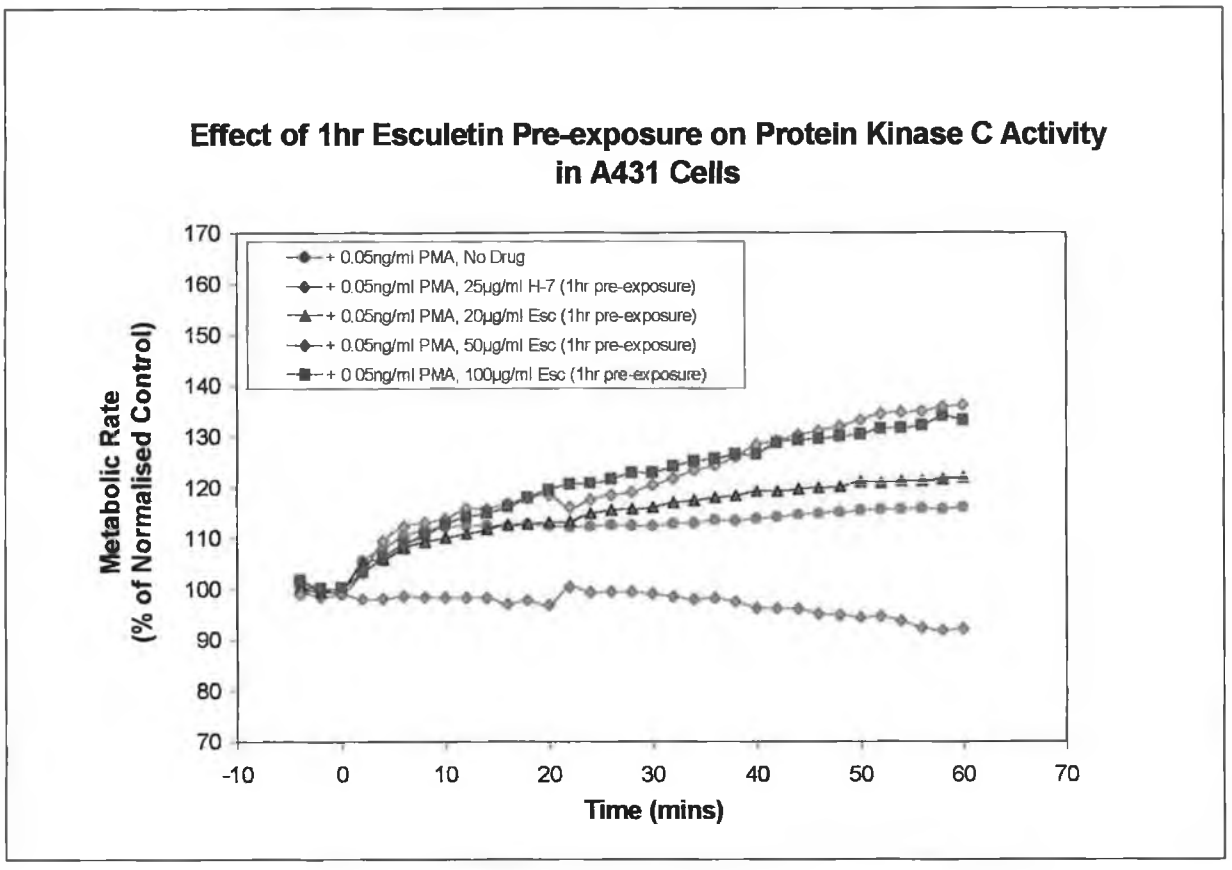


Figure 5.23: **Effect of esculetin on Protein Kinase C Activity in A431 cells. The above plot shows the experiment conducted on the Cytosensor Microphysiometer to determine the effect of 1hr esculetin exposure on the activation of the Protein Kinase C, with 0.05ng/ml PMA, in A431 cells. H-7 was used as a positive PKC inhibitor. In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to EGF exposure, using a 2 min cycle, with the acidification rate measured in the final 30 secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during and following PMA exposure were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for the cells in all experiments were –100 to -200µvolts/sec.**

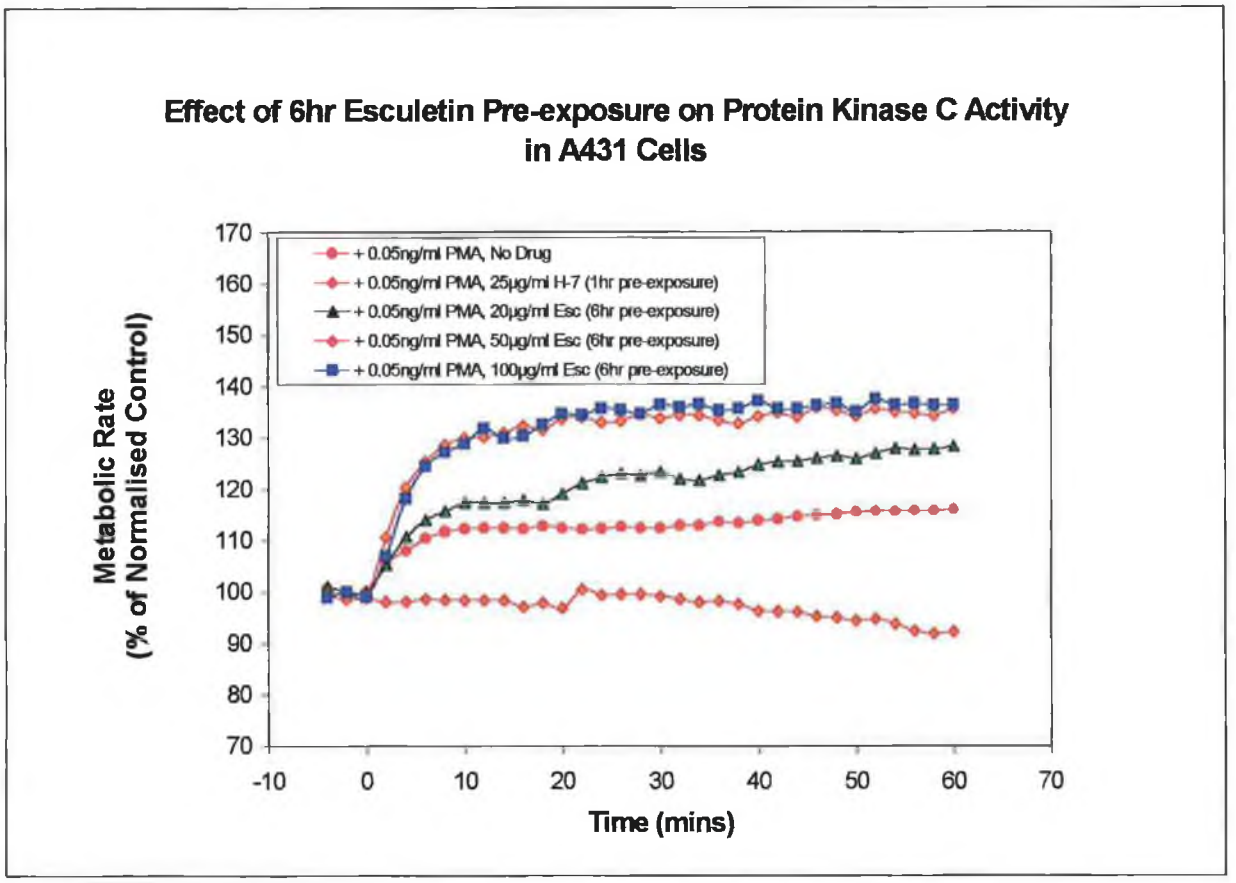


Figure 5.24: **Effect of esculetin on Protein Kinase C Activity in A431 cells. The above plot shows the experiment conducted on the Cytosensor Microphysiometer to determine the effect of 6hr esculetin exposure on the activation of the Protein Kinase C, with 0.05ng/ml PMA, in A431 cells. H-7 was used as a positive PKC inhibitor. In individual experiments, the cellular acidification rate for each sensor chamber was determined prior to EGF exposure, using a 2 min cycle, with the acidification rate measured in the final 30 secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during and following PMA exposure were expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates for the cells in all experiments were –100 to -200µvolts/sec.**

5.7. CHAPTER SUMMARY

In this chapter, the effect of two coumarin compounds, 7-hydroxycoumarin and esculetin on growth signalling pathways in tumour cells was examined. The A431 cell line was used in all experiments for examining two distinct signalling molecules – the EGF-Receptor and its associated tyrosine kinase activity, and the Protein Kinase C molecule.

The binding of EGF to its cognate receptor causes activation of the receptor tyrosine kinase activity, with consequent signalling via tyrosine phosphorylation events (Section 5.2). Growth factor-mediated tyrosine phosphorylation was examined in A431 cells using a variety of methods, namely, *in vitro* tyrosine kinase assay, ELISA, Cytosensor Microphysiometer and Western blotting. The work focussed particularly on the assays examining phosphorylation in intact cells, as these assays mirror more effectively the natural situation, as well as being more labour- and cost-effective when screening for tyrosine kinase inhibitors.

The above methods were used to examine the effect of coumarins on tyrosine phosphorylation, as a result of growth factor stimulation. ELISA and Western Blotting results revealed that 7-hydroxycoumarin and esculetin were both capable of inhibiting tyrosine phosphorylation in A431 cells following EGF binding. This inhibition was dose- and time-dependent, and esculetin was shown to be more effective than 7-hydroxycoumarin at this task. Neither compound was as potent as commercial TK inhibitors, genistein or tyrphostin.

Western blot analysis illustrated that autophosphorylation of the EGF-receptor and phosphorylation of its downstream targets was reduced in cells treated with coumarins. It is unclear whether direct inhibition of receptor tyrosine kinase activity is responsible for this effect – results from the direct *in vitro* assay did suggest that 7-hydroxycoumarin can inhibit the EGF-RTK activity directly. However, alternative explanations can be offered to account for the observed inhibition of tyrosine phosphorylation. In particular, activation of negative regulators of the EGF-RTK by coumarins would explain the observed results. Protein Kinase C is a well-documented regulator of EGF-RTK activity and we examined the effect of coumarins on its activity, using the Cytosensor Microphysiometer. Pre-exposure of A431 cells to a range of concentrations of 7-hydroxycoumarin and esculetin caused augmentation of the activation of PKC by PMA. A synergism between PKC and coumarins has been previously reported in immune cells (Zlabinger *et al.*, 1993) and our results support such an effect. As PKC is a negative regulator of EGF-RTK, the coumarins may function by indirect inhibition of the EGF-RTK through the PKC molecule, rather than direct inhibition of

EGF-RTK, leading to the observed decrease in tyrosine phosphorylation following EGF stimulation.

Regardless of whether they directly or indirectly inhibit the EGF-RTK, the decrease in tyrosine phosphorylation effected by coumarin compounds may offer an explanation for the precise mode of their anti-tumour action. Anomalies in tyrosine phosphorylation are extremely prevalent in tumour cells, and are responsible for persistent growth signalling in these cells. Interruption/disruption of this persistent signalling suppresses this abnormal proliferation, and therefore any compound which decreases tyrosine phosphorylation has anti-proliferative properties.

Chapter 6

Investigation of the effects of coumarins on monocyte functions in model monocyte systems

6.1. INTRODUCTION TO TUMOUR IMMUNITY

Changes in the membrane components of cancerous cells compared to their normal counterparts, known as either tumour–specific or tumour-associated antigens, provoke the immune system to respond to the presence of these "foreign" cells in the body. In mounting an efficient immune response against tumour cells, the immune system employs the assistance of all major immune cell types – T-cells, B-cells (through antibody-production), macrophages and natural killer (NK) cells. The major methods used in tumour rejection are outlined in Table 6.1.

However, the effectiveness of these mechanisms must be questioned when one considers the fact that in many hosts, tumour cells escape this immune surveillance and continue to grow and metastasise. The various modes by which tumour cell evasion of the immune system occur, include antigenic modulation and/or production of immunosuppressive products (*e.g.* prostaglandins) by tumour cells. In addition, the size or location of the tumour mass may preclude its destruction by the immune system. Certain immune cells including suppressor T-lymphocytes (T_s cells), and even tumour-associated macrophages (TAMs) may also lead to tumour enhancement rather than regression. Therefore, despite the presence of a natural system for foreign cell elimination, the immune system must often be manipulated to effectively mount an anti-tumour response. This is the underlying basis of cancer immunotherapy, which can use both passive (*e.g.* immunotoxin administration), or active (*e.g.* cytokine therapy) immune responses to provide a potential fourth treatment modality - to join surgery, chemotherapy and radiotherapy - in the management of cancer. The principal means of manipulation of our innate defences to effect an anti-tumour response are outlined in Table 6.2 (Benjamini *et al.*, 1996; Elgert, 1996; Scott & Welt, 1997; Andreesen *et al.*, 1998; Chen & Wu, 1998; Salgaller & Lodge, 1998). Although still in their infancy, the human clinical trials of many of these options have proved quite promising.

Mechanisms of Immune Response to Tumours
Humoral:
1. Antibody (Ab) & complement-mediated lysis of tumour cells
2. Opsonisation and destruction of tumour cells by phagocytosis (Ab- and complement–mediated)
3. Antibody-mediated loss of tumour cell adhesion
Cellular:
1. Destruction by activated macrophages
2. Destruction by Natural Killer (NK) cells
3. Destruction by cytotoxic T-lymphocytes (CTL's)
4. Antibody-dependent cell-mediated cytotoxicity (ADCC)

Table 6.1: Important immune mechanisms in the destruction of tumour cells.

Immunotherapy Approaches
1. Cytokine Therapy [IFNs, IL-2] administration
2. Tumour Vaccines
3. Immunotoxins
4. Adoptive Immunotherapy (*e.g.* LAK cells)

Table 6.2: Immunotherapy approaches to the treatment of cancer

As outlined in Table 6.1, various immune cell types are essential to the innate anti-tumour defence mechanism within humans. However, in terms of significance, many investigators believe the cells of the macrophage/monocyte lineage to be of most importance in the whole area of tumour immunity. The advantages of these cells over other immune cells in providing an anti-tumour effect have been cited recently (Bonta & Ben-Efraim, 1993; Zlabinger, 1997) and are included as Table

6.3. As a result, stimulation of monocyte/macrophage anti-tumour mechanisms is an attractive option as an immunotherapeutic approach to cancer management.

Advantages of Macrophages/Monocytes in Tumour Immunology

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1. Anti-tumour activity spectrum of these cells is wide & not species-specific
2. Activated macrophages react against "immunogenic", "weakly immunogenic" & "non-immunogenic" tumours in an indiscriminate manner
3. Macrophages recognise only foreign cells, never normal cells (self/non-self discrimination).
4. Malignancy progression is often accompanied by a decline in immune cell competence – macrophages are the last immune cell type to be affected during immune suppression.
5. Macrophage and monocyte cells can be easily isolated from cancer patients, activated *in vitro*, and re-injected into patients as an immunotherapy option.
6. No reports of induced tumour cell resistance to macrophages have been published to date.

Table 6.3: Advantages of Monocyte/Macrophages cells over other immune cells in evoking an anti-tumour response (adopted from Bonta & Ben-Efraim, 1993; Zlabinger, 1997).

6.2 INTRODUCTION TO THE MONONUCLEAR PHAGOCYTE SYSTEM (MPS)

6.2.1. Cells of the MPS Lineage

The Mononuclear Phagocyte System (MPS) is the general term used to describe the cells of the macrophage/monocyte lineage within the immune system. This includes the precursor bone marrow monoblasts and promonocytes, as well as their progeny, the peripheral blood monocytes and tissue macrophages. Macrophages are found widely located within the body in lymphoid organs (spleen, lymph nodes and thymus), lungs (as alveolar macrophages), liver (Kupffer cells), gastro-intestinal tract, central nervous system (microglia), bone (osteoclasts), skin (histiocytes) and synovial fluids (type A cells). The MPS system has been extensively reviewed (Adams & Hamilton, 1992; Auger & Ross, 1992; Lewis, 1995), the salient points of which are included here.

Macrophages originate in the bone marrow from precursor stem cells (colony forming unit, granulocyte-macrophage, CFU-GM), which are believed to be the common progenitor cell for both macrophages and granulocytes. The CFU-GM stem cells give rise to monoblasts, which progressively differentiate into pro-monocytes and monocytes. The monocytes rapidly enter the circulation and migrate into the tissues within a four day period to yield the appropriate tissue macrophages. The various stages of this cell differentiation are distinguishable at both a morphological and histochemical level *e.g.* transition from monocyte to macrophage is accompanied by an increase in the number and activity of mitochondria, a decrease in peroxidase activity, and changes in the expression of various surface receptors.

Cells of the MPS engage in a broad range of physiological and pathological processes, as summarised in Table 6.4, with active destruction of foreign cells the primary function. Macrophages, on stimulation with an appropriate agent, are activated to become microbicidal and tumouricidal machines. As part of this function they are involved in the secretion of a wide range of biologically important products, including cytokines, enzymes, oxygen radicals, coagulation factors (for a complete list consult Table 1.7, Auger & Ross, 1992), as well as in antigen processing and presentation. In addition, macrophages are central components of the inflammatory response, and are inherently involved in the production of cytokines and angiogenic factors necessary for correct wound healing. Their phagocytic capabilities also allow them to participate in normal homeostatic and metabolic functions such as removal of effete red blood cells, and iron and lipid metabolism, respectively. Finally, macrophages play an important developmental role, particularly in the selection and destruction of cells with potential reactivity to self-antigens.

Functions of MPS Cells
1. Destruction of micro-organisms
2. Tumour cell control
3. Inflammation and Wound Healing
4. Secretion
5. Homeostasis

Table 6.4: Normal functions of cells of the Mononuclear Phagocyte System.

6.2.2. Activation of MPS Cells

As mentioned above, monocytes/macrophages must be activated to elicit an anti-microbial or anti-tumour killing effect, and the requirements for this event have been reviewed extensively (Adams & Hamilton, 1992; Lewis, 1995). The acquisition of cytocidal capacity usually involves a multi-step cascade of events, whereby a cell goes through a "priming" step, before it can become fully activated to cytocidal competence. Activation of mammalian macrophages *in vivo* can occur via two major routes. In the first, direct interaction with micro-organisms, or microbial cell wall constituents, such as muramyl dipeptide (MDP), or microbial products, such as lipopolysaccharide (LPS), activate macrophages. LPS has been used as the model macrophage activating signal over many years, with its activity attributed to the lipid A portion of the molecule. Macrophage binding of LPS initiates intracellular phosphoinositide (PI) breakdown, stimulates Protein Kinase C (PKC) activity, and results in the expression of a large number of cytokines, including TNF-α, IL-1, IL-6, M-CSF, GM-CSF and IFNα/β. The second macrophage activation pathway involves the use of lymphokines such as interferon-γ (IFN-γ) or macrophage-activating factor (MAF). IFN-γ is a 55kDa glycoprotein produced by T-lymphocytes and NK cells. It possesses anti-viral and immunomodulatory functions, and activates monocytes/macrophages following interaction with its cell surface receptor. IFN-γ activation of MPS cells induces the production of many secreted molecules, including IL-1, CSF-1, IL-2-Receptor, and reactive oxygen species (ROS). The secretion of prostaglandin and the activation of integrin genes (LFA-1 adhesion molecule) also occur. IFN-γ also increases the expression of MHC Class I and II molecules, which is important for antigen presentation and MHC Class I-restricted T-cell cytotoxicity. Various synthetic molecules *e.g.* phorbol esters and ionophores, have been shown to activate macrophages *in vitro*. They achieve this effect by activating the signalling pathways utilised by the *in vivo* macrophage activators, thus mimicking their effect, *e.g.* phorbol myristate acetate (PMA) causes stimulation and translocation of PKC, and can thus partly mimic macrophage activation by LPS.

Suppression of macrophage activity can also be accomplished by biological molecules, with prostaglandins (especially PGE_2) the most significant negative regulators of immune activation. Glucocorticoids and transforming growth factor-β (TGF-β) also exert suppressive effects on immune cells, including MPS cells.

6.2.3. MPS Cells in Tumour Immunity

6.2.3.1. Overview

MPS cells have become the focus of much investigation in tumour immunity studies due to observations of their ability to elicit cytostatic and cytotoxic effects on tumour cells *in vitro* (Grabstein *et al.*, 1986; Hasday *et al.*, 1990). In addition, the recruitment of MPS cells into tumours is also a well-known event (Mantovani *et al.*, 1992). The anti-tumour effects of monocytic/macrophage cells, like their anti-microbial effects, are mediated through the secretion of a broad range of products in the tumour mass environment. The more important anti-tumour mechanisms are as follows:

1. Production of cytostatic and cytolytic cytokines
2. Production of reactive oxygen species (ROS)
3. Production of reactive nitrogen intermediates (RNI)
4. Production of enzymes

The execution of these tumouricidal mechanisms is achieved by cell activation through sequential "priming" and "triggering" stimuli as detailed in section 6.2.2. A brief outline of the most important facets of each of these mechanisms is outlined in the subsequent sections (section 6.2.3.2 – 6.2.3.5).

6.2.3.2. Cytokines as anti-tumour agents

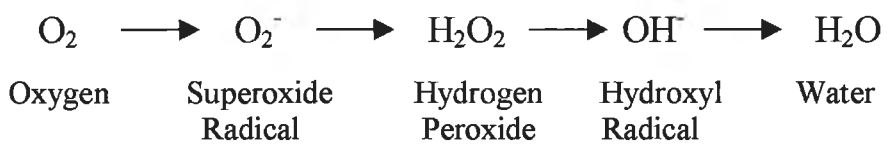
Briefly, cytokines can be defined as multifunctional peptide molecules ranging in size from 6-60kDa, which act as intercellular signals, usually in an autocrine or paracrine manner. They include monokines, produced by monocytes, lymphokines produced by T- & B-lymphocytes, interferons, haematopoietic CSFs and connective tissue growth factors. These molecules play a crucial role in the regulation of many important physiological processes, including immunity, haematopoiesis, tumourigenesis, homeostasis, cellular growth and differentiation and tissue repair (Detrick & Hooks, 1997). The importance of these molecules in tumour immunity lies firstly in their capacity for general immune system-activation (*e.g.* IL-1 production leads to stimulation of T-cells and APC cells; IFN-α release potentiates NK cell activity *etc.*), and secondly, in the existence of individual cytokines with distinct anti-tumour capabilities (*e.g.* TNF-α has direct cytocidal properties). Two cytokines of importance in monocyte anti-tumour mechanisms (TNF-α and IL-1) are described below.

Tumour necrosis factor (TNF-α) is a cytokine (17.3 kDa) secreted primarily by cells of the MPS system, which has been shown to have a broad range of immunomodulatory and physiological effects (Dietrich & Hooks, 1997). TNF-α production is regulated by a collection of substances: PMA, LPS, IL-1, IL-2, IFN-γ and TNF-α (all positive mediators), and TGF-β, prostaglandins, glucocorticoids and IL-4 (all negative regulators). It is an important effector molecule, playing a huge part in the activation of neutrophils, eosinophils, macrophages and NK cells. It has been shown to have direct anti-tumour effects both *in vivo* (Carswell *et al.*, 1975) and *in vitro* (Sugarman *et al.*, 1985, Feinman *et al.*, 1987). This activity can be synergistically increased with IFN-γ and IL-1 (Feinman *et al.*, 1987; Fidler & Ichinose, 1989).

Interleukin-1 (IL-1), as well as increasing the tumouricidal effects of TNF, acts independently in an anti-tumour fashion (Nakane *et al.*, 1990). In addition, it can cause increased binding of NK cells to tumour target cells. However, it is the myriad biological actions of this cytokine which it are of most importance – IL-1 is capable of inducing IL-2, IL-6, IL-8, IFNs, TNF, CSFs and TGF-β, each with resultant independent or synergistic anti-tumour/inflammatory effects, that highlight the significance of this molecule in the overall anti-tumour response (Dietrich & Hooks, 1997).

6.2.3.3. Production of Reactive Oxygen Species (ROS)

When appropriately activated, cells of MPS origin, like other phagocytes, undergo changes in their metabolism: their rates of oxygen uptake increase, and within seconds, activation of a special metabolic pathway takes place (this overall process has been reviewed by Babior, 1984). The ultimate fate of oxygen is its reduction to water, however, it is converted *en route* to a series of intermediate oxygen compounds, namely superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-), as follows:



This "respiratory burst" is achieved through activation of NADPH-oxidase, a membrane-bound enzyme complex, which is responsible for the production of superoxide from molecular oxygen. Superoxide dismutates with itself to form hydrogen peroxide. Hydrogen peroxide can cause DNA strand breaks in tumour cells, at concentrations produced physiologically (Chong *et al.*, 1989). The conversion of hydrogen peroxide to oxidised halogens (*e.g.* hypochlorous acid [HOCl]), through the action of myeloperoxidase, is thought to cause extreme oxygen-mediated damage by these compounds (Babior, 1984; Rodgers, 1995). Production of the toxic hydroxyl radical and hydroxyl anion from hydrogen peroxide, via an iron-dependent reaction, is also an important harmful ROS generator. Therefore, it is evident that measurement of ROS species can be an important indicator of the cytocidal capabilities of monocyte/macrophage cells, and augmentation of ROS release by agents is an attractive anti-tumour strategy.

6.2.3.4. Production of Reactive Nitrogen Intermediates

Production of reactive nitrogen intermediates (RNI) by mammalian cells is quite a recent (1980s) discovery. RNI are formed through the conversion of L-arginine by nitric oxide synthase (NOS), with the nitric oxide radical (NO•) the principal product. NO• can react *in vivo* to form many nitrogen-based products, with nitrite and nitrate principal among them. The NOS pathway is known to be of particular importance in inflammation (Dietert *et al.*, 1995), and also notable in tumour immunity, with NO• reported to have a direct tumouricidal effect (Hibbs *et al*, 1988). NO• has been produced *in vitro* by activated mammalian macrophages, on stimulation with a range of cellular/biological stimulators (Keller *et al.*, 1990; Dietert *et al.*, 1995), although to date few *in vivo* studies have been completed.

6.2.3.5. Production of Enzymes

A variety of enzymes are secreted as MPS products, and include lysozyme, lysosomal acid hydrolases, neutral proteases and lipases (Auger & Ross, 1992). A number of these enzymes are important from a destructive/phagocytic viewpoint (*e.g.* lysosomal enzymes), while others function in wound-healing through remodelling of the extracellular matrix (*e.g.* neutral proteases such as collagenases). The release of enzymes from MPS cells has been regularly cited as a possible mechanism of anti-tumour attack, but evidence for this event is relatively weak. However, as mentioned previously, some immune cells appear to display a paradoxical role in tumour immunity

– this is evident with regard to tumour-associated macrophages, where many of their products appear to benefit the growth and metastasis of the tumour mass, rather than inhibiting these processes (Mantovani *et al.*, 1992).

The expression of matrix metalloproteinase (MMP) enzymes has been reported for various immune cells including neutrophils, monocytes and macrophages (Garbisa *et al.*, 1986; Opdenakker *et al.*, 1991; Xie *et al.*, 1994b), with stage-specific expression patterns evident in cells of the MPS lineage (Shapiro *et al.*, 1991). The normal expression of these enzymes in MPS cells probably relates to their requirement to traverse basement membranes, in response to inflammatory/chemotactic signals. However, as outlined in chapter 4, the expression of MMP enzymes by tumour cells is correlated with their invasive potential. Therefore, MMP production by MPS cells, especially tumour-associated macrophages, can, in theory, benefit tumour cell metastasis. In practice, the coexistence of PBMCs was found to increase the invasion of bladder cells *in vitro*, and this effect coincided with the secretion of MMP-9 by the PBMC (Kageyama *et al.*, 1997). Thus, examination of MMP release from MPS cells, and its inhibition may be an attractive anti-tumour option.

6.3. COUMARINS AND THE IMMUNE SYSTEM

Coumarin has been pharmacologically classified as a biological response modifier. The immunomodulatory effects of coumarins have been briefly introduced in sections 1.7.3.1 and 1.7.3.2, with regard to their clinical role in oedema and chronic infection treatment. As outlined in section 1.8.5.2, coumarin has a stimulatory effect on many cells in the immune system. Much of the early work (1970s) on coumarin was carried out to explain the observed anti-oedema effect of coumarin. The benzopyrones, including coumarin, were shown to exert their effect by increasing the number and activity of macrophages in oedematous tissue (Piller and Casley-Smith, 1975; Piller 1977a, 1977b, 1977c, 1978). Koh and Willoughby (1979) found similar number and activity increases in mouse macrophages, in studies comparing coumarin and levamisole as macrophage activators. However, Filice and Remington (1981) reported that neither coumarin, nor warfarin, could elicit a microbicidal or tumouricidal activity from mouse macrophages challenged with *Toxoplasma gondii,* or tumour cells, respectively. More recently, Marshall and co-workers (1991b), using monoclonal antibodies and FACS analysis, reported an increase in the expression of DR and DQ antigens on normal donor peripheral blood mononuclear cells (PBMC), following *in vitro* exposure to coumarin. Enhanced expression of these antigens is consistent with an activated state.

The increase in DR antigen expression was also in evidence *in vivo*, when monocytes taken from patients with renal cell carcinoma, undergoing treatment with coumarin and cimetidine, were examined for surface antigen expression (Marshall *et al.*, 1987a).

The effect of coumarins on the activity of other immune cells has also been examined. It has been shown that coumarin increases the ratio of T_H: T_S cells *in vitro*, and activates natural killer (NK) cells *in vivo* (Thornes & Lynch, 1983; Thornes, 1997). Marshall and co-workers showed that coumarin did not alter the NK cell activity *in vitro* (Marshall *et al.*, 1989b). Marshall *et al.* (1987c) found no quantitative changes in peripheral blood lymphocytes or natural killer cells, in patients with advanced malignancies undergoing coumarin treatment. Coumarin did produce a modest increase in the mitogenic response of lymphocytes to phytohaemagglutinin (PHA) but not to concanavalin A (Con A) or pokeweed mitogen (PWM) both *in vivo* and *in vitro*, (Berkada *et al.*, 1983; Marshall *et al.*, 1989b). Coumarin was also shown to augment the *in vitro* production of lymphokine activated killer (LAK) activity in peripheral blood monocytes (Triozzi *et al.*, 1990).

As outlined in section 6.2.2, the most relevant secreted MPS products for tumour immunology are cytokines, reactive oxygen species and reactive nitrogen species. A number of studies have been performed in order to determine the effect of coumarins on the secretory products of monocytes. Stulmeier *et al.* (1991) showed that coumarin, 7-hydroxycoumarin and 4-hydroxycoumarin all potentiated the LPS-stimulated release of IL-1β from monocytes. The effect of coumarins on LPS-induced IL-6 and TNF-α production from donor monocytes was also assessed. While a synergistic effect was also observed for the production of these cytokines, it was not as prevalent as for IL-1 [the coumarin/LPS synergism was evidenced for IL-1 secretion in 95% of donors, but only in 53% and 32% of donors, for IL-6 and TNF-α, respectively]. In all cases of cytokine production, 7-hydroxycoumarin was the most effective synergiser, and in no instance were any of the coumarins capable of inducing significant cytokine production by themselves. Stuhlmeiers colleagues continued work in this area, probing the signalling pathways involved in the LPS/coumarin synergism (Zlabinger *et al.*, 1993). They found that the LPS-induction of IL-1 production from monocytes was coupled to both PKC and lipoxygenase pathways. The augmenting effect of 7-hydroxycoumarin on LPS-induced secretion of IL-1 also involved these two signalling pathways.

The generation of reactive oxygen species (ROS) by PMA-stimulated MPS cells was shown by Marshall and co-workers (1989b) to be influenced by coumarin species. Coumarin inhibited the generation of superoxide and hydrogen peroxide in a dose-dependent manner in freshly isolated

human monocytes. This effect was not observed for macrophages (monocytes cultured for 72hrs) stimulated with PMA. Triozzi and colleagues (1990) found coumarin inhibited the IL-2-induced generation of ROS by 58% in human peripheral blood monocytes. However, neither of these studies addressed the issue of the free-radical scavenging abilities of the coumarin compounds (Paya *et al.*, 1992).

To date, no studies have been achieved on the effect of coumarins on the production of reactive nitrogen intermediates (RNI) by MPS cells.

Finally due to their extensive networking with cytokine pathways, arachidonic acid metabolites are also important in tumour immunity (Bonta & Ben-Efraim, 1993). Prostaglandins, in particular, are known to exert a strong immunosuppressive effect in humans. In contrast, leukotrienes generate increases in the release of TNF-α from macrophages. Coumarin, 7-hydroxycoumarin and 4-hydroxycoumarin were shown to inhibit the conversion of arachidonic acid to prostaglandins, with equipotency to the inhibition attained by acetylsalicylic acid (Lee *et al.*, 1981). Triozzi and colleagues (1990) found that coumarin completely inhibited the IL-2-induced production of prostaglandin E_2 by human peripheral blood mononuclear cells. Zlabinger *et al.* (1993) made a brief observation that coumarin synergistically enhanced the LPS-induced production of leukotriene B_4 in human monocytes.

Many of the experiments achieved to date in studying the effect of coumarins on immune cells have been achieved with peripheral blood monocytes from either normal donors or patients. The use of human mononuclear cells in any study introduces many problems for the experimenter. Firstly, there is the problem of sourcing: unless a laboratory has regular access to blood bank or hospital blood samples, a consistent, fresh (<6hrs old) supply of mononuclear cell samples, in sufficient numbers for experiments, can be difficult to achieve. Once availability is overcome, the next problem to be addressed is the issue of cell uniformity: many inter-experimental differences in cell response arise due to donor heterogeneity. There is also the issue of cell subsets (as a result of distinct differentiation stages) even in cells from a single donor. Purification of the mononuclear fraction from blood samples or buffy coats, introduces further problems – apart from loss of large numbers of cells, there is the worry that activation of monocytes by, for example, endotoxin contamination, can occur. In addition, 100% purification is rarely achieved, thus, low levels of contamination with other blood cells must be taken into account in experimental designs.

These problems have been largely overcome by two methods:

1. Use of animal (usually mouse) sources of macrophages. This can allow access to large numbers of cells for exploratory work, however, problems with cell uniformity can still exist.
2. Use of immortalised cell lines. These overcome availability and heterogeneity problems, and studies can be performed on human cells. However, many "monocytic/macrophage" cell lines are leukemic in origin - therefore, there is the issue of deciding if they are appropriate models of "normal" monocytes. In addition, there is the possibility that the process of immortalisation may cause loss of some normal functions.

6.4. CHAPTER OUTLINE

In this chapter two human cell lines were examined to determine their suitability for use as "models" of human monocytes. The HL-60 cell line, derived from a single patient with acute promyelocytic leukemia has been widely used in immune cell studies. It can be induced to differentiate *in vitro* from its promonocytic state to a number of different cell types – granulocytes, monocytes, macrophages and eosinophils – by exposure to a range of chemical and biological agents (Collins, 1987). In addition to this cell line we also chose to study the 28SC cell line: these cells, recently deposited with the American Type Culture Collection (ATCC), are monocytic in nature, having been established from human peripheral blood mononuclear cells (Collins & Largen, 1995). We were particularly interested in these, as they were not leukemic in origin.

Both cell lines were exposed to a variety of immune cell stimulators and modulators, and the secretion of various anti-tumour products, namely reactive oxygen species, reactive nitrogen intermediates and proteases examined. The effect of coumarins on the secretion of these products was then investigated.

6.5. RESULTS AND DISCUSSION

6.5.1. Characterisation of HL-60 and 28SC Cells

6.5.1.1. Staining of Cytocentrifuged Cells

The HL-60 (pro-monocytic) and 28SC (monocytic) cells were assessed morphologically and cytochemically by methylene blue/eosin (MB/E), and non-specific esterase (NSE) staining, respectively. MB/E staining provides for a morphological assessment of gross cellular structure. NSE is an enzyme found on the external surface of the plasma membrane of monocytes and macrophages, and is commonly used as a positive cytochemical stain for these cell types. The staining of cytocentrifuged cells was accomplished as outlined in section 2.2.7.3, and the results are shown in Figure 6.1. It can be seen morphologically, that the two cell types are quite similar, however the 28SC cells possess a more highly "ruffled" membrane, which is indicative of a phagocytic cell. These cells, as expected, express high levels of non-specific esterase, a monocyte marker, on their plasma membrane as indicated by the intense black staining (Figure 6.1D), compared to the negative pro-monocytic HL-60 cells (Figure 6.1C).

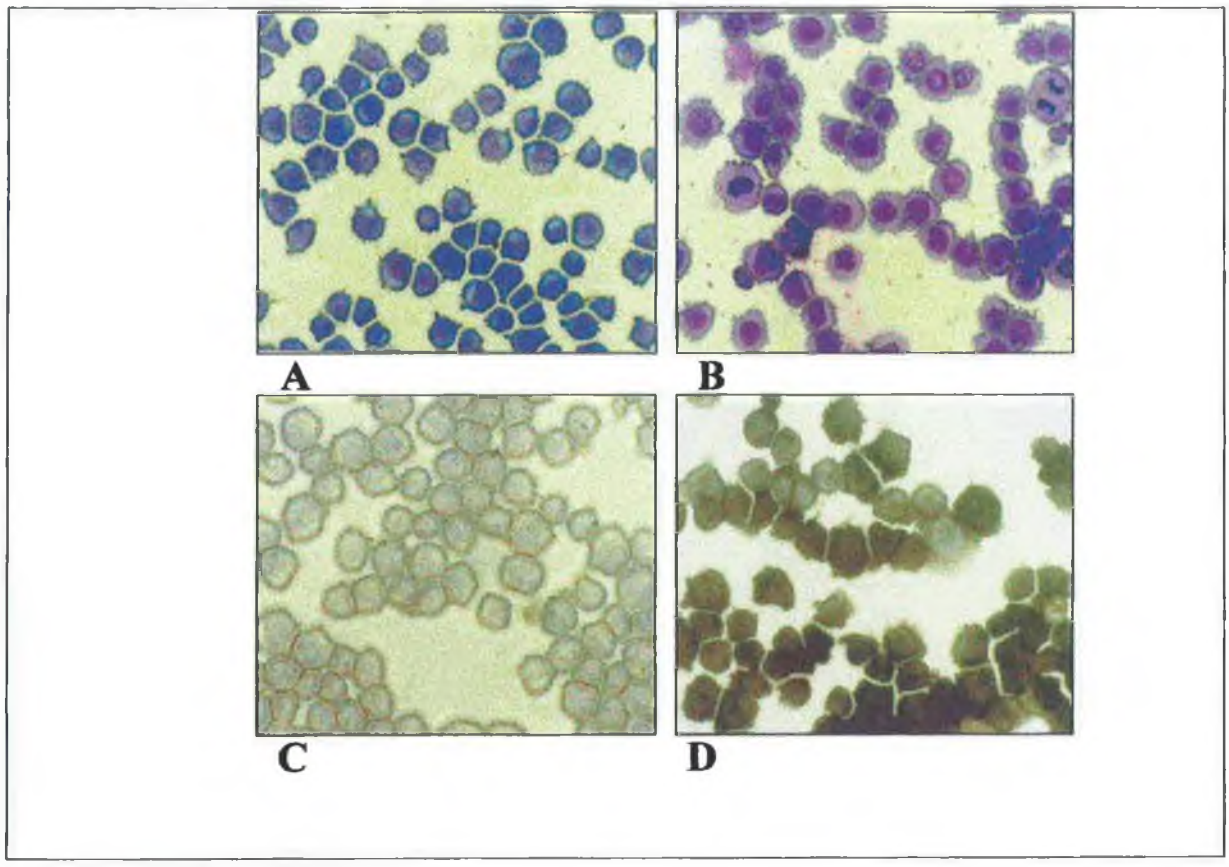


Figure 6.1: Cytochemical staining of HL-60 or 28SC cells. Both cell lines were subjected to either Methylene blue/eosin (MB/E) or Non-specific esterase (NSE) staining as outlined in section 2.2.7.3. The results for HL-60 are shown in A (MB/E staining) and C (NSE staining), and for 28SC in B (MB/E staining) and D (NSE staining).

6.5.1.2. Nitroblue Tetrazolium (NBT) Reduction

This test is commonly used to determine the respiratory burst in monocytic cells. Cells are activated with PMA, and their ability to reduce a tetrazolium salt ascertained, as evidence of the magnitude of the activation. This experiment was carried out as outlined in section 2.2.7.3.4 and the results are presented in Table 6.5. As shown neither cell line responded greatly to the applied PMA stimulus. However, the HL-60 cell line was ten times more responsive than the 28SC cell line to PMA. As this NBT reduction technique is quite a crude and subjective method, we were interested in examining the activation of these two cell lines with PMA in more detail, and proceeded to do so using the Cytosensor Microphysiometer as outlined in section 6.5.2.

Cell	*% Cells Reducing NBT*
HL-60	8.62 ± 1.31 (0.11 ± 0.18)
28SC	0.83 ± 0.60 (0.12 ± 0.21)

Table 6.5: **NBT reduction in HL-60 and 28SC cells. Cells were stimulated with 100ng/ml PMA as outlined in section 2.2.7.3.4, and following cytocentrifugation, ~200cells were counted. The number of cells containing blue/black deposits (*i.e.* positive NBT reduction) was expressed as a percentage of the total number of cells counted. The experiment was carried out on 5 separate occasions and the mean ± s.d. shown in the above table. Values for unstimulated controls are displayed in brackets.**

6.5.2. Activation of MPS cells on Cytosensor Microphysiometer

The Cytosensor Microphysiometer has been used in the past to examine activation in a variety of immune cell types. Nag *et al.* (1992) examined the specific response of T-cells to a pre-formed MHC-Class II-peptide. The activation of T-cells by interaction with anti-CD3 and anti-TCR monoclonal antibodies, as well as IL-2 exposure, have also been assessed on the Cytosensor Microphysiometer (Wada *et al.*, 1994). Activation of B-lymphoma cells, through binding of anti-idiotypic antibody and antigen binding receptor ligands, has also been studied on this instrument (Renschler *et al.*, 1995). The effect of various inflammatory mediators on the activation of neutrophils (PMNs) has been investigated using microphysiometry - in this experiment the potency of different immune cell activators was compared. Leukotriene B_4 (LTB_4) and FMLP (n-formyl-methionine-leucine-phenylalanine) were shown to be extremely potent PMN activators, while IL-8 and platelet-activating factor (PAF) illustrated only weak activating capabilities (Gronert *et al.*, 1998). The effect of lipoxins on monocytes and monocytic cell lines has also been examined on the Cytosensor Microphysiometer, but these inflammatory mediators were found to have no effects on the acidification rates of these cell types (Romano *et al.*, 1996). Finally, this instrument has also been used to examine the effect of lipoprotein on the metabolic state of cultured mouse macrophages (DeVries *et al.*, 1998).

Having observed the poor stimulation of metabolic activity for both the HL-60 and 28SC cell lines using the NBT reduction technique, we proceeded to examine the activation of the HL-60 and 28SC cell lines by PMA, using the Cytosensor Microphysiometer. The experiment was achieved as outlined in section 2.2.7.4, and the results for the exposure of HL-60 and 28SC cells to a range of PMA doses (0-5000ng/ml), are shown in Figures 6.2 and 6.3, respectively.

Figure 6.2 illustrates the stimulation of HL-60 cells in response to PMA exposure. As shown, a dose-dependent increase in the metabolic activity of these cells was observed. In the range 0.5-5ng/ml PMA, an initial "burst" to ~106% (0.5ng/ml PMA) and ~109% (5ng/ml PMA) was observed, but this was only a transient increase in metabolic activity, and the metabolic activity of both cell populations returned to their baseline levels within 60 mins of stimulus application. The metabolic rate of cells exposed to 50ng/ml PMA immediately increased to ~109% of its "basal" level following stimulation, but sustained this increased metabolic rate at ~ 110-113% over the 60 min post-exposure period. Exposure of cells to both 500 and 5000ng/ml PMA, initially caused a gradual increase in metabolic activation over the first 15mins post-exposure, however the increase was sustained at a level 110-115% above the basal metabolic rate over the 60 mins following PMA

stimulation. Overall, activation of HL-60 cells was optimal in the range 50-500ng/ml PMA – the metabolic rate of cells exposed to 5000ng/ml, did begin to return towards baseline levels, 40mins post-activation.

Figure 6.3 illustrates the activation of 28SC cells in response to PMA exposure. As outlined, these cells were not as responsive to stimulation by PMA as the HL-60 cells – the metabolic rate of the cells did not increase by greater than ~6% for any of the PMA concentrations tested in the range 0-5000ng/ml. Cells exposed to 5ng/ml PMA did not respond at all to the applied stimulus. At 50ng/ml PMA, the metabolic rate of the cells increased to ~106%, and gradually decreased to baseline levels of the 60mins post-stimulation. Cells exposed to both 500 and 5000ng/ml did not initially respond to the stimulus, but a slow, gradual increase in their metabolic rate was observed over the 60 mins post-stimulation period.

The low responsiveness of these cells to the cell activator PMA is unusual, as it acts by activating the ubiquitous Protein Kinase C (PKC), causing its translocation to the cell membrane, with subsequent initiation of cell signalling events. However, the existence of PKC isozymes has been widely established, and these isozymes are classified as one of three types: classical, novel and atypical (see Section 5.2.4), on the basis of their responsiveness to phorbol esters and their Ca^{2+}-dependency (Mellor & Parker, 1998). Members of the atypical PKC sub-classification are PMA-unresponsive. The presence/absence of different PKC isoforms has been investigated for various monocyte-like cells. Changes in the levels of PKC isoforms have been reported in HL-60 cells on differentiation to granulocytes or monocytes (Makowske *et al.*, 1988; Solomon *et al*, 1991). A similar study of PKC isoforms in U937 cells also showed a differential expression of isozymes in undifferentiated and differentiated cells (Kiley & Parker, 1995). Overexpression of PKC-ζ (PMA-unresponsive) in U937 cells was associated with a more differentiated phenotype in those cells (Ways *et al.*, 1994). A decrease in the expression of Ca^{2+}-dependent PKC isoforms in macrophages compared to monocytes has also been recently demonstrated (Monick *et al.*, 1998). Therefore, it is possible that the more differentiated 28SC cells possess PKC isozymes which are PMA-unresponsive, while the PKC isozymes present in HL-60 cells belong to the more classical PMA-responsive family. A full isozyme analysis by immunoblotting would clarify this issue. Another simpler alternative, is that PKC expression/activity (irrespective of isozymes) is decreased in 28SC cells; such a loss of enzyme activity is not unusual during establishment of immortalised cells in culture. Whatever the reason for their non-responsiveness to PMA, this facet of these cells may preclude their use in the subsequent experiments of this chapter.

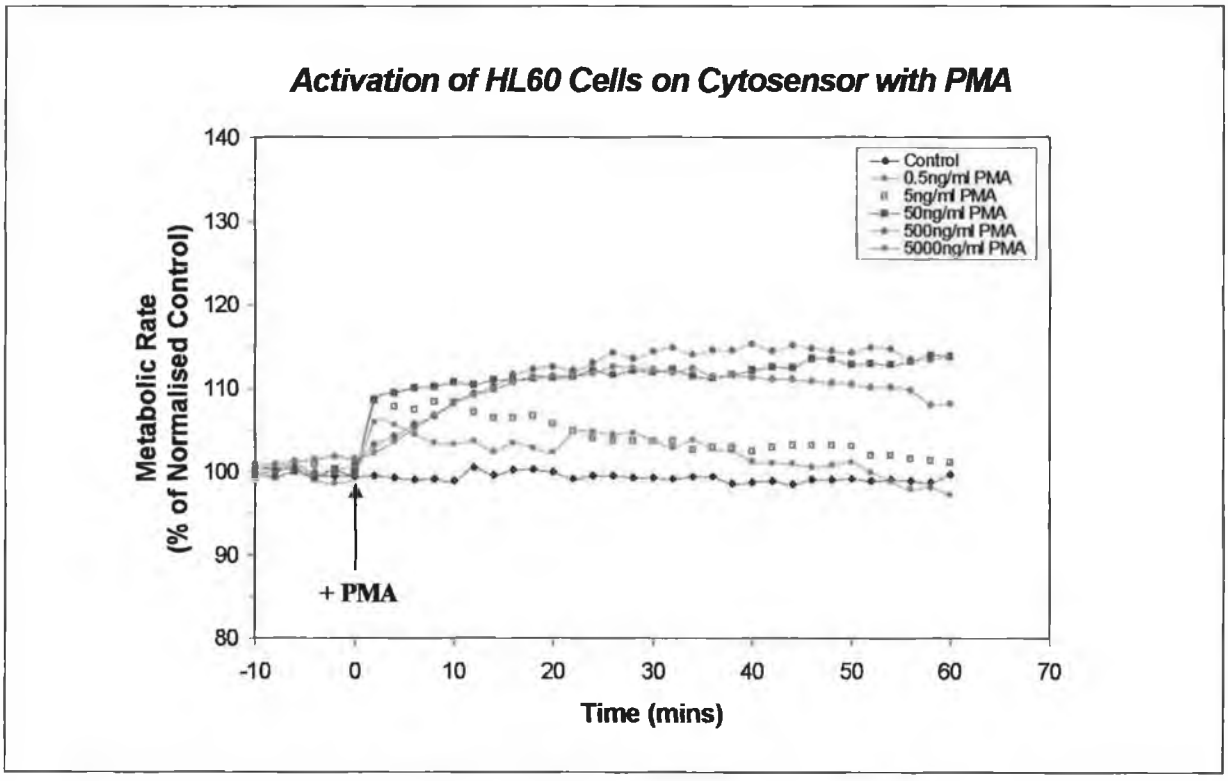


Figure 6.2: **Exposure of HL-60 cells to concentrations of PMA in the range 0-5000ng/ml over 60mins. The above plot shows the mean of three experiments conducted on the Cytosensor Microphysiometer to determine the activation of HL-60 cells in response to PMA (Section 2.2.7.4). In individual experiments, the cellular acidification rate was determined prior to PMA exposure, using a 2 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during PMA-exposure expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates (-μvolts/sec) for the HL-60 cells in the experiments were –80 to -130μvolts/sec.**

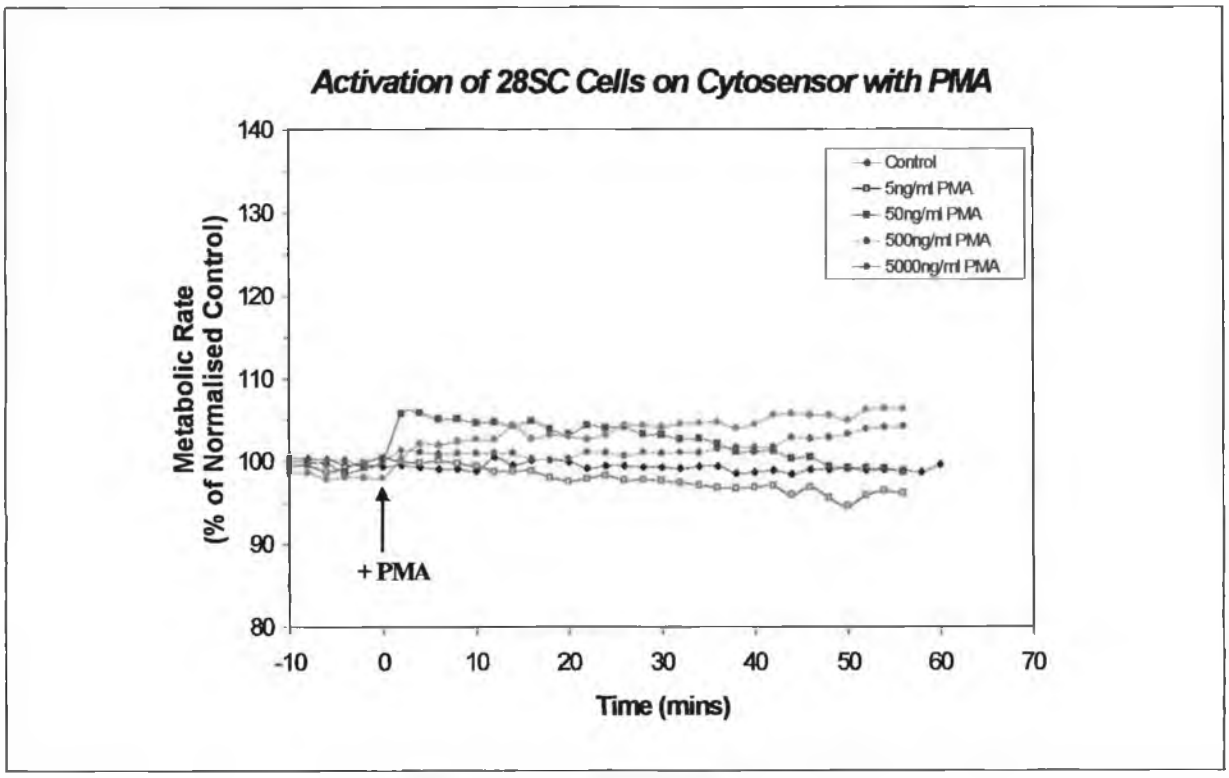


Figure 6.3: **Exposure of 28SC cells to concentrations of PMA in the range 0-5000ng/ml over 60mins. The above plot shows the mean of three experiments conducted on the Cytosensor Microphysiometer to determine the activation of HL-60 cells in response to PMA (Section 2.2.7.4). In individual experiments, the cellular acidification rate was determined prior to PMA exposure, using a 2 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during PMA-exposure expressed as a percentage of this normalised value. These values of metabolic rate (mean of three experiments) were plotted vs time in the above graph. Typical basal acidification rates (-μvolts/sec) for the 28SC cells in the experiments were –100 to -160μvolts/sec.**

6.5.3. Generation of Reactive Oxygen Species by Monocytic Cells – Chemiluminescent Studies

As outlined in Section 6.2.3.3, the measurement of ROS is an important method for ascertaining the cytocidal capacity of a phagocyte. Measurement of ROS species can be accomplished by a number of methods. The most common method entails use of colourimetric assays for the assessment of superoxide and hydrogen peroxide release from stimulated immune cells, as detailed by Rodgers (1995). An alternative method is based on the fact that the production of ROS produces molecules in electronically excited states; on relaxation to their ground state, these compounds emit photons, known as chemiluminescence (CL). Luminescence measurements are non-destructive, and unlike the available colourimetric techniques, allow for continuous monitoring of the phagocyte chemiluminescence emission (Allen, 1986). Due to their sensitivity, they are usually carried out immediately following cell activation, whereas colourimetric measurements require build-up of the oxygen species to detectable levels, an event that can require up to 24hrs.

6.5.3.1. Selection of Optimal Chemiluminescent Probe for Monitoring of CL Emission

The native CL emitted as a result of phagocyte stimulation can be amplified (100-10000-fold) through the use of various chemiluminescence probes. A choice of chemiluminescent probes are available to aid the measurement of CL emission from activated cells and two of these, luminol and lucigenin, were tested to determine their suitability for use in the assay system (Figure 6.4). From this it can be seen that only luminol was suitable for the use in monitoring the CL emission from PMA-stimulated HL-60 cells, and this probe was used in all subsequent assays. It has been previously shown that luminol and lucigenin measure temporally different ROS-generating activities (Allen, 1986), and this may account for the lack of any CL response with lucigenin as a probe, in the time interval observed. Allen (1986) also showed the choice of stimulus to affect the observation of CL emission with different chemiluminescent probes.

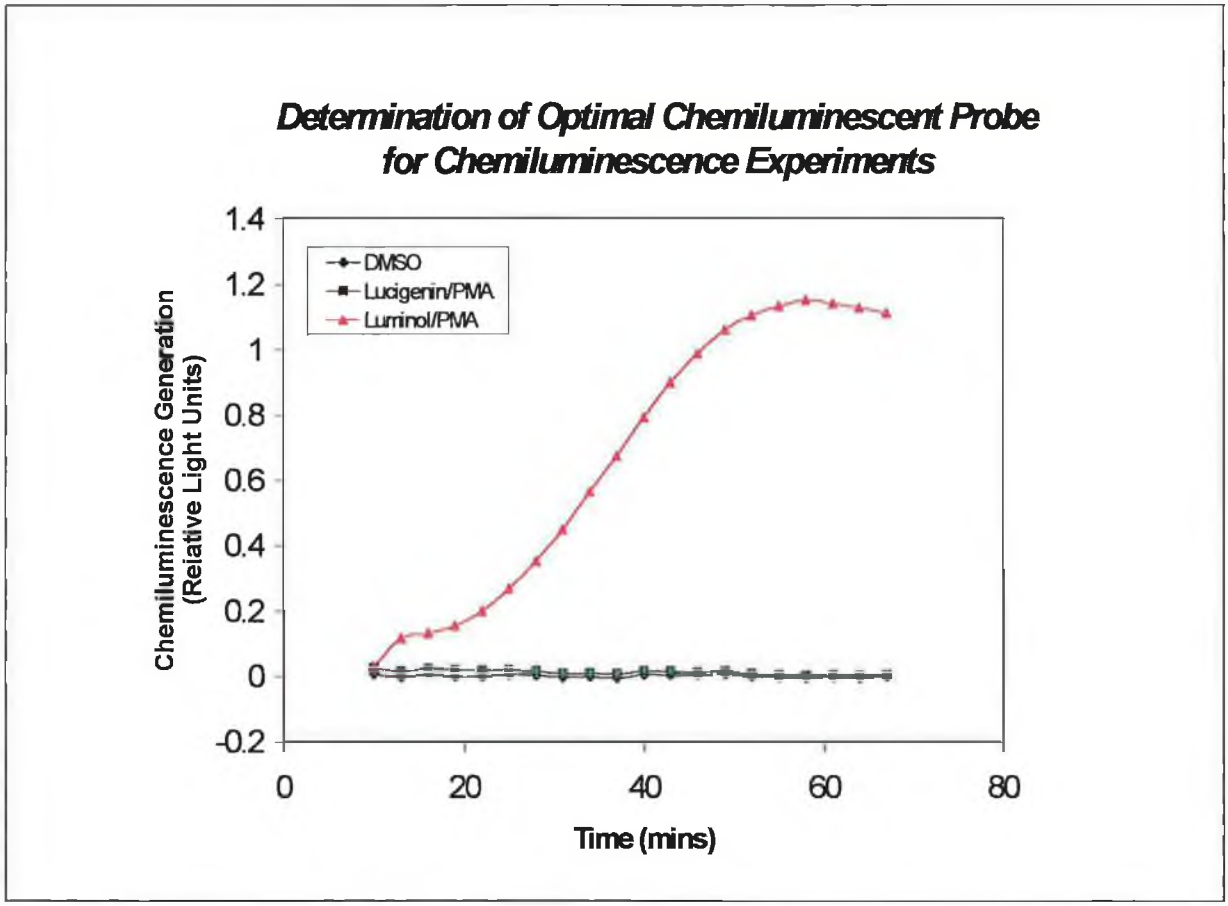


Figure 6.4: **Determination of the optimal chemiluminescent probe for the monitoring of CL emission from HL-60 cells exposed to PMA. 2 X 10^5 HL-60 cells were stimulated with 500ng/ml PMA at time = 0. CL emission from the cells was monitored for 60mins. following an initial 10min. lag period where no measurements were recorded. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 10-70mins, following PMA stimulation. The light generated, expressed as Relative Light Units (RLU), was plotted vs time in the above graph. The different probe concentrations tested were 80μM luminol and 100μM lucigenin as recommended (Allen, 1986)**

6.5.3.2. Optimisation of Cell Concentration for CL Generation

Different concentrations of HL-60 cells were incubated with 500ng/ml PMA as stimulant, in order to determine the optimum cell concentration for use in CL-emission experiments. The results are illustrated in Figure 6.5, as light-generation curves, and in Table 6.6, as integrated units (area under curve). As can be seen from Figure 6.5, light emission from HL-60 cells exposed to 500ng/ml PMA, increased as a function of the number of cells per assay in the cell concentration range 0-5 X 10^5 cells/assay. At a concentration of 5 X 10^5 cells per assay, the light generation curve started to plateau at ~ 50 mins, probably as a result of exhaustion of the CL probe luminol. Concentrations of cells of 1 X 10^6 cells per assay, exhibited an overall decreased light generation, as a result of 1) exhaustion of luminol, and more importantly 2) re-absorption of the generated-light by the cells. The optimal cell concentration chosen was 2 X 10^5 cells per assay, as the light generation event had not plateaued after the observed 60min. time period, and this concentration of cells was used in all subsequent experiments. Solvent (0.1% DMSO) controls were included in all experiments and as shown in Table 6.6, caused no stimulation of light generation by the cells.

Three different concentrations of 28SC cells were exposed to 500ng/ml PMA but as shown in Table 6.7, light emission was almost non-existent. This result was not wholly surprising when one recalls the low increases in metabolic rates observed for this cell line, using the Cytosensor Microphysiometer, on PMA stimulation (section 6.5.2). As explained in that section, this non-responsiveness to PMA may be due to the specific PKC isoforms present in these cells, or a complete loss of PKC activity as a result of cell immortalisation.

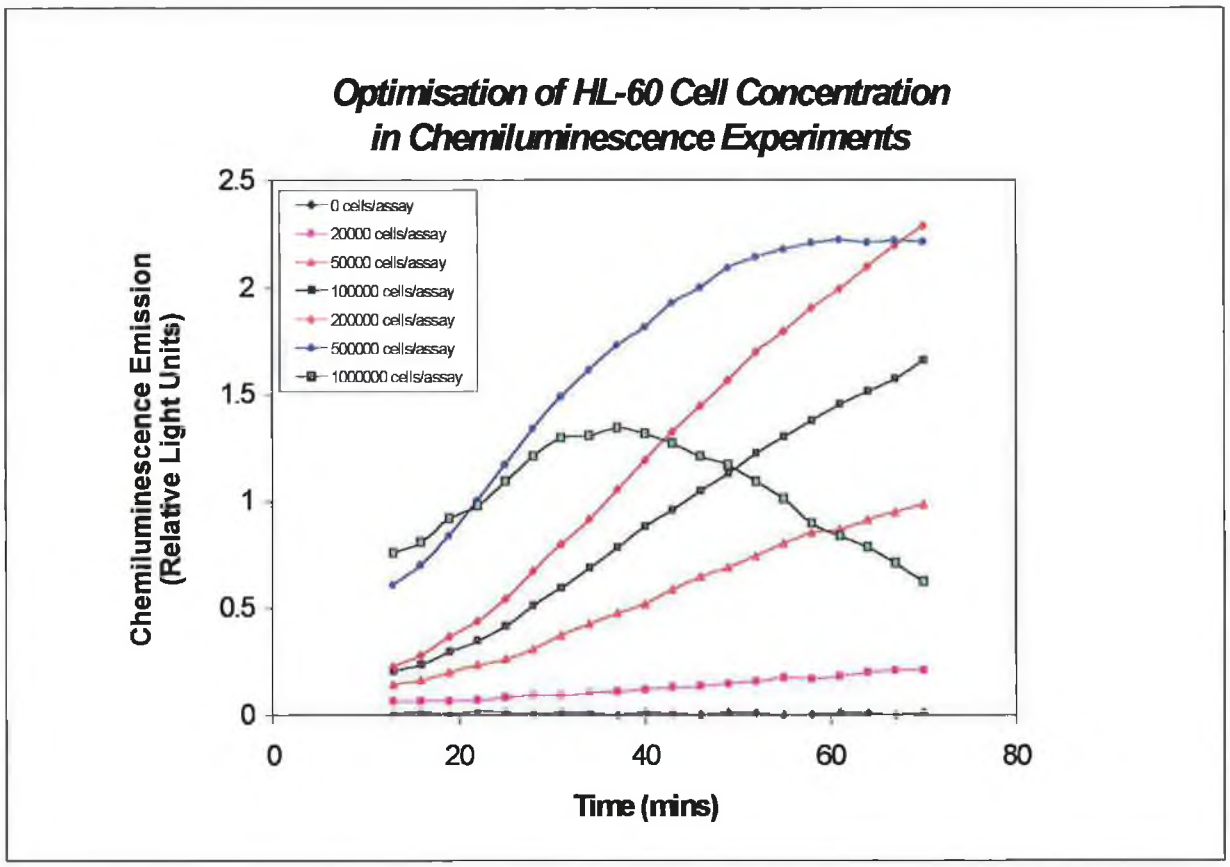


Figure 6.5: **Optimisation of cell concentration for chemiluminescence experiments on PMA-stimulated HL-60 cells. Various concentrations of HL-60 cells were incubated with 80μM luminol, and stimulated with 500ng/ml PMA, at time = 0. CL emission from the cells was monitored for 60mins. following an initial 10min. lag period where no measurements were recorded. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 10-70mins, following PMA stimulation. The light generated, expressed as Relative Light Units (RLU), was plotted vs time in the above graph. Concentrations of HL-60 cells in the range 2 X 10^4 cells/assay – 1 X 10^6 cells/assay were tested, and a sample curve is illustrated for each cell concentration. The CL emission for each cell concentration was tested in quadruplicate, and determined on three separate occasions.**

Cells per assay (HL-60)	*+ 500ng/ml PMA (Integral RLU)*	*+ 0.1% DMSO (Integral RLU)*
2 X 10^4	531.60 ± 61.54	77.77 ± 0.12
5 X 10^4	2059.67 ± 92.74	59.65 ± 2.53
1 X 10^5	3342.30 ± 89.86	36.92 ± 4.46
2 X 10^5	4319.70 ± 253.50	36.89 ± 6.55
5 X 10^5	5672.30 ± 208.14	18.67 ± 0.63
1 X 10^6	3531.30 ± 271.48	5.86 ± 0.43

Table 6.6: **Optimisation of cell concentration for chemiluminescence experiments on PMA-stimulated HL-60 cells. Concentrations of HL-60 cells in the range 2 X 10^4 cells/assay – 1 X 10^6 cells/assay were tested. Experimental conditions are outlined in the legend of Figure 6.5. This table outlines the integrated (area under light generation curves) Relative Light Units for the curves shown in Figure 6.5. All experiments were replicated 4 times, and determined on three separate occasions and the table values shown are mean ± s.d. for all such experiments. Solvent control samples (0.1% DMSO) are also included.**

Cells per Assay (28SC)	*Integral RLU*
0	3.41 ± 0.50
1 X 10^5	12.18 ± 0.62
2 X 10^5	16.71 ± 0.32
5 X 10^5	18.65 ± 5.73

Table 6.7: **Optimisation of cell concentration for chemiluminescence experiments on PMA-stimulated 28SC cells. Concentrations of 28SC cells in the range 1 X 10^5 cells/assay – 5 X 10^5 cells/assay were tested. Experimental conditions are identical to those used for HL-60 cells, outlined in the legend of Figure 6.5. This table details the integrated (area under light generation curves) Relative Light Units. All experiments were carried out in triplicate, and determined on three separate occasions and the table values shown are mean ± s.d. for all such experiments.**

6.5.3.3. Optimisation of Cell Stimulation in Chemiluminescent Experiments

From the experiments outlined in sections 6.5.2 and 6.5.3.2, PMA appeared to act as an appropriate activator of HL-60 cells, with activation leading to the production of ROS, as shown by CL-emission. Figure 6.2 shows that optimal activation of HL-60 cells was achieved with 500ng/ml PMA. We wished to confirm that this was the most appropriate PMA concentration for optimal CL-emission. Therefore we carried out suitable experiments as illustrated in Figure 6.6. As shown, the PMA concentration increased CL emission from HL-60 cells in a dose-dependent manner, with a large emission of light (> 1000 RLU) created at PMA concentrations > 5ng/ml. We therefore arbitrarily chose 500ng/ml PMA as the concentration to use in all subsequent experiments, as it had been the optimal stimulatory concentration for HL-60 cells as observed on the Cytosensor Microphysiometer (section 6.5.2). In the CL experiments, this relatively low stimulant concentration caused a large light emission (4313.46 ± 203.67 Integrated RLU).

In section 6.2.1, the concept of sequential "priming" and "activation" of MPS cells was introduced. Therefore, we examined the effect of other stimulators or modulators on the CL emission of HL-60 cells, and the results of these experiments are shown in Table 6.8. Firstly, we utilised the common phagocyte stimulator, opsonised zymosan (OZ), [a cell wall preparation of *Saccharomyces cerevisiae*, prepared as outlined in section 2.2.7.2], as a stimulator of CL emission from HL-60 cells. We found this effectively activated HL-60 cells to generate ROS, and emit chemiluminescence, but was not as competent as PMA at this task (Table 6.8). When HL-60 cells were incubated for 24hrs with either 10ng/ml LPS or 100U/ml IFN-γ (as a "priming" step), the light emission from HL-60 cells on stimulation with 500ng/ml PMA increased significantly (Table 6.8). Similar results were obtained for "primed" HL-60 cells activated with 250μg/ml OZ (Table 6.8). These results reflect well those of other investigators using CL-based assays to examine the modulation and stimulation of macrophage cell lines (De Baetselier & Schram, 1986). It should also be noted here that incubation of IFN-γ (100-1000U/ml) with HL-60 cells for 5 days has been previously shown to induce differentiation to monocytes (Harris *et al.*, 1985), therefore the increase in CL may be accompanied by early differentiation changes.

In sections 6.5.2 and 6.5.3.2, it was demonstrated that PMA does not effectively activate 28SC cells. Therefore, we examined the effect of other immune cell modulators and stimulators on the generation of ROS (as measured by CL emission). The results of these experiments are outlined in Table 6.9. As shown, "priming" of 28SC cells by pre-incubation with either LPS or IFN-γ, did result in an increase in the emission of light from PMA-stimulated 28SC cells compared to

"unprimed" cells. However, the light emission was still extremely low, and insignificant, when compared to those values obtained for similarly treated HL-60 cells (Table 6.8). 28SC cells were shown to be capable of CL emission when exposed to the phagocytic stimulator, opsonised zymosan (Table 6.9). The generation of ROS (as assessed by CL emission) was significantly improved for OZ compared to PMA (Figure 6.7). Pre-incubation with 10ng/ml LPS only slightly augmented the OZ-induced light emission (Table 6.9). However, pre-incubation of 28SC cells with 100U/ml IFN-γ, caused a significant (4.7-fold) increase in the CL-emission from these cells when stimulated with opsonised zymosan (Table 6.9).

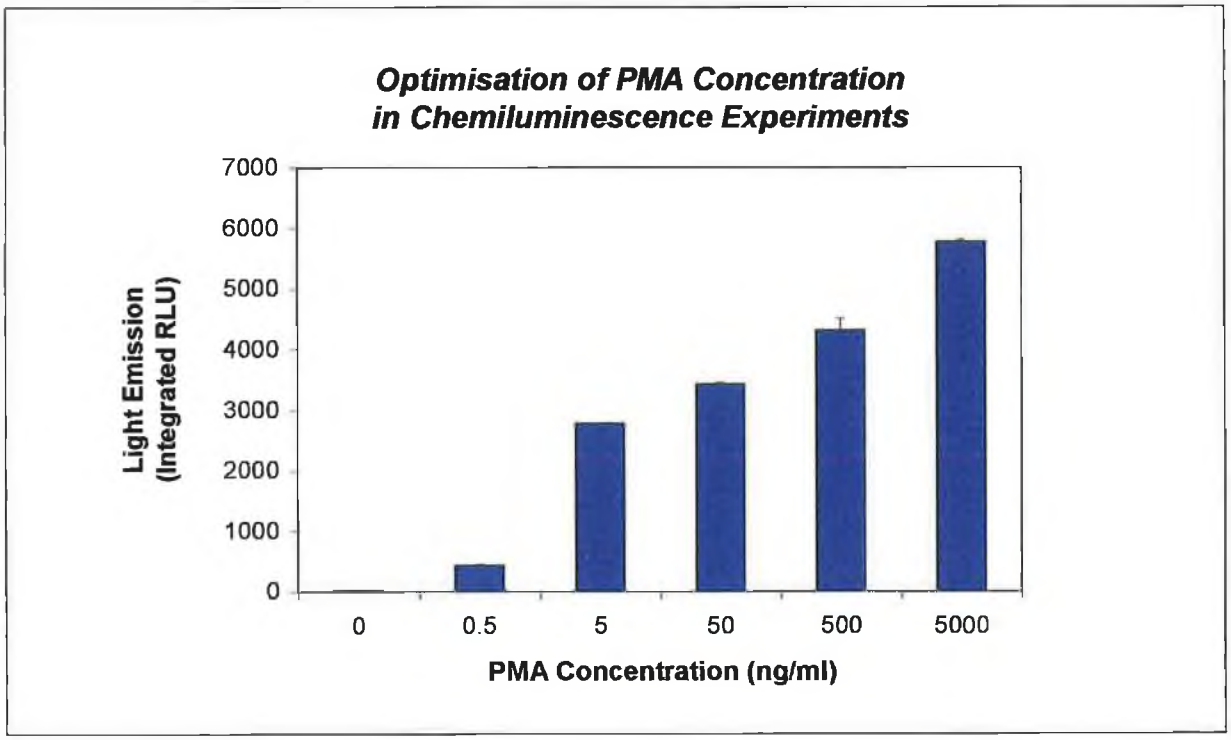


Figure 6.6: **Optimisation of PMA concentration for chemiluminescence experiments on HL-60 cells (2 X 10^5 cells/assay). HL-60 cells were incubated with 80μM luminol, and stimulated with various concentrations of PMA, at time = 0. CL emission from the cells was monitored for 60mins. following an initial 10min. lag period where no measurements were recorded. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 10-70mins, following PMA stimulation. The light generated, expressed as Integral Relative Light Units (RLU), was plotted vs cell concentration in the above graph. Concentrations of PMA in the range 0-5000ng/ml PMA were tested. The CL emission for each concentration was tested in quadruplicate, and repeated on four separate occasions.**

Stimulatory Agent	*HL-60 Cells*	*HL-60 Cells + 10ng/ml LPS*	*HL-60 Cells + 100U/ml IFN-γ*
0.1% DMSO	36.89 ± 6.55	47.74 ± 4.60	60.46 ± 1.95
500ng/ml PMA	4313.46 ± 203.67	8991.68 ± 79.88	13929.30 ± 560.94
250μg/ml OZ	1490.60 ± 54.61	4443.65 ± 568.09	15530.00 ± 231.00

Table 6.8: **Effect of various modulators and stimulators on the generation of light from HL-60 cells. HL-60 cells were incubated for 24hrs in the absence or presence of LPS (10ng/ml) or IFN-γ (100U/ml), prior to activation with either 500ng/ml PMA or 250μg/ml OZ. Measurement of chemiluminescence was achieved exactly as outlined in the legend of Figure 6.6. All modulator/stimulator combinations were tested in triplicate, on at least three separate occasions, and the mean ± s.d. provided in the table.**

Stimulatory Agent	*28SC Cells*	*28SC Cells + 10ng/ml LPS*	*28SC Cells + 100U/ml IFN-γ*
0.1% DMSO	0.70 ± 1.54	1.39 ± 0.75	6.01 ± 1.02
500ng/ml PMA	16.71 ± 0.32	27.16 ± 2.27	72.70 ± 1.68
250μg/ml OZ	413.90 ± 30.75	455.88 ± 35.52	1955.75 ± 59.99

Table 6.9: **Effect of various modulators and stimulators on the generation of light from 28SC cells. 28SC cells were incubated for 24hrs in the absence or presence of LPS (10ng/ml) or IFN-γ (100U/ml), prior to activation with either 500ng/ml PMA or 250μg/ml OZ. Measurement of chemiluminescence was achieved exactly as outlined in the legend of Figure 6.7. All modulator/stimulator combinations were tested in triplicate, on three separate occasions, and the mean ± s.d. provided in the table.**

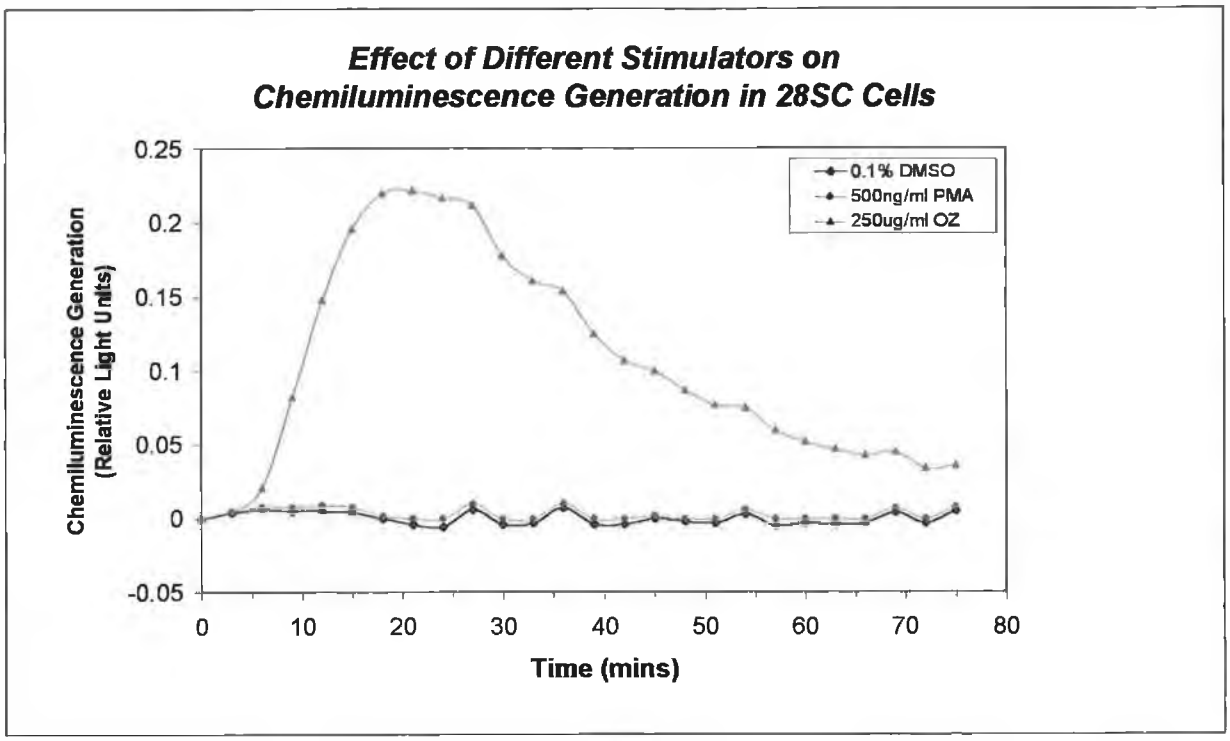


Figure 6.7: **28SC cells (2 X 10^5 cells/assay) were incubated with 80μM luminol, and stimulated with either 500ng/ml PMA, or 250μg/ml OZ, at time = 0. CL emission from the cells was monitored continuously for 72mins. following stimulation. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 0-72mins. The light generated, expressed as Relative Light Units (RLU), was plotted vs time in the above graph. Concentrations of PMA in the range 0-5000ng/ml PMA were tested. The CL emission for each concentration was tested in triplicate, and determined on three separate occasions.**

6.5.4. Time-dependent Decrease in the Response of HL-60 cells to PMA

Results from the previous sections, illustrated that of the two cell-lines tested, the HL-60 cells were more appropriate for use as a model for assaying ROS generation by chemiluminescence. However, as we were carrying out experiments with these cells we perceived that they too could prove problematic. We noticed that over time, the HL-60 cells were losing their ability to emit chemiluminescence in response to stimuli such as PMA. As shown in Figure 6.8, during culture, the HL-60 cells appeared to lose their capacity for light-generation. Immediately following resuscitation from liquid nitrogen, the HL-60 cells were immune to the stimulatory effects of PMA, but by Day 5, the cells could respond to PMA-stimulation with the emission of large levels of chemiluminescence. This response was sustained at high levels until about Day 10-12, after which time the luminescence response started to decrease rapidly over time to almost base-line levels by Day 20 (Figure 6.8 & Table 6.10). The most likely explanation of this effect is the differentiation of the HL-60 cells over time in culture. The HL-60 cells are pro-monocytic in nature, but are known to differentiate over time to cells more monocyte/macrophage-like in nature. Such a differentiation step is often accompanied by alterations in the levels of cellular enzymes, including peroxidases. In fact, the level of peroxidases peaks in pro-monocytes, and falls on differentiation of these cells to monocytes and macrophages (Adams & Hamilton, 1992; Auger & Ross, 1992), an event which might explain the decrease in ROS generation in HL-60 cells over time. In addition, as mentioned earlier, differentiation of cells may result in a change in the levels of PMA-responsive PKC isozymes, resulting in the observed decrease in luminescence.

We determined whether this decrease in ROS generation, was mirrored by a general decrease in cell activation in PMA-stimulated HL-60 cells, using the Cytosensor Microphysiometer, as outlined in Section 6.5.2. The results are shown in Figure 6.9. It can be seen that the loss of chemiluminescence as a result of extended culture (Figure 6.10), was mirrored by a decreasing metabolic activation of HL-60 cells, in response to PMA over time, with an eventual complete loss of activation, following 20 days of culture. As a result of this observation, all further chemiluminescence experiments (Section 6.5.5) used only HL-60 cells, within Days 5-12 of their resuscitation.

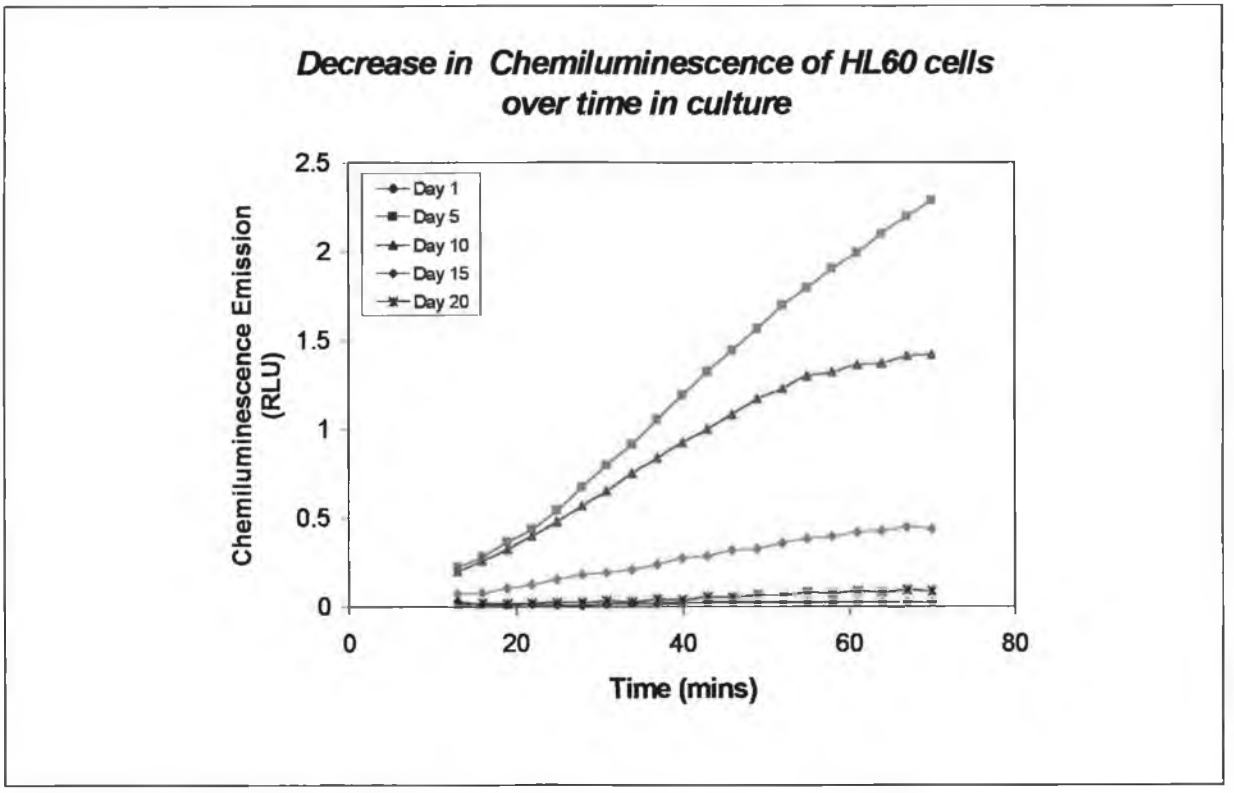


Figure 6.8: **Effect of culture time on the CL emission from PMA-stimulated HL-60 cells. HL-60 cells were resuscitated from liquid nitrogen on Day 1, and cultured as outlined in section 2.2.3.3. For experiments, HL-60 cells, cultured for the appropriate length of time, were incubated with 80μM luminol, and stimulated with 500ng/ml of PMA, at time = 0. CL emission from the cells was monitored for 60mins. following an initial 10min. lag period where no measurements were recorded. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 10-70mins, following PMA stimulation. The light generated, expressed as Relative Light Units (RLU), was plotted vs time. This experiment was repeated on three separate occasions to ensure the observed luminescence decrease was accurate. The results of one experiment are shown in the above graph.**

Days following Resuscitation	*Integral RLU*
Day 1	92.99 ± 15.67
Day 5	4566.4 ± 314.99
Day 10	3378.00 ± 52.78
Day 15	884.54 ± 18.09
Day 20	228.04 ± 9.88

Table 6.10: **Effect of culture on luminescence generation in PMA-stimulated HL-60 cells. Experimental conditions are outlined in the legend of Figure 6.8. This table outlines the integrated (area under light generation curves) Relative Light Units for the curves shown in Figure 6.8. All experiments were carried out in triplicate, on three separate occasions and the table values shown are mean ± s.d. for these experiments.**

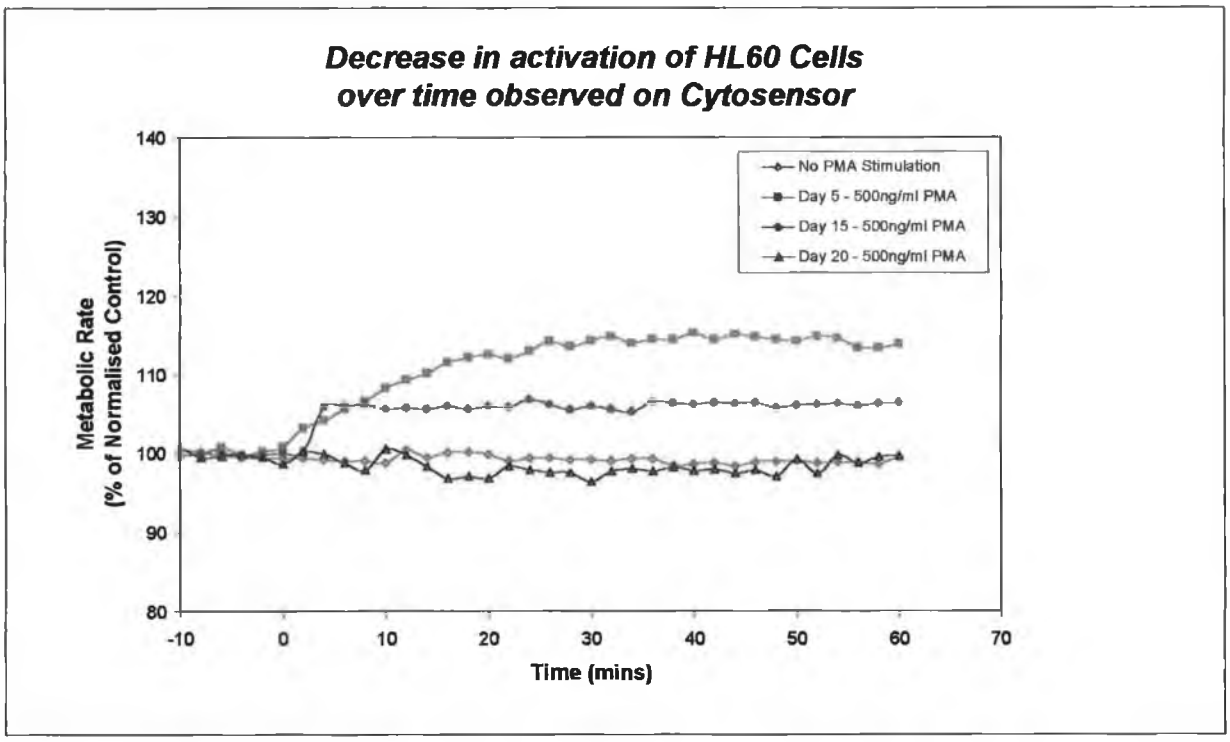


Figure 6.9: **Decreased response of HL-60 cells to PMA-stimulation, as a function of their time in culture, ascertained using the Cytosensor Microphysiometer. HL-60 cells were resuscitated from liquid nitrogen on Day 1, and cultured as outlined in section 2.2.3.3. For experiments, HL-60 cells, cultured for the appropriate length of time, were assembled onto the Cytosensor Microphysiometer, as outlined in Section 2.2.7.4, and stimulated with 500ng/ml PMA, over 60mins. The above plot shows one of three experiments conducted to determine if extended periods of culture affected the activation of HL-60 cells in response to PMA (Section 2.2.7.4). In individual experiments, the cellular acidification rate was determined prior to PMA exposure, using a 2 min pump cycle, with the acidification rate measured in the final 30secs. of this cycle. This rate was normalised at 100% and all subsequent acidification rates during PMA-exposure expressed as a percentage of this normalised value. These values of metabolic rate were plotted vs time in the above graph. Typical basal acidification rates (-μvolts/sec) for the HL-60 cells in the experiments were –80 to -130μvolts/sec.**

6.5.5. Effect of Coumarin Compounds on ROS Generation from Monocytic Cells – Chemiluminescence Studies

From the results obtained in Sections 6.5.3 and 6.5.4, it was decided that the HL-60 cell line (Day 5-12) was the more appropriate model, of the two cell lines tested, for assessing the effects of coumarin compounds on ROS generation. It was decided to assess the effect of coumarin compounds on the generation of ROS species in two different scenarios – 1) HL-60 cells activated with 500ng/ml PMA, and 2) HL-60 cells primed with 100U/ml IFN-γ for 24hrs, and activated with 500ng/ml PMA. However, prior to carrying out these experiments, it was necessary to complete a number of control experiments, as detailed in the following paragraphs.

As outlined in section 6.3, the coumarin compounds have been shown previously to decrease the production of ROS from fresh donor human blood mononuclear cells (PBMC), but not cultured PBMC, on PMA stimulation. (Marshall *et al.*, 1989b). However, the experimental set-up in these studies involved the measurement of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) production, with coumarins present in the reaction mixture. As compounds of the coumarin family have been previously shown to act as free-radical scavengers (Paya *et al.*, 1992), it is unclear whether the observed reductions in ROS generation are a result of this scavenging capability, or due to other effects. Therefore, for our assay system we initially evaluated the effect of coumarins (coumarin, 7-hydroxycoumarin and esculetin) on the generation of ROS from PMA-stimulated HL-60 cells, by including or omitting these compounds in the assayed reaction mix (Figure 6.10). As can be seen from Figure 6.10, concentrations of >50μg/ml of coumarin, and > 10μg/ml 7-hydroxycoumarin, caused decreases in the observed emission of chemiluminescence (generation of ROS) from PMA-stimulated HL-60 cells over a 1hr period. Therefore, the decreases in ROS production observed by Marshall and colleagues (1989b) over a 24hr period, are probably due to this "scavenging effect". With regard to esculetin, all tested concentrations of this drug (0.1-50μg/ml), had severe effects on CL-emission, again probably as a result of free-radical scavenging. It was therefore concluded that all further luminescence readings would be acquired in the absence of coumarins in the assay wells. Therefore, all cells exposed to coumarins were washed twice in drug-free salt solution, prior to cell stimulation, to ensure complete removal of drug traces from the extracellular medium.

Another necessary control experiment was that to determine the effect of the coumarin compounds on the natural "basal" level of chemiluminescence emission in HL60 cells. This was achieved to ensure that incubation of coumarins with HL60 cells did not themselves "activate" the

cells for increased luminescence emission. As shown in Table 6.11, the low levels of natural luminescence of HL-60 cells did not change significantly in cells exposed to the different coumarin compounds for 24hrs. This insignificance is clearly demonstrated when one compares the levels of "basal" luminescence emitted from these drug-treated cells, with the luminescence emitted when cells are stimulated with 500ng/ml PMA. A 1000-5000-fold difference in light emission is observed, which confirms the fact that the coumarin compounds do not themselves activate CL-emission from HL-60 cells.

Having completed these control experiments, we next investigated the effects of coumarin compounds on the generation of ROS in PMA-stimulated HL-60 cells (500ng/ml PMA), which had been incubated in the absence or presence of IFN-γ for 24hrs (Figures 6.11-6.16). Incubation with coumarin compounds was achieved for either 1 or 24hrs. Coumarin (1 or 24hr pre-exposure) did not effect ROS generation in either unprimed or IFN-primed HL-60 cells (Figures 6.11 and 6.12), with CL-emission was between 95-110% of control emission. The only exception was for cells exposed to 100μg/ml for 24hrs (CL-emission was suppressed ~35-40%). 1hr 7-HC pre-exposure did not significantly alter the generation of ROS in unprimed or IFN-primed HL-60 cells (Figures 6.13 and 6.14), with increases of ~6-14% observed. 24hr exposure to 7-HC did cause a dose-dependent suppression of ROS-generation (Figure 6.13). IFN-priming reversed this suppression at lower 7-HC concentrations (Figure 6.14). Esculetin pre-exposure (1 and 24hrs) caused a dose-dependent suppression of ROS-generation, with the effects more potent on 1hr exposure (Figures 6.15 and 6.16). IFN-priming partially reversed this effect in cells pre-exposed to esculetin for 1hr, but had little effect on cells exposed to esculetin for 24hrs (Figures 6.15 and 6.16).

From these results, a number of points can be made. Firstly, the coumarin compounds investigated did not appear at any concentration to display significant stimulatory effects to the monocytic cells. Previously, various reports have described the general stimulatory effect of coumarins on MPS cells (Piller, 1978; Koh & Willoughby, 1979, Marshall *et al.*, 1991b). This general effect does not appear to translate into a stimulation of ROS generation in this "model" monocyte system. However, Filice and Reminton (1981) disputed the fact that coumarin could elicit a microbicidal or tumouricidal response at all in mouse macrophages. Overall, we were surprised at the obtained result, as the applied stimulus (PMA) activates the Protein Kinase C signalling pathway, and coumarin has been previously shown to augment LPS-induced cytokine production, by networking with the protein kinase C and lipoxygenase pathways (Zlabinger *et al.*, 1993). In addition, our results from Chapter 5 suggested that the coumarins interact synergistically with PKC, enhancing its effect. Hence, we expected an increase in ROS generation to be observed.

Another unusual result was the observation that IFN-γ was capable of partially reverting the coumarin-induced suppression of CL-emission, suggesting that the two compounds may effect similar intercellular signalling pathways. IFN-γ is known to signal through its receptor, activating JAKs and STATs [c.f. Section 5.2.1], an event that involves tyrosine phosphorylation (Leonard & O'Shea, 1998). However, it is believed that the protein kinase C pathway is also involved (Adams & Hamilton, 1992), complicating the question of the cellular target of coumarins.

Finally, it must be admitted that the experimental set-up, with washing of cells to remove excess coumarins, would not have removed intracellular coumarin, therefore scavenging of ROS by intracellular coumarin may still have occurred. This might explain the unexpected results detailed above, as the scavenging effects of coumarins may be much stronger than their PKC effects. Scavenging might also explain the dose-dependency of CL-suppression, and the increase in CL-suppression on 24hr exposure to drugs. Regardless, of whether this is the reason for the dose-and time-dependent suppression of PMA-stimulated CL, the fact remains that *in vivo* ROS scavenging would also occur. Thus, the anti-tumour effect of coumarins is unlikely to be due to augmentation of ROS-generation by immune cells.

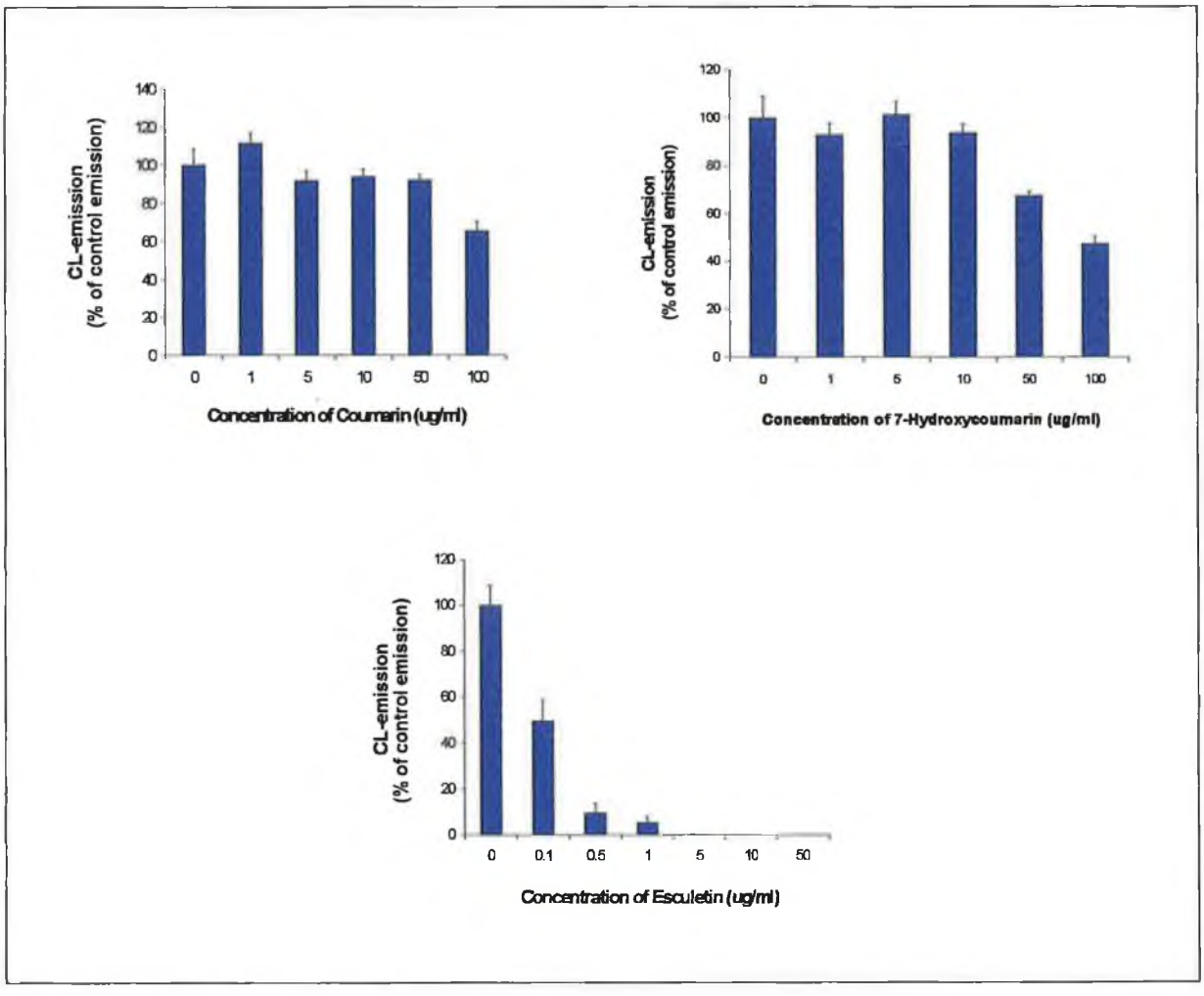


Figure 6.10: **Effect of coumarins on CL-emission from PMA-stimulated HL-60 cells. HL-60 cells (2 X 10^5/assay) were incubated with 80μM luminol in the presence or absence of various coumarin concentrations, and stimulated to emit chemiluminescence, with 500ng/ml PMA, at time = 0. CL emission from the cells was monitored for 60mins. following an initial 10min. lag period where no measurements were recorded. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 10-70mins, following PMA stimulation. The light generated from control HL-60 cells (unexposed to drugs), expressed as Integral Relative Light Units (RLU), was normalised at 100%, as the control CL-emission. CL-values from all drug treated cells were expressed as a percentage of the control CL-emission. These values are plotted vs drug concentrations in the above bar charts. This experiment was carried out in triplicate and repeated on three separate occasions. The CL-emission from control cells was 3024.43 ± 508.03 RLU.**

Drug Concentration (μg/ml)	*Coumarin*	*7-Hydroxycoumarin*	*Esculetin*
0μg/ml Drug (+ PMA)		4319.70 ± 253.50	
0		36.89 ± 6.55	
0.1	N.D.	N.D.	21.57 ± 0.52
0.5	N.D.	N.D.	5.14 ± 2.57
1	43.01 ± 3.32	12.58 ± 0.87	3.00 ± 0.29
5	37.96 ± 0.90	12.97 ± 0.58	6.54 ± 1.44
10	41.04 ± 5.05	20.91 ± 3.52	9.24 ± 0.06
50	42.68 ± 5.76	6.81 ± 0.14	10.50 ± 1.21
100	51.28 ± 0.87	11.86 ± 2.77	N.D.

Table 6.11: **Effect of coumarin compounds on the background luminescence of HL-60 cells. In these experiments, HL-60 cells (2 X 10^5/assay) were incubated ± various drug concentrations for 24 hrs. These cells were then washed, incubated with 80μM luminol, and their "basal" level of CL-emission determined over 60 mins. CL emission from the cells was monitored for 60mins. following an initial 10min. lag period where no measurements were recorded. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 10-70mins. The light generated was expressed as Integral Relative Light Units (RLU) in the above table. The CL emission for each drug concentration was tested in triplicate, and determined on three separate occasions. For comparative purposes, the CL-emission of HL-60 cells exposed to 500ng/ml PMA is also shown in the table.**

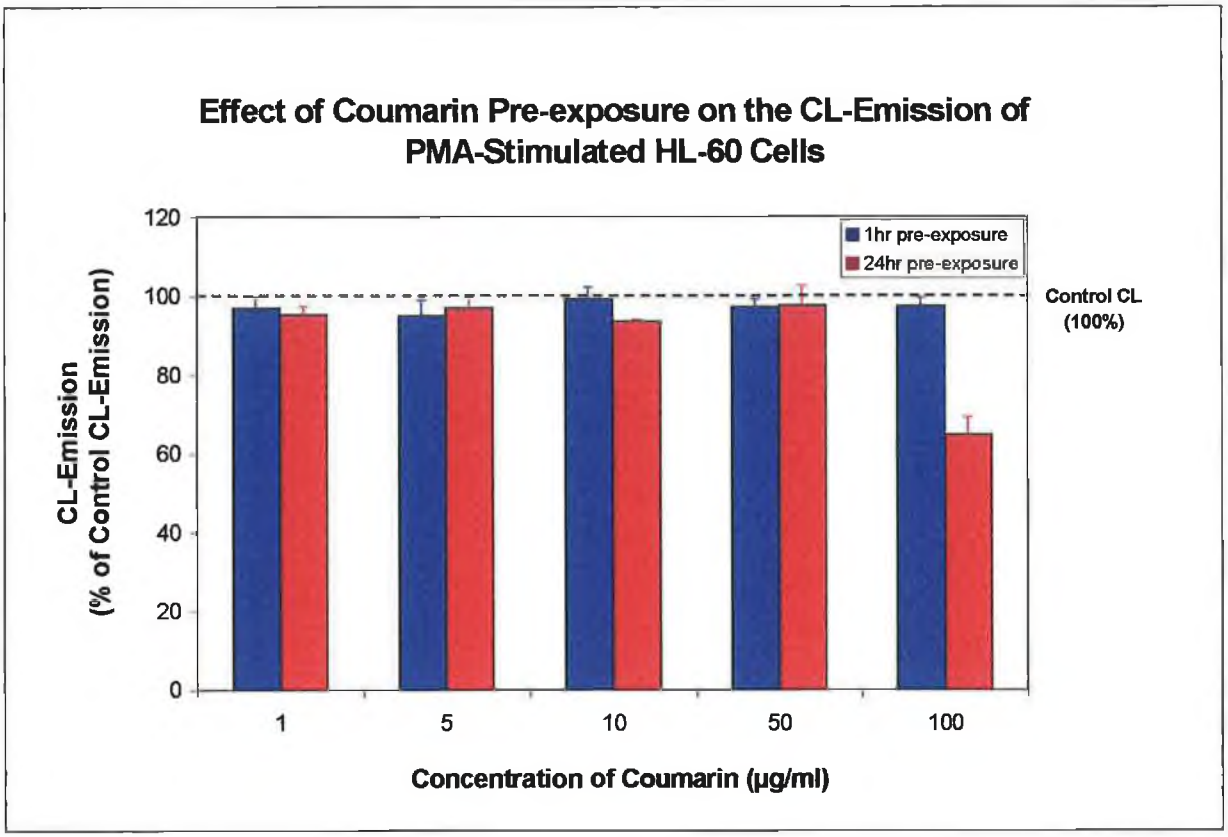


Figure 6.11: **Effect of coumarin on the emission of chemiluminescence from HL-60 cells. Cells were pre-exposed to coumarin (1-100µg/ml) for either 1hr or 24hrs. These cells were then used in the following experiment: 2 X 10^5 cells/assay were incubated with 80µM luminol and stimulated to emit chemiluminescence, with 500ng/ml PMA, at time = 0. CL emission from the cells was monitored for 60mins. following an initial 10min. lag period where no measurements were recorded. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 10-70mins, following PMA stimulation. The light generated from control HL-60 cells (unexposed to drugs), expressed as Integral Relative Light Units (RLU), was normalised at 100% as the control CL-emission. CL-values from all drug treated cells were expressed as a percentage of the control CL-emission. These values are plotted vs drug concentrations in the above bar charts. This experiment was carried out in triplicate and repeated on three separate occasions. The CL-emission from control cells was 4026.93 ± 349.15 RLU (1hr) & 5033.63 ± 327.85 (24hr)**

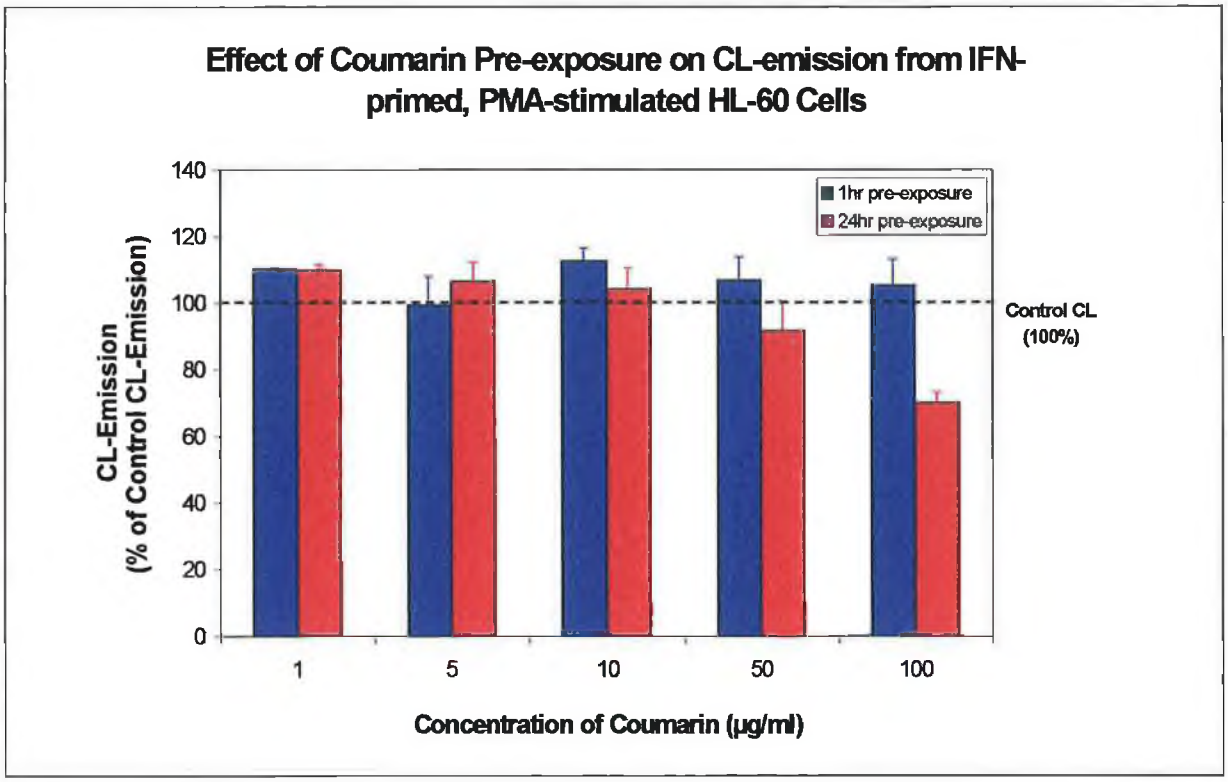


Figure 6.12: **Effect of coumarin on the emission of chemiluminescence from HL-60 cells. Cells were "primed" with IFN-γ (100U/ml) for 24hrs prior to PMA stimulation, and were pre-exposed to coumarin (1-100µg/ml) for either 1hr or 24hrs of this time period. These cells were then used in the following experiment: 2 X 10^5 cells/assay were incubated with 80µM luminol and stimulated to emit chemiluminescence, with 500ng/ml PMA, at time = 0. CL emission from the cells was monitored for 60mins. following an initial 10min. lag period where no measurements were recorded. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 10-70mins, following PMA stimulation. The light generated from control HL-60 cells ("primed" with IFN-γ, but unexposed to drugs), expressed as Integral Relative Light Units (RLU), was normalised at 100% as the control CL-emission. CL-values from all drug treated cells were expressed as a percentage of the control CL-emission. These values are plotted vs drug concentrations in the above bar charts. This experiment was carried out in triplicate and repeated on three separate occasions. The CL-emission from control cells was 13440.5 ± 1378.27 (1hr) & 12702.92 ± 1454.21 (24hr) RLU.**

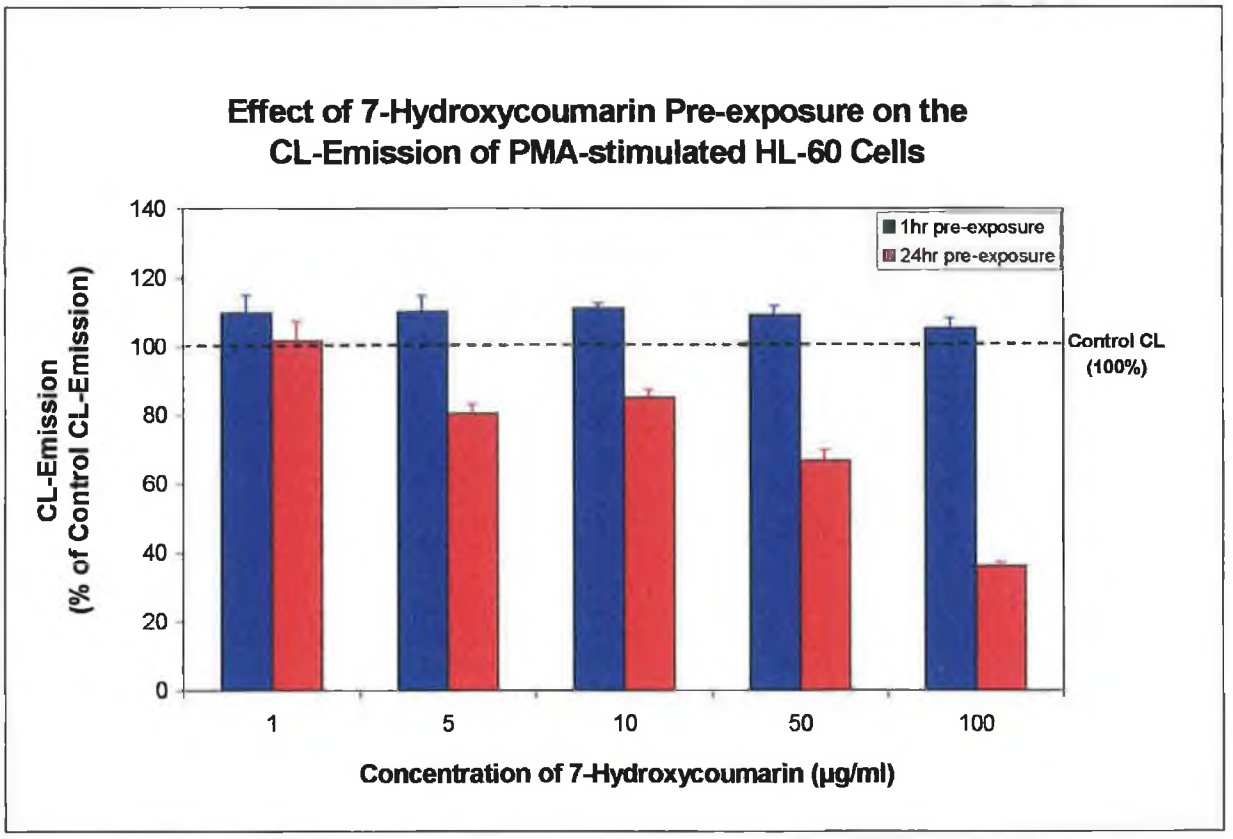


Figure 6.13: **Effect of 7-hydroxycoumarin on the emission of chemiluminescence from HL-60 cells. Cells were pre-exposed to 7-hydroxycoumarin (1-100µg/ml) for either 1hr or 24hrs. These cells were then used in the following experiment: 2 X 10^5 cells/assay were incubated with 80µM luminol and stimulated to emit chemiluminescence, with 500ng/ml PMA, at time = 0. CL emission from the cells was monitored for 60mins. following an initial 10min. lag period where no measurements were recorded. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 10-70mins, following PMA stimulation. The light generated from control HL-60 cells (unexposed to drugs), expressed as Integral Relative Light Units (RLU), was normalised at 100% as the control CL-emission. CL-values from all drug treated cells were expressed as a percentage of the control CL-emission. These values are plotted vs drug concentrations in the above bar charts. This experiment was carried out in triplicate and repeated on two separate occasions. The CL-emission from control cells was 3743.81 ± 426.09 (1hr) & 5179.60 ± 729.14 (24hr) RLU.**

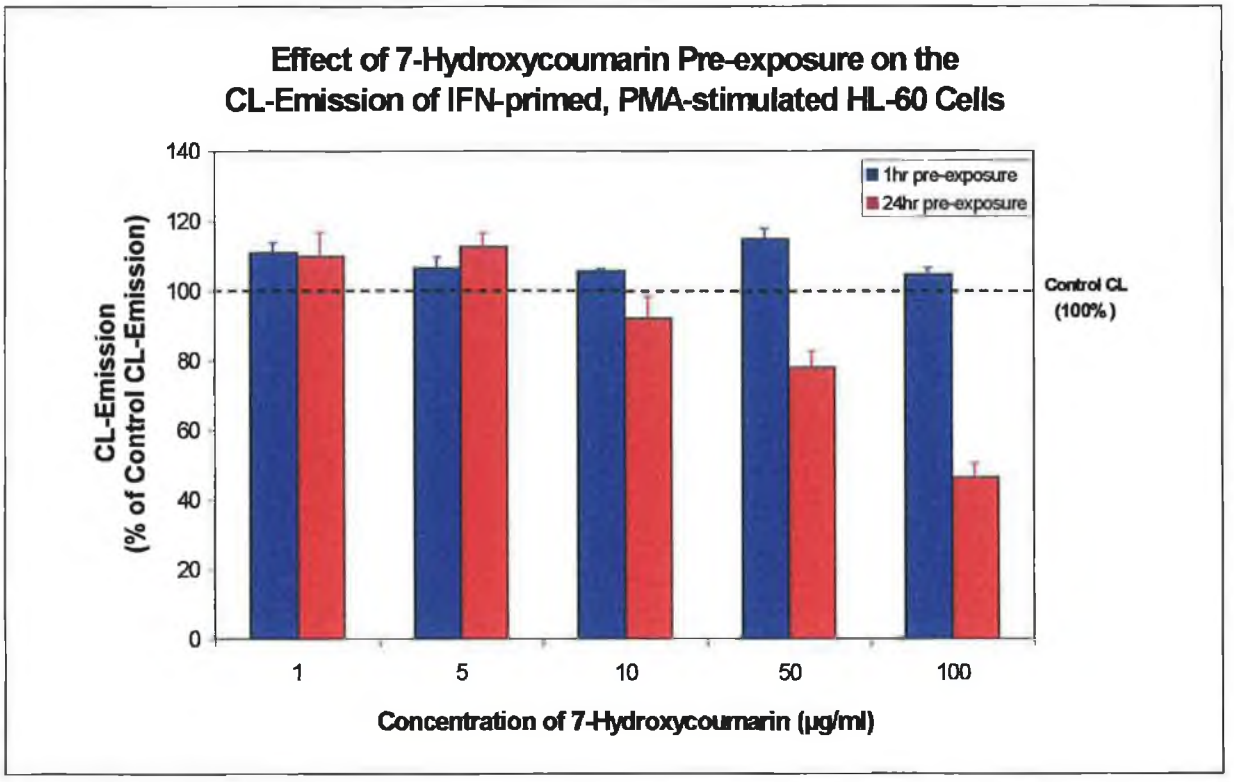


Figure 6.14: **Effect of 7-hydroxycoumarin on the emission of chemiluminescence from HL-60 cells. Cells were "primed" with IFN-γ (100U/ml) for 24hrs prior to PMA stimulation, and were pre-exposed to 7-hydroxycoumarin (1-100µg/ml) for either 1hr or 24hrs of this time period. These cells were then used in the following experiment: 2 X 10^5 cells/assay were incubated with 80µM luminol and stimulated to emit chemiluminescence, with 500ng/ml PMA, at time = 0. CL emission from the cells was monitored for 60mins. following an initial 10min. lag period where no measurements were recorded. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 10-70mins, following PMA stimulation. The light generated from control HL-60 cells ("primed" with IFN-γ, but unexposed to drugs), expressed as Integral Relative Light Units (RLU), was normalised at 100% as the control CL-emission. CL-values from all drug treated cells were expressed as a percentage of the control CL-emission. These values are plotted vs drug concentrations in the above bar charts. This experiment was carried out in triplicate and repeated on three separate occasions. The CL-emission from control cells was 10648.25 ± 493.58 (1hr) & 12095.88 ± 1269.82 (24hr) RLU.**

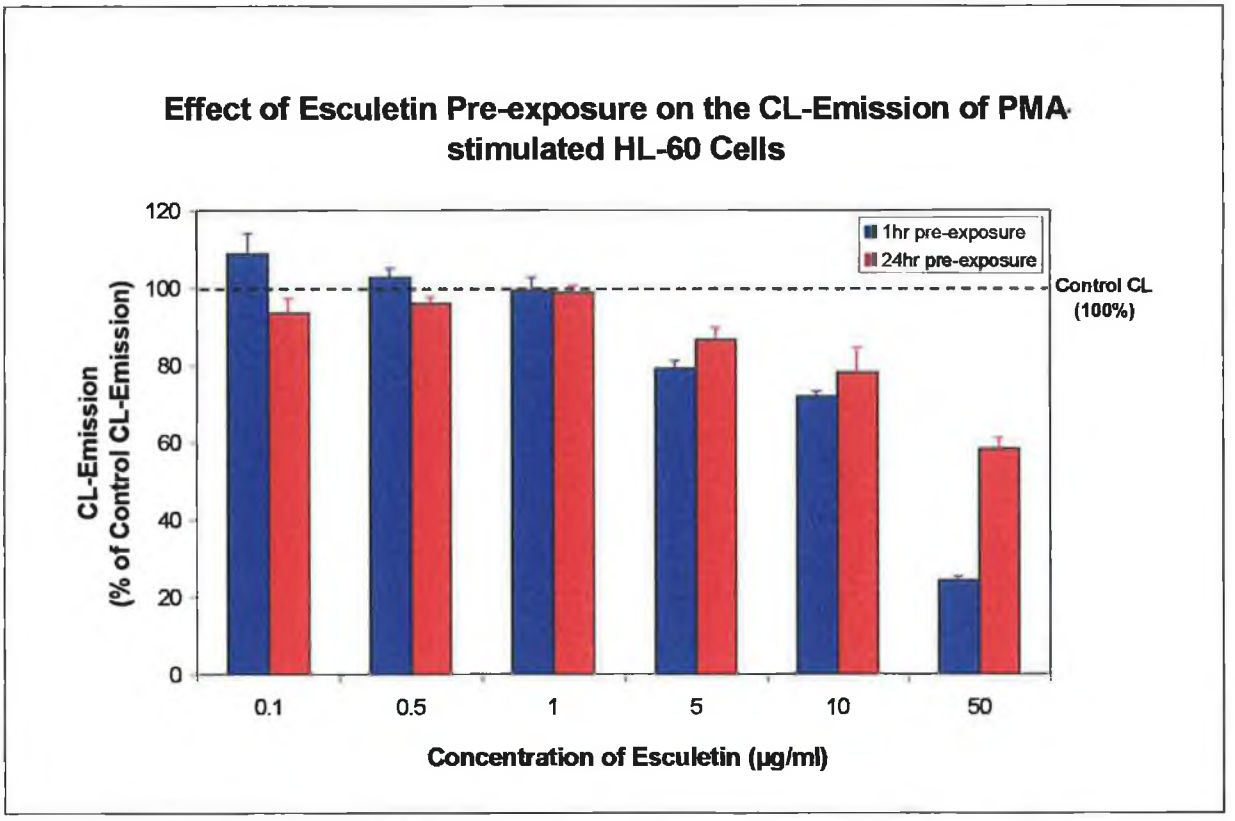


Figure 6.15: **Effect of esculetin on the emission of chemiluminescence from HL-60 cells. Cells were pre-exposed to esculetin (0.1-50µg/ml) for either 1hr or 24hrs. These cells were then used in the following experiment: 2 X 10^5 cells/assay were incubated with 80µM luminol and stimulated to emit chemiluminescence, with 500ng/ml PMA, at time = 0. CL emission from the cells was monitored for 60mins. following an initial 10min. lag period where no measurements were recorded. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 10-70mins, following PMA stimulation. The light generated from control HL-60 cells (unexposed to drugs), expressed as Integral Relative Light Units (RLU), was normalised at 100% as the control CL-emission. CL-values from all drug treated cells were expressed as a percentage of the control CL-emission. These values are plotted vs drug concentrations in the above bar charts. This experiment was carried out in triplicate and repeated on three separate occasions. The CL-emission from control cells was 3835.86 ± 435.22 (1hr) & 3534.21 ± 973.36 (24hr) RLU.**

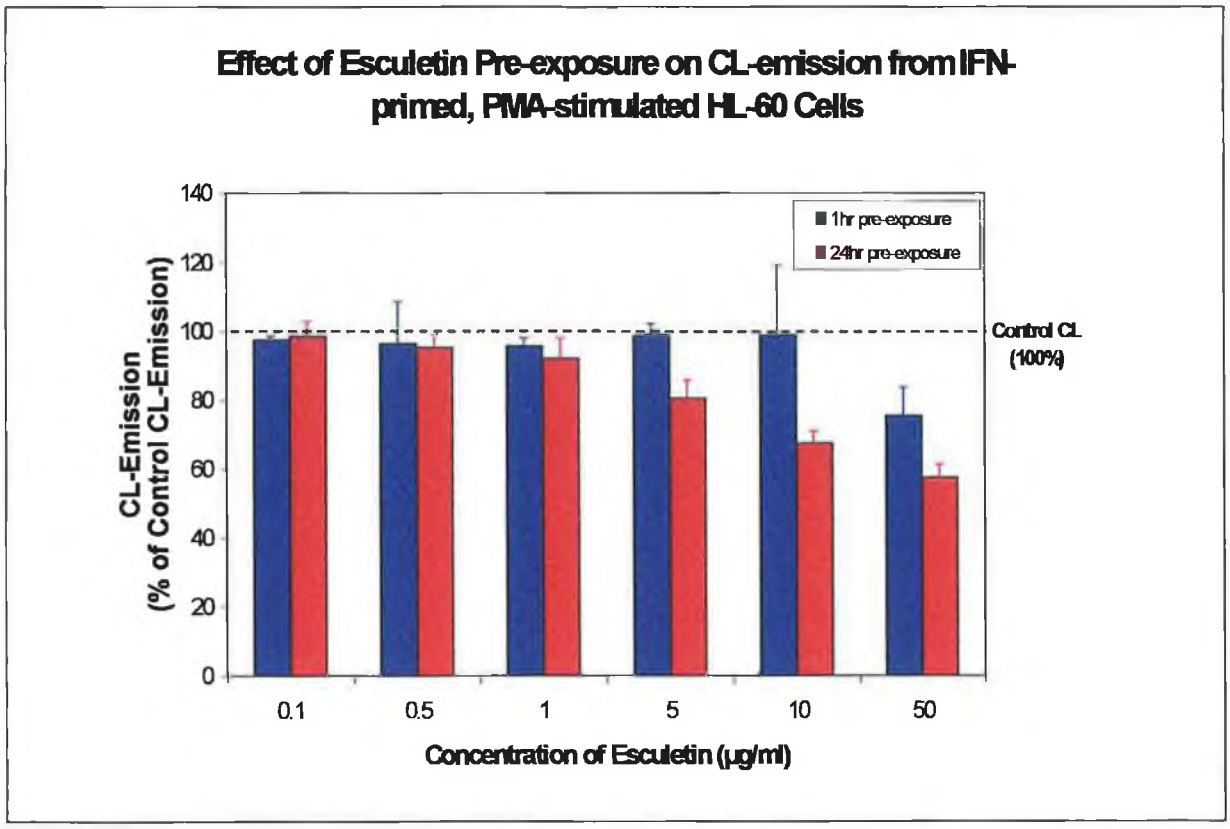


Figure 6.16: **Effect of esculetin on the emission of chemiluminescence from HL-60 cells. Cells were "primed" with IFN-γ (100U/ml) for 24hrs prior to PMA stimulation, and were pre-exposed to esculetin (0.1-50μg/ml) for either 1hr or 24hrs of this time period. These cells were then used in the following experiment: 2 X 10^5 cells/assay were incubated with 80μM luminol and stimulated to emit chemiluminescence, with 500ng/ml PMA, at time = 0. CL emission from the cells was monitored for 60mins. following an initial 10min. lag period where no measurements were recorded. CL measurements were made as outlined in section 2.2.7.5, with light emission recorded for 30 secs., every three minutes, for time = 10-70mins, following PMA stimulation. The light generated from control HL-60 cells ("primed" with IFN-γ, but unexposed to drugs), expressed as Integral Relative Light Units (RLU), was normalised at 100% as the control CL-emission. CL-values from all drug treated cells were expressed as a percentage of the control CL-emission. These values are plotted vs drug concentrations in the above bar charts. This experiment was carried out in triplicate and repeated on three separate occasions. The CL-emission from control cells was 9980.9 ± 806.20 (1hr) & 11762.50 ± 1667.50 (24hr) RLU.**

6.5.6. Production of Reactive Nitrogen Intermediates (RNI) from Monocytic Cells

As outlined in Section 6.2.3.4, the secretion of reactive nitrogen intermediates is believed to be one of the mechanisms, through which monocytes/macrophages exert an anti-tumour effect. We therefore investigated the production of RNI from HL-60 and 28SC cells in response to various immune cell stimulators/modulators, using the Greiss method to measure nitrite production (Section 2.2.7.6). The results of this study are shown in Table 6.12 – it can be clearly seen that none of the tested treatments resulted in the production of nitrite from either cell line. No further experiments with coumarins were completed.

Cell/Treatment	*Abs. @ 560nm*	*μM Nitrite*
HL-60 Medium only	0.002	< 1μM
HL-60 + 500ng/ml PMA	0.002	< 1μM
HL-60 + 10ng/ml LPS + 500ng/ml PMA	0.002	< 1μM
HL-60 + 100U/ml IFN-γ + 500ng/ml PMA	0.001	< 1μM
28SC Medium only	0.001	< 1μM
28SC + 500ng/ml PMA	0.001	< 1μM
28SC + 10ng/ml LPS + 500ng/ml PMA	0.001	< 1μM
28SC + 100U/ml IFN-γ + 500ng/ml PMA	0.001	< 1μM

Table 6.12: Experiments to examine the production of Reactive Nitrogen Intermediates (RNI) from HL-60 and 28SC cells in response to various immune modulators and stimulators. Cells (1 X 10^5 cells) were exposed to 500ng/ml PMA ± 10ng/ml LPS or 100U/ml IFN-γ, for 24hrs. The production of RNI by these treatments was then assessed by measurement of nitrite, using the Greiss method, as outlined in Section 2.2.7.6. A standard curve of nitrite was prepared in the range 0-100μM, and used to convert absorbance values at 560nm, to μM nitrite. As shown by this table, neither HL-60 nor 28SC cells were stimulated to RNI production by any of the treatments tested.

Although there are numerous reports on the production of RNI from animal macrophages (Ding *et al.*, 1988; Keller *et al.*, 1990; Dietert *et al.*, 1995), the generation of these anti-tumour products from human cells is still under investigation. Cancer patients receiving IL-2 treatment have been shown to develop a significant increase in serum and urine nitrate levels, and a number of different cell types can produce RNI in response to cytokines and LPS (O'Donnell & Liew, 1994). However, the relevance of RNI production in human monocytes/macrophages is still undetermined.

6.5.7. Production of Proteases from Monocytic Cells:

As outlined in Section 6.2.3.5, the production of various proteases by MPS cells provides a paradoxical view of the role of these cells in tumour immunity. The release of MMP products by MPS cells has been widely reported, and it is now believed they may aid the invasion of tumour cells rather than prevent it (Kageyama *et al.*, 1997). As we had shown that coumarins could decrease both activity and expression of MMP-2 from A431 tumour cells in Chapter 4, we chose to examine the effects of coumarins on the MMP enzymes released by monocyte cells.

MMP-9 (92-kDa Type IV collagenase) is the most predominantly expressed matrix-metalloproteinase of cells of the monocytic/macrophage lineage (Xie *et al.*, 1994b, 1998). MMP-2 is also produced in smaller amounts (Xie *et al.*, 1994b). Shapiro *et al.* (1991) has shown MMP expression to be dependent on the degree of differentiation for monocytic/macrophage cells.

We examined the MMP-expression of both HL-60 cells and 28SC cells, in the absence or presence of stimulating factors (500ng/ml PMA, and 250μg/ml OZ, respectively), as outlined in Section 2.2.7.7. We found both cell lines expressed a number of MMP enzymes (Figure 6.17). HL-60 cells constitutively expressed three proteases – MMP-2 (72kDa), MMP-9 (92kDa) and a third unidentified protease, with a molecular weight of > 205 kDa. This profile was similar to that reported by other investigators (Saarialho-Kere *et al.*, 1993; Xie *et al.*, 1994b). Stimulation of HL-60 cells with 500ng/ml PMA caused a ~4-fold increase in the levels of MMP-9, and a ~ 2-fold increase in MMP-2 expression, with expression of the third enzyme unchanged (Figure 6.17). Constitutive expression of both MMP-2 and MMP-9 were observed for 28SC cells – however, levels of MMP-9 were extremely low in comparison to HL-60 cells (Figure 6.17). No change in levels or profiles of MMPs occurred on stimulation of these cells with 250μg/ml OZ.

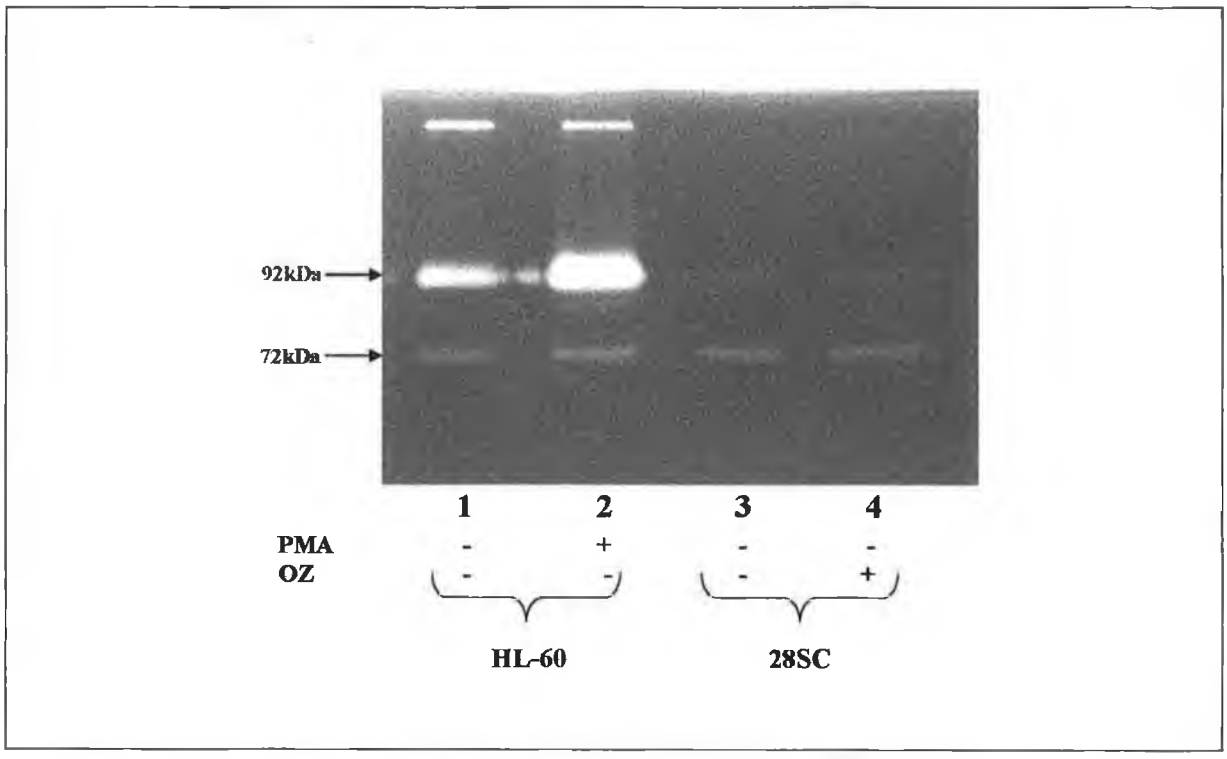


Figure 6.17: Protease production from Monocytic Cells. The production of proteases by HL-60 and 28SC cells was assessed in the absence or presence of appropriate stimuli (500ng/ml PMA and 250μg/ml OZ, respectively), as outlined in Section 2.2.7.7. Conditioned media was prepared from 1 X 10^6cells, exposed to appropriate stimulus for 24hr, and analysed for protease secretion by gelatin gel analysis.

From the results in Figure 6.17, it was decided to examine the effects of coumarins on the expression of proteases from HL-60 cells which were stimulated with 500ng/ml PMA for 24 hrs. The effect of coumarins on the expression of all three proteases (MMP-2, MMP-9 and 210kda protein) was quantitated by densitometric analysis of substrate gels (Figures 6.18-6.20). As illustrated in these results, and as expected from similar experiments in Chapter 4, all coumarin compounds caused a dose-dependent suppression of protease expression in PMA-stimulated HL-60 cells. As outlined in Chapter 4, this decrease may be due to one of a number of possibilities:

1. Effects on lipoxygenase signalling pathways, which are known to be networked with collagenase expression (Reich & Martin, 1996). Arachidonic acid metabolites play a huge role in immune responses and coumarins have been shown to affect prostaglandin synthesis (Lee *et al.*, 1981).
2. Chelation of metal ions *e.g.* Ca^{2+}/Cu^{2+}, interferes with MMP activity, and the coumarins have reported chelatory properties (Huitnik & Diehl, 1974).

Regardless of the mechanism of action, the coumarins do decrease the expression of MMPs from monocytes. Consequently, this may be a significant anti-tumour mechanism of these compounds, especially with regard to tumour-associated macrophages, as these often aid tumour invasion through MMP release, and thus, inhibition of this process may be a valuable anti-tumour mechanism.

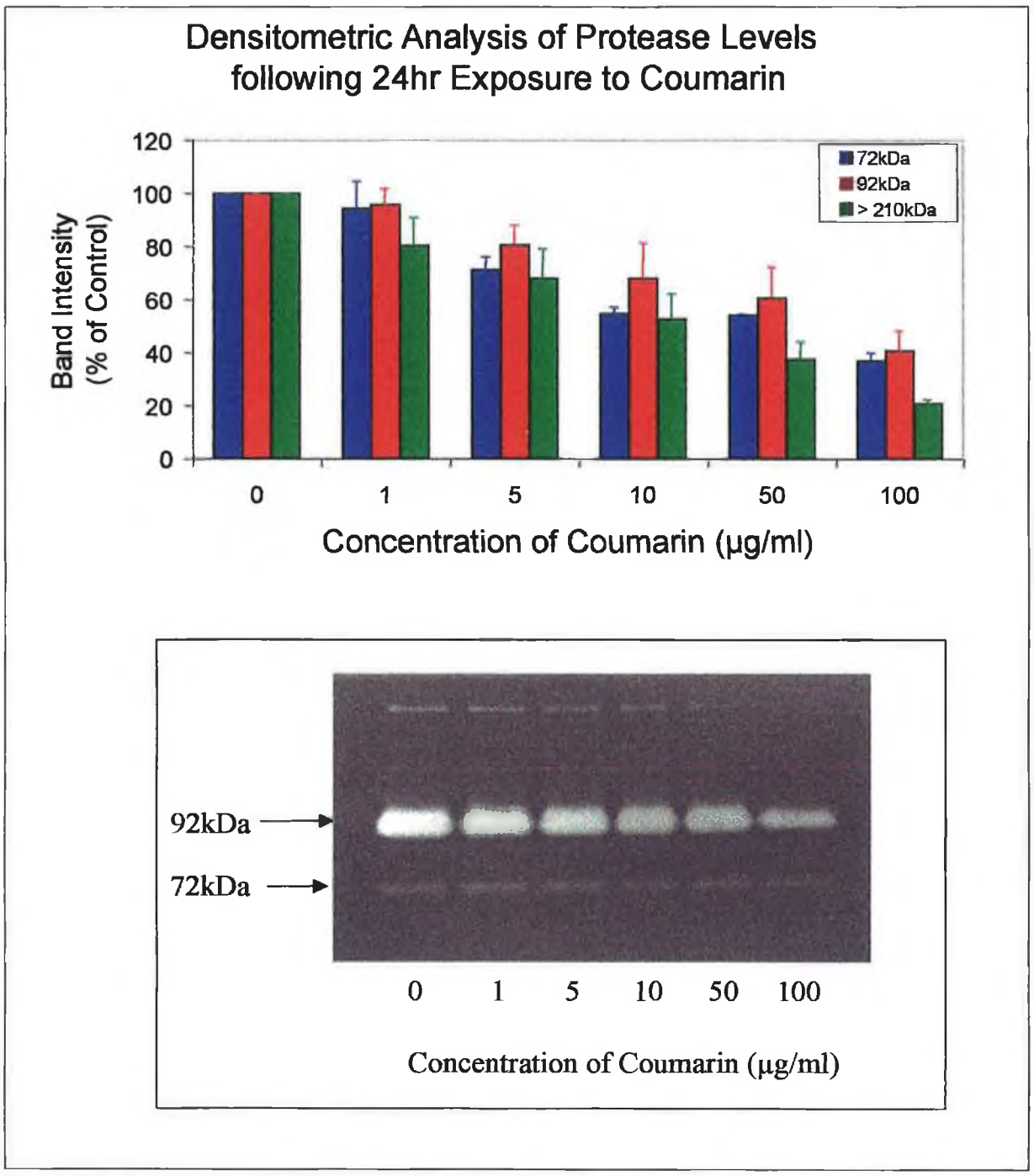


Figure 6.18: Effect of 24hr exposure to coumarin on the PMA-stimulated expression of proteases from HL-60 cells. The top graph depicts the densitometric analysis from three different experiments (mean ± s.d.) for HL-60 cells exposed to coumarin in the range 0-100µg/ml. A representative gel is also shown (bottom).

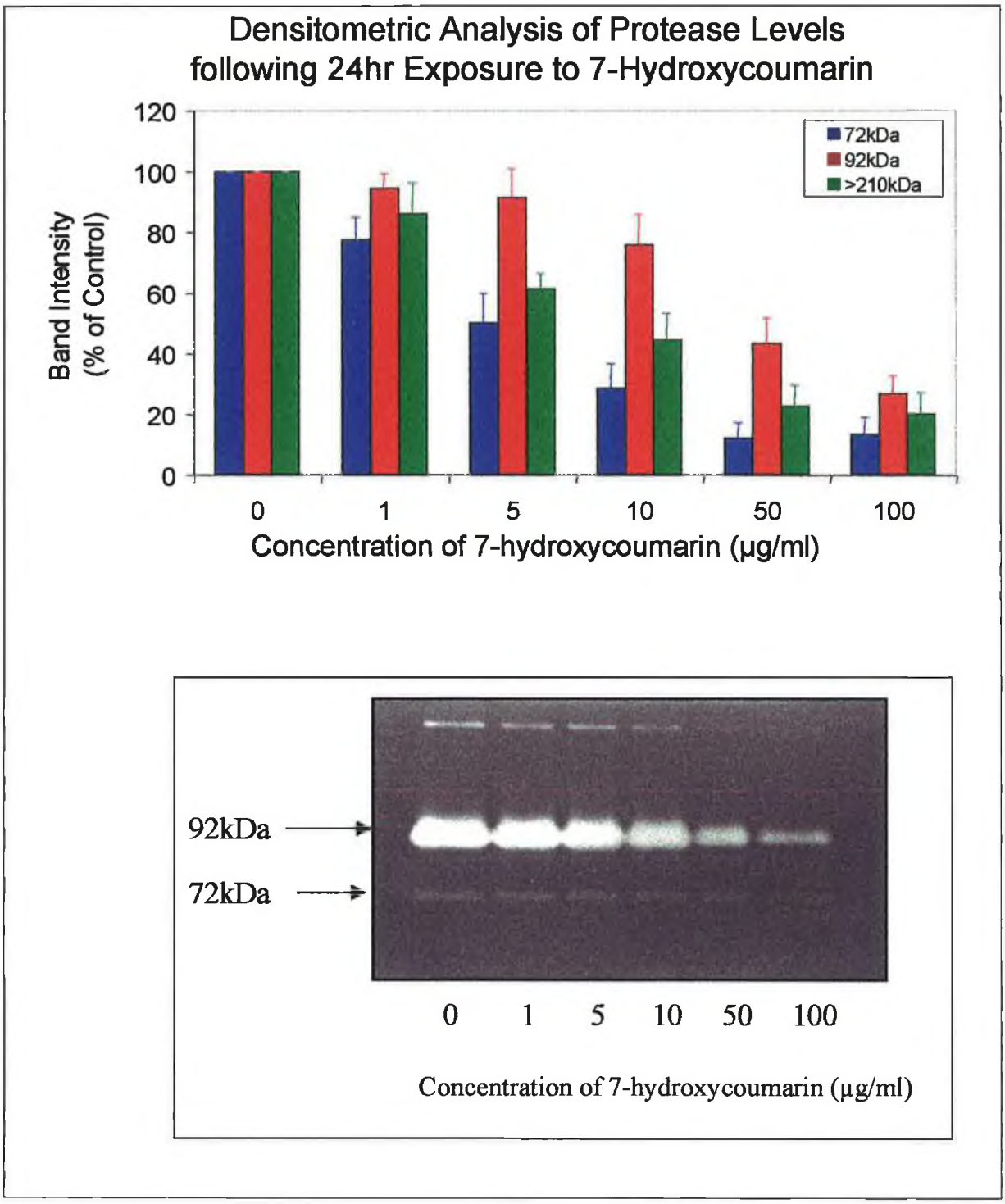


Figure 6.19: Effect of 24hr exposure to 7-hydroxycoumarin on the PMA-stimulated expression of proteases from HL-60 cells. The top graph depicts the densitometric analysis from three different experiments (mean ± s.d.) for HL-60 cells exposed to 7-hydroxycoumarin in the range 0-100µg/ml. A representative gel is also shown (bottom).

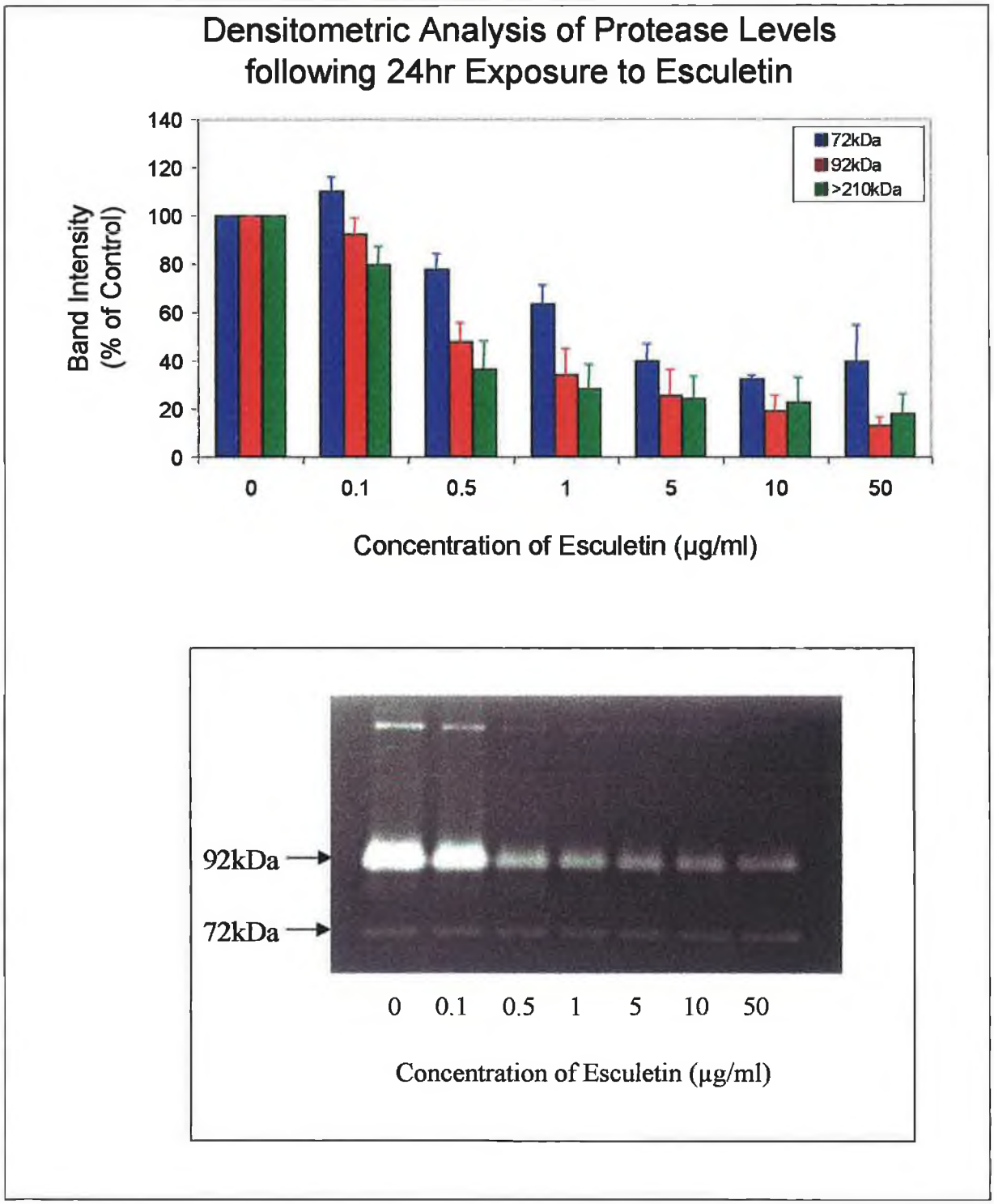


Figure 6.19: Effect of 24hr exposure to esculetin on the PMA-stimulated expression of proteases from HL-60 cells. The top graph depicts the densitometric analysis from three different experiments (mean ± s.d.) for HL-60 cells exposed to esculetin in the range 0-50µg/ml. A representative gel is also shown (bottom).

6.6 CHAPTER SUMMARY

In this chapter, two monocytic-like cell lines (28SC and HL-60) were assessed to determine their suitability as appropriate "models" of monocytes, and a number of monocytic "functions" were examined. Cytochemical staining confirmed the ATCC designation, with the 28SC cells monocytic in nature, while the HL-60 cells were pro-monocytic. However, functional activation of the 28SC cell line, both on the Cytosensor Microphysiometer and in terms of their ability to generate ROS, illustrated that this cell-line did not respond to stimulation with PMA. This was an unusual result as PMA acts as a general PKC activator. It is probable that the inactivity of the cells in response to PMA was due to either expression of PMA-unresponsive PKC isoforms or complete loss of PKC activity due to cell immortalisation. The cells did respond to activation with OZ, which illustrates that some monocytic functions (microbicidal) are present. As we were interested in tumouricidal activities, we excluded the 28SC cells in further experiments.

The second cell line examined was the promonocytic HL-60 cell, which is leukemic in origin. It was efficiently activated by PMA, both on the Cytosensor Microphysiometer, and in ROS generation studies, with 500ng/ml PMA the optimal stimulating concentration. However, problems were also encountered in the use of this cell line: it was discovered that reproducible activation of cells was only possible between Days 5-12 post-resuscitation. The decline in response of HL-60 cells to PMA over time was probably due to changes in isozyme (PKC) and enzyme (peroxidase) expression, as a result of differentiation.

The HL-60 cell line (Day 5-12 cells) was used to examine the effect of coumarins on ROS generation. None of the coumarins tested (coumarin, 7-hydroxycoumarin and esculetin) were shown to activate ROS generation by themselves. All coumarins were shown to scavenge the ROS generated in response to PMA, and therefore extensive washing of cells was required to assess other effects of coumarin exposure on PMA-stimulated ROS generation. In this assessment the coumarin compounds did not appear at any concentration to display significant stimulatory effects to the monocytic cells, which is in disagreement with previous studies detailing the stimulatory effect of coumarins on MPS cells (Piller, 1978; Koh & Willoughby, 1979, Marshall *et al.*, 1991b). We did expect to observe increases in ROS-generation in response to PMA-stimulation (on the basis of augmentation of PKC activity) but the opposite was observed for higher concentrations of 7-HC and esculetin; this result may be due to scavenging by coumarins. IFN-γ partially reverted the ROS suppression at lower drug concentrations through an unidentified mechanism. Overall, it is doubtful that the anti-tumour mechanism of coumarins is mediated through ROS mechanisms

The effect of coumarin compounds on protease expression in monocytic cells was also examined. As expected the coumarins decreased the expression of monocytic MMPs and this may be important in the anti-tumour effect of coumarins.

Chapter 7

Conclusions

7.1. SUMMARY OF WORK ACHIEVED

The purpose of this research was an examination into the anti-cancer properties of coumarin compounds at a cellular level, in order to comprehend the observed clinical properties of these natural products. In particular, the direct vs immunomodulatory effects of these compounds on cells was evaluated.

The initial work undertaken, examined the effect of a number of coumarin compounds on the growth, metabolism and metastatic potential of human tumour cell lines. Previous work by investigators has concentrated on examining the growth inhibitory properties of coumarin and its main human metabolite, 7-hydroxycoumarin (Moran *et al.*, 1987; Marshall *et al.*, 1994). However, work on the metabolism of coumarin by humans, has revealed the existence of other minor metabolites, which may also be important chemotherapeutically. We therefore examined the effect of 6-hydroxycoumarin and esculetin on various cellular functions, and compared their activity to coumarin and 7-hydroxycoumarin. With regard to growth inhibition, 6-hydroxycoumarin was almost comparable to 7-hydroxycoumarin in its potency, but neither was as effective as esculetin, in an anti-proliferative role. Unlike the mono-hydroxycoumarins, esculetin was non-specific in its potent, inhibitory effect. Coumarin itself displayed only low levels of growth inhibition. The potency of esculetin was also evident in studies on metabolism, where again it caused large decreases in the cellular metabolism as determined by the MTT and Cytosensor studies. Both mono-hydroxycoumarins also caused decreased cellular metabolism in cells, but 6-hydroxycoumarin exerted milder effects than 7-hydroxycoumarin. The detrimental effects of all three coumarins were time- and dose-dependent, and prolonged exposure (12-24hrs) caused irreversible effects.

The search for novel cancer chemotherapeutic agents has aroused interest in properties other than growth inhibition, which may be useful at controlling the spread of cancer. Thus, a preliminary examination of the effectiveness of coumarins at preventing metastasis was conducted. Various proteases are implicated in the process of cancer cell invasion and metastasis, with matrix metalloproteinases (MMPs) especially important in this event. The four tested coumarin compounds were found to dose-dependently decrease the activity and expression of MMPs, with decreasing order of effectiveness as follows: esculetin > 7-hydroxycoumarin >>6-hydroxycoumarin > coumarin. It is possible that this effect of 7-hydroxycoumarin might aid its *in vivo* anti-tumour effect. It is unclear how this inhibition works, although the mechanism may be related to

1. Interaction with the lipoxygenase signalling pathway, which is important in MMP expression.
2. Chelation of metal ions, necessary for MMP activity
3. Suppression of *ras* activity which is necessary for MMP expression.

These effects may be important in explaining the overall mode of action of coumarin in tumour cells (Section 7.2).

The second study undertaken aimed at determining more precisely the cellular target of coumarin action. Previous investigators had reported on the association of coumarin and 7-hydroxycoumarin with growth signalling pathways *e.g.* Seligers work on autocrine loops (Seliger & Pettersson, 1994a). Given the importance of signalling anomalies in cancer cells, we were interested in determining if the cellular target of 7-hydroxycoumarin was a signalling pathway component. Given that tyrosine phosphorylation is essential to the emanation of growth signals, and the fact that esculetin has been reported to inhibit cellular tyrosine kinases, we decided to concentrate on this aspect of signalling. Both 7-hydroxycoumarin and esculetin were found to inhibit tyrosine phosphorylation in EGF-stimulated tumour cells in a time- and dose-dependent manner. It appears that this effect may be achieved by reduction of the tyrosine kinase activity of the EGF-Receptor. Direct *in vitro* assays suggested a direct inhibition of the RTK activity by 7-hydroxycoumarin. However, activation of Protein Kinase C (a negative regulator of EGF-RTK) was shown to be augmented by 7-hydroxycoumarin and esculetin exposure, and may be an indirect means of inhibition of the EGF-RTK. Regardless of the mechanism, this information is useful in the proposal of a mode of action of 7-hydroxycoumarin (Section 7.2)

The final investigation regarded the effect of coumarins on monocyte functions in a model system. While the model "monocyte" system used was not ideal, it did yield some useful information regarding the coumarin compounds. These compounds are effective scavengers of superoxide radicals, and therefore it is unlikely that they aid in the secretion of ROS as part of an anti-tumour immune response. RNI do not appear important either, and it appears likely that the existence of an anti-tumour immunomodulatory effect lies principally in the augmentation of cytokine production as determined by Zlabingers research group (Stuhlmeier *et al.*, 1991; Zlabinger *et al.*, 1993). The expression of proteases by immune cells was also reduced by coumarins, which may be of importance in reducing the invasion of cancer cells, which can be aided by production of proteases by tumour-associated immune cells (Mantovani *et al.*, 1992).

7.2. MODE OF ACTION OF COUMARIN IN CANCER CELLS

While the pleiotropic effects of the coumarin compounds in the biological arena are well-established, as yet no cellular target(s) of coumarins has been identified comprehensively. This work aimed at inching closer to identifying such a target. The information obtained from this work in conjunction with data reported by other investigators enables a number of plausible cellular targets to be suggested, and each of these will now be briefly discussed (see also Figure 7.1). In all instances, the discussion will concentrate on 7-hydroxycoumarin.

1. Growth Factor Receptors and Tyrosine Kinases

The work presented in Chapter 5 of this thesis outlines the fact that the activity of the EGF-Receptor tyrosine kinase is affected adversely by exposure to 7-hydroxycoumarin and esculetin. The inhibition of the RTK may be direct as determined by direct *in vitro* assays, or may be through an indirect Protein Kinase C effect (Point 2). In addition to this report, 7-hydroxycoumarin has previously been shown to negatively regulate the PDGF-autocrine loop in glioblastoma cells (Seliger, 1997). The downregulation of RTK-induced tyrosine phosphorylation is important for the downstream growth factor signalling, and if inhibited, causes gross perturbation and downregulation of its signalling targets (particularly at the mRNA level). This might explain previous reports of downregulation of *ras* and *c-myc*, in 7-hydroxycoumarin-treated cells (Shima *et al.*, 1989; Zanker, 1993; Seliger & Pettersson, 1994b; Lu *et al.*, 1996).

2. Protein Kinase C

Cytosensor results from Chapter 5 illustrate that both 7-hydroxycoumarin and esculetin are important in augmenting the activity of Protein Kinase C. In the case of the cell system examined, this may be important in the negative regulation of the EGF-Receptor and its associated signalling networks. Augmentation of Protein Kinase C by coumarins has been previously reported in immune cells (Zlabinger *et al.*, 1993), and was important for caused increased cytokine release from LPS-stimulated cells. Protein Kinase C interacts extensively with many signalling intermediates and therefore 7-hydroxycoumarin may influence cellular events by interacting with this molecule.

3. Metal Chelation

The metal chelatory actions of coumarins have been previously documented (Huitnik & Diehl, 1974), and although it is unlikely that they can fully explain the anti-tumour effects of coumarins, it

is possible that they may augment effects. It has been previously suggested that chelation of Ca^{2+} and Mg^{2+} may explain the decrease in metalloproteinase activity and expression observed for both tumour and immune cells. In addition, the essential functioning of tyrosine kinases and other signalling enzymes within the cells is dependent on the presence of Mg^{2+} and Mn^{2+} ions. While it seems doubtful that chelation alone could explain the effects of coumarins in cells, chelation of cations by aspirin has been recently proposed to explain the therapeutic effects of aspirin like compounds (Wang, 1998).

The above three suggestions (see also Figure 7.1) are possible targets for the 7-hydroxycoumarin molecule intracellularly. However, more detailed investigations are required to fully ascertain if one, all or any of these, are the important targets in tumour cells.

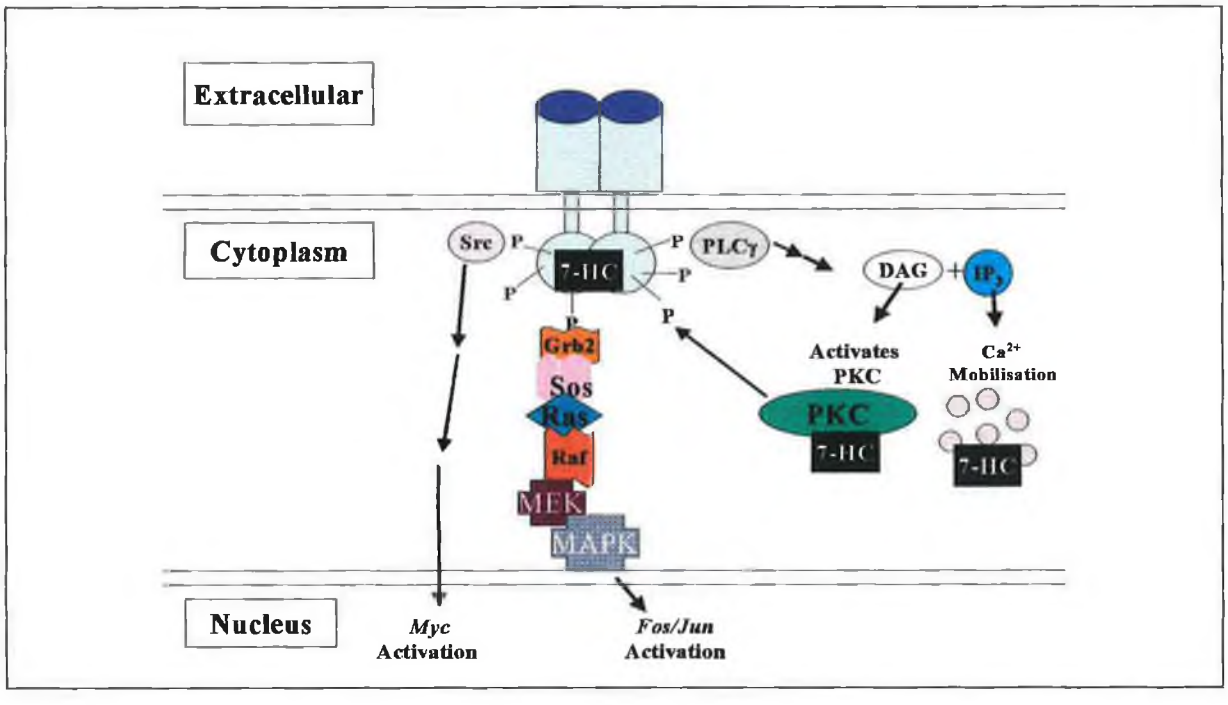


Figure 7.1: **Proposed Mode of Action of 7-Hydroxycoumarin (7-HC) in Cancer Cells. 7-HC may function by inhibiting the RTK activity of growth factor receptors. This will prevent growth signals reaching other signalling intermediates such as Ras, or activation of transcription factors such as *Myc*. Alternatively, Protein Kinase C may be the target. As a negative regulator of EGF-RTK activity, this also affects downstream signalling targets. Finally, the target may be metallic cations *e.g.* Ca^{2+}/ Mg^{2+}, which are also necessary signalling components.**

Chapter 8

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Appendix 1

Validation of the Cytosensor Microphysiometer in Toxicity Testing

A1.1. INTRODUCTION TO VALIDATION STUDIES UNDERTAKEN

The Cytosensor Microphysiometer (Chapter 3) is a useful alternative method for toxicity testing (Parce *et al.*, 1989; Bruner *et al.*, 1992; Wada *et al.*, 1992). Its detection method, reporting decreases in basal metabolic rates of cells, certainly appears useful for predicting the toxicity of various agents, and its use in screening compounds, perhaps as one of a battery of *in vitro* alternatives to animals for toxicity testing, seems plausible. Very little validation work is recorded towards achieving such an application for this instrument. Validation of an alternative toxicity testing method involves demonstration of its reliability, and its relevance for the particular purpose. In addition, the method must be reproducible across several laboratories (Bruner *et al.*, 1998).

When we initiated our work with this instrument, previous toxicological assessment of the cytosensor had been carried out principally by a combined laboratory effort between Molecular Devices Corporation and The Proctor & Gamble Company, with their focus being ocular irritancy (Parce *at al.*, 1989; Bruner *et al.*, 1992). We decided to reproduce their microphysiometry work in our laboratory, and assess the applicability of the method to the toxicity testing of the coumarin compounds, the focus of our interest. We also decided to compare the Cytosensor Microphysiometer to the MTT assay (a well-established *in vitro* toxicity test) as part of the validation procedure.

A1.2. PROCEDURES

A1.2.1. Cytosensor Studies

The studies used the exact experimental set-up described by Bruner and co-workers (1992), during their investigational toxicity work on the cytosensor into ocular irritancy, with the exception of the cells used on the instrument – they worked with normal human keratinocytes, while we used A431 epidermal cells (vulval tumour cells) and NIH3T3 fibroblasts, in our work.

Briefly, the experiment was undertaken as follows: Half-log serial dilutions of the test compounds were prepared in the low-buffered running medium (formulated as outlined in Section 2.2.4.5.1). The cells were assembled on the cytosensor, and their acidification rates allowed to stabilise prior to exposure to any test substance. The cells were sequentially exposed to increasing concentrations of test substance over successive pump cycles. Each pump cycle (Figure A1.1)

involved the following: during a flow-off period, the test substance (300µl) was injected through a dosing loop into the chamber to begin the cycle (equivalent to time zero). Flow of running medium was immediately started at a speed of 100µl/sec for 120 sec, and then halted for 200sec to allow exposure to the cells. The flow was restarted at 100µl/sec for 380 sec to wash out the test material. Stopping the flow for 200 sec, during which time the acidification rate was determined, completed the sequence. The exposure sequence was repeated with increasing concentrations of test material until the acidification rate reached zero. The acidification rates were expressed as a percentage of the control rate and plotted versus the concentration of test compound (as shown in Figure A1.2B). From these plots the MRD_{50} value *i.e.* the concentration of test material (g/ml) sufficient to reduce the metabolic rate of the cells by 50% of the control rate, was determined. All experiments were performed in triplicate.

A1.2.2. MTT Assay

The basis of this assay has been described previously in Section 2.2.4.4, and it was carried out as outlined in Section 2.2.4.4. Cells were exposed to half-log serial dilutions of the test compounds. After 24 hours incubation, detection of viable cells was determined according to the procedure in Section 2.2.4.4. The average absorbance for wells exposed to test compounds was expressed as a percentage of the control wells, and this value was plotted versus concentration of test compound (as shown in Figure A1.2A). From these plots the IC_{50} value *i.e.* the concentration of test material (g/ml) sufficient to reduce the activity of the mitochondrial dehydrogenases by 50% of the control rate, was determined.

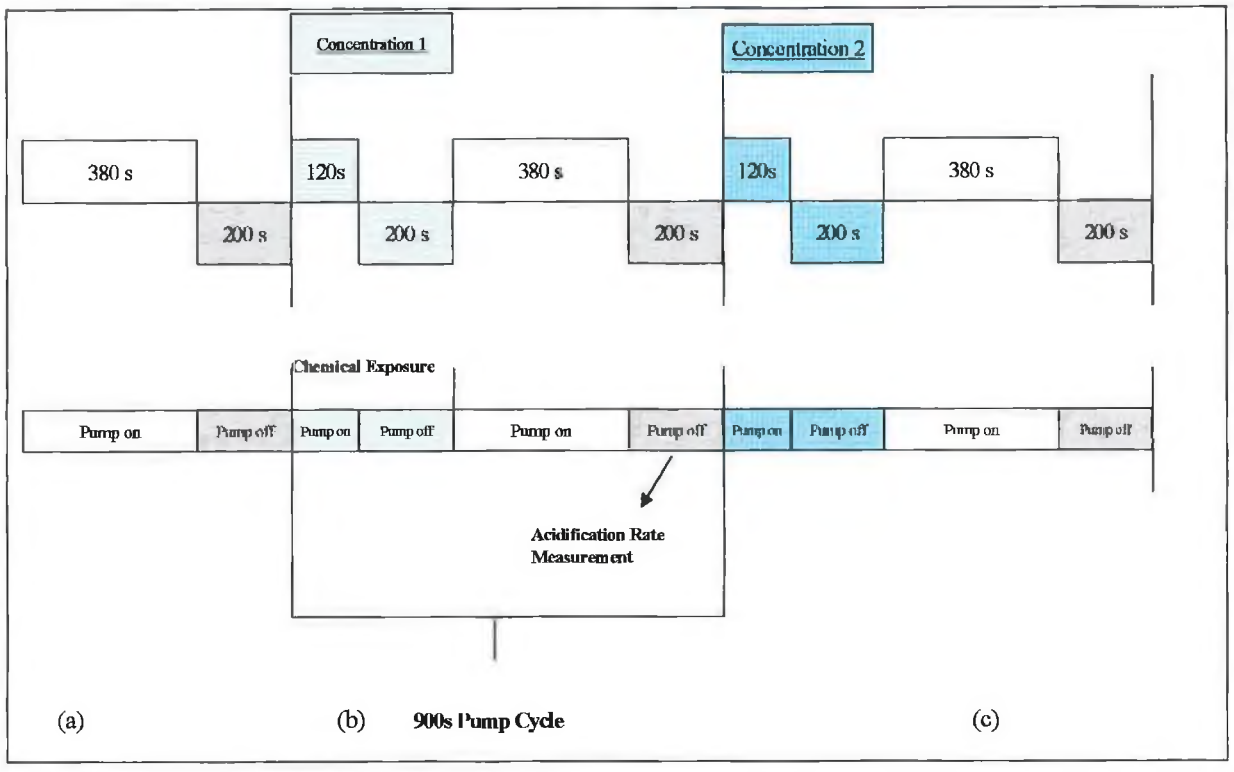

Figure A1.1: **Typical Pump schedule during a toxicity run.**

(a) Prior to exposure to test material the cells are bathed in low-buffered medium and during a 200s pump-off period, their basal acidification rate is measured.

(b) As a result of a valve switch, the test material is flushed into the sensor chamber from 0-120s at a rate of 100µl/min. After 120s the pump is switched off for 200s, to allow exposure of the cells to the sample. The pump is then turned on again and as a result of a simultaneous valve switch, fresh medium (without test material) is introduced to the cells in the chamber (again at a rate of 100µl/min), for 380s. This step ensures complete flush-out of the test compound. The pump-flow is again interrupted for 200s to allow measurement of the cellular acidification rate following chemical exposure, to complete the 900s cycle.

(c) Cessation of the acidification rate measurement of (b) marks the commencement of a new 900s pump cycle, with the introduction of a higher concentration of the test compound into the sensor chamber.

This repetition of cycles with augmenting concentrations of test compound continues, until the acidification rate of the cells reaches zero.

A1.3 RESULTS AND DISCUSSION

A1.3.1. Validation of previous toxicity work

Previous work in this area carried out by the collaborating Molecular Devices Corp./Proctor & Gamble Co. laboratories, examined a range of compounds with known *in vivo* irritancy levels (Parce *et al.*, 1989; Bruner *et al.*, 1992). For our validation of their work and the instrument, we chose seven such compounds, namely (in increasing order of irritancy), dimethyl sulfoxide (DMSO), methanol, ethanol, acetone, n-butanol, sodium dodecyl sulphate (SDS) and Triton X-100. These seven compounds were subjected to both Cytosensor and MTT analysis, as described in Section A1.2. Figure A1.2. shows the results obtained from these studies for the A431 epidermal cell line. In both graphs (A & B) the curves for the most irritating test compounds lie to the left of those for the least irritating compounds. The results obtained for the NIH3T3 fibroblastic cell line (not shown) were almost identical.

These graphs were used to determine IC_{50} values (that concentration of compound which causes a 50% reduction in the activity of mitochondrial dehydrogenases), and MRD_{50} values (that concentration of compound that causes a 50% reduction in the metabolic rate of the cells), for MTT assays and Cytosensor studies, respectively, by interpolation, and these values are presented in Table A1.1.

As can be seen from this data, the cytosensor analysis yielded comparable results to those of the MTT assay for six of the seven compounds analysed, and as such, would appear useful as an alternative method for toxicity prediction of compounds. In the case of n-butanol, the MTT assay returned an IC_{50} value ten times lower than that obtained from the cytosensor analysis ($7\ 0 \times 10^{-4}$ g/ml vs 7.5×10^{-3} g/ml, respectively). In this case the disparity between the two results may arise from the differences in exposure time (24hrs vs 320s) during the assays and may indicate a limitation of the cytosensor analysis (see Section A1.3.2.).

The advantages of the cytosensor analysis technique over the MTT assay include speed (compare 4 vs 24hrs), automation and the fact that each experiment, unlike the MTT assay, is internally controlled. By this we mean that each chamber acts as its own control – *i.e.* the metabolic rate of the cells in any one chamber post-drug exposure, is expressed as a percentage of the pre-exposure metabolic rate of the same set of cells, rather than by comparison to a separate set of cells. This latter scenario occurs with the MTT assay: due to the semi-invasive nature of the technique,

responses in drug-treated cells are expressed as a percentage of a separate sample of untreated cells. Using this "external" control, a source of error may arise, if, for example, the seeding densities of control and drug-treated cells were not identical.

In their exploratory toxicity experiments with the cytosensor, Parce *et al.* (1989) compared the MRD_{50} values of the tested compounds (using the negative logarithm of their values) with their *in vivo* ocular irritancy. They found a high correlation between the two sets of data, with the least irritating materials causing damage to cellular metabolism at relatively high concentrations, while more irritating materials altered cellular function at much lower concentrations. A similar comparison of our experimental data is outlined in Table A1.2. As can be seen, a 4-fold log range of MRD_{50} values existed between the most mild (DMSO) and most severe (Triton X-100) compounds.

One important issue (and flaw) in the whole validation process described above was the *relevance* of this test to the compounds of interest to us *i.e.* the coumarin compounds. The seven compounds examined were used in order to reproduce and validate previous cytosensor results, and compare them to an established *in vitro* test (MTT Assay). As an objective this was achieved. However, we were concerned that the compounds used were irrelevant to the focus of our work, chiefly because of their chemical nature. All seven compounds examined were either solvents or detergents, and as such their detrimental action on cells involves disruption of the cellular membrane. However, if interested, as we were, in a method for assessing toxicity of non-solvent type compounds, the issue of adequate penetration and exposure time of the investigational compound to a cell becomes important.

It should be recalled that the procedure validated above involves a short (320s) exposure of compound to cells. In the case of a solvent/detergent compound, as its toxic effect is essentially externally-mediated, a procedure involving a short exposure time (mins) may be sufficient to detect the toxic effect(s). For compounds with an internal target, such a short exposure may not provide for elucidation of toxicity (in many cases it may take hours or days for toxicity to manifest itself). We therefore assessed the above procedure for its utility in detecting the toxic effects of coumarin compounds on cells.

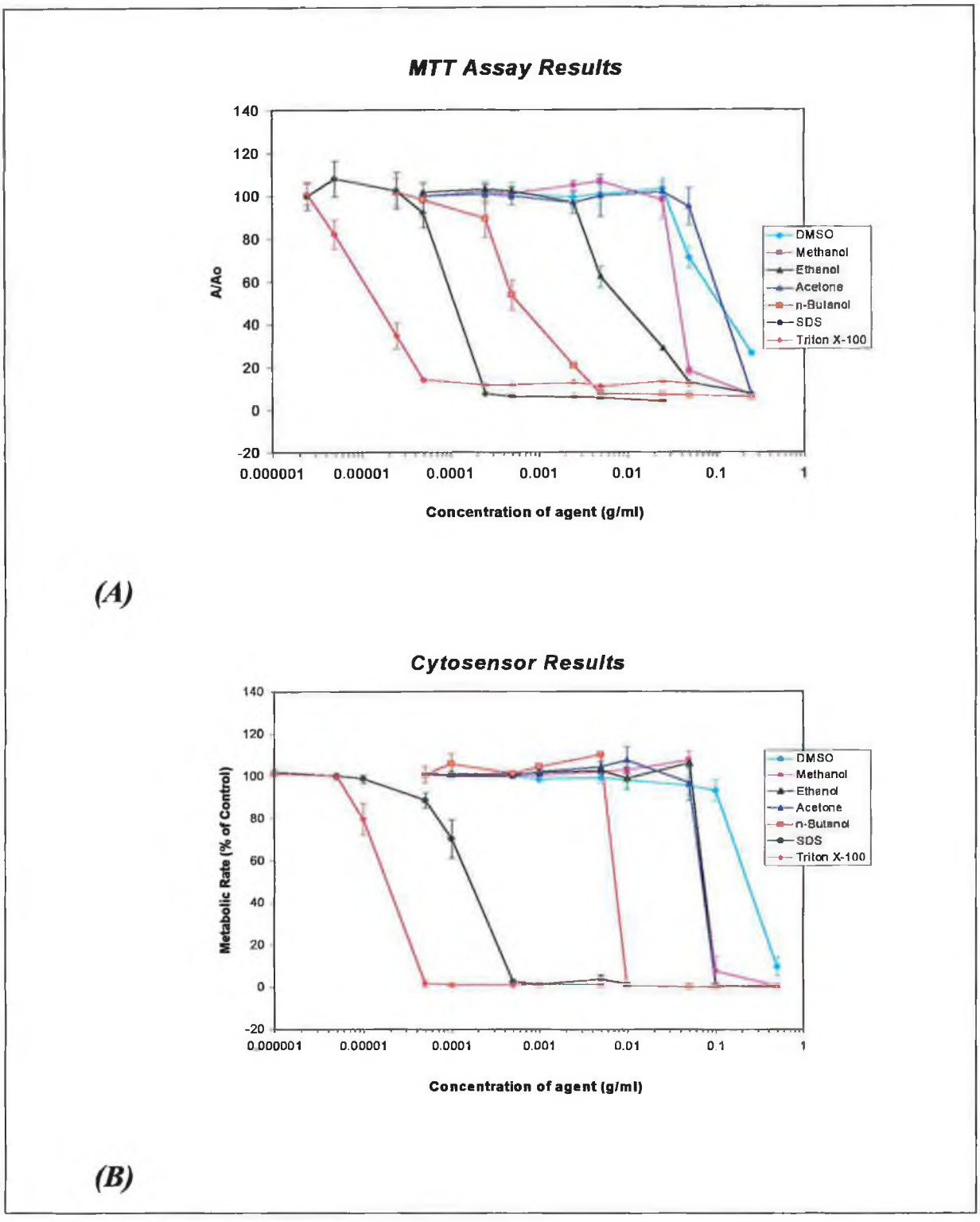

Figure A1.2: **Exposure of A431 cells to seven different toxic irritants as part of the cytosensor validation experiments. The MTT assay was used for comparison with the cytosensor in these experiments.**

Compound	*MRD_{50} [Cytosensor] (g/ml)*	*IC_{50} [MTT Assay] (g/ml)*
DMSO	2.25×10^{-1}	1.50×10^{-1}
Methanol	7.50×10^{-2}	4.00×10^{-2}
Ethanol	7.50×10^{-2}	1.20×10^{-2}
Acetone	7.00×10^{-2}	1.50×10^{-1}
n-Butanol	**7.50×10^{-3}**	**7.00×10^{-4}**
SDS	1.75×10^{-4}	1.50×10^{-4}
Triton X-100	2.00×10^{-5}	1.50×10^{-5}

Table A1.1: **Comparison of the MRD_{50} values from the Cytosensor analysis to the IC_{50}values from MTT assays. The values (g/ml) were similar for six of the seven compounds. The dissimilar results obtained for n-butanol are highlighted.**

Compound	*In Vivo Irritancy*	*$-1og_{10}(MRD_{50})$*
DMSO	Mild	0.648
Methanol	Mild-Moderate	1.12
Ethanol	Mild-Moderate	1.12
Acetone	Mild-Moderate	1.15
n-Butanol	Moderate	2.12
SDS	Moderate-Severe	3.76
Triton X-100	Severe	4.70

Table A1.2: **Comparison of *in vivo* irritancy data with MRD_{50} values from the cytosensor analysis.**

A1.3.2 Assessment of validated technique for general toxicity assessment.

Cells were exposed to coumarin compounds (either coumarin, 7-hydroxycoumarin, 4-hydroxycoumarin or esculetin) under identical conditions as those outlined above (detailed in Figure A1.1). The results of this experiment (sample one of three) are shown in Figure A1.3A. From these results it is clear that the cells were unaffected by exposure to the four coumarin compounds in the concentration range 0-200μg/ml. From previous proliferation studies which we had accomplished in Chapter 4 with these compounds (especially with regard to esculetin), we were unconvinced that these results accurately reflected the effect of these compounds on cells. We therefore decided to modify the pump cycle to lengthen the exposure time of the cells to the various compounds. Different formats were tested, all enabling increased cellular penetration by the drugs, and all demonstrated that the coumarins, given adequate exposure time, did suppress the basal metabolic rate of cells. Figure A1.3B shows results obtained with the following 35 min pump cycle:

0-30 mins – Exposure to test compound at 20μl/min

30-34 mins – Wash-out with running medium at 100μl/min

34-35 mins – Pump off : Acidification Rate measured.

As detected in this diagram, the increased exposure of the cells to the test compound, via the modified pump cycle, enabled us to observe the suppressive effect of esculetin in particular, on the basal metabolic rate of the cells. Thus the method outlined in Section A1.3.1. is, as we anticipated, unsuitable for the detection of non-solvent-based toxic effects.

As a result of this information, we decided to address the issue of using the cytosensor microphysiometer as a toxicity tool in an alternative format. We decided to adopt the approach used by various groups with microcalorimetry and oxygen electrodes, whereby they monitored continuously the metabolic rate of cells, and also determined in real-time, the effect of compounds on the basal cellular metabolism (Ara *et al.,* 1994; Schon & Wadso, 1988). The complete use of such a set-up is described procedurally in Section 2.2.4.5., and the experimental results given completely in Chapter 4. A sample set of results is shown here in Figure A1.4.

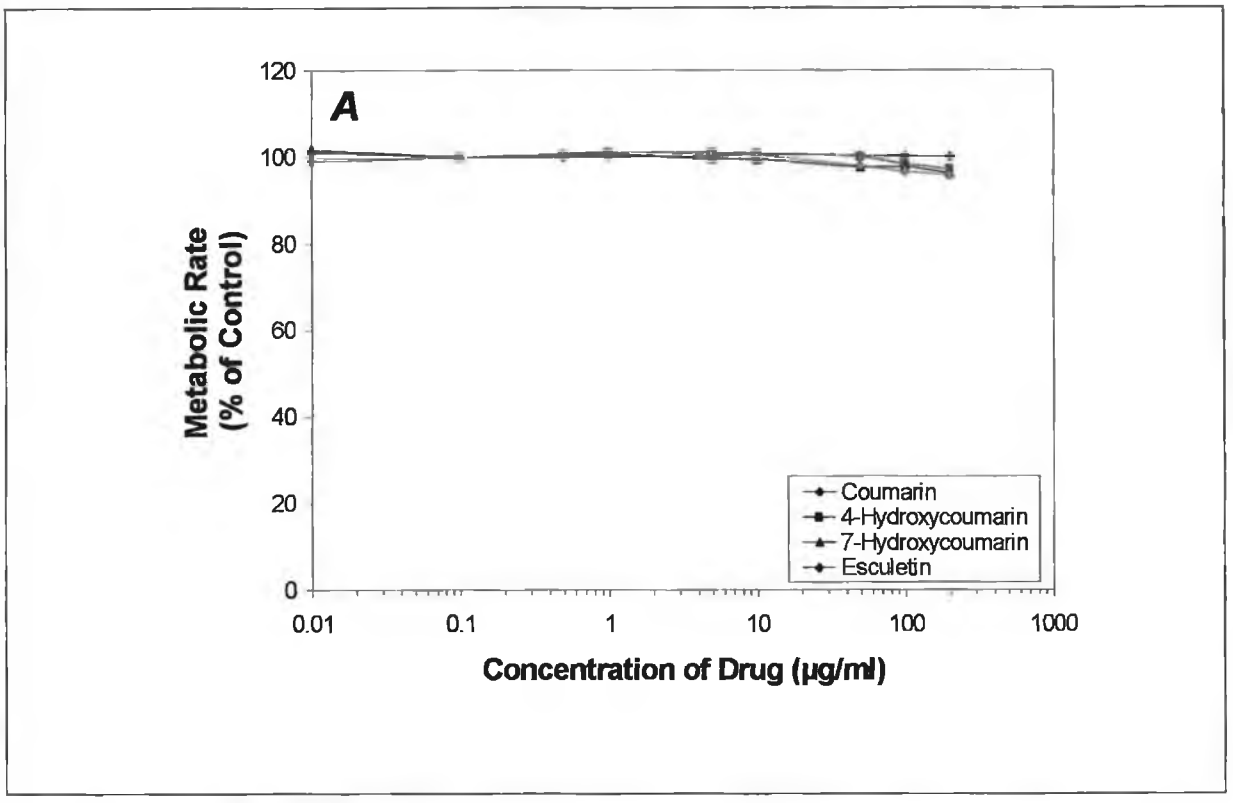

Figure A1.3A: Examination of the effect of exposure time on the detection of toxic effects in non-solvent/non-detergent compounds. A431 cells were exposed to four different coumarin compounds using the same exposure cycle as was used in Section A1.3.1 for solvent compounds. The pump cycle is outlined in Figure A1.1 , and briefly involved exposure of cells to coumarins for 320s in total. As shown in this diagram this short exposure time did not cause any decrease in acidification rate for the exposed cells. Thus, it was decided to expose the cells to coumarins for longer time periods to allow time for drug penetration, as shown in Figure A1.3B

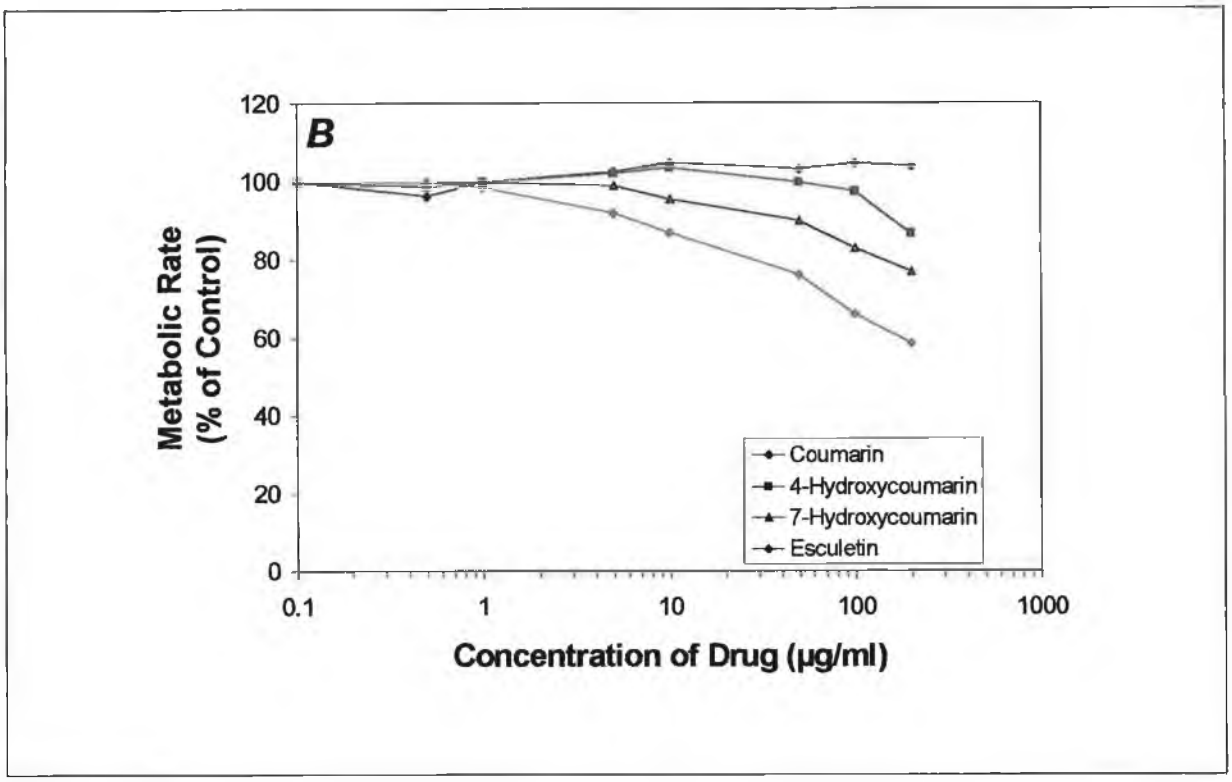

Figure A1.3B: Examination of the effect of exposure time on the detection of toxic effects in non-solvent/non-detergent compounds. A431 cells were exposed to four different coumarin compounds using a longer exposure cycle than that used in all previous studies, and in Figure A1.3B. The pump cycle involved exposure of cells to coumarins for 30 mins. in total, as oulined in Section A1.3.2. As shown in this diagram this longer exposure time did cause a decrease in acidification rate for the exposed cells, as a result of increased drug penetration.

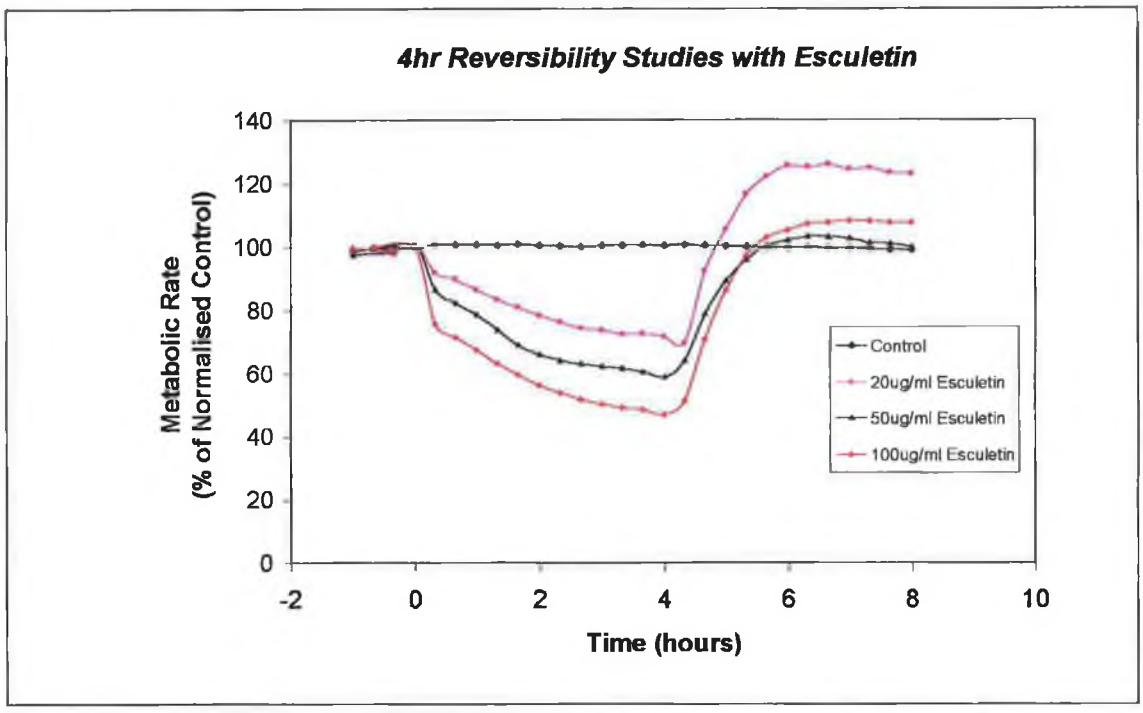

Figure A1.4: Continuous exposure of A431 cells to Esculetin over 4 hours. The acidification rate (every 20 minutes) was normalised versus the basal rate before exposure. This value was plotted versus time for each concentration to generate the above dose-response curves. The reversibility of the toxic insult was examined by allowing the cells to recover in drug-free medium following this exposure period.

This real-time toxicity-testing format (Figure A1.4) has many advantages over other *in vitro* formats. Firstly, it is a continuous monitoring system, unlike most *in vitro* toxicity tests, which are end point methods. Therefore, the time-frame over which the toxic effect of a compound occurs can be determined easily using the cytosensor format. With end-point tests, this information could only be gained by tedious, multiple end-point kinetic determinations. Secondly, as shown in Figure A1.4, the reversibility of a toxic insult to cells can be easily examined. The format of traditional reversibility tests often involves taking sub-populations of treated cells and examining their recovery from toxic insult. This introduces an element of error, if the sub-population chosen is inherently sensitive/resistant to the test material. The advantage of the cytosensor system format is that the total population of exposed cells is monitored for recovery, thus eliminating this selection error. Thirdly, the Cytosensor system is a flow-through perfusion system, and this format mimics

the *in vivo* blood-stream delivery of toxic compounds more closely than the static conditions of many traditional *in vitro* toxicity formats. Finally, the non-invasive nature of the testing format means all experiments are internally controlled, and if required, permits the re-use of the cells in other experiments post-test.

A1.4. CONCLUSION

This appendix outlines the work achieved to investigate the usefulness of the Cytosensor Microphysiometer as a tool for *in vitro* toxicity testing. Initially, a previously-developed cytosensor toxicity test for compounds (Parce *et al.* 1989) was compared to an established *in vitro* toxicity assay (the MTT Assay) to validate its effectiveness as an alternative method. The cytosensor was found comparable to the MTT assay for the prediction of toxicity for the seven compounds examined. However, as this cytosensor assay had been formulated to address *in vivo* ocular irritancy, we were concerned about its relevance and general applicability to toxicity testing of compounds with intracellular toxic effects. We found that the method of Parce and colleagues was unsuitable for use as a general toxicity assay, on the basis that with its short exposure times it did not allow for adequate penetration and targeting of a compound within the cell. Therefore a new continuous method was implemented for the determination of toxic effects and this was utilised in assessing the detrimental effects of coumarins on cellular metabolism (outlined fully in Chapter 4). The various advantages of this method over existing *in vitro* toxicity tests were also outlined.

Appendix 2

A2.1. CHARACTERISATION OF 6-HYDROXYCOUMARIN

The purity and identity of the compound, 6-hydroxycoumarin, synthesised according to the method outlined in Section 2.2.1, by Dr. Oliver Egan, BEST Centre, D.C.U., was assessed using IR and NMR spectra. (Figures A2.1-A2.3)

A2.1.1. Infrared (IR) Spectrum Analysis:

An IR spectrum of 6-hydroxycoumarin is shown in Figure A2.1. The presence of a hydroxy-group on a parent coumarin compound was confirmed by the presence of bands at 3188cm^{-1} (-OH), and 1673cm^{-1} (-C=O). A series of bands at ~1300cm^{-1} are representative of the C-O-C stretch on the pyrone ring.

A2.1.2. NMR Analysis: ^{1}H & ^{13}C- NMR Spectrometry

A proton spectrum of 6-hydroxycoumarin was determined in DMSO, exposed to a magnetic field of 400MHz (Figure A2.2). Together with the ^{13}C –NMR spectrum (Figure A2.3), a C-H correlation was obtainable, with the aid of the spectral tables of Silverstein *et al.*, (1991). The proton spectrum reveals the presence of 6 hydrogen peaks (4 doublets and two single peaks) assigned to carbon atoms as follows: ^{2}C-H δ 6.4ppm (doublet), ^{3}C-H δ 7.95ppm (doublet), ^{5}C-H δ 7ppm (singlet), ^{6}C-OH δ 9.75ppm (singlet), ^{7}C-H δ 7.3 ppm (doublet) and ^{8}C-H δ 7ppm (doublet).

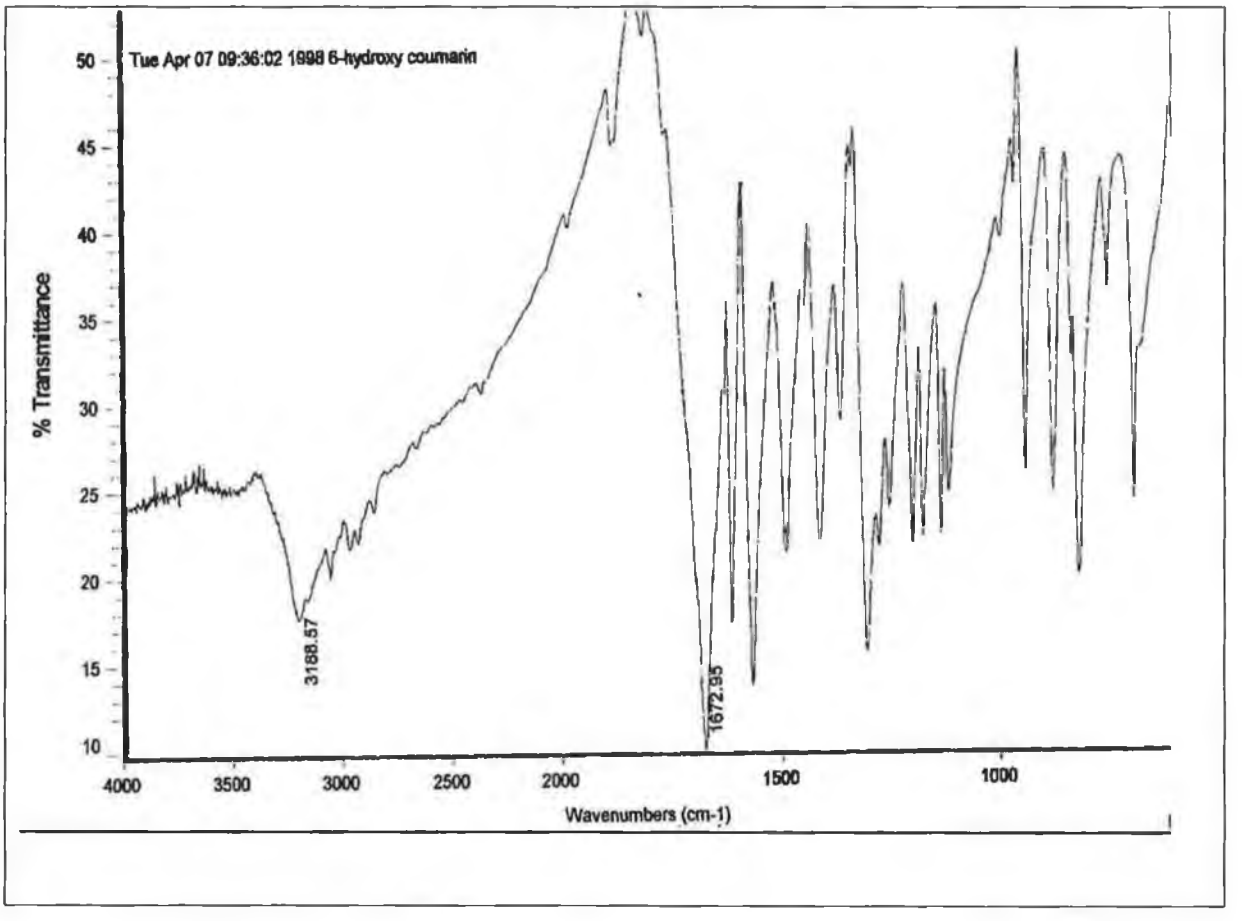

Figure A2.1: Infra-red spectrum for 6-hydroxycoumarin, showing the presence of OH ($3188cm^{-1}$) and –C=O ($1673cm^{-1}$) bands.

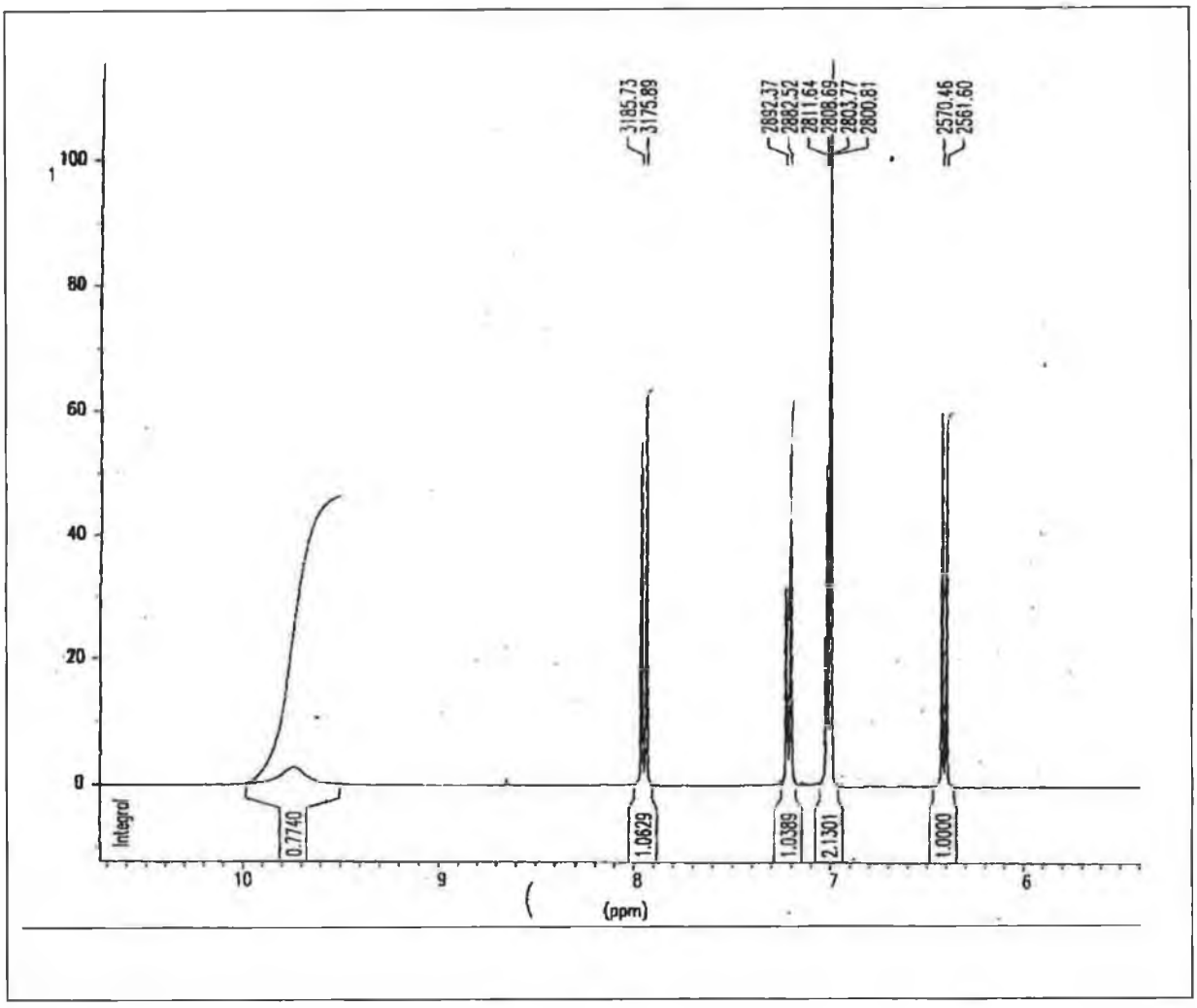

Figure A2.2: **Proton NMR spectrum of 6-hydroxycoumarin in deuterated DMSO showing the presence of 6 protons at δ= 6.4, 7 (2 H-nuclei), 7.3, 7.95 and 9.75ppm (-OH), corresponding to protons on Carbon atoms 2, 5, 8, 7, 3 and 6 (OH), respectively.**

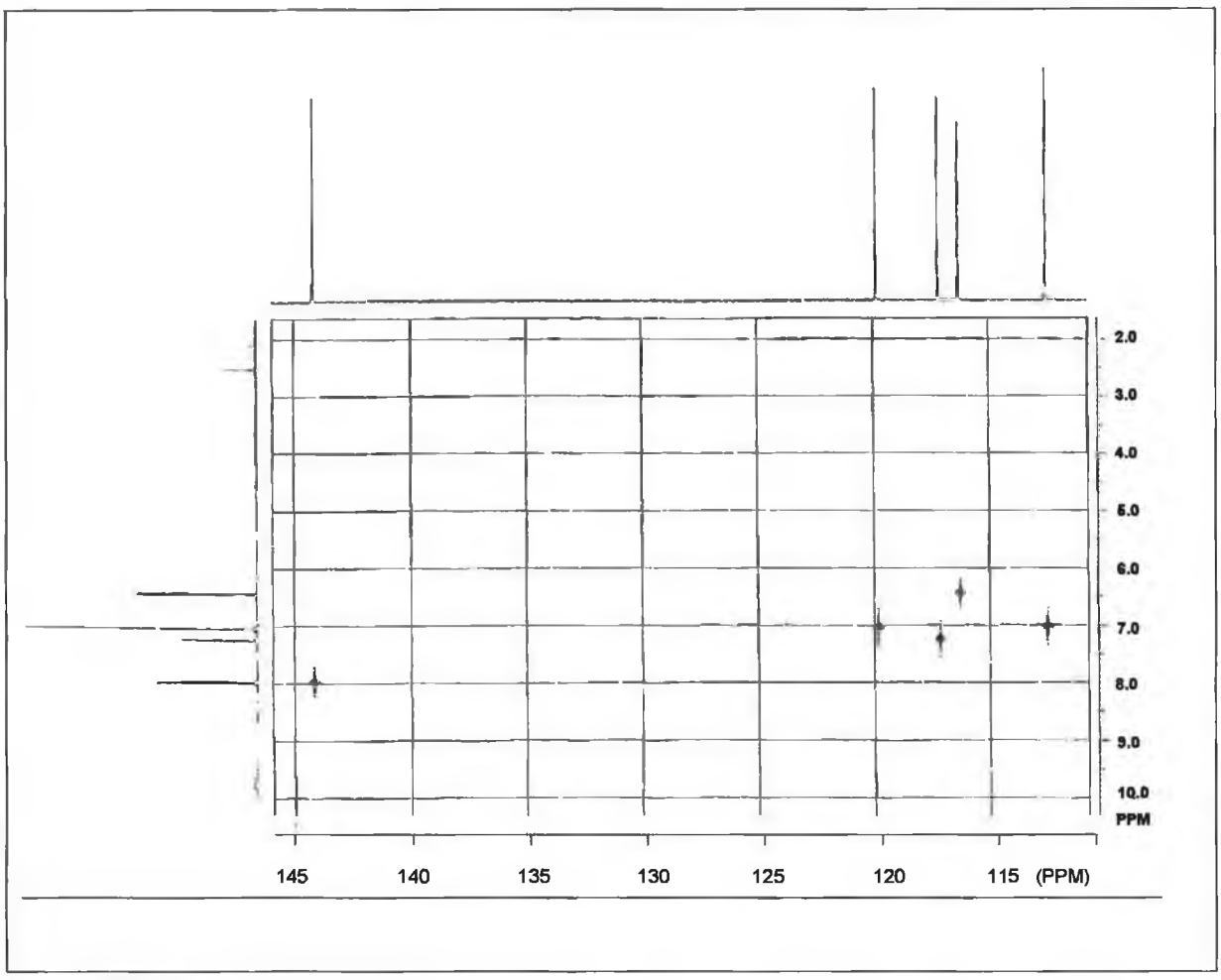

Figure A2.3: **C-H correlation chart – peaks in the ^{1}H-NMR (y-axis) and ^{13}C-NMR (x-axis) were correlated, in order to ensure correct assignment of H-atoms to the coumarin structure and ensure correct identification of 6-hydroxycoumarin.**

A2.2 IN VITRO CELL PROLIFERATION ASSAYS

The growth curves for seven cell lines (PC3, T-24, A549, HL-60, SW480, SW620 & NIH3T3) following exposure to coumarin compounds (coumarin, 6-hydroxycoumarin, 7-hydroxycoumarin and esculetin) as outlined in Section 2.2.4.2, are shown in Figures A2.4-A2.7. These results are discussed in Chapter 4 in conjunction with those from Figure 4.1 and Table 4.4.

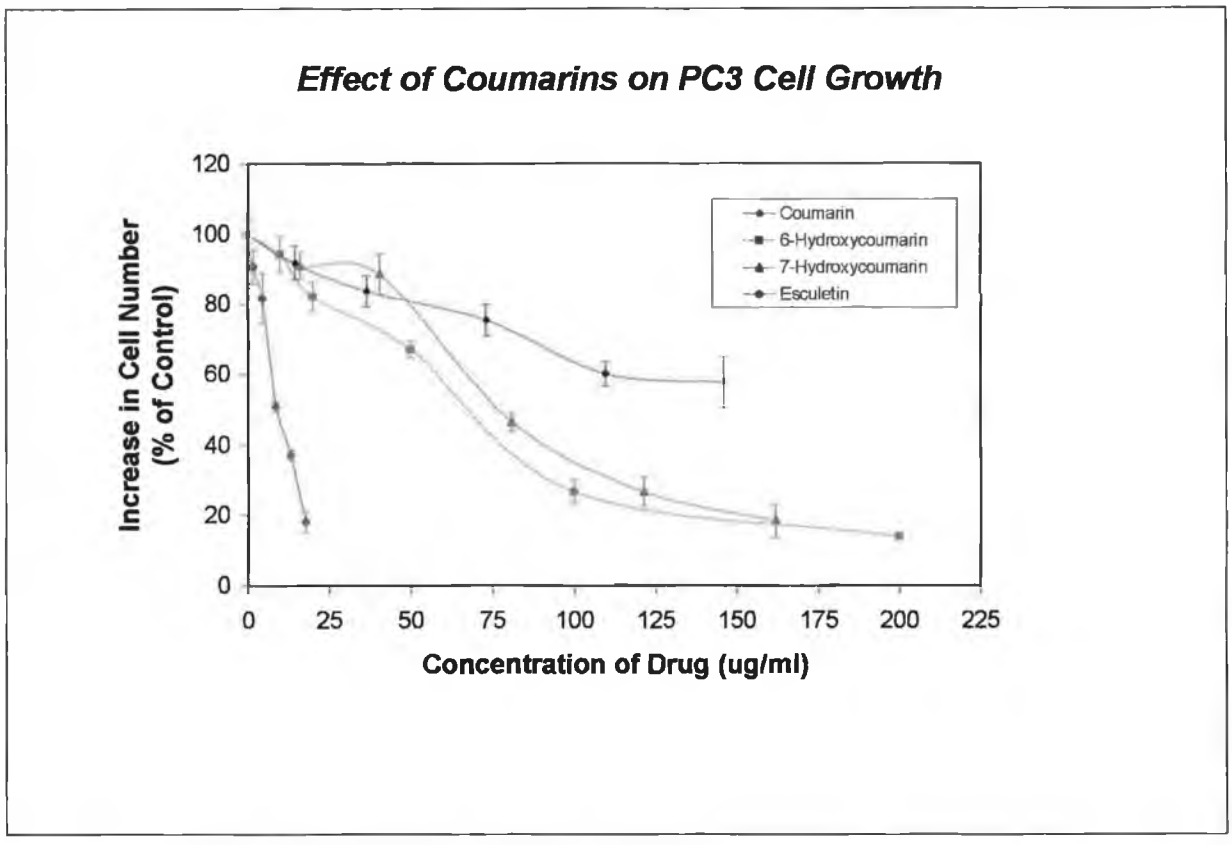

Figure A2.4: ***In Vitro*** **Cell Proliferation Assays following exposure of PC3 cells to 4 coumarin compounds for 96 hours. All experiments were conducted in duplicate on three separate occasions. The increase in cell number for drug-treated cells was expressed as a percentage of the untreated control cells and plotted versus the appropriate drug concentration.**

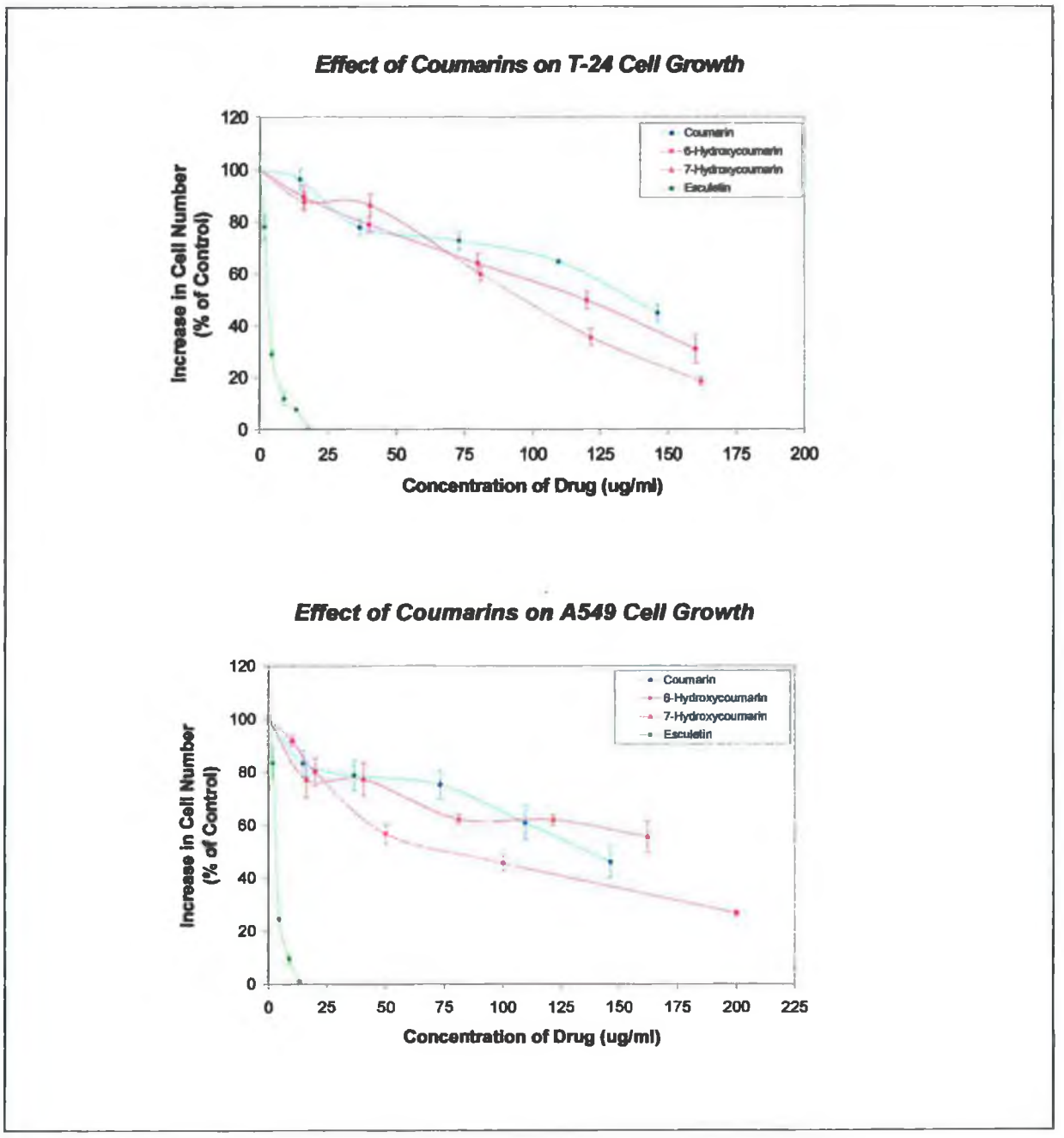

Figure A2.5: ***In Vitro*** **Cell Proliferation Assays following exposure of cells (T-24, top and A549, bottom) to 4 coumarin compounds for 96 hours. All experiments were conducted in duplicate on three separate occasions. The increase in cell number for drug-treated cells was expressed as a percentage of the untreated control cells and plotted versus the appropriate drug concentration.**

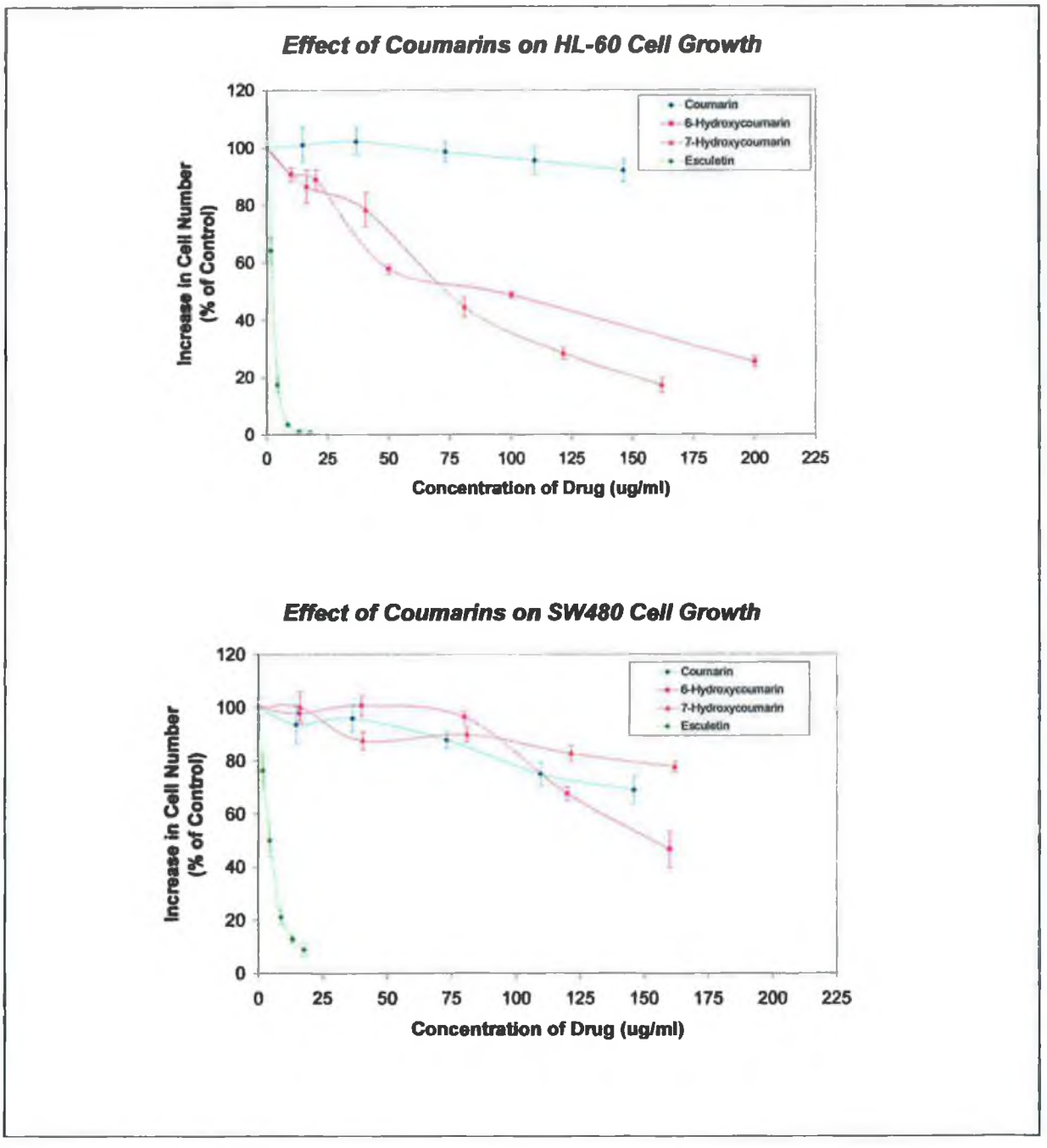

Figure A2.6: ***In Vitro*** **Cell Proliferation Assays following exposure of cells (HL-60, top and SW480, bottom) to coumarin compounds for 96 hours. All experiments were conducted in duplicate on three separate occasions. The increase in cell number for drug-treated cells was expressed as a percentage of the untreated control cells and plotted versus the appropriate drug concentration.**

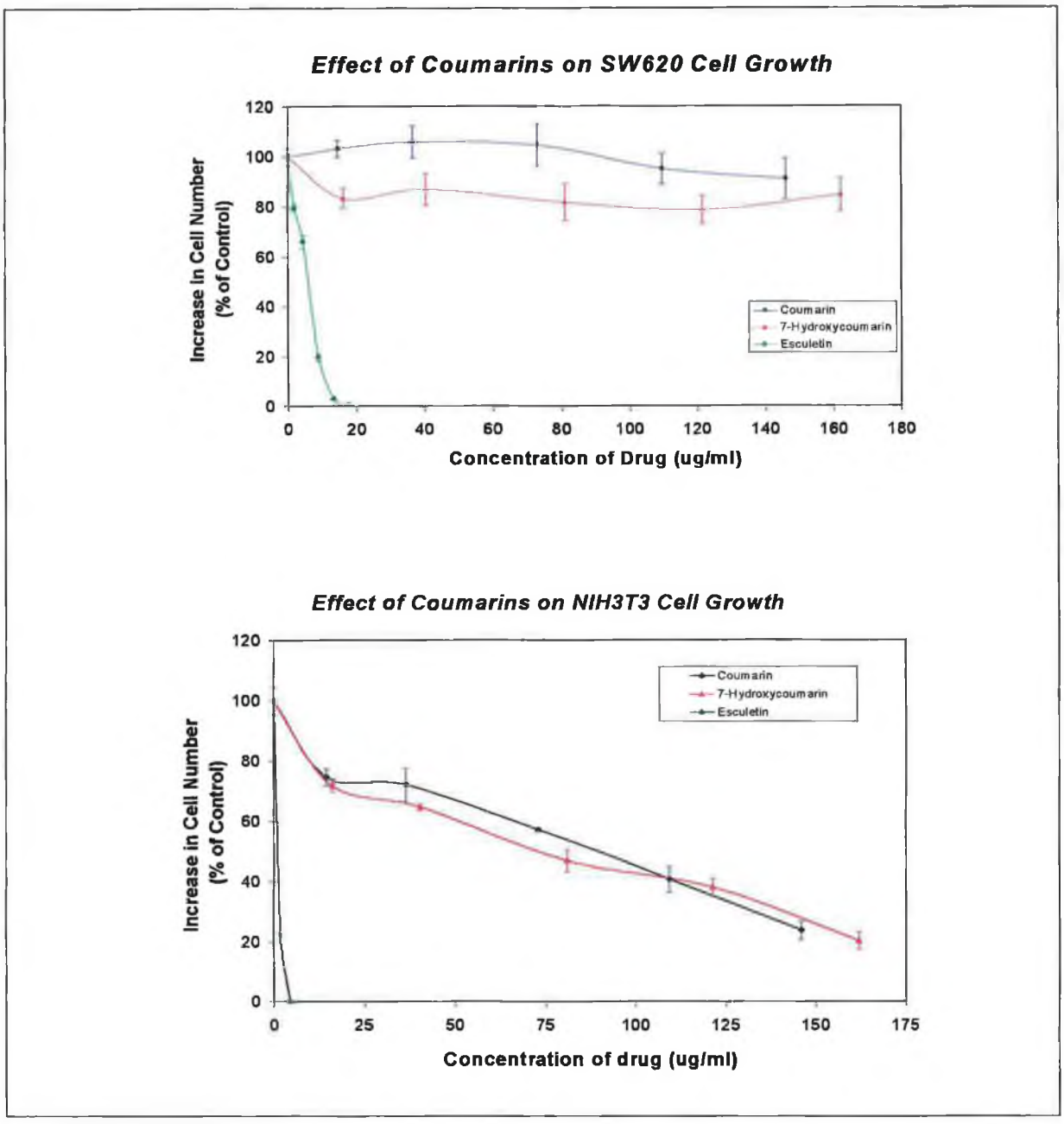

Figure A2.7: ***In Vitro*** **Cell Proliferation Assays following exposure of cells (SW620, top; NIH3T3, bottom) to coumarin compounds for 96 hours. All experiments were conducted in duplicate on three separate occasions. The increase in cell number for drug-treated cells was expressed as a percentage of the untreated control cells and plotted versus the appropriate drug concentration.**