INVESTIGATION OF XENOBIOTIC METABOLISM IN MAMMALIAN CELLS IN CULTURE

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

Signed: Geraldine Grant Date: 30 8 93

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The use of permanent cell lines in toxicity testing is a long established procedure Cell culture offers two major advantages over research conducted *in vivo* or with tissues, (1) it reduces the ethical difficulties, (2) allows environmental factors to be controlled
However, many compounds e g premutagens and precarcinogens are metabolically converted by monooxygenases and other xenobiotic-metabolizing enzymes into their ultimate mutagenic/carcinogenic form, cultured cells rapidly lose the ability to express many of these enzymes Therefore, a toxicological test system which intends to detect mutagens or carcinogens must be capable of producing these compounds in their 'active' form
To overcome this lack of activation capability external activation systems have been employed (e g. hepatocytes and S9 fraction) but such systems show great variability
Some established cell lines do, however, retain a degree of their original metabolic ability making them very useful tools in toxicity testing

This project involved A) the investigation of primary culture of Non Small Cell Carcinoma of the lung The aim of this investigation was to improve the success rate of establishing cultures, which may then be used in the determination of toxicity of chemotherapeutic agents on tumours, B) Determination of the levels of drug metabolizing activity (both phase 1 and phase 2) in a number of established cell lines under inductive pressure in order to establish the possible relevance of these lines to toxicity testing, C) To investigate the possible role of drug metabolizing enzymes in multiple drug resistance, D) To construct, by transfection, cell lines expressing the Cytochrome P4501A1 gene

This thesis is dedicated to
my parents.

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Abbreviation

ABBREVIATIONS

Ah	Aromatic hydrocarbon
AHH	Aryl hydrocarbon hydroxylase
AMP	Ampicillin
Arnt	Aromatic hydrocarbon nuclear translocator protein
ATCC	American tissue culture collection
BA	Benzanthracene
BCPI	5-Bromo-4-chloro-3-indolyl phosphate
BTE	Basal transcriptional element
$CaCl_2$	Calcium chloride
CM	Conditioned medium
CO	Carbon monoxide
DMEM	Dubeccos minimum essential medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoximide
DNCB	Dinitrochlorobenzene
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis[β-aminoethylether]-N,N,N',N'-tetraacetic acid
FCS	Foetal calf serum
FITC	Fluorescein Isothiocyanate
GH	Growth hormone
GSH	Glutathione
GST	Glutathione-S-transferase
HBS	HEPES balanced salts
HCL	Hydrochloric acid
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HSP90	Heat shock protein
L-Glut	L-glutamine
MC	3-Methylcholanthrene
MDR	Multiple drug resistance
MEM	Minimum essential medium

$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_2$	Sodium bicarbonate
NaOH	Sodium hydroxide
NEAA	Non essential amino acids
PAH	Polycyclic aromatic hydrocarbon
PB	Phenobarbital
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIPES	Piperazine-N,N'-bis[2-ethanesulfonic acid]
SDS	Sodium dodecyl sulphate
SP	Sodium pyruvate
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TE	Tris-EDTA
TEMED	N,N,N',N',-Tetramethyl-ethylenediamine
TRIS	Tris[hydroxymethyl]aminomethane hydrochloride
TV	Trypsin versene
UP WATER	Ultra pure water
UV	Ultra violet
X-Gal	5-Btp,p-4-chloro-3-indolyl-β-D-galactopyranoside
XPBS	X phosphate buffered saline
XRE	Xenobiotic regulatory element

1.0 INTRODUCTION.

1.1 XENOBIOTIC METABOLISM - An overview.

Our modern industrial society has achieved spectacular technological progress However, this progress is often at the expense of our environment and health Exposure to toxic compounds is not just a consequence of the modern era The cocktail of components, volcanic gases which formed the original cell must also have contained a complement of noxious ingredients Natural toxins may be considered as a successful evolutionary adaptation by species to predators They are a form of chemical warfare practised by one species on another to ensure their own subsistence Higher organisms have developed a sophisticated and flexible immune systems to eliminate large foreign particles *e g* pathogens *etc* , small molecules *e g* chemicals are not dealt with by this pathway The survival of man and all other species depends on their ability to successfully adapt to the presence of these chemicals in our food and the environment The ultimate problem facing organisms is a limitless number of small molecules of all possible physicochemical descriptions, inert, reactive, electrophilic, nucleophilic, lipophilic, hydrophillic *etc* These properties dictate how a molecule is absorbed and distributed in addition to the type of biotransformation necessary for detoxication The lipophilic compounds pose the greatest problem as they can penetrate the physical barriers of skin and cell membrane Both reactive and inert lipophilic molecules must be transformed into hydrophillic species so that they can be eliminated Otherwise these molecules will remain in the cell exerting their effect indefinitely, as occurs with chemicals that are resistant to metabolism and detoxication *e g* polychlorinated biphenyls and dioxins

To deal with this problem species have adapted This adaptation comes in the form of two highly efficient biological defence systems which have evolved over millions of years for this purpose, along with the obvious physical barriers (Gonzalez and Nebert, 1990) They are (1) the anti-oxidant system which protects against ionizing radiation, oxygen radicals and oxidants, and (2) the detoxication enzymes system These two biological defence systems are closely related, as products of the detoxication system *e g* (quinones) may become oxygen radical generators - substrates for the anti-oxidant system (Kappus, 1986) Cytochrome P450 is prominent in both systems absorbing dioxygen and its more dangerous singlet form in the antioxidant system and as a reductase and oxygenase in the detoxication system

The anti-oxidant system contains cytochrome P450 as mentioned above, superoxide dismutase and catalase which remove excess dioxygen and superoxide anion, Glutathione-S-Transferase (GST), Glutathione (GSH) peroxidase and phospholipid glutathione peroxidase which remove epoxides and lipid peroxides, GSH and GSH reductase, ascorbate, tocopherols and ubiquinone which protect tissues component from oxidants The detoxication system is made up of the cytochrome P450-dependent and independent oxygenases, flavoprotein oxidoreductase, oxidases, glutathione-S-transferase, epoxide hydrolase, sulphotransferases and glucuronlytransferases *etc* (Parke *et al ,* 1990)

These enzymes are capable of catalyzing a diverse group of chemical transformations They are extremely flexible where substrate structure is concerned, have loose constraints on substrate recognition, overlap, of activity between these enzymes is often observed This lack of selectivity is the key to the success of these enzymes in detoxifying a vast library of structurally diverse compounds To achieve this flexibility multiple forms or isozymes of these enzymes have evolved Each isoform can display different substrate preferences, but may also share some substrates

Exposure of an organism to certain compounds *(e g* benzo[a]pyrene) results in an induction response akin to that of the immune response, which induces the production of groups of enzymes which deal with the metabolism of that compound

The transformations catalyzed by detoxication enzymes are divided into two classes, Phase I which modifies xenobiotics resulting in the insertion of a polar functional group into that molecule Phase II enzymes recognise these newly exposed functional groups and conjugates hydrophilic moieties to them These transformed molecules can then be transported by the cell's regular transport mechanism out of the cell (Eckert *et al ,* 1986)

However a guaranteed scheme for the detoxication of every xenobiotic is not possible due to the limited resources available to the cell As a result the detoxifying enzyme system can accidentally catalyze the activation of some foreign compounds to their more toxic forms, rather than their less toxic forms For example, in the case of vinyl chloride a single metabolic step catalysed by a cytochrome P450 enzyme leads to the mutagen - chloroethylene oxide, 1,2-bromoethane on conjugation with glutathione via Glutathione-S-Transferase, produces 1-bromo-2-(*S*-glutathionyl)ethane an unstable intermediate which degenerates to a reactive episulfomum ion The activation of the polycyclic aryl hydrocarbon benzo[*a*]pyrene to the highly tumourigenic 7,8-diol-9,10-epoxide is carried out by the combined efforts of cytochrome P4501A1 and epoxide hydroxylase (Armstrong, 1987)

Over two dozen enzymes can reasonably be said to be involved in the detoxication of foreign compounds to the extent that it is their primary function Of these, those of importance to this work are the cytochrome P_{450} dependent enzymes, UDP-gluconyltransferase and Glutathione-S-Transferase

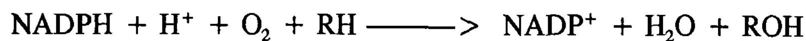
REACTIONS CLASSED AS PHASE I OR PHASE II

Phase I	Phase II
Oxidation	Glucuronidation/glycosidation
Reduction	Sulphation
Hydrolysis	Methylation
Hydration	Acetylation
Dethioacetlyation	Amino acid conjugation
Isomerization	Glutathione conjugation
	Fatty acid conjugation
	Condensation

1.2.0 PHASE I METABOLISM

Phase 1 metabolism involves cytochrome P450 dependent **and** independent oxidations, reduction, hydrolysis, hydration as well as isomerization and other rarer reactions of hydrophobic compounds found in the cell

The Mixed Function Oxidase system (MFO) is a battery of enzymes situated in the endoplasmic reticulum of the cell especially the liver, lung, kidney and intestine These enzymes carry out oxidation reactions on hundreds of structurally diverse compounds whose common denominator is their lipophilicity These reactions are dependent on a terminal oxidase - cytochrome P450 The reaction being,



$$NADPH + H^+ + O_2 + RH \longrightarrow NADP^+ + H_2O + ROH$$

Note The oxygen in this equation is molecular oxygen and not derrived from water

EXAMPLES OF THE GENERAL TYPE OF OXIDATION REACTIONS CATALYZED BY THE CYTOCHROME P450-CONTAINING MONOOXYGENASES.

REACTION		EXAMPLES OF COMPOUNDS METABOLISED
Aliphatic hydroxylation	R-CH_2-CH_2-CH_2→R-CH_2-CHOH-CH_2	Various side chain hydroxylation of *n*-propylbenzene,Pentobarbitone
Aromatic hydroxylation	R-⬡ →R-⬡ -OH	Benoz[a]pyrene, Naphthalene lignocaine → 3-hydroxy-hgnocaine *
Epoxidation	R-CH=CH-R'→R-CH-CH-R' O	Aldrin→Dieldrin, Naphthalene→Naphthalene epoxide, Benzo[a]pyrene→7,8,-Diol-9,10,-epoxides $
N-, O-, *S*-dealkylation	R-(N,O,S)-CH_3→R-(NH2,OH,SH) + CH_2O	*O*-dealkylation of *p*-nitroanisole,codeine,morphine and phenacetin *N*-dealkylation of aminopyrene,diazepam *S*-dealkylation of methylmercaptan and 6-methylthiopurine
Deamination	R-CH_2-NH_2→R-C-H + NH_3 O	Amphetamine → Phenylacetone
N-hydroxylation	R-NH-C-CH_3→R-NOH-C-CH_3 O O	Aniline → Phenylhydroxylamine,3-methylpyridine '
Sulfoxidation	R-S-R → R-S-R' O	Endosulfan → endosulfan sulfate, Chloropromazine and DMSO → Sulfone
Desulfuration	R_1R_2P-X → R_1R_2P-X + S S O	Parathion → Paraoxon
Oxidative Dehalogenation	R-CHX-H → R-CHX-OH → R-C=O-H + HX	General anaesthetics - halothane → CF_3-COOH

X = Halogen

* Hydroxylation of aromatic compounds can take place by one of two mechanisms One involves direct insertion of the oxygen atom to the carbon-hydrogen bond, the other results in the addition of the oxygen to the C=C producing arene oxide intermediate which in some cases are toxic This intermediates can then rearrange to form an aromatic hydroxyl compound

$ The products of epoxidation are often unstable due to the strain on their bond angles They readily decompose becoming substrates for epoxides hydratase forming dihydrodiols and other hydroxylated products

' In the presence of NADPH and O_2 either the mixed function oxidases or separate flavoprotein *N*-oxides will carry out this reaction depending on the substrate in question *N*-oxidation produces hydroxylamines like that of 2-acetlyaminofluorene which is thought to be a proximate carcinogen *Table reproduced from Sipes et al ,1986*

1.2.1 THE COMPONENTS OF THE MFO SYSTEM

i) CYTOCHROME P450

The cytochrome P450 superfamily of enzymes are haemproteins ranging in size from 45 kD to 60 Kd which contains one molecule of iron protoporphyrin IX prosthetic group per polypeptide chain (Figure 1) (Guengerich, 1990)

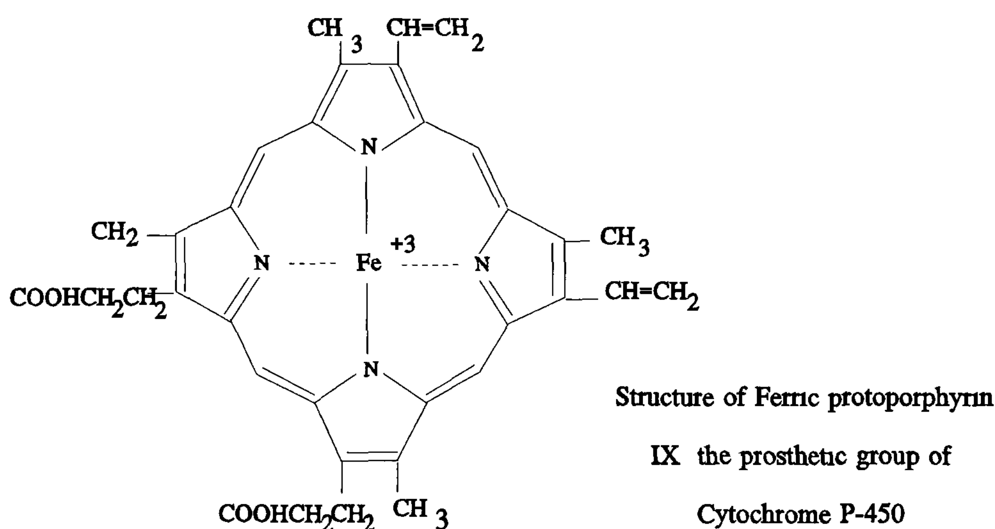


Figure 1

These enzymes exist as aggregates of approximately six monomers in the endoplasmic reticulum (Black, 1992) The use of the term "cytochrome" in this instance is inaccurate, as the P450 does not transfer electrons to another acceptor as is seen with other cytochromes but to a final substrate, however, the name has stuck over the years This haemprotein acts as both an oxygen <u>and</u> substrate binding site for MFO reaction In conjunction with a flavoprotein reductase (NADPH-cytochrome P450 reductase) it undergoes cyclic oxidation\reduction of the haem iron

Relatively little is known about the structure of this protein due to the fact that it is difficult to isolate for X-ray crystallography and too large a protein for NMR Attempts at isolation using limited proteolysis of microsomes did not release a soluble functional catalytic domain, but resulted in the conversion of P450 to the inactive form P420 Until recently, these findings suggested that P450 is deeply imbedded into the lipid bilayer membrane of the endoplasmic reticulum (De Pierre *et al* , 1977)

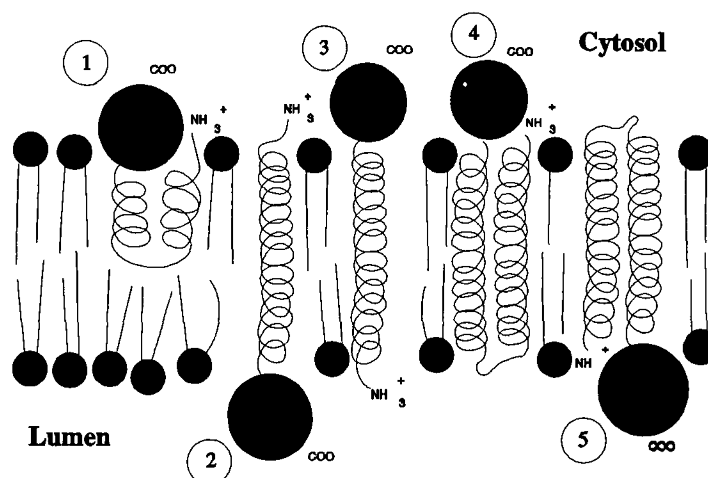


Figure 2 Suggested membrane topology of eukaryotic cytochrome P450 **(Black, 1992).**

However, recent studies have indicated that the protein is anchored to the membrane by insertion of its short amino-terminal segment, which is less than 29 residues (Fig 2) A number of configurations have been suggested (figure 2) No 1 with the carboxy terminal on the cytosolic face and a non-transmembrane hairpin segment with the NH_2 terminal on the cytosolic face also, No 2 a transmembrane protein with the carboxy terminal on the lumen face and the NH_2 terminal on the cytosolic face, No 4 a transmembrane - hairpin anchor with the carboxy and NH_2 termini on the cytosolic face, No 5 which is the inverse of 4 , but the majority lean toward the transmembrane orientation of No 3 *i e* a single trans-membrane anchor This NH_3 region serves as an insertion signal, anchor and halt-transfer signal (preventing further translocation of the protein), while the carboxy terminal protrudes into the cytoplasm (Vergeres *et al* , 1989 Black, 1992) Without accurate details on the 3D structure of P450 a prediction of the catalytic specificity of cytochrome P450 is impossible due to the vast variation in sequence and difficulty in isolating protein (Guengerich, 1990, Gotoh *et al* , 1983)

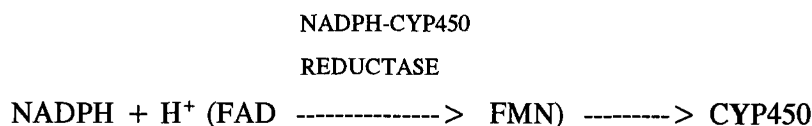
There are over 200 known P450s at present Each isoform has the same characteristic absorbance maximum when the Fe(II) group is complexed with carbon monoxide, *i e* near 450nm, due to axial ligation with a well conserved cysteine thiolate of the protein sequence (Guengerich, 1991)

The NH_3 anchor terminal is conserved to a large degree, but the molecular and structural design which governs the specificity of the P450 enzymes has yet to be uncovered Small changes in the protein sequence change the specificity dramatically *e g* in the P450 associated with progesterone 21-hydroxylase activity any two substitutions within the three sites 113, 115 and 118, result in a change to estradiol 2-hydroxylation (Guengerich, 1991)

Therefore it is difficult to assign catalytic activity based purely on protein structure P450s which are 90% identical still have over 50 substitutions to alter catalytic profile, and P450s which are structurally diverse may carry out identical reactions Also small changes in *in vitro* conditions may also effect substrate specificity

i) **NADPH CYTOCHROME-P450 REDUCTASE (NADPH cytochrome c reductase)**

This reductase contains one molecule of FAD and FMN per mole of apoprotein In humans this molecule has a molecular weight of 76,656 D and is anchored to the endoplasmic reticulum (next to the cytochrome P450) by a hydrophobic amino terminal peptide with the remainder of the molecule protruding onto the cytoplasmic surface of this membrane (Yamano *et al*, 1989) The human cDNA displays 83% similarity with that of the rat The gene is located on chromosome 7 in humans (7p15-q35) (Yamano *et al*, 1989) Cytochrome P450 accepts its reducing equivalents from the flavoprotein as follows



This reaction requires the presence of Mg^{++} (Peters *et al*, 1970)

The reductase acts as a transducer *i e* moving reducing equivalents sequentially on to Cytochrome P450, one electron at a time The exact electron flow has yet to be determined The redox state of the flavoproteins during oxidation is not known however it is believed that FAD accepts the reducing equivalents from NADPH + H^+ and FMN donates them to CYP450 (Poulos *et al*, 1992)

ii) **LIPID**

A Lipid component is essential for the function of P450 Reconstitution experiments which lacked a lipid component, resulted in non-functional protein

The Lipid component is believed to be involved in substrate binding, electron transfer, conformational change and providing an anchor for the interaction of Cytochrome P450 and its NADPH reductase (Nisimoto *et al*, 1983, Vergeres *et al*, 1989)

iii) Cytochrome b_5

As far as is known cytochrome b_5 has no uniform effect on P450-dependent oxidations It has been reported in many roles but the precise function of this element here is unknown

The main biological function of hepatic microsomal b_5 is to participate in the desaturation of long-chain fatty acid acyl-CoA derivatives, providing reducing equivalents for the desaturase enzymes Unlike P450 it is relatively insensitive to induction by exogenous drugs and chemicals In uninduced liver microsomes, the molar ratio of P450 to b_5 is approximately 2 1,during induction this increases to 6 1

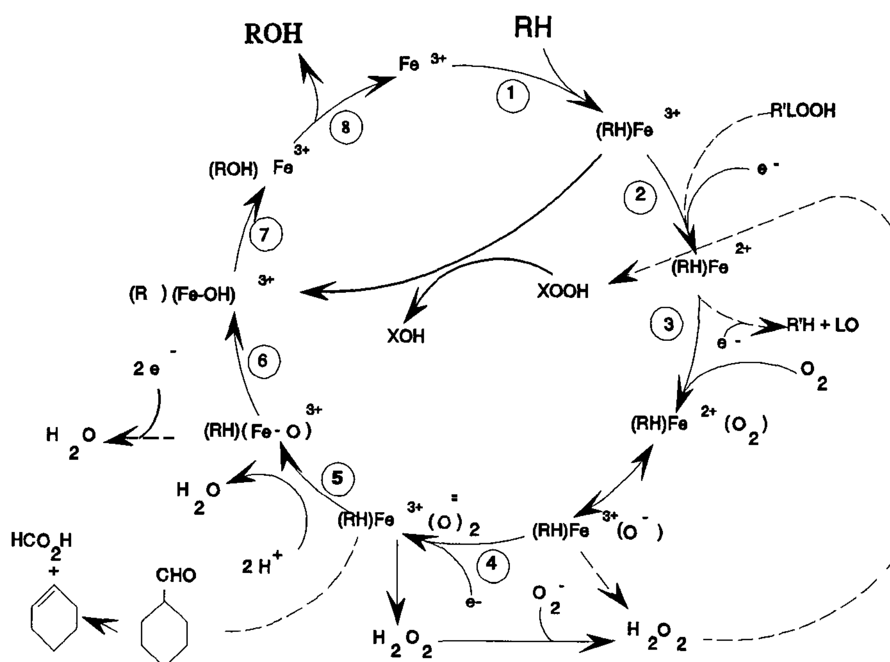


Figure 3: The catalytic cycle, electron flow, of cytochrome P450.

RH: substrate; ROH: monooxygenase product; R'LOOH: lipid hydroperoxide; R'H & LO corresponding reduction products; XOOH: peroxy compound that serves as and alternate oxygen donor to molecular oxygen (Coon *et al.*, 1992)

1.2.2 THE CATALYTIC CYCLE

a) **Step 1**

Substrates bind to the ferric form (Fe^{+3}) of the haem moiety within the Cytochrome P450 molecule Binding can be of three types I, II, IIb (or reverse II), as seen by the spectral changes observed as the substrates bind to P450 These spectral changes result from interference with the spin states of the electrons in the d orbitals of the iron molecule contained within the P450 as influenced by the 2 axial ligands belonging to the P450, as the equatorial 4 pyrroles of the protoporphyrin IX complex cannot be changed As substrate binds to the P450 molecule the electrons occupying the d orbitals of the haem iron are exposed to electron repulsion by the electrons of the binding ligands This results in a splitting in the energy levels of the ferric ion d-orbitals resulting in either low or high spin configurations (figure 4)

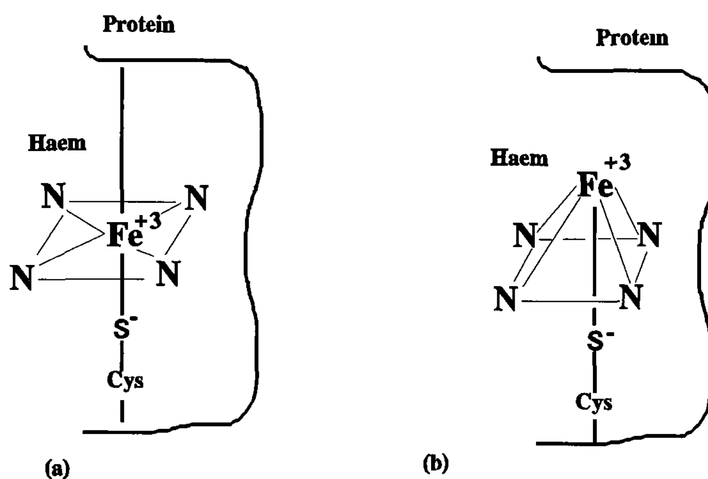


Figure 4: Haem iron co-ordination in Cytochrome P450.

a) Low spin orientation with only one unpaired d electron.

b) High spin - with five unpaired d electrons.

Cytochrome P450 exist mainly in the low spin state demonstrating an absorbance peak at 418 nm Type I substrates *e g* benzphetamine, caffeine and DDT, bind to the protein moiety and induce conformational change This change in environment affects the haem iron d orbital electrons resulting in a change from low-spin to high-spm and a corresponding absorbtion maximum at 390 nm Binding to the haem iron as in the case of type II nitrogenous substrates *e g* aniline, nicotine and type IIb ligands *e g* ethanol and acetamlide, results in low-spin configuration and an absorbance max at 418 nm
The catalytic activation of oxygen occurs on the iron atom at the site of the exchangeable sixth ligand (White and Coon, 1980)
The importance of the axial ligands as determinants of haemoprotein function is highlighted when it is considered that P450, haemoglobin, peroxidases and catalase, all contain protoporphyrin IX as their prosthetic group, the orientation of the haem relative to the fifth ligand is the same and yet each performs different biological functions (Ortiz de Montellano *et al* , 1983)

b) **Step 2**

This is the first electron reduction, by NADPH cytochrome P450 reductase, of the substrate-bound ferric form of cytochrome P450, resulting in the ferrous form
At this stage the first reductive reaction is also observed The peroxide shunt, where peroxy compounds *e g* alkyl hydroperoxide, donate an oxygen atom for substrate hydroxylation This pathway dose not require molecular oxygen or NADPH for its electrons The stepwise one electron transfer has also been proposed Lipid and xenobiotic hydroperoxides (R'LOOH) are cleaved, resulting in hydrocarbon formation *e g* Cumyl hydroperoxide undergoes this reductive metabolism yielding acetophenone and methane (R'H and LO) The ethanol inducible P450 2E1 has been strongly implicated in this reaction (Coon *et al* , 1992)

In adrenal mitochondria, which are more concerned with endogenous metabolism than xenobiotic metabolism, the sequence of events is different A further carrier iron-sulphur protein, adrenodoxin, is required, before the electrons reach the P450 molecule

The spin-redox coupling in steps 1 + 2 is complex due to the fact that the NADPH cytochrome P450 reductase contains FAD and FMN and has the capacity to carry 4 electrons

However, it is believed that the fully reduced state of the low potential flavin, is the electron-donating species (Gibson and Skett, 1986)

c) **Step 3**

Binding of molecular oxygen to ferrous cytochrome P450-substrate complex, producing an unstable oxy-ferrous substrate complex

d) **Steps 4 - 8**

These steps involve, electron rearrangement, introduction of the second electron, oxygen insertion into substrate, followed by product release The origin of the second electron is debatable It may originate from the NADPH Cytochrome P450 reductase once again, or NADH Cytochrome b_5 reductase

Uncoupling of the P450 electron cycle occurs when the reducing equivalents (the electrons) are short circuited away from substrate hydroxylation to oxygen reduction

This produces superoxide (O_{2-}) hydrogenperoxide and $4e^-$ reduction of water at the 6th step The peroxide shunt donates an oxygen atom for hydroxylation requiring neither molecular O_2 or NADPH electrons (Coon *et al* , 1992) These radicals may convert xenobiotics into products, some of which may be of a toxic nature (Bast, 1986)

REDUCTIVE METABOLISM

Under certain conditions of low oxygen tension cytochrome P450 enzymes will donate one or even two of their electrons (as seen in Fig 1 above) to certain xenobiotics Both the flavoprotein enzyme, NADPH-cytochrome P450 reductase, and the terminal oxidase, cytochrome P450, are involved in these reductions This alternative action of P450 can result in activation of compounds to their more toxic products or reactive intermediates *e g* carbon tetrachloride and halothane Intestinal microflora also carry out reductive metabolism, often resulting in reactivation of previously deactivated xenobiotics

Reduction catalysed by the NADPH-cytochrome P450 reductase results in the activation of some quinone anticancer agents These compounds undergo one-electron reduction of their quinone group to produce the semiquinone free radical species This semiquinone metabolite is unstable in the presence of air and is rapidly re-oxidized to form the parent quinone and the superoxide anion radical It is this anion which binds with the DNA delivering the cytotoxic effect Drugs like adriamycin and mitomycin C are activated this way (Gibson and Skett, 1988)

ETHANOL METABOLISM

Ethanol metabolism is usually carried out by alcohol dehydrogenase, however on induction with ethanol, 80% of its clearance is carried out by the P450-dependent enzymes

This phase I battery of enzymes also contains a number of non P450-dependent enzymes as can be seen below

1.2.3 OXIDATIONS OTHER THAN CYTOCHROME P450 DEPENDENT

Alcohol dehydrogenase	Carbonyl reductase
Aldehyde dehydrogenase	Dihydrodiol dehydrogenase
Xanthine oxidase	Glutathione peroxidase
Amine oxidase	Monoamine oxidase
Aromatases/amidases/esterases	Aldehyde oxidase
Alkylhydraxine oxidase	D-Amino acid oxidase
Flavin-containing monooxygenases	Quinone reductase
	Epoxide hydroxylase

Aldehydes, ketone and alcohols are often the byproducts of xenobiotic metabolism Aldehyde reductase, alcohol dehydrogenase, ketone reductase and a number of aldehyde oxidases are involved in the further metabolism of these compounds

These enzymes are localized within the cytosol of the liver, kidney and lung

Alcohol dehydrogenase is one of the most important members of this group Located mainly in the liver it accounts for the majority of ethanol metabolism This enzyme will also metabolise other alcohols however, not always with the same detoxifying effects *i e* the oxidation of methanol and ethylene glycol resulting in the toxins formate and oxalate

Aldehyde dehydrogenase is made up to two forms One form oxidises formaldehyde complexed with glutathione and is called formaldehyde dehydrogenase

The other aldehyde dehydrogenase oxidizes free aldehydes and has a broad substrate specificity It is this second dehydrogenase that is mainly involved mostly in xenobiotic metabolism

Xanthine oxidases metabolise xanthine-containmg drugs to their corresponding uric acid derivative *e g* caffeine, theophylline, theobromine

Amine oxidases are a group of enzymes which require NADPH and molecular oxygen, but they are not mixed-function oxidases They can be subdivided into

1) monoamine oxidases (MAO) responsible for the metabolism of endogenous catecholamine

2) diamine oxidases responsible for the deamination of endogenous diamine *e g* histamine and are not primarily involved in drug metabolism

3) Finally the microsomal **flavin containing monooxygenase.**

Also known as microsomal mixed function amine oxidase and *N*-oxygenase (Jakoby and Ziegier, 1990) The broad substrate specificity and wide tissue distribution of this enzyme makes it a major participant in the oxidative metabolism of drugs and xenobiotics It is a polymeric protein made up of monomers of Mw 65 Kd, containing one molecule of FAD per mole of protein monomer It is present in all tissue with substantial concentrations in the liver It can utilize both NADH and NADPH The reaction sequence is flavin reduction, oxygen binding, electron transfer to oxygen forming a peroxy-flavin complex, substrate binding, oxygenated product release and dissociation of $NADP^+$ yielding the oxidized enzyme This sequence can be short circuited to H_2O_2 when an oxidizable substrate is not present

Many endogenous nucleophiles contain 1 or more anionic groups Nucleophiles such as these are not metabolised by this enzyme which suggests a self-preserving mechanism of the body as their metabolism would be unfavourable

This is the only known mammalian flavoprotein hydroxylase

Aromatases convert cyclohexanecarboxylic acid containing xenobiotics to their corresponding benzoic acid This enzyme is expressed in the liver and kidney For metabolism to occur the substrate must first be converted to its Coenzyme-A derivative FAD and oxygen are also required as co-factors

HYDROLYSIS

Nonspecific hydrolysis reactions occur in the blood plasma Specific esterases /amidases reactions are reserved to the liver Ester and amide hydrolysis reactions release a carboxylic group, in the case of esters, an alcohol group is released, whereas amides produce an ammonium group

Esterase can be categorized into four main classes

1) arylesterases - which act mainly on aromatic esters

2) carboxylesterases - which hydrolyse aliphatic esters

3) acetylesterases where the acid in the ester is acetic acid and
4) cholinesterases - where the alcohol in the ester is choline

HYDRATION

A large number of xenobiotics, such as esters, amides and substituted phosphates that are composed of ester-type bonds, are susceptible to hydrolysis The precarcinogenic polycyclic hydrocarbon epoxides in particular undergo this reaction forming trans-diol compounds *e g* benzo[a]pyrene

OTHER REACTIONS OF PHASE I INVOLVED IN DRUG METABOLISM

Reaction	Example
Ring cyclization	Proguanil
N-Carboxylation	Toxamide
Dimerization	*N*-OH-2-Acetylaminofluroene
Transamidation	Propiram
Isomerization	α-Methylfluroene-2-acetic acid
Decarboxylation	L-Dopa
Dethiacetylation	Spironolactone

Some of these Phase I enzymes are primarily involved in endogenous metabolism

1.2.4 ENDOGENOUS METABOLISM BY PHASE I ENZYMES

Enzyme	Endogenous substrate
Mixed function oxidase	Steroids Sterols Thyroid hormones Fatty acids Prostaglandins Vitamin D Leukotrienes
Monoamine oxidase	Monoamine neurotransmitters
Diamine oxidase	Histamine Putrescine Cadaverine
Xanthine oxidase	Xanthine
Hydroxysteroid oxidoreductase	Steroids
Acetylcholinesterase	Acetylcholine
Reductase	Steroids

PROSTAGLANDIN SYNTHETASE DEPENDENT CO-OXIDATION OF DRUGS

Prostaglandin synthetase is present in most mammalian cell types

This enzyme catalyses the formation of prostaglandins from arachidonic acid (AA) via a two step pathway functioning as both a fatty acid cyclooxygenase to form prostaglandin G_2 and a hydroperoxidase, reducing prostaglandin G_2 to form prostaglandin H_2 Many xenobiotics and drugs are co-oxidized during the prostaglandin specific hydroperoxidase action of this enzymes The mechanism of this reaction is unknown but it is believed in the case of paracetamol to involve a 1-electron oxidation followed by the formation of a quinone radical which can either reacts with glutathione, or is reduced along with glutathione to reform paracetamol (Gibson and Skett 1986) Compounds like aminopyrme, benzphetamine, oxyphenbutazone, paracetamol and chemical carcinogens like benzo[a]pyrene etc are metabolised by this pathway The overall contribution of this co-oxidation to the elimination of these compounds is not known

NON-P450 DEPENDENT REDUCTIVE METABOLISM

This form of reductive metabolism takes place using cytochrome b_5 as the transducer and NADH + H Substrates like epoxides, heterocyclic azo and Nitro compounds, and halogenated hydrocarbons utilize this pathway Reduction can convert some epoxides back to their original compounds *e g* benzo[a]-anthracene-8,9-epoxide to benzanthracene

EPOXIDE HYDROXYLASE

This enzyme is found in almost all tissues investigated, with highest concentrations found in the liver endoplasmic reticulum, nuclear membrane and cytosol This is a monomeric protein composed of units of MW 48,000-54,000 daltons Epoxide hyroxylase contains no flavin It is inducible by a variety of hepatocarcinogens, barbiturates and fluorenes *e g* DEN an alkylnitrosamine elevates the mRNA levels of microsomal epoxide hydroxylase (Kondo, *et al* , 1990)

Epoxides formed by the microsomal mixed function oxidase system follow a number of paths in the body

i) *in vivo* nonenzymic rearrangement to phenols

ii) irreversible binding to nucleic acids and phenols

iii) glutathione conjugation

iv) enzymic hydration to dihydrodiol or further oxidation to diol epoxide

Epoxides are reactive electrophilic species

Epoxides hydratase catalyses the nucleophilic attack of water on one of the two electron deficient carbon atoms of the oxirane ring, a reaction which is sterio specific and regio selective Epoxide Hydroxylase plays a major role in the activation of compounds to their toxic metabolites This enzyme is also believed to be a pre-neoplastic antigen and as such an early cancer marker (Sipes *et al* , 1985) This protein in rat nodules and cancer cells is believed to be effected by constitutive internal stimuli associated with cell growth (Kondo *et al* , 1990)

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1.3.0 PHASE II

Phase II reactions are conjugation reactions which require energy to drive them To provide this energy the cofactors or substrate are activated to high energy intermediates Phase II enzymes react with phase I metabolites thereby producing "bulky" water-soluble metabolites which can readily be excreted However, a Phase I functionised metabolite is not always required and phase II conjugation can occur directly

PHASE TWO REACTIONS

REACTION	ENZYME	FUNCTIONAL GROUP	COFACTOR INVOLVED
Glucuronidation	UDP-Glucuronyltransferase	-OH -COOH $-NH_2$ -SH	UDP GLUCURONIC ACID
Glycosidation	UDP-Glycosyltransferase	-OH -COOH -SH	
Sulfation	Sulfotransferase	$-NH_2$ $-SO_2NH_2$ -OH	PAPS - 3'PHOSPHOADENOSINE-5'PHOSPHOSULPHATE
Methylation	Methyltransferase	-OH $-NH_2$	SAM - S-ADENOSYLMETHIONE
Acetylation	Acetyltransferase	$-NH_2$ $-SO_2NH_2$ -OH	ACETYL CO-A
Amino Acid Conjugation		-COOH	AMINO ACIDS
Glutathione Conjugation	Glutathione-S-transferase	Epoxide Organic halide	GLUTATHIONE
Fatty acid Conjugation		-OH	STEARIC AND PALMITIC ACID
Condensation		Various	

1.3.1 GLUCURONIDATION: CONJUGATION WITH SUGARS

Glucuronidation *is* the conjugation of a compound with the co-factor UDP-glucuronic acid which is a product of intermediary metabolism This reaction is carried out on both endogenous and exogenous compounds by the isoenzymes UDP-glucuronosyltransferases and is one of the major conjugation reactions in drug metabolism The resulting glucuronides are then transported from the cell and eliminated from the body in the normal manner

These enzymes are expressed in many species and have a broad range of substrates This quality makes conjugation with glucuronic acid an important conjugation reaction

Glucuronidation conjugates alcohols, phenols, hydroxylamines, carboxylic acids, amines, sulfonamides and thiols

These isoenzymes are glycoproteins which range in molecular weight between 50,000-60,000 daltons They are endoplasmic-reticulum and nuclear envelope-bound, which allows for closer association with phase 1 metabolites Substrate specificity varies from isoform to isoform, with some displaying activity toward a single compound and others having a broad substrate band (Burchell, 1991) The liver is the most important tissue with regard to UDP-glucuronosyltransferase activity, but activity has also been noted in the kidney, intestine, skin, brain and spleen Due to their membrane orientation these transferases demonstrate latency on examination *i e* the enzyme requires activation e g freeze thawing before it will demonstrate its full activity They are believed to be transmembrane molecules, consisting of four domains A) a conserved UDP-glucuronic acid binding site, B) a variable substrate binding region, C) a conserved NH_3 terminal and D) a transmembrane anchor Catalysis is believed to take place on the lumen side of the endoplasmic reticulum *i e* behind a membrane It is this membrane barrier which may explain the latency of this enzyme *in vitro* (Burchell, 1991) As already mentioned in connection with P450, with deactivation, there is also often activation The susceptibility of certain glucuronides to enzymatic and chemical degradation makes them ideal candidates to transport potentially reactive compounds from the liver to the target tissue For example *N*-glucuronides of *N*-hydroxy-arlyamines have been implicated in bladder cancer, compounds like 2-naphthylamine, 4-amino-biphenyl, and related compounds which, having reached the bladder are unstable in the acidic environment and are thus hydrolysed to their unstable carcinogenic *N*-hydroxylamine (Sipes *et al*, 1986)

Comparable to the P450 superfamily, the UDPGT superfamily is subject to induction by the same PAH inducers *i e* via the Ah receptor

This induction ties in with the observation that cells appear to respond to the presence of a given toxin by inducing a battery of enzymes involved in the metabolism of that compound in this case *e g* PAH However, these enzymes also display antioxidant induction, a phenomenon not observed in P450 This induction is mediated through ARE (antioxidant responsive elements) identical to those upstream of the GST Ya subunit (Bock, 1991) There are four main classes of glucuronide conjugates

i) **O-Glucuronides**

Formed from phenols, alcohols, and carboxylic acids The carboxylic acids form ester glucuronides and the phenols and alcohols form ether glucuronides During these reactions the α-glucuronic acid is inverted to the β-glucuronide β-glucuronides which are excreted into the intestine become substrates for the β-glucuronidases which hydrolyse the conjugate back to its original compound As a result of this hydrolysis the plasma half life of some drugs is increased

ii) **N-Glucuronides**

Formed from amines (usually aromatic, and some tertiary) amides and sulphonamides Some N-glucuronides form spontaneously *i e* they do not need the presence of an enzyme

iii) S-Glucuronides

Thiols which react with UDPGA in the presence of UDP-gluconyltransferase forming S-Glucuronides

iv) **C-Glucuronides**

Direct attachment of UDPGA to the carbon skeleton has also been observed Most species use UDPGA conjugation as their major conjugation sugar, with the exception of Insects where glucose is employed more readily The reactions and products are analogous with UDP-glucose being the conjugate rather than UDPGA UDP-glucose reactions have also been seen in plants and mammals to a small extent UDP-xylose and UDP-ribose also contribute to conjugation N-ribosides are the most prevalent and may be spontaneous, but O-xylosides need the involvement of a microsomal transferase

1.3.2 SULPHOTRANSFERASES

Sulphation is the major conjugation pathway chosen by phenols, and aliphatic alcohols and amines Thiols also use this pathway but only to a small extent The sulfotransferases are found in the cytosol of liver, kidney, intestinal tract and lungs Their primary function is to transfer inorganic sulfate to hydroxyl groups present to it Many low-molecular-weight endogenous compounds *e g* catecholamine, hydroxy steroids, and bile acids are conjugated in this way

Where as UDPGA is the active donor in glucuromdation, PAPS is the active donor in sulfation *i e* 3'-phosphoadenosine- 5'-phosphosulfate This cofactor is synthesized from inorganic sulfate and ATP in a two stage process in the cell cytosol

There are many different sulphotransferases isoenzymes *e g* phenol-, alcohol-, steroid-, and arylamine-sulphotransferase With the exception of those involved in steroid metabolism sulphotransferases are relatively non-specific and vary in activity due to the age and sex of the animal in question In general sulphate conjugation predominates at low substrate concentration and glucuronidation at high concentration due to substrate inhibition of the transferases (Sipes *et al* , 1986, Gibson and Skett, 1986)

1.3.3 METHYLTRANSFERASE

This reaction is mainly involved in endogenous metabolism However, some exogenous compounds are conjugated by non-specific methyltransferases, in lung and by the physiological methyltransferases

The nature of the methyltransferase reaction is similar again to that of other conjugation reactions, in that the methyl group is transferred to the xenobiotic from a high-energy cofactor, *S*-adenosylmethionine or SAM

Methylation differs from most other conjugation reactions in that it <u>can</u> lead to the masking of functional groups, reducing their water solubility and preventing further conjugation and reducing their ability to be excreted This property can lead to the reprecipitation of the compound thereby increasing its exposure time

METHYLTRANSFERASES

Enzyme	Substrate	Site
Phenylethanolamine N-methyltransferase	Noradrenaline	Adrenals
Non-specific *N*-Methyltransferase	Various(desmethylimipramine)	Lung
Imidazole *N*-methlytransferase	Histamine	Liver
Hydroxyindole *O*-methyltransferase	*N*-Acetylserotonin	Pineal gland
S-Methyltransferase	Thiols	Liver Kidney Lung
Catechol *o*-methyltransferase	Catechols	Liver Kidney Skin Nerve Tissue

NON-SPECIFIC *N*-METHYLTRANSFERASE FOUND IN THE LUNG CAN REVERSE THE *N*-DEMETHYLATION REACTIONS OF PHASE I METABOLISM The S-methyltransferases, present in the microsomal fraction, are believed to have evolved to detoxify hydrogen sulfide produced by anaerobic bacterial in the intestinal tract These enzymes also handle the thio ethers of glutathione conjugation, another phase II enzyme (Sipes *et al* , 1986)

1.3.4 N-ACETYLTRANSFERASE

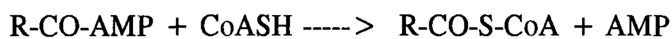
Acetylation reactions require as their co-factor, acetyl-CoA which comes from the glycolysis pathway, or direct interaction of acetate and coenzyme A Acetylation takes place mainly in the liver cytosol and is found in the Kupffer cells, not the hepatocytes It is also found in the reticuloendothelial cells of the spleen, lung and gut This is a major detoxification pathway for aromatic amines and sulphonamides N-acetyltransferase polymorphism for selected substrates has been reported in humans, mice, rabbits, and species of monkeys

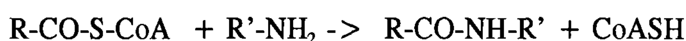
Subjects are classified as "rapid" or "slow" acetylators, based on their ability to acetylate isoniazid Acetylation is another example of a conjugation reaction which can mask functional groups Some N-acetylated derivatives are less water soluble than their parent compounds The N-acetylated sulphonamides are of particular interest as they are appreciably less soluble in water than the parent drug and have been implicated in renal toxicity - the earlier N acetylated sulphonamindes precipitated in the renal tubules resulting in toxicity (Sipes *et al* , 1986)

1.3.5 AMINO ACID CONJUGATION

Amino acid conjugation is a specialized form of N-acylation, where the drug and not the endogenous co-factor is activated (in this case the amino acid) Glycine, glutamine, ornithine, arginine and taurine are usually involved as follows,

R-COOH + ATP ---------> R-CO-AMP + PPi


R-CO-AMP + CoASH -----> R-CO-S-CoA + AMP


R-CO-S-CoA + R'-NH_2 -> R-CO-NH-R' + CoASH

The amino acid chosen for conjugation is related to the intermediary metabolism of the species *i e* Ureotelic animals (those that excrete urea) tend to use glycine, and uricotelic species (those that excrete uric acid) use ornithine This pathway displays a high affinity for substrate, but a low capacity As the pathway is saturated by substrate other mechanisms are employed *e g* methylation *etc* (Sipes *et al*, 1986)

1.3.6 GLUTATHIONE CONJUGATION

Glutathione is a tripeptide made up of glutamate, cysteine and glycine The function of this molecule is to conjugate with electrophilic compounds which may be harmful to the body Many of the drugs metabolized by the Phase I enzymes result in strong electrophiles These metabolites then react with glutathione to form, in most instances, non-toxic conjugates The list of compounds which are conjugated with glutathione includes

Haloalkanes
Epoxides
Nitroalkanes
Alkenes
Aromatic Halo-
Aromatic Nitro- Compounds

Glutathione-S-transferases the family of enzymes responsible for these conjugation reactions, are located in the cytosol of liver, kidney, gut and other tissues They consist of three classes of isoenzymes with differing substrate specificity *See section 1 6 0, for more information on Glutathione-S-Transferase*

1.3.7 FATTY ACID CONJUGATION

The fatty acids involved in this process are stearic and palmitic acid These enzymes are situated in the liver microsomal fraction Little is known about this mechanism, except that it has been implicated in the metabolism of 11-hydroxy-delta 9 tetrahydrocannabinol (Gibson and Skett, 1986)

1.3.8 CONDENSATION REACTIONS

Condensation reactions have been observed with amines and aldehydes, eg dopamine These reactions are important from the point of view that condensation of *e g* dopamine results in the alkaloid tetrahydropapaveroline, a potent dopamine antagonist However, it remains to be proven whether these reactions are mediated enzymatically or if they are purely chemical

1.3.9 ENDOGENOUS PHASE II REACTIONS

Glucuronidation is also a common pathway for many endogenous compounds including steroid hormones, catecholamines, bilirubin and thyroxine as can be seen in the table below

PHASE II METABOLISM OF ENDOGENOUS COMPOUNDS

REACTION	SUBSTRATES
Glucuronidation	Steroids Thyroxine Bilirubin Catecholamines
Sulphation	Steroid Carbohydrates
Methylation	Biogenic amines
Acylation	Serotonin
Ammo acid conjugation	Bile acids
Glutathione conjugation	Arachidomc acids metabolites Leukotrienes

1.3.10 PHASE III

It has been proposed that a further phase of metabolism for conjugates exist, a third phase in metabolism This metabolism is believed to take place in the intestine and is carried out by the intestinal microflora (Sipes *et al* , 1987, Gibson and Skett, 1986)

SUMMARY

The ultimate action of the phase I battery of enzymes is to expose -OH, -COOH, $-NH_2$ and -SH moieties on lipophilic xenobiotics This action results in increased polarity of these molecules and makes them ideal substrates for the phase II enzyme system These enzyme have broad substrate overlap capabilities Unlike many of the Phase I metabolites, Phase II metabolites have no common link Their one common feature is the requirement for an energy-rich 'activated' intermediate *i e* co-factors like UDPGA, PAPS, SAM, Acetyl-CoA, or an activated drug in the case of amino acid conjugation Phase II metabolites are ***usually*** more water soluble and more readily excreted

1.4.0 INDUCTION OF THE XENOBIOTIC METABOLIZING ENZYME SYSTEM

The induction of xenobiotic metabolism was originally observed (before the discovery of cytochrome P450) in rats which had been chronically dosed with barbiturates It was observed that the sleeping time of pre-dosed animals was reduced by half in comparison to animals which were not pre-dosed Reduction in the carcinogenicity of the amino-azo dyes when administered together with 3-Methylcholanthrene (MC) was also noted The effect of these compounds was recognized to be of importance, due to the fact that biological responses were altered by their presence

After the discovery of cytochrome P450 in the early 60s (Omura and Sato, 1964) further analysis of this phenomenon in the presence of PB (phenobarbital) demonstrated that the concentration of cytochrome P450 and most importantly P450 associated enzymatic activity, increased two to three fold (Nebert & Gonzalez, 1987) Later studies using a number of other inducers *e g* 3-MC, also demonstrated increases in P450 In some cases the characteristic cytochrome P450 carbon monoxide spectral peak at 450nm was observed to shift from 450 to 448nm This observation indicated that a single protein/mechanism was not involved, but probably a number of proteins and mechanisms, which has shown to be the case

1.4.1 MECHANISMS OF INDUCTION.

A number of modes of induction have been documented to date They are increased transcription of mRNA, increased stabilization of mRNA, increased protein synthesis/mRNA translation, and increased protein stabilization The early work in this area was mainly carried out on the PB and 3-MC-like inducers At least five inducer categories are known, *e g* polycyclic aromatic compounds (PAH), phenobarbital (PB), glucocorticoids, ethanol, and peroxisome proliferators Not all P450s are inducible and not all members of the same P450 family are necessarily inducible Many of these inducers have been reported to aid their own elimination by inducing those P450 enzymes responsible for their metabolism, *e g* Polycyclic hydrocarbons, some of which also induce the phase II conjugating enzymes which completes their metabolism (Coon *et al* , 1992) Often the phase I enzymes are induced to a greater extent than the phase II, which can lead to an increased concentration of reactive intermediates awaiting conjugation In some instances this may not be of benefit to the cell Induction is not an exclusively mammalian trait and has been reported in other species

1.4.2 GENERAL MECHANISM OF INDUCTION for P450.

No single mechanism accounts for P450 induction, not even within any single major inducer category Every stage from transcription-elongation through to incorporation of the haem molecule into P450 protein is subject to regulation The combined effects of these stages leads to increased enzyme activity (Okey, 1990)

Not all inductions draw the same cellular response, but *de novo* protein synthesis is required for there to be true induction, not simply activation of an already present inactive enzyme In order for *de novo* protein synthesis to occur there must be increased levels of mRNA coding for <u>functional</u> protein

There are some methods of induction which do not involve increased transcription, however the steady state mRNA levels are altered (Conney, 1967) Other systems concentrate at the post transcriptional level, increasing the stabilization of the current mRNA and on the stability of the protein

The haem moiety also plays a role in regulating induction at the post transcriptional level As drug induced P450 apoprotein incorporates haem into new P450, the haem pool is reduced This reduction results in increases expression of 5-amino-levulinate synthase (responsible for the first step in haem synthesis) thereby allowing the haem supply to keep pace with its utilization (Okey, 1990)

1.4.3 SITES OF INDUCTION

The majority of research to date has been carried out using liver derived cell lines and hepatocytes However, induction does occur to some extent in every mammalian tissue studied (Okey, 1990)

1.4.4 RECEPTOR MEDIATED INDUCTION - PAH:

The most extensively studied receptor-mediated method of induction is that seen in the P450 IA family - the induction of CYP1A1 - *see section 1 5 1 2 - Genetic regulation*

1.4.5 PHENOBARBITAL LIKE INDUCTION:

Phenobarbital and phenobarbital-like inducers work in liver more so than in extra-hepatic tissue Induction by this category results in an overall increase in P450 levels and smooth endoplasmic reticulum (Remmer & Merker, 1983) The main family of enzymes effected are the cytochrome P450IIB family, IIC6 and IIIA1 As is the case with the receptor mediated induction, phenobarbital induction also requires *de novo* protein synthesis

However, unlike the receptor mediated induction pathway, a mechanism or mode of recognition for this induction has not yet been discovered This is mainly due to the fact that there are, in addition to phenobarbital, a number of phenobarbital-like inducers All these compounds have different structures, making a common receptor hard to envisage Further hampering the investigations is the low level of response to induction by all these compounds, even phenobarbital itself This weak response makes a model system for study difficult to develop A potent inducer which gives maximum response has yet to be found

In cell culture, phenobarbital and PB-like compounds have been found to act in an uncharacteristic manner They have been implicated in the induction of enzymes normally associated with PAH and PCN/glucocorticoid induction The reason for this behaviour is unknown, but may be a result of culture conditions (Okey *et al* , 1990)

1.4.6 PCN/GLUCOCORTICOID - type induction.

Pregnenalone-16α-carbonitrile (PCN), macrolide antibiotics, antifungal agents, some PB-like inducers, steroid antagonists, endogenous and synthetic glucocorticoids, produce a spectrum of induction unlike that of 3MC/PB (Lu *et al* , 1972) These compounds induces CYP2A1 gene by increasing mRNA and by stabilizing current IIA1 proteins However, the catalytic role of this enzyme has not yet been uncovered It is thought that it may play a role in some steroid hydroxylation and metabolism of some of its inducers, it may also be involved in the metabolism of cyclosporin (Kronbach *et al* , 1988)

1.4.7 ETHANOL INDUCTION:

Exposure to ethanol increases the rate of oxidation of several drugs as well as oxidation of ethanol itself (Peterson *et al* , 1982) The P450 induced by ethanol is a member of the II family, IIE1 This family has been reported to be PB inducible, however, IIE1 is <u>not</u> PB inducible This induction is not via increased mRNA transcription, but by stabilization of mRNA and protein

Induction of IIE1 has been observed in unchecked diabetic rats, where the ketone level is increased, this is unusual as drug metabolism normally decreases in diabetes (Bellward *et al* , 1988)

1.4.8 MIXED INDUCTION:

There are many examples of compounds that induce P450 overlap *i e* inducing species belonging to more than one category or gene family Compounds like PCBs (polychlorinated biphenyls) along with mono-*ortho* or di-*ortho* chlorine substituent are capable of acting both as PB <u>and</u> 3-MC inducers However the entire complement of induction is not achieved, as some genes normally induced by one or other of these compounds will not be induced by a mixed inducer Isosafrole induces P450IA1, IA2, II1 and IIB2 in rat liver, but will only induce P450IA2 in mouse

The mechanism of this induction is not mediated via the Ah receptor, but isosafrole has been reported to bind tightly to IIB2 resulting in the stabilization of this protein

The inducer Aroclor 1254 induces a broad spectrum of drug metabolising enzymes However, this compound is not made up of one inducer, it is a cocktail of a number of Pb and 3-MC inducers compiled for maximum effect

1.4.9 THE CONSEQUENCES OF INDUCTION:

There are a number of practical concerns about P450 induction

(1) it may alter the efficiency of clinically administered drugs

(2) it may alter the balance between toxification and detoxification

Patients treated with PB show increased clearance rates for several other unrelated drugs *e g* antipyrine, phenytoin, oral contraceptives and warfarin (Park & Breckenridge, 1981) The severity of this increased clearance and whether an increased clearance may compromise treatment, depends upon the therapeutic index of the drug in question For example, the administration of the anticoagulant warfarin in conjunction with PB, results in a requirement for increased warfarin concentration due to the accelerated clearance rate of the compounds, induced by PB When PB administration is stopped, the warfarin metabolism rates return to their normal levels and the resulting concentrations of warfarin in the bloodstream could result in fatal haemorrhage

Similar effects have been recorded with phenytoin and PB administration resulting in increased cyclosporin metabolism (Okey, 1990)

1.5.0 THE CYTOCHROME P450 MULTIGENE FAMILY

The P-450 superfamily includes many different genes and their gene products. To date the superfamily contains more than 80 members and 30 sub-families (Gonzalez, 1992). Individual P450 protein sequences are very different from each other, apart from a 26-residue region near the carboxy-terminus of the protein which is essentially conserved (Nebert *et al.,* 1989). This common denominator suggests a single shared ancestral gene dating back 2 billion years, from which all P450s diverged, by gene duplication and mutation (Nebert, 1989). The cytochrome P450 multigene family has evolved a great many new genes in the past 800 million years. This new development especially in the class II family coincides with the commencement of "animal-plant warfare". As animals began to ingest plants, the plants developed toxins as a form of self defence. Under this pressure, animals developed new enzymes to metabolise these toxins as can be seen in the cytochrome II sub-family. The number of genes present in the sub-families of particular species seems to be a function of the environment in which the species finds itself, *i.e.* the compounds that it comes in contact with and its necessity to metabolise them. It is also believed to be a function of the generation time *i.e.* the time which has elapsed since one species, diverged from another.
Therefore, the human sub-family should not contain identical genes or numbers of sub-families to other species, in some cases a corresponding human gene may not exist (Nebert *et al.,* 1989).

As particular P450s become obsolete, the corresponding gene can accumulate mutations and become inactive. The early stages of gene inactivation can lead to genetic polymorphism, a major issue in P450-dependent metabolism. This polymorphism can result in clinical difficulties *e.g.* in administration of drugs which have low therapeutic indices and in individuals being resistant or sensitive to environmentally based diseases *e.g.* cancer (Gonzalez, 1990).
The mammalian P450 superfamily can be subdivided into two classes; those exclusive to synthesis of steroids and bile acids and those which are involved in metabolism of xenobiotics. The cytochrome P450 subgene families exclusively involved in steroid metabolism are the CYP11, 17, 21 and 19. Their sequences are well conserved and display rigid substrate and product specificities. Mutations in the CYP21A2 gene results in serious birth defects, indicating the importance of these genes and their conservation (Gonzalez, 1992).
The P450 families I, II and III metabolize drugs and carcinogens, with some steroid

metabolism overlap

The P450IV family carries out metabolism of fatty acids and prostaglandins The remaining genes contribute mainly to steroid metabolism, are expressed m extra-hepatic tissue, or are expressed in bacteria, yeast or other organisms

1.5.1 NOMENCLATURE

A strict consensus as to the nomenclature of the cytochrome P450 has not evolved until recently As a result there is confusion as to the classification of the genes and their protein products For example the Aryl Hydrocarbon Hydroxylase enzyme has been described as AHH, P-450c, P450form 6, P1-450, CYP1, CYP2, P(1)450, P450DX, Cyp-1, P450C1, P450C1A1 and CYPIA1 To resolve this identification problem, the following system was proposed For chromosomal loci, the root symbol is "*CYP*" for human, and "*cyp*" for mouse This is followed by an arabic numeral denoting the P450 family, a capital letter for the subfamily (lowercase for mouse), followed by an Arabic numeral for the specific gene For the gene product i e the protein, the gene family is represented by Roman numerals Therefore, referring to the phenobarbital 2B gene family in humans, the correct nomenclature would by for the gene *CYP*2B1, and the protein P450IIB1 (Nebert *et al*, 1989, Gonzalez, 1990)

As new genes are being discovered constantly and new information about already classified genes comes to light, regular revision of the nomenclature is essential to avoid confusion To belong to the same family a protein must be <u>more</u> than 40% homologous to the other genes of that family For an enzyme to be allocated within a particular family, it must exhibit <u>less</u> than 40% homology to genes from any other family There are two exceptions to this rule 1) The microsomal enzymes of the XI family, scc and 11β, These two proteins are only 37 5 - 38 8% homologous 2) The other exception is the II family, which contains a subfamily IID which lowers the homology of the family from greater than 41 6% to greater than 32 1% when it is included

When mammalian genes are accounted for alone, sequences within the same subfamily are greater than 59% homologous However, when other species and allelic variants are included this value drops to less than 47%

1.5.2 THE IA FAMILY

This sub-family contains two members, P450IA1 and IA2

- ENZYME ACTIVITY:

This family of enzymes is involved in oxidative metabolism of exogenous chemicals *e g* polycyclic hydrocarbons, aromatic amines, heterocyclic amines *etc* Both enzymes have overlapping specificities, but their shared activities are slower than their specific reactions The P450IA1 enzyme mainly carries out the metabolism of PAH (polycyclic hydrocarbons), noticeably the hydroxylation of benzo[a]pyrene which leads to its carcinogenic metabolite benzo[a]pyrene-7,8-diol-9,10-epoxide (Pelkonen and Nebert, 1982)

P450IA2's preferred hydroxylation activity is towards aryl ammes and amides *e g* β-naphthylamine and 2-acetamidofluorene (Ikeya *et al*, 1989) IA2 also participates in the metabolism of aromatic amines, caffeine, oestradiol and certain drugs *e g* phenacetin (Fisher *et al*, 1992)

- TISSUE DISTRIBUTION AND INDUCTION.

The P450IA1 enzyme levels are low in both liver and extra-hepatic tissue Enzyme levels are not uniform in the liver acinus, demonstrating highest levels in the cells surrounding the terminal hepatic venules and intercalated veins (McKinnon *et al*, 1991) This family possesses a unique mode of induction by compounds like PAH *i e* benzo[a]pyrene and 3-methylcholanthrene (3-MC) IA1 also exhibits a high degree of activity toward these PAH compounds

IA2 is constitutively expressed in liver and comprises 5% of the total cytochrome *i e* mitochondria electron transport *etc*, in that tissue There is very little expression of this gene in extra-hepatic tissue It is inducible in liver and to some extent in kidney, lung, spleen, small intestine, large intestine and foetal tissue, but this induction of enzymic activity is variable and low except when induced with high levels of TCDD (2,3,7,8-tetrachlorodibenzo-*p*-doxin) Isosafrole is the most potent CYP1A2 gene inducer which is surprising as it is not a 1A1 inducer (Nebert *et al*, 1987, Fujii-Kuriyama *et al*, 1992)

- GENE STRUCTURE:

The CYP1A genes have been assigned to chromosome 9 in the mouse, with the genes lying in tandem between the Mpi-1 and Pkm-3 loci (Hildebrand *et al* , 1985a) and to chromosome 15 in humans 15q22-qter near MPI (Jaiswal *et al* , 1985a) Each gene has seven exons and contains a noncoding first exon (Gonzalez *et al* , 1985, Tukey *et al* , 1984, Hildebrand *et al* , 1985) The two forms, P450IA1 and IA2, are 75% homologous at the ammo acid level The human CYP1A1 gene was sequenced and shown to span 6 3 kbp with a 1,569 bp mRNA This gene has a translation start codon 27 bp into the second exon and is composed of 523 amino acid residues with a molecular weight of 59,300 daltons (Yabusaki *et al* , 1984, Jaiawal *et al* , 1985b) The CYP1A2 human gene spans 7 8 kb with a 3 3 kbp mRNA, encoding 515 amino acids to give a molecular weight of 58,294 daltons (Ikeya *et al* , 1989)

Conservation of nucleotides and number of bases between human CYP1A1 and 1A2 in the regions of exons 2,4,6 and 5 is very strong Comparison of human 1A2 regulatory upstream exon 1 with that of mouse or rat reveals 68% similarity in the 150 bases 5' from the mRNA cap site, 80% similarity between -841 to -758 in human and -1529-1439 in mouse The pribnow/canonical (involved in binding RNA polymerase) 5 bp box (CACGC) which is found upstream in all mammalian 1A1 genes so far, is believed to interact with the PAH-receptor complex This region is absent in the 1A2 gene There are however highly conserved regions in human 1A2 intron 1 in rat and mouse This conservation possibly indicates a different location for the regulatory elements of 1A2 (Ikeya *et al* , 1989)

- GENETIC REGULATION OF 1A1:

The genetic regulation of the rat CYP1A1 gene is the best characterised of the cytochrome P450s This regulation consists of at least two different *cis*-acting regulatory DNA sequences, which are located 5' upstream of the gene and their associated, *trans*-acting DNA binding factors The upstream DNA elements are, The XRE or inducible enhancer xenobiotic responsive element, is present in multiple copies (up to five) The other element BTE (basal transcriptional element) is present in only one copy, close to the TATA box (25 bp upstream of the RNA polymerase II start site) and is concerned with constitutive expression (Fujii-Kuriyama *et al* , 1992) The presence of both elements is required for constitutive and inductive expression The associated binding proteins are the Ah receptor (section 1 4 0), the Ah receptor nuclear translocator protein (arnt), both associated with the XRE

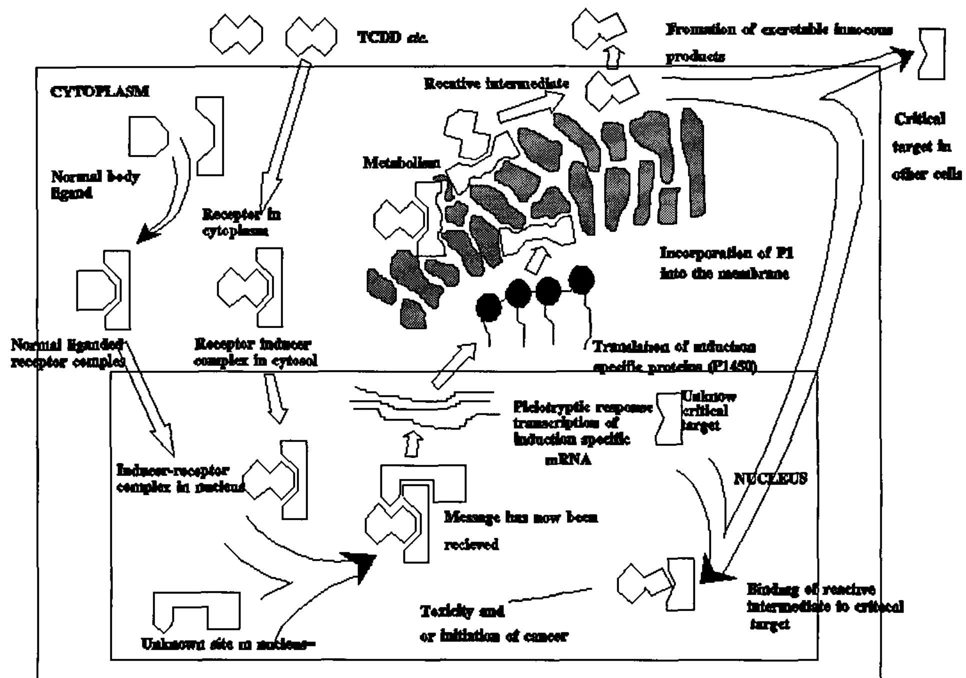


Figure 5 Regulation of the rat CYP1A1 gene by the Ah receptor in a cell (Nebert and Jaiswal, 1987)

The two proteins associated with the BTE are the BTEB or basal transcriptional element binding protein and Sp1 As can be seen from figure 5 the overall mechanism of regulation and induction of CYP1A1 is complex and mediated by the binding of inducers (exogenous or endogenous) to a cytosolic receptor, although an endogenous ligand has not yet been discovered This receptor complex is translocated to the nucleus where it binds to the major groove of the DNA at the sites mentioned above This binding causes a conformational change in the DNA probably resulting in greater access for RNA polymerase to the gene and increased transcription to mRNA For more information on the regulation on CYP1A1 gene expression, see section 4 2

-REGULATION OF CYP1A2:

In contrast to CYP1A1 gene regulation of CYP1A2 is not well known or understood Study of this regulatory mechanism was hampered until recently by the lack of an expression system Now, however expression has been achieved in HepG2 and V79 Cells

Induction of CYP1A2 is not always simply a case of increased rates of transcription CYPIA2 mRNA is stabilized by inducer compounds like 3-Methylcholanthrene and TCDD in rat liver *in vivo*, and *in vitro* as seen in rat hepatocytes On the other hand, in mouse liver transcriptional rates of mRNA are increased by induction Post-transcriptional mechanisms may be involved, *e g* mRNA processing, mRNA transport, and mRNA stability MCF-7 (a human breast carcinoma cell line) and HepG2 (a human hepatoma cell line) were negative when probed for 1A2 activity However following transfection of the full IA2 gene coupled to CAT activity, the MCF-7 line remained negative whereas HepG2 were now positive This implies a possible tissue-specific regulatory mechanism, or a specific inhibition in MCF-7 (Jaiswal *et al*, 1985c, Tukey *et al*, 1988)

It has been reported that hepatic induction of IA2 mRNA occurs at lower TCDD levels than those required for IA1 induction and that IA2 has been detected in the centrilobular hepatocytes of untreated "nonresponsive" mice in the absence of IA1 As these genes are both believed to be regulated by the Ah receptor and lie in tandem, it is hard to explain why they should respond differently to the same inducer It is possible that the TCDD-Ah receptor complex may have greater affinity for regulatory regions, as yet unidentified, in the IA2 gene than for the regulatory regions in the IA1 gene (Tuteja *et al*, 1985) since the 5' flanking region of the CYP1A2 gene appears not to contain the same regulatory sequences as CYP1A1 gene (Ikeya *et al*, 1989) It has also been suggested that the transcriptional factor, which is sensitive to protein synthesis inhibition and plays an important role in superinduction of CYP1A1 via the Ah receptor, is not important in 1A2 regulation, this again highlights the differing modes of regulation of these two family members (Teifeld *et al*, 1989)

1.5.3 THE CYPIIA SUBFAMILY

The CYP2A gene famıly ın the rat has 3 members, CYP2A1,2A2 and 3A3, codıng for 3 proteıns There are 2 ın the human famıly closely assocıated wıth the rat CYP2A3 whıch are CYP2A6 and 2A7 There ıs one mouse gene, encodıng two proteıns, also assocıated wıth CYP2A3

- ENZYME ACTIVITY:

The P450IIA1 proteın ın rat (a1), ıs assocıated wıth hydroxylatıon of testosterone at the 7α and the 6α posıtıon, the latter beıng the less potent of the two reactıons IIA2 (a2) also metabolıses testosterone, but to a lesser extent

In the mouse the correspondıng IIA subfamıly - type I and type II carry out testosterone 15α-hydroxylatıon (Gonzalez 1990)

The two human partıal cDNAs have been ısolated to date CYP2A6 ıs belıeved to be synonymous wıth the rat IIA3 proteın, and carrıes out the metabolısm of Coumarın and dıethylmtrosamıne Lıttle ıs known about the P450IIA7 proteın (Gonzalez, 1992)

- TISSUE DISTRIBUTION AND INDUCTION:

Both P450IIA1 and IIA2 are expressed only ın the lıver, where as IIA3 ın rat ıs expressed only ın lung The CYP2A1 gene ın rats ıs expressed ın ımmature male and female, but ıs absent ın male rats at puberty P450IIA2 ıs expressed only ın males at the onset of puberty Control of these developmental and sex specıfic expressıons ıs carrıed out by hormones of the pıtuıtary gland, the exact regulatıon of these genes has not yet been ıdentıfied (Gonzalez 1990)

P450IIA1 and IIA3 are ınduced by 3-Methylcholanthrene (MC) where as IIA2 ıs not

The mouse IIA genes type I and type II and are expressed ın kıdney and lıver

Type I ıs expressed ın male mıce and type II ın female kıdney (Gonzalez, 1990) To date the human forms have only been found to be present ın the lıver (Gonzalez 1992)

- GENE STRUCTURE:

In the rat CYP2A1 and 2A2 genes have been ısolated and sequenced They exhıbıt an 88% degree of homology and have 9 exons The thırd gene CYP2A3 has been sequenced and expresses 71% and 73% homology wıth 2A1 and 2A2 respectıvely Thıs suggests that 2A1 and 2A2 dıverged at a later date than 2A3 The mouse 2A gene subfamıly contaıns two genes whıch have been assıgned to chromosome 7

The two mouse genes, type I and type II, are 98% homologous to each other, 90% homologous to CYP2A3 and 70% and 75% similar to CYPA1 and CYPA2 respectively Types I and II are expressed in the liver and kidney, where as IIA3 is found only in lung (Gonzalez 1990)

In humans this subfamily has been located on the long arm of chromosome 19 The human CYP2A7 gene displays 85% homology to rat 2A3, 69% homology with 2A1 and 65% with 2A2 (Miles *et al* , 1989, Yamano *et al* , 1989) The regulation of these genes has yet to be decided

1.5.4 THE P450IIB SUBFAMILY

The P4502B subfamily of genes has been isolated in man, mouse and rabbit In the rat this subfamily contains three forms CYP2B1 (b), 2B2 (e) and 2B3 In the rabbit there are 2 forms, CYP2B5, IIB4 and its pseudogene CYP2B4p The human subfamily contains three forms, CYP2B6, 2B7 and 2B8, and the mouse subfamily contains 2B9 and 2B10

- ENZYME ACTIVITY:

The IIB family of enzymes demonstrate broad substrate overlap carrying out the following reactions, O-dealkylation of 7-pentoxyresorufin, benzphetamine *N*-demethylation, aflatoxin B1 metabolism, cyclohexane oxidation, chloroethylnitrosourea hydroxylation, oestradiol-2 hydroxylase, aminopyrene demethylation, 7-ethoxycoumarin demethylation, 4-ipomeanol activation, *p*-xylene activation, parathion oxidation, pentoxy-resorufin metabolism, and 1-nitropropane denitrification (Wolf *et al*, 1990, Platt *et al*, 1989) The P450IIB1 in rats demonstrated 2-10 times the activity of IIB2 in these reactions Rat IIB1 has also been reported to selectively carry out the stereospecific D-ring hydroxylation of the steroids, androstenedione and testosterone (Waxman *et al* , 1989) IIB1 is also reported to activate aflatoxin B_1 to its mutagenic form, N-methyl-*N'*-nitro-N-nitrosoguanidine (Doehmer *et al*, 1988) IIB1 also activates the anticancer agent cyclophosphamide to its toxic anti-tumor form and also activates ifosfamide to its mutagenic form

The human form P450IIB6 also carries out the activation of the anticancer agent cyclophosphamide to its anti-tumorigenic form 4-hydroperoxycyclophosphamide (Gonzalez 1992)

- **TISSUE DISTRIBUTION:**

The rat P450IIB1 is constitutively expressed in lung and testis and only inducible by phenobarbital in the liver P450IIB2 is constitutive in the small intestine and liver, but this activity cannot be induced in that tissue, suggesting that these genes have different control mechanisms P450IIB3 is constitutively expressed and is not inducible

The human forms IIB6 and IIB7 are expressed m the liver and lung respectively P4502B8 is expressed in liver and intestine (Gonzalez 1990)

- **GENE STRUCTURE:**

The rat CYP2B1 and 2B2 genes contain nine exons These genes are located on chromosome 19cen-q13 3 in humans and Proximal 7 (Coh) in mice

CYP2B1 and 2B2 genes display 97% homology in the rat CYP2B3 is 77% homologous to both 2B1 and 2B2, implying that 2B3 diverged from the root gene of 2B1 and 2B2

In the rabbit the two CYP2B genes recorded exhibit 95% homology Expression of several other rabbit 2B cDNA's has been reported, but these have not been fully studied and may be allelic variants (Gonzalez 1990)

- **GENE REGULATION:**

Induction of enzyme activity by phenobarbital is via the activation of transcription (Okey 1990) It has been reported that thyroid and pituitary and especially growth hormone (GH), suppress the constitutive and phenobarbital induced expression of the rat CYP2B1 and 2B2 proteins The levels of both CYP2B mRNAs are suppressed by the presence of GH

However thyroid hormone has a differential effect on the suppression of P450IIB1 and IIB2 Suppression of P450IIB1 by TH is not as strong as that of GH whereas P450IIB2 demonstrates equal levels of inhibition by both GH and TH Simultaneous induction of GH and phenobarbital results in similar suppression profiles However, pretreatment with TH is required before addition of PB to witness this TH mediated suppression This TH suppression only can be reversed by addition of cycloheximide suggesting *de novo* synthesis of a repressor protein (Yamazoe *et al* , 1987, Murayama *et al* , 1991) (*see section 1 4 0)*

1.5.5 THE P450IIC GENE SUBFAMILY

This family contains many members as can be seen below

CYP450 IIC subfamily	SPECIES	ACTIVITY/ SUBSTRATE	TISSUE
IIC1	Rabbit	Pb inducible	Liver
IIC2	Rabbit	Pb inducible	Liver
IIC3	Rabbit	Pb inducible	Liver
IIC4	Rabbit	Pb inducible	Liver
IIC5	Rabbit	Progesterone 21-hydroxylase	Liver
IIC6	Rat		Male and Female Liver
IIC6P	Rat	Pseudogene	
IIC7	Rat		Male and Female Liver
IIC8	Human	Tolbutamide R-mephenytoin	Liver Intestine
IIC9	Human	R-mephenytoin tolbutamide S-Warfarin	Liver Intestine
IIC10	Human		
IIC11	Rat	16-α steroid metabolism	Male specific Liver
IIC12	Rat	15-β steroid metabolism	Female specific Liver
IIC13	Rat		Male specific Liver
IIC14	Rabbit		
IIC15	Rabbit		
IIC17	Human		Liver
IIC18	Human		Liver
IIC19	Human		Liver

- TISSUE DISTRIBUTION:

This sub-family has been detected as constitutively expressed only in the liver to date

- ENZYME ACTIVITY:

These enzymes exhibit broad overlapping substrate specificities and some display high levels of steroid metabolism *e g* testosterone

They also as a group carry out the metabolism of benzo[a]pyrene, benzphetamin demethylation, aflatoxin B1 activation, tienilic metabolism, 2-hydroxylation of oestradiol and progesterone, dimethylbenzanthracene activation, warfarin 7-hydroxylation and triacetyloleandomycin metabolism (Wolf *et al* , 1990)

In the rat there are two adult male-specific P450s IIC13 and IIC11, a female specific IIC12 and two which are nonspecific IIC6 and IIC7

The rabbit expresses seven genes in total, 4 of which are phenobarbital inducible enzymes P450 2C 1, 2, 3 and 4 (see table on previous page) The three remaining rabbit forms are IIC5, (a progesterone 21-hydroxylase) and two others, IIC14 and IIC15 These enzymes carry out aminopyrene, ethylmorphine and chlorcyclizine metabolism

Human liver expresses two enzymes P450IIC8 and IIC9, these enzymes catalyze the hydroxylation of S-mephenytion and S-warfarin Metabolism of this latter anticoagulant is deficient in some individuals *i e* P450IIC9 is polymorphic in some people and is therefore of clinical importance These human enzymes also carry out mrvanol metabolism, benzphetamine demethylation, tolbutamide, hexobarbital, ethotoin mephobarbital, methsuzimide and phensuximid metabolism

- GENE STRUCTURE:

The CYP2C subfamily is made up of 9 exons The gene cluster, has been located on chromosome 10 10q24 1-24 3 in man and on chromosome 19 in mice In the rat these enzymes are under developmental and sex-specific control, however this is not the case in humans (Gonzalez 1990)

-GENETIC REGULATION:

Regulation of the CYP2C genes is complex, as can be seen in the rat The CYP2C6 and the 2C7 genes are only activated at puberty in the rat The 2C11 and 2C12 genes are controlled by testosterone levels to a degree, but this has no effect on the rat CYP2C6 and 2C7

CYP2C11 is expressed in mature male rats, only when they have been exposed to testosterone during the neonatal period (Waxman *et al* , 1985)

CYPIIC12 expression is effected partially by oestradiol Exposure during the neonatal period to sex-hormones results in development which could be attributed to growth hormone secretions For example exposure to androgen results in the pattern of growth hormone secretion as seen in adult males which is pulsatile whereas in females this pattern is constant (Jansson *et al* , 1985)

Hypophysectomy (removal of the pituitary gland) of male rats resulted in a loss of IIC11 mRNA, and this was only restored on intermittent *i e* pulsatile, injection of growth hormone

Constant exposure to growth hormone resulted in increased mRNA levels of the female specific gene IIC12 Each of these treatments required a 2-6 day period before an effect was seen suggesting the involvement of an intermediary protein factor to elicit the response This response may be in the form of mRNA stabilisation (Gonzalez 1990)

1.5.1.6 THE P450IID GENE SUBFAMILY

-ENZYME ACTIVITY AND TISSUE DISTRIBUTION:

In rat and human these enzymes carry out the oxidation of the drugs debrisoquine in the intestine, bufuralol in the liver and sparteine in the kidney It has been reported that 5-10% of all CYP2D6 alleles are defective in caucasians, resulting in the condition called debrisoquine/sparteine polymorphism, these individuals being poor metabolisers (PMs) of the debrisoquine molecule This polymorphism is believed to be linked with susceptibility to leukaemia, bladder and malignant melanoma (Smith *et al* , 1991, Wolf 1991) This enzyme also oxidizes at least 20 other drugs, all basic amines, including the vasodilator perhexiline (Guengerich 1989)

The human locus also codes for 2 other IID forms, CYP2D7 and 2D8P Both of these genes lie upstream of 2D6 but are defective and not expressed The 2D6 protein also carries out the metabolism of a number of clinical cardiovascular and psychiatric drugs *e g* metroprolol, despramme, MPTP, codeine *etc* , (Wolf, 1991)

The mouse CYPIID9 and IID10 do not carry out debrisoquine metabolism, or share any of the above mentioned metabolic traits, demonstrating strong species variation However mouse does exhibit a male specific 16α-testosterone hydroxylase (Gonzalez, 1992)

-GENE STRUCTURE:

To date there are five cytochrome P450 genes identified in this sub-family in rats - CYP2D1, 2, 3, 4 and 5 These genes show 75%-95% homology and lie in tandem on a 60 Kb segment of DNA The male specific testosterone 16α hydroxylase belongs to this sub-family of enzymes and exhibits 70-82% homology with the five genes found in rat discussed above

The human sub-family IID has three members, two of which lie in tandem on the long arm of chromosome 22 These genes are 92%-97% homologous and are detectable in only some human livers This is due to the fact that these genes have potential for non-lethal inactivation mutations

The human gene CYP2D8 has potential for non-lethal gene-inactivation mutations CYP2D7 contains a base deletion which gives rise to a protein-coding frame shift In liver samples where debrisquinone metabolism was not detected, the IID6 gene has been found to contain three distinct mutations (1) partial or complete deletion of the coding sequences, (2) a base pair deletion at position 2637 in exon 5 which leads to premature termination, and (3) a G-A transition at the intron 3/exon 4 junction which results in incorrect splicing of the mRNA

The mouse genes cyp2D9 and 2D10 have been located on chromosome 15 (Gonzalez 1990)

1.5.7 THE P450IIE GENE SUB-FAMILY

The IIE sub-family contains four genes to date, one human CYP2E1 (j), one rat CYP2E1 and two rabbit CYP2E1 (3a) and 2E2

-ENZYME ACTIVITY:

The P450IIE1 enzyme is inducible by ethanol and also carries out the metabolism of ethanol, acetone, acetoacetate, acetol, N-nitrosodimethylamine, paracetamol, and ketone bodies

The human P450IIE1 form also carries out oestradiol and ethinlyloestradiol 2-hydroxylation (Wolf *et al* , 1990) P450IIE1 demethylation of N-nitrosodimethylamine results in metabolites which may be involved in carcinogenesis P450IIE1 has also been reported to be involved in the activation of numerous low-molecular weight toxins and carcinogens *e g* *N*-nitrosamines, benzene, and urethane (Gonzalez, 1992, Kato *et al* , 1992)

-TISSUE DISTRIBUTION:

P450IIE1 is constitutively expressed in the human liver where its expression is influenced by inducers, many of which are substrates for the enzyme Studies in rodents have also shown extrahepatic expression especially after induction, noticeably in the leucocytes of uncontrolled diabetics (Gonzalez 1992)

-GENE STRUCTURE:

These genes like all other CYP2 genes have 9 exons They are approximately 10 Kb long The CYP2E genes have been localized to chromosome 7 in the mouse and chromosome 10 in humans These genes are among the best conserved P450 isoforms in the CYP2 family

-GENETIC REGULATION

Induction of 2E by ethanol, acetone, pyrazole or methylpyrazole causes an increase in protein synthesis without increasing mRNA level (Gonzalez 1990) It is believed that increased stabilization of the protein combined with a decrease in protein turnover accounts for the overall induction of this enzyme Starvation or diabetes induction does however increase mRNA levels of IIE sub-family in rats How this occurs is unknown Combination of acetone treatment and starvation in rats resulted in synergistic effect on IIE induction

Genetic polymorphisms and racial variation in distribution have been detected with regard to the CYP2E gene in humans These polymorphisms have been suggested to be linked with increased susceptibility to lung cancer in Caucasians and to gastric or oesophageal cancer among Japanese (Kato *et al* , 1992)

1.5.8 THE P450III FAMILY

The CYPIII family of enzymes was first isolated by induction with the synthetic glucocorticoid pregnenolone 16α-carbomtrile PCN There are two known forms expressed in the rat, P450IIIA1 and IIIA2, three forms in human IIIA3, A4, A5 and one known form in rabbit IIIA6

-TISSUE DISTRIBUTION:

In the rat there are currently known to be two P450III isoforms in the liver They are P450IIIA1(PCN1, P-450p) which is expressed only on induction with glucocorticoids and phenobarbital (Okey 1990) P450IIIA2(PCN2) is the constitutively expressed in male rat liver and transiently expressed in young female rats, this enzyme is not detectable in foetal rats (Gonzalez *et al* , 1986) P450IIIA2 is not inducible by PCN, but mRNA concentrations are increased in the presence of phenobarbital There are three human adult forms of IIIA family which are expressed in the liver and one form expressed in fetal liver (Kitada *et al* , 1987, Gonzalez 1992) The human forms are IIIA3(HLp), IIIA4(nf-25,pcn1,nf-10) and IIIA5(pcn3)

Rabbit liver also constitutively expresses a P450IIIA6 (3c) form which is equally similar to rat P450IIIA1 and IIIA2

-ENZYME ACTIVITY:

The rat form P450IIIA1 is involved in the hydroxylation of testosterone at the 6-β position, ethylmorphine N-demethylation, erythromycin N-demethylation and mephenytom 4-hydroxylation (Gonzalez *et al*, 1990) The human equivalent, P450IIIA4, carries out testosterone 6β-hydroxylation, 17, 2- and 4- α-estradiol, d-benzphetamine demethylation, aldrin epoxidation and nifedipine oxidation These human enzymes are also involved in dehydroepiandrosterone 3-sulfate, benzo[a]pyrene and 7-ethoxycoumarin hydroxylations (Guengerich *et al*, 1986, Gonzalez *et al*, 1990)

-GENE STRUCTURE

The structure of this gene family has yet to be examined The human III loci has been located on chromosome 7 7q21 3-q22 and the mouse loci on chromosome 6 (Gonzalez 1990)

- GENE REGULATION:

These genes are induced by the presence of glucocorticoids (Elshourbagy *et al*, 1980) The rat form CYP3A1 is induced by glucocorticoids and phenobarbital, whereas the constitutively expressed form CYP3A2 is inducible only by phenobarbital

This suggests that these genes are regulated differently (Gonzalez 1990) Triacetyloleandomycin (TAO) has been reported to induce the CYP3A6 form in rabbit, by stabilization of the mRNA In rats this induction is mediated by increased transcription of both CYP3A1 and CYP3A2 *In vitro* however this induction by TAO is not seen, however dexamethazone will induce the same pattern *in vivo* and in culture possibly through a different mechanism (Watkins *et al*, 1986)

1.5.9 THE P450 IVA FAMILY

There are seven members of this sub-family, three rat genes CYP4A1, 4A2, 4A3 and four in rabbit, CYP4A4, 4A5, 4A6 and 4A7 These enzymes were discovered due to their inducibility by hypolipidemic drugs like clofibrate (Gonzalez, 1990)

-ENZYME ACTIVITY

In the rat, the P450IVA1 protein has been associated with the metabolism of arachadonic acid and the metabolism of lauric acid at the ω position (Tamburini *et al*, 1984) In lung of pregnant and progesterone treated rabbits this family is associated with the ω-hydroxylation of prostaglandin, possibly the IVA4 protein (Matsubara *et al*, 1987)

-TISSUE DISTRIBUTION:

This family of enzymes is expressed in the liver and kidneys of nonpregnant rabbits (Matsubara *et al*, 1987) In rats P450IVA1 is inducible in liver and kidney (Hardwich *et al*, 1987) P450IVA2 is inducible in liver only and constitutive in kidney (Gonzalez 1990)

- GENE STRUCTURE:

The IVA1 gene has 13 exons, and as such is believed to have evolved early in P450 gene superfamily development (Gonzalez, 1990)

- REGULATION:

Addition of clofibrate results in rapid transcription of the CYP4A1 gene It is possible that these enzymes are part of a family of peroxisomal enzymes which are inducible by hypolipidemic drugs It has been reported that this response may be via a receptor (Lalwani *et al*, 1987)

1.5.10 THE P450 IVB SUBFAMILY

IVB genes have been isolated in humans, rat and rabbit CYP4B1 in humans is constitutively expressed in lung however, it is not well expressed in liver Rabbit P450IVB1 (form 5) activates 2-aminofluorene, whereas human P450IVB1 does not This difference in metabolism illustrates how important it is to recognise species variation (Nhamburo *et al*, 1989)

1.5.11 METABOLIC P450s INVOLVED IN STEROID SYNTHESIS.

There are five families of Cytochrome P450 which are solely involved in the metabolism of steroids and are not involved in xenobiotic metabolism The distribution of these enzymes is governed by the steroid requirements of the tissue (Gonzalez, 1992)

The P450XI family contains two mitochondrial enzymes, P450 XIA1 which encodes for steroid 11-β-Hydroxylase and 2 forms of XIB1 which codes for cholesterol side-cham cleavage The bovine XIB1 was sequenced and found to express a 39 residue signal peptide similar to other mitochondrial proteins (Gonzalez, 1990)

The Human CYP11B1 gene has been reported to contain nine exons and is induced on addition of the peptide hormone ACTH (adrenocorticotropin) through a cAMP mediated mechanism (John *et al* , 1986) The Human CYP11A1 and 11B1 genes have been located to chromosomes 15 and 8 respectively (Gonzalez 1990)

The P450XVIIA1 protein codes for steroid 17-α-hydroxylase and 17,20-lyase activities This protein has been isolated in human, pig, rat and chicken (Nebert *et al* , 1989) The human form of XVIIA1 contains eight exons and spans 7 kbp It is located on chromosome 10 and its expression is also ACTH responsive (Gonzalez, 1990)

The P450XXIA1 protein codes for progesterone 21-hydroxylase It is the best known of the P450s involved in steroid metabolism because of it clinical relevance Deficiency of this enzyme is a major contributor to congenital adrenal hyperplasia (Miller *et al* , 1987) The CYP21 gene locus contains two genes which code for proteins P450XXIA1 and XXIA2 In mouse, pig and cow, the enzyme activity is associated with the XXIA1 gene product, the XXIA2 gene is non functional Whereas in humans, this activity is coded for by the XXIA2 gene with the XXIA1 gene inactive (Parker *et al* , 1985) In humans these genes are located in tandem on the short arm of chromosome 6

The P450 aromatase - XIXA1, originally isolated from human placenta, is involved in aromatization of C19 androgens to C18 estrogens This gene has been assigned to chromosome 15 There is very little other information on this family (Gonzalez, 1992)

1.5.12 CYTOCHROMES EXPRESSED IN YEAST

Saccharomyces cerevisiae expresses a P450LI (14DM) which demethylates lanosterol at the 14α position This enzyme is very similar to mammalian P450, for example it expresses a hydrophobic membrane binding protein which anchors the protein to the membrane *Candida tropicalis* also contains a P450 which is 66% similar to the CYPLI protein Exposure of *Candida tropicalis* to n-alkanes results in increased transcription of a P450alk gene, however this protein is less than 30% similar to CYPLI This enzyme was assigned to a separate family P450LII (Chen *et al* , 1988)

1.5.13 CYTOCHROMES EXPRESSED IN BACTERIA:

P450CI -P450$_{cam}$ isolated from *Pseudomonas putita* is a soluble enzymes which converts camphor to 5-exohydroxycamphor

This enzyme was used extensively in the elucidation of the electron transport pathways and oxygen metabolism To function correctly this enzyme requires putidaredoxin reductase-a flavoprotein and a sulphur containing protein-putidaredoxin (similar to the mitochondrial P450 which require the presence adrenodioxin) for the transfer of electrons from NADPH to its active site (Unger *et al* , 1986)

The P450cam gene is located only 22 base pairs from the gene coding for putidaredoxin reductase, implying the presence of an operon (Gonzalez 1990)

Bacillus megaterium contains a unique P450 - P450LII, in that this enzyme is self-sufficient, consisting of a protein incorporating both hame-P450 and NADPH-P450 This gene carries out the hydroxylation of long chain fatty acids and is inducible by barbiturates via cis- and trans- acting elements (Wen *et al* , 1989)

1.5.14 CYTOCHROME P450 COMPANION PROTEINS WHICH ARE REQUIRED FOR ACTIVE PROTEIN:

1.5.14.1 NADPH-P450-(OXIDO)REDUCTASE is the endoplasmic reticulum membrane bound flavoprotein responsible for the transfer of electrons from NADPH to Cytochrome P450 There is only one form of P450 oxidoreductase and it is compatible with all P450s NADPH-P450-oxidoreductase has been isolated from rat, yeast, pig, rabbit, trout and humans In each of these species FAD, FMN binding sites are strongly conserved, even when the remainder of the sequence bears as little as 33% similarity (Yabusaki *et al* , 1988) Phenobarbital exposure results in induction of the reductase, along with P450IIB1, IIB2, IIIA1 and IIIA2 (Hardwick *et al* , 1983, Gonzalez *et al* , 1986) Dexamethazone and 3-Methylcolanthrene do not have an effect on reductase levels (Gonzalez, 1990)

1.5.14.2 ADRENODOXIN AND ADRENODOXIN REDUCTASE support the mitochondrial P450s The Adrenodoxin molecule accepts electrons from the reductase and passes them onto the mitochondrial P450 The gene coding for adrenodoxin reductase is located on chromosome 17 Adrenodoxin is expressed in the adrenal cortex, testicular Leydig cells, ovary, placenta and areas of the brain This iron-sulphur protein is expressed as a pre-protein from which a 19-kD signal protein is removed on transport into the mitochondria

Adrenodoxin is the product of a single gene There are number of mRNAs produced but not all of these are translated to mRNA (Gonzalez 1990)

1.6.0 THE GLUTATHIONE-S-TRANSFERASE FAMILY OF ENZYMES.

The G-S-T family of enzymes were first discovered in 1961 by Booth *et al*, (1961) The sole function of these enzymes was originally believed to be intracellular transport of proteins and endogenous ligands which were marginally insoluble They transported compounds such as bilirubin, hame bile acids, steroid hormones, leukotriene A4, and prostaglandins These compounds were not substrates for the GST enzymes but simply bound to them for transport This original assumed function lead to the name ligandin (Litwack *et al*, 1971) However, it is now known that these enzymes are also involved in the detoxication of foreign compounds, mutagens, *etc*, by conjugation with the tripeptide - glutathione (Glutathione is a tripeptide made up of Glutamate, Cysteine and glycine which is synthetised by glutathione synthetase - see figure 6) Glutathione is present at a concentration of approximately 5mM in cells GSTs are also believed to confer cellular protection against reactive electrophiles in the same conjugation manner (Oesch *et al*, 1991) There are three main classes of GSTs π, μ and α, which are made up of subunits arranged as hetero- or homo- dimers of 25-29 kD The classification is based on the enzymes N-terminal ammo acid sequence, substrate specificity, sensitivity to inhibitors and immunological response A fourth class, θ also exists which is an endoplasmic reticulum-bound timer, made up of 17 kD subunits (Tsuchida and Sato, 1992, Hayes *et al*, 1991)

Little is known about the exact structure of the subunits, but it is believed that in each subunit there are two domains One of these is the COOH terminus, which is known as the "H-site" where the electrophilic substrates bind, this site is specific for each subunit The other site is the glutathione binding site called the "G-Site", which is common to each unit This 'G-site' domain has been localised to amino acid residues 53-72 in GST π m pig (Gulick *et al*, 1992) Each subunit contains its own "H" and "G" sites Homo- and heterodimers are made up of subunits from the same class, with the homodimeric reaction rate being faster than that of the heterodimer (Tsuchida and Sato, 1992) The μ and π classes have proven particularly efficient in the detoxication of K-region and bay-region diol epoxide carcinogens from polycyclic aromatic hydrocarbons like benzo[a]pyrene (Oesch *et al*, 1991, Hesse and Jernstrom, 1984) The α class of enzymes are more efficient at conjugating with cytotoxic and mutagenic lipid

peroxidation products (Osech *et al.*, 1991).

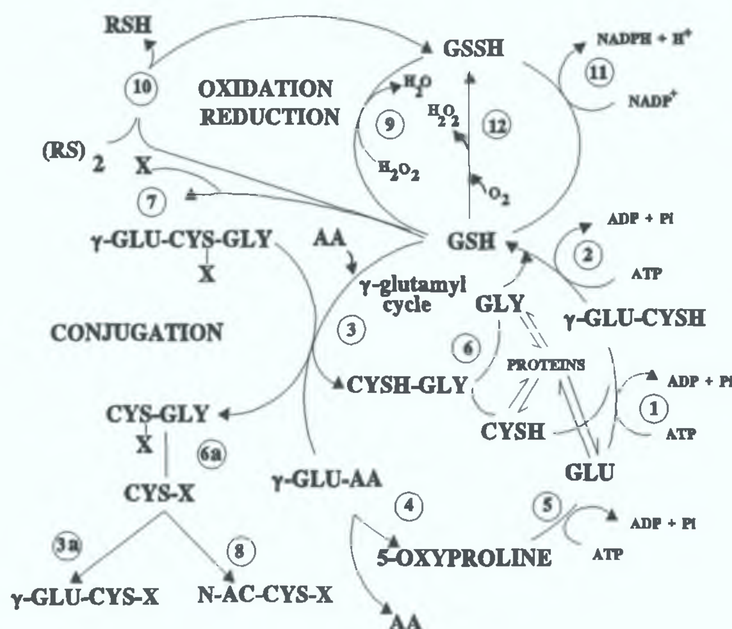


Figure 6 Glutathione metabolism cycle.

Reactions: 1: γ-GLU-CYSH synthetase; 2:GSH synthetase; 3 + 3a:glutamyl transpeptidase; 4:γ-glutamyl cyclotransferase; 5: 5-oxyproline; 6 + 6a: dipeptidase; 7: GSH S-transferase; 8: N-acetylase; 9: GSH peroxidase; 10: transhydrogenase; 11: GSSG reductase; 12: oxidation of GSH by O_2 conversion of GSH to GSSG mediated by free radicals. (Meister and Anderson, 1983).

The most extensive studies of the GST enzymes have been on rat tissue. There are 19 known molecular forms of cytosolic GST and 14 different subunits have been reported (Tsuchida and Sato, 1992). In subunit 1, three 5' flanking regulatory regions have been identified. The region including -867 to -857 ensures constitutive expression of the gene; -908 to -899 contains an XRE (xenobiotic responsive element); and -722 to -682 is an ARE (antioxidant responsive element) similar to that found upstream from UDP-glucuronosyltransferase (Tsuchida and Sato, 1992). In human five α forms, two π and eleven μ forms have been identified.

GSTs have been reported in human kidney, lung, brain, skin, intestine, adrenal glands, testis, prostate, uterus, heart blood vessels and skeletal muscle (Tsuchida and Sato, 1992).

The expression of the various isoforms is tissue specific and, in the case of π and α, developmentally regulated, π is expressed in most organs, with the exception of adult liver The molecular regulatory mechanism of these enzymes remains to be identified, but a TRE-like sequence has been reported upstream of GST π which is involved in basal expression of the gene, similar to subunit 1 in rat (Tsuchida and Sato, 1992)

The expression and regulation of GST isoforms found in mouse are different to those found in rat and human tissue Two α forms, comprising of dimeric combinations of 3 subunits, have been reported, one π and four μ forms have also been described

1.6.1 GLUTATHIONE-S-TRANSFERASE AND CANCER:

Chemical carcinogenesis is thought to be initiated by the covalent modification of DNA with electrophilic metabolites derived from carcinogens For many toxic xenobiotics and electrophiles, GST conjugation represents a major detoxification pathway The role of GST here is to eliminate electrophiles by conjugation, thereby preventing their carcinogenic and mutagenic roles However, it has been observed that some glutathione and cysteine conjugates are of toxicological concern (Pickett *et al* , 1989, Bladern *et al* , 1988) The conjugates produced can form unstable thiols which degenerate to alkylating agents and toxic metabolites For example, 1,2 Dibromoethane on conjugation with glutathione becomes S-[2-(N^1-guanyl)ethyl]-glutathione which binds to DNA Trichloroethene also, in its conjugated form is unstable and more susceptible to breakdown into unstable DNA binding thiol metabolites (Tsuchida and Sato, 1992)

Altered expression of GST has been reported m tumours in a variety of organs and species GST π has been noted as a marker in rat hepatocarcinogensis (Sato, 1988), as its expression increases in parallel with the progression of the disease However, this elevation in expression is not seen in human hepatocarcinogensis (Hayes *et al* , 1991), except in liver tumours resulting from metastasis (Tsuchida and Sato, 1992) The α class of GST is the predominant form expressed in normal human hepatocytes, with expression being localized to adult biliary epithelium Increases in α subunit B_1 expression have been noted in liver damage brought on by alcohol abuse, hepatitis and paracetamol poisoning Levels of π are increased in bile, secreted by the Kupffer cells, sinusoidal macrophages and hepatocytes in alcohol abused livers (Hayes *et al* , 1991)

Altered expression of the isoform GST π has also been implicated in colon (Kodate *et al* , 1986), stomach, bladder (Singh *et al* , 1990), squamous uterine cervix (Riou *et al* , 1991), oesophagus and pancreatic adenocarcinomas (Hayes *et al* , 1991) and in leukaemic bone marrow (Tidefelt *et al* , 1992, Hall *et al* , 1990) In some breast cancers GST π has been noted to mirror oestrogen receptor levels (Hayes *et al* , 1991)

Decreased expression was also noted in liver and kidney tumours (Howie *et al* , 1990)

The basis of elevated GST π levels in "cancer" has been the subject of some speculation Tsuchida and Sato (1992) have commented on the coinciding increased GST π expression in large colonic polyps with the activation of the "ras" oncogene and the loss of tumour suppressor genes which are also seen in these larger polyps However, it has also been proposed that these elevations of GST π are due to the multiple biochemical changes on going within the cancer cells (Riou, 1991) Hayes *et al* , (1991), also suggested that elevated expression may be due to clonal expansion of those particular cells which express π, *e g* pancreatic cancer arise form acini and ductular cells which express GST π in their normal state, the elevated expression reported in pancreatic cancer could be due simply to the increase in the number of these cells They also noted that the use of serum detection for marker GST π in neoplasia has inherent problems, since in the clotting process GST π is released from platelets, and GST α subunit B^1 is released from red blood cells For this reason, a plasma detection system was introduced The results suggested that GST π levels were elevated in the plasma of patients suffering with gastrointestinal malignant growths, especially those that had metastasised, and also in adenocarcinoma of the lung However, due to the implication of GST π in so may diseases, the results obtained from plasma or serum test may be difficult to interpret accurately for a single disease

The GST α class constitutes 3% of the total cytosolic protein of hepatocytes The half life of these subunits is 60 mins and as such they are used as markers of liver damage (Hayes *et al* , 1991) Elevation of the B_1 subunit has been noted in paracetamol poisoning, cirrhosis and thyrotoxicosis However the reason for this fluctuation is unclear In contrast, diminished GST α levels have been reported in stomach and kidney tumours (Tsuchida and Sato, 1992)

Expression of class μ activity towards trans-stilbene has been reported to be decreased in lung cancer patients (Seidegard *et al* , 1990, Tsuchida and Sato, 1992) A survey of patients with lung cancer revealed that levels of GST μ were lower in lung cancer patients who smoked than in smokers with no lung cancer They also reported that levels of GST μ were lower in patients with squamous cell carcinoma than those with adenocarcinoma

The GST μ gene is dominantly inherited and may be a marker of susceptibility to lung cancer and adenocarcinoma of the stomach and colon (Tsuchida and Sato, 1992)

Over all, the involvement of GST isoforms in cancer debatable As already mentioned, elevation and depression of individual isoforms may simply be a reflection of the biochemical changes occurring within a tumour due to clonal expansion, or they may play an intrinsic role in the mechanism and progression of the disease As yet, only a few isoforms have been investigated with respect to their part in cancer The involvement of other forms needs to be clarified before a conclusion as to the role of GST in cancer can be made

1.7.0 ACTIVATION AND CANCER:

As already mentioned, some P450s activities lead to activation of certain compounds to their toxic carcinogenic metabolites *e g* benzo[a]pyrene-1A1, aromatic amines-1A2 and the food derived 2-amino-3-methylimidazo[4,5-*f*]-quinoline - 3MC-like inducers (Parke *et al* , 1990) The induction process therefore, 1) may lead to an increased activation of compounds or, 2) at the opposite end of the scale induction may lead to increased clearance of the toxic metabolites The balance between toxification and detoxification very much depends on the time and the place of induction, where the compound finds itself during that time and the genetic variance of the individual involved Genetic variability in the oxidative activation of chemical carcinogens may explain the inter-individual differences in response to carcinogens (Nakachi *et al* , 1991)

Activation of P450 is involved at the therapeutic level The chemotheraputic agent cyclophosphamide requires activation before it can effect its toxic anti-tumor activities (Doehmer *et al* , 1989) Pretreatment with PB results in an increased activation of this agent

The clinical administration of different inducers may allow for increased toxification or detoxification depending on the reaction required In essence it may be possible to exploit P450 induction as a method for increasing the efficacy or reducing the side effects of therapeutic agent The induction process has been maintained throughout evolution This adaptive response is necessary in a constantly changing enviroment

1.8.0 DRUG RESISTANCE IN TUMOUR CELLS.

Tumour resistance to chemotherapy may be a primary or secondary phenomenon Tumour cells which show no initial response to therapy are deemed to be primarily resistant Others, which exhibit an initial response of tumour remission, can relapse and present with secondary resistance This resistance may be due to extracellular or intracellular factors Extracellular factors influence the drug before it reaches its target The timing and frequency of drug administration is critical to ensure that as many tumour cells as possible, at the correct point in the cell cycle are exposed to drug Inaccessibility of tumour is a problem for larger tumours in that the drug cannot reach the cells at the centre of the tumour The blood brain barrier also poses a major problem as drugs cannot pass through it to exert their effect Other extracellular mechanisms of drug resistance have also been reported *e g* drug antagonism and antibody production (Priestman, 1980) However, it is the intracellular mechanisms of drug resistance which are of interest here This form of resistance can be due to a variety of mechanisms, for example altered xenobiotic metabolism *e g* an increase in aldehyde dehydrogenase as seen in cyclophosphamide resistance (Waxman, 1990), increased P450 dependent metabolism of procarbanzine, etoposide and the P450 reduction of adriamycin and mitomycin C (Doehmer, to be published), and increased DNA repair by enzymes such as guanine O^6 -methyltransferase (Waxman, 1990) Classical multiple drug resistance (MDR) involves either primary or secondary resistance <u>and</u> cross resistance to particular subsets of structurally unrelated drugs Those drugs normally associated with classical MDR are large molecules with hydrophobic and hydrophilic moieties adriamycin, vinca alkaloids, peptide antibiotics like actinomycin D and epipodophyllotoxins like VP-16 and VM-26 This multiple drug resistance does not involve nucleotide analogues or metabolic inhibitors *e g* 5-fluorouracil and methotrexate or the alkylating agents bleomycin or cisplatin (Clynes *et al*, 1990) The mechanism most frequently associated with this classical resistance is the overexpression of the membrane glycoprotein with a molecular weight of 170kd - the "P-glycoprotein" The proposed function of this protein is as an "active transport system", an efflux pump which results in decreased accumulation of toxins within the cell (Endicott and Ling, 1989) This protein is associated with a broad range of structurally unrelated drugs as mentioned above and is expressed in a variety of normal tissue as well as resistant tumours

The possiblity that the drug metabolising battery of enzymes may be involved in drug resistance is logical when the function of these enzymes is considered

Some of the drugs involved in chemotherapy require metabolic activation before they can exert their toxic affect *e g* cyclophosphamide which requires activation by liver cytochrome P450 2B enzymes for toxicity (Doehmer *et al* , 1990) and aldehyde dehydrogenase for its detoxification (Hilton *et al* , 1982, 1984) Altered expression of either of these enzymes would effect the cell's response to the presence of cyclophosphamide Decreased expression of the enzyme CYP1A1 activity in adriamycin resistant MCF-7 cells has also been reported by Ivy *et al* , (1988) However, given their potential for a role in resistance, very few reports have actually cited the involvement of such enzymes in *in vitro* models of MDR

The *in vivo* situation may be different, as the expression of these enzymes would be as per normal, however, the levels of these enzymes are depleted in culture, so that *in vitro* models may not reflect the *in vivo* situation accurately

As mentioned above, the cytosolic, phase II, glutathione-S-transferase conjugating family of enzymes and the associated enzymes display altered expression in a number of tumours This transformation has also been reported in many resistant tumour cell lines (Waxman, 1990, Moscow, to be published) Waxman suggested that the possible function of these enzymes in alkylating agent resistance are

1) enhanced inactivation of electrophilic alkylating agents *e g* melphalan by direct conjugation
2) denitrosation of nitrosurea by *e g* GST μ in rats
3) scavenging of reactive organic peroxides by selenium dependant GSH peroxidase and,
4) quenching of chloroethylated - DNA monoadducts, platinum -DNA adducts and DNA hydroperoxides (Waxman, 1990)

Increased expression of GST μ and α is believed to be involved in resistance to nitrogen mustards, nitrosureas, benzo[a]pyrene and alkylating agents (Moscow *et al* , 1989, Hosking *et al* , 1990) For example, resistance in the CHO (Chinese hamster ovary) cell line to the alkylating agent, chlorambucil is attributed to a greater than 40 fold increased expression of GST α than its parental line CHO, as inhibition of GST α circumvented resistance, using the nonsteroidal antiinflammatory drug indomethancin (Hall *et al* , 1989) Manoharan *et al* , (1987) noted that transfection of the GST α class Ya subunits into COS cells resulted in chlorambucil resistance attributable to GST α In 1991, the same authors also noted that tandem expression of Ya (an α subunit) with Yb (a π subunit) resulting in a heterodimer with subunits from each class, also conferred resistance to this agent Yang *et al* , (1992) also found that in NIH-3T3 chlorambucil-resistant N50-4 cells, elevated levels of GSTα and GSH played major roles in the mechanism of resistance Transfection of GST α cDNA into BL-10 cells resulted in increased resistance to bleomycin (Giaccia *et al* , 1991)

However, Townsend *et al*, (1992) found that although transfection of GST α and μ into MCF-7 cells resulted in increased resistance to ethracrynic acid, it did not confer cross resistance to any other agents

The enzymes associated with the glutathione cycle have also been implicated in the mechanisms of resistance (Fig 6) Increased expression of GSH-peroxidase and GSH-reductase along with increased and also decreased GSH concentration, have been reported to play a role in resistance to adriamycin, cisplatin or x-ray irradiation in a variety of cell lines (Hosking *et al*, 1990, Batist *et al*, 1987, Cole *et al*, 1990)

However, the isoform mostly associated with the resistance phenomena is GST π

Elevated expression of this isoform has been connected with increased resistance to CDDP (cis-diaminodichloroplatinum) in CHO cells tranfected with the cDNA of this isoform and in a head and neck squamous cell carcinoma cell line (Miyazaki, *et al*, 1990, Teicher *et al*, 1987), to alkylating agents in the resistant selected human melanoma cell lines from G3361 (Wang *et al*, 1989), and to BCNU (1,3-bis(2-chloroethyl-1 nitrosourea) in a human malignant astrocytoma cell line (interestingly, this resistance is due to GST μ rat astrocytomas)(Ali-Osman *et al*, 1990) In NIH 3T3 c-H-*ras*-transformed cells, transfection of GST π resulted in resistance to ethracrynic acid and adriamycin, but <u>not</u> the alkylating agents (Nakagawa *et al*, 1990) Combined elevation of GST π and its selenium-independent peroxidase activity has also been reported in a human adriamycin-resistant breast carcinoma cell line MCF-7, although the exact mechanism of resistance is unknown (Batist *et al*, 1987)

A number of cell lines made resistant to adriamycin display increased GST π expression Adriamycin-resistant colon cancer and lung cancer cells with increased GST π expression have also been reported, but the controlling mechanism of resistance was once again undecided (Chato *et al*, 1992, Cole *et al*, 1990, Moscow, 1993) Kuroda *et al*, (1991) followed the expression of GST π in cell lines developed from a neuroblastoma before and after chemotherapy treatment and found increased levels in the treated cell line Fairchild *et al*, (1990) transfected a variety of GST isoforms into the cell line MCF-7 with no significant effect on the level of resistance to adriamycin, cisplatin or melphalan, except when co-transfected with the MDR1 gene Nonetheless, GST π did confer resistance to the carcinogen benzo[a]pyrene

Transfection with GST π seems only to confer low levels of resistance to adriamycin but no cross resistance *i e* it does not confer multiple drug resistance (Moscow *et al*, 1993) It is possible that the increased level of GST π that is observed in some MDR cell lines may be a symptom rather than the cause of resistance

The GST π may conjugate and transport the toxins to the P-glycoprotein for export and these genes may be regulated together (Fairchild *et al*, 1990) Evidence for the conjugation or binding of drugs commonly associated with MDR has not been reported (Moscow, 1993, Black *et al*, 1990) Increased GST expression levels have been reported in many cases of single compound resistance, but cross resistance to other agents does not seem to be involved Overall, the GST isoenzymes seem to play an important role in the phenotype of resistance They are overexpressed in many single-agent resistant and multiple drug resistant lines In some instances they play a major role in single agent resistance, but in the majority of cases they appear to be distantly involved and are not capable of supporting resistance alone

In this thesis we were interested in examining the xenobiotic metabolic pathways associated with the lung The liver is the organ most often associated with this form of metabolism due to is high concentrations of the enzymes involved and its central role in the regulation of the blood content The lung is also of importance as it is the port of entry for many airborne and gaseous compounds Metabolism of xenobiotics within the lung often leads to toxicity, although certain pulmonary cells are readily damaged than others (Baron and Voigt, 1990) The compartmental nature of the lung, its many cell types each expressing different isoforms at variable concentrations, make an accurate picture of the xenobiotic metabolizing profile of this tissue difficult (Guengerich, 1990) It is believed that many of the xenobiotic pathways previously reported to be absent in the total lung are probably present, but have not been detected as of yet However, recent studies by Baron and Voigt, (1990) have detected varying xenobiotic metabolizing activity to different extents in bronchial epithelial cells, Clara and ciliated bronchiolar epithelial cells and type II pneumocytes and other alveolar wall cells

In the rabbit lung four isoforms of P450 have been isolated They are P450-2 (IIB4), 5(IVB1), and 6 (IA1) and IVA4 The P450-2 form carries out *d*-benzphetamine N-demethylation, P450 5, carries out 2-aminofluorene N-hydroxylation and P450 6, carries out benzo[a]pyrene hydroxylation and 7-ethoxyresorufin O-dethylation IVA4 carries out the hydroxylation of prostaglandin E1 The activity of this enzymes is greatly induced in pregnant rabbits in the liver as well as in the lung (Yamamoto *et al*, 1984) P4502 and 5 are not inducible, but P450-6, whose activity in normal circumstances is low is greatly induced in the presence of PAH, however polyhalogenated biphenyl inducers appear to inhibits induction, as does Aroclor 1260 inhibit normal expression of P4502 and 5

Immunohistochemical staining of rat cells revealed the presence of NADPH-cytochrome P-450 reductase and cytochromes P450 BNF(IAI), PB-B(IIB1) and PCN-E(IIB4) in bronchial epithelial cells, both ciliated and nonciliated (Clara) bronchiolar epithelial cells, and type II pneumocytes as well as other cell in the alveolar wall Higher concentrations of reductase and PB-B were detected in Clara cells Benzoαpyrene was hydroxylated in situ by bronchial and bronchiolar epithelial cells and alveolar wall cells, especially type II pneumocytes (Voigt *et al*, 1990) Three other forms were also detectable CYP2A3, 3A and 2F Hamster lungs have also been reported to contain inducible CYP1A1 and 4B form has also been detected in Guinea pigs (Shimada *et al*, 1992)

In humans lung no isoform of P450 has been purified, however, the presence of three major forms of P450 have been detected, which are P450IA1 and P4502F1 and 4B1 (Shimada *et al*, 1992) The importance of these enzymes, especially P450IA1 is apparent due to their possible involvement in the etiology of lung cancer, especially that associated with tobacco (Kawajiri and Fujii-Kuriyama, 1991) P450IA1 mRNA has been detected in human lung, lymphocytes and placenta In lung tissue from active cigarette smokers levels of P450IA1 are mRNA elevated in comparison with nonsmokers (McLemore *et al*, 1989, 1990) This increased expression is linked with increased activity of this enzyme, resulting in activation of benzo[a]pyrene, a precarcinogen found in cigarette smoke, to its tumourogenic form This CYP1A1 gene demonstrates genetic polymorphism in its 3' -flanking region, resulting in 3 phenotypes Individual with genotype C (homozygous rare allele) were at a higher risk of developing lung cancer than those with genotypes A (homozygous) or B (heterozygous) due to the fact that their CYP1A1 gene is far more inducible than A or B (Nakachi *et al*, 1991)

Other phase I xenobiotic enzymes have been reported in lung tissue, NADPH cytochrome P450 reductase, flavin-containing monooxygenase, epoxide hydroxylase, alcohol dehydrogenase and aldehyde dehydrogenase (Guengerich 1990)
The phase II enzymes N-methyltransferase, acetyltransferase and GST have also been detected in the lung As already discussed, the polymorphic GST μ isoform is most relevant with respect to lung cancer, as expression of this isoform is linked with successful elimination of the toxic metabolites of benzo[a]pyrene (Tsuchida and Sato, 1992)

The aims of this project were

a) to examine and improve the techniques employed in this laboratory for primary culture of human lung tumours

b) To investigate the xenobiotic metabolising profile of a number of continuous lung cell lines with a view to their application in *in vitro* toxicity testing

c) To research the introduction of cDNA coding for specific P450 into continuous cell lines and to apply them to *in vitro* toxicity testing, and to compare their ability to activate certain compounds (as would be seen *in vivo*) with the parental nonexpressing lines, and

d) To investigate the possible role of xenobiotic metabolising enzyme in the phenomon of drug resistance, as seen in the adriamycin resistance lines developed in this laboratory

SECTION 2: MATERIALS AND METHODS.

2.0 TISSUE CULTURE TECHNIQUES.

2.0.1 Preparation of glassware:

All glassware used ın cell culture was maıntaıned for that purpose only Thıs was to ensure that no other laboratory chemıcals (whıch maybe resıdual on general glassware) ınterfered wıth the preparatıons whıch came ın contact wıth the cells All glassware and lıds were soaked ın hot tap water contaınıng 2% (v/v) RBS 25 (Chemıcal products, Belgıum) for 1-2 hours The glassware was then scrubbed clean and rınsed thoroughly m tap water to remove all RBS resıdue Followıng thıs the glassware was rınsed three tımes wıth deıonısed water (Mıllıpore MıllıRO 10 - reverse osmosıs treated water Thıs water ıs 99% free of partıculate matter, bacterıal and organıcs and has 90% of monovalent ıons and 95% polyvalent ıons removed) The glassware was then rınsed once wıth ultrapure water (ElgaStat U H P - reagent grade water produced by organıc absorptıon, ıonexchange, ultra mıcrofıltratıon and photo-oxıdatıon)

- For spınner cell flasks Unıts for suspensıon cell culture were washed ın the manner mentıoned above and allowed to dry at 50°C The spınner flask was then coated wıth a film of "repelcote" (BDH 63216) by rotatıng 10 mls of the lıquıd around the flask untıl all the surface area had been covered, ıncludıng the central spınner bar The flask was then allow to dry completely m a fume hood and then rınsed three tımes wıth ultrapure water

All glassware was then autoclaved wet (to ensure steam generatıon) at 120°C and 21 lb per cm^2 pressure for 20 mıns

2.0.2 Basal media preparation:

All medıa *ı e* powder and lıquıd was prepared from 10X concentrate from Gıbco stocks m 5 lıtre quantıtıes and dıluted usıng freshly-autoclaved cooled ultrapure water Basıc medıum were used and supplemented wıth the followıng components 1M HEPES (Sıgma H9136) pH 7 3 - Autoclaved, 7 5% $NaHCO_3$ (RdeH 31437) - Autoclaved, 5N NaOH (RdeH 30620) Autoclaved, 1 5 M HCl (BDH 10125) - Autoclaved

Each medıum was made up accordıng to the followıng approprıate recıpe, the medıum pH was brought to between pH 7 44 and 7 55 usıng sterıle 1 5 N NaOH and 1 5 M HCl

Medium	Cat. No.	Volume conc.	Vol. H_2O	Vol. HEPES	Vol. $NaHCO_3$
DMEM 10X	Gibco 042-02501	500 mls	4 3 L	100 mls	45 mls
MEM 10X	Gibco 042-01430 M	500 mls	4 3 L	100 mls	45 mls
RPMI-1640 10X	Gibco 042-2511M	500 mls	4 3 L	100 mls	45 mls
Hams F12	Gibco 047-1700	1 pack for 5L	4 7 L	100 mls	45 mls

The medium was then filter sterilized in a laminar flow cabinet using a 0 2 μ filter for 10 L capacity (Gelman Cat No 12158 - Micro-culture capsule w/filling bell) into numbered autoclaved bottles which had been treated as per section 2 0 1

Each bottle was checked for sterility by taking a

1) 3 ml sample at filter sterilization into a sterile universals
2) 1 ml sample onto a fresh sterile autoclaved Columbian based agar plate (Oxoid cm 331) containing 6% blood
3) 1 ml sample into 5 ml sterile autoclaved Thioglycolate broth (Oxoid cm 173),
4) 1 ml sample into 5 ml sterile autoclaved Sabauraud broth (Oxoid cm 147)

Each sample was placed at 37°C for at least 3 days to determine the sterility of the individual medium bottles

2.0.3 Preparation of Trypsin.

Trypsin was prepared from a 10X 2 5% sterile stock (Gibco 043-5090H) The 10X stock was filter sterilized through a 0 2 μ (low protein binding filter - Millex GV SLGV 025BS) diluted to 0 25% with sterile PBS (Oxoid Br14a dissolved in 100 ml ultrapure water and autoclaved) For trypsin versene (TV) 1 ml of 1% sterile EDTA(Sigma E-5134) was added Trypsin is aliquoted and stored at -20°C until required

2.0.4 Routine management of cells in culture.

Routine maintenance of cell culture encompassed feeding, sub-culturing, freezing and thawing All these procedures were carried out aseptically in a laminar flow hood which had been swabbed with 70% IMS (industrial methylated spirits) All articles entering the laminar flow were also swabbed with 70% IMS to ensure a sterile atmosphere All flasks and plates used were tissue culture treated (Greiner and Costar)

2.0.4.1 Feeding cells in culture.

For adherent cells: Feeding attached cells involved aseptic removal of waste medium, in a laminar flow, into a sterile waste bottle exclusive to each cell line. The cells were then overlaid with fresh complete medium containing the required supplements, as indicated below.

For spinner flasks: Feeding suspension cultures required that the flask be removed from the agitation apparatus approximately 3-4 mins before feeding, to allow the cells to settle. Once the cells had settled at the bottom of the flask, the medium was be removed by pipette and replaced with fresh complete medium as indicated below. Each cell line required a specific complete medium *i.e.* a 1X basal medium supplemented with components for that cell's individual requirements. The following table demonstrates the components required for 100 ml complete medium for each of the cell lines used in this study.

Cell line	1X Basal Medium	L-Glut	FCS	SP	NEAA	Other
DLKP	DMEM	1 ml	5%	-	-	-
Hep-G2	DMEM	1 ml	10%	-	-	-
H411E	DMEM	1 ml	5%	-	-	-
SKMES-1	DMEM	1 ml	10%	1 ml	-	-
SKLU1	MEM	1 ml	10%	1 ml	1 ml	-
HTB-120	DMEM:Hams-F12	1 ml	10%	-	-	-
SCC-9	DMEM:Hams-F12	1 ml	10%	-	-	-
DLRP	DMEM:Hams-F12	0.5 ml	10%	-	-	-
NRK	DMEM	1 ml	10%	-	-	-
V-79	DMEM	1 ml	5%	-	-	-
SD1 + XEM2	DMEM	1 ml	5%	-	-	400μg/ml geneticin
EP-16	RPMI-1640	1 ml	10%	-	-	-
V-79-1A1	DMEM	1 ml	5%	-	-	400μg/ml geneticin
HTC-BUdr	DMEM	1 ml	10%	-	-	-
DLKP-A	DMEM:Hams-F12	0.5 ml	10%	-	-	2 μg/ml adriamycin
SKMES1-A	DMEM	1 ml	10%	1 ml	-	1 μg/ml adriamycin
SKLU1-A	MEM	1 ml	10%	1 ml	1 ml	0.5 μg/ml adriamycin
704A1	DMEM+HAT	1 ml	10%	-	-	-
703D4	DMEM+HAT	1 ml	10%	-	-	-

NEAA 100X Non essentıal amıno acıds, Gıbco 043-01140 H, SP 100 mM Sodıum pyruvate, Gıbco 043-01360 H, Dulbecco's HAT medıum (Hypozanthene, amınopterın and thymıdıne) 1X stock, Gıbco 041-00170 M, L-glut 200 mM L-glutamıne, Gıbco 043-05030 H,

2.0.4.2 Experimental constituents of medıa:

Genetıcın G148 Sıgma (G-5103) was dıssolved at 100X stock ın complete medıum and filter sterılızed PB Phenobarbıtal (Clonmel Chemıcals) was dıssolved at 100X ın 5N NaOH and filter sterılızed The phenobarbıtal was then dıluted ın complete medıum and the pH adjusted to 7 44-7 55 range B[a]P (Sıgma B-1760) Benzo[a]pyrene was dıssolved as 2mg/ml ın DMSO and used dırectly at 5μl per 2 ml of medıum Benzanthracene (Sıgma B-2750) was dıssolved ın neat DMSO (Sıgma D-5879) and used dırectly at a concentratıon of 0 01% ın the medıum Cyclophosphamıde (Sıgma C0768) was dıssolved ın medıum and filter sterılızed before use

2.0.4.3 Induction of cell lines:

The cells to be examıned were seeded ınto 100 mm petrı-dıshes at a concentratıon of 5 x 10^5 cells/ml 20 mls complete medıum was added to each plate After ıncubatıng the plates overnıght at 37°C ın 5% CO_2, the waste medıum was removed and the ınducer contınıng medıum added (ınducer were made up ın complete medıum as per sectıon 2 0 4 3) The plates were then returned to the ıncubator for the approprıate length of tıme For benzanthracene and DMSO ınductıon the exposure lenght was for 18 hours at 5% CO_2 and 37°C For phenobarbıtal, exposure tıme was for 72 hours at 5% CO_2 and 37°C Cell lysates were then ısolated as per sectıon 2 12 1

2.0.4.4 Subculturing cells in culture.

<u>For adherent cells ın a 25 cm² flask</u> 1) Waste medıum was removed from the flask (as for feedıng cells) ınto a waste bottle and the cells were overlaıd wıth 2 mls of TV pre-warmed to 37°C The flask was gently rotated and the TV then remove to a waste bottle To the rınsed flask, 4 mls of warmed TV was then added and the flask removed to the ıncubator at 37°C for 3-5 mıns The cells were monıtor under the mıcroscope constantly, as some cells requıred less tıme to become trypsınızed than others When the cells had become detached from the flask bottom and had formed a sıngle cell suspensıon, 2 mls of complete medıum was added and the total 6 mls removed to a sterıle unıversal (Greıner) whıch had been labelled wıth the name of the cell lıne

The universal was centrifuged for 5 mins at 1000 rpm The supernatant was poured off and the pellet was resuspended in complete medium A fresh flask was re-seed with the required number of cells, feed with complete medium and return to the incubator (LEC 37°C incubator)

For suspension cultures The suspension was removed aseptically from the spinner flask, by pipette to sterile 50 ml universals and centrifuge at 1000 rpm for 5 mins The pellet was then rinse with 3 mls of pre-warmed TV and re-pellet at 1000 rpm for 5 mins The resulting second pellet was resuspend in 10 mls of fresh warmed TV and place at 37°C for 5 mins, or until the cells had attained a single cell suspension To this was added 4 mls of complete medium and the suspension centrifuged at 1000 rpm for 5 mins The supernatant was removed to a sterile waste bottle and the pellet resuspended in fresh complete medium The spinner flask was then re-seeded with the required density of cells and the appropriate volume of complete medium was added

2.0.4.5 Freezing cells in culture.

Purpose To allow long term storage of cultured cells and ensure adequate stocks of cells are available, within a given passage number range, for particular work

A large quantity of cells was required for this procedure Cells were grown to 80% confluence having been fed 12-24 hours previous to this procedure to ensure they were in good condition

Cells were trypsinized and pelleted as per section 2 0 4 3 The cell pellet was resuspended in a volume of 100% serum which ensured a single cell density of 5 x 10^7 viable cells/ml (as determined by trypan blue (Gibco 043-05250 H) exclusion) 10% v/v DMSO in 100% serum was added drop-wise, with continuous shaking of the cell suspension, to give a final 1 1 dilution This suspension was removed to a sterile labelled cryovial (Greiner 122 278) and placed in the vapour phase of a liquid nitrogen container for 3 hours After 3 hours the vials were placed directly into the liquid nitrogen where they were stored A vial of cells was thawed 2-5 days after freezing to determine the sterility and viability of the stocks

2.0.4.6 Testing viability of cells.

It is very important to test cell suspensions before and after freezing and also before seeding toxicity plates to determine the exact number of viable cells available for study This is quantified by trypan blue exclusion

Viable cells did not stain strongly in trypan blue however, damaged or dead cells will and stained dark blue, which can be viewed under the microscope To determine the number of viable cells a 250μl sample of the cell suspension was mixed with 50μl of trypan blue dye This mix is allowed to stand for 2-3 mins and a 100 μl aliquot placed in the 0 01 mm deep depression of a haemocytometer The four sets of 16 squares at the corners of the central graded area of the slide were counted, taking account of the total number of cell (clear and blue) and the viable (clear) cell so as to determine the percentage survival The number achieved was multiplied by 1 2 X 10^4 (the 1 2 takes into account the dilution caused by the trypan blue) to give the number of cells per ml

2.0.4.7 Thawing frozen stocks.

The vial required was removed from the liquid phase of the liquid nitrogen container and placed in a shatter proof container (ELKA/50 ml tube 00-2094) at 37°C for 2-3 mins until the contents had thawed The vial was then removed to the laminar flow, swabbed with 70% IMS and opened aseptically The contents was placed into a sterile universal containing 5 mls complete medium

The umversal was centrifuged at 1000 rpm for 5 mins and the pellet resuspened in 10 mls fresh cold complete medium A 1 ml aliquot of the suspension was taken to determine viability by trypan blue exclusion (section 2 0 4 5) and the remainder of the suspension re-seeded into a 25 cm^2 flask The cells were monitor by microscope for 1-2 hours and the medium replaced with fresh complete medium 24 hours later to ensure all traces of DMSO are removed

2.0.4.8 Mycoplasma testing using the Hoechst DNA staining method.

This method used the cell line NRK as an indicator, due to its maintenance of morphology during fixation

This procedure was carried out away from routine cell culture designated areas, to avoid possible contamination of clean cell stocks

1) Cells to be tested were subcultured a minimum of three times out of antibiotics, after thawing and resistant lines were fed and subcultured three times in medium not containing drug
2) 5 mls of conditioned medium (CM) from these lines *i e* medium that has been in contact with the cells for 2-3 days, was removed asceptically

3) 1 ml of the cleared CM was added asceptıcally to a 35 mm petrı dısh containıng a coverslıp (pre-treated as for ımmunofluroscence slıdes) on whıch NRK cells had previously been seeded at 2 x 10^3 cells/ml (1 day prıor to testıng)

4) The slıdes were ıncubated at 37°C and 5% CO_2 for 2-5 days untıl the cells had reached 50% confluence

5) The waste medıum was removed and the slıdes washed ın PBS twıce, ensurıng that the slıdes dıd not dry out at any tıme

6) Slıdes were then wash once wıth cold fresh 50 50 v/v PBS/Carnoys fixatıve (1 3 glacıal acetıc acıd (BDH 27013), methanol (BDH 10158) at -20°C)

7) To thıs 2 mls of carnoys fixatıve was added and left for 10 mıns

8) The fixatıve was removed and the samples allowed to aır dry

9) 2 mls of Hoechst was then added (50 ng/ml ın PBS, filtered through a 0 22 μ filter and stored ın alıqouts at -20°C away from lıght) for 10 mıns at low lıght ıntensıty

10) Slıdes were then washed wıth dıstılled water and mounted ın mountıng medıum (0 022M cıtrıc acıd, 0 055M dısodıum phosphate and 50% glycerol - pH 5 5 and filter sterılised usınng a 0 22 μ filter)

11) The slıdes were vıewed by mercury fluorescent mıcroscopy - extranuclear staınıng ındıcated a posıtıve contamınated sample

2.1 PRIMARY CULTURE:

Samples were transported from surgery ın sterıle complete medıum at 4° C, whıch contaıned DME/Hams F12 50/50 v/v 1% L-glutamıne,2% Pen/strep (Gıbco 5000 IU/ml pemcıllın/5000 Ug/ml streptomycın), 250 μg/ml fungızone (Gıbco 043 05290 D) and 10% FCS (Foetal calf serum)

1) In the laboratory, samples were removed asceptıcally from the collectıon medıum, ın the lamınar flow and placed ın sterıle PBS (The collectıon medıum was retaıned)

2) Usıng sterıle scalpels the samples were then dıssected ınto 1-4 mm^3 pıeces

3) The dıssectıon medıum was retaıned and the remaınder of the sample placed m 0 2% Dısphase (Boehrınger Mannheım - Neurtal Protease - 165-859) and 200 unıts Collagenase type IV (Sıgma C-5138), for up to 4 hours wıth constant agıtatıon

For single cell cultures Collection medium, dissection media and enzymatic desegregated medium were all pelleted at 1000 rpm for 4 mins to retrieve cells Pellets were resuspended in complete medium and seeded into 25 cm^2 flasks at a density of no less than 1 x 10^6 cells/ml and placed at 37° C

For explant culture At stage 2, using a sterile forceps individual 1-2 mm^3 samples of pure tumour were placed in a 25 cm^2 flask which had been pre-wetted with 0 5 ml of complete medium Once the explants had been placed, the flask was placed upright with the cap loosely closed for 30 mins in a laminar flow to allow the samples to settle After the 30 mins, 1 ml of complete medium was gently added to the flask, taking care not to disturb the fragments The cap was then secured and the flask moved to 37°C

Each sample was recorded and re-fed with complete medium every 3-4 days depending on its growth progress This growth was traced by microscopic evaluation Samples were kept for at least 3 weeks, longer when growth was substantial

2.1.1 Fibroblast eradication:

Over growth by normal fibroblasts within these cultures was a major obstacle to the success of primary epithelial tumour culture growth
The following procedures were employed to attempt to elevate this problem

1) Differential attachment Fibroblasts cells are believed to attach at a faster rate to tissue culture flasks (Freshney, 1987) Therefore 2-4 hours after initial seeding of the samples when the fibroblasts have attached, the supernatant was removed and reseeded into a new flask

2) Differential detachment Epithelial cells grow in close association with each other via desmosomes Therefore they are more resistant to detachment than fibroblast
Cultures of mixed epithelial and fibroblasts were exposed to a) 0 02% EDTA at room temperature for 3-5 mins, or b) 0 02% EDTA and 0 01% Trypsin for 5-6 mins at 37° C

3) Selective toxicity of Geneticin G148 Sulfate Mixed populations of cells were exposed to 100 μg/ml geneticin for 48 days

All cultures were continiously monitored by microscope through out the procedure, then rinsed vigorously by pipetting with PBS, followed by 3 rinses with PBS to remove the detached cells Cultures were re-fed with complete medium and returned to 37°C

2.2 SERUM BATCH TESTING:

One of the major problems associated with the use of FCS in cell culture, is the batch to batch variation that exists In extreme cases this variation may result in a serum which will not support the growth of cells, in more moderate cases growth may be retarded In instances where growth is the determining characteristic this factor becomes very important To avoid artifactual results screening of a number of batches for growth of the particular cells is recommended

2.2.1 Pretreatment of cells

Cells were pre-treated to ensure all cells were at the same stage of growth by, seeding cells into tissue culture treated vessels, at low density, four days prior to experimentation On day four cells were fed with complete medium Cells were required to be approximately 75% confluent on the day subculturmg for experimentation

Cells were seeded at low density 10^4 cells/ml in medium containing no FCS, into 96 well tissue culture treated plates (100 μl/well)

FCS batches were added to the pre-seeded wells over the range 2%, 10% and 20% (v/v) complete medium in 100 μl quantities, resulting in a 1/2 dilution of the FCS samples and a final concentration range of 1%, 5% and 10% FCS, in each well Plates were placed in a 5% CO_2 humid environment at 37°C for 5 days Growth was ascertained by the acid phosphatase enzyme activity (Martin *et al , 1991)*

2.2.2 Acid phosphatase enzyme assay for cell growth:

1) After mcubation with the desired compound for the required length of time, 96 well plates were removed from the incubator and the medium removed
2) The wells were washed with PBS (100μl) twice and the PBS removed
3) To each of the rinsed now empty wells 100 μl of 10 mM para-nitrophenolphosphate (Sigma Substrate 104) in pH 5 5 buffer (0 1 M sodium acetate 0 1% triton X-100 (BDH 30632)) was added
4) Plates were then covered in aluminium foil and placed in the dark at 37°C for 2 hours

5) After 2 hours 50 μl of 1 N NaOH was added to each well

6) Growth was determined by the absorbance at 405 nm and 620 nm in a dual wavelength elisa plate reader (Titertek Multiskan plus at 405 and 620 nm)

Results were recorded as a percentage of a control *i e* cells grown in a FCS with known acceptable growth pattern The FCS which performs within the required growth profile is ordered in bulk for a block of work to avoid artifactual variation which may result due to the FCS

2.3 TOXICITY TESTING:

Procedure for the detection of toxicity of chemicals on continuous cell lines in culture

Day 1 Pre-treated Cells (see section 2 3 1) were seeded into 96 well tissue culture plates at 10^3 cells/well *i e* 10^4 cells/ml in 100 μl/ml as for serum batch testing and allowed to attach overnight in 5% CO_2, in a 37°C humid environment (Jouan CO_2 incubator)

Day 2 Test compounds were added dissolved in the appropriate solvent, pH returned to appropriate 7 45-7 55, diluted to 2x the desired concentration and filter sterilised (ensuring that the solvent concentration is below 0 01% of the final volume) Test compounds were then added in 100μl quantities to the test wells and the plates returned to the CO_2 incubator

- Toxicity tests were monitored each day until the controls growth *i e* cells not exposed to compound, had reached approximately 80 % confluence At this point the test is terminated and growth determined by acid phosphatase test as in section 2 3 2

2.4 IMMUNOFLUORESCENCE ANALYSIS

2.4.1 Pretreatment of slides

Slides were soaked in a solution of 5% (v/v) of RBS for 1-2 hours followed by rinsing m distilled (RO) water for 10 mins The slides were then kept in methanol at 4° C until required Slides were flamed and placed in sterile petri dishes just prior to use

Cells were seeded at a concentration of 10^5 cell/ml of complete mediuml, 20μl per well, on eight well H T C blue, autoclavable, 6 mm sterile slides The slides were then incubated at 37° C in a humidified, 5% CO_2 atmosphere for 24 hours For non adherent cells cells were mounted on non-sterile Gold star microslide at a concentration of 10^5 cell/ml in PBS and allowed to air dry on the day

2.4.2 Procedure for surface membrane antigen Ep-16:

The incubated slides were washed in phosphate-buffered saline (PBS) three times and excess saline was removed using filter paper, taking care not to disturb the attached cells Slides were then placed in 3% (v/v) formaldehyde at room temperature for 5 mins The slides were then submerged in acetone at -20° C for a further 5 mins Aliqouts of 20 μl of EP16 conditioned medium (from an anti-human epithelium hybridoma (A T C C HB 155)) which contained the primary antibody, were added to each well Slides were then incubated at 37° C in a humid environment for 1 hour Following this, the slides were washed with PBS for 10 mins , three times Secondary antibody (FITC-labelled antimouse IgM (1/30 dilution)Sigma) was added to each well in 20 μl aliqouts and incubated at 37° C, in a humid environment, in the dark, for 1 hour Slides were then washed in PBS for 10 mins , three times keeping the light intensity low Mounting was in glycerol PBS 90 10 (v/v) and coverslips were sealed using clear nail varnish

2.4.3 Procedure for intracellular antigen

Slides were washed in PBS three times and excess saline removed using filter paper, as described above The slides were placed in methanol at -20° C for 7 mins and then removed to acetone at -20° C for 2 mins

Primary antibodies (conditioned medium from the lines 704A1, 703D4 expressing two murine IgG2Ak monoclonal antibodies and Anti-cytokeratin N° 18 (Bohreingher Mannheim 1 μg/ml)) was added to each well m 20 μl quantities Slides were then incubated in a humid environment at 37° C for 1 hour. Following incubation the slides were rinsed three times for 10 mm each time in PBS The secondary antibody (goat anti-mouse FITC- as above) was added in 20 μl quantities and the slides were then incubated in the dark for 1 hour Slides were washed in PBS three time after incubation and mounted as above mentioned

All slides were viewed both with and without oil immersion on a fluorescent microscope

2.4.4 Immunohistochemistry to detect for expression of enzyme:

This procedure was used to screen transfectants for gene expression

Aliqouts of 5 x 10^4 cells/ml in complete medium were seeded on to slides (as described above) in growth medium and allowed to attach over night at 37° C, 5% CO_2, in a humid environment Following this, slides were rinsed with PBS (15 mM sodium phosphate pH 7 4, 0 15 M NaCl), fixed in acetone methanol (1 1) at -20° for 5- 10 mins , and allowed to air dry for 5 mins at room temperature

Slides were then incubated with 400μl quantities of complete medium containing 5μg/μl of specific P_{450} antibody (Oxygene 001A1-P-RT polyclonal - rabbit anti-Rat Cytochrome P450IA1), at room temperature, in a humid environment for 35 mins Excess primary antibody was removed by washing in PBS for 10 mins , three times, shaking slowly Excess PBS was removed and 400μl of full medium containing secondary antibody (goat anti-rabbit FITC (1 80)Sigma F-0511) is applied Slides were then incubated at room temperature in a humidified atmosphere for 35 mins Excess secondary antibody was removed by rinsing for 10 mins in PBS three times, shaking slowly

Slides were mounted in PBS/glycerol (Sigma G5516) (as above) and viewed within one week

2.5 ISO-ENZYME ANALYSIS:

Iso-enzyme analysis was carried out using the Corning Authentikit System

2.5.1 Cell extraction procedure:

1) A pellet of 10^7 cells was resuspended in 10 ml cold Ca(II)-free, Mg(II)-free Earl's Balanced Salt solution and re-pelleted at 1000 rpm for 5 mins
2) The resulting pellet was resuspended in 1 ml extraction buffer (50 mM Tris, pH 7 5, 1 mM EDTA, 2 0% Triton X-100) and left to stand for 15 mins or until all the cells had been lysed (as determined by microscopic analysis)
3) The Cells were then sheared by continuous vigorious pipetting, ensuring that excessive foaming of the samples did not occur, as this could lead to denaturing of the protein
4) The suspension was then centrifuged at 2000g for 10 mins at 4°C
5) 100 μL of the resulting supernatant was removed and a 100 μl of authentiKit stabilization buffer was added (This extract could be stored at -20°C for up to 6 months)

2.5.2 To test the extracts for enzyme activity

a) A vial of each of the six lyophilized enzyme substrates was reconstituted in 2 5 ml of 0 1 M PHAB AuthentiKit buffer and placed on ice for use within 30 mins

The enzyme activities for which the cell extracts were tested were NP - Nucleoside phosphorylase, G-6-P - Glucose-6-phosphase dehydrogenase, MD - Malate dehydrogenase, MPI - Mannose phosphate isomerase, PEP-B - peptidase B, LDH - Lactate dehydrogenase

For each enzyme

b) 0 2 mls of the reconstituted reagent was transfered to a test tube and heated to 37°C in a water bath

c) 10 μl of cell extract was added to each of the tubes 10 μl deionised water was added in place of cell extract as a control

d) The sample tubes were incubated for 10 minutes at 37°C

e) The reaction was stopped by addition of 1 0 ml of stoppmg reagent (0 05 N HCl, 1% Triton X-100 in distilled H_2O) and the absorbance of the samples read at 565 nm within 30 mins Each sample was required to give an absorbance reading between 0 1 and 0 5 units, to carry out electrophoresis

Electrophoresis was carried out according to the AuthentiKit proceedure using the prepared agarose gels After electrophoresis gels were overlaid with the individual substrates and incubated for 20 mins , except for Pep B which required 40 mins incubation at 37°C in the incubator chamber After incubation the gels were rinsed in deionized H_2O for 15- 30 mins to ensure that residual substrate was removed and to give a clear background The gels were then left to dry for 30 mins at below 65°C

2.6 CLONING IN AGAR

Cells were seeded at a range of densities from 10^3 to $2x10^5$ cells/ml in DME medium containing 10% FCS, and 2% P/S in 30mm plates The lower 1 5 ml agar layer contained 0 6% Difco Bacto-agar (Detroit, U S A) and was poured at 44°C The upper layer in which the cells were suspended contained 0 3% agar (this agar was at 40 5°C due to the presence of cells and FCS) Cultures were incubated at 37° C, 5% CO_2 in a humid atmosphere Colonies were allowed to develop over a period of 10 - 14 days Colonies greater than 50 mm in diameter were counted on an inverted microscope (CK Olympus Tokyo) at 100x or 40x

2.7 HISTOPATHOLOGY

Cells were analyzed for characteristic properties of malignancy as seen m tissue sections, such as multiple nucleoli and tri- or multipolar mitoses (Gilvarry *et al* , 1990) Cells were trypsinized and seeded on sterile 8-well slides (Dynatech) at a concentration of 2 5 x 10^3 cells/well Slides were incubated for 24-48 hours at 37° C, 5% CO_2 in a humid atmosphere Slides were rinsed in phosphate-buffered saline and the cells were fixed with absolute alcohol before staining with Carazzi's haematoxylin and 0 5% eosin Glass slides were fixed in xylene for 1 hour followed by mounting in DPX (BDH Chemicals)

2.8 CHROMOSOMAL ANALYSIS

Chromosome harvest was achieved by addition of colcemid (0 02 ug/ml) to log phase cultures for the first two hours of incubation Culture flasks were shaken, the detached cells collected in centrifuge tubes and centrifuged at 1000 rpm for five minutes The cells were then subjected to hypotonic treatment which consisted of exposure to 0 075 M KCl for 20-35 minutes Initial fixation in methanol acetic acid (3 1) was followed by three washes in fresh fixative Air-dried preparations were either stained conventionally m 3% giemsa (Gurr 35086) or incubated overnight at 60° C and G banded according to the method of Seabright (1971)

2.9 DNA ISOLATION:

2.9.1 Preparation of competent cells for transformation:

1) 20 ml sterile L-broth (Oxoid cm1) was inoculated with either JM109 or C600 *E coli*, in a 50 ml universal and grown over night at 37° C in a shaking incubator at 250 rpm
2) 4 mls of this overnight culture was innoculated into 400 ml sterile L-Broth m a sterile 2 Litre baffled flask and placed at 250 rpm at 37°C until the absorbance at 590 nm was exactly 0 375
3) This culture was then aliquotted into pre-chilled 50 ml universals and left on ice for 5-10 mins
4) The cells were then centrifuged at 3000 rpm @ 4°C for 7 mins
5) The supernatant was removed and the pellet resuspended in 10 mls ice-cold sterile $CaCl_2$ (60 mM $CaCl_2$ (Sigma C7902), 15% Glycerol, 10 mM PIPES (Sigma P9291) @ pH 7 0
7) Cells were centrifuged again for 5 mins at 2500 rpm @ 4°C, the resulting pellet was resuspended in 2 ml ice-cold $CaCl_2$
8) This suspension was left on ice for 12 - 24 hours
9) After the 12-24 hours cooling the suspension was dispensed in batches into -70°C pre-chilled sterile eppendorfs and stored at -70°C

2.9.2 Transformation of bacteria with plasmid:

1) 10 ng of DNA to be transformed was placed in a sterile eppendorf

2) 100 μl competent cells were taken dırectly from the freezer, thawed quıckly and added to the DNA, the contents of the eppendorf were swırled gently

3) Thıs eppendorf was placed on ıce for 30 mıns

4) 1ml L-broth was added to thıs suspensıon, mıxed gently by ınversıon and ıncubated at 37°C and 250 rpm for 1 hour

5) The cells were then pelleted for 1-2 mıns at 13,000 rpm and resuspended ın 200 μl L-Broth

6) 200 μl of thıs culture was then lawned on "resıstance plates" contaınıng the selectıve agent coded for ın the plasmıd whıch had been transformed

7) These plates were then ıncubated at 37°C overnıght

8) Colonıes were checked for plasmıd by mını-prep

2.9.3 Small scale preparation of plasmid DNA - miniprep:

1) One colony (from the transformed plates, sectıon 2 7 2) was removed to 2 ml selectıve L-broth (*ı e* contaınıng ampıcıllın (amp Sıgma A-9393)), 100 μg/ml ıf the plasmıd expresses amp resıstance) and ıncubated over-nıght at 250 rpm and 37°C

2) 1 5 ml of thıs overnıght culture was placed ınto a sterıle eppendorf and the cells pelleted at 12,000 rpm for 30 seconds at 4°C (the remaınıng 0 5 ml was stored at 4°C untıl requıred)

3) The supernatant was removed completely and the pellet resuspended ın 100 μl of ıce cold solutıon 1 (50mM glucose, 25mM Trıs-HCl pH 8 0 (Sıgma T8524) 10 mM EDTA pH 8 0, autoclaved and stored at 4°C

4) 200 μl of fresh solutıon 2 (0 2 N NaOH, 1% SDS (Sıgma L-5125)) was added to the eppendorf

5) To thıs was added 150 μl ıce cold solutıon 3 (60 ml 5M potassıum acetate /11 5 ml glacıal acetıc acıd (BDH 27013) made up to 100ml wıth UPH_2O) and the eppendorf was ınverted for 10 seconds and stored on ıce for 3-5 mıns

6) Thıs solutıon was then centrıfuged at 12,000 for 5 mıns at 4°C and the supernatant removed to a clean eppendorf

7) To thıs was added an equal volume of equılıbrated (see sectıon 2 7 4) phenol(Sıgma P-1037) chloroform (BDH 10077) Isoamylalcohol (RdeH 60217) (25 24 1 v/v/v)

After mixing the solution was then centrifuged for 2 mins at 12,000 rpm The upper clear layer was transfered to a clean eppendorf and this process was repeated with chloroform isoamylalcohol (50 1 v/v) twice more to remove all phenol

8) 2 volumes of 100% ethanol were then added and the solution allowed to stand at room temperature for 2 mins

9) This was followed by centrifugation at 12,000 g for 5 mins 4°C

10) The supernatant was removed and the tube inverted on tissue paper and allowed to dry

11) The semi-dry pellet was rinsed once with 1ml 70% ethanol at 4°C - to remove the supernatant and allowed to dry at room temperature for 10 mins

12) The pellet was resuspended in 50 μl TE (10mM Tris-HCl, pH 8 0, 1mM EDTA pH 8 0) and stored at -20° C

2.9.4 Phenol equilibration:

1) Frozen phenol was melted at 68° C in a water bath in a fume hood To the phenol was added an equal volume of 0 5 M Tris-HCl, pH 8 0 and the mixture was stirred using a magnetic stirrer for 15 mins The phases were left to settle and the upper aqueous layer was then removed by pipette

2) An equal volume of 0 1 M Tris-HCl, pH 8 0 was added and stired for 15 mins, the layers were allowed to settle and the upper aqueous layer removed again This process was repeated with 0 1 M Tris-HCl, pH 8 0 until the phenol reached pH 8 0, tested using litmus paper

3) 0 1 M Tris-HCl pH 8 0 containing 0 2% β-mercapto-ethanol (Sigma M7154) was then added to a volume of on tenth of the total volume and the phenol was then stored in a dark bottle at 4° C for 1 month only

2.9.5 Large-scale DNA preparation:

1) 10 mls sterile selective L-broth was inoculated with a loop of transformed bacteria and incubated at 37°C at 250 rpm overnight

2) 1 Litre sterile selective L-broth was then inoculated with the overnight culture and incubated overnight at 250 rpm and 37°C

3) The cells were centrifuged for 20 mins at 4°C at 12,000 g

4) The resultıng pellet was resuspended ın 3 mls of Solutıon 1 (25% sucrose ın 0 05 M Trıs-HCl pH 8 0) and transfered to a 30 ml sorvell tube

5) To thıs was added 1ml solutıon 2 - 0 25 M Trıs-HCl contaınıng 25g/l lysozyme (Sıgma L6876, prepared just prıor to use) and the resultıng solutıon was left on ıce for 15 mıns

6) When thıs tıme had elasped, 7 mls of solutıon 3 (2% Trıton X-100 ın 0 05 M Trıs-HCl, 0 0625 M EDTA pH 8 0) was added and the contaıner was placed on ıce for a further 15 mıns

7) Thıs solutıon was then spın-cleared at 48,000 g for 45 mıns at 4°C and removed to a sterıle unıversal

8) 1 g per ml of solutıon of CsCl (C4036) was added per ml of supernatant and then 0 5 ml ethıdıum bromıde (10 mg/ml)

9) To clear the solutıon the unıveral was spun at 8000 g for 5 mıns and the supernatant transferred to a 20 ml heat seal tube (Beckman)

10) The tubes were balanced to wıthın 0 01 g and centrıfuged for 40 hours at 125,000g and 21°C ın a Beckman 70ıTı rotor

11) The heat seal tube was vıewed ın front of a UV source The lower plasmıd band was removed usıng a 20 gauge syrınge takıng care not to contamınate thıs band wıth the upper genomıc band

12) In the case of DNA used for transfectıon, thıs band was mıxed wıth CsCl/TE pH 8 0 (1g/ml) and recentrıfuged at 125,000g for 40 hours at 21°C ın a Beckman 70ıTı rotor ın a Beckman XL80 ultracentrıfuge After thıs centrıfugatıon the lower plasmıd band was reısolated as above - thıs ensured very clean DNA for transfectıon

13) The ısolated band was then added to an equal volume of ısopropanol and the upper layer removed, thıs process was repeated untıl the lower layer was clear Thıs removed the ethıdıum bromıde from the sample

14) The resultıng sample was then dıalysed agaınst TE (pH 8 0) for 40 hours wıth regular changes of the buffer

15) The dıalysed sample was then phenol extracted as ın sectıon 2 7 3 steps 7-12

2.9.6 Plasmıd isolation using PZ523™ spin columns:

Extractıon was carrıed out accordıng to the procedure supplıed wıth the columns - (5 prıme → 3 prıme, Inc , Boulder, CO U S A)

2.9.7 Removal of contaminating RNA from DNA samples:

2.9.7.1 RNAse procedure:

1) DNA was disolved in 5 ml of TE pH 8 0
2) To the disolved pellet was added 10 μl of 10 mg/ml DNAase-free RNAse (5'-3' Inc , Boulder, CO, U S A , 5305-461036) and the resulting solution incubated for 30 mins at 37°C
3) After incubation the DNA solution was extracted once with 5 mls of phenol chlorofrom isoamylalcohol (25 24 1, v/v/v)
4) The aqueous phase was removed and extracted with 5 ml chloroform isoamylalcohol (24 1 v/v)
5) The aqueous phase was isolated again and 10 M ammonium acetate added (RdeH 32301) to a concentration of 2 0 M
6) O 6 volume of 100 % isopropanol was then added at room temperature and mixed by inversion The resulting solution was left at room temperature for 30 mins
7) After 30 mins the solution was centrifuged at 13,000 rpm for 30 mins at 20°C and the pellet washed with 5-10 ml ice cold 70% ethanol (Merck 986)
8) The sample was repelleted at 13,000 rpm for 5 mins and the pellet was allowed to air dry

2.9.7.2 Polyethylene glycol (PEG) procedure:

1) The DNA pellet was resuspended in 20 μl TE pH 8 0 and add 4 μl of 4 M NaCl were added followed 20 μl 13% PEG (Polyethylene glycol RdeH 32309)
2) The resulting mixture was placed on ice for 20 mins and centrifuged at 13,000 for 30 mins
3) The pellet was washed with 70% ethanol twice and allowed to air dry
4) The washed pellet was resuspended in TE pH 8 0 and stored at -70°C

2.9.2.3 Sephadex G-50 (Superfine Pharmacia) procedure:

1) Sephadex G-50 (Pharmacia 17-0041-01) was equilibrated with sterile H_2O and allowed to settle

2) The water was removed and replaced with TE, the suspension was allowed to settle the process was and repeated

3) The equilibrated G-50 was added to a 1 5 ml eppendorf which had previously been punctured, plugged with glass wool which had been coated with repelcote as per section 2 0 1 and autoclaved The filled column was centrifuged for 4 mins at 1600 g and this step repeated until the height of the column remained constant at 1 ml A layer of TE was placed on top of the column, the eppendorf was sealed with parafilm and stored at 4°C until required

4) Just prior to use the column was spun dry, and 100 μl of contaminated DNA sample diluted in TE was layered on top of the G-50 The column was centrifuged at 1600 g for 4 mins The eluate which contained the cleaned DNA was recovered

2.10 DNA ANALYSIS - GEL ELECTROPHORESIS:

Analysis of DNA samples was carried out using 1% Agarose which was dissolved in 1X TBE (5X, 5g-Tris 27 5 g Boric acid 20 ml 0 5M EDTA, pH 8 0) and the solution was brought to the boil When the molten gel had cooled to approximately 37°C, 3μl of 10mg/ml ethidium bromide were added and the gel was poured into to the casting well of the a BRL Horizon 58 mini gel system, the combs were then inserted and the gel was left to solidify

The samples are diluted 6 1 with 6X loading buffer (0 25 % Bromophenol blue (Sigma B5525), 40% sucrose)in an eppendorf Samples were then centrifuged down to ensure all the volume was at the bottom of the eppendorf The gel was loaded using a Gilson P-10, overlaid with 1X TBE and run at "low" for approximately 1-2 hours The DNA was then visualized as bright pink bands by placing the gel on a transilluminator (UVP transilluminator) Samples were compared with lambda markers cut with the appropriate restriction enzyme to ensure bands of the desired size The DNA was illuminated by the UV light due to the presence of the ethidium bromide in the gel which attaches in the grooves of the DNA

2.10.1. Restriction digestion of DNA.

Restriction enzyme digestion of DNA was carried out m this work to linearise plasmid DNA for transfection, for diagnostic purposes to ensure the identity of a plasmid and to produce lambda markers for gel electrophoresis

1) For a 50μl digest, 10 μl of DNA (1μg/μl) to be digested was aliquotted asceptically with a sterile yellow tip in a sterile eppendorf

2) To this eppendorf was added 5μl of sterile 10X specific restriction enzyme buffer (supplied with the enzyme)

3) 30μl of sterile UP water was then added to ensure the correct volume, followed by 5μl of the specific enzyme

4) The eppendorf was incubated at 37°C for 2 hours

5) A 5 μl sample was then run on a minigel as per section 2 9 to ensure all the DNA had cut and gave the correct banding pattern

The restricted DNA was recleaned for transfection by phenol extraction as per section 2 7 3 steps 7-12

2.11 POLY-ACRYLAMIDE GEL ELECTROPHORESIS:- using LKB mini-gel system.

For this project 10 % polyacrylamide gels as can be seen from the table below, were used throughout with 0 75 mm gaskets on ethanol cleaned plates

Gel component	10% Gel	5% Stacking Gel
30% Acrylamide stock	5 0 ml	0 8 ml
UP Water	6 8 ml	3 6 ml
Tris-HCl 1 875 pH 8 8	3 0 ml	-
10% SDS	150 μl	50 μl
10% Ammonium persulphate (fresh)	50 μl	17 μl
TEMED (Sigma T-7024)	10 μl	10 μl
Tris-HCl 1 24 M pH 6 8	-	0 5 μl

Stock 30% acrylamide contained 29 1 g/100ml acrylamide (RdeH 62021) and 0 9 g NN-Methylene bis-acrylamide (Sigma M2022), which was filtered through Watman 3mm filter paper

Equal proteın concentratıon of samples were made up 50 50 wıth loadıng buffer (2 5 ml 1 25 M Trıs-HCl pH 6 8, 10% SDS, 2 5 ml β-mercaptoethanol, 2 9 ml glycerol, 0 1% bromphenol blue - made up to a total of 50 mls wıth water) Samples were boıled for 2 mıns and loaded onto the gels Gels were run accordıng to the procedure of Laemmlı (1970) for 50 mıns at 250 M 45 mA for 2 gels ın 1X runnıng buffer (10X - 1 9 M glycıne (RdeH 33226), 0 25 M Trıs, 0 1% SDS - pH 8 3)

2.12 WESTERN BLOT ANALYSIS:

2.12.1 Sample preparation:

Membrane and cytosolıc preparatıons for western blot analysıs were prepared by the followıng procedure The cell lınes to be tested were grown m theır specıfic medıum untıl they were 80% confluent Cells were fed the evenıng prıor to extractıon

1) Cells were trypsınızed ın the usual manner and the pellet rınsed three tımes wıth PBS contaınıng 2 mM PMSF (Phenylmethylsulfonyl Fluorıde Sıgma 7626)
2) Cells were ruptured by sonıcatıon on ıce at 045 mA, 20 pulses followed by a 10 second rest, three tımes
3) Homogenate was spun at 4000g for 10 mıns at 4°C
4) Supernatant was then centrıfuged at 100,000 g for 1 hour at 4°C
5) Both pellet and supernatant were retaıned The pellet was resuspended ın PBS-PMSF and lyophılısed Supernatant was used dırectly

Gels were run accordıng to sectıon 2 11

2.12.2 Blotting:

1) The acrylamıde gel was removed after electrophoresıs to blottıng buffer (20 mM Trıs, 150 mM glycıne -make up fresh each tıme)(Towbın *et al , 1979)*
2) The nıtrocellulose (Hybond- C Amersham) was soaked ın blottıng buffer for 5 mıns
3) 4 sheet of Watman 3mm filter paper whıch were the same sıze as the nıtrocellulose pıece to be blotted, were stacked and soaked ın blottıng buffer The filter paper was placed on the cathode plate of the semı dry blotter

4) The nıtrocellulose was placed over the filter paper stack, ensurıng that no bubbles had developed

5) The gel was placed on the nıtrocellulose and covered wıth 4 more sheets of filter paper treated as before, once agaın ensurıng that no bubbles have entered the system

6) The transfer took place at 0 34 mA/015 V for 15-30 mıns (or as long as was requıred for the sıze proteın to be transfer and the thıckness of the gel) 20-25 mıns was the requıred transfer tıme for the proteıns transfered ın thıs study

7) The filter paper was removed and the was nıtrocellulose marked at the locatıon of the gels lanes and the molecular weıght markers

8) Thıs blot was placed ın blockıng buffer (TBS (500mM NaCl, 20 mM Trıs - pH 7 5), 3% Marvel (Cadbury's Marvel Skımmed mılk) for at least 1 hour or over nıght at 4°C

9) After blockıng the buffer was removed and prımary antı-body (1 200 dılutıon for GST antı α, μ and π antı-bodıes (BıoTrın) and 1 100 for Oxygene P450IA1 antı-body) was added ın TBS wıth 1% Marvel for 1 hour at room temperature

10) After ıncubatıon the blot was washed for 10 mıns three tımes ın TBS contaınıng 0 05% tween 20 (Aldrıch 9005-64-5) at room temperature

11) Secondary antı-body - Goat antı-rabbıt IgM 1 18,000 dılutıon wıth TBS contaınıng 1% marvel, was then added for 1 hour

12) The blot was washed agaın for 10 mıns three tımes ın TBS contaınıng 0 05% tween-20

13) The blot was then removed from TBS 0 05% tween-20 and placed ın 100 ml solutıon X - (10 ml (a), 4 ml (b), 400 μl (c) and 88 6 ml (d))
    a) 0 1% Nıtroblue tetrazolıum (NBT)(Sıgma H5514) ın 10 mM Trıs-HCl pH 8 9 (1 tablet ın 10 mls)
    b) 5mg/ml 5-bromo-4-chloro-3-ındolylphosphate- BCIP (Sıgma B0274) ın DMF (Dımethyl formamıde Sıgma D8654)
    c) 1M $MgCl_2$
    d) 0 5 M Trıs-HCl pH 8 9

14) Thıs proceedure developed the colour of the blot, whıch was then rınsed ın dıstılled water and stored dry away from the lıght

2.13 TRANSFECTION OF MAMMALIAN CELLS:

2.13.1 Calcium Phosphate mediumed transfection:

Day 1 Pre-treated cells (see section 2 3 1) were seeded in complete medium at 5 x 10^5 cells per 10 mm petri dish, so as to ensure that the cells are 70% confluent on day 2 Plasmid DNA was diluted to 1 μg/ml in TE The required quantity of prepared DNA (see section 2 7 5) (10μg) was diluted with 410 μl sterile H_2O in a sterile 3 ml tube and stored overnight at 4°C

Day 2 1) The tubes containing the DNA were incubated at 37°C for 1 hour 480 μl 2X HBS (2X HBS pH 7 12 (280 mM NaCl, 1 5 mM Na_2HPO_4, 50 mM HEPES) filter sterilizd and stored at 4°C) was placed into a sterile tube and left at room temperature

2) 60 μl 2 M $CaCl_2$ was added dropwise into the tube containing the DNA with continual mixing using a vortex

Immediumtely after the DNA-$CaCl_2$ mixture was added to the 2X HBS dropwise with continuous mixing using a vortex and allowed to stand at room temperature for 30 mins in the laminar flow

3) After 30 mins DNA-$CaCl_2$ mixture was added to the cells drop by drop with constant gentle aggitation of the universal, to ensure even mixing

4) The plate was returned to the incubator for 4 hours

5) To aid the entry of DNA the cells were glycerol shocked after the 4 hour incubation The DNA-$CaCl_2$ was removed and 5 ml of 10 % sterile glycerol in 1 x HBS was added The glycerol was left on the cells for exactly 3 mins and then removed by repeated washing with serum free medium

6) The transfected cells were fed with complete medium and returned to the incubator

Day 3 The plates were passaged to low density and allowed to attach

Day 4 The plates were fed with complete medium containing the selective agent - see section 2 0 4 3

Surviving colonies were cloned out by cloning cylinders after 10-15 days

2.13.2 Electroporation mediated transfection:

1) Pre-treated cell were isolated (after rinsing twice in ice cold XPBS) at a concentration of 1 x 10^7 cells/ml in XPBS(137 mM NaCl, 2 7 mM KCl, 4 3 mM Na_2HPO_4 $7H_2O$, 1 4 mM KH_2PO_4 - pH 7 3)

2) 0 5 ml of cell suspension was removed to a precooled electroporation cuvette (BioRad 165-2088) and placed on ice

3) 10 μg of prepared DNA (2 9 5) was added to the cell suspension and the cuvette "flicked" to ensure correct mixing The cuvette was then left to stand on ice for 5 mins

4) The cuvette was placed in the electroporation apparatus and one pulse was delivered (for DLKP - resistance = infinity, capacitance 2000, one pulse only required)

5) The cuvette was then returned to ice for a further 10 mins

6) The transfected cells were diluted 20 fold in nonselective complete medium

7) These cells were grown for 48 hours after which time the medium was replaced with complete medium containing selective agent and sruvivmg colonies were isolated as described in 2 13 3

2.13.3 CLONING COLONIES:

To clone out the plasmid containing colonies,

1) The colonies to be cloned were located by microscopy and marked A sterile steel cloning cylinder (6 mm) (which had been dipped m sterile silicon grease to create a seal with the base of the petri dish) was placed over the colony to isolate it

2) The medium inside the cloning ring was removed aseptically and replaced with 100 μl prewarmed trypsin using a 200 μl Gilson A second 100 μl trypsin was added and the plate left at 37°C for 2-3 mins

3) The trypsin was gently pipetted in the cylinder and removed to a 24 well plate 3 mls complete medium was added and the cells were allowed to attach overnight

4) The next day the medium was replaced with fresh complete medium and the clones left to grow

2.13.4 To test for frequency of transfection using plasmid PCH110.

1) Cells were transfected with PCH110 as in sections 2 13 1 and 2 13 2 A sample of the transfected cells, of a known quantity, were kept separate for this test

2) At the 48 hour point these cells were rinsed twice with 10 mls PBS

3) The cells were placed in fixative for 10 mins, twice, by adding 10 ml fixing solution (25% gluteraldehyde-0 4ml (Sigma G-6257, 0 5 M sodium phosphate buffer pH 7 3-10ml, 0 1 EGTA (Sigma E-4378), pH 8 0-2 5ml, 1 0 M $MgCl_2$-0 1 ml and 37 0 ml deionised water)

4) After fixation the cells were washed twice for 10 mins, with rinsing solution (40 ml 0 5M phosphate buffer pH 7 3, 0 4 ml 1 0 M $MgCl_2$ (Sigma M0250), 20 mg Sodium deoxycholate, 40μl NP-40, 160 nl deionised water)

5) The cells were stained overnight at 37°C, with 2 5 ml staining solution (10ml rinsing solution, 25mg/ml in DMF of X-Gal, 16 5 mg potassium ferricyanide (Sigma P8131), 16 5 mg potassium ferrocyanide (Sigma P9387))

6) After staining the cells were rinsed with rinsing solution for 10 mins twice and stored in rinse solution at 4°C sealed with parafilm to prevent evaporation

7) The stained colonies were viewed under an inverted microscope - colonies containing the plasmid stained bright blue, plasmid negative colonies remained transparent

2.14 POLYMERASE CHAIN REACTION FOR DETECTION OF PLASMID:

The plasmid PSVP4501A1 contains no gene for a selective agent Therefore it was necessary to co-transfect this plasmid with pSV2Neo, which contains geneticin G-418 resistance, to enable selection of the cell containing the plasmid To achieve this the plasmids were transfected at a ratio of 20 1 PSV450IA1 PSV2Neo This proceedure operates on the principle that, if a cell takes up and expresses geneticin resistance, then due to the twenty times greater concentration of PSV450IA1, then it is assumed that the PSVP450 plasmid was also taken up
However, expression of the PSV2Neo was detected in DLKP co-transfected cells, PSV450IA1 was not detected It was essential to investigate the PSV2Neo positive cells to see if they had taken up the PSV450IA1 plasmid or not The method chosen to carry this out was Polymerase Chain Reaction (PCR) Primers coding for 20-30 bp of the CYP1A1 cDNA segment which is present in the plasmid were chosen, from the cDNA sequence of rat CYP1A1, previously published sequence of Yabusaki *et al*, 1984, primers of 20-30 bp were chosen and sent by E-Mail for verification to "Genebank" at Heidlberg

Verification involves comparing the chosen sequence with all published sequences present in the "Genebank" memory including the human CYP1A1 gene sequence The resulting print-out gives the percentage homology between the primer sequence, which you have chosen and those sequences present in the memory, in decending order of similarity From these results it is possible to determine wheither the primer you have chosen will cross-react with any other gene appart from the gene of interest, as this would result in a false positive Primers were chosen so that they would detect rat CYP1A1 sequence but not the corresponsding human sequence One of the primer sequences chosen for this experiment was a previously published rat CYP1A1 cDNA sequence (Yang *et al*, 1991) -5'-TCT GGT GAG CAT CCA GGA CA -3', which runs from 1650-1670 and demonstrates very low homology with any human genes and no cross reactivity with human CYP1A1 The second primer was chosen at random from the sequence and verified through "Genebank" to exhibit similar low levels of homology with human genes as the first primer and no cross reactivity with human CYP1A1 It is 5'- CAC AGA GCT CTT GGC CGT CAC CAC ATT -3' This primer runs from 111-140 bp The area within the plasmid spanned by these primers can be seen in figure 7

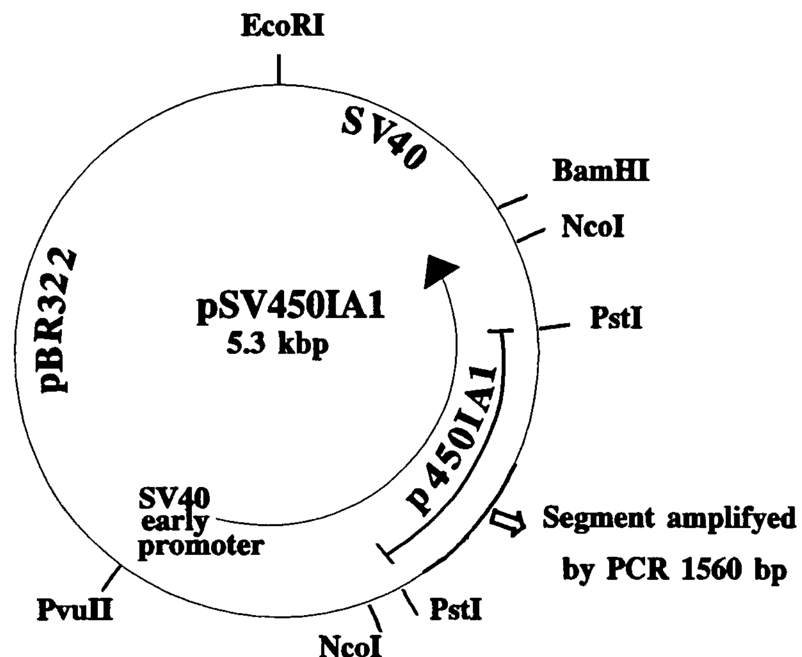


Figure 7 PSV450IA1 plasmid demonstrating primer positions

The primers were made by British Bio-technology Product Ltd, Oxon

2.14.1 Polymerase chain reaction (PCR):

All components were mixed gently in a sterile eppendorf with the Taq polymerase added last The eppendorf was then spun down at 5000 g for 30 sec and a drop of mineral oil added to prevent evaporation The eppendorf was then place on a Techne PCR machine and run at the following parameters 1) 94°C for 1 5 mins 2) 30 cycles of (94°C for 1 5 mins, 54°c for 1 min and 72°C for 3 mins) this was then followed by 72°cC for 7 mins The resulting amplified DNA run out on a 1% agarose minigel with EcoRI cut lambda size markers (as per secion 2 9)

COMPONENT	VOLUME μL	FINAL CONCENTRATION
$MgCl_2$ B free	5	1X
$MgCl_2$ (Promega A351)	3	1 5 mM
dNTP	16	0 4 mM
Primer 1	1	50 ng/ml
Primer 2	1	50 ng/ml
Control primer 1	1	50 ng/ml
Control primer 2	1	50 ng/ml
Plasmid DNA	10	1-2 μg
Sterile water	10	
Taq DNA polymerase (Promega M1861)	1	5 units

$MgCl_2$ Free buffer contained 500 mM KCL, 100 mM Tris-HCl pH 9, 1% triton X-100 (Promega M190A)

2.15 ENZYMOLOGY

All enzyme assays were carried out on cell homogenate Cells were grown to subconfluency and rinsed twice with PBS Cells were then trypsinized, rinsed once with PBS and resuspened in fresh PBS containing 2mM PMSF The cell suspension was then somcated as previously described (2 12 1) The direct sonicated homogenate was used in all assays The protein concentration was determined by BioRad protein reagent (BioRad 500-006) Enzyme blanks in all cases contain no substrate For time zero, a test-tube containing all the components of the assay was set up, the stopping reagent for each individual assay was added and the assay run as normal The addition of the stop reagent prevented any reaction from taking place The controls contained no cell homogenate

2.15.1 Spectrophotometric detection:

2.15.1.1 Aminopyrine *N*-demethylation

The N-demethylation of aminopyrine results in the stochiometric production of one molecule formaldehyde per molecule of aminopyrine which can be detected using the Hantszch reaction (Nash, 1953)

1) Reaction contained 1 0 ml of co-factor solution as follows 0 1 M Tris-HCl pH 7 4 -8 5 ml, 0 15 M $MgCl_2$/0 1 M semicarbazide- 1 0 ml, 0 5 M nicotinamide - 1 0 ml (BDH 44068), 40 mg glucose-6-phosphate(Sigma G-7879), 2 Units glucose-6-phosphate dehydrogenase, 8 mg $NADP^+$
2) 1 ml of co-factor solution was added to 0 5 ml 20 mM aminopyrine (Sigma D-8015) and pre-warm at 37°C for 2 mins
3) To start the reaction 0 5 ml of cell homogenate was added and incubated at 37°C for 30 mins
4) To terminate the reaction 0 5 ml 25% zinc sulphate (Sigma Z-4750) was added and the tubes placed on ice for 5 mins
5) 0 5 ml of saturated barium hydroxide (Sigma B-2507) was then added and the solution allowed to stand for 5 mins, then centrifuged for 5 mins at 1000 rpm
6) 1 ml of supernatant was then removed and added to 2ml Nash reagent (30g ammonium acetate (RdeH 32304), 0 4 ml acetylactone (Sigma A-3511), made up to 100 ml with water) followed by incubation at 6°C for 30 mins
7) The tubes were then cooled and read at 415 nm

Results were determined relative to a formaldehyde (BDH 10113) standard curve

2.15.1.2 Assay for the glucuronidation of 2-aminophenol.

This assay is based on the principle that a diazonium salt is formed on the addition of a sodium nitrite to an acidified solution of an aromatic amine This diazonums salt reacts with *N*-naphthyethylene diamine and produces an coloured azo compound, the concentration of which can be determined spectrophotometrically at 540 nm

1) 1 ml of co-factor solution (0 1 M Tris-HCl pH 8 0 - 8 0 ml, 0 15 $MgCl_2$ - 0 1 ml, 1% w/v Triton X-100 - 0 5 ml, 0 02 M Ascorbic acid 1 0 ml, 10 mg UDP-glucuromc acid (Sigma U-6751)) was added to 0 5 ml of 1 mM 2-aminophenol (BDH 26008) The reaction was initiated with 0 5 ml cell homogenate and incubated at 37°C for 30 mins with constant shaking

2) To stop the reaction 1 ml of ice-cold 20% (w/v) Trichloroacetic acetic (RdeH 27242) in 0 1 M phosphate buffer pH 2 7 was added and the tubes placed on ice for 5 mins

3) The resulting solution was centrifuged at 1000 rpm for 5 mins and 1 ml of the supernatant removed to 0 5 ml (fresh) 0 1% sodium nitrite and left to stand for 2 mins

4) To this 0 5 ml of 0 5% ammonium sulfamate was added and left to stand for a further 3 mins

5) 0 5 ml of 0 1% N-naphthylethylene diamine (Sigma N-9125) was then added and the solution left to stand at room temperature in the dark for 60 mins

6) The absorbance was read at 540 nm An aniline (BDH 27229) standard curve was used to determine the concentration of azo produced

2.15.1.3 Assay for Glutathione-S-transferase:

This assay is based on the conjugation of glutathione to 2,4-di-nitrochlorobenzene forming 2,4-dinitrophenyl-glutathione which absorbs light at 340 nm and has an extinction co-efficient of 9 6 mM^{-1} cm^{-1} (Gibson *et al*, 1987)

1) 2 cuvettes containing 0 1 ml 30 mM glutathione (Sigma G-4251), 0 1 ml of 30 mM dinitrochlorobenzene (in ethanol) and 2 2 ml of 100 mM potassium phosphate buffer pH 6 5 for 2 mins, were placed in the constant temperature chamber of a dual beam (Shimadzu UV 160A) spectrophotometer

2) 0 6 ml of water was added to the reference cuvette and 0 6 ml of cell homogenate to the sample cuvette

3) The reaction was then followed at 340 mm over 3 mins giving a change in absorbance per min which is related to conjugation by the extinction co-efficient

2.15.1.4 Determination of NADPH-cytochrome-c-reductase

This reaction is based on the reduction of the ferric cytochrome to the ferrous form which has a characteristic absorbance at 550 nm and an extinction co-efficient of 19 6 μmoles conjugated per min per mg of cell homogenate protein (Gibson *et al*, 1987)

1) 250 μl of ferric cytochrome c at 5mg/ml (Sigma C3006), 2 15 ml of 0 1 M Tris-HCl pH 7 4 and 0 1 ml of cell homogenate were added into 2 cuvette in a dual beam spectrophotometer (Shimatzo UV 160A)

2) 25 μl of 2% NADPH (Sigma N-1630) was added to start the reaction and the increase in absorbance recorded at 550 nm

2.15.1.5 Spectral determination of Cytochrome P450:

This procedure is based on the characteristic absorption spectrum of reduced, carbon monoxide (CO) complexed haem of cytochrome P450 which has an extinction co-efficient of 91mM-1cm^{-1}

1) 2 cuvettes were placed in the reference and sample compartments of a dual beam spectrophotometer 2 mls of cell homogenate diluted to 2 mg/ml in 0 1 M Tris-HCl pH 7 4 containing 20% v/v glycerol (which prevents the denaturation of P450 to 420 nm) was added and a base line recorded between 400 and 500 nm

2) A few grains of solid sodium dithionite were added to each cuvette to reduce the haem CO gas (IIG) was bubbled through the sample cuvette only for 1 min and the difference spectrum between 400 and 500 nm was recorded

The resulting difference spectrum looked like figure 2 15 1 5

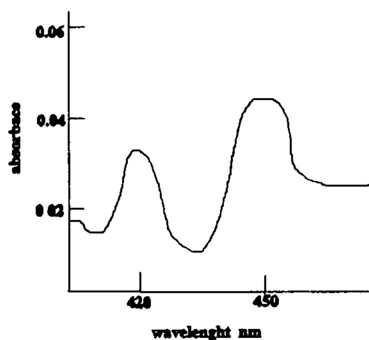


Figure 2.15.1.5: Difference spectrum of reduced Carbon monoxide cytochrome P450

2.15.2 FLUROMETRIC ASSAYS

2.15.2.1 Determination of UDP-glucuronlytransferase activity.

This method is based on the conjugation of 4-methylumbelliferone (Sigma M-1508) to 4-methylumbelliferyl-β,D-glucuronide (Sigma M-1930) which fluoresces at an excitation wavelenght of 315 nm and emits at a wavelenght of 365 nm in an alkiline pH (10 3) environment (Lilienblum *et al* , 1982)

1) Each reaction was made up as per table below UDPGA (UDP-glucuronic acid), 4-MU (4-methylumbeliferone) and pre-warmed to 37°C in a water bath

Component	Volume μl	Concentration
$MgCl_2$	100	25 mM
Tris-HCl pH 7 4	125	0 4 M
Brij	100	0 25%
UDPGA	100	3 mM
4-MU	25	10 mM
Cell Homogenate	50	

2) The reaction was started by the addition of UDP-GA (Sigma U6751) and incubated at 37°C for 30 mins
3) To stop the reaction 0 5 ml 0 5 M perchloric acid was added and the sample extracted with 2 ml chloroform
4) Each tube was then centrifuge at 1000 rpm for 2 mins 1 ml of the resulting aqueous phase was removed into 2 ml of 1 6 M glycine/NaOH pH 10 3
5) The samples were read in a four sided clear cuvette at excitation 315 nm and emission 365 nm in a Shimadzu LD50 fluorometer
6) The standard curve consisted of 4-methyl β,D-glucuronide in 1 6 M glycine/NaOH pH 10 3

2.15.2.2 Detection of O-deethylation of 7-ethoxycoumarin.

This method is based on the O-deethylation of 7-ethoxycoumarin (Sigma E1379) to umbelliferone (Sigma U7626) which has a characteristic fluorescence at 456 nm when excited at 368 nm (Lilienblum *et al* , 1992)

1) Each reaction was set up as below and pre-incubated at 37°C for 2 mins in a water bath

Component	Volume μl	Concentration
$MgCl_2$	100	50 μmol/ml
NADPH	50	10 μmol/ml
NADH (Sigma N8129)	50	10 μmol/ml
BSA (Sigma A2153)	100	10 mg/ml
Phosphate buffer pH 7 2	150	260 μmol/ml
Cell homogenate	50	
7-EC	25	40 μmol/ml

2) The reaction was started by the addition of 7-EC (7-ethoxycoumarin in acetone)

3) To stop the reaction 65 μl ice cold 15% TCA (trichloroacetic acid RdeH 27242) and 1 5 ml chloroform were added and mixed by vortexing at 37°C for 10 mins

4) The samples were then centrifuged at 1000 rpm for 5 mins 1 ml of the organic layer was removed to 2 5 ml 0 01 NaOH/1 M NaCl and mixed at 37°C for 10 mins

5) The fluorescence of the aqueous layer was then read in a four sided cuvette in a fluorimeter at excitation 368 nm and emission 456 nm,

6) The standard curve was composed of umbelliferone in 0 01 NaOH/1 M NaCl

2.15.2.3 Determination of Aryl Hydrocarbon Hydroxylase activity.

The assay is based on the hydroxylation of benzo[a]pyrene (Sigma B1760) at the 3 position which produces 3-hydroxy-benzo[a]pyrene which in a basic environment fluoresces at 522 nm, when subjected to excitation at 396 nm (Nebert and Gelboin, 1968)

1) Each reaction contained the following components m the following volumes The test tubes were pre warmed for 2 mins at 37°C is a water bath The reaction was started by the addition of benzo[a]pyrene in DMSO

Component	Volume μl	Concentration
NADPH	50	10 μmol/ml
$MgCl_2$	50	60 μmol/ml
Tris-HCl pH 7 5	340	147 μmol/ml
B[a]P	25	100 nmol/ml
Cell homogenate	50	

2) The reaction was terminated by the addition of 0 5 ml of ice cold acetone (BDH 10003), followed by the addition of 1 6 ml hexane (BDH 4368) The samples were then vortexed and placed in a 37°C water bath for 10 mins

3) Each tube was centrifuged at 1000 rpm for 5 mins and 0 5 ml of the organic phase removed

4) 0 5 ml organic phase was mixed with 2 5 ml of 1 N NaOH and the resulting fluorescence read at an excitation at 396 nm and emission at 522 nm in a four sided clear glass cuvette

5) 3-Hydroxybenz[a]pyrene (available from the NIC Chemical Repository, Midwest research Inst Kansas, Missouri) in 1 NaOH served as the standard

2.15.2.4 Inhibition of AHH enzyme activity by antibody addition.

To determine the effect of antibodies directed against componants of cytochrome P450 on the activity of AHH Antibodies were supplied by Oxygene (Oxygene Dallas Western Blot Kit, 800-446-9943) to rat CYPIA1 (001A1-P-RT (polyclonal)), rat CYPIIB1 (002B1-P-RT (polyclonal) and rat NADPH cytochrome P450 reductase (Reduc-P-RT (polyclonal) The antibody was added to the AHH assay components, at the required concentration (*i e* 25, 50 and 100 μg/ml final concentration in the assay) before prewarming to 37°C and placed on ice for 30 mins (Wiebel *et al*, 1984) After this time had elapsed, the assay was carried out as per 2 15 2 3

2.16 PHOTOGRAPHY.

2.16.1 Photomicrography.

1) The camera was loaded with the appropriate film and the ASA set to the appropriate number according to the films specifications
2) A halogen lamp and a green filter were used for black and white photography A halogen lamp and a blue ND10 filter were used for colour photograph
3) The microscope was adjusted to ensure that there was no vibration which would interfere with the picture
4) The shot was focused through the camera, switching the microscope to camera and increasing the light to 6V (in the green range) A range of exposures were used on each occasion to ensure a good photograph

2.16.2 Fluorescence photomicrography.

1) The mercury lamp was switched on 15 mins before use
2) The Heochst B2 filter was used for FITC staining
3) The shot was focused using the halogen lamp, then turned off and the mercury lamp shutter opened
4) The photograph was taken as per section 2 16 1
5) Fluorescent photographs in this thesis were taken using a UFX photomicrograph attachment with a photomultiplier which is automatic giving clear intense shots

2.16.3 Black and white film processing.

In this thesis Kodak - TriX-pan film was used for black and white photography and processed as follows

1) The film was removed in total darkness from its container and wound onto the reel of the developing tank
2) The developing tank was secured ensuing that it was light sealed and 300 mls of D-79 (Kodak dilute stock 1 1 with tap water) was added for 11 mins
3) To stop the developing the D-76 was removed and tap water added
4) The tank was then emptied and 300 mls of Kodak unifix liquid at 1 3 dilution of stock was added to the developing tank to fix the film for 11 mins in the dark
5) The film was then rinsed in Kodak Photoflo 1 capful diluted to 300 mls in tap water and air dried

2.16.3 Paper processing

Using the film developed in the section 2 15 2

1) The chosen negative was placed in the enlarger and the lights switched off
2) The negative was focoused by adjusting the lens in the enlarger (Meopta) with the aperture fully open to ensure maximum light
3) When the image produced was at the desired size and focus, the light were switched to red - safe light

4) A test strip of photographic paper was placed over the image and covered with a piece of card The enlarger was switched on and the paper exposed in 1 second time units, piece by piece resulting in a gradient of exposure
5) The enlarger was switched off and the paper placed in the developer bath (Kodak Dektol Stock diluted 1 2 with tap water) for 1 min
6) This test strip was placed in a stop bath containing tap water to stop the developing
7) The film was then fixed in Kodak unifix stock diluted 1 7 with tap water for 2-5 mins
8) The paper was rinsed in water containing photoflo for at least 10 mins
9) Prints were dried in a print drier at medium heat

The resulting gradient indicated the correct length of exposure required to achieve the best prints from the negative

If the gradient was too dark, the aperture was decreased step by step, testing each time, in the above manner for the best exposure

Once the correct parameters had been chosen, the complete negative was enlarged using this procedure replacing step 4 with one complete exposure at the chosen time and aperture size The paper was then processed as in steps 5-9

2.17. Safety.

In this body of work a number of hazardous chemicals were used In all instances when chemicals were ordered, the company's data sheet was also requested which contained their recommendations for laboratory use and disposal

All solvents were stored in a fire-proof cabinet and used only in the fume hood Solvent waste was stored in a cool, dark, isolated building and removed by Minchem , Dún Laoghaire

All hazardous chemicals were stored in a locked cupboard.

The lowest possible concentrations of chemicals were used and inital dilutions of hazardous chemicals were carried out m the fume-hood Highly toxic chemicals *e g* PAH - benzo[a]pyrene, benzanthracene and ethidium bromide, were dealt with individually The quantities of PAH waste were recorded, stored m isolation and all articles which came in contact with them were disposed of by incineration by Bioburn

When working with ethidium bromide a white coat and double gloves were worn, and the work area covered with a disposable bench top Ethidium bromide waste was isolated and either incinerated by Bioburn, or treated with hyperchlorite which is believed to deactivate it (Sambrook *et al* , 1989)

General cell culture waste which contained FCS, medium which had been in contact with cells, articles which came in contact with these solutions, and medium which contained anticancer drugs *e g* adriamycin, VP-16 *etc* , were autoclaved at 121°C and 21 lb per cm^2 pressure for 20 mins, to render them safe When working with ultra violet light, a protective face-visor was worn together with a closed, long sleeved white coat and disposable gloves to prevent exposure to the light

During routine work in the laboratory a white coat and gloves were worn

SECTION 3.0 RESULTS.

3.1 PRIMARY CULTURE.

93 human tissue samples were processed for primary culture as described in section 2 The results are summarized in figure 8 As can be seen, failure to establish cell lines from these tissue samples was due to the total lack of attachment (66%), lack of further growth following attachment (30%), fibroblast overgrowth after initial attachment (64%) or differentiated after attachment (24%)

SURGICAL PROCEDURE	DESCRIPTION OF SAMPLES
LOBECTOMY	-where lobe of lung is removed, sample is tumour
BRONCHOSCOPY	-investigation of the inside of bronchus using a bronchoscope - samples were small biopsies of bronchial tissue
MEDIASTINOSCOPY	-(MS) the mass of tissue which separates the sternum in the front and the vertebral column behind and contains the heart and blood vessels, trachea, oesophagus, thymus, lymph nodes - samples were usually lymph nodes
SARCOID	-a highly malignant form derived from connective tissue which shows rapid metastasis to the lymph system - sample was lymph node taken at Mediastinoscopy
OESOPHOAGEAL	-tumour derived from oesophageal tissue - sample was from at bronchoscopy
OAT CELL CARCINOMA	-small cell carcinoma - grows in suspension in culture
PLEURAL FLUID	- Fluid taken from the pleural cavity which contains malignant epithelial cells
HODGKINS LYMPHOMA	- enlargement of the lymph nodes and associated tissue - samples were lymph nodes taken at MS

Table 1: Surgical procedures from which the samples (Figure 8) used for primary culture were obtained

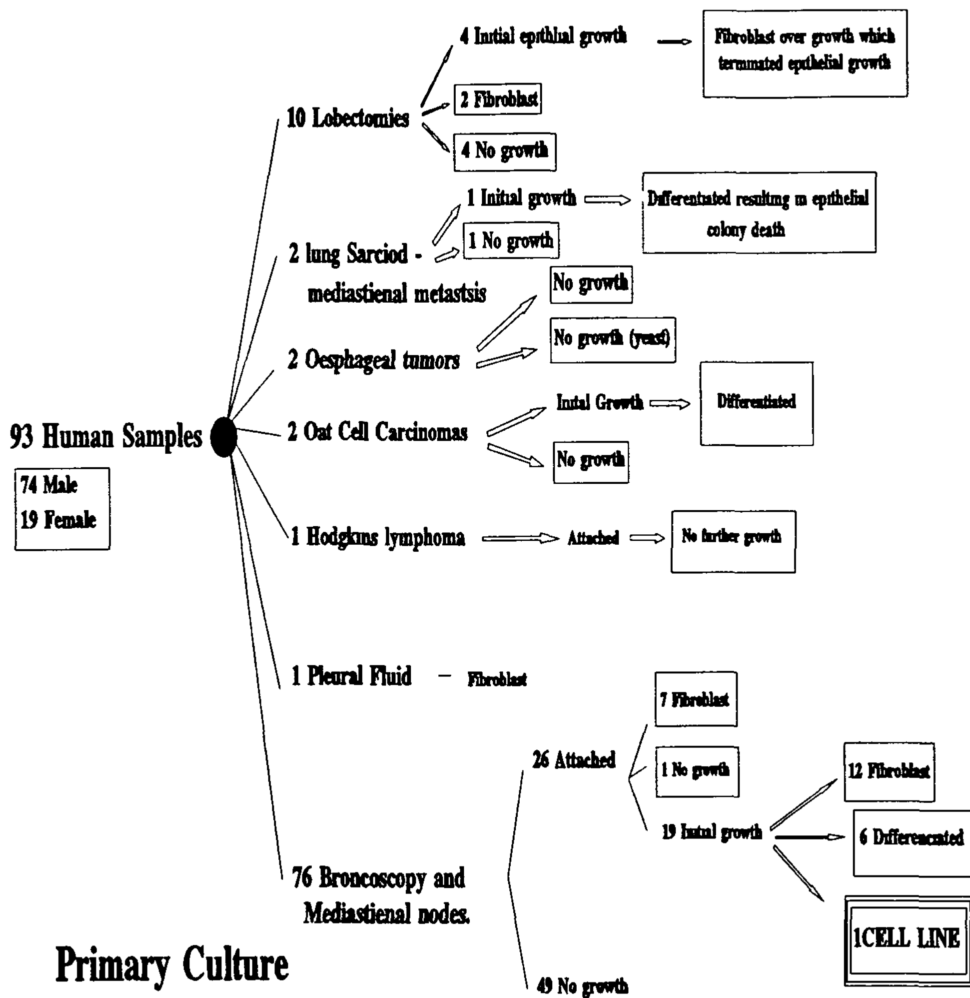


Figure 8: Results of growth progress of samples received.

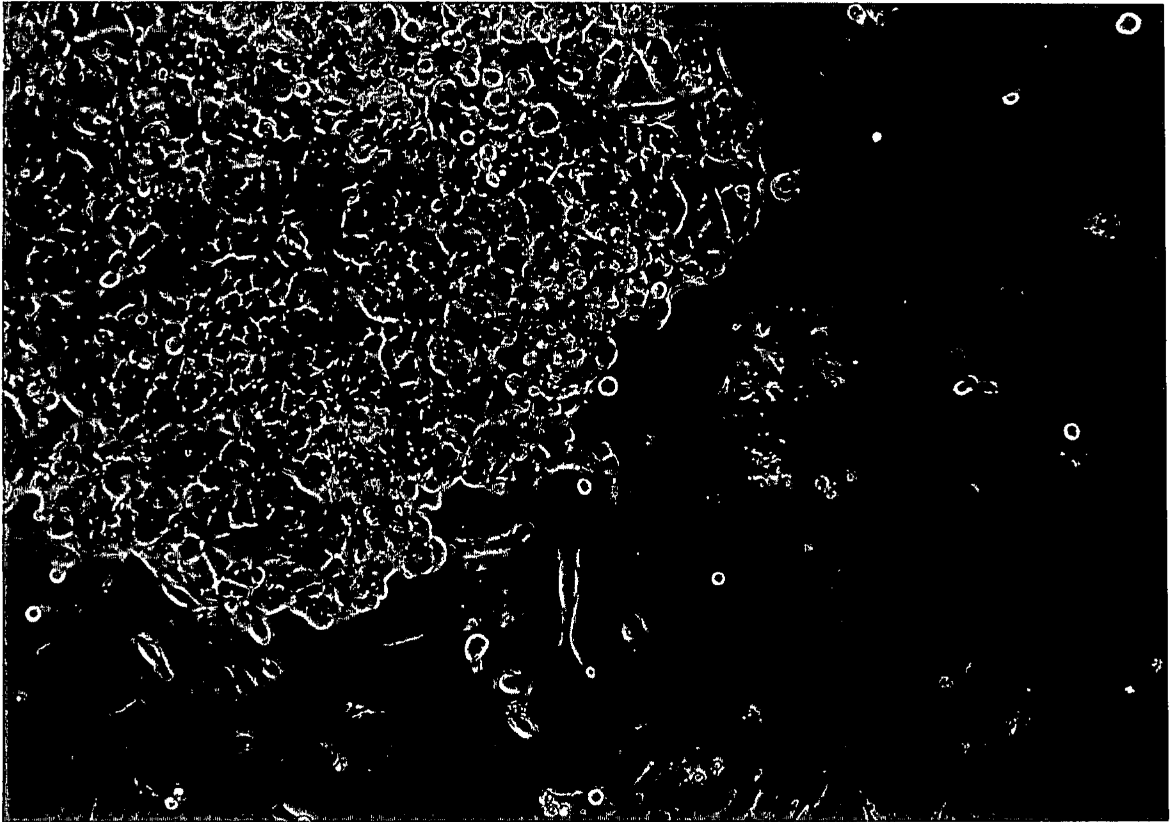


Figure 9: Primary culture of a secondary tumour of the lung, from a 52 year old male patient Derived from mediastinal node biopsy The sample displayed initial growth as shown, note fibroblast growth approaching This sample eventually differentiated and was engulfed by fibroblast cells

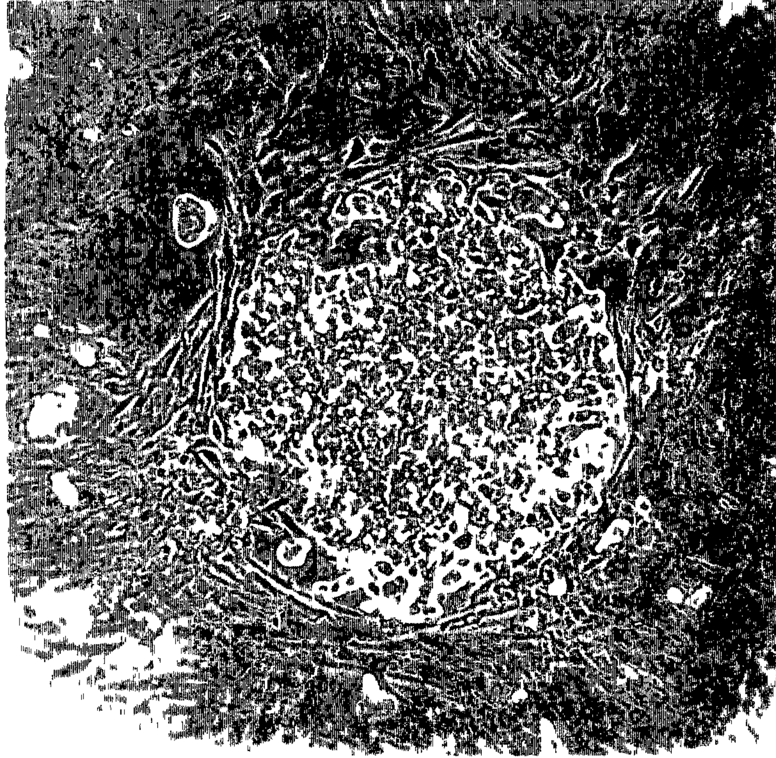
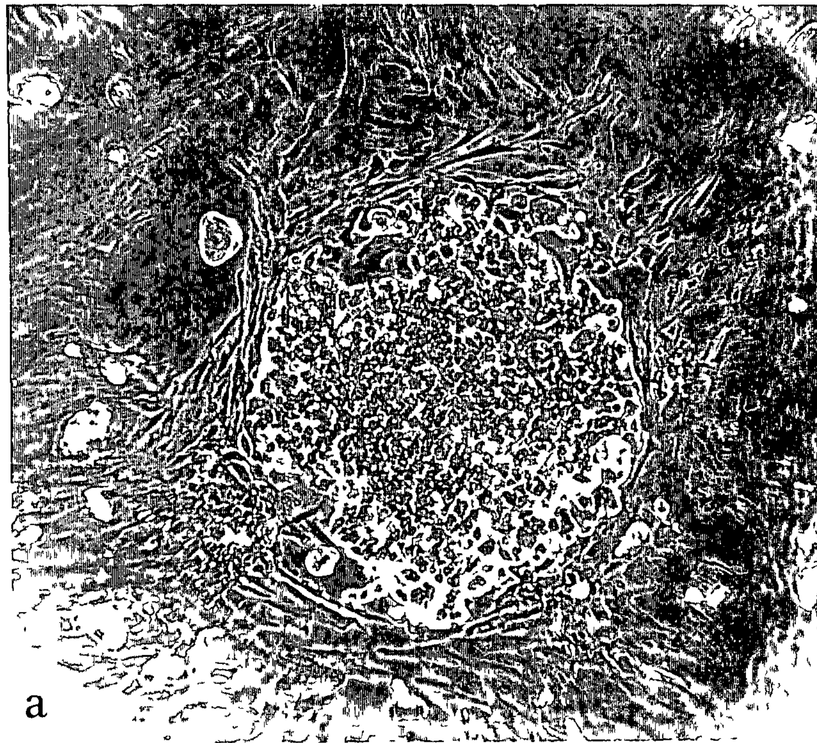


Figure 10: Explant of primary lung carcinoma showing good initial growth, but surrounded by fibroblast cells Once again this sample eventually became overwhelmed by the fibroblasts



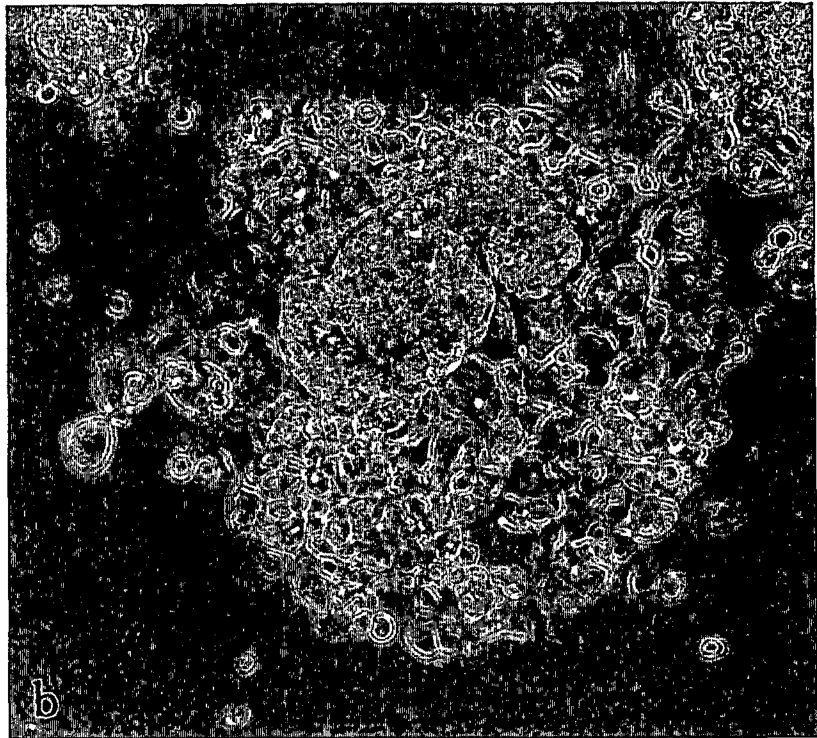


Figure 11: Time course displaying differential trypsinization using trypsin/EDTA (see section 2 1 1) to eliminate contaminating fibroblasts from a primary culture derived from a lobectomy a) demonstrates contamination by fibroblasts which was engulfing the epithelial cells preventing further growth of the colony b) The colony after treatment with trypsin/EDTA (section 2 1 1), note the disappearance of the fibroblast

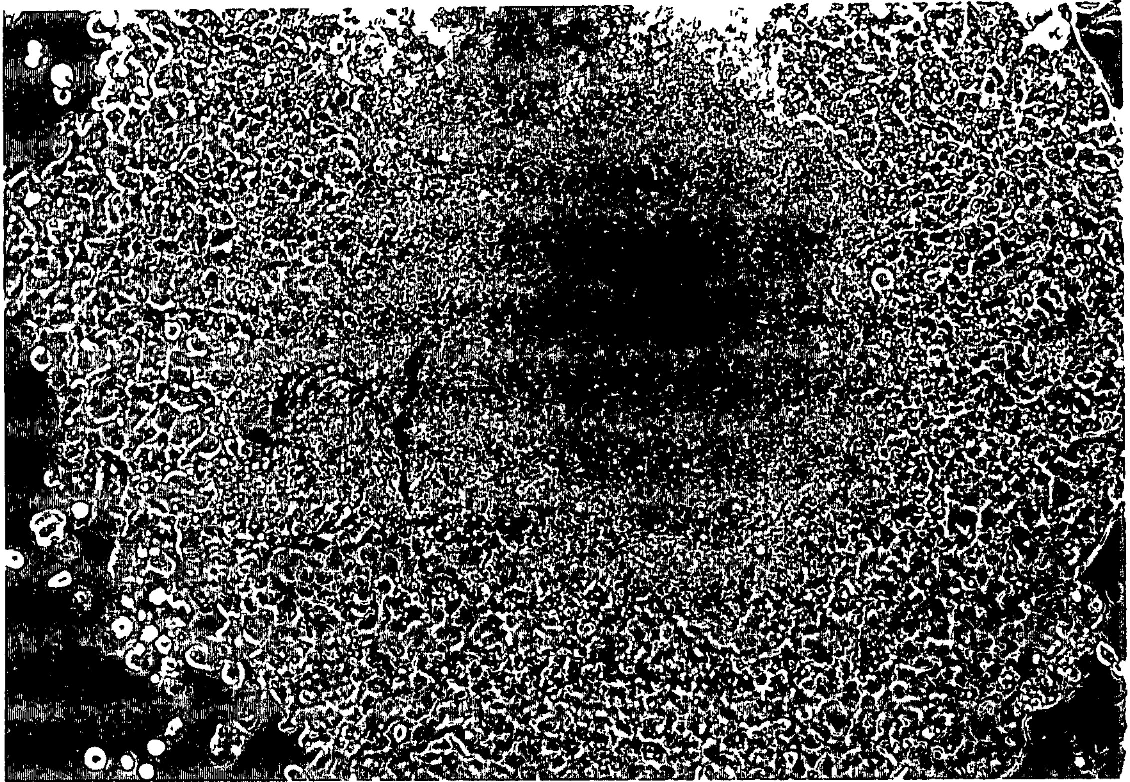


Figure 12: Primary explant of Mediastinal Nodes after 5 days in Culture

3.1.1 ESTABLISHMENT OF DLKP

3.1.1.1 CLINICAL HISTORY

Biopsies of tumour were taken from a male patient, aged 52 years with a history of smoking Samples were taken from the mediastinum and a lymph node metastasis Patient presented with possible poorly differentiated squamous cell Carcinoma of the Lung and had received no prior treatment

Day 1: The sample was a large intact piece of tumour with no viable necrotic tissue, or red blood cell contamination Sample was set up as per methods *i e* mechanically using scalpels and enzymatically using a combination of 200 units collagenase/2% dispase in PBS Four 25cm^2 were seeded with the dissection media on day 1 To test the effect of initial cell density on growth, two flasks each at the following densities 10^6 cells, 5 x 10^5 cells, 2 5 x 10^5 cells were used

Day 4: in culture, most of the cells had attached in each flask, but were not actively dividing One flask was changed from DME/Hams F12 with 10% FCS to MEM with 10% FCS to see if decreasing the nutrient level would encourage the cells to divide

Day 7: The flasks which were seeded at lower than 5 x 10^5 cell have shown no growth indicating that the cell density is too low to support growth at this early stage in establishment

Day 10: All flasks with initial cell densities greater than 5 x 10^5 cell had begun to grow *i e* those flasks seeded at 10^6 cells/flask

By week 10 this sample had been passaged 12 times and could be designated a cell line (Figure 13) To establish the characteristics of these cells the following experiments were carried out

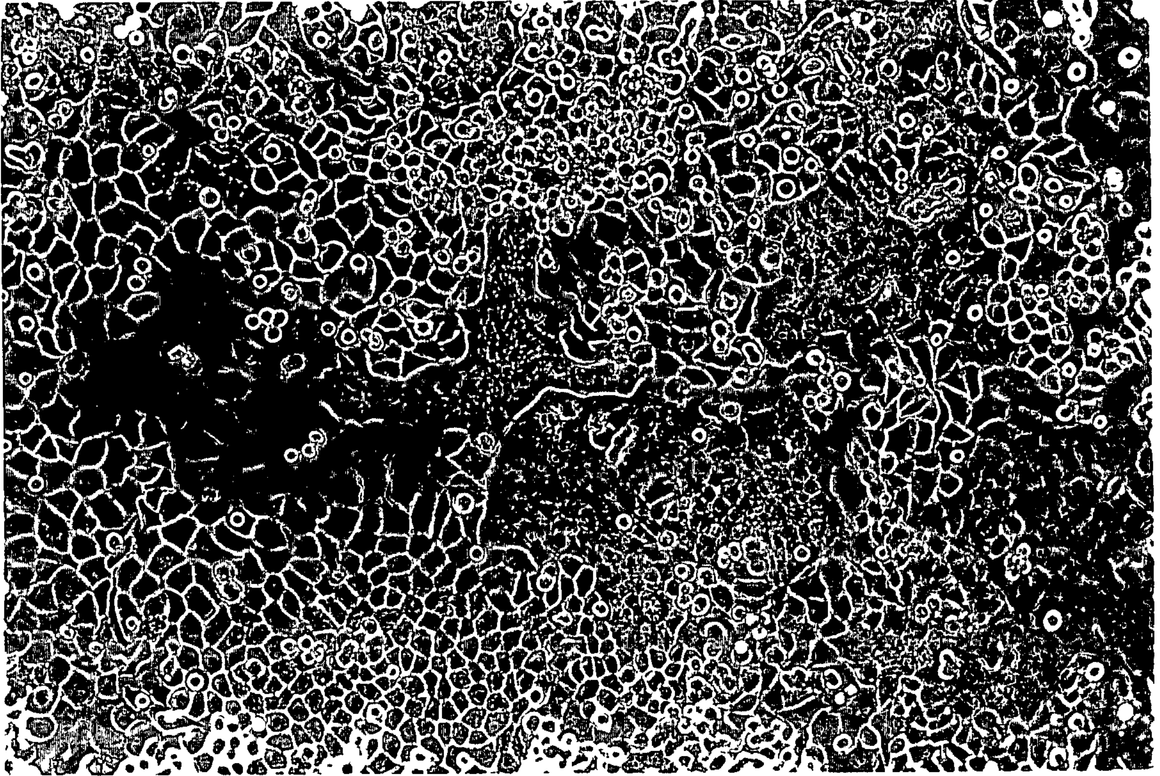


Figure 13: P_0 DLKP Note different cell type populations apparent

3.1.1.2 IMMUNOFLUORESCENCE

The immunofluorescence staining pattern of DLKP cell line was investigated using two mouse IgG2Ak monoclonal antibodies 704A1, 703D4 (Mulshine *et al*, 1983) These antibodies are reported to distinguish between small cell lung carcinoma and non-small cell lung carcinoma To investigate the epithelial nature of the cells an IgM antibody Ep-16, a monoclonal antibody which recognises a surface antigen on epithelial cells (Hamburger *et al*, 1985), and anti-cytokeratin Pan and N° 18 which binds to the keratin intermediary filaments of epithelial cells, were used

The 704A1 and 703D4 antibodies bind to most human non-small cell lung cancer i e adenocarcinoma, epidermoids and large cell, but not to small cell cancer tumours These antibodies recognize human melanomas, osteogenic sarcoma, renal cell carcinoma but not many other human tumours e g breast, colon, neuroblastoma, lymphoid or normal tissue
The small cell lung carcinoma line HTB-120 and MRC-5 the human fibroblast cell line were used as negative controls due to their non large cell characteristics, Figures 19 & 16 respectively Figures 15 & 18 illustrate DLKP's positive staining with 704A1 and 703D4 respectively Figure 14 is the positive control for 704A1 - squamous cell carcinoma of the lung SKMES-1, figure 17 is the positive control for 703D4 - adenocarcinoma of the lung SKLU1

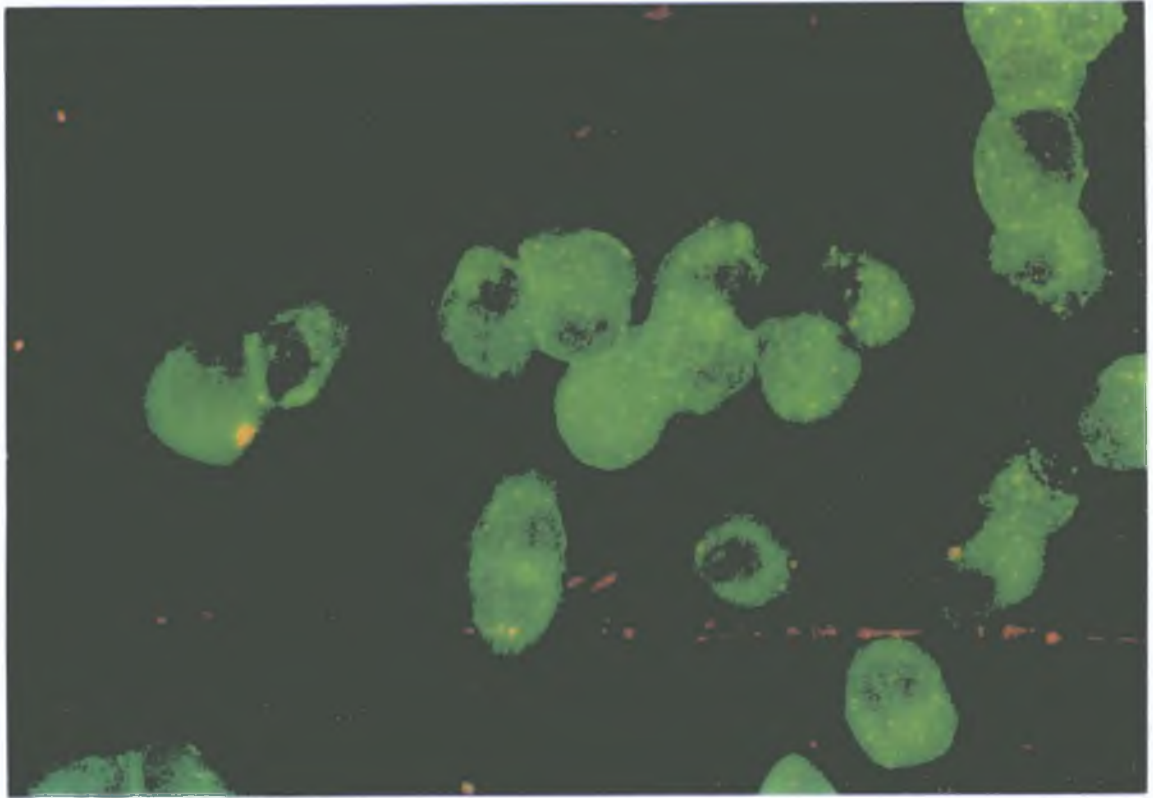


Figure 14: SKMES-1 Primary antibody 704A1, secondary antibody FITC. Positive for non small cell lung cell.

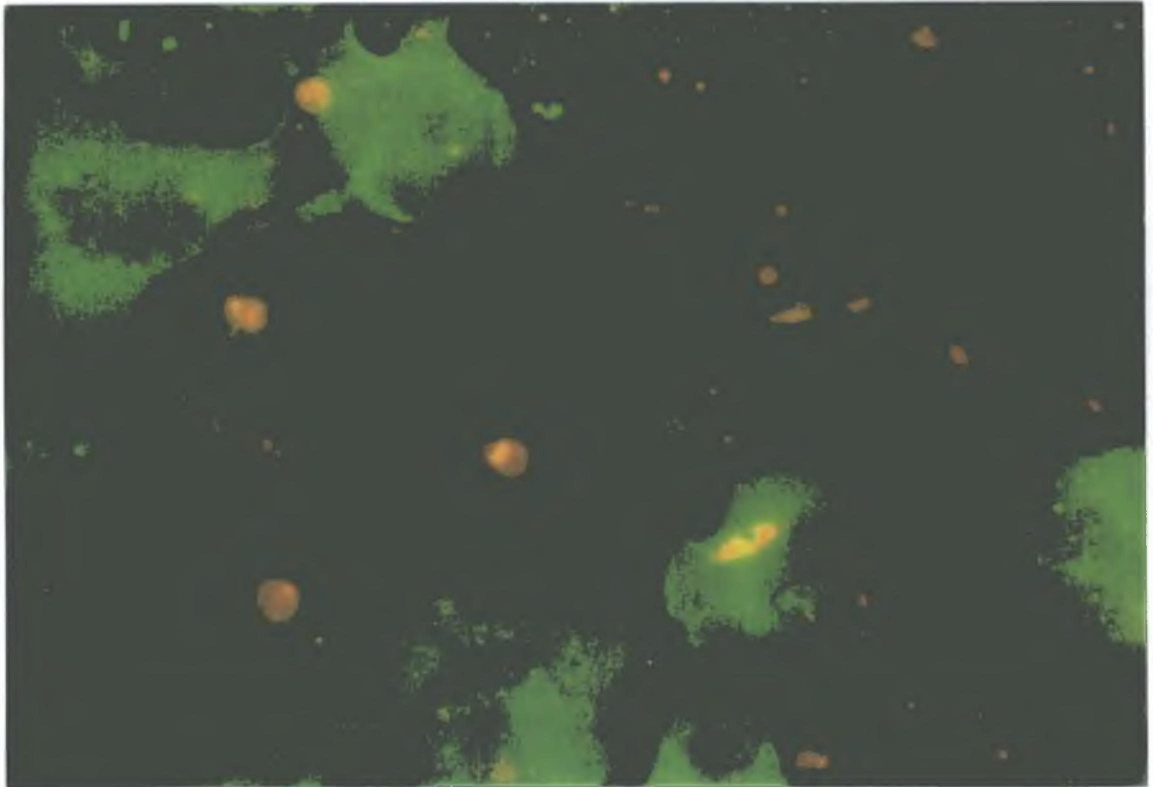


Figure 15: DLKP Primary antibody 704A1, secondary antibody FITC. Positive for non small cell lung cell.

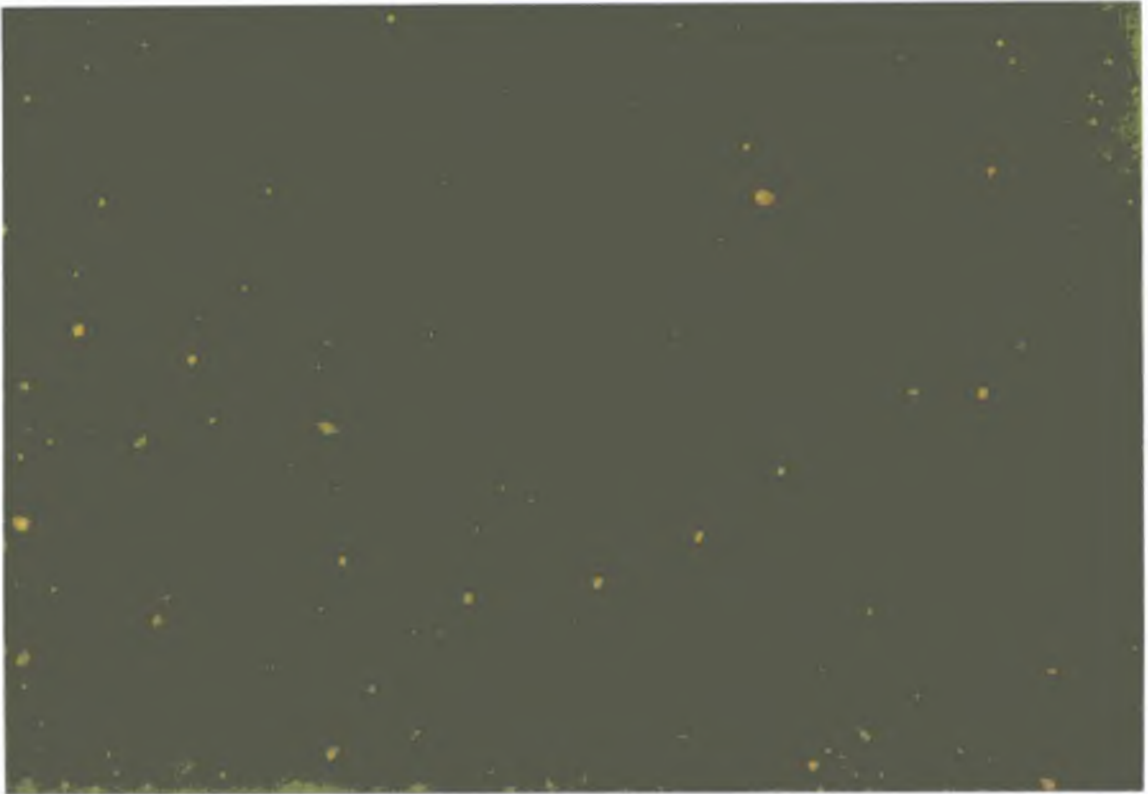


Figure 16: MRC-5 Primary antibody 704A1, secondary antibody FITC. Negative for non small cell lung line.

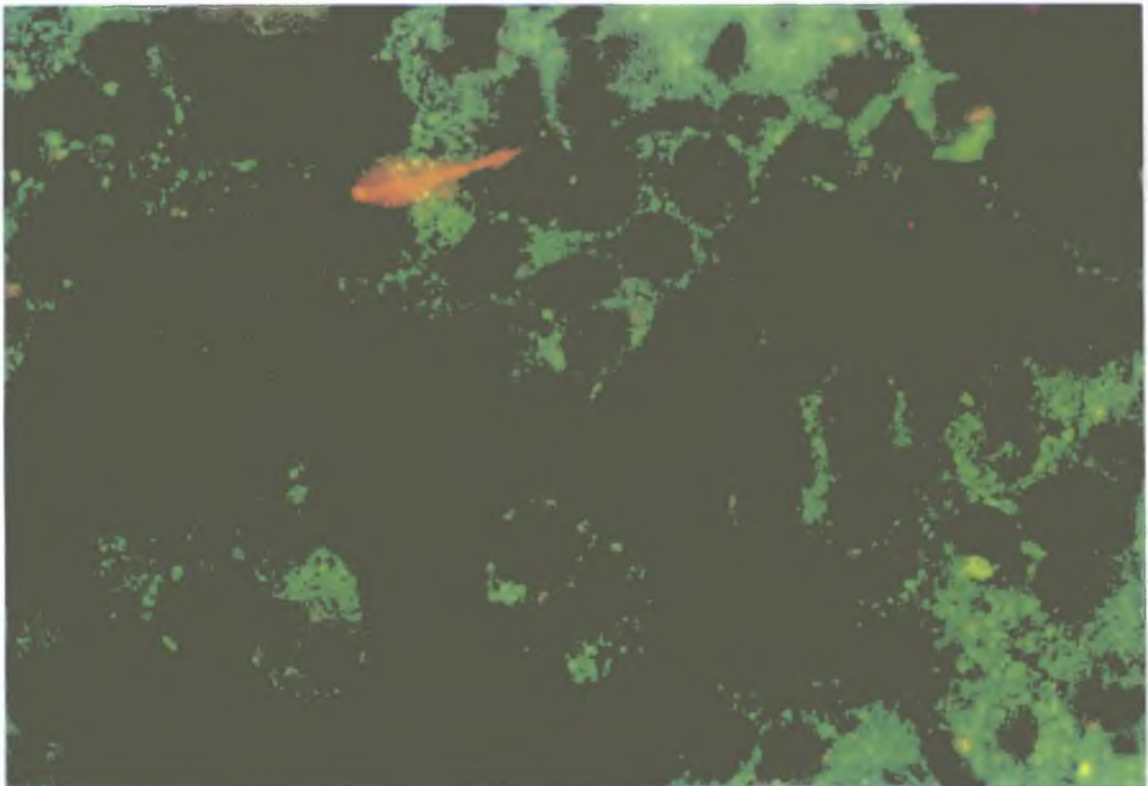


Figure 17: SKLU1 Primary antibody 703D4, secondary antibody FITC. Positive for non small cell lung cell.

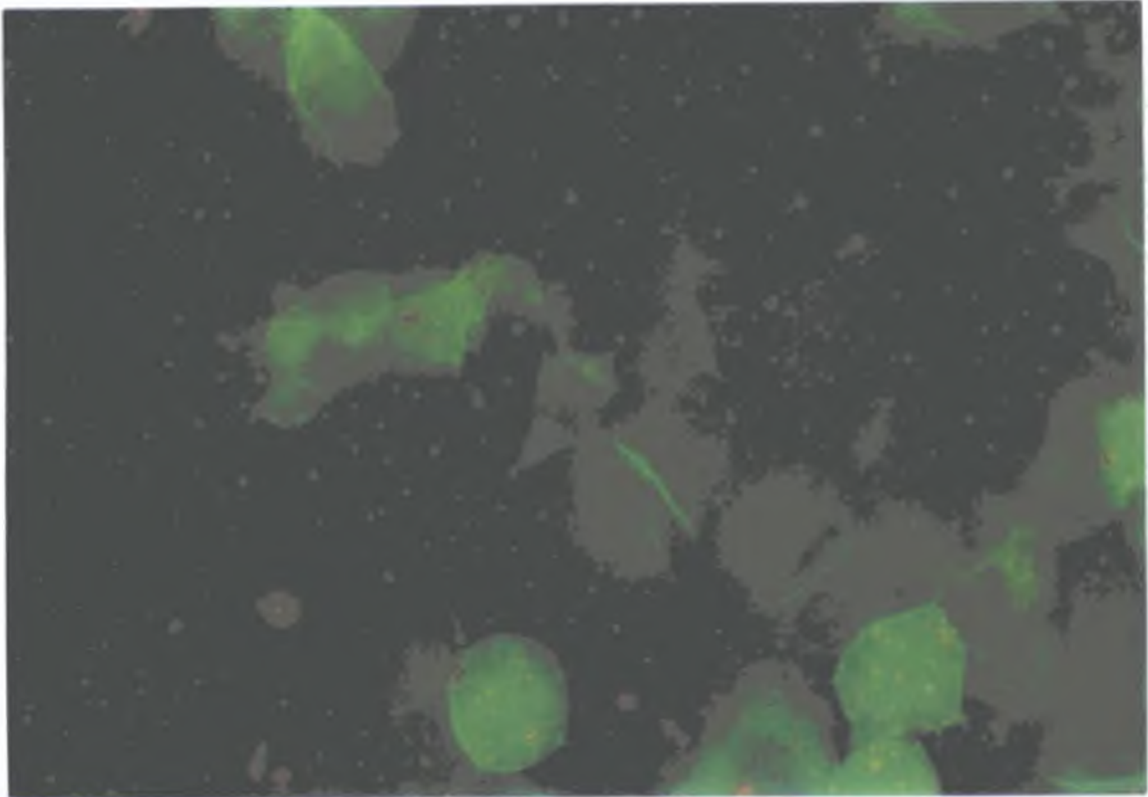


Figure 18: DLKP Primary antibody 703D4, secondary antibody FITC. Positive for non small cell carcinoma cell.

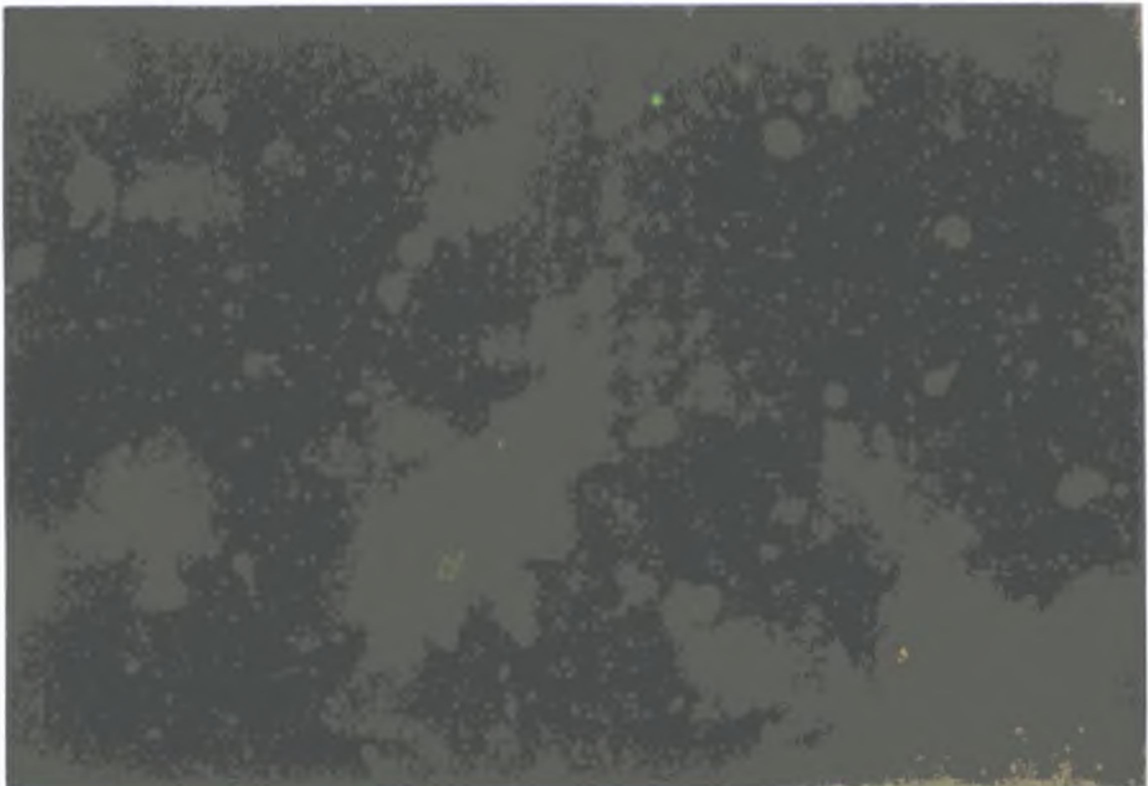


Figure 19: HTB-120 Primary antibody 703D4, secondary antibody, negative for non small cell lung line.

Ep-16 monoclonal antibody is directed against a specific membrane protein on the surface of epithelial cells figure 20 displays the positive control SCC-9 - squamous cell carcinoma of the lung. Figure 21 shows DLKP staining with Ep-16 which in comparison with figure 22 - MRC-5 (human foetal normal lung fibroblast line which is the negative control) DLKP is slightly positive.

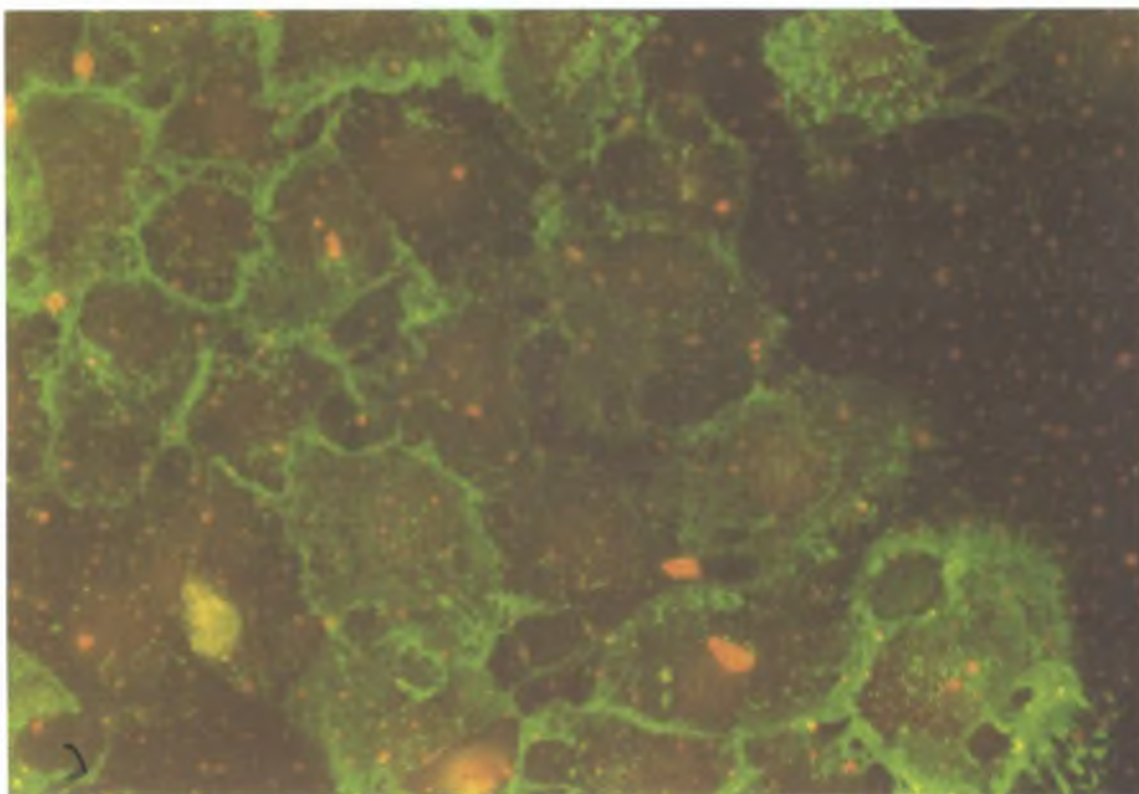


Figure 20: SCC-9 Primary antibody EP16, secondary antibody FITC. Positive for epithelial cell line.

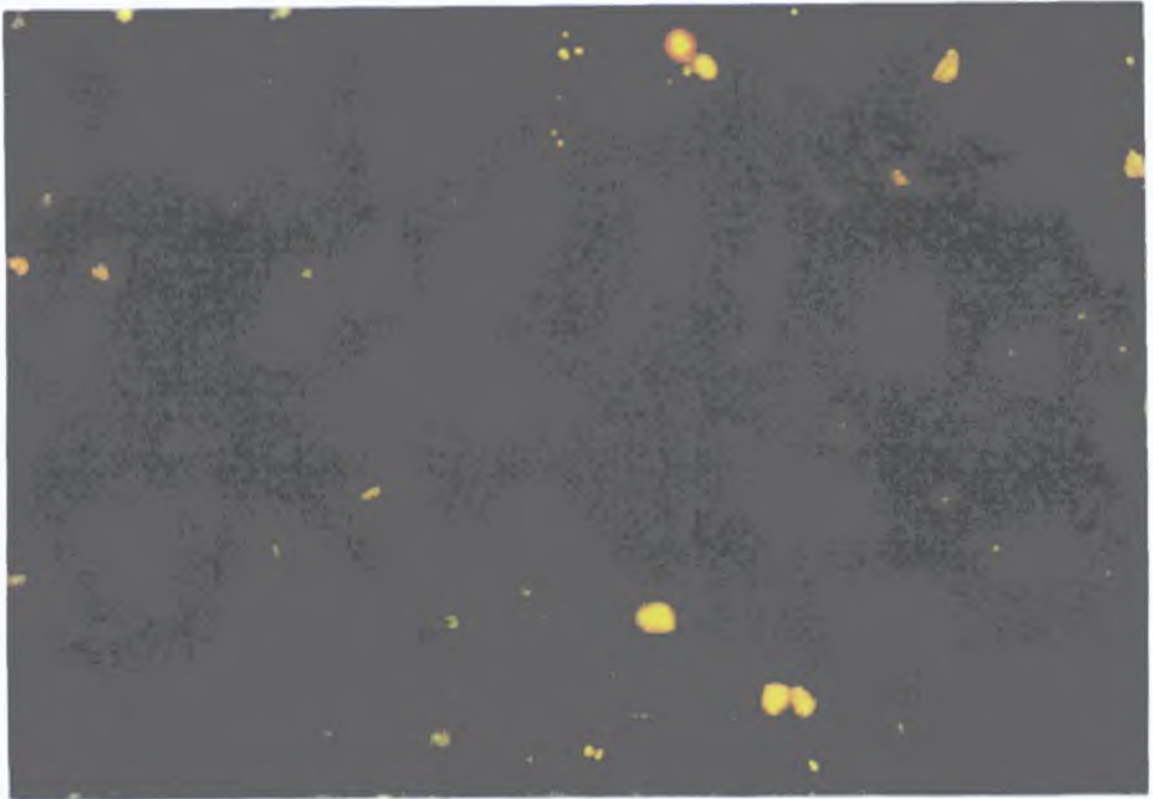


Figure 21: DLKP Primary antibody EP16, secondary antibody FITC. Weakly positive for epithelial surface antigen - possible due to poor differentiation.

Figure 22: MRC-5 Primary antibody EP16, secondary antibody FITC. Negative for epithelial surface antigen.

Anti-cytokeratin is directed against a combination of the intermediate filaments in anti-cytokeratin Pan and the specific cytokeratin No. 18. Figure 23 is SCC-9 the positive control for this anti-cytokeratin antibody. Figure 25 shows the fluorescence of DLKP with anti-cytokeratin N° 18. In comparison with Figure 24 MRC-5 the anti-cytokeratin negative control DLKP appears to be negative.

Figure 23: SCC-9 Primary antibody anti-cytokeratin, secondary antibody FITC. Positive for keratin N° 18.

Figure 24: MRC-5 primary antibody anti-cytokeratin, secondary antibody FITC. Negative for keratin- negative control.

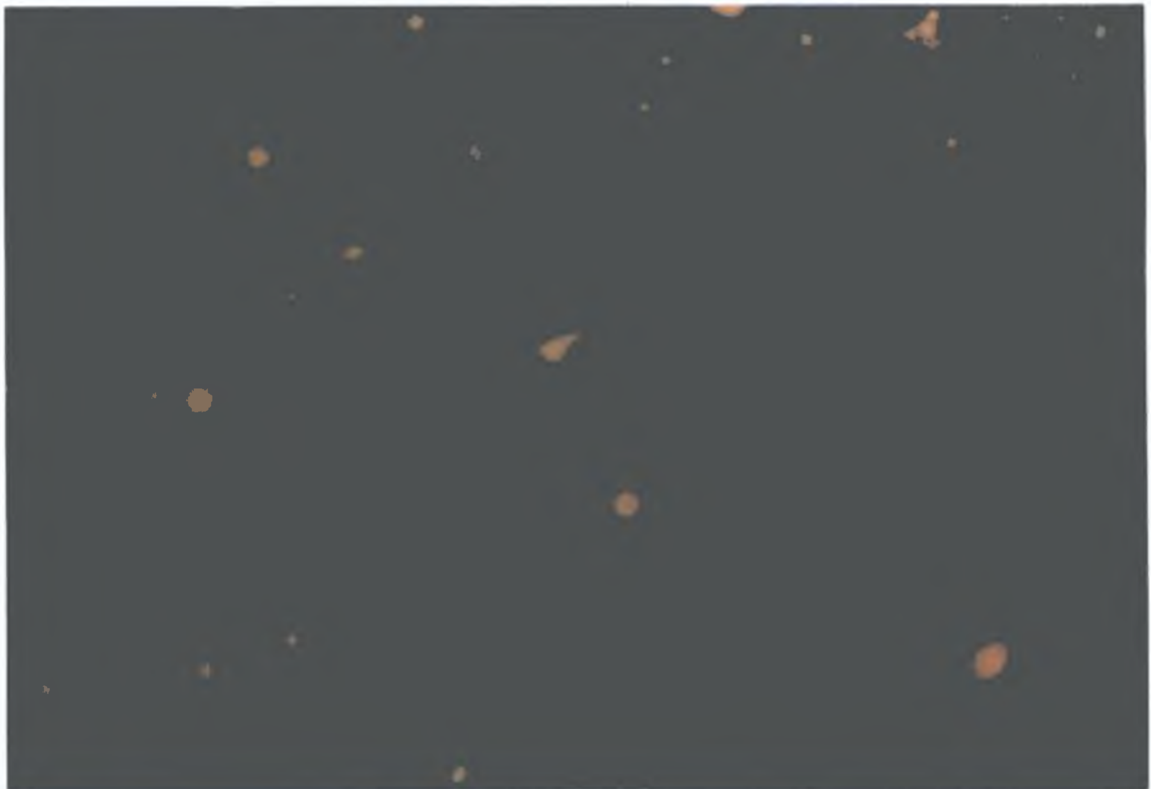


Figure 25: DLKP primary antibody anti-cytokeratin, secondary antibody FITC. Result: negative - also possibly due to their poor differentiation state.

3.1.1.3 ISOENZYME ANALYSIS

Isoenzyme patterns were determined using the Authentikit system available from Corning medical and Scientific, Corning Limited, Halstead, Essex, England Enzymes used in the studied were as follows, lactate dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase, peptidase B and nucleoside phosphorylase The method used to differentiate between species using this procedure, was distance migrated from the central origin The cell lines used for comparison were NRK (normal rat kidney), RPMI (human nasal carcinoma), CALU-3 (human adenocarcinoma), HTB-120 (human small cell carcinoma)

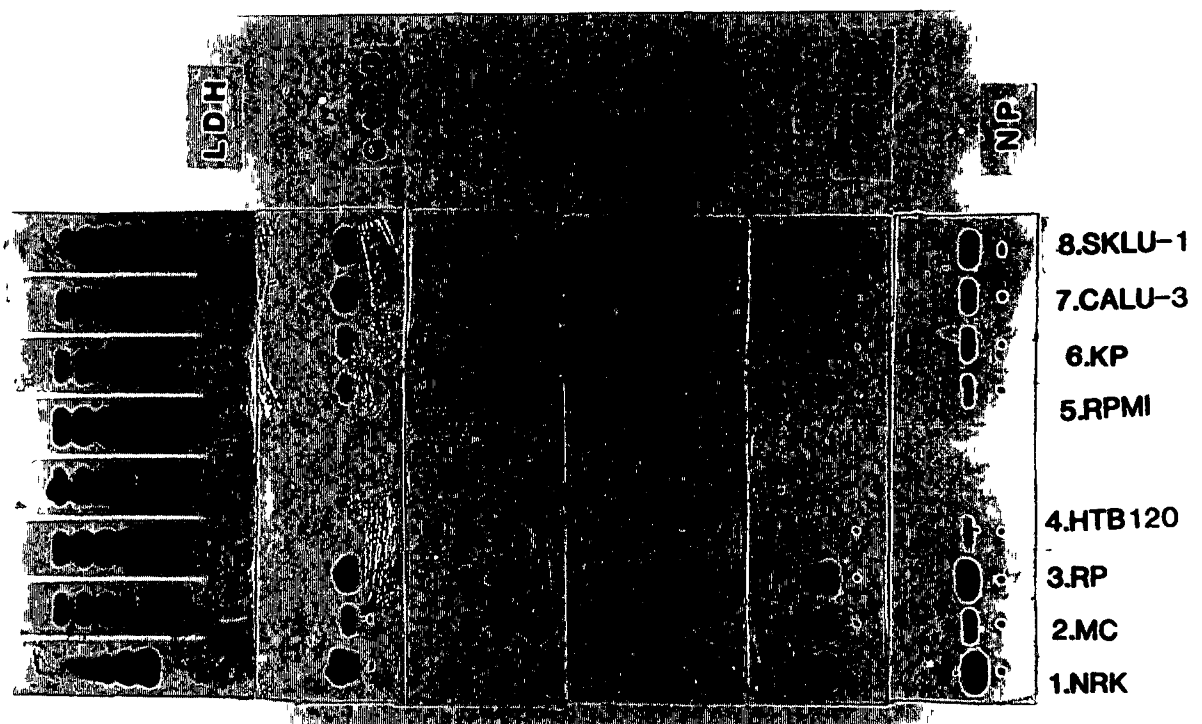


Figure 26: Isoenzyme analysis of DLKP in comparison with other human and nonhuman cell lines to determine species

3.1.1.4 DNA FINGERPRINTING

DLKP was analyzed by DNA fingerprinting to establish its genetic distinction from other cell lines and its genetic identity with the tumour from which it was established The DNA fingerprinting was carried out by Cellmark diagnostics, Abingdon, Oxforshire DNA from DLKP and the original tumour were restricted and resolved by electrophoresis and blotted The blots were exposed to Mix A which contained a cocktail of 3 single locus probes and in the Mix B containing two single locus probes The results were as figure 27a and b

In the report which was received with the autoradiographs from Cellmark it was pointed out that the bands in the tumour sample were not very clear due to the fact that the sample had degraded to a large extent In Mix B three strong bands were visualised and matched in the DLKP extract Mix A is not as clear and demonstrates a high molecular weight band in the DLKP sample which is not present in the tumour However, this may be explained by the fact that tumour sample was quite degraded on arrival at Cellmark and that high molecular weight alleles are degraded preferentially in DNA samples Mix A and B both displayed three band of low molecular weight in the tumour sample, two of which are also visible in the DLKP extract It was decided that the possibility of 9 bands matching purely by chance between the cell line and the tumour samples were very large and that the tumour and cell line DLKP matched

Note In order to show all the minor bands present in the DLKP lanes (figure 27 a+b) it would have been necessary to overdevelop the photograph In this case it would not have been possible to see any of the bands present in the tumour lanes The photograph of the autoradiographs presented is a compromise showing as many bands from both tumour and cell line samples as possible

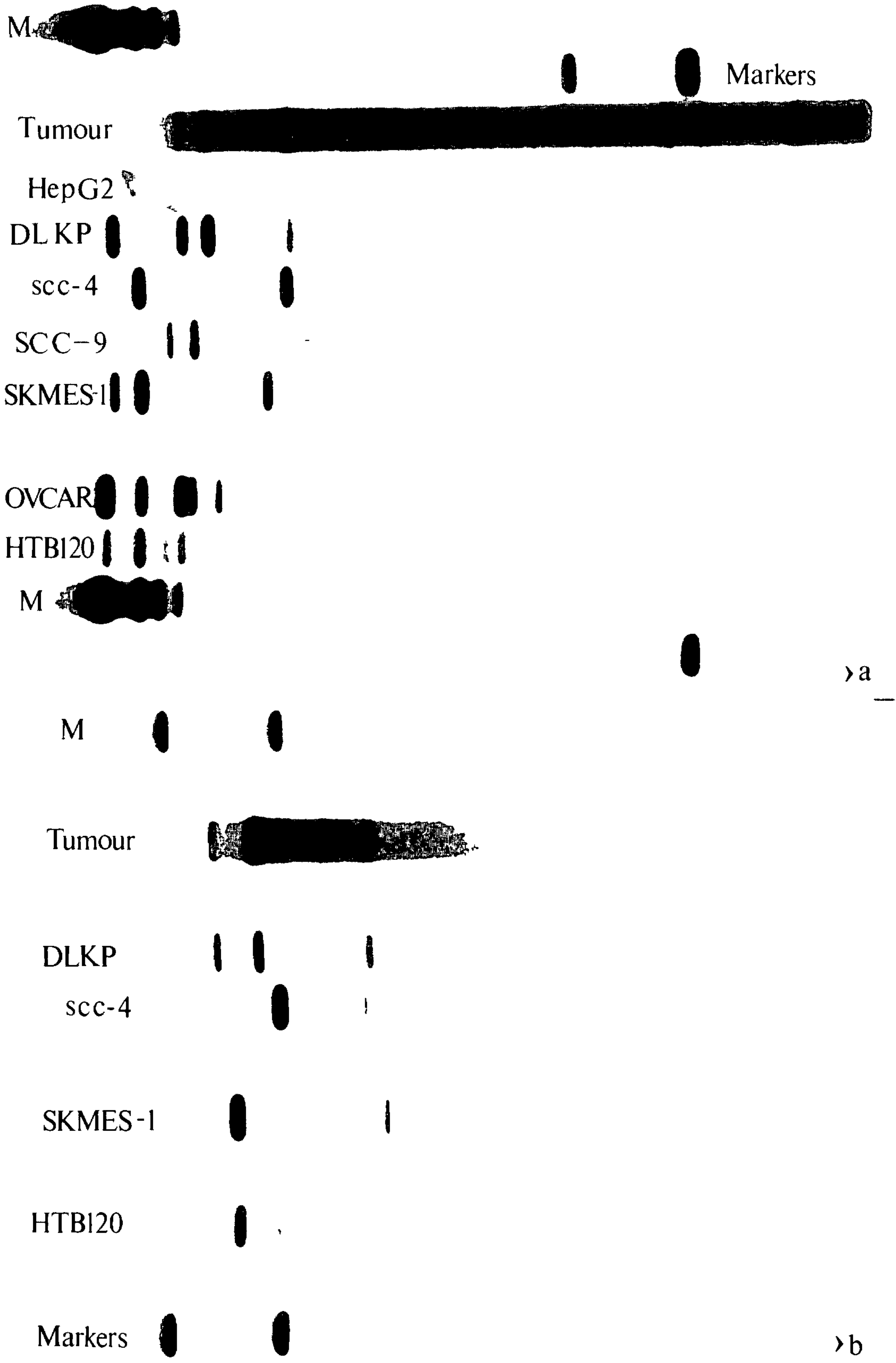


Figure 27a and b DNA fingerprints using a mixture of 3 single locus probes and b 2 single locus probes, of DLKP(lane 1) in comparison with the original tumour (lane 2) and other cell lines present in the laboratory at the time of culturing to ensure cross contamination had not occurred The tumour had partially degraded before the DNA was extraced and gave a smearing effect Note the 3 bands on each gel in the tumour which correspond with the cell line There is one high molecular mass band in the cell line, mix a, which is not present in the tumour, this is believed to be due to degredation of high molecular weight material on transport

3.1.1.5 HISTOPATHOLOGY

DLKP cells were analyzed for characteristic properties of malignancy, as seen in tissue sections (Gilvarry et al 1990) Properties such as multiple nucleoli and tri- or multipolar mitoses Figure 28 show a tri-polar mitosis in DLKP stained with giemsa providing supportive evidence for the malignant nature of DLKP

Figure 28: Tri-polar mitosis in DLKP cells stained with Geimsa

3.1.1.6 CLONING IN AGAR / COLONY FORMING EFFICIENCY

Another test employed to determine the malignant nature of cells in culture is their ability to grow in agar DLKP cells were seeded into soft agar at 10^3, 10^4, and 10^5 cells per ml as described in section 2 6 Colonies were allowed to develop over a period of 10 - 14 days Colonies greater than 50 μm in diameter were counted on an inverted microscope (CK Olympus Tokyo) at 100x or 40x The resulting colony forming efficiency is 7 3 $\pm$ 1 4 % at 10^3 cells/ml and 7 5 $\pm$ 2 4 % at 10^4 cells/ml

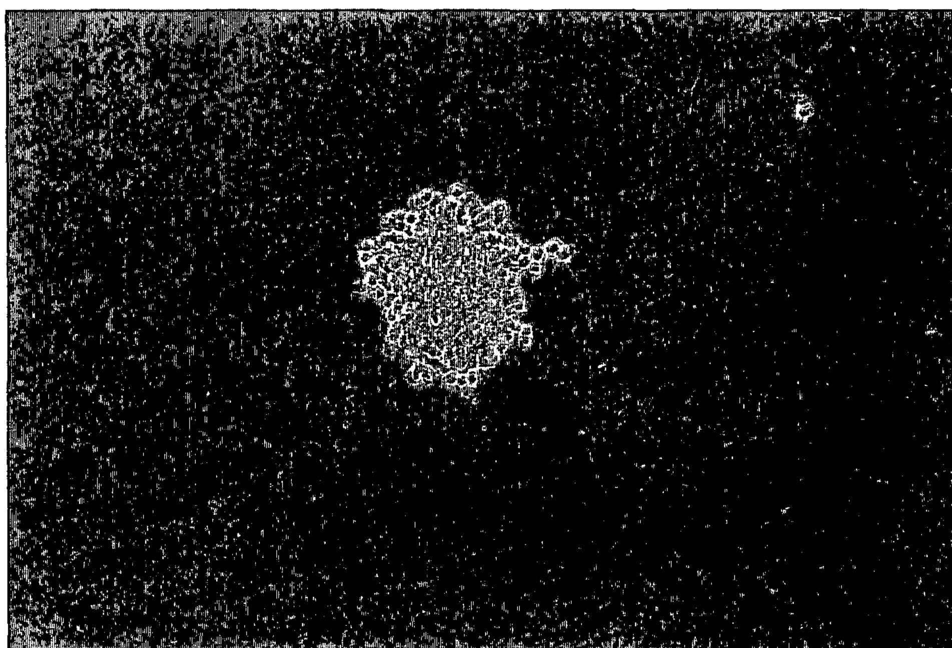


Figure 29: DLKP cell colony in agar - a characteristic of malignant cells

3.2 ANALYSIS OF CELL LINES FOR XENOBIOTIC METABOLIZING ACTIVITY.

The followıng cell lınes ın the ınduced and unınduced form, were ınvestıgated for theır expressıon of CYP1A1, UDP-Gluconyltransferase, 7-Ethoxycoumarın-O-Deethylase, total Glutathıone-S-Transferase actıvıtıes and NADPH Cytochrome P450 Reductase Inıtıally these tests were to be carrıed out usıng spectrophotometrıc analysıs, however after the assays had been set up and calıbrated usıng rat lıver - an abundant source of cytochrome P450 and other drug metabolısıng enzymes, ıt was found that these assays were not sensıtıve enough to be used on cell lıne At that poınt we changed to the above mentıoned assays whıch are of a fluorometrıc form and capable of detectıon ınto the nanomolar range As further conformatıon of the results obtaıned for enzymes actıvıtıes, western analysıs was also carrıed out

Each cell lınes was treated wıth ınducers as follows Cells were seeded at a densıty of 5 X 10^5 cell/ml ınto 100 mm^2 petrı dıshes ın the approprıate medıa (see sectıon 2 0 4 1) and placed ın a 5% CO_2 ıncubator at 37°C overnıght Benzanthracene (BA) (dıssolved ın DMSO) and DMSO was dıluted ın complete medıa at 20 μmolar and 0 01 % respectıvely and the cells fed wıth thıs medıa After 18 hours the cells were harvested and assayed as per sectıon 2 15 Phenobarbıtal (PB) was dıssolved 0 5 N NaOH and dıluted to 2mM m complete medıa The pH of the medıa was corrected to 7 44 - 7 55 and the cells were exposure as for benzanthracene, over 72 hours After the 72 hours the cells were harvested and assay as per sectıon 2 15

The followıng abbrevıatıons are used m these tables - GST Glutathıone-S-transferase, DNCB Dı-nıtro-chloro-benzene conjugatıon, GST actıvıty ıs expressed ın nmoles of DNCB conjugated per mın per mg of proteın

NADPH cytochrome c reductase actıvıty ıs expressed as the rate of reductıon of cytochrome c per mın per mg of cell homogenate *ı e* nmoles reduced per mın per mg of proteın N A - No actıvıty N D not determıned

Each assay ıs the result of two sets of three assays, carrıed out on dıfferent days

CELL LINE	DESCRIPTION & SOURCE
H411E	Rat hepatoma, Reuber H35 - ATCC CRL 1548
HEP-G2	Human Hepatocellular carcinoma - ATCC HB 8065
V79 and XEM2	Chinese Hamster fibroblasts parental line and Transfected with Rat *CYP*IA1 Cells and plasmid from J Doehmer Germany
HTC-BUdr	Rat hepatoma ECACC N°85061110
DLKP	Squamous Cell Carcinoma of the Lung - NCTCC DCU
DLKPA	Adriamycin resistant variant of DLKP - NCTCC DCU
SKMES-1	Human squamous cell carcinoma of the lung - ATCC HTB 58
SKMES-1A	Adriamycin resistant variant of SKMES-1 - NCTCC DCU
SKLU1	Human adenocarcinoma of the lung poorly differentiated - ATCC HTB 57
SKLU1A	Adriamycin resistant variant of SKLU1 - NCTCC DCU

Table 2.

ARYLHYDROCARBON HYDROXYLASE ACTIVITY IN H411E CELLS EXPOSED TO INDUCERS.

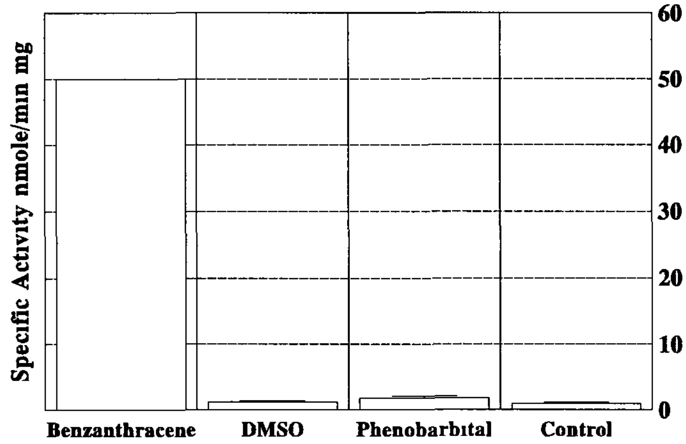


Figure 30 Aryl Hydrocarbon Hydroxylase activity in H411E cells exposed to a number of inducers - see section 2 15 2 3

ARYLHYDROCARBON HYDROXYLASE ACTIVITY IN HEPG2 CELLS EXPOSED TO INDUCERS.

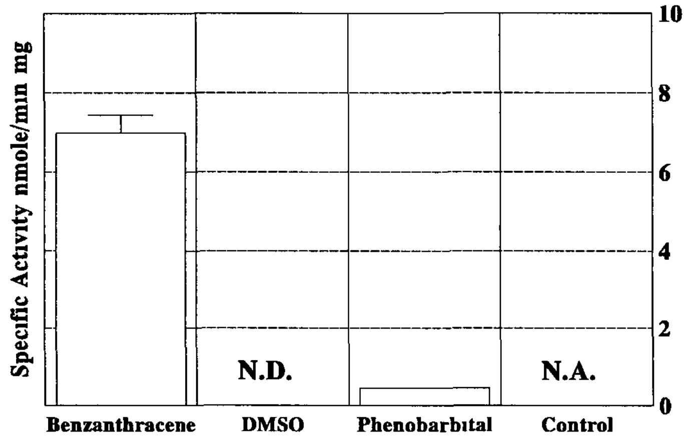


Figure 31 Aryl Hydrocarbon Hydroxylase in HepG2 cells exposed to a number of inducers - see section 2 15 2 3

DE-ETHYLASE ACTIVITY IN H411E CELLS EXPOSED TO INDUCERS.

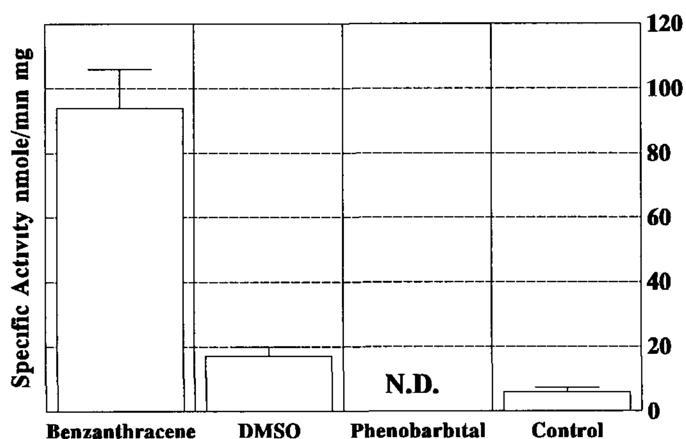


Figure 32 Ethoxycoumarin O-deethylase activity in HepG2 exposed to a number of inducers - See section 2 15 2 2

DE-ETHYLASE ACTIVITY IN HEPG2 CELLS EXPOSED TO INDUCERS.

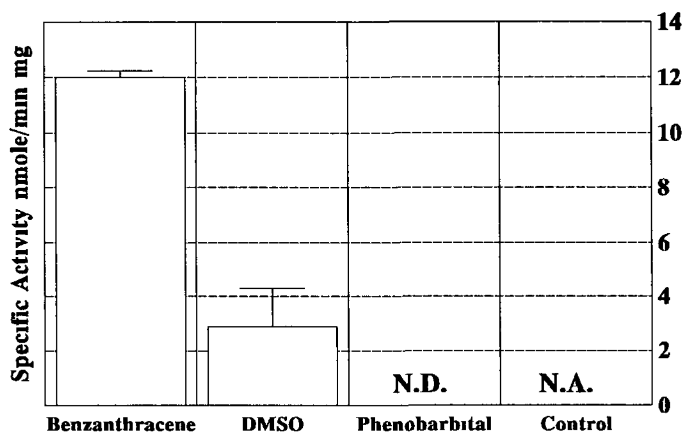


Figure 33 Ethoxycoumarin O-deethylase activity in HepG2 cells exposed to a number of inducer - See section 2 15 2 2

UDP-GLUCONYLTRANSFERASE ACTIVITY IN H411E CELLS EXPOSED TO INDUCERS.

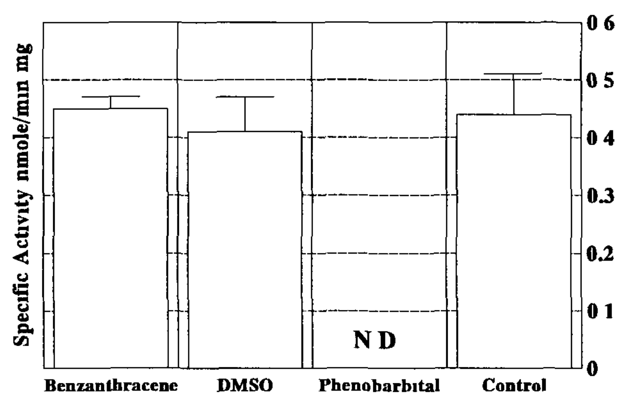


Figure 30 UDP-gluconyltransferase activity in H411E cells exposed to a number of inducers - see section 2 15 2 1

GLUTATHIONE-S-TRANSFERASE ACTIVITY IN H411E CELLS EXPOSED TO INDUCERS.

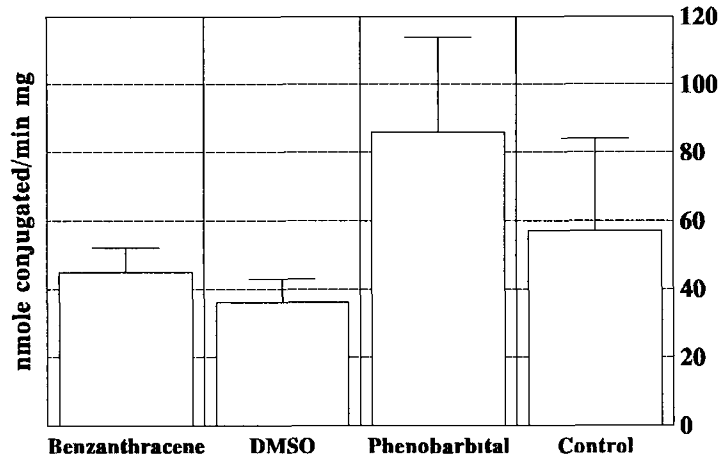


Figure 35 Total DNCB Glutathione-S-transferase activity in H411E cells exposed to inducers - See section 2 15 1 3, DNCB, Dinitrochlorobenzene

GLUTATHIONE-S-TRANSFERASE ACTIVITY IN HEPG2 CELLS EXPOSED TO INDUCERS.

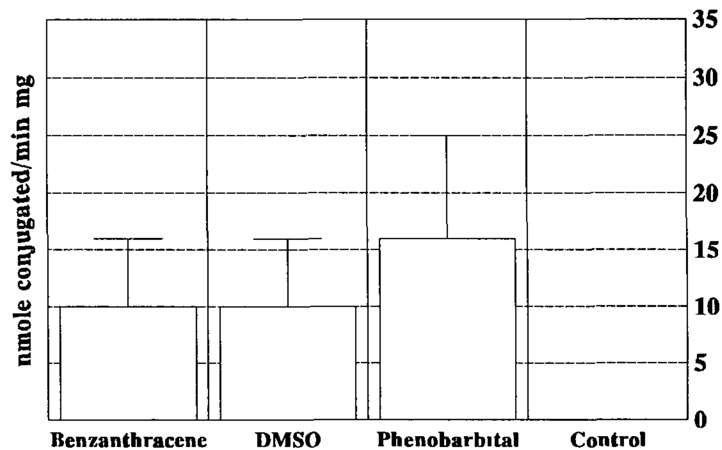


Figure 36 Total DNCB Glutathione-S-transferase activity in HepG2 cells exposed to inducer - See section 2 15 1 3

GLUTATHIONE-S-TRANSFERASE ACTIVITY IN SKLU-1 AND SKLU-1A CELLS.

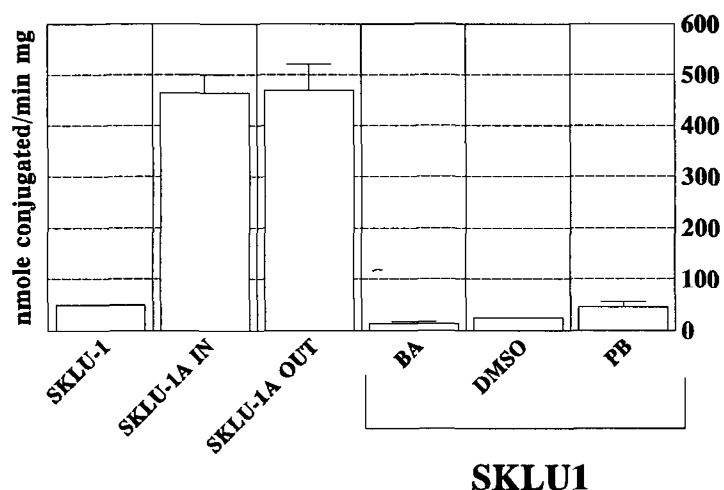


Figure 37 Total DNCB Glutathione-S-transferase activity in SKLU1 cells exposed to inducers and SKLU1-A cells - See section 2 15 1 3

GLUTATHIONE-S-TRANSFERASE ACTIVITY IN SKMES-1 AND SKMES-1A CELLS.

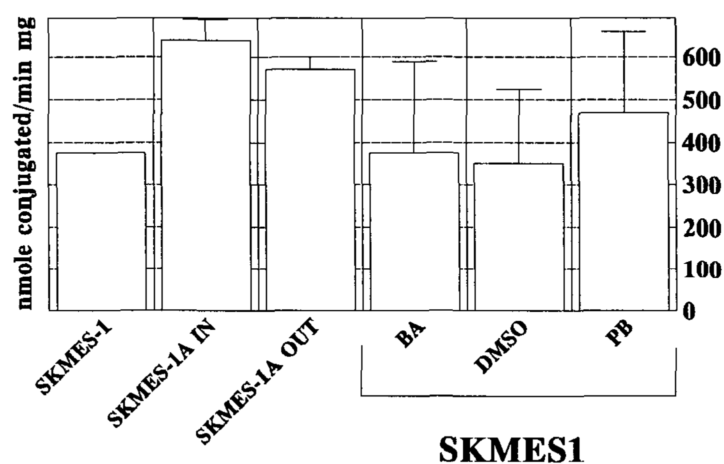


Figure 38 Total DNCB Glutathione-S-transferase activity in SKMES1 cells exposed to inducers and SKMES1-A cells - See section 2 15 1 3

GLUTATHIONE-S-TRANSFERASE ACTIVITY IN DLKP AND DLKPA CELLS.

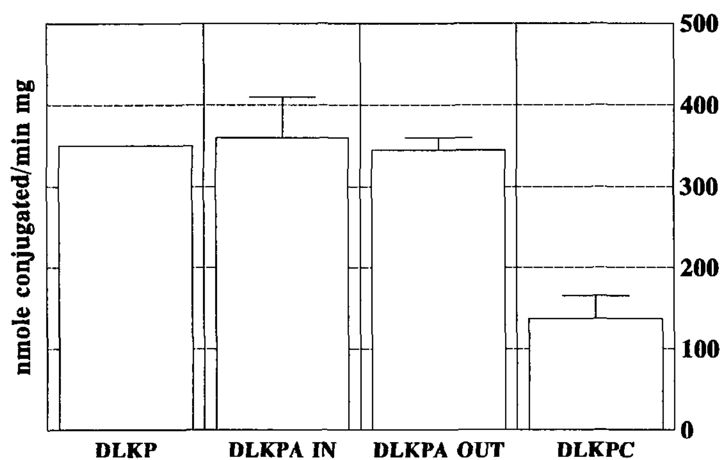


Figure 39 Total DNCB Glutathione-S-transferase activity in DLKP cells exposed to inducers and DLKPA cells - See section 2 15 1 3

PKC is a carboplatin resistant varient of DLKP At the time of testing this line DLPKC was resistant to 10 2 μg/ml of carboplatin

NADPH CYTOCHROME C REDUCTASE ACTIVITY IN H411E CELLS EXPOSED TO INDUCERS.

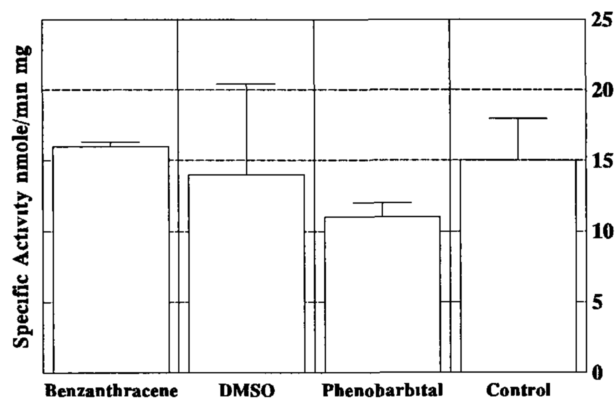


Figure 40: NADPH cytochrome P450 (c) reductase activity in H411E cell exposed to inducers - See section 2 15 1 4

NADPH CYTOCHROME C REDUCTASE ACTIVITY IN HEPG2 CELLS EXPOSED TO INDUCERS.

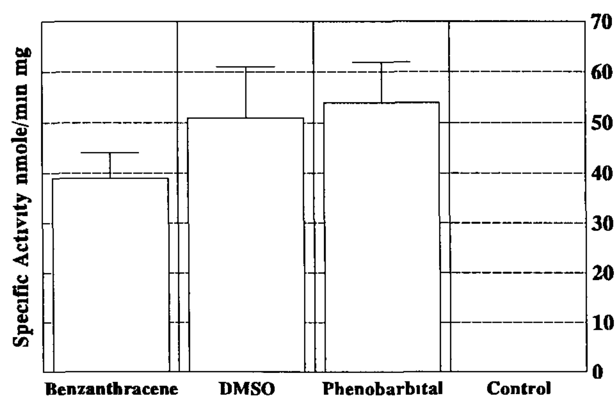


Figure 41 NADPH cytochrome P450 (c) reductase activity in HepG2 cells exposed to inducers - See section 2 15 1 4

NADPH Cytochrome c Reductase IN SKLU1 CELLS EXPOSED TO INDUCERS.

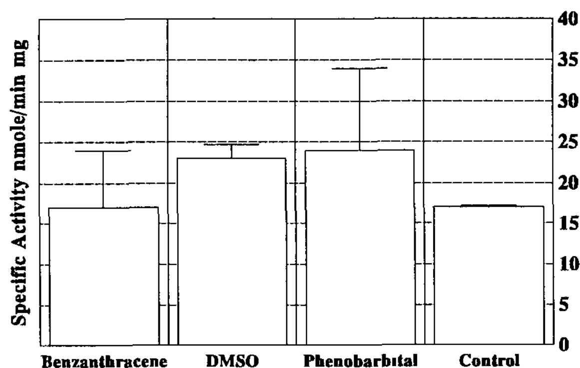


Figure 42 NADPH cytochrome P450 (c) reductase activity in SKLU1 cells exposed to inducer - See section 2 15 1 4

NADPH cytochrome c reductase activity in SKMES1 exposes to inducers and SKMES1A cells

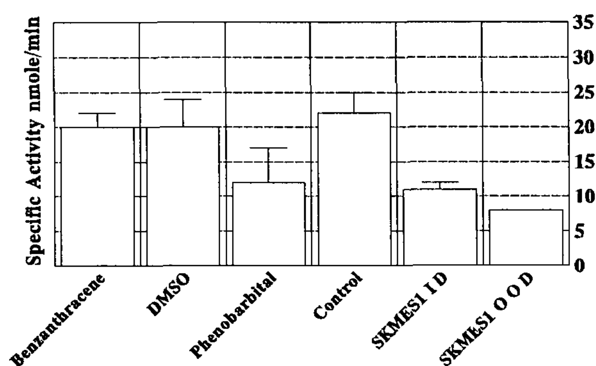


Figure 43 NADPH cytochrome P450 (c) reductase activity in SKMES-1 cells exposed to inducers and SKMES-1A cells - see section 2 15 1 4

NADPH Cytochrome c Reductase activity in DLKP and DLKPA cells.

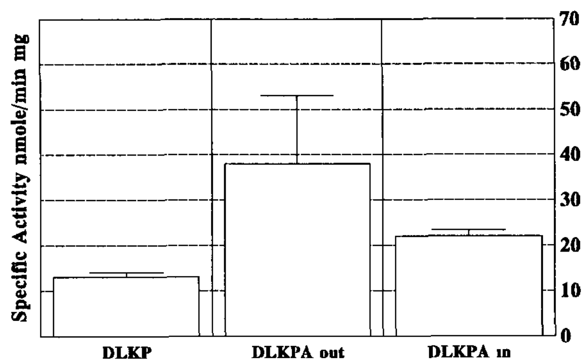


Figure 44 NADPH cytochrome P450 (c) reductase activity in DLKP cells exposed to inducers and DLKPA cells see section 2 15 1 4

The following seven tables 3 to 9 display the numerical specific activities obtained for each assay and cell line mentioned The graphical representation can be seen in the preceeding pages Figures 30 to 44 Benzanthracene (BA) and DMSO induction were carried out for 18 hours at 20 μmolar and 0 01 % respectively

Phenobarbital (PB) was disolved 0 5 N NaOH and the cell media pH corrected to 7 4 Exposure was over 72 hours at a concentration of 2mM

G-S-T Glutathione-S-transferase, Di-nitro-chloro-benzene conjugation, expressed in nmoles conjugated per min per mg of protein

NADPH cytochrome c reductase activity is expressed as the rate of reduction of cytochrome c *i e* nmoles reduced per min per mg of protein N D not determined

Each assay is the result of two sets of three assays, carried out on different days

AHH Aryl Hydrocarbon Hydroxylase, UDPGA UDP-glucuronyltransferase, De-ethylase 7-ethoxycoumarin *O*-deethylase, Reductase NADPH cytochrome P450 reductase, G-S-T Glutathione-S-Transferase

CELL LINE: H411E

INDUCER	ASSAY	SPECIFIC ACTIVITY nmoles/min mg pr
Benzanthracene	AHH	50 ± 2
	UDPGT	0 45 ± 0 02
	DE-ETHYLASE	94 ±12
	REDUCTASE	16 ± 0 3
	G-S-T	45 ± 8
DMSO	AHH	1 2 ± 0 2
	UDPGT	0 41 ± 0 06
	DE-ETHYLASE	17 ± 3
	REDUCTASE	14 ± 6 4
	G-S-T	36 ± 7
Phenobarbital	AHH	1 8 ± 0 3
	UDPGT	N D
	DE-ETHYLASE	N D
	REDUCTASE	11 ± 1
	G-S-T	86 ± 28
Control	AHH	0 9 ± 0 2
	UDPGT	0 44 ± 0 07
	DE-ETHYLASE	6 ± 1 4
	REDUCTASE	15 ± 3
	G-S-T	57 ± 27

Table 3: Numerical results of individual assays for H411E See above for details

CELL LINE: HEPG2

INDUCER	ASSAY	SPECIFIC ACTIVITY nmoles/min mg pr
Effect of Benzanthracene on HepG2	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	7 ± 0 45 No Activity 12 ± 0 25 39 ± 5 10 ± 6
Effect of DMSO on HepG2	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No Activity No Activity 2 9 ± 1 4 51 ± 10 10 ± 6
Effect of Phenobarbital on HepG2	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	0 45 ± 0 002 N D N D 54 ± 8 16 ± 9
Control HepG2	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No Activity No Activity No Activity

Table 4: Numerical results of assays carried out on HepG2 - for details see table 3

CELL LINE: HTC

INDUCER	ASSAY	SPECIFIC ACTIVITY nmoles/min mg pr
Effect of Benzanthracene on HTC	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No Activity No Activity No Activity N D 100 ± 10
Effect of DMSO on HTC	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No Activity No Activity No Activity N D N D
Effect of Phenobarbital on HTC	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No Activity No Activity No Activity N D N D
Control HTC	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No Activity No Activity No Activity - -

Table 5: Numerical results of enzyme assays on HTC For details see table 3

CELL LINE: DLKP and DLKPA - adriamycin resistant form

INDUCER	ASSAY	SPECIFIC ACTIVITY nmoles/min mg pr
Effect of Benzanthracene on DLKP	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No activity No activity No activity N D N D
Effect of DMSO on DLKP	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No activity No activity No activity N D N D
Effect of Phenobarbital on DLKP	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No activity No activity No activity N D N D
Control DLKP	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No activity No activity No activity 13 ± 1 350 ± 17
ENZYME ACTIVITY OF DLKPA	**ASSAY**	**SPECIFIC ACTIVITY nmole/min mg pr**
DLKPA in 2 μg/ml Adriamycin	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No activity No activity N D 22 ± 1 360 ± 50
DLKPA out of drug	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No activity No activity N D 38 ± 25 345 ± 17
DLKPC - carboplatin resistant 10 2 μg/ml	G-S-T	137 ± 25

Table 6: Numerical results of assays carried out on DLKP and DLKPA - details above

CELL LINE· SKMES-1 and SKMES-1A -adnamycın resıstant form

INDUCER	ASSAY	SPECIFIC ACTIVITY nmoles/mın mg pr
Effect of Benzanthracene on SKMES1	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No actıvıty No actıvıty No actıvıty 20 ± 2 375 ± 200
Effect of DMSO on SKMES1	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No actıvıty No actıvıty No actıvıty 20 ± 4 350 ± 175
Effect of Phenobarbıtal on SKMES1	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No actıvıty No actıvıty No actıvıty 12 ± 5 470 ± 188
Control SKMES1	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No actıvıty No actıvıty No actıvıty 25 ± 15 375 ± 10
ENZYME ACTIVITY OF SKMESA	**ASSAY**	**SPECIFIC ACTIVITY nmoles/mın mg pr**
SKMESA ın 1 μg/ml Adnamycın	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No actıvıty No actıvıty No actıvıty 11 ± 1 637 ± 50
SKMESA out of drug	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	N D N D N D 8 ± 0 000 570 ± 30

Table 7: Numerıcal results of assays carrıed out on SKMES1 and SKMES1A - For detaıls see table 3

CELL LINE SKLU1 and SKLU1-A - adnamycin resistant form

INDUCER	ASSAY	SPECIFIC ACTIVITY nmoles/min mg pr
Effect of Benzanthracene on SKLU1	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No activity No activity No activity 17 ± 7 14 ± 2
Effect of DMSO on SKLU1	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No activity No activity No activity 23 ± 1 25 ± 0 000
Effect of Phenobarbital SKLU1	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No activity No activity No activity 24 ± 10 46 ± 10
Control SKLU1	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No activity No activity No activity 17 ± 0 2 50 ± 3
ENZYME ACTIVITY OF SKLU1A	**ASSAY**	**SPECIFIC ACTIVIY nmoles/min mg pr**
SKLU1A in 1 μg/ml Adriamycin	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	No activity No activity N D N D 465 ± 35
SKLU1A out of drug	AHH UDPGT DE-ETHYLASE REDUCTASE G-S-T	N D N D N D N D 470 ± 50

Table 8: Numerical results of assays carried out on SKLU1 and SKLU1A cells - details see table 3

CELL LINE	ASSAY	SPECIFIC ACTIVITY nmole/min mg
V79	AHH	No activity
V791A1	AHH	6 46 ± 0 086

Table 9: Results of AHH assays carried out on V79 and V791A1

3.2.1. CYTOCHROME P450IA1 ANALYSIS BY WESTERN BLOT.

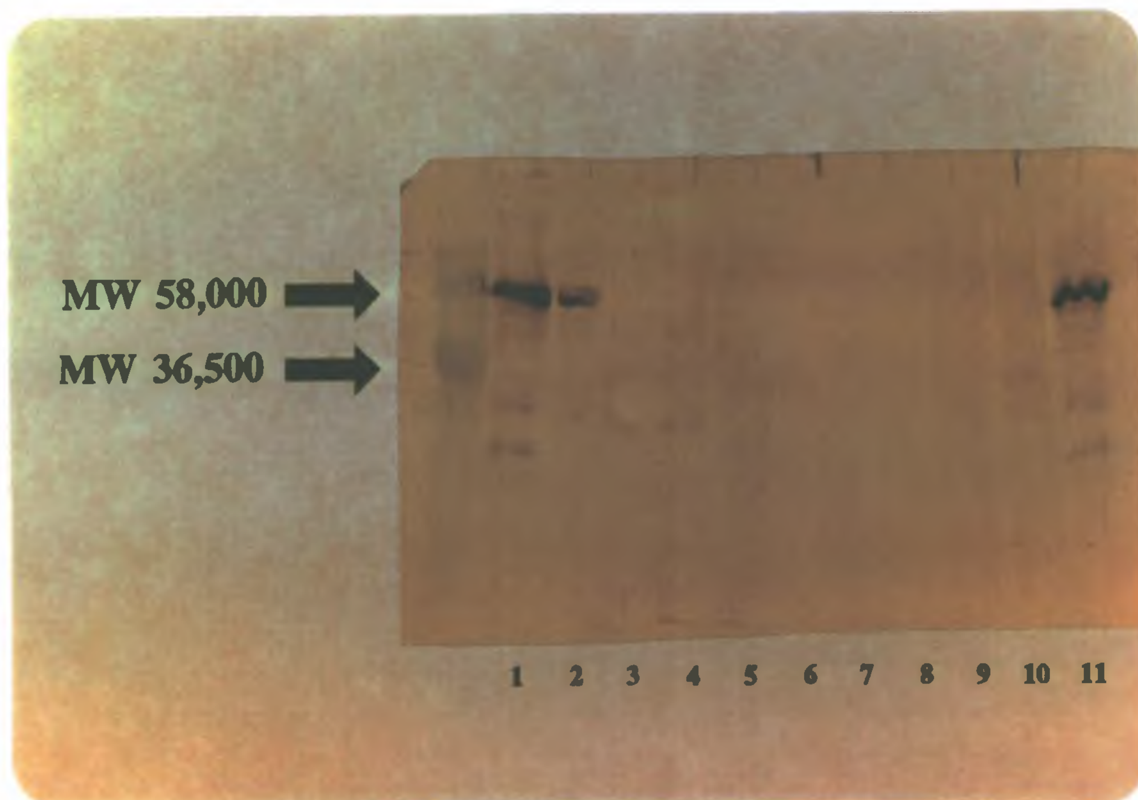


Figure 45: Analysis of cell lines H411E, SKMES-1A and SKLU1 for the presence of cytochrome P450IA1 protein. Cell lines were exposed to inducers as in section 2.0.4.3 and analyses as in section 2.12. 1+11: Standard, induced rat liver microsome; 2: H411E BA induced; 3: H411E DMSO induced; 4: H411E uninduced control; 5: H411E phenobarbital induced; 6: SKLU1 BA induced; 7: SKLU1 DMSO induced; 8: SKLU1 uninduced control; 9: SKLU1 phenobarbital induced; 10: SKMES-1A in 0.1 μg/ml adriamycin.

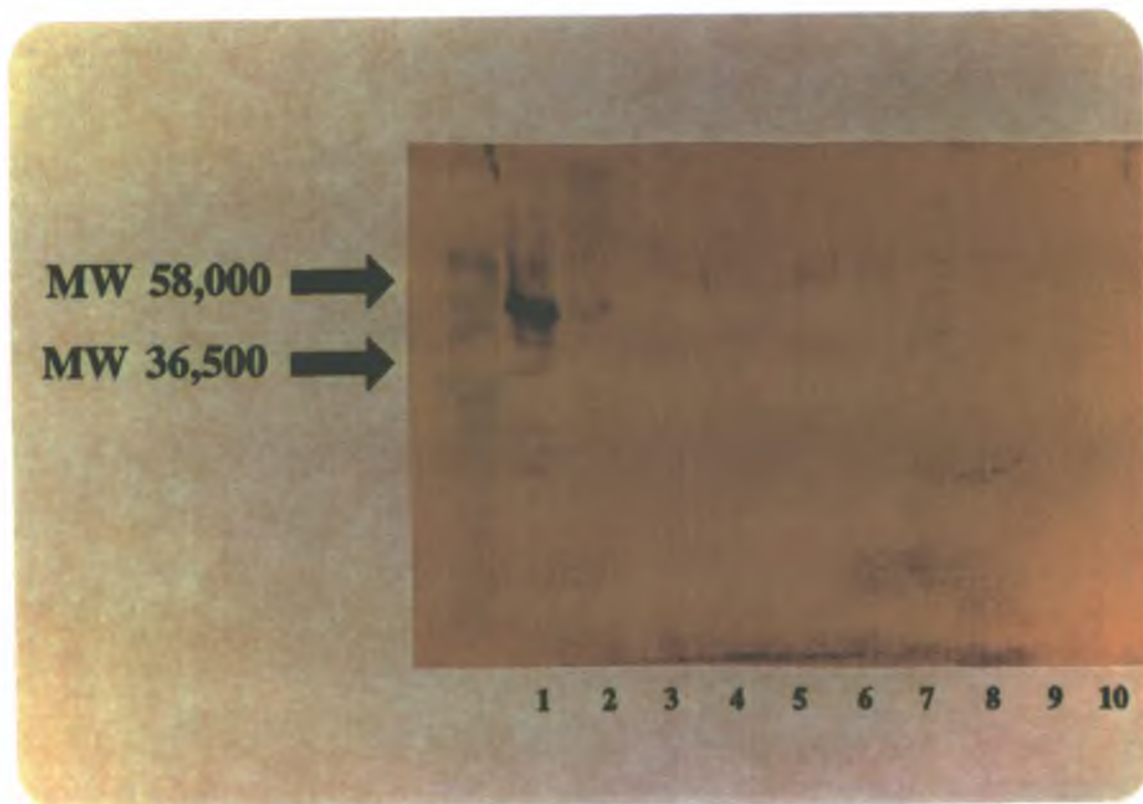


Figure 46: Analysis of cell lines H411E, SKMES-1 and DLKP for the presence of cytochrome P450IA1 protein. Cell lines were exposed to inducers as in section 2.0.4.3 and analyses as in section 2.12. 1: Standard, induced rat liver microsome; 2: H411E BA induced; 3: DLKP BA induced; 4: DLKP DMSO induced; 5: DLKP uninduced control; 6: DLKP phenobarbital induced; 7: SKMES-1 BA induced; 8: SKMES-1 DMSO induced; 9: SKMES-1 uninduced control; 10: SKMES-1 phenobarbital induced.

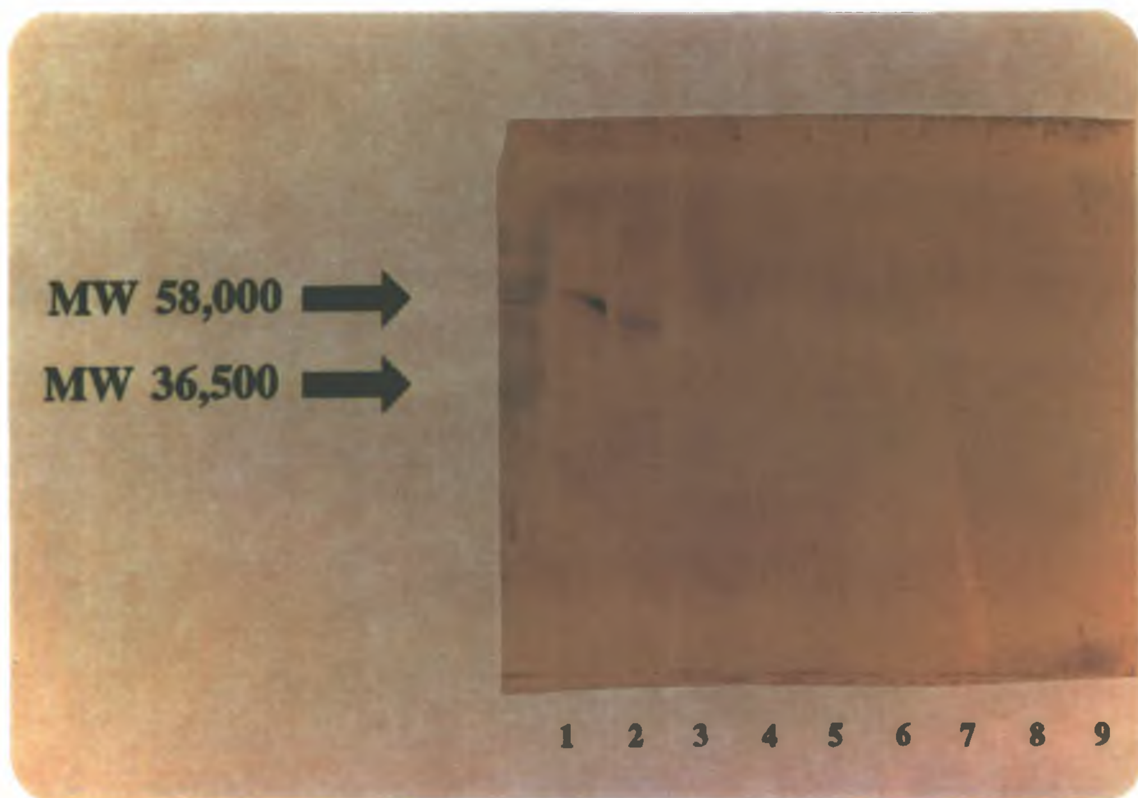


Figure 47: Analysis of cell lines HepG2 and DLPRSCC for the presence of cytochrome P450IA1 protein. Cell lines were exposed to inducers as in section 2.0.4.3 and analyses as in section 2.12. 1: Standard, induced rat liver microsome; 2: HepG2 BA induced; 3: HepG2 DMSO induced; 4: HepG2 uninduced control; 5: HepG2 phenobarbital induced; 6: DLPRSCC BA induced; 7: DLPRSCC DMSO induced; 8: DLPRSCC uninduced control; 9: DLPRSCC phenobarbital induced;

3.3 EFFECT OF ANTIBODIES ON H411E EXPRESSION OF AHH.

The effect of addition of antibodies Rat CYP1A1, Rat CYPIIB1 and cytochrome P450 reductase on Aryl Hydrocarbon Hydroxylase and cytochrome P450 reductase, is seen in Figure 48 Antibody was introduced into the assay mixture before the sample and allowed to stand on ice for 30 min before assay The assay was then run as per usual - see section 2 15 2 3 As can be seen the greater majority of AHH activity in the induced H411E cells can be attributed to CYP1A1, however there is a 20% portion of this activity which is carried out by other enzymes Addition of CYP2B1 antibody results in a decrease in AHH activity in these cells also, further enforcing the CYP1A1 results These results demonstrate the shared enzyme activity and broad substrate specificity characteristic of the different CYP families

Effect of antibodies on H411E induced AHH expression

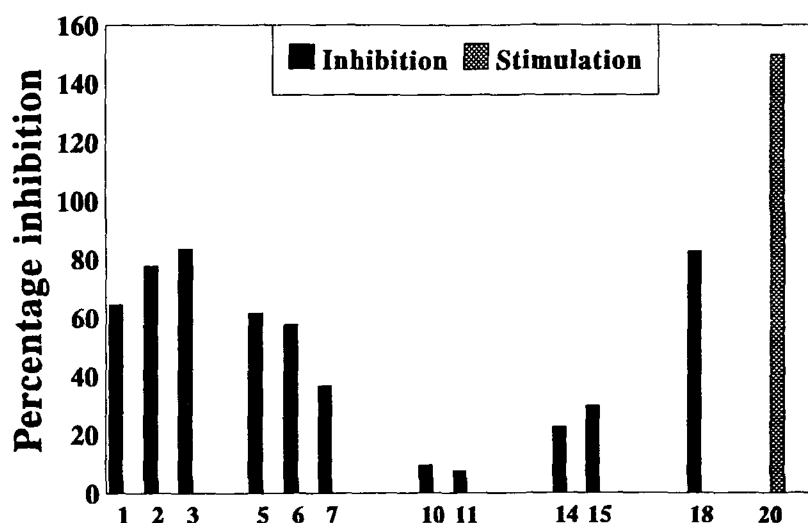


Figure 48: The effect of antibody addition on the expression of AHH in induced H411E cells Induction was carried out as in section 2 0 4 3 Antibody addition was as in section 2 15 2 4, and AHH assay was carried out as in section 2 15 2 3 2

1:	**Addition of 25 μg/ml** P450IA1 antibody to H411E cells which had been induced over 18 hours with BA
2:	**50 μg/ml** P450IA1 antibody to cells induced over 18 hours with BA
3:	**100 μg/ml** P450IA1 antibody to cells induced over 18 hours with BA
5:	**25 μg/ml** P450IA1 antibody to cells induced with DMSO for 18 hours
6:	**25 μg/ml** P450IA1 antibody to uninduced H411E cells
7:	**100 μg/ml** P450IA1 antibody cells induced with PB for 72 hours
10:	**50 μg/ml** P450IIB1 antibody to cells induced with BA for 18 hours
11:	**100 μg/ml** P450IIB1 antibody to cells induced with BA for 18 hours
14:	**50 μg/ml** NADPH cytochrome P450 reductase antibody to cells induced with BA for 18 hours
15:	**100 μg/ml** NADPH cytochrome P450 reductase antibody to cells induced with BA for 18 hours
18:	**100 μg/ml** of both P450IA1 and P450IIB1 antibodies to cells induced with BA for 18 hours
20:	**100 μg/ml** P450IIB1 to cells induced with PB for 72 hours

Standard deviation were less than 10% in each of these assays

3.4 TRANSFECTION OF MAMMALIAN CELLS IN CULTURE

The transfection of V79 and DLKP cells was carried out using the rat CYP1A1 gene which had been cloned in to a pSV2 vector (Dogra *et al*, 1990) The choice of V79 for this experiment was because it had been successfully transfected by Dogra *et al*, *(1990)* and served as a positive control, as it had already been positively transfected Transfection was carried out using Calcium Phosphate procedure in a co-transfection with pSV2Neo at a 20 1 ratio respectively However this method resulted in a low efficiency

CELL LINE	GENE	EFFICIENCY	No. POSITIVE
V79	1A1/Neo	$16/1 \times 10^6$	1*
V79	Neo	$29/1 \times 10^6$	-
DLKP	1A1/Neo	$5/1 \times 10^6$	-
DLKP	Neo	$60/1 \times 10^6$	-

Table 10

Electroporation of the Line DLKP proved to be more efficient In this transfection also included the marker plasmid pCH110 encoding β-Galactosidase which could be visually scored after transfection by reaction with X-Gal, which on metabolism turns positive cells *i e* those cells that have taken up plasmid blue

Efficiency of transfection: $3\ 1 \times 10^3/1 \times 10^6$ cells containing the β-Gal Plasmid which had been transfected at a concentration of 20 times less than the Rat CYP1A1
DLKP was transfected on 8 different occasions with CYPIA1 plasmid using both calcium phosphate and electroporation On each occasion geneticin resistant colonies and β-glactosidase positive colonies observed - highlighting that the transfections had been successful on each occasion However, in the over 47 geneticin resistant DLKP colonies which were cloned, no CYP1A1 expressing clone was isolated

Our main problem at this point was that we could prove the presence of the pSV2Neo plasmid and the PCH110 plasmid, but there was no visual marker for the CYPIA1 plasmid.

The fact that the PCH110 and pSV2Neo plasmids were at a concentration 20 times lower than that of the CYPIA1 would strongly suggest that the CYPIA1 would have more than lightly been transfected also and its lack of expression was due to other factors which would be of great interest (Figure 49). Also the fact that I had isolated a CYP1A1 positive clone which demonstrated that the plasmid was functional V791A1-7 (Figure 50d). However there was a possibility that the plasmid just never went into the DLKP cells at all. Therefore it was necessary to find a way of proving the presence of this plasmid in at least one of the clones positive for PSV2NEO. To do this the technique Polymerase Chain Reaction (PCR) was employed. A pair of primers were picked which reacted only with the rat CYPIA1 gene present in the plasmid. The product of these primers would be a 1540 bp length of DNA from position -110 to position -1650.

DNA samples were extracted and PCR carried out. As can be seen form Figure 51, the DLKP transfectant is positive for the presence of plasmid as there is a single band present at 1540 bp, therefore there is a reason for its non expression of CYP1A1 protein and the corresponding AHH activity.

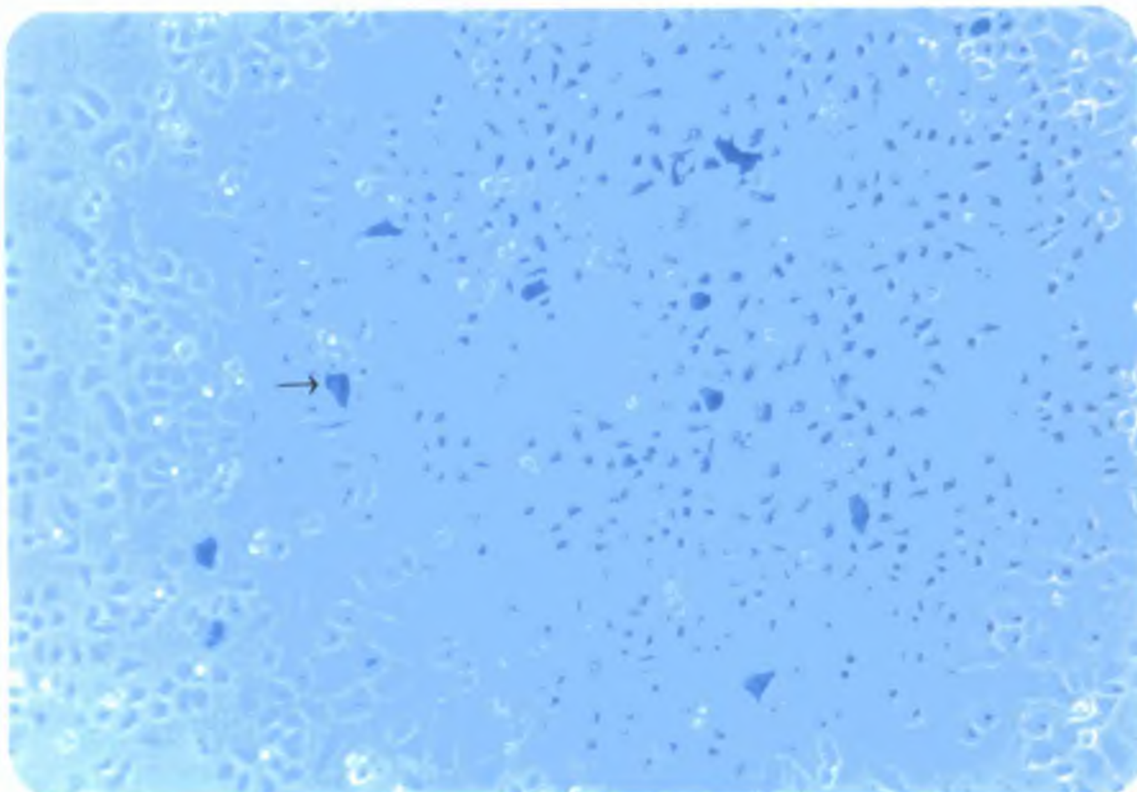


Figure 49: PCH110 positive transfectant of DLKP electroporated with 1 ug of PCH110. Blue cells are positive for the plasmid.

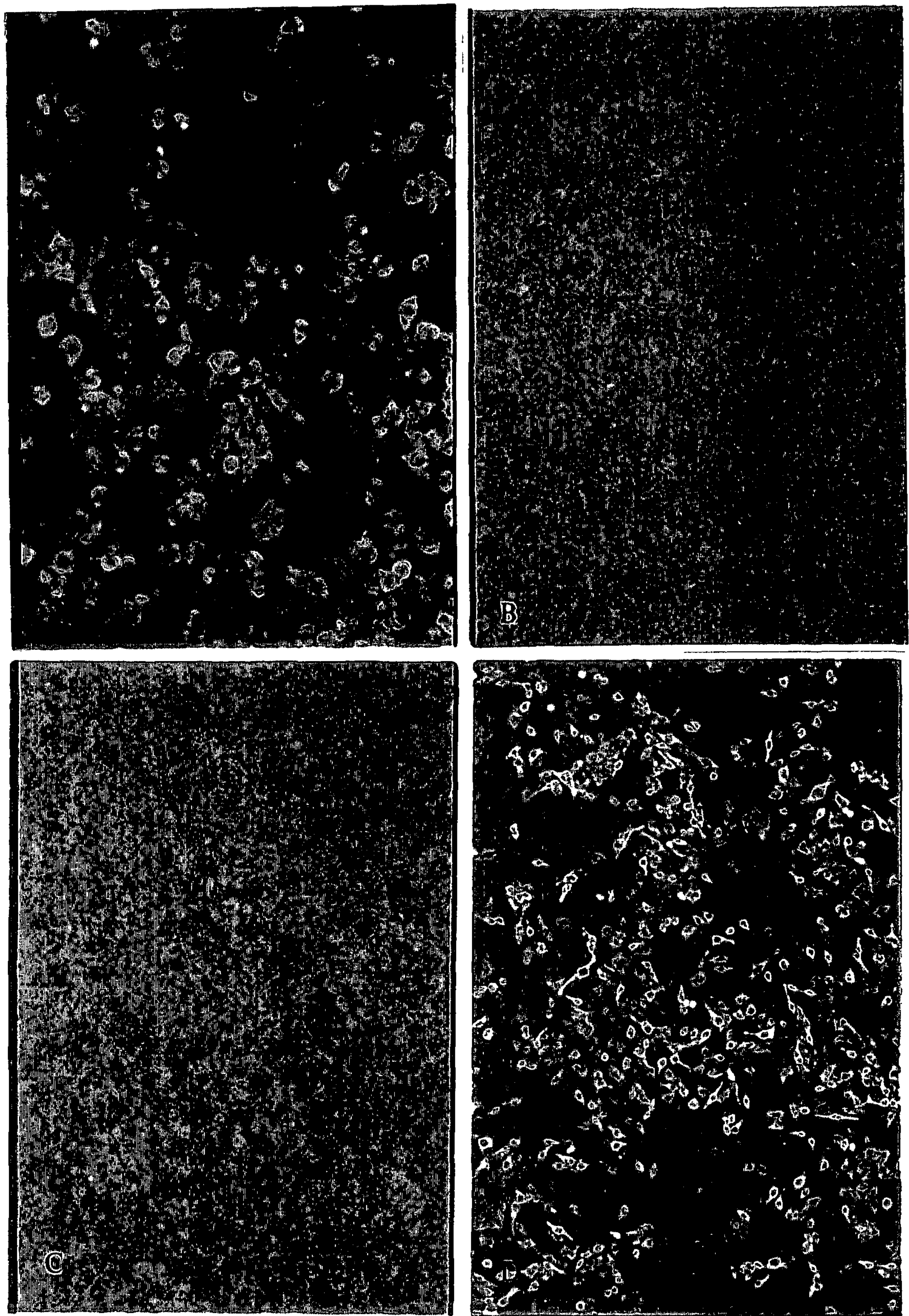


Figure 50: Immunofluorescence of V-79 cells transfected with the rat *CYPIA1* gene and DLKP, as in sectio 2 5 2 A H411E BA induced (section 2 0 4 3) B DLKP, C V79 D V791A1-7 As can be seen both H411E and V791A1-7 are postive for P450IA1 enzyme DLKP and V79 parental line are negative

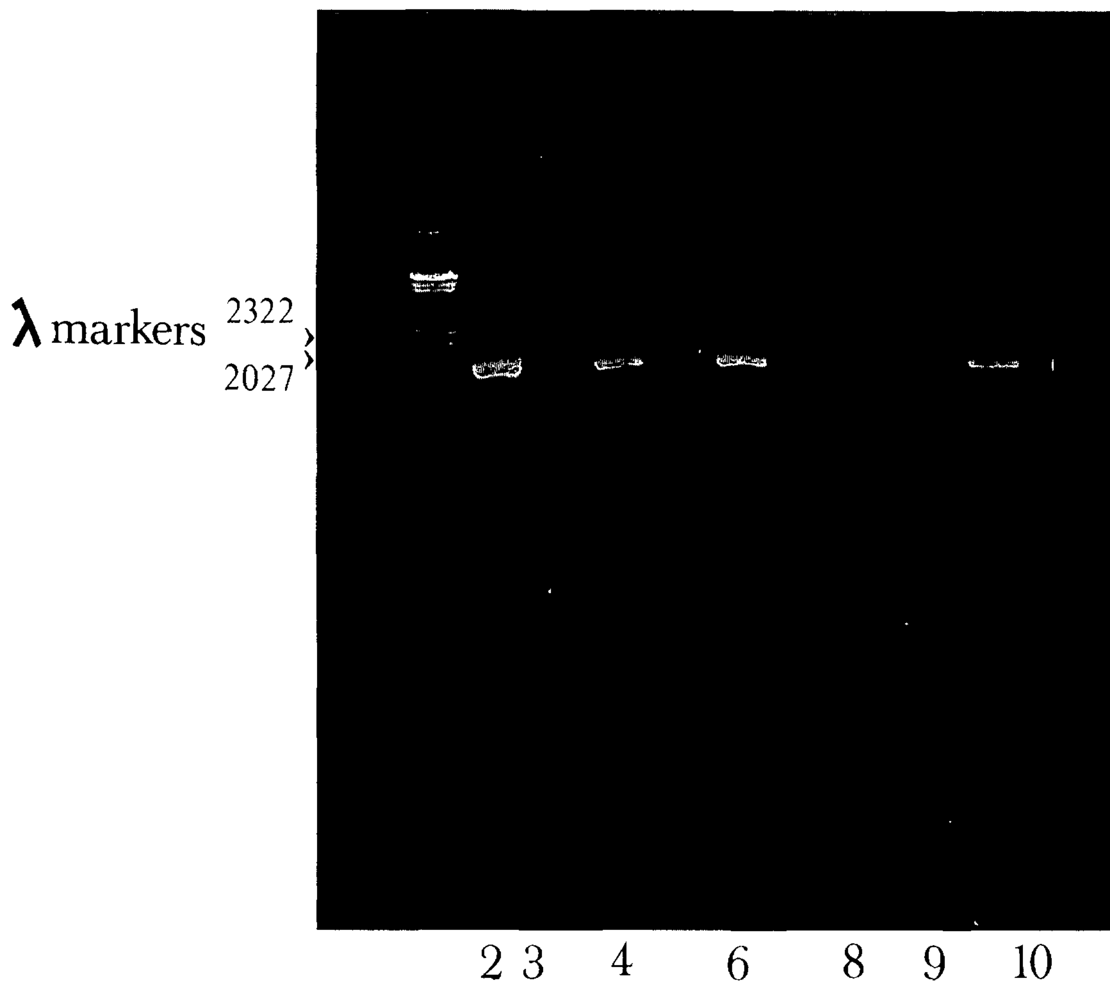


Figure 51: Gel of transfected and parental cell lines subjected to PCR Lane 2 contains plasmid alone Lane 3 - V79 Lane 4 - XEM2 Lane 6 - V791A1-7 Lane 8 - DLKP spiked with plasmid Lane 9 - DLKP parental Lane 10 - DLKP1A1 which is positive for the 1540 bp section of DNA proving the presence of the plasmid even though it is not expressed

3.5 GLUTATHIONE-S-TRANSFERASE WESTERN BLOT ANALYSIS.

Cytosolic preparations of the following cell lines were extracted to examine by immunoblot for the expression of GST μ,α, and π, using polyclonal antibodies raised in rabbit. This was to reinforce the result already obtained from the DNCB total GST assays already performed on the cells Figures 35 to 39.

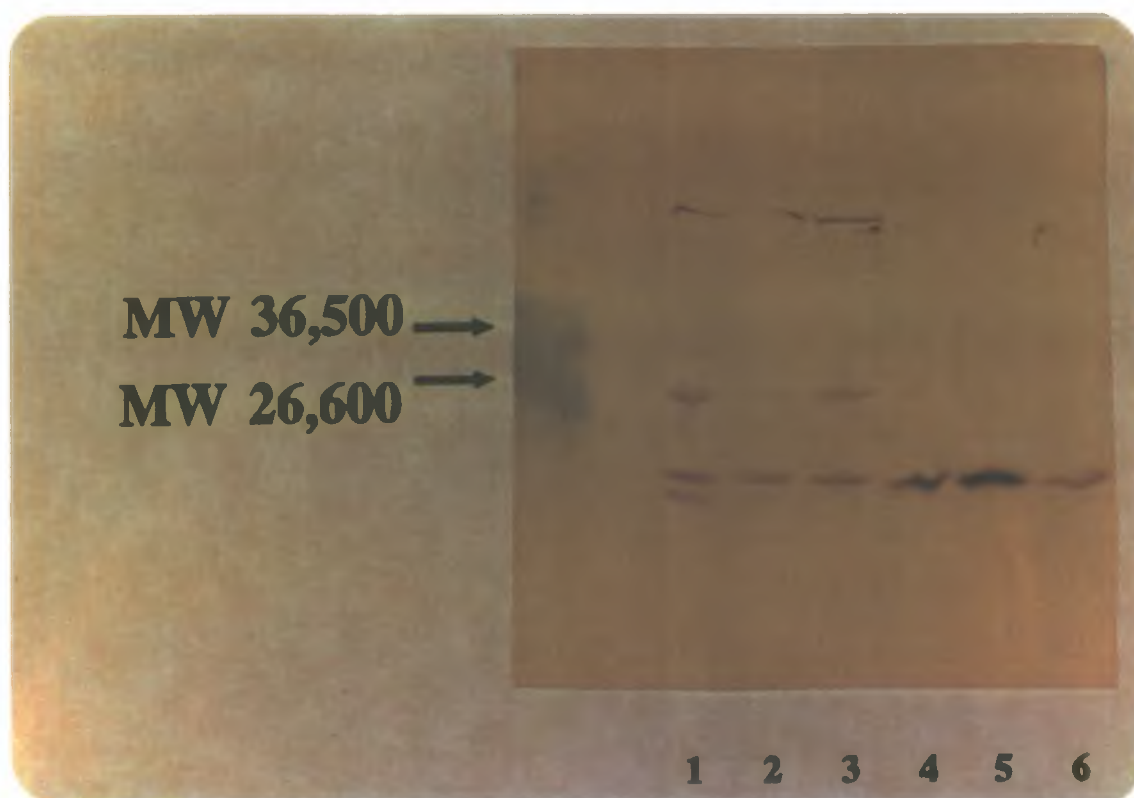


Figure 52: Western Blot analysis of DLKP and its adriamycin resistant line DLKPA for GST π. 20 μg of protein was separated per lane as per section 2.12. 1 + 2: DLKP parental line; 3 + 4: DLKPA in 2 μg/ml adriamycin; 5: Standard GST π antigen from Biotrin; 6: DLKPA out of adriamycin. As can be seen there is very little difference between the parental and the resistant lines with regard to GST π level. This pattern can also be seen in the DNCB GST enzyme assay figure 39. These lines were also probed for GST α and μ and were negative for both.

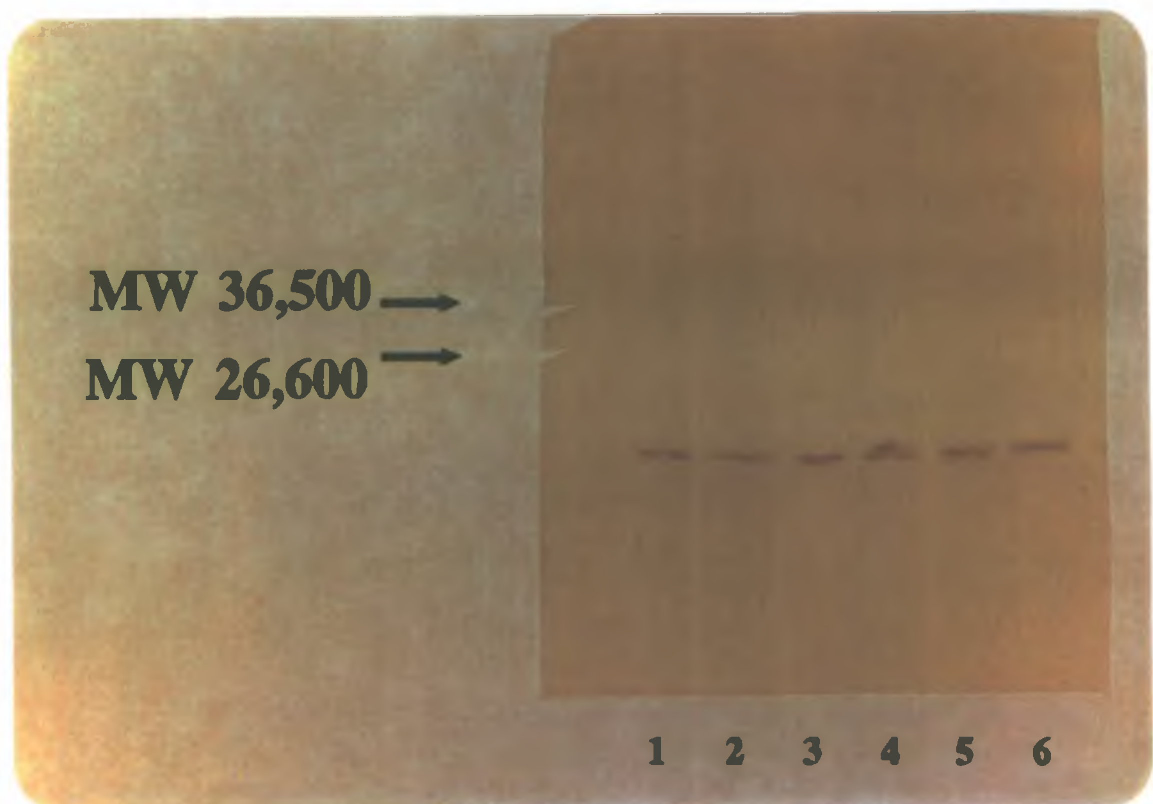


Figure 53: Western Blot analysis of SKMES-1 and its adriamycin resistant form SKMES-1A. for presence of GST π. 10 μg protein was separated per lane as in section 2.12. 1 + 2: SKMES-1 parental line; 3 + 4: SKMES-1A in 1μg/ml adriamycin; 5: SKMES-1A out of adriamycin; 6: Standard GST π antigen Biotrin. These results confirm the results obtained by DNCB enzymatic analysis figure 38, that there is little difference in the levels of GST π brtween SKMES-1 and its resistant counterpart. Blots for GST α and μ were negative for this cell line.

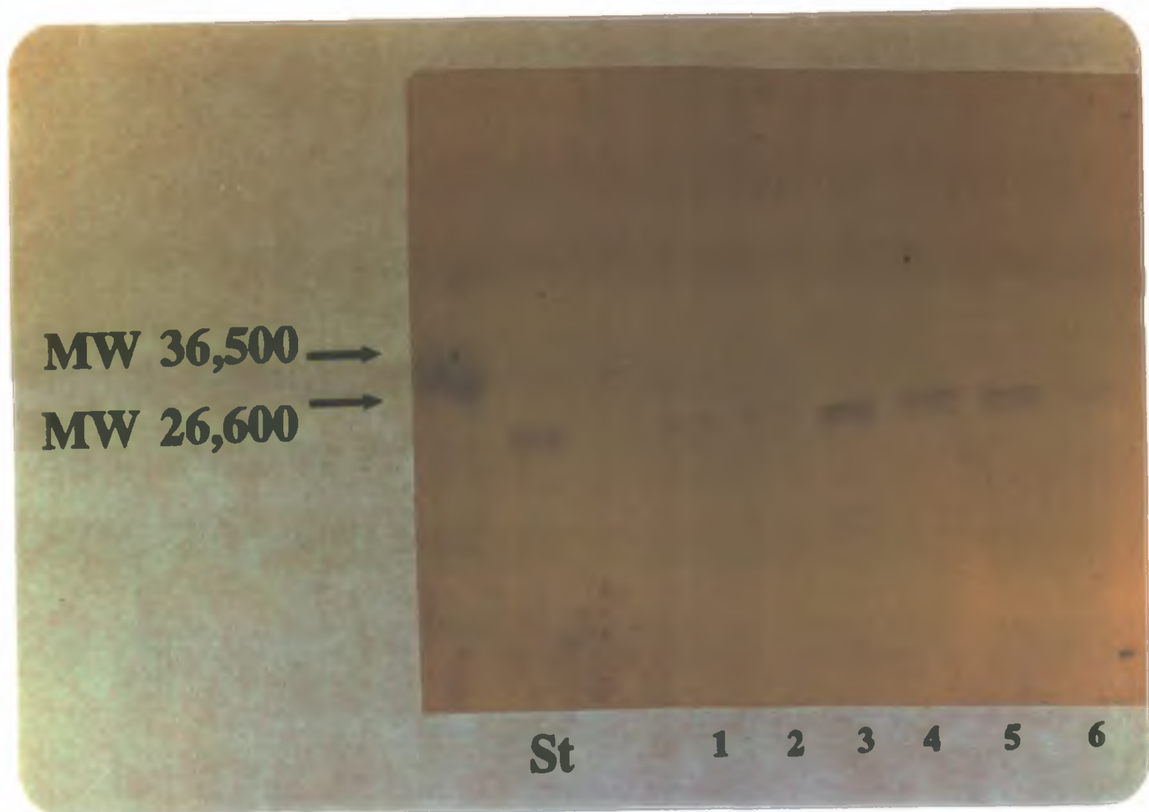


Figure 54: Western Blot analysis of SKLU1 and its adriamycin resistant form SKLU1A for GST π. St standard is the GST π antigen. 1 + 2: SKLU1 parental line; 3 + 4: SKLU1-A in 0.5 μg/ml adriamycin; 5 + 6: SKLU1-A out of adriamycin. These results confirm those of figure 37, that there is an elevated level of expression GST π protein and activity in SKLU1-A cells in comparison with the parental sensitive line. It is possible that this elevated level of expression is affecting the resistance of the SKLU1-A cell lines. Blots for GST α and μ were negative for this cell line.

3.6 TOXICITY TESTING OF A NUMBER OF CELL LINES.

The Chinese Hamster cell line V79 or the human squamous cell carcinoma DLKP contains no detectable Cytochrome P450 activity The V79 line was transfected with the Rat Cytochrome P_{450} 1A1 gene (Dogra *et al* , 1990) The line that resulted XEM2 expressed Aryl Hydrocarbon Hydroxylase activity V79 was also transfected with rat P450IA1 in this laboratory and a clone V791A1-7 was isolated which expressed higher activity of AHH than XEM2 The connection between CYP1A1 activity with Multiple Drug Resistance (MDR) has been made on a number of occasions It has been suggested that depression of CYP1A1 and elevation of expression of CYP1A1 could contribute to MDR It was decided to investigate the toxicity of a number of anticancer agents on these three lines, V79, XEM1, V791A1-7, using adriamycin, vincristine, and VP16 Figures 55-57

To demonstrate the activating ability of the transfected lines XEM2 and V791A1-7 over the parental line, the toxicity profile of these three lines on exposure to benzo[a]pyrene is seen in figure 58

The activation ability of CYP2B1 is investigated in figure 59 Cyclophosphamide is an anticancer agent which requires activation before it becomes toxic The cytochrome P450 dependent enzymes 2B1 is largely responsible for this action The SD1 cell line is V79 transfected with the CYPIIB1 gene and expressing the protein CYP2B1 To show its activation ability the toxicity profile of V79 was compared with SD1 DLKP and DLKPA were also exposed to cyclophosphamide and appear to activate this drug to the same degree as SD1

Figure 60 displays the resistant nature of SKLU1A over its parental line SKLU1 Figures 61 and 62 demonstrate the effect of BSO, which inhibits glutathione production and prevents GST action on this resistance As already demonstrated in figure 37, SKLU1A displays a different pattern of GST π than its parental line SKLU1 and it was possible that this elevation in GST activity played a part in the resistance of the cells

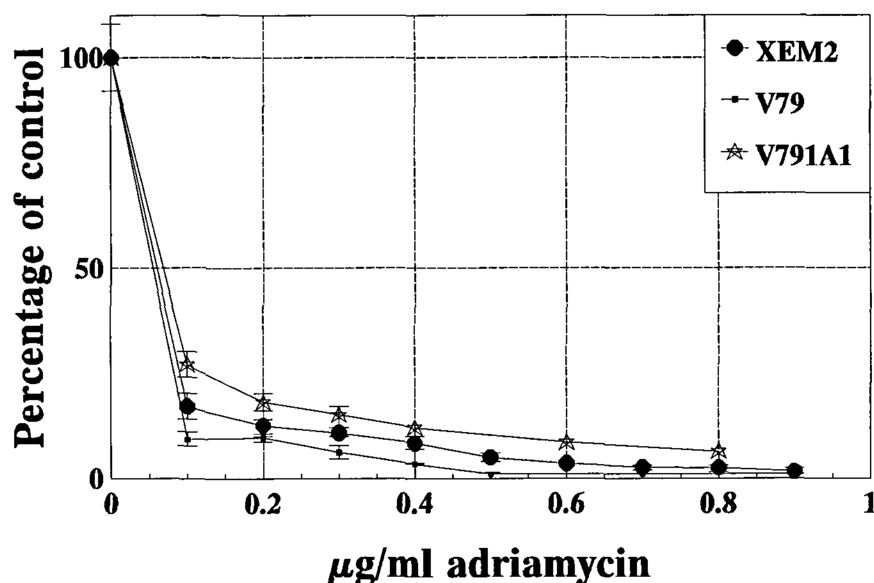


Figure 55: Toxicity of adriamycin on V79, XEM2 and V791A1-7 cells Cells were pretreated as per methods and seeded at a density of 5 x 10^3 cells per ml into 96 well tissue culture plates on day one Twelve hours later, when the cells had attached adriamycin was added The plates were incubated at 37°C and 5% CO_2 for 5 days Results were determined as a percentage survival in comparison with a control which was not exposed to drug End-point was the acid phosphatase assay V79 is a hamster foetal lung line which contains no CYP1A1 XEM2 is V79 which has been transfected with rat CYPIA1 gene and expresses CYP1A1 protein - AHH activity (Doehmer *et al*, 1990) V791A1-7 is V79 line transfected with rat CYPIA1 in this laboratory This V791A1-7 expresses functional CYP1A1 protein at a higher activity than XEM2 As can be seen from the results, each line has a similar toxicity profile The IC_{50}s for each line are within standard deviation of each other and no major difference is apparent The levels of expression of CYP1A1 have been linked with the phenomena of Multiple Drug resistance In some cases increased expression of this enzyme has been associated with increased metabolism of anticancer agents to their less toxic form resulting in decreased toxicity Other have suggested that decreased expression of this enzyme leads to non activation of certain cytotoxic agents therefore these agents are not converted to their cytotoxic form and no tumour kill is recorded

Toxicity of Vincristine on V79, XEM2 and V791A1-7 Cells.

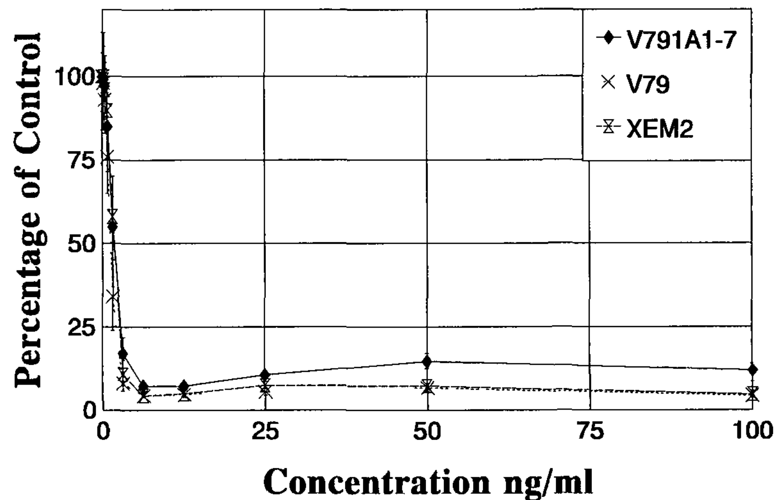


Figure 56: Toxicity of Vincristine on the foetal hamster cell line V79 - parental, XEM2 - V79 which was transfected with rat cytochrome P450IA1 gene (Doehmer *et al*, 92) and V791A1-7 - V79 cell line transfected in this laboratory with rat CYPIA1 gene Cells were pretreated as per materials and methods, seeded at density of 5 x 10^3 cell/ml in 96 well plates Vincristine was added 12 hours later when cells had attached Plates were incubated at 37°C in 5% CO_2 for 5 days Acid phosphatase assay was the endpoint indicator As can be seen from the graph there is little difference in the toxicity or metabolism of vincristine between the cells containing CYP1A1 enzyme activity *i e* XEM2 and V791A1-7, and V79 the parental line which does not

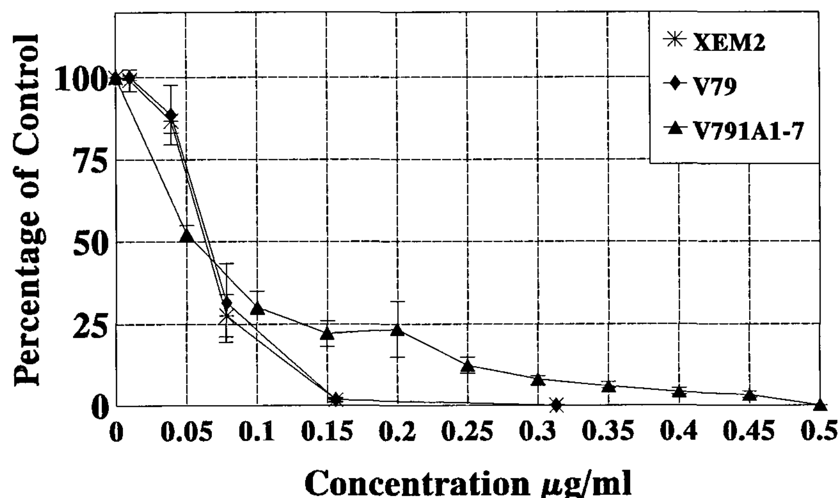


Figure 57: Toxicity of VP16 on V79, XEM2 and V791A1 cells Cells were pretreated at as per methods Cells were seeded at a concentration of 5 x 10^3 cells per ml into 96 well tissue culture plates on day one VP16 was added on day two 12 hours after the plates were seeded, to allow the cells to attach Plates were incubated for 5 days at 37°C in 5% CO_2 Toxicity was determined as percentage survival in comparison to a control which was not exposed to toxin End-point was determined by Acid phosphatase assay As can be seen from the graph, there is no obvious difference in toxicity between the V79 foetal hamster cells and XEM2 There appears to be an increased resistance to Vp16 in V791A1-7, however the IC_{50} of all three line are very similar

XEM2 - V79 cells transfected with rat CYPIA1 gene which codes for CYP1A1 protein *i e* AHH activity (Doehmer *et al*, 1990), V791A1-7 transfected with rat CYPIA1 gene in this laboratory and expressing higher levels of 1A1 activity

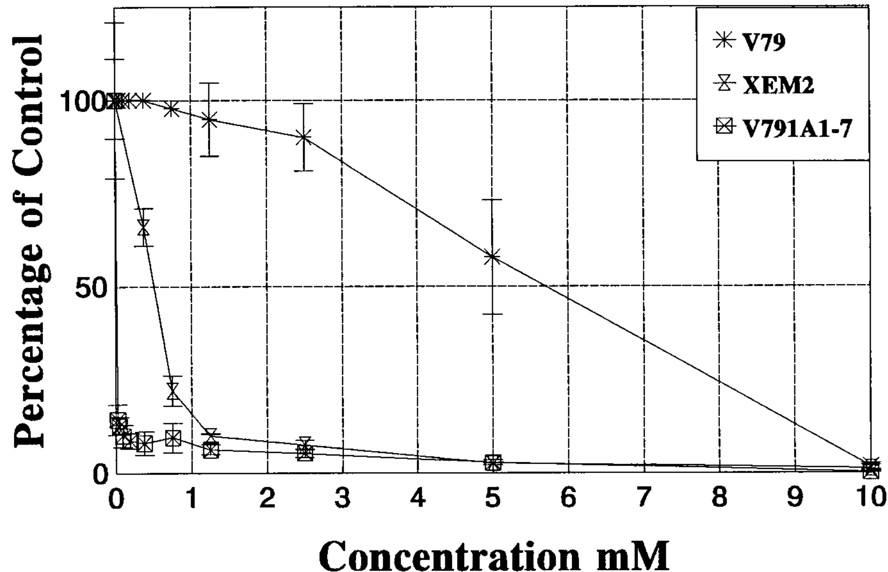


Figure 58: Toxicity of Benzo[a]pyrene on V79, XEM2 and V791A1-7 Cells were seeded on day one at 5 x 10^3 cells per ml into a 96 well tissue culture plate Benzo[a]pyrene was added 12 hours later when the cells had attached Benzo[a]pyrene was dissolved in DMSO so that the final volume would not exceed 0 01% in the assay Plates were incubated at 37°C and 5% CO_2 for 5 days Toxicity was determined by percentage survival in comparison with a control which had not been exposed to benzo[a]pyrene End-point was acid phosphatase As can be seen the parental Line V79 which expresses no CYP1A1 enzyme activity survives completely at the concentrations which kill the other two lines IC_{50} 5 5 mM XEM2 - V79 transfected with rat CYPIA1 gene (Doehmer *et al*, 1990) expresses the protein CYP1A1 or Aryl hydrocarbon hydroxylase This enzyme is responsible to a large extent for the activation of Benzo[a]pyrene to a toxic 3-hydroxybenzo[a]pyrene As a result the XEM2 cells are killed by their own metabolite IC_{50} 0 5 mM The V791A1-7 cells are transfected with rat CYP1A1 enzyme activity also - in this laboratory, these cells express a higher level of CYP1A1 activity and as such display a greater level of toxicity - IC_{50} 0 015 mM

Toxicity of Cyclophosphamide DLKP, DLKPA, V79 and SD1 Cells.

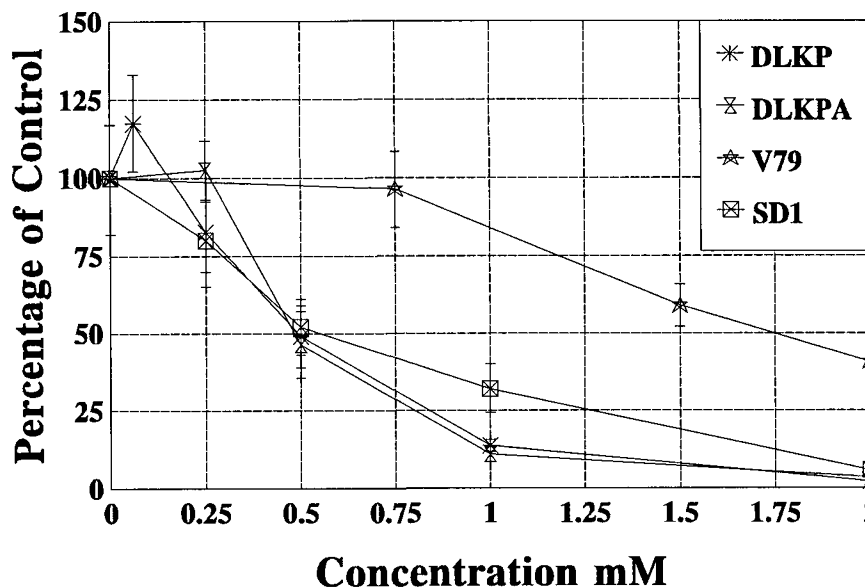


Figure 59: Toxicity of the anti cancer agent cyclophosphamide on four cell lines Cyclophosphamide in its unmetabolised form is nontoxic The enzyme which carries out this function is a cytochrome P450 dependent hydroxylase - CYP2B1 V79 is a foetal hamster cell line which contains no 2B1 activity, SD1 is the V79 cell line which has been transfected with rat CYPIIB1 gene and expresses the CYP2B1 enzyme DLKP is a human lung squamous cell carcinoma, DLKPA is the adriamycin resistant form of DLKP Each line was seeded at 5 x 10^3 cells per ml in a 96 well plate on day one Cyclophosphamide was added on day two at the concentrations seen in the graph Percentage survival in comparison with a control, was measured using the Acid Phosphatase assay As can be seen from the graph, the transfected SD1 cell line activates cyclophosphamide to its toxic form much more efficiently than the parental line V79 The lung cell lines SKLU1 and SKMES did not activate cyclophosphamide but, both DLKP and DLKPA did, which is surprising, as they do not appear to express any Cytochrome P450 dependant enzymes as can be seen in table 6 section 3 2

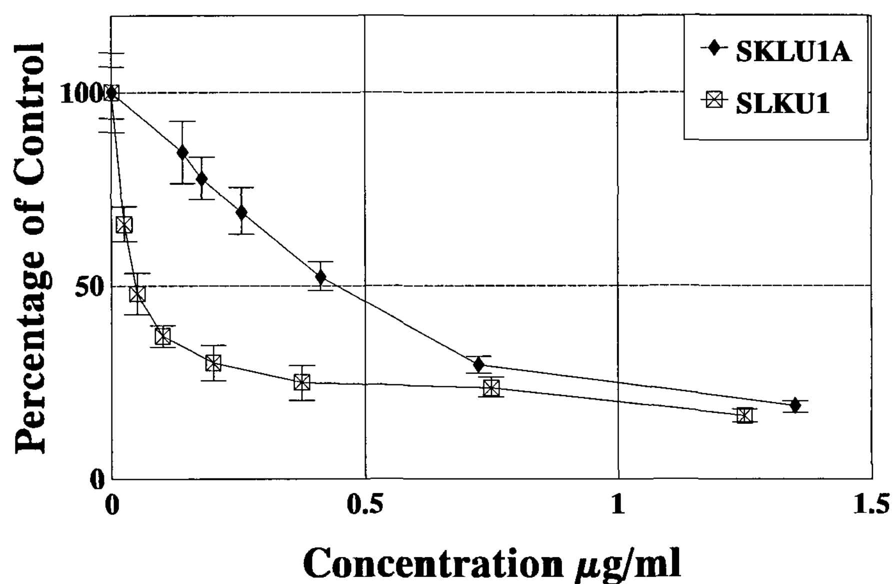


Figure 60: Toxicity of Adriamycin on SKLU1 - human adeno cell carcinoma of the lung and SKLU1A - the adriamycin resistant (0 2 µg/ml) form of SKLU1 Cells were seeded in 96 well tissue culture plates at 5 x 10^3 cell per ml on day one Adriamycin was added 12 hours after seeding when the cells had attached Plates were incubated at 37°C and 5% CO_2 for 5 days Toxicity was determined as a percentage survival in comparison with a control which had not been exposed to adriamycin As can be seen the resistant line had an LD_{50} much greater approximately 6 times greater than that of its parental line SKLU1

3.7 INVESTIGATION OF SKLU1A - ADRIAMYCIN RESISTANT CELLS

SKLU1A cells have an IC_{50} of 0 04 μg/ml of adriamycin The mechanism of drug resistance displayed by these cells is unknown Expression of P-glycoprotem has been investigated, but no detectable levels of this protein were recorded Therefore it was decided to investigate the xenobiotic metabolic profile of these cells The AHH level - the enzyme mostly associated with resistance was investigated in both an enzyme assay and western blot - no expression was detected However on assaying for GST total using DNCB elevated levels of GST were detected - see Figure 37 Western blot analysis confirmed the increased expression of GST in SKLU1A over SKLU1 cells and that this expression was of the isoform π, a form often associated with cancer (Tsuchida and Sato 1992, Moscow to be published) To investigate the effect of this elevation of GST π on MDR the GSH BSO was employed If the mechanism of resistance is GST dependant, then addition of BSO should circumvent this resistance by inhibiting the production of GSH and thereby inhibiting GST action Figures 61 and 62 depict the effect of BSO, adriamycin, and BSO in the presence of adriamycin on SLKU1 - parental control cells, and SKLU1A - the adriamycin resistant form

As can be seen from figure 62 addition of BSO combined with adriamycin results in cell death however not as much as BSO alone In the parental line the pattern is different, BSO alone causes a significant cell kill, however addition of BSO with adriamycin resulted in far greater cell death than BSO alone This implies that in the parental line the residual resistance at 0 05 μg/ml is circumvented by addition of BSO, however this does not seem to be the case in the resistant form figure 62 The resistant cells appear to be far more sensitive to BSO than the parental line when no adriamycin is present, however on addition of adriamycin, a degree of resistance seems to return to the cells This is in contradiction to what we expected, and warrants further investigation Perhaps the loss of GST activity in the presence of both BSO and adriamycin opens the way for another mechanism of resistance to operate, a mechanism quite apart from a GST orientated one This also suggests that the SLKU1A mechanism of resistance is not GST dependant, and that the elevated GST π levels maybe a symptom rather than the cause of resistance as suggested by Center M S 1993 (in publication)

Toxicity of SKLU1 cells to BSO - in the presence of adriamcyin

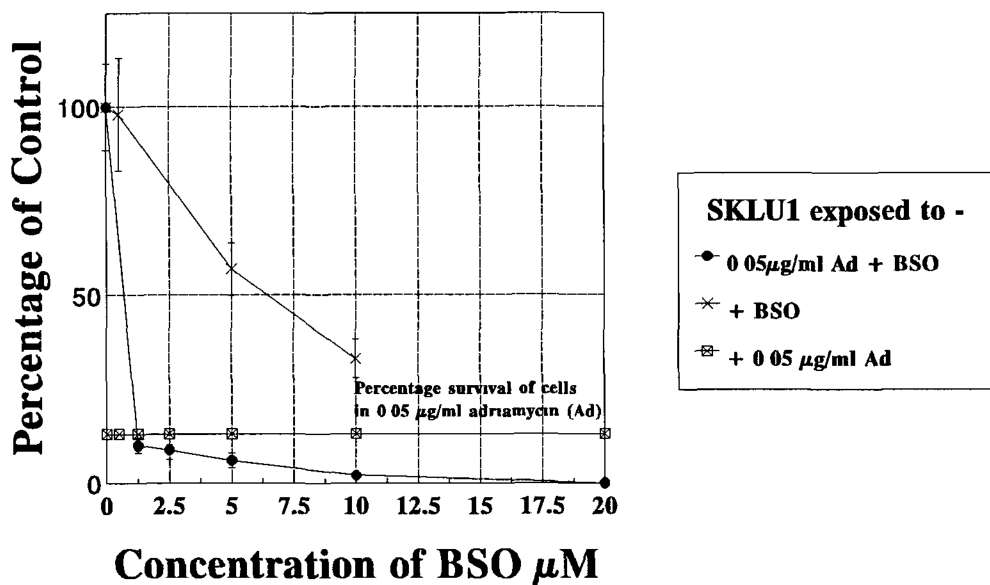


Figure 61 Toxicity of BSO on SLKU1 parental non resistant cell line to demonstrate the effect of inhibition of G-S-H on the parental line

Toxicity of SKLU1A cells to BSO - in the presence of adriamcyin

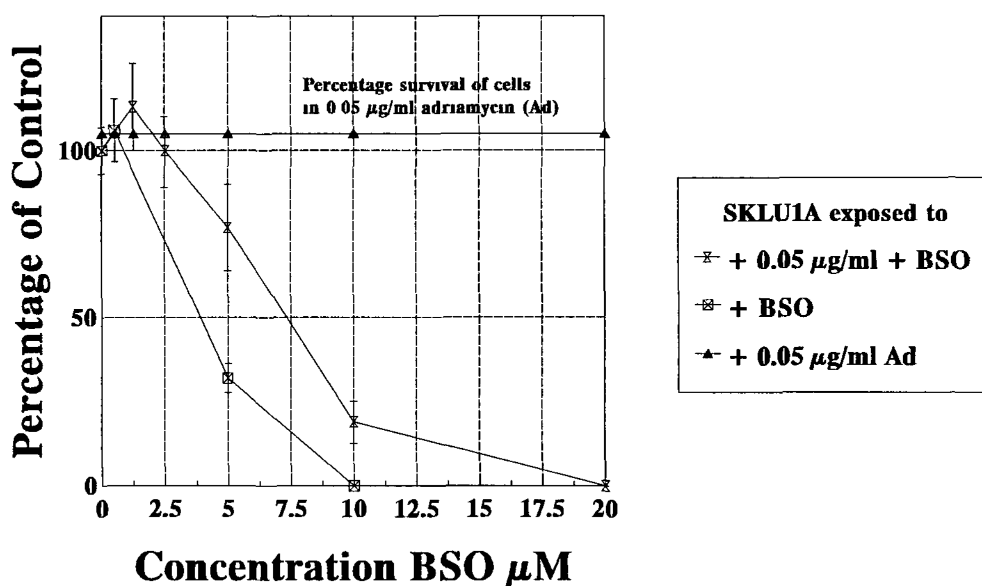


Figure 62 Toxicity of BSO on SKLU1A adriamycin resistant cells to investigate if BSO would circumvent resistance in these cells by inhibiting G-S-H production

4.0 DISCUSSION

4.1 *In Vitro* toxicity testing - application to Cancer therapy.

"The objective of toxicity testing is to develop an adequate database to make reasonable and reliable judgments concerning the safe use of chemicals for societal needs" (Frazier *et al* , 1991) However, due to the sheer volume of new chemicals which require testing, the cost and ethical implication involved with whole animal studies, other methods for the determination of toxicity are required

The advancement of cell, tissue and organ culture in the last few years has provided a feasible solution to this problem Cell culture is not only a valuable tool in the assessment of general toxicity, but also has important implications in experimental and clinical situations

Different forms of cancer react with varying degrees of success to chemotherapy and in different people, similar cancers also exhibit individual degrees of sensitivity Primary culture provides the possibility for individual toxicological and chemosensitivity prediction and a model of the disease for study (Salmon *et al* , 1978, Yamashita *et al* , 1989, Kobayashi *et al* , 1989, Von Hoff, 1990)

In vitro chemosensitivity predictive assay technology *i e* the use of primary culture on biopsies to predict a course of chemotherapy, has been applied, for a number of tumours *e g* bladder tumours using trypan blue dye exclusion (Irisawa *et al* , 1992), renal, urothelial and testicular tumours assaying intracellular ATP levels (Yamada *et al* , 1992), small cell lung cancer using the MTT assay (Campling *et al* , 1991), Gastrointestinal tumours using a combination of a three-dimensional histoculture and MTT endpoint and cell suspension assay with MTT endpoint (Furukawa, *et al* , 1992, Yamaue *et al* , 1992)), Osteosarcoma using ABA - Adriamycin binding assay (Baldini *et al* , 1992)

In the case of lung cancer the development of a reliable *in vitro* system which will further the investigation of the disease is required For Small Cell Lung Cancer (SCLC) patients there is an accepted chemotherapeutic regime due to the *in vivo* sensitivity of the disease to these agents However, many patients who initially respond to chemotherapy, develop resistance to this treatment over time, whilst a percentage of the population are resistant at the onset (Gazdar *et al* , 1990) For Non Small Cell Lung Cancer - adeno-, squamous and large cell undifferentiated carcinoma of the lung surgery is the only course normally followed, as these cancers are relatively chemo-resistant (Vescio *et al* , 1990, Gazdar *et al* , 1992)

As a result of resistance presenting during treatment, precious time is lost because the patient is following a course of therapy to which their cancer is insensitive

To address this problem the use of *in vitro* primary cell culture has been implemented This involves toxicity testing with chemotherapeutic agents, using the patient's own tumour cells obtained at biopsy (Salmon *et al* , 1978, Kobayashi *et al* , 1989) The results obtained should in principle enable the oncologist to tailor the chemotherapeutic agent to which the individuals tumour is most sensitive, assuming a direct correlation with the *in vivo* behaviour (Van Hoff *et al* 1990, Gazdar *et al* , 1990, Wilbur *et al* , 1992)

Our interest in this area was to improve the longevity of samples so that as much work as possible may be carried out on each sample We concentrated on non-small cell lung carcinoma for a number of reasons, 1) the fact that therapy for this particular cancer has remained static for a number of years (Wilbur *et al* , 1992), 2) the inherent chemo-resistance displayed by nonsmall cell carcinomas 3) and (probably the most important factor) - the availability of samples Over the three years of this part of the project, the majority of the samples received were small solid biopsies These samples were either from bronchoscopy or mediastinoscopy, or pure samples of tumour taken during lobectomy (Figure 8) The samples were immediately placed in 4°C DME/Ham's F12 (50/50 v/v) medium containing 10% FCS (see section 2) Samples were then removed to the laboratory where they were enzymatically disaggregated, seeded into flasks and placed at 37°C

There are many problems associated with the primary culture of tumour biopsies and the subsequent toxicity tests These include heterogeneity of the cell population within the tumour *i e* a mixed population of normal, tumour and blood cells *etc* , clump artifacts which result from difficulties in dissociating the tissue, density of cells required to assay, length of incubation (before results become available) inconclusive end point determination, destruction of the cell-cell interaction as experienced in the *in vivo* situation (Hoffman, 1991) We encountered a number of these problems in our endeavours to establish new non small cell lung carcinoma cell lines, with a major problem being that a great many of the samples received through bronchoscopy/mediastinoscopy were necrotic Of these samples only 34%(26) attached, the remaining 66%(49) displayed no attachment and no growth Of the 26 samples that did attach, 1 showed no further signs of growth, 7 were purely fibroblasts The remaining 69 2% continued to grow 12 of these sample became fibroblastic, 6 differentiated and died off in culture However one sample was passaged to nontransformed cell line status, a poorly differentiated squamous cell carcinoma of the lung derived from a mediastinal node biopsies The cell line was designated DLKP

Fibroblast invasion of epithelial colonies is a fundamental problem with primary culture Generally the tumour samples we obtained were heterogenous *i e* made up of many cell types including normal epithelial and fibroblast These fibroblasts are activated *in vitro* when the tissue becomes traumatized to protect it (analogous to scar tissue formation *in vivo)* The surgical procedure and mechanical disaggregation which the samples were subjected to is likely to have activated these cells *in vitro* In the normal course of events these cells should die off within a few passages due to the fact that they are normal cells and normal untransformed cells do not survive in continuous culture beyond about 12 passages at a maximum However, except in the case of very large samples, the number of surviving epithelial tumour cell is severely compromised by these rapidly proliferating normal cells (figure 9 and 10) The result is over-growth of the culture by the fibroblasts cells which surround any malignant epithelial colonies that have developed, preventing them form expanding and leading to colony death It has also been reported that fibroblast produce a soluble anti-tumour substance that is active against a wide range of cell lines established from human and mouse solid tumours, thereby inhibiting epithelial development (Kirk *et al* , 1993)

A number of avenues were explored to prevent fibroblasts killing off potential cell lines 1) Differential attachment *i e* fibroblast cells are believed to attach at a faster rate than epithelial cells (Freshney, 1987) Due to the fact that the samples were small, the large number of cells required for the work was not often available, however on those occasions when there were enough cells, we observed no difference in attachment time 2) Differential trypsinization using 0 02% EDTA + 0 01% Trypsin at 37°C (figure 11) In theory the fibroblasts detach faster than the epithelial cells because they have less inter cell association through desmosome We found that the fibroblast were selectively removed but they returned, often resulting in complete over-run of the culture 3) Treatment of cultures with the antibiotic Geneticin, G-418 sulphate, at a concentration of 100μg/ml for 2 days was found to select melanocytes from fibroblast by Halaban and Alfano (1984) While the samples which showed fibroblast contamination did show a reduced fibroblast population using this procedure, due to the inherent variability of each individual sample, many epithelial samples were also terminated

We had limited success with fibroblast eradication, but there are several procedures which we did not have time to explore *e g* selective coating of plates, anti-fibroblast anti-body treatment (Paradkeva *et al* , 1985) and growth on polypropylene (Wilbur *et al* , 1992)

As can be seen from figure 8, 40%(4) of the lobectomy samples showed initial growth, but in each case this resulted in fibroblast-associated termination of the culture

50%(1) of the metastatic sarcoid tumours attached and displayed initial growth, but differentiated within 2 weeks

Neither of the Oesophageal tumours or the Hodgkins Lymphoma sample received showed growth of any form *i e* epithelial or fibroblast

Of the two oat cell carcinomas (small cell carcinoma) one was promising and displayed initial growth but after 3 weeks it was apparent that the cells were not dividing and had differentiated, (there was no attachment as these cells grow in suspension) The environment into which the cells were introduced - a tissue culture treated 25 cm^2 flask, favours attachment dependant cell Squamous cell carcinoma are attachment-dependent but so too are normal cells and normal fibroblast Tumour cells will support their own growth in suspension and in agar, while normal cells will not The DST - drug sensitivity test, capillary cloning system, employed by Van Hoff, requires less cells than a 96 well plate assay and was extendable to multiple tumour types This procedure involves the growth of the cells in a suspension culture in a capillary tube, the end point of the assay is determined by the number of colonies which result (Van Hoff *et al* , 1990) As this system is a toxicity assay further growth of the cells is not required However, we want to keep cultures growing for longer periods of time, perhaps this system would serve as an initial starting culture Primary single cell sample cultures could in principle be established without the threat of fibroblast and normal cell contamination due to the suspension nature of the culture The sample could then moved on to larger flasks for continued culture Wilbur *et al* employed polypropylene culture tubes which inhibits the attachment and growth of normal cells (Wilbur *et al* , 1992) Gazdar and Campling established cultures directly into flasks and plates but restricted their work to suspension culture of small cell lung carcinoma, for which a serum free medium has been developed (Gazdar *et al* , 1990, Campling *et al* , 1991)

Disaggregation of the sample using a less aggressive approach may reduce the activation of fibroblast and increase the viability of the epithelial tumour cells At present we use a combination of collagenase IV and diaphase, other enzymes like hyuronidase or other forms of collagenase maybe more effective at dissociating the tissue, and less activating of fibroblasts Reducing the amount of mechanical disaggregation using scalpels and the use of magnetic stirrers to break up the sample There are many angles from which to approach the many problems attached to this area The possible clinical ramification of this work makes it important that each alternate possibility is explored

4.1.1 CHARACTERISATION OF DLKP

As can be seen from figure 8, of the 93 human biopsies samples obtained, one non-small cell poorly differentiated squamous cell carcinoma cell line was developed This sample was from a secondary metastasis from the lung, obtained from a mediastinal investigation from a male patient aged 54, with a history of smoking, who had been diagnosed with poorly differentiated squamous cell carcinoma On transfere to culture, immediate growth was observed and there was little to no fibroblast involvement The sample has been in continuous culture in the laboratory and has reached passages upward of 60

To characterise this cell line it was decided to investigate a number of features which are characteristic of squamous cell carcinomas of the lung

4.1.1.1 CLONING IN AGAR

Our first objective was to ensure that the cell line showed malignant behaviour *in vitro* Normal cells cannot grow in agar whereas tumour cells can (Bergh *et al*, 1981, Luster *et al*, 1985) DLKP cells were seeded at varying density and cultured for 10 days, after which a colony forming efficiency of 7 4% was observed at 10^3 cell per ml (Figure 29)

4.1.1.2 HISTOLOGY AND CYTOGENETICS:

Histological analysis also identified multiple nucleoli, tri- and multipolar mitosis (figure 28), features normally associated with malignancy (Gilvarry *et al*, 1991) Cytological analysis also revealed eleven structurally abnormal chromosomes or marker chromosomes, three of which del(1)(q11), del(2)(p11 1) and del(2)(q11 1) are in common with another non small cell squamous carcinoma line, DLRP, also developed in this laboratory Abnormalities of chromosome 1 are often associated with malignancy Over-representation of chromosome 7 was also observed along with duplicate copies of markers 1,4 and 5 DLKP exhibits two populations with modal numbers of 56 and 115 (Law *et al*, 1992)

- The sum of these results confirms the malignant nature of DLKP

Once this was established, it was necessary to prove that this line was 1) of human origin, and 2) derived form the biopsy we had received and not a contaminant derived from another cell line in the laboratory

4.1.1.3 ISOENZYME ANALYSIS:

To verify the human origin of the sample isoenzyme analysis was carried out (Figure 26) This consisted of comparing six enzymes which displayed varying gel electrophoresis patterns depend on species The enzymes chosen were Lactate Dehydrogenase (LDH), Glucose-6-phosphate Dehydrogenase (G-6-D), Malate Dehydrogenase (MD), Mannose Phosphate Isomerase (MPI), Peptidase B (PB) and Nucleoside Phosphorylase (NP) LDH is a tetramer made up of 2 subunits A and B These subunits can be arranged as LD1-5, AAAA, which migrates to the cathode, AAAB, AABB, ABBB, BBBB which migrate to the anode (another form CCCC is seen only in testis and sperm) LDH isoenzymes are present in all tissues but the ratio of the different form varies In some instance not all isoforms can be detected As can be seen from the figure 26 the enzyme LDH in the rat demonstrated by the cell line NRK (normal rat kidney), only 3 isoenzymes are detectable, whereas the other lines tested all 5 isoenzymes are detectable MD is composed of two isoenzymes, one form migrates to the anode which is the cytosolic form, the other mitochondrial form migrates to the cathode Migrational distance is the deciding factor with this enzyme Once again NRK line shows different migration distances from the human lines tested From these results we can conclude that our line is not of rat origin G-6-P is made up of 2 identical subunits and migrates towards the anode In humans there are two forms A & B Type B is mainly expressed in the Caucasian population and migrates slower than type A which is expressed in all other populations This particular isoenzyme is useful in detecting HeLa contamination in cell lines As can be seen there is no HeLa contamination due to the slow migration of this enzyme in the DLKP sample MPI, PEP-B and NP all exhibit species-dependent migrational profiles In comparison with other cell lines assayed for these enzymes as seen in Figure 26, SKLU-1 human adenocarcinoma,CALU-3 human adenocarcinoma,RPMI human nasal carcinoma,HTB-120 human small cell carcinoma,and NRK rat kidney,it can be concluded that DLKP is of human origin

4.1.1.4 DNA FINGERPRINTING:

To confirm that the exact origin of DLKP was the surgical sample obtained, DNA from DLKP and the original tumour which had been frozen in liquid nitrogen, were subjected to DNA fingerprinting by Cellmark Diagnostics (Figure 27a +b), using cocktails of single locus probes Due to poor condition of the samples when they arrived for analysis the bands in the profile from the tumour sample are not very clear and there is an amount of background due to extensive DNA degradation There is one high molecular weight band in the cell line which is not present in the tumour

This may be an artifact of degradation as high molecular weight alleles are degraded preferentially in DNA samples However, 9 bands in total can be matched between the tumour and in the cell line Based on these results Cellmark determined that tumour sample and cell lines were the same

- To this point we have concluded that the cell line DLKP is a malignant line, of human origin, and derived form the surgical samples we received To determine the cell type we used immunofluorescent labelling

4.1.1.5 IMMUNOFLUORESCENCE ANALYSIS:

Cytokeratin is a component of the intermediate filaments in epithelial cells Keratins are particularly prominent in cells which are subjected to mechanical stress *e g* epithelial cells There are 19 distinct forms of keratin in human epithelial cells and different cell types display individual patterns of keratins This provides a distinctive "fingerprint" which can then be used to trace cells, especially metastatic tumour cells to their tissue of origin

In characterization of DLKP, this technique was employed DLKP was exposed to anti-cytokeratin No 18 and compared with SCC-9 (derrived form a squamous carcinoma of the tongue) and MRC-5 a human foetal lung cell line As seen from the results Figure 23 SCC-9 is positive for cytokeratin, MRC-5 is negative (figure 24) DLKP stained negatively (figure 25) for this form of cytokeratin This experiment was repeated using anti-cytokeratin Pan (a combination of all the cytokeratins) as the primary antibody, the result obtained was the same, negative for DLKP, which is surprising The premises for using these antibodies is that a given epithelial cell type generally retains its specific cytokeratin polypeptide patterns in neoplasms derived from those cells, irrespective to the degree of differentiation and distance metastasized However, it has been reported that cytokeratins are not present in all epithelial cell types or in all poorly differentiated carcinomas arising from epithelial cells that contain these filaments (Erlandson, 1984, Wheater *et al*, 1985) Erlandson also reported that the cytokeratin patterns may vary with different stages of differentiation of a given cell type and that some cells may produce other intermediary filament, vimentin, instead of cytokeratin Another study on intermediary filaments of lung tumour cell lines over a four year period, reported that 1) classic small cell lung cancer cell lines normally expressed cytokeratins 8, 18 and occasionally 19, however, variant-type SCLC, however, expressed no cytokeratins, but did express neurofilaments In non small cell lung cancer cell lines, expression of cytokeratins 7, 8, 18 was detected and in some cases anti-cytokeratins 4, 10 and 13 were also noted(Broers, *et al*, 1988) They also noted that in the four years some of the variant SCLC reverted to

classıc demonstratıng a correspondıng re-expressıon of cytokeratıns   Boyer *et al* , (1990) reported that remodellıng of tumour cells cytoskeleton may be ınvolved ın the detachment of malıgnant cells from the prımary tumour allowıng for ınvasıon of neıghbourıng tıssues   They found that exposıng a rat bladder carcınoma cell lıne to a soluble ınducer (acıdıc FGF) or to collagen resulted ın transformatıon of the cell ınto a fibroblast lıke cell wıth the abılıty to penetrate ınto the collagen gel

These reports ındıcate that not all tumour cells behave ın the same manner wıth respect to cytokeratın expressıon and that over tıme ın culture and durıng progressıon of the dısease especıally metastsıs rearrangement of cell components may occur

DLKP was orıgınally dıagnosed as a poorly dıfferentıated squamous cell carcınoma of the lung whıch has metastasızed to the lymph nodes   It ıs possıble that ın the progressıon of dısease the tumours cell's orıgınal ıntermedıate filament proteın expressıon may have been rearranged, or ıt ıs also possıble that sımılar to the varıent SCLC lınes mentıoned by Broers *et al* , (1988), DLKP may sımply not express cytokeratın   However, before comıng to any final conclusıon ıt would be necessary to test DLKP wıth the complete range of specıfic monoclonal antıbodıes specıfic for the dıfferent forms of cytokeratın

EP-16 antıbody ıs specıfic for antıgen on the surface epıthelıal cells (Hamburger *et al* , 1985) Once agaın SCC-9 staıned posıtıve (figure 20) and MRC-5 negatıve (figure 22)   In thıs ınstance DLKP staıned posıtıve (figure 21), but weakly posıtıve whıch reınforces the result obtaıned from the antı-cytokeratın results

704A1 and 703D4 are IgG monoclonal antıbodıes developed by Mulshıne *et al* , *(1983),* whıch are specıfic for non small cell lung carcınoma

Comparıson of DLKP wıth the establıshed Squamous cell carcınoma of the lung -SKMES-1 whıch ıs posıtıve (Fıgure 14), the Adenocarcınoma of the lung -SKLU1 also posıtıve (Fıgure 17) and HTB-120 the small cell carcınoma of the lung - the negatıve control (figure 19), DLKP staıns posıtıvely for both antıbodıes 704A1 and 703D4 (Fıgures 15 & 18)

Together all these results confirm the poorly dıfferentıated non small cell carcınoma of the lung hıstology, as per the pathology report

4.1.1.5 ENZYMATIC ANALYSIS OF DLKP.

Analysıs of xenobıotıc metabolısm ın DLKP revealed no detectable expressıon of P450-dependent Ethoxycoumarın O-deethylase (table 6),UDP-Gluconyltransferase (table 6),or the Cytochrome P450 1A1 enzyme (table 6), on ınductıon wıth known ınducers or ın the controls

by the fluorescence assay techniques used

Glutathione-S-Transferase class π is the only isoform of GST expressed using a DNCB conjugation assay, confirmed by western blot analysis (Figure 52)

There is no detectable expression of the μ or α isoforms of GST The absence of the isoform μ is of particular interest in that the loss of GST-μ expression has been implicated a marker for greater susceptibility to lung cancer among smokers (Tsuchida and Sato, 1992, Campell *et al*, 1990, Peters *et al*, 1990, Seidegard *et al*, 1990)

In conclusion DLKP is a human poorly differentiated squamous cell carcinoma of the lung, which resulted from a metastasis to the lymph nodes This line has been in culture at present to passage 95 and has a doubling time of 17 hours It expresses no detectable cytochrome P450 dependant xenobiotic metabolising enzymes The phase II GST isoenzyme π is expressed, but GST μ linked with improved resistance to lung cancer, is not expressed

4.2 EXPRESSION OF ENZYMES OF XENOBIOTIC METABOLISM IN CELL LINES

4.2.1

The aim of this section of the project was to investigate the drug metabolising profile of a panel of lung cell lines 1) DLKP the novel human squamous lung cell line which was established and is characterised as part of this thesis, as in the previous section, 2)SKMES1 a human squamous cell carcinoma lung cell line obtained from the ATCC and 3) SKLU1 an adenocarcinoma of the lung also from the ATCC As has been mentioned in the introduction the xenobiotic status of cells in culture is very different to that *in vivo* Cytochrome P450 levels and their response to inducers, decline within the first 24-48 hours of culture for unknown reasons (Grant *et al* , 1985,86,87, Bolluinne *et al* , 1987, Guillouzo *et al* , 1985) This decline in expression results in a severely compromised xenobiotic metabolizing profile for these cells and a frustrating problem for those wishing to employ them in *in vitro* toxicity testing

In principle cell culture provides an ideal substitute for whole animal experimentation It eradicates the problem of interspecies variation of xenobiotic metabolism and allows for parameters to be varied with a greater degree of accuracy than whole animal tests However, as has been discussed, cultured cells do not behave exactly as their *in vivo* counterparts

Although in culture the xenobiotic metabolizing profile is severely altered, some cell lines do retain the ability to express particular enzymes *e g* H411E, the rat hepatoma cell line which on exposure to the polycyclic aromatic hydrocarbon (PAH) - benzanthracene, expresses CYP1A1 (figure 30) (Wiebel *et al* , 1984, Zacharewshi *et al* 1989) Most human cells in continuous culture retain some degree of P450 inducibility, generally the PAH-type induction *e g* the human liver line HepG2 (figure 31) and the human breast carcinoma cell line MCF-7 (Pasco, 1988, Tukey, 1988)

It is this residual expression that interested us and it was decided to investigate other cell lines for their ability to express xenobiotic metabolising enzymes The reasoning behind this was that if the metabolic pathway(s) available in a cell line were known, then a compound could be exposed to this/these enzymes and the resulting metabolites either isolated or allowed to exercise their toxicity on the cells which had metabolised that compound Therefore if a single pathway was expressed in a given cell line, such a cell line would be useful in determining if a given compound was A) metabolised by that particular enzyme or B) a candidate for activation by that enzyme to a more toxic form

The analysis encompassed both phase one and phase two enzymes and especially those enzymes linked with activation and carcinogenesis, *e g* AHH - phase one P450 enzyme associated with activation of polycyclic hydrocarbons in lung cancer The lung as a whole *in vivo* expresses considerable levels of xenobiotic metabolising enzymes This expression is essential for the survival of the tissue due to its direct exposure to the environment The xenobiotic metabolising enzymes reported so far in the lung are, cytochrome P450s including AHH, NADPH cytochrome P450 reductase, epoxide hydrolase, glutathione S-transferase, UDP-gluconyltransferase and sulfotransferase Each individual lung cell type contributes a different degree or assortment of activities *e g* bronchial epithelial cells, Clara and ciliated bronchiolar epithelial cells and type II pneumocytes *etc* , (Baron 1990) Clara cells have been reported on immunocytochemical analysis to be positive for two cytochrome families IIB, IVB and NADPH cytochrome P450 reductase However they were negative for CYP1A1 (Chichester *et al* , 1991) CYPIA activity was localised in the peripheral airways in alveolar epithelium of types I and II and in ciliated columnar and cuboidal bronchiolar epithelium This activity is of particular importance given its implication in the activation of the polycyclic hydrocarbons present in cigarette smoke and smog - benzo[a]pyrene, to its active carcinogenic form (Anttila *et al* , 1991a, Kouri *et al* , 1982, McLemore *et al* , 1989, 1990, Pelkonen et al , 1986, Nakachi, *et al* , 1991)

Elevated expression of GST π has been reported in several forms of cancer (compared with the corresponding normal tissue which also expressed π) GST π has also been implicated in multiple drug resistance (Tsuchida and Sato, 1992, Hayes *et al* , 1991, Tidefelt *et al* , 1992, Moscow *et al* , 1989) In the case of lung tumours, GST π is expressed in both adenocarcinomas and squamous cell carcinomas, but not in small cell lung cancers (Tsuchida and Sato, 1992)

In order to investigate xenobiotic metabolism we initially chose to use a spectrophotometric method for detection the cytochrome P450 dependent activity and UDP-gluconyltransferase activity, as these were simple quick assays For the P450 dependent activity the assay chosen was the *N*-Demethylation of antipyrene (Nash *et al* , 1953) based on the formation of formaldehyde, UDP-gluconyltransferase activity was detected using 2-ammophenol (Bratton and Marshall 1939) Both of these methods were set up using rat liver microsomes However, when the cell samples were subjected to analysis by these methods, no activity was detectable, even in the positive controls At this point it was decided that perhaps these methods were not sensitive enough for the purposes which we intended Therefore three alternative fluorometric assays were investigated.

The P450 dependent 3-hydroxylation of benzo[a]pyrene (AHH) (Nebert and Gelboin, 1968), the P450 *O*-de-ethylation of 7-ethoxycoumarin (ECOD) and the phase II glucuronidation of 4-methylumbelliferone (Liliemblum *et al*, 1982) All assays detected product formation in the nmolar to pmolar range which was essential for analysing cultured cells due to the nominal levels present

As can be seen from the results in figures 30 to 33 the liver lines H411E and HepG2, on induction, exhibited elevated levels of AHH and ECOD which was expected as had previously been described above These result were then confirmed by western analysis (Figures 45 & 47) The phase II enzyme UDP-gluconyltransferase was also detectable in H411E but the level was extremely low and unaffected by the presence of inducers as shown in figures 34

Tables 5 to 8 show the results obtained for the other lines tested, none of the other lung lines or the rat hepatoma HTC demonstrated detectable levels of these three enzymes This deficiency of AHH activity in these lines even under induction was confirmed by western analysis as shown in figures 45-47

This lack of expression, although not unexpected, was interesting due to the fact that lung cells do express xenobiotic metabolising enzymes extensively *in vivo* However judging by the reactions of many other cell types on transfer into culture as already mentioned, the profile of these lung lines is not exceptional

To overcome the lack of expression of xenobiotic metabolising enzymes by the lung cell lines we had chosen, it was decided to investigate the possibility of transfecting a xenobiotic metabolising enzyme in an expression vector into DLKP This procedure has been explored using a number of cell line (Crespi *et al*, 1991, Aoyama *et al*, 1990, Battula *et al*, 1987, Ellard *et al*, 1991, Puga *et al*, 1990, Montisano *et al*, 1985) Initially cDNAs were hosted in yeast and COS cells, but expression was found to be variable and unstable (Odea *et al*, 1985) Vaccinia Virus infection was also examined and successfully expressed a number of P450s Using this procedure Aoyama expressed five of twelve forms of human hepatic cytochrome P450, IA2, IIA3, IIB7, IIIA3 and IIIA4 in HepG2 cells Each transfectant was capable of activating aflatoxin B_1 to its mutagenic metabolite (Aoyama *et al*, 1990) The mouse forms IA1 and IA2 were also actively expressed in HeLa cells (Battula *et al*, 1987) However these transfectants by their nature are only short lived and useful only on an immediate basis

Stable transfection of a xenobiotic metabolising enzyme into a human lung line had not been attempted The vector which we decided to use was the rat cytochrome P450IA1 (courtesy of J Doehmer) which had been cloned into the PBR322 plasmid by Dr J Doehmer
This plasmid had been successfully expressed in Chinese hamster cell V-79 (Dogra *et al*, 1990) As the plasmid contained no selective gene it was therefore co-transfected with the pSV2Neo plasmid (which codes for resistance to the antibiotic G418 (geneticin), and the PCH110 a plasmid which contains the β-galactosidase gene, which allows visual conformation of transfection) at a ratio of 20 CYPIA1 1 pSV2Neo PCH110 It was decided to transfect this plasmid into DLKP, and to include V79 in the procedure as a positive control - as it had been successfully transfected with this plasmid before As can be seen from Table 10, the use of calcium phosphate technique to transfect the V79 and DLKP cells produced poor efficiency of transfection Two positive V79 control transfectants were isolated, out of 63 geneticin resistant transfectants tested, however, only one was stable in culture and resulted in the line V791A1-7 Of the 47 DLKP1A1 - geneticin resistant clones which were isolated, none expressed the AHH protein as detectable by immunofluorescence and AHH activity After a number of attempts to improve the efficiency of the $CaPO_2$ technique it was decided to apply another technique - electroporation The efficiency of transfection increased dramatically for the PCH110 and geneticin when electroporated at 25 μF, as can be seen from Table 10 Efficiency of transfection increased from 0 0016% using calcium phosphate precipitation to 0 31% efficiency with electroporation However, cloning of the transfectants and analysis of their CYP1A1 expression once again yielded no positive clones
DLKP transfection was repeated eight times and 47 geneticin resistant clones were isolated and tested for AHH expression At this stage we began to wonder that perhaps there was another element (an endogenous element) preventing expression

Regulation of the CYP1A gene family has been studied extensively in cultured cells The 1A1 gene has been focused on due to its unique method of regulation, induction and expression in cell culture
Transcription of the 1A1 gene is governed by interaction of cis-acting factor(s) (*i e* DNA elements within the locus which effect the expression of the gene) with DNA sequence elements lying within 1 kilobase upstream of the RNA polymerase II start site (see figure 63) These elements can only increase the transcription of the 1A1 gene in the presence of inducer *e g* PAH

The xenobiotic regulatory element (XRE) (also known as the DRE (dioxin responsive element) and AhRE (aromatic hydrocarbon responsive element) in the mouse), is believed to be the most effective The XRE has the following sequence

This sequence bears some similarity to the GRE (glucocorticoid regulatory element) but the XREs are not responsive to glucocorticoid hormones (Fujii-Kuriyama, *et al* , 1992)

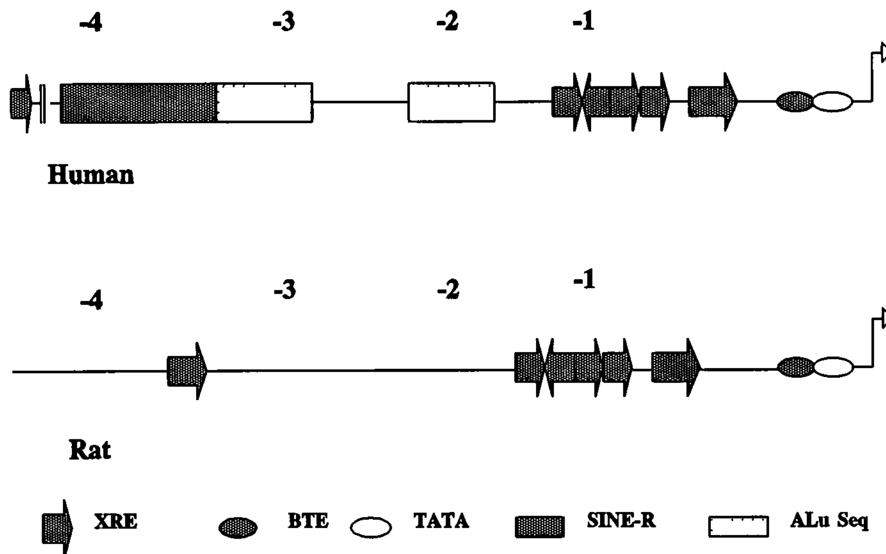


Figure 63 Regulatory DNA elements in the upstream region of human and rat CYP1A1 gene Numbers represent sizes in kb (Kubota *et al* , 1991)

There are 5 copies of this XRE element at -537, -1010, -1089, -1233, and -3586 bp positions The effect of these elements is additive and independent of their orientation around the gene (Kubota *et al* , 1991) These XRE elements are also known to regulate a number of other inducible genes as well as the 1A1, *e g* the phase II enzymes UDP-Gluconyltransferase and Glutathione-S-transferase (Negishi and Nebert, 1979)

A second regulatory element believed to be involved m constitutive expression of the CYP1A1 gene is the BTE (basal transcriptional element) This element, is situated close to the TATA sequence (conserved AT rich septamer found approximately 25 bp before the startpoint of each eucaryotic RNA plolymerase II transcription unit Probably involved in positioning RNA ploymerase II enzyme for correct initiation of genes)

The BTE is believed to play a common regulatory role in many genes, predominantly P450 genes, all of which demonstrate different modes of regulation, see figure 63 (Yanagida *et al* , 1990) Regulatory elements similar to XRE and BTE, have been found in the mouse, rat and humans In humans the two intermediately repetitive sequences (Alu and SINE-R) are located at -3068, -2116, and -1876 bp for the Alu sequence and from -3126 bp for the SINE-R element, figure 63 (Kubota, *et al* , 1991)

XRE induced expression of the 1A1 genes requires the presence of the BTE sequence, *i e* both sequences must be present This implies a possible co-operation between the trans-acting factors of XRE and BTE

<u>Trans-acting protein factor(s)</u> bind to the DNA at the XRE and BTE sites *i e* the cis-acting elements, in the nucleus (Durrin & Whitlock 1987, Fukjisawa-Sehara *et al* , 1987, Denison *et al* 1988, Fukjisawa-Sehara *et al* , 1988) The XRE binding proteins were isolated from nuclear extracts from 3MC-treated Hepa-1 cells

In untreated cells these proteins were found in the cytoplasm and they demonstrated no XRE binding ability However, on addition of 3MC, binding was seen Specific binding of an ^{125}I-labelled ligand to a factor that interacts with the XRE suggests that this factor is the TCDD(Ah) receptor complex, leading to the possibility that the XRE binding protein/factor and the hitherto uncharacterised Ah receptor are the same factor The Ah (TCDD) receptor is a soluble intracellular protein which regulates the induction of CYP1A1 by recognizing and binding <u>A</u>romatic <u>h</u>ydrocarbon type inducers which results in the transformation of the inducer-receptor complex This complex is then moved to the nucleus in a heat sensitive step, binds to specific DNA sites and stimulates mRNA production (Okey, 1990)

Ah receptor is very similar to receptors for steroid hormones and may be a member of this family of receptors However, evolutionary data suggests that this receptor is much older than the receptors for steroid hormones (Okey, 1990) This receptor has also been implicated in the induction of other proteins apart from those involved in drug metabolism It has been suggested that these proteins may be fundamental in the regulation of cell proliferation and differentiation (Poland and Knutson, 1982 Paulson, *et al* , 1990) The Ah locus in mouse is believed to be made up of at least three genes and lies on chromosome 12 (Okey, 1990)

The unbound cytosolic XRE binding factor is associated with a HSP90 (heat shock protein) On induction, the inducer binds to the XRE binding factor/HSP90 complex causing a conformational change and resulting in the dissociation of the HSP90 (Perdew, 1988a).

The newly liganded factor is then translocated to the nucleus Exactly how this translocation is achieved is unknown Ah receptor-positive and translocator-negative (cells that could form receptor-ligand complexes but could not translocate the complex to the nucleus) cells were investigated in fusion-complementation studies with receptor negative cells Fully functional *i e* inducible cells resulted, indicating the involvement of a trans-acting translocator factor - (the Arnt protein, Fujii-Kuriyama *et al* , 1992) - the Ah receptor nuclear translocator protein The Arnt protein is 789 ammo acids in length, has the potential to form helix-loop-helix domains and is comparable with a family of homo and heterodimeric transcription factors It is proposed that this Arnt protein dimerizes with the XRE/liganded factor and translocates the entire complex to the nucleus

The receptor-complex binds within the major groove of the DNA helix causing a structural change in chromatin This conformation change results in increased access to the chromatin and increased transcription (Durrin and Whitlock, 1989, Whitlock *et al* , 1989, Hankinson *et al* , 1991, Reyes *et al* , 1992) Cytosine methylation at this recognition site diminishes the response to dioxin by impeding the Ah receptor-enhanced interaction thereby eliminating expression of the gene (Shen *et al* , 1989) Methylation of DNA's cytosine resulting in 5-methylcytosine has been associated with the silencing of genes in cell culture (Holliday, 1989)

This activation takes place without the synthesis of new protein and is sensitive to *in vitro* methylation of DNA (Shen and Whitlock, 1989, Fujii-Kuriyama *et al* , 1992) Isolation of the Ah receptor is an on-going project Partial purification of the receptor has been achieved, which should lead to a better understanding of the entire mechanism (Bradfield, *et al* , 1990, Poland *et al* , 1987, Perdew & Poland, 1988b)

Unlike the XRE binding protein which is found in the cytosol as well as the nucleus, the BTE binding factor is found only in the cell nucleus To date two different cDNAs for rat BTE binding proteins have been cloned One, designated Sp1 due to its 98% homology with the human Sp1, is a 788 amino acid transcription factor The other, is a novel 224 amino acid protein called BTEB or BTE-binding protein Both these proteins possess the 'basal' transcription factor - zinc finger domain, which are arranged in a tri-repeating pattern It is these domains which are believed to be responsible for recognizing specific GC rich binding sites for these proteins in the BTE Both the BTE-binding protein and Sp1 demonstrate independent transcriptional activity with respect to CAT activity from pSV2CAT, when the promoter contains tamdem repeats of GC box sequence Expression of P450IA1 in a vector containing a single GC box and the BTE, is activated by the Sp1 protein alone

The introduction of the BTE-binding protein gene into this vector along with CYP1A1 and the single GC box, seems to partially inhibit the Sp1 induction, perhaps by competition for the BTE BTE-binding protein requires the presence of tandem GC boxes before it can produce a response (Fujii-Kuriyama *et al* , 1992)

In the absence of a purified Ah receptor and knowledge of exact sequence of induction, other possible regulatory mechanisms have been studied The presence of a labile inhibitor protein which represses transcription of CYP1A1 *in vitro*, was proposed when the phenomenon of "superinduction" was observed in cells which were induced with PAH combined with the protein synthesis inhibitor cycloheximide (Bhat *et al* , 1987, Foldes and Bresnick, 1989, Israel *et al* , 1985) The mechanism of this "superinduction" is believed to include the alteration in the protein-protein interactions involved in the activation of the CYP1A1 transcription by the ligated Ah receptor (Lusska *et al* , 1992) and post-transcriptional mRNA stabilization (Nemoto and Sakurai, 1991)

As can be seen in the above paragraphs, the sequence of events which lead to CYPIA1 regulation and induction are complex and to date not all the component or stages have been realised This ever increasing list of documented factors, both cis- and trans- acting, cytosolic and nuclear, which have been reported to act as regulators of CYP1A1 expression, any one of these factors or a concert of them may be the reason for the decline of this particular enzyme in culture

Labile protein(s) in the nucleus which bind to the Ah liganded receptor site preventing the action of the XRE regulatory system and the initiation of transcription were reported by Foldes *et al* , (1988), and Lusska *et al* , (1991) Similarly a repressor protein which interferes with liganded Ah-receptor binding to the XRE by binding to the Ah-receptor, was also noted in dominant Hepa-1 mutants by Watson *et al* , (1992)

Ivy *et al* 1988, also reported in the breast cancer cell line MCF-7 induction of AHH activity was observed when the potent inducer TCDD was used, however, in a variant of this line (which had been selected for resistance to adriamycin), this induction of AHH activity using the same inducer (TCDD) was no longer observed To uncover the possible mechanism of this inhibition they followed the expression of the reporter gene CAT linked to the CYP1A1 control region of the plasmid which was transfected into the wild type MCF-7 and the resistant MCF-7 cells They found that CAT activity was detectable only in the wild type cells, suggesting that the defect in the resistant cell was not due to a structural P4501A1 gene mutation, but involved a product of the resistant line which was regulating the AHH-inducible expression

Puga *et al* , (1989) observed that on transfection of the Cyp1a1 murine cDNA into the mouse hepatoma Hepa-1c1c7 c37 cell line the construct must contain the AhRD -aromatic hydrocarbon responsive domain, for a sizable level of expression It was also noted that the expression of endogenous Cyp1a1 mRNA in the c37 parental line is decreased by the presence of exogenous CYP1A1 or 1A2 enzyme activity, but that induction of endogenous mRNA for *cyp*1a1 was restored by the presence of the exogenous genes Puga *et al* , concluded that a functional product or post-transcriptional signal of either the *cyp*1a1 gene or the CYP1A2 gene may play a role in an autoregulatory loop controlling the constitutive expression of the Cyp1a1 gene Hankinson *et al* , (1991) found that a dominant induction defective mutant of Hepa-1 when fused with wild type Hepa-1 cells resulted in prevention of induction They proposed that a repressor protein which these dominant mutants express, effects the P4501A1 induction process and that this protein is involved in the translocation of Ah receptor-ligand complexes from cytoplasm to nucleus As a result there is no induction of this protein

As can be seen from these reports there are a number of elements believed to be affecting endogenous P450IA1 production and at a number of levels

It is possible that a form of inhibitory regulation of this gene may be in operation in DLKP The fact that PCH110 and Geneticin resistance are substantially expressed in each of the transfections, even though their concentration is 20 times lower than that of the CYP1A1 plasmid indicates that some specific regulator controlling factor may be involved

But before we can ask questions about the lack of expression of CYP1A1 it is necessary to prove that the plasmid actually entered the cell As this plasmid contains no selective gene within itself *e g* geneticin or colchicine resistance, it was necessary to co-transfect it with pSV2Neo so as the transfection positive cells could be selected over the non transfected This was also the purpose of the PCH110 plasmid, as this plasmid contains β-galactosidase activity cells which had taken up this gene could be visualised as they turn blue on metabolism of X-Gal by the β-galactosidase At this point we could positively say that the transfection had worked due to the visualization of PCH110 blue cells and the presence of geneticin resistant cells Therefore since the CYPIA1 plasmid was at a concentration 20 times that of either of the other two plasmids it is assumed that it too will be present in those clones selected for This is an accepted system and had we recorded expression no further investigation would have been required However expression was not recorded and there was the very real possibility that the plasmid had simply never entered the cell or had failed to integrate and had been degraded Accordingly no expression would be recorded Therefore it was necessary to prove the presence of the plasmid in the DLKP transfectant To do this it was decided to carry out PCR

using one primer previously chosen by Yang *et al*, (1991) and one primer chosen by myself from the sequence of CYP1A1 recorded by Yabusaki *et al*, (1984) These primers spanned a distance of 1540 bp from 110 to 1650 bp within the gene segment present in the plasmid pSVP4501A1 As can be seen in figure 51 the DLKP transfected with CYP1A1 reacted positively therefore proving that the plasmid had actually entered the cell

With this result confirmed the next question which arises is why there is no expression of this plasmid There are a number of possible reasons for this lack of expression The plasmid may have integrated at a site in the chromosome which would not allow its transcription But for this to happen 47 times is hard to accept Another possibility is that the plasmid itself may be damaged The full inserted sequence spans 2 2 kb (Dogra *et al*, 1990), the segment chosen for PCR spans 1 54 kb and was intact however the plasmid may have mutated upstream of the -110 start site for the PCR sector, perhaps within the SV40 promoter site The V791A1-7 transfectant expresses activity demonstrating that the plasmid can actually produce protein but the efficiency of transfection with the V-79 was also very low, only 2 transfectants out of 63 tested, one of which was unstable

It is possible that in the endogenous situation the gene may be have been methylated (as reported by Shen *et al*, 1989) thereby preventing binding of transcription factors, the incorporating plasmid may have also been methylated which would result in its silence

To uncover possible reasons for the silence of this endogenous and plasmid gene in DLKP, cell fusion experiments could be carried out, where DLKP is fused with another line which expresses AHH activity This experiment would highlight the presence of a trans-acting inhibitor if expression in the AHH positive line is prevented Treatment of DLKP with 5-azacytidme which would remove the methyl groups on the DNA which might be preventing access of transcription factor Treatment of DLKP with cycloheximide (thereby preventing production of new protein) may uncover the presence of an inhibitor which may be preventing transcription of this protein

Sequencing of the plasmid would be of interest to ensure that no mutations which may affect expression may have occurred Cloning the rat CYP1A1 gene into another vector with a different promoter would also demonstrate if the SV40 promoter was damaged or unresponsive in this instance

However, as the expression of the gene within the plasmid is governed by the SV40 promoter, it is unlikely that the upstream elements of the endogenous gene would affect transcription of the plasmid gene In preliminary RT-PCR experiments carried out by Lorraine McElroy in this laboratory, it has been discovered that mRNA encoding for endogenous cytochrome P450IA1

is present in DLKP However, a functional translation product of this mRNA has not been detected by either enzyme activity or immunoanalysis, as can be seen in table 6 and figure 46 This suggests that regulation of this gene in DLKP may be taking place at some post-transcriptional level and that this regulation is affecting both endogenous and plasmid expression mRNA turnover is determined in part by the structure of each mRNA molecule and a host of substances extrinsic to an mRNA itself *e g* translational factors, hormones and occasionally viruses, issue direct or indirect regulatory signals that affect mRNA turnover (Ross, 1989) In the V79 cell, the mRNA produced by the plasmid appears to be intact, as it produces functional aryl hydrocarbon hydroxylating protein in the transfected V791A1-7 cells Therefore, the plasmid and its independent promoter is capable of producing mature mRNA in V79 cells However, RT-PCR tells us only of the presence of the mRNA sequence which lies between the chosen set of primers and not the size or condition of the full specific mRNA To ascertain this information, it would be necessary to carry out Northern Blot analysis to investigate if mRNA present is the correct size to produce P450IA1 At that point it would be possible to determine if the regulation was occurring at the mRNA processing level, or at a later stage *e g* interference at the translation stage, or alteration in the stabilization of the mRNA *e g* increased turnover of the mature mRNA by elevated levels of ribonuclease which would degrade the specific mRNA before it is translated, or decreased binding to the poly(A) tail of the mRNA by PABB (poly(A) binding protein) thereby increasing the instability of the mRNA (Shapiro *et al* , 1987) Futher analyses would result in a better understanding of the regulation of this gene and its expression in cell culture

4.2.2 Glutathione-S-Transferase in cell culture

The aim of this section of the project was to investigate the expression of the phase II enzyme family, the Glutathione-S-transferases, in cell culture, with special interest in their expression and their connection with Multiple Drug Resistance

The glutathione-S-transferases are a family of multifunctional proteins which act as enzymes and binding proteins in various detoxication processes GST catalyze the reaction of nucleophilic reduced glutathione with electrophilic compounds, often biotransformed compounds from xenobiotics including carcinogens and endogenous substances This conjugation in some cases prevents inicitation of the carcinogenic process by inactivation of the electrophilic product or ultimate carcinogen However, recent studies have shown that some glutathione and cysteine conjugates can be more toxic than their original compounds (Tsuchida and Sato, 1992)

The expression and role of the GST classes μ,π and α in cell culture and in cancer is a complex issue It has been reported that in pre-neoplastic cells and neoplastic cells, specific molecular isoenzymes of GST are expressed and that these isoforms participate in the mechanisms of their resistance to drug (Tsuchida and Sato, 1992, Moscow - to be published) Increased expression of α and μ GST isoenzymes during rat chemical hepatocarcinogensis have been observed (Sato *et al* , 1988) GST π has been identified as a marker for rat hepatic pre-neoplastic and neo-plastic lesions (Sato *et al* , 1989), but human hepatocellular carcinomas do not express GST π Increased expression of human GST π has also been demonstrated in pre-neoplastic and neoplastic lesions in a variety of tissues A significant increase in GST π expression levels is evident in metastatic liver tumours which originate from the gall bladder, stomach, and colon Increased levels of GST π expressed in colon carcinomas and squamous cell carcinomas of the uterine cervix have also been reported (Soma *et al* , 1986, Kodate *et al* , 1986, Shiratori *et al* , 1987) In primary human lung cancers elevated levels of π have been observed in adenocarcinomas and squamous cell carcinomas, but not in small cell lung cancers (Di ILio *et al* , 1988)

Expression levels of GST-μ have also been implicated as a possible marker for increased susceptibility to lung cancer, especially among the smoking population This is due to the isoforms π and μ which are known to have high activity towards epoxides of Benzo[a]pyrene, and these epoxides are associated with an increased incidence of lung cancer (Anttila *et al* , 1991) Deletion of the gene coding for GST μ could result is a greater concentration of toxic benzo[a]pyrene metabolites in circulation (Seidegard *et al* , 1988,1990)

This phenotype expressed in combination with the cytochrome P450IA1 C phenotype which codes for increased inducibility of the P450IA1 protein which metabolises Benzo[a]pyrene would result in severely increased susceptibility to lung cancer (Nakachi *et al*, 1991) At the other end of the scale reduction in expression of GST isoforms has also been implicated in greater risk of adenocarcinoma of the stomach and colon (Strange *et al*, 1991)

In the analysis presented in this thesis as can be seen from the results Table 3-8 and Figures 35-39 total GST activity as determined by conjugation with 2,4-Dinitro-chlorobenzene, varies from cell line to cell line Without samples of normal tissue from the original source it is impossible to say if this expression is as a result of culture conditions or the malignancy of the cells Increased expression of GST total was noted on induction with Phenobarbital (PB) in all the cell lines tested This phenomenon had been previously reported in rat hepatocytes In these cells PB induced the transcription of π,μ and α This increase in expression is attributed to increased transcription in the short term and stabilization of the GST mRNA in longer term cultures (Vandenberghe *et al*, 1991) This induction indicated that although the CYPIA1 gene and the UDP-gluconyltransferase induction mechanism (which share common XRE upstream from their genes) had been effected by culture conditions, that this PB mechanism of induction continued to operate *in vitro* an it had *in vivo*

With respect to multiple drug resistance, the expression of GST has been studied extensively in MDR cancer cell lines Elevated levels of GSTs have been recorded in MDR cell lines and several mechanism of their action have been proposed, 1) enhanced activation of electrophilic alkylating agents by direct conjugation, 2) GSH dependent denitrosation of nitrosoureas thereby decreasing nitrosurea toxicity, 3) removal of reactive peroxides, 4) quenching of chloroethylated-DNA monoadducts, platinum-DNA adducts, and DNA hydroperoxides (Waxman 1990), 5) and working in concert with the P-170 glycoprotein by conjugating with anticancer agents and transporting them to the endoplasmic reticulum for export out of the cell (Moscow *et al*, 1993) GST isoforms α, μ have been associated with the *in vitro* development of resistance to nitrogen mustard, nitrosoureas, and benzo[a]pyrenes (Moscow *et al*, 1989) However, it is the π form that is present in most human tumours

The detection of high levels of GST π in most established cell lines and in many cancer tissues as well as in drug resistant cell lines, would suggests an important role for the enzyme in acquired and natural resistance

GST π expression in the adriamycin resistant lung lines in (figures 37 - 39) as confirmed by immunoblot analysis (figures 52 - 54) highlights this statement These results are in agreement with the previously mentioned reports of GST π expression in lung cancer

Elevation of π expression was seen in the two adriamycin resistant lines SKLU1-A and SKMES-1A, with the former demonstrating the greatest increase of 10 fold over its parental line and the latter displaying only 2 fold increase This result is of great interest in that the mechanism of resistance in this line has yet to be uncovered However, to date even though GST levels are increased in a number of MDR lines, no conclusive evidence exist that any of the anticancer drugs normally associated with MDR are conjugated with GSH Adriamycin, VP16 and vincristine, are conjugated by the cytosolic GSTs normally associated with cancer (Black *et al*, 1990, Moscow *et al*, 1993) In an attempt to uncover the relevance of GSTs in MDR, transfection of GST π subunit cDNA into MCF-7 human breast carcinoma cell line, which contain very low levels of endogenous GST, did not stimulate significant resistance, even on co-transfection with P-glycoprotein (Tsuchida and Sato, 1992, Moscow *et al*, 1993)

If the resistance of the cell line SKLU1A is mediated by the GST isoform π then inhibition of the action of this enzyme should circumvent this resistance

Buthiomne sulfoximime (BSO) is an inhibitor of gamma-glutamyl cysteine synthase, the enzyme which produces glutathione (GSH) the substrate essential for GST to function (Waxman 1990, Moscow *et al*, to be published)

To investigate the possible role of GST in the mechanism of SKLU1-A resistance, SKLU1A (the adriamycin resistant cell line) and SKLU1 (the parental non-resistant line) were exposed to BSO The results are represented in figures 61 and 62 As can be seen from the graphs, the treatment of SKLU1A with BSO alone resulted in and IC_{50} of 3 75 μM, for SKLU1 the IC_{50} in the presence of BSO alone was 6 25 μM

The SKLU1A cells demonstrated 100% survival in 0 05 μg/ml of adriamycin, whereas the parental line displayed a survival of only 11% (possibly demonstrating a basal level of resistance to adriamycin), in comparison with an untreated control These test were to identify the concentrations of adriamycin and BSO which could be used in the main experiment

To test the effect BSO on resistance both lines were exposed to non toxic BSO in the presence of 0 05 μg/ml adriamycin The parental line demonstrated a decrease in survival below the 11% survival already reported in 0 05μg/ml adriamycin when a range of BSO was used This pattern of increasing circumvention of basal resistance displayed by SKLU1 can be seen (figure 61)

The resistant line on the other hand on exposure to BSO in the presence of 0 05 μg/ml adriamycin displays a decrease in sensitivity rather than an increase as expected for BSO - the combination of the two drugs seemed to confer a type of resistance for BSO on the SLKU1A cells, a result similar to that found in the BSO treatment of GST transfected yeast (Moscow *et al*, 1993) These finding imply that in the SLKU1A cells which have acquired resistance to adriamycin, that the over-expression of GST π is perhaps a symptom of its MDR phenotype, rather than the cause, as has been suggested recently by a number of groups (Moscow *et al*, 1993, Tsuchida and Sato, 1992, Waxman 1990)

4.2.3 TOXICITY TESTING of V-79, XEM2 and V791A1

This final section of my project was to investigate the consequences of the presence of CYP1A1 and CYPIIB1 in V79 cell lines.

The cytochrome P450 superfamily and in particular the CYP1A1 - AHH enzyme, have been implicated in carcinogenesis and MDR (Guerngerich, 1988; Levine *et al.*, 1984; Manson *et al.*, 1981;Kawajiri *et al.*, 1991;McLemore *et al.*, 1989, 1990; Kouri, *et al.*, 1982; Anttila *et al.*, 1991; Clynes, 1993). It is also suggested that the MDR and cytochrome P450 gene families may have overlapping regulatory elements and that induction of the mdr gene can be mediated via by the CYP inducers 3MC (3-Methylcolanthrene) and AAF (2-Acetlyaminofluorene) but not via the Ah receptor (Burt *et al.*, 1988; Gant *et al.*, 1991).

The connection between xenobiotic drug metabolism and MDR is one that as a concept is not hard to accept, given that the aim of these enzymes is to rid the cell of exogenous and harmful compound. Therefore as with their accidental activation of compounds, it is to be expected that these enzymes should be involved in the expulsion of agents that are detrimental to the cell, but beneficial to the whole body. Both elevated and depressed levels of xenobiotic metabolising enzymes have been reported. Ivy *et al.*, (1988) reported that an adriamycin resistant MCF-7 cell line expresses a trans acting inhibitor of CYPIA1 expression thereby preventing any metabolism by this enzyme and resulting in a level of resistance to benzo[a]pyrene. Over-expression of xenobiotic metabolising enzymes *in vitro* results in increased metabolism of anticancer agents which might decrease their plasma half-life *in vivo* (Doehmer, to be published).

It has been reported that in toxicity tests using adriamycin on V-79 and XEM2, that a degree of resistance approximately 10% was observed in XEM2 (J. Doehmer unpublished results) XEM2 as mentioned previously contains the transfected rat cytochrome P450IA1 cDNA and expresses AHH activity.

As can be seen from Figures 55 - 57 toxicity test were carried out on three lines containing rat CYPIA1 using three chemotherapeutic agents, adriamycin, vincristine and VP-16. The three lines were V79 (the parental non expressing line), XEM2 (the rat CYPIA1 expressing line transfected by J. Doehmer) and V79IA1-7 (the V79 line which was successfully transfected with rat CYPIA1 in this laboratory). The figures show that for vincristine there is essentially no difference between the lines. For Adriamycin (figure 55) I found that there was once again very little difference between the cell lines with V79IA1-7 displaying slightly more resistance than XEM2, but when the error is taken into account once again the sensitivity is very similar.

For VP16 (figure 57), however, there is a difference in IC_{25} between V79IA1-7 at 0 125 μg/ml and the other two lines both at 0 75 μg/ml (An IC25 was used here as it was a more sensitive range) Overall the presence of P450IA1 in these lines has little effect on their sensitivity for the drugs tested However, when these lines were exposed to benzo[a]pyrene figure 58 the effect of the presence of P450IA1 is obvious Where V79, the parental cell line displays a IC50 of 5 75 mM both of the transfected-lines are much more sensitive (IC50 0 5mM for XEM2 and 0 0025mM for V791A1-7) due to their metabolism of benzo[a]pyrene to its toxic metabolites This toxicity profile demonstrates the value of transfection mediated xenobiotic metabolising expressing cell lines as they expose the toxic nature of potential toxic compounds that m normal *in vitro* experiments may have gone undetected due to the lines lack of expression of CYP1A1 Figure 59 also highlights the transfection mediated activation of a drug - cyclophosphamide, which in its unmetabolised form, as demonstrated by V79 cells, is not toxic except at very high concentrations However, in the presence of CYP2B1 (transfected into V79 cells resulting in the line SD1 (Doehmer *et al*, 1988)) it is hydroxylated to its toxic form as it would be *in vivo* Figure 59 also includes the toxicity profile of cyclophosphamide to DLKP and DLKPA As can be seen from this graph both DLKP and DKLPA activate cyclophosphamide This was a surprising result as to this point neither line had displayed any other xenobiotic metabolising enzyme other than glutathione-S-transferase The fact that both the resistant and the parental lines display similar toxicity profile suggests that this expression is not as a result of exposure to adriamycin, or linked with the MDR phenotype of DLKPA It does suggest to me that this expression of this enzyme (which is phenobarbital inducible) is endogenous to DLKP and DLKPA, it is of interest that the phenobarbital-induction of GST is also expressed in these cells Perhaps the phenobarbital inducible enzymes have survived translocation into culture in this line - DLKP Another possibility is spontaneous breakdown of cyclophosphamide, however the activation is uniform and comparable to the transfected line SD1 I would expect if spontaneous breakdown were occurring, that this activation should also be observed in V-79, SKMES-1 and SKLU1 cells which were also tested, this however did not occur Therefore there is an agent actively metabolising cyclophosphamide in DLKP possibly P450IIB1

5.0 Conclusions and suggestions for further work.

The aims of this project were to 1) improve the methods for primary cultures of non-small cell lung carcinomas, with a view to *in vitro* chemosensitivity testing 2) Investigate the possible use of existing cell lines as activation system for *in vitro* toxicity testing 3) To establish a cell line expressing activation ability and prove that lines value in *in vitro* toxicity testing and finally 4) to investigate the possible connection between MDR as seen in SKLU1A adriamycin resistant cell and expression of xenobiotic metabolising enzymes in these cell
Along the way these goal have changed slightly The primary culture of non-small cell lung carcinoma evolved into a larger project than had been intended The overall success rate of this section of the project was low The survival rate of samples was small and as time moved on the mam emphasis changed from *in vitro* chemosensitivity testing to improving the survival time of the sample However a poorly differentiated squamous cell carcinoma of lung line - DLKP, was established, which is characterised and is being applied for other work in this laboratory There are many parameters which could be varied in attempt to achieve this which time did not allow for in my project For future work the use of polypropylene coated dishes could be investigated This compound is reported to inhibit the attachment of normal - fibroblast cells, as a result the malignant tumour cells will be selected The use of 'start up cultures' in capillary tubes which would keep the density of cells high and the volume low is another possibility This method would allow for any autocrine growth factors being expressed by the malignant cells to exert their effect on the cells in a suspension environment more akin to the *in vivo* situation This method would also favour the growth of malignant cells over the normal fibroblast which are so devastating to malignant growth in the attachment-dependent system currently in use

Section two covered the investigation of established lung cell lines for expression of xenobiotic metabolising enzymes This investigation was carried out on the cells in the absence and presence of known inducers of xenobiotic metabolising enzyme As already mentioned some cells on transfer into culture do retain their inductive ability, however none of the cell lines tested in this section expressed this facility, in activity assays or immunocytochemical analysis As a result of this lack of expression of xenobiotic metabolising enzyme these cells line were perfect candidates for transfection of xenobiotic metabolising enzyme cDNA, as they had retained their ability to express NADPH cytochrome P450 reductase

DLKP was chosen as the recipient of the gene - cytochrome P450IA1 the gene coding for aryl hydrocarbon hydroxylase, the activity associated with the activation of a carcinogen present in cigarette smoke and smog - benzo[a]pyrene and the induction of lung cancer However, even though the cells took up the plasmid (as seen by PCR of the gene) and incorporated it into its DNA, no expression of protein was seen There are a number of reasons why this could have happened If the plasmid is incorrectly orientated in the cells DNA then transcription would not occur, but for this to have happened consistently is difficult to accept If the plasmid was damaged or mutated then no protein would be produced, however, a functional V79 transfectant was produced using the same plasmid which demonstrates the competence of the plasmid and rules out damage to the plasmid This leaves the possibility of factors within DLKP which effect the expression of this gene These factors may include methylation of the DNA after incorporation, which could be investigated using 5-azacytidine, a chemical which removes the methyl groups from cytosines An inhibitory trans acting factor may also be present which would prevent the expression of this gene, cell fusion of DLKP with a CYPIA1 expressing line would demonstrate this possibility, if inhibition of expression resulted in the fused cells, then it could be concluded that a trans acting inhibitor of CYPIA1 expression exist within DLKP Also the preliminay RT-PCR which has demonstrated the presence of CYP1A1 mRNA in the parental line indicating that transcription is taking place, however the size and condition of this mRNA needs to be ascertained before we can speculate as to the possible mechanism of regulation of this protein These studies would hopefully lead to the expression of CYPIA1 in DLKP - a human lung line They would also lead to a greater understanding of the mechanism of this gene which is of great importance in lung cancer If successful these studies would also result in an activating lung line which would be of importance in *in vitro* toxicity testing - one of the main aims of this project

The final section of this project involved the investigation of the adriamycin resistant human adenocarcinoma SKLU1A to decide if the resistance in these cells is GST π mediated, as the resistant form of this cell line displays increased expression of this GST isoform The results obtained suggested that the mechanism is not GST mediated Therefore the resistance in this cell line must be due to some other factor *e g* altered topoisomerase activity, or altered drug transport Future work on these aspects may reveal the mechanism of resistance operating in the cell line

SECTION 5

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