

# REGULATION OF MULTIDRUG RESISTANT GENE EXPRESSION IN RODENTS

This thesis is my own composition and describes a project carried out by myself; experiments performed by other people are appropriately acknowledged.

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1991.



*The Road goes ever on and on  
Down from the door where it began.  
Now far ahead the Road has gone,  
And I must follow, if I can,  
Pursuing it with eager feet,  
Until it joins some larger way  
Where many paths and errands meet.  
And wither then? I cannot say.*

J. R. R. Tolkien

## ACKNOWLEDGEMENTS.

Numerous people have helped in many ways throughout the course of my project. I would like to mention them here by way of thanks.

Firstly, to Roland, who supervised the work and sent me on a superb ski-ing trip to the Rockies!

Also to all the staff at the Imperial Cancer Research Fund for their support, but especially to Colin for providing samples, helping with the animal work, giving excellent technical advice, proof-reading this text and always being willing to lend a hand when needed. His assistance was invaluable.

To the staff at the animal house at Clare Hall for some of the animal treatments.

To David and Linda, for their expertise in tissue sectioning, staining and immunohistochemistry.

To Veronica for patiently teaching me the fine art of tissue-culture and supervising the early days of the project.

To Brent for performing the corticosterone assay and Sandy for doing some of the photography.

To good friends I have made whilst in Edinburgh, for the pleasure of their company.

And finally to Billy, whose unfailing care and support I will always cherish.

## ABSTRACT

P-glycoprotein (P-gp) is a member of a family of energy-dependent transport proteins. The expression of P-gp isoforms in different tissues has been previously examined, but the physiological functions of the genes have not yet been established. Three gene isoforms have been found in rodents. These appear to be functionally distinct and only two of them are related to the multidrug resistance (mdr) phenotype displayed by tumour cells refractory to chemotherapy.

Gene specific probes for each mouse isoform has allowed an analysis of mdr gene expression in normal mouse tissues. The major mdr mRNA species expressed in mouse liver is a 4.5 kb transcript encoding mdr 2. The function and factors regulating expression of this gene are unknown. Mdr 1 and mdr 3 are also expressed in mouse liver, but at lower levels.

The exogenous and endogenous factors controlling the expression of mdr 1, 2 and 3 mRNA in mouse liver were examined. The regulation of mdr genes in the rat liver by xenobiotics, including cytochrome P450 and glutathione S-transferase (GST) inducers, was also studied. The hepatotoxins 2-acetylaminofluorene, aflatoxin B<sub>1</sub> and diethylnitrosamine induced rat hepatic mdr gene expression. Diethylnitrosamine also induced hepatic and renal mdr 1 expression in the mouse. The compound, 1,4-bis [2-(3-dichloropyridyloxy)] benzene (TCPOBOP) caused a suppression of hepatic mdr 2 and mdr 3 levels in mouse whilst inducing cytochrome P450 levels to a high extent. Long-term suppression of mdr 2 levels was also demonstrated. The anti-cancer drugs vincristine and etoposide, as well as the phenolic antioxidant butylated hydroxyanisole, known to elevate GST levels, induced mdr 2 gene expression in the mouse.

Using hypophysectomised animals, it was shown that the pituitary regulates the expression of both mdr 2 and mdr 3 in mouse liver. Animal models, in which specific pituitary hormones were ablated, demonstrated that neither growth hormone nor thyroid hormone depletion reduces the expression of mdr 2. Elevation or depletion of hormones produced by the adrenal gland, using chemical treatment, also did not affect mdr 2 gene expression. The regulation of mdr 2 and 3 gene expression by the pituitary

appears to be complex and may involve more than one hormone.

Using a cell culture model, it was demonstrated that non-metabolised carcinogens do not compete with known substrates for transport across the plasma membrane.

All these findings are important in elucidating the regulation of mdr genes in rodents.

## PUBLICATIONS ARISING FROM RESEARCH

Prolonged suppression of multi-drug resistance gene expression in the mouse liver.

Russell, A. L., Henderson, C. J., Smith, G. and Wolf, C. R.

Manuscript submitted.

The effect of selenium deficiency on hepatic type-1,5-iodothyronine deiodinase activity and hepatic thyroid hormone levels in the rat.

Beckett, G. J., Russell, A. L., Nicol, F., Sahu, P., Wolf, C. W. and Arthur, J. J.

Manuscript submitted.

Sexual differentiation and regulation of cytochrome P450 CYP2C7.

Henderson, C. J., Russell, A. L., Allan, J. A. and Wolf, C. R.

Biochica Biophysica Acta (in press).

Complimentary DNA sequence, deduced amino acid sequence, predicted gene structure and chemical regulation of mouse Cyp2e1.

Freeman, J. E., Stirling, D., Russell, A. L. and Wolf, C. R.

Biochemical Journal (in press).

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## ABBREVIATIONS

2-AAF	2-acetylaminofluorene
4-AAP	4-acetamidophenol
ACTH	adrenocorticotrophic hormone
adr(R)	adriamycin (resistant)
AFB <sub>1</sub>	aflatoxin B <sub>1</sub>
Ah	aromatic hydrocarbon
AHHase	arylhydrocarbon hydroxylase
Ara	Araclor 1254
ATP	adenosine triphosphate
B(a)p	benzo(a)pyrene
BHA	butylated hydroxyanisole [2-(3)-tert-butyl-4-hydroxyanisole]
BSA	bovine serum albumin
C	C57BL/6
CaM	calmodulin
CBP	corticosteroid binding globulin
cDNA	complementary deoxyribonucleic acid
CDTA	trans-1,2-diaminocyclohexane-NNN'N'-tetraacetic acid
CF	clofibrate
CHO	chinese hamster ovary
CH <sup>R</sup> C5	colchicine resistant chinese hamster ovary cell line
Clo	clotrimazole
co	corn-oil
con (C)	control
CsA	cyclosporin A
CTC	carbon tetrachloride
CYP	cytochrome
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
D	DBA2/N
DEN	diethylnitrosamine
depc	diethylpyrocarbonate
dex (D)	dexamethasone

dGTP	deoxyguanine triphosphate
dH <sub>2</sub> O	distilled water
DHPG	9-[1,3-dihydroxy-2-propoxy(methyl)]-guanine
DMN	dimethylnitrosamine
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dox	doxorubicin (adriamycin)
Dox-5	doxorubicin resistant human ovarian cell line
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
Ery	erythromycin
EtOH	ethanol
F	female
FCS	foetal calf serum
FIAU	1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil
GSH	glutathione
GST(s)	glutathione-S-transferase(s)
GH	growth hormone
GHRH	growth hormone releasing hormone
HCC	hepatocellular carcinoma
H/E	haematoxylin/eosin
HN	hyperplastic nodule
HSV1-TK	herpes virus thymidine kinase
HSV-tk	herpes virus gene
Hsp	heat shock protein
hypox	hypohysectomy
kg	kilogram
lit	little
IgG	immunoglobulin G
M	male
3-MC	3-methylcholanthrene
mdr	multidrug resistance/multidrug resistant

MOPS	3-[N-morpholino]propanesulphonic acid
MTT	3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl) tetrazolium bromide
Mr	molecular weight
mRNA	messenger ribonucleic acid
NADP	nicotinamide adenine dinucleotide phosphate
NaPPi	sodium pyrophosphate
$\alpha/\beta$ -NF	$\alpha/\beta$ -naphthoflavone
nit	nitrate
4-NQO	4-nitroquinolene oxide
OD	optical density
PA	polyclonal antibody
PAH	polycyclic aromatic hydrocarbon
PAS	periodic acid Schiff
PB (P)	phenobarbital
PBS	phosphate buffered saline
PCN	pregnenolone 16 $\alpha$ -carbonitrile
PCR	polymerase chain reaction
Pgp	p-glycoprotein
PKC	protein kinase C
PLPD	periodate lysine paraformaldehyde
Prog	progesterone
PVP	polyvinylpyrrolidone
Pyr	pyrazole
Rif	rifampicin
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS/PAGE	SDS/polyacrylamide gel electrophoresis
sham	sham-operated
SKOV3	human ovarian carcinoma cell line
SKVLB	vinblastine resistant SKOV3
Std	standard
TAO	triacetyloleandomycin
TCDD	2,3,7,8-tetrachlorobenzo-p-dioxin
TCPOBOP	1,4-bis [2-(3,5-dichloropyridyloxy)] benzene

TBST	tris buffered saline plus Tween 20
TEMED	NNNN'-tetramethyl ethylene diamine
Topo II	topoisomerase II
Vinb	vinblastine
Vinc	vincristine
VP16	etoposide
XRE	xenobiotic responsive element
PMSF	propyl methyl sulphonyl flouride
NP 40	Nonidet P 40
HRP	horseradish peroxidase
Eth Br	ethidium bromide

# CHAPTER 1

## INTRODUCTION

### 1.1 INTRODUCTORY COMMENTS

Cells protect themselves from toxic insults in a variety of ways. Use of chemicals often results in their becoming ineffective due to cellular resistance mechanisms and understanding such mechanisms has become increasingly significant in medicine. Resistance may be both intrinsic or acquired. Intrinsic resistance may be due to a variety of biochemical reasons as shown in Table 1.1. For example, the mammalian lung copes with oxygen-induced free radicals by producing antioxidants including superoxide dismutase and glutathione peroxidase (Halliwell and Gutteridge, 1985). Drugs and toxins produce biochemical changes including enzyme induction, DNA repair and increased detoxification capacity. Certain bacteria produce dihydrofolate reductase in response to trimethoprim treatment (Young and Amyes, 1986).

Environmental factors can elicit an adaptive response that also confers chemical resistance. Prokaryotes have various stress response mechanisms. Many proteins are regulated by stress and the responses may inter-relate.

Heat-shock to cells causes the expression of three families of heat-shock genes, Hsp 90, Hsp 70 and Hsp 20. Hsp 70 and Hsp 72 bind peroxisome proliferators, for example clofibrate, which may inhibit their activity (Alvares *et al*, 1990).

A fundamental association between adaptive response to environmental stress and drug resistance seems to exist. The heat-resistant chinese hamster ovary (CHO) cell line over-expresses Hsp 70 and also shows resistance to adriamycin and the epipodophyllotoxin VM-26 (Wallner and Li, 1986). These are both well known substrates for P-glycoprotein (P-gp), which is involved in the mdr phenotype. It has been shown that P-gp expressing cells contain a heat-shock consensus sequence in their

**TABLE 1.1  
EXAMPLES OF INTRINSIC RESISTANCE**

EXAMPLE	ORGANISM	PROTEIN OR OTHER FACTORS	TYPE	DRUG
Antibiotic producers	<i>Streptomyces griseus</i>	Aminoglycoside phosphotransferase	Novel metabolic inactivation	Streptomycin
Brain tumours	Man	Sanctuary site	Drug delivery	Anticancer drugs
Chemoprevention	Mammals	Dietary manipulation	Adaptive change	Carcinogen (aflatoxin B <sub>1</sub> )
Heat shock	Chinese hamster fibroblasts	Transient hyperthermia	Stress response	Adriamycin, ethanol
Poor debrisoquine metabolizers	Man	Cytochrome P-450	Population polymorphism	Reduced sensitivity to carcinogens

Adapted from Hayes and Wolf, 1990

promoter region (Chin *et al*, 1990). Carcinogens also induce heat-shock proteins (Carr *et al*, 1986).

The second type of resistance, that of acquired resistance, describes the emergence of a population of previously drug-sensitive cells. The development of cancer is a step-wise process that involves in part such a selection. Major alterations are observed in the levels of drug metabolising enzymes in hepatocyte nodules selected by exposure to carcinogens. The changes include a decrease in the expression of the cytochrome P450 and sulphotransferases and over-expression of glutathione-S-transferases (GSTs), UDP glucuronyl transferase, epoxide hydrolase, DT-diaphorase,  $\gamma$ -glutamyl transferase and P-gp.

Such a diversity of events suggests the existence of a common control mechanism responsible for coordinating the expression of these enzymes. An initial mutation event, which could involve a stress response regulatory gene, may initiate a cascade of events as described above. An understanding of these enzymes is crucial to the prevention or treatment of cancer.

Cytochrome P450s and glutathione S-transferases (GSTs) are subject to regulation by a variety of endogenous and exogenous factors. The aim of this project was to study the factors controlling the regulation of P-gp, as well as trying to relate its regulation to that of P450s and GSTs in order to establish whether areas of common regulation exist.

In this introductory chapter, literature dealing with the properties of the P-gp, cytochrome P450 and GST gene families in rodents will be discussed. The regulation of cytochrome P450s and GSTs by xenobiotics and endogenous substances will be briefly reviewed. Questions regarding the regulation of P-gp will be raised and the strategy for answering them described.

The following chapters will contain more detailed background information regarding different aspects of the project and discuss the findings of the experimental work conducted. The final chapter will summarise the results and discuss them more fully in addition to suggesting future topics for study.

## 1.2 MULTIDRUG-RESISTANCE

### 1.2.1 THE MULTIDRUG RESISTANCE PHENOTYPE AND P-GLYCOPROTEIN

Multidrug-resistance (mdr) describes the simultaneous expression of cellular resistance to a wide range of structurally unrelated drugs. Such a phenotype is frequently seen in clonal mammalian lines selected for resistance to a single cytotoxic agent. The complexity of the mdr phenotype is illustrated in Table 1.2, where it is shown that mdr cells can be cross-resistant to structurally and functionally distinct compounds.

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**TABLE 1.2**  
**CROSS-RESISTANCE PHENOTYPE OF MDR CELL LINES**

DRUG	FOLD RESISTANCE*	
	CH <sup>R</sup> C5	CEM/VLB <sub>100</sub>
Colchicine	180	45
Colcemid	16	NA
Vinblastine	30	420
Doxorubicin	25	110
Gramicidin D	~5000	NA
Methotrexate	NA	3

Resistance was raised against colchicine, in the case of CH<sup>R</sup>C5, and vinblastine in the case of CEM/VLB<sub>100</sub>.

\* compared to parental wild-type cell line

NA not assayed

Adapted from Gerlach *et al*, 1986

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Mdr cells frequently display collateral sensitivity to certain hydrophobic compounds. Resistance appears to be due to a lower cellular accumulation of the drugs involved.

Many mammalian cell lines selected for resistance to a single cytotoxic agent, for example actinomycin D, adriamycin, colchicine or Taxol, show an altered sensitivity to an unexpectedly wide range of cytotoxic

compounds (Biedler *et al*, 1983; Gupta, 1983 a, b; Ling *et al*, 1983; Beck, 1984a; Curt *et al*, 1984). Usually, the cell lines display the highest degree of resistance to the selecting agent and lower resistance to other compounds.

In general, among the anti-cancer drugs, resistance to the anthracyclines, vinca alkaloids, podophyllotoxins, actinomycin D, maytansine and Taxol are most often observed in mdr cell lines. Cross-resistance to alkylating agents such as melphalan, nitrogen mustard and mitomycin C and to anti-metabolites such as methotrexate occurs less frequently and usually at a lower level.

Cytogenetic studies demonstrate the frequent presence of homogeneously staining regions and double minutes in mdr cell lines (Baskin *et al*, 1981), which are karyotypic features of gene amplification (Cowell, 1982). Gene amplification of P-gp sequences in a variety of cell lines has been noted (Riordan *et al*, 1985).

A reduced accumulation of drug has been a consistent feature of many cell lines, relative to their drug sensitive counter-part (Beck, 1984; Riordan and Ling, 1985). This may be effected by reduced influx, increased efflux, altered compartmentalization or by a combination of these mechanisms. A unifying model to account for all the observed reduced accumulation events in these cell lines has not yet been conclusively demonstrated.

Many studies have concentrated on elucidating the biochemical differences between mdr and sensitive cells. The most consistent biochemical change is the increased expression of a plasma membrane glycoprotein, termed p-glycoprotein (P-gp). Only minor differences are observed in the lipid constituents of the membranes of mdr cells (Bosman, 1971; Ling *et al*, 1977). However, changes in cytoplasmic components have been noted. The most consistent of these is an increase in a small cytoplasmic protein of 19 kD and isoelectric point (pI) of 5.7. This is present in elevated amounts in chinese hamster and mouse cells resistant to vincristine (Meyers and Biedler, 1981; Meyers *et al*, 1985). This protein is called VP19 and is phosphorylated (Biedler *et al*, 1983).

The association of P-glycoprotein with mdr was first described in colchicine- resistant chinese hamster ovary (CHO) cells and in actinomycin

D-resistant Syrian hamster cells (Juliano and Ling, 1976). Similar alterations were later described in many other drug resistant cell lines (Peterson and Biedler, 1978; Garman and Center, 1982; Beck *et al*, 1979).

In isolated plasma membranes of highly resistant CHO cells, P-gp was found to be the major membrane component (Riordan and Ling, 1979). The reported molecular weight (Mr) for P-gp is 170,000 kD, but this can vary from between 140,000-220,000 kD depending on the SDS-PAGE system, the solubilisation and electrophoresis conditions used. An Mr of 170,000 kD was obtained using a modified Fairbanks system for SDS-PAGE (Fairbanks *et al*, 1971; Debenham *et al*, 1982; Kartner *et al*, 1983 a, b). This buffer system gives sharper resolution of glycoproteins than the more conventionally used discontinuous systems such as that of Laemmli (1970).

The improved sensitivity of detection of P-gp by monoclonal antibodies (Kartner *et al*, 1985) along with Western blotting (Towbin *et al*, 1979) made possible a comparative study of different mdr cell lines (Kartner *et al*, 1983a, 1985). It was found that homologous P-gps are expressed in mdr cells of different origins and that the level of P-gp expression was always elevated over drug sensitive parental levels by an amount consistent with the degree of resistance.

Monoclonal antibodies have also been applied to the identification of cDNA clones encoding P-gp. Riordan *et al* (1985) identified a 600 bp clone from a  $\lambda$ gt11 library that was shown to code for three epitopes of P-gp. A 4.7 kb mRNA specific to mdr cells was found using this cDNA insert which was consistent with the molecular size of the P-gp polypeptide. The amount of message expressed also correlated with the level of P-gp expression in cell lines with different degrees of mdr.

Using Southern blotting with P-gp genomic DNA from sensitive and resistant cell lines, it has been shown that there is amplification of the genomic DNA coding for P-gp in some mdr cells (Riordan *et al*, 1985). Increased P-gp can also occur in the absence of gene amplification (Fuqua *et al*, 1987).

Cloning P-gp genes was a major advancement in the understanding of the structure and function of P-gp. The sequence of human P-gp (mdr 1 gene) encodes a molecule of 1280 amino acids and is tandemly repeated.

Each half of the molecule consists of a hydrophobic domain containing three pairs of membrane-spanning  $\alpha$ -helices and a highly conserved hydrophilic cytoplasmic domain containing an adenosine triphosphate (ATP) binding site (Gros *et al*, 1986; Chen *et al*, 1986; Gerlach *et al*, 1986).

The transmembrane domains are thought to form a pore through which P-gp effluxes drugs and endogenous substances. How energy is transduced for transport is unknown although the binding of ATP analogues to P-gp and the ATPase activity of the purified protein has been confirmed (Cornwell *et al*, 1987; Hamada and Tsuruo, 1988). It has also been shown that a mutation of one or both nucleotide-binding consensus sequences results in a lack of drug resistance in mdr transfected cells (Rothenburg and Ling, 1989). These sites may functionally interact to effect drug efflux.

The mechanism of drug binding is not yet known, but direct binding of drugs to P-gp may be an important step. Choik *et al* (1988) reported a mutation in a human mdr cell line that resulted in a change from valine to glycine at position 185. Mutants displayed an increased resistance to colchicine, etoposide (VP16) and a decreased resistance to vinblastine, vincristine and actinomycin D. This finding infers that subtle alteration in these sequences can produce significantly different mdr phenotypes.

Direct binding of drug analogues to P-gp has been studied. Photoaffinity vinblastine analogues can label the protein and the binding is inhibited by other drugs such as vincristine and daunomycin, as well as chemosensitisers such as verapamil (Gottesman, 1988b). The conclusion from these findings is that these compounds may be competing for a common binding site.

It is possible that differential expression of mdr genes could alter the profile of transported drugs. Co-amplification of other genes, as well as the mdr genes, may also modify the profile. Van der Bliek *et al* (1986) reported the co-amplification and overexpression of at least six classes of genes in one CHO cell line. P-gp is phosphorylated at both serine and threonine residues (Endicott and Ling, 1989) and changes in the extent of such phosphorylation may also modulate activity.

### 1.2.2 P-GLYCOPROTEIN AS PART OF A TRANSPORT SUPER-FAMILY

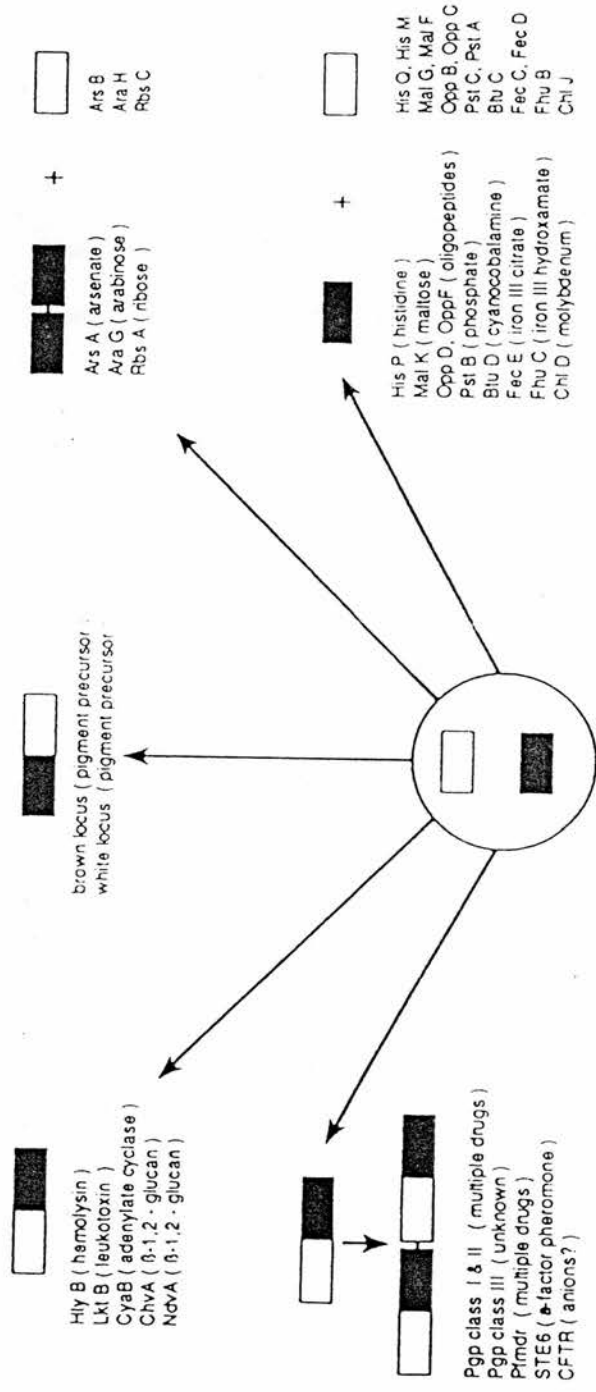
Both bacterial and eukaryotic transport proteins share extensive sequence homology to P-gp, as displayed in Figure 1.1, and is therefore considered a member of a super-family of proteins that transport a wide range of substrates. Members of this family have high sequence homology in the nucleotide binding consensus sequence (Walker *et al*, 1982).

The transporters fall into two general classes. The first represents proteins which have linked hydrophobic and hydrophilic domains. P-gp appears structurally most like this class and can be viewed as a tandemly duplicated hemolysin B (Hly B)-like protein (Gros *et al*, 1986; Gerlach *et al*, 1986). The hydrophobic domains of P-gp and Hly B-like proteins have low sequence homology but their location and the number of membrane-spanning helices argue for a similar membrane disposition and pore-forming function. The brown and white loci of *Drosophila* are also part of the Hly B-like protein class but the order of the two domains is reversed (Dreesen *et al*, 1988). The brown and white proteins import pteridine pigment precursors into cells whereas the Hly B class export substrates.

Riordan *et al* (1989) has recently described the cystic fibrosis gene product, referred to as the cystic fibrosis transmembrane conductance regulator (CFTR). This is also a member of this class of transporters and has a tandemly duplicated structure. The CFTR protein is presumed to be involved in the transport of anions across cell membranes.

The second group of transporters are members of multi-component periplasmic protein binding permeases (PPBP) systems of bacteria. The permease system is composed of a substrate binding protein that presents the substrate to a membrane complex consisting of two integral membrane proteins capable of forming several transmembrane helices and a membrane associated cytoplasmic hydrophilic protein that binds ATP (Ames, 1986). There is evidence that two ATP binding subunits might also be required in the PPBP systems ( Prossnitz *et al*, 1989).

**FIGURE 1.1 SUPERFAMILY OF TRANSPORT PROTEINS**



The P-glycoprotein superfamily of transport proteins. The names of proteins and the substrates they transport (in parentheses) are shown under a boxed representation of the domain structure of the proteins. Filled boxes represent the hydrophilic domain or polypeptide containing the ATP-binding consensus sequences, which is highly homologous among all members of the superfamily. Open boxes represent a hydrophobic domain or polypeptide which contains multiple putative membrane-spanning  $\alpha$ -helices that share limited sequence homology only between proteins within each group.

Redrawn from Juranka et al, 1989

### 1.2.3 THE P-GLYCOPROTEIN MULTI-GENE FAMILY

P-gp is a member of a small, highly conserved multigene family with three genes in rodent and two genes in primates (Ng *et al*, 1989). The family appears to have arisen from one or more gene duplication events (Van der Bliek *et al*, 1988a). Each P-gp isoform is highly conserved in protein sequence and genomic structure.

Analysis of the 3' untranslated region of various P-gp genes has allowed identification of equivalent isoforms between species (Ng *et al*, 1989). Each gene within a species encodes a unique 3' untranslated region but certain differences are conserved across species.

The P-gp isoforms can be grouped into three classes as shown in Table 1.3.

---

**TABLE 1.3**  
**CLASSIFICATION OF THE P-GLYCOPROTEIN GENES**

SPECIES	CLASS I	CLASS II	CLASS III
Hamster	pgp 1	pgp 2	pgp 3
Mouse	mdr 3	mdr 1	mdr 2
Human	mdr 1	-	mdr 3 (mdr 2)

Redrawn from Juranka *et al*, 1989

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Various studies indicate that transfection of class I and II P-gp can confer the mdr phenotype (Guild *et al*, 1988; Ueda *et al*, 1987) whereas type III transfection does not result in drug resistance (Rothenburg and Ling, 1989; Gros *et al*, 1988). Structural features of this isoform clearly imply that it is a membrane transport protein, similar to class I and II isoforms and it would be interesting to determine the compounds it may transport.

Human class I and mouse class II P-gp genes have been studied to determine their genomic intron/exon organization (Chen *et al*, 1989;

Raymond and Gros, 1989). Both genes contain 27 exons with identical relative location within the coding region (Ng *et al*, 1989; Chen *et al*, 1989; Raymond and Gros, 1989). P-gp may have originated from a duplication event or by the fusion of two closely related but independently evolving genes.

The number of P-gp genes in vertebrates has been estimated (Ng *et al*, 1989). Primates (human, rhesus monkey), rabbit, chicken and fish contain two genes; rodents (hamster, mouse, rat) and cow contain three genes and pig has five genes. Invertebrates also contain P-gp sequences. *Drosophila melanogaster* contains two genes, and three genes have been cloned from the nematode *Caenorhabditis elegans*. McGrath and Varshowsky (1989) reported a P-gp gene in *Saccharomyces cerevisiae*. The P-gp cloned is the product of the STE6 gene; a locus required for the secretion of the  $\alpha$ -factor mating pheromone. This represented the first identification of a normal function for a P-gp gene product. STE6 is part of a signal sequence-independent protein translocating system. This is shared by the products of the bacterial Hly B, cya B and the lkt B genes. The mammalian class III P-gp gene may have a similar protein translocating function.

The malarial parasite, *Plasmodium Falciparum*, is known to have two genes (pfmdr) (Foote *et al*, 1989; Wilson *et al*, 1989). The pfmdr is amplified in some chloroquine-resistant plasmodium cells. Resistance is associated with increased drug efflux (Krogstad *et al*, 1987) and verapamil can reverse such resistance.

#### 1.2.4 EXPRESSION AND REGULATION OF P-GLYCOPROTEIN

Regulation of P-gp may occur at various levels including transcription, translation and post-translation. Most regulation studies have been done in cell lines but the mechanisms may also apply to normal and malignant tissues.

Following early drug selection steps, human cell lines exhibit increased levels of P-gp, mRNA and protein, but without increased gene copy number. Control of transcription is suggested as the reason (Shen *et al*, 1986). Bradley *et al* (1988) showed that P-gp may also be over-

expressed in resistant human and rodent cell lines due to gene amplification. Bradley *et al* (1989) has more recently reported increased P-gp in the absence of gene copy number or mRNA levels, suggesting that such mechanisms as protein stabilisation or increased translation may occur.

Localisation of P-gp in normal human and rodent tissues has been performed using specific DNA probes and antibodies. It has been found that the P-gp genes are differentially expressed in a tissue-specific fashion.

Such studies demonstrate the consistently high expression of P-gp in the adrenal cortex, liver, kidney, intestine, muscle fibres and gravid uterus (Baas and Borst, 1988; Thiebaut *et al*, 1987; Croop *et al*, 1989). P-gp is localised to the apical surface of the small and large intestine, renal proximal tubules, bile and pancreatic ducts and the bile canalicular face of hepatocytes. This distribution implies a secretory function for P-gp and a role in clearing the cell of xenobiotics. P-gp is also found in the cells lining the brain capillaries and may be involved in the blood/brain barrier (Cordon-Cardo, 1989). In the adrenal cortex, P-gp may be responsible for the transport of corticosteroids.

Recently Croop *et al* (1989) examined the distribution of P-gp in mouse tissues by Northern blot analysis using gene specific probes. Class I (mouse *mdr 3*) was found at highest levels in the intestine and at intermediate levels in heart, brain and kidney. The highest level of class II (mouse *mdr 1*) were present in the gravid uterus and adrenal with medium levels in kidney and heart. Medium levels of class III P-gp (mouse *mdr 2*) were found in adrenal, liver, spleen, heart and muscle. The expression of class III P-gp is also found in a subset of muscle fibres (Georges *et al*, 1989).

The class III P-gp is the only gene to date that has been shown to undergo differential splicing. Three different class III transcripts have been identified from a human liver library (Van der Bliet *et al*, 1988b). It is not known whether the variants are expressed at the protein level and what effect the changes would have on function.

P-gp increases dramatically in the uterus during pregnancy and is localised to the luminal surface of the secretory epithelial cells of the endometrium. It was also demonstrated that female mice treated with

oestradiol and progesterone demonstrated induced P-gp mRNA. This leads to the conclusion that P-gp may be under steroidal control (Arceci *et al*, 1988).

P-gp expression also seems to be a specific response of cells to toxic or metabolic stress. In carcinogen-induced preneoplastic and neoplastic liver nodules and hepatocytes from regenerating rat liver, increased mRNA levels have been observed (Thorgeirsson *et al*, 1987; Fairchild *et al*, 1987b).

The three classes of P-gp genes express different lengths of transcripts. In mouse, class I (*mdr 3*) produces a 6 and 5 kb transcript, class II (*mdr 1*) produces a 5 kb transcript and class III (*mdr 2*) is 4.5 kb in length (Croop *et al*, 1989).

#### 1.2.5 SIGNIFICANCE OF P-GLYCOPROTEIN IN CANCER

Increased levels of P-gp have been seen in human cancers, for example leukaemias, lymphomas, sarcomas and carcinomas ( \* ).

In many cases, an increased level of P-gp is detected in tumours following relapse from chemotherapy compared to the biopsies before treatment. However, in some tumours high levels of P-gp are found even before therapy due to the cells expressing a phenotype which has intrinsically high P-gp levels (Goldstein *et al*, 1989).

Chan *et al* (1989) reported an elegant study on soft tissue sarcoma in childhood which indicated that the level of P-gp was a good prognostic marker of relapse-free response. However, the answer does not appear so straight-forward for other types of cancer, and the universal significance of P-gp to clinical response has not yet been convincingly shown.

Reversing the *mdr* phenotype using chemosensitisers to aid cancer treatment is being investigated. Ford and Hait (1990) recently reviewed the pharmacology of drugs that alter *mdr* in cancer therapy. These compounds were shown to fall into six broad categories.

Verapamil, a calcium channel blocker has been shown by many investigators to reverse *mdr* in cell lines (Beck, 1984; Radel *et al*, 1988) by altering drug accumulation (Tsuruo *et al*, 1981). Safa *et al* (1987, 1988) demonstrated that verapamil binds to P-gp and can inhibit the binding of

\* Tsuruo *et al*, 1987; Goldstein *et al*, 1989; Gerlach *et al*, 1987; Kakehi *et al*, 1988.

many chemotherapeutic drugs. However, verapamil is extremely cardiotoxic in the concentration range needed for antagonism of mdr. The use of less toxic enantiomers of verapamil and its analogs may provide a means of utilising these compounds effectively at a clinically useful level (Pirker *et al*, 1990). Other calcium channel blockers have been studied and found to be active.

Calmodulin antagonists, such as phenothiazines, are effective chemosensitizers and can enhance drug accumulation in mdr cells (Ford *et al*, 1990; Chafouleas *et al*, 1984).

Inaba's group (1984) investigated the chemosensitizing effects of anthracycline analogs on mdr cells and found significant enhancement of cytotoxicity. Similarly a number of relatively non-toxic Vinca alkaloid analogs effectively antagonized cross-resistance of mdr cell lines (Inaba and Nagashima, 1986).

Prompted by the high levels of mdr 1 mRNA found in the pregnant murine uterus (Arceci *et al*, 1988), Yang *et al* (1989) studied the effects of steroids on mdr. It was found that progesterone and deoxycorticosterone increased vinblastine accumulation and caused an equivalent reversal of vinblastine resistance in murine macrophages as verapamil. The results suggested that progesterone may bind to a hydrophobic pocket present in the P-gp molecule and that certain steroid hormones may be natural substrates for P-gp. Anti-oestrogens, for example tamoxifen, can partially overcome resistance (Ramu *et al*, 1984).

Cyclosporins are yet another group of chemosensitising agents. Cyclosporin A (CsA) has immunosuppressive activity and can inhibit an early stage of T lymphocyte activation (Handschumacher *et al*, 1984). CsA has been found to reverse resistance in mdr cells (Twentyman *et al*, 1987, 1988). However cyclosporins also appear to increase the effect of chemotherapeutics on certain sensitive cell lines (Chambers *et al*, 1989). The mechanism by which the cyclosporins sensitise mdr cells remains unclear, but it may be through an indirect effect of drug metabolism or potentiation of drug toxicity.

CsA does not consistently alter drug accumulation. Silbermann *et al* (1989) reported the increased accumulation of daunomycin in a multidrug

resistant cell line but not its sensitive counterpart due to CsA administration. In contrast, Chambers *et al* (1989) showed that CsA had no effect on doxorubicin accumulation in CH<sup>R</sup>C5 cells. Nooter *et al* (1989) reported increased daunomycin accumulation and toxicity in P388/Dau cells.

The final group of sensitisers are the hydrophobic cationic compounds such as quinidine (Tsuruo *et al*, 1984) and the antibiotic erythromycin (Hofslis and Nissen-Meyer, 1989). Whether these compounds act through a common mechanism is unclear, but their high degree of hydrophobicity may cause non-specific perturbations in the cell membrane leading to increased drug accumulation.

### **1.3 ALTERNATIVE MECHANISMS INVOLVED IN MULTIDRUG-RESISTANCE**

Apart from an increase in P-gp expression, an expanding number of biochemical and molecular changes are observed in cell lines selected for resistance to multiple cytotoxic drugs. These alterations have been described for non-Pgp expressing cells and those that over-express P-gp.

Clinical drug resistance in human tumours probably comprises a combination of cellular mechanisms of resistance. The two best characterised additional resistance mechanisms are firstly, changes in the expression or activity of enzymes involved in the phase II glutathione detoxification pathway and secondly, alterations in topoisomerase II.

Glutathione-S-transferases (GSTs) are enzymes that catalyse the conjugation of electrophilic substances and endogenous xenobiotics to glutathione (GSH), a tripeptide thiol, in order to form soluble stable metabolites which can be more easily excreted (Jackoby, 1978; Chasseaud, 1979).

Many researchers have reported an alteration in GST and GSH-related enzymes in cell lines resistant to alkylating agents (Lewis *et al*, 1988; Saburi *et al*, 1989). Cowan *et al* (1986) also noted an increase in the activity of the drug metabolising enzymes DT-diaphorase and glucuronyl transferase when comparing the biochemical changes in mdr human breast cells to those seen in carcinogen induced resistance to xenobiotics in rats. It

is believed that these changes are not simply coincidental to the selection of resistance but have a significant function or regulatory role in the process.

The role of GST in mdr involving non-alkylating agents is still under debate. The role of GST  $\pi$  in mdr and correlation with P-gp is considered in the following chapters. A fuller discussion of the GST multi-gene family will therefore be given in section 1.4.

Topoisomerases are enzymes that catalyse the unwinding of DNA, which is necessary for replication (Liu, 1983). Topoisomerase II (topo II) is thought to be a target for DNA intercalating and non-intercalating drugs such as doxorubicin, mitoxantrone and etoposide (Chen *et al*, 1984). Several mdr cell lines display reduced Topo II activity in the absence of P-gp or changes in drug accumulation (Pommier *et al*, 1986; deJong *et al*, 1990). Ganapathi *et al* (1989) described a series of cells, selected for increased resistance to doxorubicin, which exhibited increased levels of P-gp and reduced Topo II mediated cleavage of DNA. This suggests that the alteration with this enzyme can be multi-factorial.

Other changes described in cross-resistant cells appear only to occur with over-expression of mdr 1 which suggests that this may be the result of being passively transcribed along with amplification of mdr 1. Meyers *et al* (1985, 1987) has shown that sorcin, a 22 kD anionic calcium binding protein, is over-produced in vincristine resistant mouse and chinese hamster lung and ovary cells.

Ivy *et al* (1988) reported an alteration in the expression of cytochrome P4501A1 in MCF 7 adriamycin resistant cells. Burt and Thorgeirsson (1988) reported the co-induction of the mdr 1 gene with cytochrome P450 genes in rat liver by exposure to xenobiotics. They concluded that P-gp was in fact under the control of the Ah locus, as is P450 1A2 (Gonzalez *et al*, 1984). The regulation of mdr genes by compounds known to operate through the Ah locus will be discussed in the course of this thesis. A fuller description of P450 genes and their regulation will therefore be given in section 1.5.

## 1.4 GLUTATHIONE-S-TRANSFERASE DRUG METABOLISING ENZYMES

GSTs are a multi-gene family of enzymes that catalyse the conjugation of GSH to electrophilic reactive intermediates. The reaction constitutes the first step in the mercapturic acid pathway which is involved in the excretion of foreign compounds. The GSH conjugate may be excreted directly into bile or further metabolised to mercapturic acid.

All the GSTs have in common a dimeric structure with subunits of molecular weight between 23,000 and 26,000 kD. Eleven subunits have been characterised for the rat as outlined in Table 1.4.

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**TABLE 1.4**  
**GSH TRANSFERASE SUBUNITS IN THE RAT**

NUMERICAL	CLASS	"Y" DESIGNATION	MOL. MASS. ( D)	REF
1	$\alpha$	Ya	25,000	1
2	$\alpha$	Yc	27,500	1
3	$\mu$	Yb <sub>1</sub>	27,500	1
4	$\mu$	Yb <sub>2</sub>	26,500	1
5				
6	$\mu$	Yb <sub>3</sub>	26,000	2,3
7	$\pi$	Yf or Yp	24,800	4,5
8	$\alpha$	Yk	25,500	1
9	$\mu$	Yn <sub>2</sub>	26,000	1
10	$\alpha$	Y1	25,500	1
11	$\mu$	Yo	26,500	6

1. Hayes (1988)
2. Abramovitz and Litowsky (1987)
3. Guthenberg *et al* (1985)
4. Kitahara *et al* (1984)
5. Hayes and Mantle (1986)

## 6. Kispert *et al* (1989)

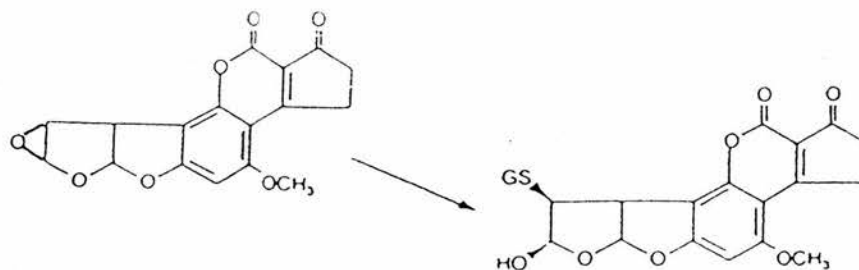
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Complete sequences are known for subunits 1, 2, 3, 4 and 7. They fall into three categories based on sequence homology. These have been named  $\alpha$  (1 and 2),  $\mu$  (3 and 4) and  $\pi$  (7) by Mannervick *et al* (1985 a, b). The genetic relationship extends the  $\alpha$  class to include 8 and 10 and  $\mu$  to include 6 and 9. Heterodimers exist within the multi-gene family (Ketterer *et al*, 1986). The most commonly used substrate for most rat subunits is 1-chloro-2,4-dinitrobenzene (CDNB). CDNB undergoes nucleophilic displacement of the chloro substituent by GSH. Other substrates may be used to determine the relative activity of a particular subunit.

A wide range of chemicals, both natural and synthetic, induce GST in the rat. These include barbiturates, polyaromatic hydrocarbons (Igarashi *et al*, 1987); certain antioxidants such as butylated hydroxyanisole (BHA) and ethoxyquin (Benson, 1979; Rushmore and Pickett, 1990; Sato *et al*, 1984) and natural products. Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is a hepatocarcinogen in the rat.

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**FIGURE 1.2**  
**GLUTATHIONE CONJUGATION OF AFLATOXIN B<sub>1</sub>-8,9-OXIDE**



Redrawn from Ketterer *et al*, 1988

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The carcinogenic metabolite, AFB<sub>1</sub>-8,9-oxide, is a substrate for subunits 1

and 2 (Coles *et al*, 1985) (Figure 1.2). Induction of subunit 1 by ethoxyquin results in a four- to five-fold induction in the biliary excretion of the AFB<sub>1</sub>-GSH conjugate and a decrease in genotoxicity (Kensler *et al*, 1986).

GSTs also have Selenium (Se)-independent GSH peroxidase activity for which lipid and nucleic acid hydroperoxides are good substrates. *In vivo* it has been proposed that GSH peroxidases are part of a system for repairing damage to membrane phospholipids by free radicals (van Kuijk *et al*, 1987). Ketterer *et al* (1987) proposed that GSTs may be a part of the repair system for thymidine hydroperoxides resulting from free radical damage to DNA. The rat nucleus appears to contain a chromatin-associated GST isozyme with high activity towards DNA hydroperoxide (Tan *et al*, 1988).

GSTs have been found in almost every rat tissue examined. Liver and testis have very high CDNB-transferase activity, as reported by Meyer *et al* (1983). In the rat liver, both  $\alpha$  and  $\mu$  are abundant whereas  $\pi$  class GST is present at very low levels. This isozyme is present in all bile duct cells and in some hepatocytes (Tatematsu *et al*, 1985);  $\alpha$  and  $\mu$  class isozymes are present in all hepatocytes but are most abundant around the central vein (Redick *et al*, 1982).

GST gene expression changes during development in the liver. Before birth, levels are low and increase after birth until, at two weeks of age, all adult subunits are expressed. The quantity continues to increase until at maturity the female expresses higher levels of  $\alpha$  class GST whereas the  $\mu$  class is more abundant in male rats (Igarashi *et al*, 1987).

#### 1.4.1 INDUCTION OF GLUTATHIONE-S-TRANSFERASE GENE EXPRESSION

GSTs are inducible by various compounds. The dietary antioxidant butylated hydroxyanisole (BHA) induces subunit 1 from the  $\alpha$  class GST (Rushmore and Pickett, 1990). Phenobarbitone and 3-methylcholanthrene induce subunit 1 from the  $\alpha$  class and subunits 3 and 4 from the  $\mu$  class in rat liver (Hales and Neims, 1977; Pickett *et al*, 1982). In rat liver preneoplastic

foci,  $\alpha$ ,  $\mu$  and  $\pi$  class GST are abundant (Kitahara *et al*, 1984). Many develop into nodules which also have increased expression of epoxide hydrolase, NAD(P)H-quinone oxido-reductase and UDP-glucuronyl transferase (Eriksson *et al*,1983). Some nodules persist and become neoplastic primary hepatomas (Tatematsu *et al*, 1983). Progress to malignancy is marked by the expression of the  $\pi$  class (subunit 7) GST making this isozyme a very useful marker for hepatocellular pre-neoplasia and hepatocarcinomas (for reviews see Sato, 1988, 1989).

Four isozymes have been purified from mouse liver, as shown in Table 1.5. These can be classed into the families  $\alpha$ ,  $\mu$  and  $\pi$  (Warholm *et al*, 1986). The  $\pi$ -type enzyme, which can be resolved into three forms, is the dominant form in the male mouse liver (McLellan and Hayes, 1987) but is absent from many extrahepatic tissues (Hayes *et al*, 1987). The  $\pi$  class isozyme expression is known to be male hormone-dependent (Hatayama *et al*, 1986).

Benson *et al* (1978, 1979) reported up to ten-fold induction of GST levels in the mouse by phenobarbitone, BHA and BHT administration. Diethylnitrosamine (Agius and Gidari, 1985) and chronic ethanol administration (David and Nerland, 1983) have been shown to induce GST expression up to two-fold. However none of these studies identified the

**TABLE 1.5**  
**GSH TRANSFERASES IN THE MOUSE**

FAMILY	"Y" DESIGNATION	MOL MASS
$\mu$	YbYb	27,000
$\mu$	YbYb	27,000
$\pi$	YfYf	24,500
$\alpha$	YaYa	26,000

Hayes *et al* (1987)  
Warholm *et al* (1986)  
McLellan and Hayes (1987)

isozymes affected by the inducers.

More recently Pearson *et al* (1988) showed that BHA induced the class  $\mu$  and  $\alpha$  GST mRNA levels in mouse liver 15- and 50-fold respectively whereas class  $\pi$  mRNAs were much less responsive.

#### 1.4.2 REGULATION OF GLUTATHIONE-S-TRANSFERASE GENES

Rushmore *et al* (1990) and Paulson *et al* (1990) identified two regions of the 5' flanking sequence of the GST Ya (alpha class) subunit gene that have elements to control the regulation of gene expression by planar aromatic compounds. One such element, spanning nucleotides -908 to -899, contains the xenobiotic responsive element (XRE) sequence:

5'-T-GCGTG-3'  
3'-A-CGCAC-5'

This consensus sequence is also found in the cytochrome P4501A1 gene (Whitlock, 1987; Deninson *et al*, 1988, 1989).

The second region has no sequence identity to the XRE but responds to the planar aromatic compounds  $\beta$ -naphthoflavone ( $\beta$ -NF) and 3-methylcholanthrene (3-MC). This element, known as the antioxidant responsive element (ARE), can respond to phenolic antioxidants such as t-butylhydroquinone in cells that lack a functional Ah receptor, but requires the presence of the Ah receptor and a functional cytochrome P4501A1 protein before responding to  $\beta$ -NF (Rushmore and Pickett, 1990). It spans nucleotides -722 to -682 of the 5' flanking region of the rat Ya gene.

A particularly interesting example of endogenous regulation is the induction of  $\pi$  class GST by interferons- $\alpha/\beta$  (Adams *et al*, 1987). Both testosterone and interferon- $\alpha/\beta$  can induce the  $\pi$  family in female mice.

Many researchers have evidence indicating that the GSTs are an important part of cellular protection against cytotoxic compounds.

Cowan (1986) reported similarities in the changes in GST expression during preneoplasia and in drug resistant cell lines, indicating that similar

mechanisms may be involved. Alteration in gene expression in preneoplastic lesions may be due to an immortalisation of a transient stress response (Adams *et al*, 1985; Hayes and Wolf, 1988). GSTs are found to be over-expressed in cells undergoing cytotoxic insult, which is also indicative of a stress response (Carmichael *et al*, 1986). The mechanism by which these changes occur is not clear.

#### 1.4.3 INVOLVEMENT OF GLUTATHIONE-S-TRANSFERASES IN DRUG RESISTANCE

The  $\pi$  class isozyme is strikingly elevated in three rat models of broad cross-resistance to multiple toxic agents. Firstly, in the Solt Farber model of hepatocellular carcinogenesis, preneoplastic nodules develop resistance to the toxic effects of the carcinogen that induce the transformation and also become cross-resistant to other structurally unrelated carcinogens (Carr, 1987). The nodules have increased amounts of the rat  $\pi$  class GST, GST-P (Kitahara *et al*, 1984).

Secondly, MCF-7 human breast cancer cell line selected for resistance to doxorubicin (Cowan *et al*, 1986) show increased expression of  $\pi$  class GST (Batist, 1986). Thirdly, v-H-ras transformed rat liver epithelial cells demonstrate an mdr phenotype along with increased GST-P expression (Burt *et al*, 1988).

Yf may play a role in the initiation of altered hepatocyte growth and its increase during preneoplasia is of great interest. Phorbol esters, such as 12-O-tetradecanoylphorbol-3-acetate (TPA), are potent tumour promoters. Alterations in gene expression following TPA has been suggested to involve a PKC signal transduction mechanism. The promoter region of the human  $\pi$  gene has a TPA response element (TRE) sequences (Cowell *et al*, 1988). Burt *et al* (1988) transfected v-Ha-ras and v-raf into rat liver epithelial cells and showed a marked induction of Yf GST and P-gp. The increase in Yf during preneoplasia and in many tumours could therefore be explained by linking it with the fact that Yf is induced by tumour promoters and genes linked to cell growth and division. The role of Yf in the carcinogenic process still requires further investigation.

To determine whether GST  $\pi$  is directly involved in drug resistance and because of the parallel over-expression of P-gp and GST  $\pi$  in models of pleiotropic resistance, transfection experiments involving the expression of the individual genes or the two genes expressed in the same cell line were conducted (Moscow *et al*, 1989b). The study demonstrated that there was no direct relationship between GST levels and mdr.

Mdr could be conferred by transfection of a full length mdr 1 cDNA but transfection of GST  $\pi$  alone could not impart resistance to doxorubicin. This is in agreement with the observations of Tsuruo who examined paired sensitive and resistant cell lines (Yusa *et al*, 1988). The apparent inability of GST  $\pi$  to enhance cell resistance to anti-neoplastic compounds does not rule out the involvement of this isozyme in drug-resistance. The overexpression of GST  $\pi$  and mdr 1 in several models of resistance indicates that common factors may exist to coordinate the regulation of both these genes and thus coordinate protection from toxic compounds. GST  $\pi$  is found elevated in some tumours compared to normal tissues (Moscow *et al*, 1989) suggesting a possible role as a marker of drug resistance and neoplasia.

## **1.5 CYTOCHROME P450 FAMILY OF DRUG METABOLISING ENZYMES**

The microsomal P450-dependent monooxygenase system has evolved to cope with the plethora of toxic chemicals that organisms have to deal with. The two main components of this system are NADPH-cytochrome P450 oxidoreductase and cytochrome P450. Cooper *et al* (1965) determined the role of P450 in the system as the terminal oxidase of the microsomal electron transport chain. In this process, two electrons are transferred from NADPH via NADPH cytochrome P450 reductase and P450 to mediate the incorporation of one atom from oxygen into a particular substrate and the other oxygen atom is reduced to form water (Black and Coon, 1987). The reductase is associated with the endoplasmic reticulum membrane and contains one flavin mononucleotide (FMN) and one flavin adenine dinucleotide (FAD) molecule per catalytic unit. Electrons pass from

FAD to FMN within reductase and then to P450.

Multiple forms of P450 exist which exhibit overlapping substrate specificities (Guengerich, 1987). P450 mediated reactions include oxidative and reductive dehalogenation, N-hydroxylation, N-oxidation, S-, N- and O-dealkylation and aliphatic and aromatic hydroxylation.

In general, the microsomal P450s, particularly those of the liver, convert hydrophobic substances into more hydrophilic ones that can be excreted from the body via the urine or bile.

Metabolism occurs in two phases. In Phase 1, a lipophilic compound may be oxidised by the addition of a hydroxyl or epoxide group. In Phase 2, the resulting electrophile is a substrate for transferase enzymes that attach either GSH sulphate or glucuronic acid, making it water soluble and hence easily excretable (Nebert and Negishi, 1984). However, with certain compounds, the high energy intermediate can attack DNA, RNA and protein causing mutation, toxicity and carcinogenesis (Conney, 1982).

Some enzymes of steroid biogenesis are P450s found in the adrenal gland, for example steroid 17  $\alpha$ -hydroxylase and steroid 11  $\beta$ -hydroxylase, the latter being a mitochondrial P450 receiving electrons from adrenodoxin and adrenodoxin reductase.

Many researchers have purified and characterised P450s which has resulted in the confusing nature of P450 nomenclature. The classification system is based on primary amino acid sequence alignment data. A recent update of this classification has been made (Nebert *et al*, 1991).

The primary hepatic drug metabolising enzymes comprise four gene families (1-4). Many P450 gene families contain multiple genes that have arisen through the process of gene duplication.

Listed below is a brief synopsis of the gene families in rat and mouse referred to later in this thesis.

### CYP1

The CYP1A1 and CYP1A2 genes are ubiquitous in mammals and have similar catalytic activities. CYP1A1 metabolises benzo(a)pyrene and other substrates (Goldstein *et al*, 1982) and can be induced by 3-MC in

many tissues (Kimura *et al*, 1986) and cigarette smoke in humans (Song *et al*, 1985).

CYP1A2 metabolises arylamine compounds including 2-acetyl amino fluorene (2-AAF) (Goldstein *et al*, 1984) and has AFB<sub>1</sub>-4-hydroxylase activity (Faletto, 1988).

## CYP2A

CYP2A1 and CYP2A2 (Nagata *et al*, 1987; Matsunaga *et al*, 1988) are 88% identical in rat but they are regulated differently during development and have distinct substrate specificities. CYP2A1 hydroxylates testosterone and other steroids at the 7 $\alpha$  position whereas CYP2A2 has a high testosterone 15 $\alpha$  hydroxylase activity (Matsunaga *et al*, 1988; Wood *et al*, 1983). Rat CYP2A3 is expressed only in lung and is induced by 3-MC (Kimura *et al*, 1989).

Cyp2a-4 and Cyp2a-5 genes in the mouse are orthologous to the rat CYP2A3 genes. The two mouse cDNAs are expressed in both liver and kidney (Squires and Negishi, 1988).

## CYP2B

Three cDNAs for CYP2B genes in the rat have been isolated; *CYP2B1*, *CYP2B2* and *CYP2B3*. Unlike *CYP2B1* and *CYP2B2*, *CYP2B3* is consistently expressed as a minor form in male and female liver and is not induced by phenobarbital (Labbe *et al*, 1988).

Southern blotting experiments suggest the possibility of multiple genes in the mouse (Simmons and Kasper, 1983). Two female specific mouse cDNAs, *Cyp2b-9* and *Cyp2b1-0*, containing full amino acid coding regions of P450s were isolated. They display 82% homology with rat *CYP2B1*. *Cyp2b-9* has been shown to have testosterone 16  $\alpha$ -hydroxylase activity (Noshiro *et al*, 1988).

## CYP2C

Members of this subfamily are mostly constitutively expressed, but some are under developmental and sex-specific regulation. Several constitutive P450s in the rat CYP2C subfamily have been isolated and characterised. CYP2C6 and CYP2C7 are developmentally regulated in male and female (Gonzalez *et al*, 1986a). These have 75% amino acid homology to each other. CYP2C11 is a male-specific P450 purified from rats which metabolises testosterone at the 16  $\alpha$ , 2  $\alpha$  and 17  $\beta$  positions (Ryan *et al*, 1984).

## CYP2D

The debrisoquine 4-hydroxylase P450, named CYP2D1, was purified from rat (Gonzalez *et al*, 1987). CYP2D2 has 78% amino acid sequence identity with CYP2D1. In total, 5 rat CYP2D genes have been isolated and sequenced.

The mouse may also have up to five genes. Recently a mouse male specific testosterone 16  $\alpha$ -hydroxylase (Cyp2d-9) was sequenced and found to have between 70-82 % amino sequence identity with the rat CYP2D genes (Wong *et al*, 1987).

## CYP2E

Patten *et al* (1986) reported a form of ethanol inducible P450 in rats, named CYP2E1. Only a single CYP2E gene has been identified in rats. This subfamily can metabolise a range of substrates including ethanol, acetone, acetol, benzene and N-nitrosodimethylamine (Koop *et al*, 1982; Johansson and Sundberg, 1988; Hong and Yang, 1985). CYP2E1 is believed to be involved in gluconeogenesis during the state of fasting (Casazza *et al*, 1984). CYP2E1 is also induced in rats with spontaneous and chemically induced diabetes (Past and Cook, 1982). Circulating acetone as well as hormonal factors have been implicated in the induction (Miller and Yang, 1984). A mouse Cyp2e1 gene has also recently been cloned (Freeman *et al*,

1991), and has been shown to share 92%, 79%, 80% and 79% sequence similarity over the coding region to rat, human, rabbit 1 and rabbit 2 CYP2E1 cDNA sequences respectively. Cyp2e1 protein was found to be induced by acetone treatment in all tissues studied in both male and female mice, but this was accompanied by decreases in Cyp2e1 mRNA levels. This suggests that acetone administration affects the stability of the protein or the efficiency of mRNA translation.

### CYP3

Enzymes in the CYP3 family, including CYP3A1 (Waxman, 1984), have activities for erythromycin demethylation, triacetyloleandomycin metabolism and testosterone 6  $\beta$ -hydroxylation (Wrighton *et al*, 1985; Gonzalez *et al*, 1986b). Lu and West (1980) identified a cytochrome P450, subsequently named CYP3A2, which could be induced by the synthetic steroid pregnenolone 16 $\alpha$  carbonitrile (PCN). CYP3A2 was isolated from an adult male liver library, using CYP3A1 as a probe and was found to be 89% amino acid sequence identical.

Humans have at least four CYP3A genes. CYP3A4 (Beaune *et al*, 1986) and CYP3A3 (Knodell, 1988) were found to be 98% similar at the amino acid level. Human CYP3A5 was identified in 10 - 20% of human liver samples examined using an anti-rat CYP3A1 antibody. CYP3A5 is the foetal form.

There is evidence to suggest that a CYP3 enzyme can be induced in humans by glucocorticoids (Molowa *et al*, 1986). Variability in CYP3 expression could have considerable clinical implications since CYP3 metabolises drugs including the calcium channel blocker nifedipine (Gonzalez *et al*, 1988), progesterone (Waxman *et al*, 1988a), cyclosporin (Kronbach *et al*, 1988) and the antibiotic erythromycin (Watkins *et al*, 1985). These compounds are all well known substrates for P-gp.

### CYP4

CYP4A1 is a clofibrate-inducible lauric acid  $\omega$ -hydroxylase isolated in

rats by Tamburini *et al* (1984). The enzyme is specific for fatty acid oxidations and oxidises arachadonic acid (Bains *et al*, 1985). The main effect of the hypolipidaemic peroxisome proliferator clofibrate is to lower triglycerides and cholesterol concentrations in the plasma but also has been found to be a rodent hepatocarcinogen. A peroxisome proliferator binding protein has been found present in rat liver (Lalwani *et al*, 1983) and the cloning of a novel member of the steroid hormone receptor superfamily activated by peroxisome proliferators has been recently reported (Issemann and Green, 1990). The identification of the mouse peroxisome proliferator activated receptor (PPAR) as a putative transcription factor activated specifically by peroxisome proliferators suggests that the receptor directly mediates the effects of this class of chemical.

Interestingly, the chemicals known to the most potent proliferators are the same as the chemicals which are most potent in receptor mediated activation of genes encoding P450 and other drug metabolising enzymes (Nebert, 1990). It was proposed by Nebert (1990) that these enzymes control the level of small organic oxygenated molecules that act as signals for growth differentiation and tumour promotion and that PPAR mediates one such pathway.

### 1.5.1 INDUCTION OF CYTOCHROME P450 GENE EXPRESSION

- (a) 3-Methylcholanthrene/2,3,7,8-tetrachlorodibenzo-p-dioxin- (TCDD) inducible

Rats fed 3-MC can metabolise certain amino azo dyes more efficiently (Conney *et al*, 1956). Aryl hydrocarbon hydroxylase (AHH) activity is induced by 3-MC, which is associated with the transcriptional activation of CYP1A1 (Nebert and Gelboin, 1970).

A polymorphism for 3-MC induction was found in mice and was due to a recessive mutation of a single gene (Thomas *et al*, 1972). Several other enzymes are under the control of this locus, named the Ah locus. The locus is controlled by a specific receptor. The potent inducer TCDD binds strongly to the receptor in responsive mice whereas non-responsive mice have a

receptor defect and hence possess a low affinity for the ligand and lack inducibility by 3-methylcholanthrene (Poland and Glover, 1975). The receptor probably has an endogenous ligand with a developmental or physiological role. The TCDD receptor can mediate transcriptional activation in hepatoma cells (Israel and Whitlock, 1984). To study the mechanism of transcriptional activation, CYP1A1 promoter-chloramphenicol transferase (CAT) expression plasmids have been constructed from cloned genes. The regulatory elements that control induction of transcription by TCDD are at -950 and -1100 from the ATG start site (Jones *et al*, 1986; Neuhold *et al*, 1986). Consensus sequences, named drug regulatory elements (DRE) and xenobiotic regulatory elements (XRE) are found in many copies in this region (Sogawa *et al*, 1986). The TCDD receptor and the glucocorticoid receptor element (GRE) have various similar physicochemical properties (Cuthill *et al*, 1987) and therefore the XRE may be evolutionarily related to the GRE. The glucocorticoid, Ah and phenobarbital receptor are all thought to be part of the steroid hormone receptor superfamily (Nebert, 1990).

The role of the TCDD receptor in gene activation is still not clearly understood. However, Deninson *et al* (1988) reported an observation in which the TCDD receptor plus ligand bound to the upstream control regions of the mouse CYP1A1 gene. It was suggested that the receptor was involved in binding the XRE.

CYP1A2 regulation has not been easily studied due to the lack of detectable expression in established cell lines. However, CYP1A2 is constitutively expressed in the liver and is inducible. It is not expressed significantly in extra-hepatic tissues even under the influence of a potent inducer (Kimura *et al*, 1986). CYP1A1 is readily inducible in all tissues but constitutive expression is undetectable. Control of this gene is primarily transcriptional, with a small contribution from mRNA stabilisation.

The control of *mdr* gene expression by the Ah locus will be a subject for discussion in Chapter 5.

(b) Phenobarbital-inducible P450 genes

Phenobarbital (PB) causes proliferation of liver endoplasmic

reticulum (ER) membrane (Remmer and Merker, 1963) and increases in the levels of various cytochrome P450s (Orrenius *et al*, 1965). CYP2B1 and CYP2B2 are coordinately regulated in liver by PB. CYP2B1 is transcriptionally inactive in untreated rats while CYP2B2 is constitutively expressed. Both mRNAs are induced in the presence of PB.

PB also induces two genes in the rat CYP3 family. *CYP3A2*, which is male specific and constitutively expressed, and the *CYP3A1* gene which is not expressed are induced by PB. *CYP3A1* is also induced by steroids.

These data suggest that the genes have common regulatory elements for PB but distinct elements for steroid inducibility and constitutive expression. Cis-acting regulatory elements have yet to be defined for specific PB receptors. Extensive metabolism of the compound by liver enzymes may account for difficulties encountered in such studies. Poland *et al* (1980, 1981) reported the potent induction in mice caused by the PB-like ligand 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP). This compound is not extensively metabolised and hence will be valuable in the study of such a receptor. The effect of TCPOBOP on *mdr* gene expression will be discussed in Chapter 5.

(c) Steroid inducible P450 genes

The rat *CYP3A1* gene is induced by glucocorticoids such as dexamethasone and pregnenolone 16  $\alpha$ -carbonitrile. Regulation is distinct from the control of classical glucocorticoid-regulated genes such as tyrosine aminotransferase (Schuetz and Guzelian, 1984). Induction of *CYP3A1* requires approximately ten-fold more steroid than is normally required. Induction results from an activation of *CYP3A1* transcription and increase in mRNA (Simmons *et al*, 1987). The rat *CYP3A1* and *CYP3A2* are stabilised by the macrolide antibiotic triacetyloleandomycin (TAO) (Watkins *et al*, 1986).

(d) Hypolipidaemic drug-inducible P450 genes

Clofibrate induces CYP4A1, enoyl CoA hydratase and fatty acyl-CoA

oxidase genes by rapid transcriptional activation (Hardwick *et al*, 1987; Reddy *et al*, 1986). This leads to an increase in fatty acid  $\beta$ -oxidation.

### 1.5.2 REGULATION OF CONSTITUTIVELY EXPRESSED P450 GENES

Four ways of constitutively-expressed P450 regulation have been reported. These include activation of genes following birth, at the onset of puberty, "sex imprinting" ( activation of expression in males or females) or specific suppression of activity in either males or females at puberty.

CYP2D1 and CYP2D2 are elevated immediately after birth in rats due to transcriptional activation correlating with changes in the methylation states of the genes (Umeno *et al*,1988). Levels peak at six days post partum.

Sex specific genes are present in the rat. Rat *CYP2C6* and *CYP2C7* are transcriptionally activated at the onset of puberty (Gonzalez *et al*, 1986a). *CYP2C11* is expressed only in the adult male liver and is dependent on testosterone exposure both in neonatal and adult life (Waxman *et al*, 1985).

CYP2C12 is expressed in a sex-specific manner in adult female rats, but present in both sexes during adolescence. Suppression of this enzyme occurs in males due to androgen exposure during the neonatal period (Dannan *et al*, 1986).

The role of pituitary hormones in the regulation of sex specific expression of P450s has been studied. Jansson *et al* (1985) reported that adult male and female rats differ in their growth hormone secretion pattern in that males have a pulsatile pattern, whereas females have a more constant secretion. The patterns are set by neonatal androgen expression and adult testosterone levels. CYP2C11 and CYP2C12 regulation both involve growth hormone secretion patterns (Kato *et al*, 1986).

The sex specific expression of certain male and female genes sometimes results from the suppression of gene expression. Expression of CYP2A is decreased in males at puberty due to the loss of the CYP2A protein associated testosterone 7  $\alpha$ -hydroxylase activity (Matsunaga *et al*, 1988). Growth hormone seems to play a role in suppressing P450 expression. Data suggests that the decrease in CYP3A2 in adult females is

due to the continuously high levels of growth hormone found in female rats (Kato *et al*, 1986).

Most P450s characterised to date are expressed in hepatocytes. In the CYP2A family, both CYP2A1 and CYP2A2 are only present in the liver (Matsunaga *et al*, 1988) but CYP2A3 is expressed only in the lung and is undetectable in the liver (Kimura *et al*, 1989). Three different regulatory mechanisms seem to exist for the CYP2A genes. First, CYP2A1 is inducible by 3-MC, second CYP2A2 is induced in males at puberty, and finally tissue specific expression of CYP2A1 and CYP2A2 (liver) and CYP2A3 (lung) exists.

P450 genes can also be post-transcriptionally regulated. One of the best examples of such regulation is the rapid induction of 2E1 protein in rats following administration of ethanol, acetone or pyrazole. The levels of mRNA for CYP2E1 do not appear to increase (Song *et al*, 1986). Song *et al* (1989) suggested that such an increase was due to protein stabilisation. An increase in the efficiency of mRNA translation could also explain such a finding. Fasting in rats causes an increase in CYP2E1 mRNA and protein in addition to elevation of ketone bodies (Hong *et al*, 1987). CYP2E1 is thought to be involved in the conversion of acetone to acetol and methylglyoxal which is metabolised to D-lactate and pyruvate (Koop and Casazza, 1985).

## 1.6 CONCLUDING REMARKS AND AIMS OF THE THESIS

P-glycoprotein is widely accepted as being causative of mdr in many cell lines and probably plays an important part in the failure of chemotherapy. However, the physiological role and natural substrates for P-gp remain largely unknown. How the expression of the mdr genes are regulated in different tissues and in different species still awaits an answer. The mechanism by which it transports drugs across the plasma membrane is still also unknown.

Studies indicate that P-gp has a major role in normal cell physiology, probably to transport diverse molecules across the cell membrane. The occurrence of an mdr-like protein in the drug-resistant parasite *P. falciparum*

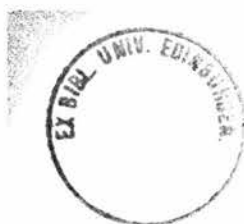
suggests that active efflux by a protein such as P-gp may be a very common defence mechanism against toxic compounds in many diverse species. Other proteins belonging to a transport super family, each with unique substrate specificity, probably await discovery.

Since little is known about the regulation of *mdr* gene expression, various factors, known to effect the expression of P450 and GST drug metabolising enzymes, were examined. *In vivo* regulation was studied in mouse and rat liver. The rat genes have only recently been cloned and sequenced (Silverman *et al*, 1991). However, much is already known about the three mouse genes, which have been cloned, sequenced and studied to some extent (Dhir, 1990; Buschman and Gros, 1991; Cohen *et al*, 1991; Gros *et al*, 1988; Devault and Gros, 1990; Gros *et al*, 1986; Raymond *et al*, 1990) . The distribution of the genes in various tissues has been published (Croop *et al*, 1989).

For our purposes, mouse liver was a good model to study for several reasons. The tissue is known to express all three genes to some extent. The expression and regulation of both cytochrome P450 and GST enzymes<sup>\*</sup> in mouse liver has been widely studied. Correlations between the expression of these drug metabolising enzyme systems and P-gp was evaluated.

Exogenous and endogenous regulation of the *mdr* genes was examined. Exogenous regulation studies involved examining the effects of P450 and GST inducing agents, carcinogens and anti-cancer drugs on the expression of the *mdr* genes in liver.

The relative levels of expression of the P450 gene families, GST  $\pi$  and P-gp appeared, from previous studies, to be particularly relevant to study in an *in vivo* model. GST  $\pi$  is associated with multi-drug resistant cell lines and is a marker for preneoplasia and hepatocarcinoma in rats. CYP1A can be induced by carcinogens and it weakly induced by 2AAF and AFB<sub>1</sub> which have been reported to induce *mdr* in the rat (Burt and Thorgeirsson, 1988). CYP3A1 is inducible by steroids such as dexamethasone and can metabolise progesterone. The *mdr* 1 gene in the mouse is upregulated by progesterone during pregnancy and it has been shown that progesterone specifically binds to P-gp. Other compounds metabolised by CYP3A1 are also substrates for P-gp, such as nifedipine and cyclosporin. A common



mechanism of regulation by such compounds may be operative.

Endogenous regulation of *mdr* gene expression involved studying the effects of pituitary hormones on *mdr* gene expression. Cytochrome P450s have been shown to be under the regulation of growth hormone, which determines the expression of sex specific enzyme forms (Mode *et al*, 1982, 1983; Skett, 1987; Zaphiropolous *et al*, 1988). Thyroid hormones are known to have an effect on the expression of hepatic enzyme systems. Hypothyroidism induced by propylthiouracil (PTU) in mice is accompanied by an increase in hepatic GST and can be reversed by intraperitoneal injection of 3,3',5-tri-iodo-L-thyronine (T3) (Williams *et al*, 1986). It is of interest to determine whether *mdr* genes were also under such types of hormonal control.

To determine whether or not carcinogens act as substrates for the P-gp 'pump', a tissue culture model was employed using a cell line known to express P-gp to a high level.

As a first approach to study the expression of P-gp, it was aimed to raise peptide antibodies against the protein and investigate their applicability in determining the expression of P-gp in cells and tissues.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 MATERIALS

##### 2.1.1 COMMERCIAL MATERIAL SOURCES

Most chemicals were supplied by BDH Limited, Burnfield Avenue, Thornliebank, Glasgow, G46 7TP and Sigma Chemical Company Limited, Fancy Road, Poole, Dorset, BH17 7NH. A comprehensive list of chemicals including tissue culture materials and suppliers is given in Appendix 1.

##### 2.1.2 NON-COMMERCIAL MATERIALS

###### (a) Antibodies and protein standards

Antisera against rat GST subunits and protein standards for use in Western Blotting and SDS PAGE were obtained from Dr. J. D. Hayes, Department of Clinical Chemistry, Royal Infirmary of Edinburgh. Use of these antibodies to discriminate between GST subunits has been described (Hayes and Mantle, 1986). Dr. L. I. McLellan of the same Department kindly provided antisera against mouse GST subunits. The specificity of these antibodies has also been tested (McLellan and Hayes, 1989).

Antisera to purified cytochrome P-450 isozymes were supplied by Dr. C. R. Wolf and have been previously detailed (Wolf and Oesch, 1983, Wolf *et al* , 1984). Monoclonal antibodies against the clofibrate-inducible CYP4 family were produced by Ciba Geigy, R-1066-4 -32, Basel, Switzerland and have been characterised (Savoy *et al*, 1990).

Peptides corresponding to various regions of the human *mdr 1* sequence (Chen *et al*, 1986) were synthesised and conjugated to thyroglobulin and bovine serum albumin by Dr. J. Rothbard, Lincoln's Inn

Fields, London. Peptide antibodies were raised against the thyroglobulin conjugates in New Zealand White rabbits at Clare Hall Laboratories, Imperial Cancer Research Fund, Blanche Lane, Potters Bar, Herts. EN6 3LR.

(b) cDNA clones

The Rat Yf cDNA clone was provided by Professor M. Muramatsu, Department of Biochemistry, Tokyo. The rat Yb1 cDNA clone was gifted by Dr. C. B. Pickett, Merck Frost Centre for Therapeutic Research, Quebec. The rat Ya cDNA was obtained from Dr. J. B. Taylor, CRC Molecular Toxicology Group, Middlesex Hospital, London. Dr. P. Gros, Department of Biochemistry, McGill University, Montreal, Quebec, Canada kindly gifted the mouse mdr 1-, 2- and 3-specific clones. The human mdr 5A clone was obtained from Dr. R. Brown, Wolfson Laboratory for Molecular Pathology, Beatson Laboratories, Bearsden, Glasgow, G61 1BD.

Further details about these clones, and the P-450 clones used, are outlined in Section 2.7.

(c) Chemicals

TCPOBOP (1,4-bis [2-(3,5 dichloropyridyloxy)] benzene) was a gift from Dr. A. Poland, McArdle Laboratory for Cancer Research, Maddison, Wisconsin, U.S.A.

Later aliquots were synthesised and kindly provided for use by Miss G. Smith, Imperial Cancer Research Fund, Molecular Pharmacology Group, University of Edinburgh.

(d) RNA

Hepatic RNA isolated from phenobarbital, dexamethasone and TCPOBOP-treated mice as well as hypophysectomised and sham-operated animals was generously donated by Dr. C. J. Henderson, Imperial Cancer Research Fund, Molecular Pharmacology Group, Edinburgh.

## 2.2 ANIMAL EXPERIMENTS

### 2.2.1 XENOBIOTIC EXPOSURE OF MICE

Mouse experiments were conducted at the Imperial Cancer Research Clare Hall Laboratories, Blanche Lane, South Mimms, Potters Bar, Herts. EN6 3LR.

Male C57BL/6 and DBA/2N mice were fed *ad libitum* on a standard laboratory chow diet and acclimatised in a 12 hour light/dark cycle for at least 4 days before treatments. The actual treatments administered are outlined in Chapter 5. Either corn-oil or phosphate-buffered saline was used as the treatment vehicle. On the final day of the experiment, the mice were sacrificed by cervical dislocation. The organs were removed and snap-frozen in card-ice or liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until required for preparation of RNA or protein extraction. For measurement of corticosterone levels, mice were sacrificed following treatment and cardiac puncture was performed as described in Section 2.13.1. The assay was kindly conducted by Dr. B. Williams, Western General Hospital, Edinburgh.

### 2.2.2 SURGICALLY TREATED MICE

Intra-auricular hypophysectomised male and female C57BL/6 mice plus sham-operated control animals, were obtained from Charles Rivers Laboratories, Willmington, Massachusetts. U.S.A.

### 2.2.3 MUTANT MICE

The mouse strain 'little' (lit/lit), deficient in pituitary growth hormone releasing factor and wild type control animals were obtained from Charles Rivers Laboratories.

Thyroid-deficient transgenic mice were obtained from Dr. R. Al-Shawi, Department of Genetics, University of Edinburgh.

### 2.2.4 CONTROL MICE

Male C57BL/6 (+/+) mice at 1, 2, 4, 6 and 10 weeks old, as well as 15 day embryo, were obtained from Clare Hall Laboratories, Imperial Cancer Research Fund, Blanche Lane, Potters Bar, Herts, U.K.

The livers were removed, snap frozen following cervical dislocation and stored at  $-70^{\circ}\text{C}$  until required.

## 2.2.5 XENOBIOTIC INDUCTION OF RATS

Wistar and Fischer male rats (~150 gm) were obtained from Banton and Kingman, UK. Animals were fed *ad libitum* and acclimatised, as described for the mice, before treatments were administered.

## 2.2.6. PREPARATION OF MICROSOMAL AND CYTOSOLIC FRACTIONS FROM MOUSE AND RAT TISSUES

Microsomal fractions from mouse liver, kidney and lung as well as rat liver were prepared as described by Meehan *et al* (1988a). Tissue was thawed at  $37^{\circ}\text{C}$ , scissor-minced and homogenised in 3 volumes of KCl-phosphate buffer (10 mM potassium phosphate, pH 7.4, 1.15% (w/v) potassium chloride, 0.1mM EDTA). A Silverson Laboratory Mixer Emulsifier was used to homogenise the tissue, whilst the samples were maintained at  $4^{\circ}\text{C}$ . The homogenate was then centrifuged in a Du Pont Sorvall RC-5B Refrigerated Centrifuge (SS 34 rotor) at 11,000 g for 20 minutes to remove nuclei and cell debris. The supernatant was retained and centrifuged at 45,000 rpm at  $4^{\circ}\text{C}$  for 80 minutes in a Du Pont Sorvall Ultracentrifuge OTD 65B (TFT 45.6 rotor). The supernatant (cytosolic fraction) was removed and aliquoted in volumes of 1 ml before being frozen at  $-40^{\circ}\text{C}$  until required. The microsomal pellet was resuspended in KCl-phosphate buffer using a Teflon-glass hand-homogeniser and re-centrifuged for 60 minutes at  $4^{\circ}\text{C}$  at 45,000 rpm. Finally, the pellet was resuspended in ice-cold sucrose buffer (0.25 M sucrose, 10 mM potassium phosphate pH 7.4, 1% (w/v) KCl, 0.1 mM EDTA), aliquoted and stored at  $-40^{\circ}\text{C}$  until required.

## 2.3 HISTOLOGICAL EXAMINATION OF RAT TISSUES

Both tissue fixation and staining was dindly performed by Miss Linda May and Dr D. Harrison, Department of Pathology, University of Edinburgh.

### 2.3.1 TISSUE FIXATION

Following treatment of Wistar rats with 2-acetylaminofluorene (2-AAF), aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and diethylnitrosamine (DEN), as outlined in Chapter 5, the liver and kidney were removed from the animal following sacrifice and fixed for histological examination.

The fixative procedure used is designed to preserve both antigenicity and ultrastructure (McLean and Nakane, 1974). The fixation used is a periodate-lysine paraformaldehyde (PLPD) fixative (0.01M periodate, 0.075M lysine HCl, 2% (w/v) paraformaldehyde and phosphate buffer, pH 7.4) which primarily stabilises carbohydrate moieties. The carbohydrates are oxidised by periodate and cross-linked by lysine.

The tissues were sectioned into small pieces and fixed in the PLPD solution for 3h before being further processed in the routine manner.

### 2.3.2 STAINING FOR MORPHOLOGY AND GLYCOGEN DISTRIBUTION

Haematoxylin and eosin (H/E) stain was used to demonstrate the structure of the liver and kidney tissue following treatment of Wistar rats with 2-AAF, AFB<sub>1</sub> and DEN.

The haematoxylin component stains the cell nuclei blue-black, whilst eosin stains cell cytoplasm and most connective tissue fibres red.

The sections were dewaxed in xylene and hydrated through graded alcohols to water. The fixation pigments were removed and the section stained in an alum haematoxylin for 30'. After washing the sections, they were stained in 1% eosin Y for 10' and washed in water for 5'. The sections were then dehydrated through alcohols, cleared in xylene and mounted in DePex (BDH).

For glycogen staining, the periodic acid-Schiff (PAS) reaction was

employed. The PAS reaction is a useful indicator of the presence of tissue carbohydrates. The sections were dewaxed and placed in distilled water, before being treated with periodic acid (0.5% (w/v), 5'). After washing in distilled water they were immersed in Schiff's solution (0.5% (w/v) basic fuchsin, 1% (w/v) potassium metabisulphite, 1% (v/v) hydrochloric acid) for 15' and washed in water for 5'. Nuclei were stained with haematoxylin. The sections were finally washed in water, rinsed in alcohol, cleared in xylene and mounted.

### 2.3.3 STAINING FOR P450 AND GST $\alpha$

The tissue sections were deparaffinised and washed in water. They were washed twice in TBS (0.01 M Tris, pH 7.6) for 5' and incubated in pre-immune rabbit serum (1:5 dilution in TBS) for 10'. After draining off excess serum, the sections were incubated in the cytochrome P450 or GST polyclonal antibody (each at a 1:50 dilution in TBS) overnight at 4°C. After washing in TBS, the sections were again blocked in pre-immune serum and then incubated in biotinylated swine anti-rabbit (DAKO) (diluted 1:500 in TBS, for 30'). The sections were washed in TBS and incubated for 30' in the avidin biotin complex (ABC). The avidin is conjugated to horseradish peroxidase (HRP) and extra sites on the avidin bind the second antibody. After TBS washing, a diaminobenzidine substrate solution (0.05% (w/v) diaminobenzidine, 0.068% (w/v) imidazole, 2% (v/v) hydrogen peroxide) was applied to the tissue sections (10'). The sections were washed, counterstained in Mayer's haematoxylin and blued-up in alkali. The sections were then dehydrated, cleared and mounted in DePex (BDH).

## 2.4 CELL CULTURE

### 2.4.1 CELL LINES

Four human and four chinese hamster cell lines were cultured during the course of the project. Two of the human and two of the hamster cell lines,

were drug resistant to varying extents and displayed the *mdr* phenotype. The human cell line MES-SA was established from a uterine sarcoma (Harker *et al*, 1983). The development and characterisation of the Dx-5 subline of MES-SA, selected by continuous *in vitro* exposure to the anthracycline antibiotic doxorubicin, has been reported (Harker and Sikic, 1985). The cross-resistance pattern of Dx-5 for various anti-tumour agents is shown in Table 2.1. Both MES-SA and Dx-5 were kindly provided by Dr. B. I. Sikic, Oncology Division, Department of Medicine, Stanford University Medical Centre.

Dr. V. Ling, The Ontario Cancer Institute, Toronto, Canada kindly provided both the human cell line SKOV 3 and the drug-resistant counterpart SKVLB (1.0), and the chinese hamster ovarian cell line Aux B1 and the drug-resistant derivative CHRC5. SKOV 3 is a human ovarian carcinoma cell line (Fogh and Trempe, 1975) obtained originally from the American Type Culture Collection. An SKVLB series of drug-resistant cell lines were obtained by selection of SKOV 3 cells for growth in increasing concentrations of vinblastine (Bradley *et al*, 1989). The levels of drug resistance in SKVLB (1.0) cells are shown in Table 2.1.

The selection of *mdr* CHO cells from the parent line, Aux B1, has been previously described (Ling and Thompson, 1974). The most sensitive cell line, CH<sup>R</sup>C5 is 300 times relatively more resistant to colchicine and 42 times more resistant to vinblastine than the wild-type Aux B1 cell line, as shown in Table 2.1. The adriamycin CHO cell line, CHO-Adr<sup>R</sup> derived from wild-type CHO cells was received from Dr. I. D. Hickson, I.C.R.F., University of Oxford, The Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford. OX3 9DU.

#### 2.4.2. CELL CULTURE TECHNIQUES AND CONDITIONS FOR CULTURE

The cell culture methods used were as described by Freshney (1987). All culture was conducted in a Class 2 Biological Safety Cabinet, MDH Ltd., Walworth Road, Andover, Hampshire. The cabinet was U.V. sterilised when not in use and cleaned at regular intervals.

TABLE 2.1

CROSS-RESISTANCE PATTERNS OF THE MDR CELL LINES STUDIED  
 Dox-5, SKVLB and CH<sup>R</sup>C5 were made resistant to doxorubicin, vinblastine and colchicine respectively.

DRUG	CELL LINE		
	DOX-5	SKVLB	CH <sup>R</sup> C5
Doxorubicin	100	260	-
Vincristine	240	10,000	-
Colchicine	25	510	300
Mitomycin C	10	-	-
Vinblastine	105	2,000	42
Colcemid	-	-	25

These values represent the fold resistance of the selected cell lines compared to parental resistance levels.

- not assayed

Harker and Sikic, 1985; Bradley *et al*, 1989; Ling and Thompson, 1974

(a) Freezing cells for storage and retrieving stocks

On reaching confluency, cells were harvested using 0.1% trypsin, 0.01% EDTA until the monolayer detached. The trypsin was then quickly diluted out by adding growth medium (specific for each particular cell line) and the cells spun down for 5 minutes at 2500 rpm in a MSE Microcentaur centrifuge. The cell pellet was then resuspended at a density of 5-10 million cells/ml in 90% new-born calf serum, 10% DMSO. Aliquots were frozen at -70°C overnight before transfer to liquid nitrogen for long-term storage. DMSO permeates cells rapidly and maintains long-term viability of cell lines. To retrieve cells from storage, an aliquot was thawed at 37°C and carefully resuspended in the appropriate growth medium (10 mls) pre-warmed to 37°C. The cells were then spun at 2500 rpm for 5 minutes and again resuspended in growth medium before being seeded into a 25 cm<sup>2</sup> tissue-culture flat-bottomed flask. The cells were allowed to adhere overnight and

refed the next morning to eliminate any dead cells or other debris from the flask.

(b) Feeding cells

Cells were fed with their own specific media as required. Most cell lines required re-feeding every 2 days to maintain optimal pH and essential growth requirements. MES-SA and Dx-5 were grown in RPMI 1640 medium (Moore *et al*, 1967) plus 10% FCS. MES-SA had a generation time of 22 hr whilst that of the Dx-5 cells was 30 hr. Dx-5 cells were routinely maintained in  $5 \times 10^{-7}$  M adriamycin (adr) at which concentration the cells were selected originally.

SKOV 3 and SKVLB (1.0) were routinely maintained in  $\alpha$ -MEM (Stanners *et al*, 1971). SKVLB (1.0) was also continuously exposed to 1  $\mu$ g/ml vinblastine whilst in culture. CHO and CHO-Adr<sup>R</sup> cells were maintained in RPMI 1640 plus 1  $\mu$ g/ml adriamycin for the drug-resistant variant. Aux B1 and CH<sup>R</sup>C5 cell cultures were grown in  $\alpha$ -MEM with 10% FCS. These lines are auxotrophic, requiring glycine, adenosine, thymidine and proline for growth. CH<sup>R</sup>C5 did not require colchicine, the drug to which the cell line is resistant, to passage it in culture in order to maintain resistance.

Glutamine (0.2 mM), penicillin (15 U/ml) and streptomycin (5  $\mu$ g/ml) were routinely added to the media of all cell lines.

(c) Sub-culture of cells

Just before reaching confluency, cells were sub-cultured by washing them twice in PBS and harvesting them in 0.1% trypsin, 0.01% EDTA until the cells detached. Fresh medium was then added to inhibit the trypsin and the cell suspension spun at 2500 rpm for 5 minutes. The cell pellet was disaggregated in fresh medium.

To ensure accurate seeding, when required, the cells were counted using a Neubauer haemocytometer and seeded into fresh flasks. Their viability could be assessed by nigrosin dye exclusion (Kaltenbach *et al*,

1958).

Most parental cell lines were sub-cultured every 3-4 days at a dilution of 1:10. The cell lines maintained in drug normally grew more slowly and were passaged once a week at a dilution not exceeding 1:10. Drug was routinely added 24 hr after the cells were sub-cultured in order to allow them to re-adhere to the flask surface.

#### (d) Sterility

Culturing of cells was carried out in a sterile cabinet and aseptic techniques were employed at all times. Solutions were either sterilised in a Laboratory Thermal Equipment 225 EH autoclave or filter-sterilised (0.2  $\mu\text{m}$  pore size) before use. Testing the sterility of media and other solutions was routinely done by inoculation of the test solution into sterile L-broth (2% Bactotryptone, 1% Bactoyeast, 2% NaCl) and incubating for 4-7 days at 37°C. A turbid L-broth test was an indication of some form of contamination and the solution could be discarded before use. Regular mycoplasma tests were done by Mr. W. Christie, MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Crewe Road, Edinburgh. The mycoplasma test involved staining with Hoescht 33258 fluorescent dye and growth on selective broth (Taylor-Robson, 1978). Mycoplasma contamination can cause such changes as chromosomal aberrations (Fogh and Fogh, 1965).

#### 2.4.3. PREPARATION OF SOLUBLE PROTEIN LYSATES FOR P-GLYCOPROTEIN ESTIMATION

Cells were grown in 25 cm<sup>2</sup> flasks, seeded at a density of  $1 \times 10^6$  cells per flask. The cells were grown overnight and re-fed with 5 ml of fresh media until confluent. For the preparation of protein lysates, cells were washed twice in ice-cold PBS to eliminate all traces of media. Cold lysis buffer (0.1 M Tris, pH 8.0, 0.5 % NP 40, 10% glycerol, 0.5 ml and 2 mM PMSF added from a 200 mM stock in ethanol just before use of the buffer) was added to each flask and the cells rocked for 10 minutes at 4°C to

detach the cells from the sub-stratum and solubilise the protein. The cells were then harvested and disaggregated in the buffer using a fine-tipped pastette. The lysate was then spun for 2 minutes at 13,000 rpm in a bench-top microfuge at 4°C. The supernatant, containing the plasma membrane fraction was retained, aliquoted and frozen at -40°C until required.

## **2.5 PROTEIN DETERMINATION OF SAMPLES**

### **2.5.1 PROTEIN ESTIMATION**

The method of Lowry *et al* (1951) was used to estimate the protein content of both tissue and cell culture samples. A range of known bovine serum albumin concentrations were used as the standards from which the protein concentration of the unknown was calculated.

All samples were diluted, usually 1:40, in 0.1 M NaOH to within a range of 0-200 µg/ml. The plot of protein concentration against OD<sub>600nm</sub> of the final complex was approximately linear over this range. A range of standards were assayed each time samples were to be measured. A Shimadzu UV 160 spectrophotometer was used to measure the optical densities. Each standard was assayed in duplicate, whilst each sample was determined in triplicate.

### **2.5.2 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS PAGE)**

Following the determination of protein concentration, all samples were assessed using SDS-PAGE according to the method of Laemmli (1970). This was performed to check the accuracy of the protein concentration estimates and to ensure the integrity of the samples.

Using SDS-PAGE it is possible to estimate the relative intensities of the proteins in the molecular weight range for P450s and GSTs, in the mouse and rat microsomal and cytosolic samples respectively, by staining the polyacrylamide gel in Coomassie Brilliant Blue R dye. P-glycoprotein is difficult to detect using this method, but can be stained for by using the

sensitive silver staining method of Switzer *et al* (1979).

Samples for assessing P450 and GST proteins were prepared for electrophoresis by diluting them to a protein concentration of 2 mg/ml with water, adding an equal volume of sample preparation solution (0.05 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.05% (w/v) bromophenol blue dye) and heating to 100°C for 5 minutes. Samples could be loaded immediately or stored frozen at -40°C until required.

Protein from tissue fractions (10µg) and cultured cell preparations (30µg) was routinely separated by SDS-PAGE to assess loadings. For immunoblotting, rat and mouse microsomes (15µg) and cytosols from liver and other tissues were loaded for analysis. Rainbow molecular weight protein markers (Amersham International plc., Bucks., U.K.) were used for molecular weight calibration purposes. The marker proteins (5µl) were diluted 1:1 in sample preparation solution and heated to 100°C for 5 minutes, prior to separation by SDS-PAGE. The marker proteins were myosin (200,000 kD), phosphorylase b (92,500 kD), bovine serum albumin (69,000 kD), ovalbumin (46,000 kD), carbonic anhydrase (30,000 kD), trypsin inhibitor (21,500 kD) and lysozyme (14,300 kD). SDS PAGE gels (0.15 cm thick) were cast using a BioRad Protean 2 Vertical Electrophoresis kit.

For detection of P450 proteins, the separating gel consisted of 9% (w/v) polyacrylamide (8.7% acrylamide, 0.3% N, N'-methylene-bis-acrylamide) in 0.375 M Tris-HCl, 0.15% SDS. For separating GST proteins, the gel was 12% (w/v) polyacrylamide. Ammonium persulphate (APS), freshly prepared, and N, N, N',N'-tetra-methyl ethylenediamine (TEMED), both 0.05%, were added to initiate polymerisation. A stacking gel (4.5% (w/v) acrylamide, 0.125M Tris-HCl pH 6.8, 0.125% SDS, 0.05% APS, 0.05% TEMED) was poured on top of the polymerised separating gel, and the sample wells formed using a teflon comb of appropriate size. The gels were run in Electrode buffer (0.05M Tris-HCl, pH 8.3, 0.05 M glycine, 0.1% SDS). Proteins were run through the stacking gel at 50mA per gel, and thereafter at 30 mA per gel constant current until the dye front was 1 cm from the bottom

of the gel (~4 hr). The apparatus was water-cooled. When separation was complete, gels were fixed in 45.5 % methanol, 9% acetic acid for 1 hr, stained in Coomassie Brilliant Blue R (0.25% (w/v) Coomassie Blue R in the same solution) for 1 hr and destained in 10% methanol, 7% acetic acid, until the background staining had disappeared and the protein bands were clearly visible.

### 2.5.3 ELECTROPHORESIS OF PROTEINS FOR P-GLYCOPROTEIN IMMUNOBLOTTING

Estimation of protein loading and integrity was done in the same manner as for P450 and GST protein except that the separating gel consisted of 7 % (w/v) polyacrylamide. Two methods were assessed for separation of proteins to be immunoblotted.

The first method involved the use of the Laemmli system previously described. The separating gel was 7% polyacrylamide and no stacking was used.

Cells were harvested using trypsin/versene and hand-homogenised using a teflon-glass homogeniser. The whole cell homogenate was then spun at 4000g, 10 minutes to pellet the unbroken cells and nuclei. The supernatant was retained and re-centrifuged (100,000g, 1 h, 4°C). The resultant pellet was resuspended and retained.

The fractions examined were as follows:

- (a) whole cell homogenate
- (b) 4000g pellet
- (c) 100,000g pellet.

All fractions were suspended in KCl-phosphate buffer previously described in Section 2.2.6. Aux B1 and CH<sup>R</sup>C5 cell samples were prepared under a variety of conditions, to assess the best method of maintaining immunogenicity. All samples were diluted 1:1 in sample preparation solution, as described in Section 2.5.2, and used directly, or after heating for 5 minutes at 100°C. Samples were also prepared with or without 2-mercaptoethanol.

Section 2.6.3 (a) describes the results obtained on these cell preparations following immunoblotting.

The second method for SDS PAGE was more successful in resolving the P-glycoprotein band and used a modification (Debenham *et al*, 1982) of the procedure of Fairbanks *et al* (1971). The modifications were the inclusion of 2% (w/v) SDS in the solubilising buffer and the addition of urea to both the sample and the polyacrylamide gel (4.5 M and 9.0 M respectively).

The cell lysate protein samples, prepared as described previously in Section 2.4.3, were diluted 1:1 in a buffer consisting of 9.0 M urea, 2% (w/v) SDS, 10% (w/v) sucrose, 50 mM dithiothreitol (DTT), 1mM EDTA, 10 mM Tris-HCl pH 8.0. This buffer was prepared, aliquoted and kept frozen at -40°C until required. Bromophenol blue was used as a tracking dye.

The sample volume was kept as small as possible to optimise the resolution of the protein bands since the gel system of Fairbanks *et al* (1971) does not employ a stacking gel.

Gels were prepared in electrophoresis buffer (1% (w/v) SDS, 2 mM di-sodium EDTA, 20 mM sodium acetate, 40 mM Tris-HCl , adjusted to pH 7.4 with glacial acetic acid (Fairbanks *et al*, 1971). The gels contained 5.6% (w/v) acrylamide and 0.21% bis-acrylamide polymerised by the addition of 0.025% (v/v) TEMED and 0.15% (w/v) APS and were routinely cast in a mini Protean 2 BioRad kit with a 10-well comb. The sample wells were rinsed with electrophoresis buffer immediately prior to loading to remove diffused urea and the solubilised samples (50-100µg of protein) loaded by underlaying.

## **2.6 IMMUNOCHEMICAL DETECTION**

### **2.6.1 ELISA ASSAY TO DETERMINE P-GLYCOPROTEIN ANTIBODY TITRES**

Rabbits were immunised with various peptide antigens, as described in Section 3.2, and bled at specific time points throughout the immunisation schedule. Serum was collected by allowing the blood to clot overnight at

4°C and centrifuging (1500 rpm, 15 minutes) it to spin out the remaining red blood cells. The serum was then aliquoted and stored at -40°C until the titre was measured. Pre-immune serum from each rabbit was collected, aliquoted and frozen at -40°C.

The protocol used to measure the serum titre was described by Campbell (1984) and involves a checkerboard ELISA for soluble antigens.

The antigen of interest was initially diluted to 50µg/ml, and serially diluted to a final concentration of 1: 64. The lowest dilution of the antigen solution (100µl) was added to each well of row A of a 96-well plate. Row B contained the next dilution and so on until row G. PBS (100µl) was added to row H. The plate was incubated overnight at 4°C to allow the soluble protein antigen to be passively adsorbed onto the plate surface. Since most solid supports adsorb proteins non-specifically by hydrophobic interactions, they will also adsorb the antibody if the plate is not fully saturated with antigen. Therefore once the antigen was bound, the plate was then incubated for 1 h at room temperature with 10 mg/ml BSA in PBS (100µl). The plate was then washed three times with PBS containing 0.05% (v/v) Tween 20, to reduce the background and discourage the formation of further hydrophobic interactions between the support and first and second antibodies. Dilutions of the rabbit serum were made, starting with a 1:100 dilution (in PBS containing 0.5 mg/ml BSA) up to 1:10,000 dilution. To all wells in column 1, 100 µl of 1:100 dilution of anti-serum was added; to each in column 2, 100 µl of 1:200 was added and so on. In column 12, a 1:100 dilution of control serum was added and the plate incubated at room temperature for 2 h. Washing was performed as before. Alkaline phosphatase anti-rabbit IgG conjugate, diluted 1:500 in 0.5 mg/ml BSA, 0.05% Tween 20 (100µl) was then added to all wells and the plate incubated for 2 h at room temperature. The wells were then washed three times in PBS containing 0.05 % Tween 20 and knocked dry.

A solution of 1mg/ml p-nitrophenyl phosphate freshly dissolved in 9.7% (v/v) diethanolamine buffer, pH 9.8 (100µl) was then added. The reaction was stopped by the addition of 3N NaOH (50µl). Absorption was read at 405 nm, in a BioRad Model 2550 EIA spectrophotometer adapted for

96-well microtite plates. The ELISA plates were a strong colour in the top left-hand corner fading to a weaker colour in the bottom left-hand corner. Row H and column 12 had little colour and provided the background. The titre of the serum was estimated by finding the highest dilution at which it gave more colour than the pre-immune serum and the wells without antigen.

## 2.6.2 WESTERN BLOTTING ANALYSIS FOR CYTOCHROME P450 AND GLUTATHIONE S-TRANSFERASE PROTEINS

Western blot analysis was performed by a modification of the method of Towbin *et al* (1979). For analysis of both the microsomal and cytosolic fractions from rat and mouse liver, kidney and lung the same basic procedure was adopted. Where polyclonal antibodies were employed, dual labelling with horseradish peroxidase (HRP)-conjugated donkey anti-serum against rabbit IgG was used. Where the first antibody was monoclonal, the second antibody was HRP-conjugated rabbit anti-serum against mouse IgG. SDS-PAGE was performed as described in Section 2.5.2.

For liver, kidney and lung microsomes, 15  $\mu\text{g}$ , 30  $\mu\text{g}$  and 50  $\mu\text{g}$  of protein were loaded respectively. For liver cytosols, 20  $\mu\text{g}$  (for Ya<sub>1</sub>, Ya<sub>2</sub> and Ya<sub>3</sub> subunits) and 5  $\mu\text{g}$  (for Yf and Yb subunits) of protein was required. For kidney and lung cytosols, 30  $\mu\text{g}$  and 50  $\mu\text{g}$  were loaded respectively.

Following electrophoresis, the gel was marked to identify it and placed on top of a 0.45  $\mu\text{m}$  nitrocellulose filter, cut to a slightly larger size than the gel. This was sandwiched between 2 sheets of Whatman 3 mm paper and "Scotch-brite" pads to keep the gel in place. The sandwich was placed in a BioRad transblotting cassette and this was transblotted overnight at 250 mA, with the nitrocellulose sheet nearest the anode, in a BioRad transblotting tank. The transblotting buffer (20mM disodium ortho-phosphate, 20% methanol) was gently agitated throughout this procedure. After transfer, the nitrocellulose was trimmed to the exact size of the gel and washed for two 10 minute periods in 50 mM Tris-HCl, pH 7.9, 0.15 M NaCl, 0.05 % Tween 20 (TBST buffer) then blocked in 3% low fat powdered milk (Marvel - Cadbury's) in TBST for 1 h. To check protein transfer from the gel, it was fixed and stained in Coomassie Brilliant Blue R and destained as described

in Section 2.5.2. Transfer was complete if no protein bands were visible.

After blocking the nitrocellulose, the filter was washed twice in TBST and incubated for 60 minutes with the anti-serum against the P450 or GST protein of interest (1:500 or 1:1000 dilution of antirabbit or antimouse antibody respectively). The nitrocellulose was then washed 4 times (10 minutes each wash) and incubated with HRP- conjugated anti-rabbit IgG or anti-mouse IgG (both 1:1000 dilution in TBST). A further four 10 minute washes in TBST followed before the immuno-reactive proteins were visualised. This was done by exposing the filter to the HRP substrate (120 mg of 4-chloro-1-naphthol in 40 ml methanol, 200 ml of TBS (50 mM Tris-HCl pH 7.9, 0.5M NaCl) and 80  $\mu$ l of 30% H<sub>2</sub>O<sub>2</sub>). The filter was shaken gently in this mixture until the immuno-reactive proteins were visible and then washed for 10 minutes in distilled water to stop the reaction. It was then labelled with 0.19 MBq <sup>125</sup>I-conjugated protein A (Amersham International plc., Amersham, Bucks, U.K.) in 50 mls of TBST for 45 minutes and repeatedly washed with TBST to remove unbound radioactivity, until the wash solution recorded background levels. Filters were air-dried, covered in Saran wrap and exposed to Kodak X-Omat AR-5 X-ray film at -70°C in a lead cassette with intensifying screens. The film was developed at an appropriate time interval using a Gevomatic 60 (Agfa-Gevaert) automatic developer.

### 2.6.3 WESTERN BLOTTING OF P-GLYCOPROTEIN

Various methods were assessed for immunoblotting P-glycoprotein using cultured cells. Aux B1 sensitive and CH<sup>R</sup>C5 mdr chinese-hamster ovary cells were used to develop the best method. Initially, a system using standard 7% Laemmli SDS-PAGE followed by wet-blotting transfer to nitrocellulose was used. Later a more successful system, using SDS-PAGE urea gels followed by semi-dry blotting, was employed.

#### (a) Laemmli SDS-PAGE/wet blotting method

Polyacrylamide gels (7%) were prepared as described in Section

2.5.3. The wet transfer of the proteins from the gel to the nitrocellulose and subsequent blocking was as described in Section 2.6.2. After blocking the filter was incubated in C219 P-glycocheck monoclonal antibody (1:100 in TBST) overnight at 4°C. The monoclonal recognises the IgG<sub>2a</sub> class of immunoglobulin. The filter was then given two 10 minute washes in TBST before being incubated in either 0.19 MBq <sup>125</sup>I conjugated anti-mouse IgG or <sup>125</sup>I conjugated protein A for 45 minutes. The filter was then repeatedly washed in TBST to remove unbound radioactivity. Filters were air-dried, covered in Saran wrap and exposed to Kodak X-Omat AR-5 film at -70°C. The film was developed between 4-14 days using an automatic developer. The results of this immunoblotting method for Aux B1 and CH<sup>R</sup>C5 cell line samples, prepared in various ways, described in Section 2.5.3, is shown in Figure 2.1.

Using the Laemmli SDS PAGE system, the detection of the P-gp was best when using a 100,000g fraction of the solubilised, non-heated mdr cells. Exposure of the blot to antimouse IgG-I<sup>125</sup> gave a more specific reaction than Protein A-I<sup>125</sup>. Protein A is characterised by its ability to interact to some degree with the IgG of almost all mammals but gives only a weak reaction with IgG<sub>1</sub>. However, the isotype of C219 is IgG<sub>2a</sub> (Kartner *et al*, 1985) and thus a reaction between the Protein A and C219 was expected. The time required for autoradiograph exposure to detect P-gp using this Laemmli system was considerable. Even using antimouse IgG-I<sup>125</sup> as the second antibody still required a considerable length of time for exposure of the autoradiograph. It was decided later therefore to adopt the urea gel system and ECL detection to improve the integrity of the signal and the speed of detection. The procedure for this technique is outlined in below.

Greenberger *et al* (1988) reported a comparative study using a polyclonal antibody against P-gp electrophoresed using the Laemmli and modified Fairbanks gel system. He found that the electrophoretic mobility and the amount of P-gp detected were remarkably dependent on the conditions of analysis. He observed that boiling P-gp containing samples before Laemmli gel electrophoresis decreased its mobility by ~15kD and

reduced the apparent amount of the protein. Figure 2.1 shows similar results obtained in this study using such a system.

(b) Modified Fairbanks urea gel SDS-PAGE/semi-dry blotting method

Western blot analysis was performed using a semi-dry electrophoresis blotter which allowed for fast, efficient electro-transfer of P-gp from polyacrylamide gels onto nitrocellulose. The Milli-blot SDE (Millipore) transfer system provided an even, uniform pressure across the entire surface of the gel, ensuring consistent transfer from the entire gel. It was found that this was a superior method for transfer of high molecular weight proteins such as P-gp, than the BioRad buffer tank transfer system.

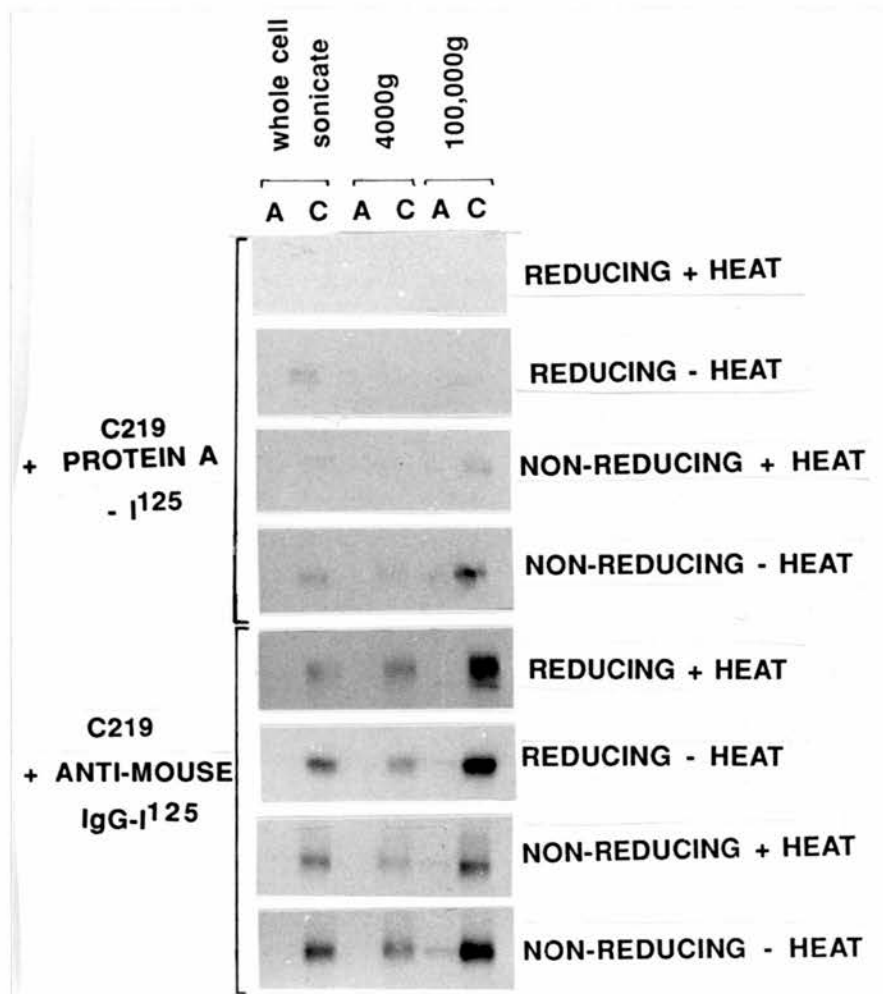
SDS-PAGE was performed using the modified urea gel system of Fairbanks *et al* (1971), as described in Section 2.5.3, loading 50-100  $\mu$ g of solubilised protein per track.

Following electrophoresis, the gel was marked for orientation and briefly soaked in cathode buffer (25 mM Tris-HCl, 20% methanol, 0.1% SDS, 40 mM 6 amino-n-hexanoic acid, pH 9.4). Six pieces of Whatman 3 mm paper were cut to the same size as the gel along with one piece of nitrocellulose. Two pieces of 3 mm paper were soaked in anode buffer no.1 (0.3 M Tris-HCl, 20 % methanol, 0.1% SDS, pH 10.4), 1 piece in anode buffer no. 2 (25 mM Tris-HCl, 20% methanol, 0.1% SDS, pH 10.4) and 3 pieces in cathode buffer. The nitrocellulose was placed briefly in distilled water to wet it. The anode and cathode of the transfer apparatus consisted of 2 graphite plates which were first washed in distilled water. The 2 anode buffer no. 1 papers were carefully placed on the anodic plate, excluding air

**FIGURE 2.1 IMMUNOBLOTTING AUX B1 AND CHRC5 CELL LINES**

Cell fractions were prepared, as described in Section 2.5.3, were Western blotted using the C219 monoclonal antibody.

- A AuxB1
- C CH<sup>R</sup>C5



bubbles, upon which the no. 2 anode buffer filter paper was placed. The nitrocellulose, gel and cathode buffer soaked papers were then assembled in order, with the cathodic plate placed on top. Transfer was routinely carried out for 90 minutes at 1 mA/cm<sup>2</sup>.

The assembly was taken apart carefully and the nitrocellulose filter washed, blocked and incubated in C219 monoclonal antibody, as described in Section 2.6.3 (a). The filter was washed in TBST for two 10 minute periods before being incubated in HRP conjugated-rabbit anti-serum raised against mouse IgG (diluted 1:5000 in TBST) for 20 minutes at room temperature. The filter was then washed 4 times in TBST for a total time of 20 minutes. The immunoreactive proteins were visualised using the ECL detection system (Amersham International plc., Amersham, Bucks., U.K.)

#### 2.6.4 ECL DETECTION OF PROTEINS

The Enhanced Chemiluminescence (ECL) Immuno-blotting detection system is a light-emitting method for detection of immobilised specific antigens conjugated indirectly with HRP-labelled antibodies.

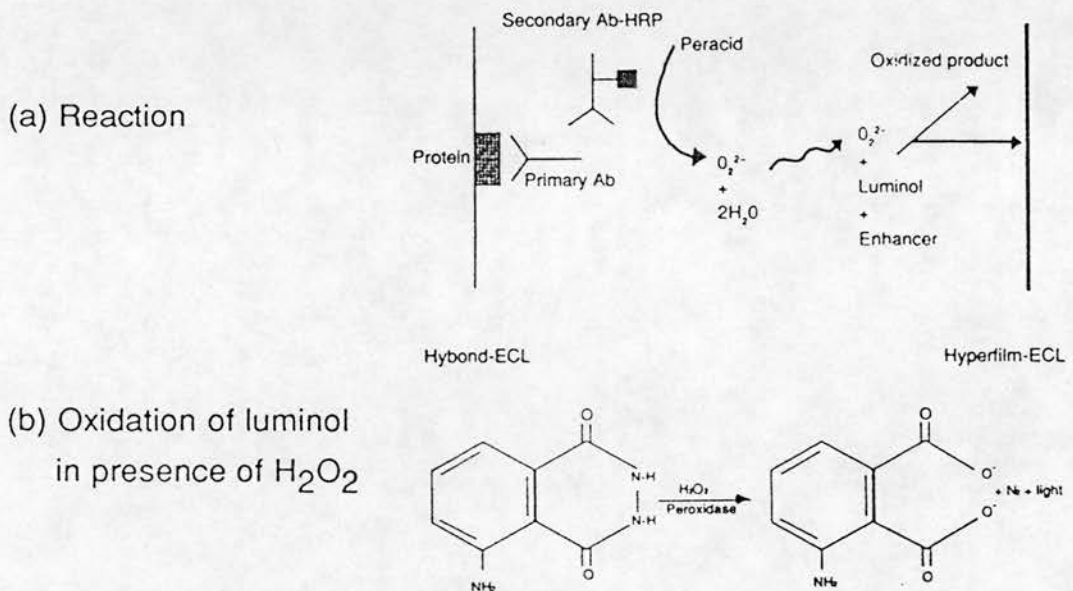
The principle of the detection is shown in Figure 2.2. The chemiluminescent reaction of cyclic diacyl hydrazines such as luminol has been extensively studied (Roswell *et al*, 1978). HRP is used to catalyse the oxidation of luminol in the presence of H<sub>2</sub>O<sub>2</sub>. Following oxidation, luminol decays to a ground state via the emission of light. This system is more sensitive than radioactive detection systems and was used for the detection of P-glycoprotein in cell line samples. Following washing in TBST, nitrocellulose filters were covered in a 1:1 mixture of the solutions provided in the kit to a final volume of 0.125 ml/cm<sup>2</sup> of membrane and incubated for 1 minute, drained, wrapped in Saran wrap and exposed to Kodak X-Omat AR-5 film for between 10 seconds to 30 minutes depending on the level of the protein present.

#### 2.7 cDNA CLONES

Hepatic GST, P450 and mdr mRNA levels in mouse and rat were

investigated by Northern blot analysis. Two cytosolic gene families were examined. The alpha class GST mRNA was measured using pGSTr155, which contained a partial cDNA insert coding for the N-terminal 129 amino acids of the rat Ya subunit. A clone containing the complete amino acid sequence for the human acidic GST cloned into pUC 19 was used to probe for homologous sequences in the mouse.

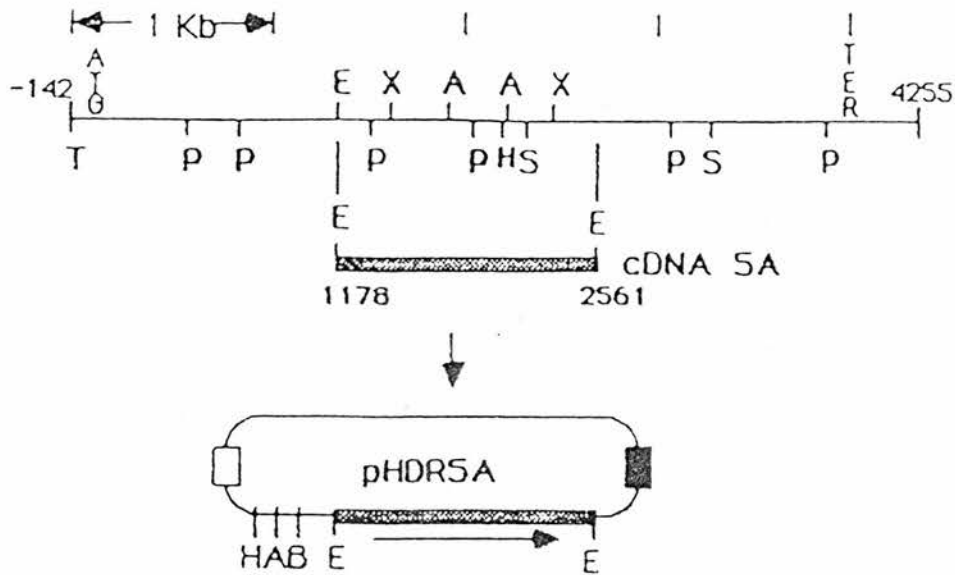
**FIGURE 2.2 ECL PROTEIN DETECTION**



Mouse and rat liver were also probed with various P450 clones. A full-length human clone of 1.75 kb was used to probe the cytochrome P450 sub-family CYP2A6. This clone encodes a protein with coumarin 7-hydroxylase (COH) activity (Miles *et al*, 1990). Yamano *et al* (1989) also described a full length human CYP2A cDNA which differed from the pMP 81 sequence by only 3 nucleotides in the coding region. The human CYP2A6 cDNA deduced protein displayed 85% and 82% homologies with rat CYP2A3 (Kimura *et al*, 1989) and mouse Cyp 2a-4 (Squires and Negishi, 1988) proteins. A full-length clone encoding the rat CYP2B1 (Friedberg *et al*, 1986) was provided by Dr. M. Adesnik, Department of Cell Biology, New York University School of Medicine, New York, U.S.A. A partial length (1.4 kb) mouse orthologue of the rat CYP2C clone (Meehan *et al*, 1988) was

also used. Dr. K. Stevenson, Imperial Cancer Research Fund, Molecular Pharmacology Group, Edinburgh kindly provided a 727 bp fragment of human CYP3A4 cloned into an EcoRI site of pUC 9. The isolation and characterisation of the full-length cDNA clone has been previously described (Stevenson *et al*, 1988). Dr. G. Gibson, Department of Biochemistry, University of Surrey, Guildford, Surrey kindly provided the full-length rat CYP 4A1 clone (Earnshaw *et al*, 1988).

**FIGURE 2.3 cDNA PROBE 5A FOR HUMAN mdr 1 GENE**



The cDNA 5A (EcoRI-EcoRI, 1383 bp) was subcloned into EcoRI site of pGEM4 (Promega Biotec).

□, T7 promoter; ■, SP6 promoter;

A, Acc I; B, BamHI; E, EcoRI; H, HindIII; P, Pvu II;  
S, Stu I; X, Xmn I.

**TABLE 2.2 INFORMATION ON CLONES**

PLASMID	VECTOR	cDNA CLONED	INSERT	REFERENCE
pGSTR155	pAT153	153 amino acids of rat Ya	452 bp	Taylor <i>et al</i> , 1984
pGP5	pUC8	complete rat Yf	734 bp	Sugoaka <i>et al</i> , 1985
pMP81	pUC19	human CYP2A3	1.75 kb	Miles <i>et al</i> , 1989
pMP23	pUC19	rat CYP2B1	1.7 kb	Friedberg <i>et al</i> , 1986
pMP63	pUC9	mouse Cyp2c	1.4 kb	Meehan <i>et al</i> , 1988
pMP26	pUC9	partial human CYP3A4	727 bp	Stevenson <i>et al</i> , 1988
pMP112	pUC9	full length rat CYP4A1	2.1 kb	Earnshaw <i>et al</i> , 1988
pMP90	pUC19	mouse actin	1.2 kb	Leader <i>et al</i> , 1985
pMDR5A	pGEM-4	partial human mdr 1	1.38 kb	Ueda <i>et al</i> , 1987
BBpG4	pGEM-4	mouse mdr 1	180 bp	Raymond <i>et al</i> , 1990
H1pG3	pGEM-3	mouse mdr 2	408 bp	as above
H3pG3	pGEM-3	mouse mdr 3	140 bp	as above

A mouse actin cDNA probe was given by Dr. J. McNab, Institute of Virology, Glasgow. Dr. R. Brown provided the mdr 5A clone for the 1383 bp EcoR1 insert of the human mdr 1 gene (Figure 2.3) cloned into pGEM.

All 3 mouse gene specific mdr clones were gifted by Dr. P. Gros, Department of Biochemistry, McGill University, Quebec, Canada, H3G 1Y6. The plasmids all have a pGEM backbone and their isolation is described by Raymond (1990). The specificity of these probes was examined before use, as shown in Section 5.3 and Figure 5.12.

The information regarding these clones is summarised in Table 2.2.

## **2.8 PLASMIDS**

### **2.8.1 PLASMID CULTURE**

Bacterial cells were transformed using the calcium chloride method of Mandel and Higa (1970). Transformed *Escherichia Coli* K12 strain HB101 were selected and maintained on L-agar plates with appropriate antibiotic selection. Tetracycline (10 µg/ml ) or ampicillin (100 µg/ml) was used depending on the plasmid construct.

### **2.8.2 STORAGE OF PLASMIDS**

Transformed bacteria were grown in L-broth overnight with appropriate antibiotic selection. Glycerol was added to a final concentration of 40% (v/v) and 1 ml aliquots were frozen at -70°C. Plasmid DNA from transformed cells was also prepared as described in Section 2.8.4 and stored at -70°C.

### **2.8.3 MEDIA USED FOR BACTERIAL CULTURE**

L-broth and L-agar were used for the culture of HB101 in liquid and solid phase respectively. L-agar plates with streaked out colonies were stored at 4°C for up to 4 weeks and then sub-cultured by selecting a single

colony and incubating it in L-broth plus antibiotic at 37°C overnight followed by re-streaking onto L-agar.

#### L-broth

Difco bactotryptone	10g/l
Difco yeast extract	5g/l
NaCl	5g/l

#### Difco agar

As for L-broth but including 15g/l Difco agar.

#### 2.8.4 PREPARATION OF PLASMID DNA

Initially the procedure used for large-scale preparation of plasmid DNA was a modification of the method of Birnboim and Doly (1979). Latterly, Qiagen kits were employed.

##### (a) Plasmid DNA preparation (Birnboim and Doly, 1979)

A single colony from an L-agar plate was inoculated into 10 ml of L-broth plus appropriate antibiotic and incubated for 6-8 hr at 37°C with shaking. This culture was seeded into a 1 litre conical flask containing 500 ml of pre-warmed L-broth plus appropriate antibiotic and shaken at 37°C overnight. The cell suspension was spun at 8,000g at 4°C for 10 minutes in a Sorvall RC 5B, SS34 rotor. The bacterial pellet was then resuspended in 20 ml of solution A (2 mg/ml lysozyme, 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM di-sodium EDTA) and kept on ice for 30 minutes to lyse the bacterial cell wall. Solution B (1% SDS, 0.2 M NaOH, 40 ml) was then added and the mixture left on ice for 5 minutes to further disrupt the bacterial

cell membrane. To precipitate the proteins, Solution C (3M sodium acetate, pH 4.8, 20 ml) was added and the mixture left on ice for a further 1 h. The heavy white precipitate which had formed was pelleted by centrifugation at 10,000 g , 4<sup>o</sup>C, 15 minutes. The supernatant, which contained the plasmid, was retained and ice-cold isopropanol (0.7 volumes) added. The DNA was precipitated by chilling the solution for 30 minutes at -20<sup>o</sup>C, followed by a 10 minute spin (10,000 g, 4<sup>o</sup>C). The pellet was resuspended in Solution D (100 mM sodium acetate, 50 mM Tris-HCl, pH 8.0, 10 ml). Cold ethanol (2 volumes) was added and the solution kept at -20<sup>o</sup>C for 1 h until re-centrifuged for 10 minutes at 10,000 g , 4<sup>o</sup>C. The pellet from this spin was resuspended in TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 6.7 ml) to which caesium chloride (74g) and ethidium bromide (10 mg/ml, 1.4 ml ) was added. After the suspension was centrifuged (200,000g, 16 hr ,20<sup>o</sup>C) the nucleic acid bands were visualised using a short-wave transilluminator. Using a hypodermic needle inserted into the side of the ultracentrifuge tube, the band of closed, circular plasmid DNA was collected as described by Maniatis (1982). Ethidium bromide was then extracted using water-saturated butanol and the solution dialysed against three changes of 1000 volumes of TE at 4<sup>o</sup>C for 16 hr with stirring. The DNA was precipitated at -70<sup>o</sup>C for 30 minutes by adding cold ethanol (2 volumes) and 3M sodium acetate, pH 4.8 (0.1 volume) and spinning at 10,000g, 4<sup>o</sup>C for 10 minutes. The plasmid was finally resuspended in an appropriate volume of TE buffer and stored at -20<sup>o</sup>C.

(b) Plasmid DNA Preparation (Qiagen Column Method)

The initial steps of the protocol were essentially the same as for part (a). Following addition of 0.2M NaOH and 1%(w/v) SDS, 2.55M potassium acetate, pH 4.8 was added to precipitate proteins. The mixture was then centrifuged at 4<sup>o</sup>C for 30 minutes, 20,000g. The supernatant was carefully but promptly removed to obtain a particle-free clear lysate. A Qiagen column

was equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50mM MOPS, 15% (v/v) ethanol, pH 7.0, 0.15% (v/v) Triton X-100) and allowed to empty by gravity flow. The supernatant, containing plasmid DNA was then applied to the column and allowed to enter the resin by gravity flow. The column was then washed with 3 x 10ml buffer QC (1.0 M NaCl, 50 mM MOPS, 15% (v/v) ethanol, pH 7.0). At pH 7.0, the solubility of DNA is reduced and it will stick to the column. The DNA was then eluted with 15 ml of buffer QF (1.25M NaCl, 50 mM MOPS, 15%(v/v) ethanol, pH 8.2). Normally plasmid DNA is highly soluble at slightly alkaline pH. The DNA was then precipitated with isopropanol ( 0.7 volumes) and centrifuged at 4°C for 30 minutes, 10,000g. The DNA pellet was finally washed in 70% ethanol, dried briefly by dessication and redissolved in a suitable volume of buffer and kept frozen at -20°C.

## **2.9 RNA PREPARATION**

To prevent degradation of RNA by contaminating nucleases, all items used in the extraction procedure were thoroughly soaked in 0.1% DEPC-treated water for 2h, rinsed and autoclaved. DEPC is a strong but not absolute inhibitor of RNAases. Rinsing and autoclaving removes traces of DEPC that might otherwise modify purine residues in RNA by carboxymethylation. All tissues were snap-frozen in liquid nitrogen immediately following sacrifice and dissection. Extractions were carried out on ice and gloves were worn at all times.

### **2.9.1 PREPARATION OF TOTAL CELLULAR RNA FROM CULTURED CELLS**

Total cellular RNA was prepared from 75cm<sup>2</sup> flasks of confluent cells in culture using the method described by Birnboim (1988) which utilises differential precipitation to separate high molecular weight RNA from other types of nucleic acid.

To detach the cells from the sub-stratum, the culture medium was poured off and the cells washed in PBS followed by the addition of RES

buffer (2 ml of 0.5M LiCl, 1M urea, 0.25% SDS, 0.02M sodium citrate, 2.5 mM CDTA, pH 6.8) with gentle shaking. The mixture was then poured into an ice-cold universal tube and the culture flask washed with a further 1 ml of RES buffer. This was pooled with the first wash and the cell suspension was sonicated for 5-10 seconds at low power to disrupt the cells before adding proteinase K (150 $\mu$ l of 1mg/ml) and incubating the mixture for 30 minutes at 50 $^{\circ}$ C. Proteinase K is a non-specific serine protease that is prepared free from nuclease activity. The sample was then cooled to room temperature and sodium acetate, pH 5.2 (200 $\mu$ l) and ice-cold ethanol ( 7ml ) were added. The mixture was cooled at -20 $^{\circ}$ C for 20 minutes and centrifuged at 10,000 rpm for 10 minutes. The pellet from this spin was dissolved in RES buffer (1ml) and extracted with chloroform/isoamyl alcohol (24:1, 100 $\mu$ l) vortexed for 2-3 minutes and centrifuged at 13,000g for 5 minutes.

The aqueous phase was precipitated in 2M acetic acid, pH 5.2 (7.5 $\mu$ l ) and 5M LiCl/ethanol, 3:2 (1ml). RNA was precipitated overnight at 4 $^{\circ}$ C and centrifuged at 13,000g for 2 minutes. The pellet was dissolved in 1mM sodium citrate, 1mM CDTA, 0.1% SDS, pH 6.8 (100 $\mu$ l) and frozen at -70 $^{\circ}$ C until required. The quality and quantity of the RNA was assessed as outlined in Section 2.10.

## 2.9.2 PREPARATION OF TOTAL RNA FROM WHOLE TISSUES

Total RNA was extracted using approximately 1g of tissue from pooled samples of snap-frozen tissue using the method of Cox (1968). Proteins dissolve readily in solutions of potent denaturing agents such as guanidium hydrochloride. Cellular structures disintegrate and nucleoproteins dissociate from nucleic acids as protein secondary structure is lost.

Tissue was removed from storage at -70 $^{\circ}$ C and kept on dry-ice until 8M guanidine HCl (20ml) was added. The tissue was then quickly scissor-minced and homogenised using a Silveson Laboratory Mixer Emulsifier, for 1 min. The homogenate was spun at 4 $^{\circ}$ C for 5 minutes at 9000 g (Sorvall

RC 5B centrifuge, SS-34 rotor) after which the fatty layer was removed. Cold absolute alcohol (0.5 volumes ) was then added to the supernatant which was left at  $-20^{\circ}\text{C}$  for 45 minutes. The solution was spun at  $9000g$ ,  $4^{\circ}\text{C}$ , 10 minutes to pellet the nucleic acids. The pellet was then resuspended in a small volume of 6M guanidine HCl using a teflon-glass hand-homogeniser. The volume was made up to 20 ml and ice-cold absolute alcohol (10 ml ) added. The solution was mixed immediately and stored at  $-20^{\circ}\text{C}$  for 45'. The nucleic acids were recovered by centrifugation at  $9000 g$ ,  $4^{\circ}\text{C}$ , 10 minutes and the ethanol precipitation step in 6 M guanidine HCl repeated once more. Following this, the recovered pellet was dissolved in DEPC-treated water ( 1 ml ) and precipitated in 7.5 M ammonium acetate (0.5volume) and cold absolute alcohol (2.5 volumes) at  $-20^{\circ}\text{C}$  for at least 2 h. Under these conditions, cellular DNA remains soluble whilst the RNA is precipitated. The solution was centrifuged at  $9000 g$ , 10 minutes,  $4^{\circ}\text{C}$  and the pellet redissolved in DEPC-treated water (1 ml). The nucleic acids were reprecipitated 2-3 times with 2M sodium acetate, pH 4.8 (0.1 volume), and cold absolute alcohol (2.5 volumes) at  $-70^{\circ}\text{C}$  for 1 h. The final pellet was spun out at  $9000 g$ , 10 minutes,  $4^{\circ}\text{C}$  and resuspended in DEPC-water. After assessing the concentration of the RNA samples, as outlined in Section 2.10.1, they were aliquoted and frozen at  $-70^{\circ}\text{C}$  until required.

## **2.10 QUANTITATION OF DNA AND RNA**

### **2.10.1 SPECTROPHOTOMETRIC QUANTITATION**

DNA or RNA concentrations were estimated by UV absorption spectrophotometry. DNA or RNA samples were diluted 200 or 250 fold respectively, mixed and transferred to a spectrophotometric quartz cuvette. The spectrophotometer was blanked against DEPC- water and the absorbance of the sample was read at 260 nm. The concentration of DNA or RNA, in  $1 \mu\text{g/ml}$  of sample, was calculated as 10 times the O.D. reading.

To assess the purity of the samples, the absorbance at 280 nm was

also measured and the ratio of absorbance at 260 nm to 280 nm calculated. For DNA and RNA the ratio should be 1.8 and 2.0 respectively. If the ratio was below 1.6 the sample was re-extracted with phenol/chloroform and ethanol precipitated to eliminate contaminating protein.

## 2.10.2 ETHIDIUM BROMIDE FLUORESCENCE QUANTITATION

### (a) DNA estimation

DNA concentrations were estimated by electrophoresing samples in 0.8% (w/v) agarose in TBE (130 mM Tris-HCl, 45 mM boric acid, 2.5 mM EDTA). Mini-gels (50mm x 75mm x 5mm) were cast, transferred to a BRL horizontal electrophoresis kit H6 (BRL, Uxbridge, U.K.) and run in TBE with ethidium bromide (0.5 µg/ml) at 20 V for 2-3 hr. A series of DNA standards were also run to estimate the size of linear DNA. A 1 kb ladder (BRL, Paisley, U.K.) with a range of fragment sizes from 75 - 12,216 bp was used. A DNA sample of known concentration was run to check the concentration of unknown samples, as calculated from spectrophotometric analysis.

DNA fragments were visualised using a short wavelength transilluminator (Model TM-40, Ultraviolet Products Incorp.). The amount of UV induced-fluorescence emitted by ethidium bromide molecules, intercalated with DNA, is proportional to the total mass of DNA.

### (b) RNA estimation

RNA loadings were assessed by electrophoretic size fractionation under denaturing conditions using a method developed by Lehrach et al (1977). Horizontal 1% (w/v) agarose gels were prepared by melting 1gm of agarose, cooling it to 60°C and adding 5 x GRB (0.1 M MOPS, pH 7.0, 40 mM sodium acetate, 5 mM EDTA, pH 8.0, 20 mls) and 37% (12.3 M) formaldehyde to give final concentrations of 1x and 2.2 M respectively.

The RNA samples were prepared by mixing the following solutions in a sterile microfuge tube:

RNA (5µg)	4.5µl
5 x GRB	2.0µl
formaldehyde	3.5µl
formamide	10.0µl

and heating for 15 minutes at 55°C. Loading dye (0.4% bromophenol blue, 0.4% xylene cyanol, 50% glycerol, 1mM EDTA, pH 8.0, 2 µl) was immediately added to the samples. Electrophoresis was carried out at 40 V for 2-3 hr. After electrophoresis, the gel was stained in ethidium bromide (0.5 µg/ml) for 30 minutes, to visualise the abundant 28 S (6333 nucleotides) and 18 S (2366 nucleotides) ribosomal RNA species. This allowed for an approximate estimation of loading equality between the samples and assessed the integrity of the RNA.

## 2.11 NORTHERN BLOTTING

The attachment of denatured RNA to nitrocellulose is presumed to be non-covalent but essentially irreversible. It is possible to hybridise RNA immobilised on nitrocellulose to several radioactive probes without significant loss of bound nucleic acid. Unfortunately, nitrocellulose is not durable enough to withstand more than 2 rounds of hybridisation and washing. This difficulty can be solved by transferring the RNA to positively charged nylon membranes. Hybond is a nylon membrane which is inherently hydrophobic and thus requires no pre-wetting. It is possible to easily and quickly cross-link nucleic acids to the nylon using UV light. Combined with the physical strength of the membrane it is ideal for multiple rounds of hybridisation. Reed and Mann (1985) have reviewed the practical information regarding membranes.

### 2.11.1 SEPARATION, TRANSFER AND HYBRIDISATION OF RNA

RNA was size-fractionated under conditions described in Section 2.10.2 (b). 15 µg samples of RNA along with 3 µl of an RNA ladder (0.24 -

9.5 kb derived from bacteriophage T7, yeast 2 $\mu$  circle and bacteriophage lamda DNA. BRL, Paisley, U.K.), were run routinely using a BRL horizontal electrophoresis unit (BRL, Uxbridge, U.K.) at 20 V for ~16 hr until the dye front was ~5 cm from the edge of the gel. The gel was then washed for 10 minutes in 10 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M Na citrate) and transferred to Amersham Hybond N membrane by capillary transfer in 2 x SSC. Following a 16-18 hr transfer, the RNA was fixed to the nylon membrane by exposing the filter to ultra-violet irradiation (254 nm) for 30 seconds using a Stratalinker 2400. This method enhances the hybridisation signal obtained with charged nylon membranes compared to conventional oven-baking (Khandjian, 1987). The aim of the procedure is to form cross-links between a small fraction of the bases in the RNA and the positively charged amino acids on the surface of the membrane (Church and Gilbert, 1984). To check the efficiency of transfer the gel was stained for 45 minutes in a solution of ethidium bromide and examined under ultra-violet illumination. The part of the membrane containing the RNA ladder was stained with methylene blue (0.04% methylene blue, 0.5M sodium acetate) for 5-10 minutes at room temperature. The details of this method are described by Herrin and Schmidt (1988). After washing in distilled water, the RNA standards were clearly visible as a series of blue bands which were used to estimate the size of the RNA transcript hybridising to the probe used.

The filter was placed in hybridisation mix (5 x SSC, 4 x Denhardt's solution, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate) for at least 4 hr at 65 $^{\circ}$ C in a glass tube in a preheated oven. To maximise the rate of annealing of the probe to target DNA, hybridisation was carried out at high ionic strength (5 x SSC). The rate of hybridisation is accelerated 10 fold in the presence of 10% (w/v) dextran sulphate according to Wahl *et al* (1979). Denhardt's reagent (0.02% (w/v) polyvinylpyrrolidone, bovine serum albumin and Ficoll 400) is used to block non-specific attachment of the probe to the filter surface.

Probes, prepared and radiolabelled as described in the following sections, were added directly to the hybridisation solution following denaturation and allowed to hybridise to the RNA for 16-18 hr at 65 $^{\circ}$ C. The filter was washed 3 times in 2 x SSC, 0.1% (w/v) SDS, 0.1% (w/v) sodium

pyrophosphate (NaPPi). Higher stringency washes of 0.2 x SSC, 0.1% (w/v) SDS, 0.1% (w/v) NaPPi were carried out for certain probes, to eliminate cross-reactivity to similar gene sequences. The filter was wrapped in Saran wrap and exposed to Kodak X-Omat AR-5 film at -70°C in a cassette with intensifying screens.

In order to reprobe the RNA, the filters were washed in stripping solution (5mM Tris HCl, pH 8.0, 2mM EDTA, 0.1 x Denhardt's) for 1 h at 65°C, according to the manufacturer's instructions.

Autoradiographs were sometimes quantitated by scanning with a Joyce Loebel chromoscan. Non-saturated blots were scanned in order to ensure linearity across the samples.

### 2.11.2 PREPARATION OF cDNA PROBES FOR USE IN HYBRIDISATION

To isolate the cDNA clones referred to in Section 2.7, the plasmids were cut with the appropriate restriction enzyme. Usually this involved only a single enzyme digest at 37°C which was left for 6 hr, in a buffer as supplied by the manufacturer of the restriction enzyme.

Loading dye (50% glycerol, 1 x TAE, 1% bromophenol blue, 1% xylene cyanol, 2µl) was added to the digests and samples were electrophoresed in TAE buffer (40 mM Tris acetate, 1 mM EDTA) in a mini-gel containing 0.8% (w/v) low melting point agarose and 0.5 µg/ml EthBr. Aliquots of both cut and uncut plasmid DNA were run to assess the digestion procedure and a 1 kb DNA ladder was also run to estimate the insert size. Segments of the gel containing the inserts were excised and sterile water added at 1.5 ml per 1 gm of gel. This solution was then heated to 100°C for 7 minutes and frozen at -20°C until required.

### 2.11.3 RADIOLABELLING OF cDNA CLONES

Small amounts of DNA (50-200 ng) were labelled to high specificity using the method of Feinberg and Vogelstein (1983). This method is used to

generate probes from denatured double-stranded DNA. The purified DNA, mixed with a molar excess of primers, is denatured and synthesis carried out using the Klenow fragment of polymerase I. This enzyme lacks 5'-3' exonuclease activity so that the product is synthesised exclusively by primer extension. The reaction is carried out at pH 6.6, thus reducing the 3'-5' exonuclease activity of the enzyme (Lehman and Richardson, 1964). These conditions favour random initiation of synthesis.

DNA was denatured by heating at 100°C for 3 minutes and kept at 37°C until the radio-labelling reaction was initiated. The following constituents were mixed and the radiolabelling reaction was allowed to proceed for 3h or 16h according to convenience.

BSA (Fraction 5, Sigma) 10 mg/ml	2µl
Oligonucleotide-labelling buffer (OLB)	5µl
water	18µl
DNA	20µl
Klenow fragment of EColi DNA pol 1	2µl
[α- <sup>32</sup> P]dCTP] sp. act. >3000 Ci/mmole; 10 µCi/ml	3µl

OLB consists of a mixture of solutions A, B and C mixed in a ratio of 2:5:1 and was routinely stored at -20°C.

#### Solution A

2M Tris-HCl, pH 8.0	625µl
water	82µl
2-mercaptoethanol	18µl
MgCl <sub>2</sub> (1M)	125µl
10 mM dATP, dGTP, dTTP	50µl (of each)

## Solution B

2M Hepes titrated to pH 6.6 with NaOH and stored at 4°C.

## Solution C

Hexadeoxyribonucleotides suspended in 3 mM Tris-HCl 0.2 mM EDTA, pH 7.0 at a concentration of 90 O.D. units/ml and stored at -20°C.

Following incorporation, the DNA was denatured for 5 minutes at 100°C and added to the hybridisation solution as quickly as possible. To estimate the percentage of radioactivity incorporated into the DNA, a 1µl aliquot was removed from the reaction mixture and spotted onto DE 18 Whatman filter paper. A chromatograph was run in 0.3 M ammonium formate, pH 8.0 for 15 minutes and exposed to Kodak X-Omat AR-5 film for 10 minutes. Radioactivity incorporated into the DNA appeared as a spot at the origin whereas unincorporated nucleotides eluted up the paper with the solvent front. Incorporation was routinely between 70-90%.

## **2.12 CYTOTOXICITY TESTING**

### 2.12.1 MTT ASSAY

The MTT assay relies on the cleavage of the ring structure of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a blue crystalline formazan product by the mitochondrial enzyme succinate dehydrogenase of viable cells. The assay can be used for both chemosensitivity and radiosensitivity testing (Carmichael *et al*, 1987 a,b) and was chosen because it is rapid, simple and can also be used to measure the toxicity of hydrophobic compounds. The assay appears to give results comparable to the clonogenic assay which is widely used to assess the chemosensitivity of tumour cells *in vitro* although it cannot distinguish between cytostasis and cytotoxicity.

The assay was performed according to the method of Carmichael *et*

al (1987a). Cells (2500) were seeded in a volume of 180µl in medium and continuously exposed to the compound of interest in Costar 96-well micro-titration plates. The outer wells were filled with water to prevent evaporation from other wells and also since growth of cells in these wells differed from the rest of the plate. The plate was blanked against 6 wells (1 column) which had medium in them. To allow the cells to adhere, plates were incubated overnight at 37°C, 5% CO<sub>2</sub> in a humid atmosphere. The following day, the compound of interest was dissolved in serum-free medium from a stock prepared in DMSO, sterile water or ethanol. Serial dilutions were made from the compound dissolved in medium. The diluted compound ( 20µl ) was then added to the wells. Each dilution was assayed in triplicate.

Plates were left 4 days at 37°C followed by the addition of MTT (50 µl of 2 mg/ml in distilled water). Following a further 4 hr incubation, the medium was aspirated off and DMSO (50µl) added to the formazan crystals which had formed at the bottom of the wells. The plate was then read at 504 nm in a Biorad Model 2550 EIA plate reader. The absorbance of each well, containing cells in the presence of the compound of interest, was calculated as a percentage of the absorbance of the wells containing cells in the absence of the compound of interest (% control absorbance). The blank column (media only) reading was automatically subtracted from the rest of the plate. Each assay was carried out in triplicate and the results combined. Hydrophobic compounds were dissolved in DMSO for use in this assay. The toxicity of DMSO was tested on the CH<sup>R</sup>C5 and Aux B1 cell lines and it was found to be non-toxic at 0.5% (v/v) and this concentration was never exceeded in the assay. When compounds dissolved in DMSO were tested, the seeding medium was aspirated from the cells before the test compound (dissolved in DMSO and diluted further in medium) was added. The rest of the assay was as described before. A control with cells exposed to 0.5%(v/v) DMSO but not test compound was also measured, to ensure non-toxicity.

## 2.13 CORTICOSTERONE MEASUREMENT

### 2.13.1 SERUM COLLECTION

In order to assay corticosterone levels in mice treated with dexamethsone and TCPOBOP, C57BL/6 male adult mice (2 per group) were treated with dexamethsone (100 mg/kg, 3 dy) or TCPOBOP (3 mg/kg, 1 dy). Controls (10 mice) were treated with corn-oil (0.2 ml, 3 dy). Mice were sacrificed by cervical dislocation following treatment. Cardiac puncture was performed on each animal and the blood collected into tubes (Brunswick, England). The blood was spun (12,000 g, 2', 4°C) and the supernatant (serum) removed and stored at -20°C until assayed for corticosterone. Serum was pooled for dexamethasone and TCPOBOP treated mice as well as controls.

### 2.13.2 CORTICOSTERONE ASSAY

A simple and highly specific radioimmunoassay for corticosterone was developed (Al-Dujaili *et al*, 1981) and used in this study. The assay utilises a specific antibody raised against corticosterone-3-(*o*-carboxymethyl)-oxime-BSA and  $\gamma$ -labelled corticosterone of high specific activity. The production and assessment of antisera used in the assay has been previously described (Al-Dujaili *et al*, 1981). Dilutions of corticosterone standards in steroid-free medium ranging from 0-1152 nmol/l were used to construct the standard curve. Corticosterone free medium was prepared by the charcoal stripping method which involves adding 10% charcoal to the plasma to be stripped and agitation for at least 5h. The solution was then spun (30', 12K) and the plasma removed and filtered.

Triplicate standard or sample aliquots (25 $\mu$ l, 1:10 dilution) were assayed; ~5000 cpm of [<sup>125</sup>I] corticosterone (100 $\mu$ l) and corticosterone antibody (100 $\mu$ l, 1:5000 dilution), each dissolved in assay buffer (0.05 M phosphate/citrate buffer, pH 3.0) were added. The assay buffer at pH 3.0 was found to be the optimum pH which produces maximum inhibition of

corticosteroid-binding globulin (CBG) with minimum effect on the antibody-antigen binding. Therefore at pH 3.0, most of the steroid is in the unbound form and the assay can measure approximately total steroid concentration.

The tubes were mixed and incubated at 4°C for 4h. The antibody-bound and free fractions were separated by adding dextran-coated charcoal suspension (0.5 ml of 0.1M phosphate buffer, pH 7.4, 0.05% (w/v) gelatin, 0.6% (w/v) activated charcoal, 0.06% dextran T70) and spinning the tubes at 4°C, 30', 2000g. The supernatant was then aspirated and the antibody-free fraction (spun down in the charcoal pellet) counted in an automatic gamma counter.

Total unbound counts were estimated by adding only the label solution to the incubation without antibody.

To calculate bound counts:

$$\begin{array}{rcl} \text{Total counts} & - & \text{unbound counts} & = & \text{Bound counts} \\ \text{(without antibody)} & & \text{(standard or sample} & & \\ & & \text{with antibody)} & & \end{array}$$

$$\% \text{ Binding} = (\text{bound counts} / \text{total counts without antibody}) \%$$

From the percentage binding values, a standard curve can be plotted against known amounts of corticosterone. From the standard curve, an estimation of corticosterone (nmol/l) for the unknowns can be determined.

## CHAPTER 3

# DEVELOPMENT OF PEPTIDE ANTIBODIES TO P-GLYCOPROTEIN

### 3.1 INTRODUCTION AND AIMS

The precise role of P-gp in **acquired** drug resistance is uncertain, however evidence suggests that it may directly bind drugs and act as an efflux pump (Safa *et al*, 1986). An alternative mechanism is that drugs bind a carrier protein and the drug-protein complex is actively transported from the cell. In either case, P-gp should be in high concentrations in the plasma membrane if it is involved in the transport of drugs out of the cell.

The first monoclonal antibody with a high affinity to P-gp which was useful for detecting this transmembrane protein was developed by Ling's group (Kartner *et al*, 1985). Several others have also described the development of monoclonal antibodies that appear useful in the detection of P-gp (Scheper *et al*, 1988; Hamada and Tsuruo, 1986; Danks *et al*, 1985). The monoclonal antibody MRK16, reactive with an epitope present in P-gp which is expressed on the external face of the plasma membrane of human mdr cells was isolated (Hamada and Tsuruo, 1986). This antibody reacts only with human P-gp and has been used to localise the protein in normal human tissues by means of peroxidase immunohistochemistry (Thiebaut *et al*, 1987) and in cultured mdr cells by use of electron microscopic immunocytochemistry (Pastan *et al*, 1988; Willingham *et al*, 1987). Using MRK16 to probe normal human tissues, the protein was found concentrated in a small number of specific sites. Most tissues contained very little P-gp. In liver, P-gp is found exclusively on the biliary canalicular front of hepatocytes and on the apical surface of epithelial cells in small biliary ductules. In kidney, P-gp was found concentrated on the apical surface of epithelial cells of the proximal tubules. Colon and jejunum both showed high levels of expression on the apical surfaces of superficial columnal epithelial cells. It was also diffusely distributed on the surface of cells in the adrenal cortex and medulla (Fojo *et al*, 1987; Thiebaut *et al*, 1987).

The localisation of P-gp in human tissues on the apical surface of certain cell types facing an excretory compartment suggests that the protein may have a role in the excretion of drugs and physiological metabolites produced by cells. Liver, kidney and bowel are the sites from which cytotoxic natural products in the diet or introduced are removed from the body. The *mdr 1* gene transporter may be protecting animals from a variety of cytotoxic compounds. In the mouse, *mdr 1* is expressed at high levels in the secretory epithelial cells of the gravid uterus (Arceci *et al*, 1988). In the adrenal gland or pregnant uterus, the *mdr 1* gene product may be involved in the transport of endogenous metabolites.

In *mdr* vinblastine-selected KB-V1 and colchicine-selected KB-C4 cells, MRK16 was used to detect P-gp in high concentrations in the plasma membranes compared to the drug sensitive parental KB-3-1 cells (Willingham *et al*, 1987).

Monoclonal antibody C219 (Kartner *et al*, 1985) has been shown to react with P-gp in many mammalian species and detects an epitope located on the cytoplasmic face of the plasma membrane, near the C-terminal end, close to one of the putative ATP binding sites. The presence of these sites in the primary sequence of the *mdr 1* gene and the requirement for ATP in the efflux activity of P-gp in intact cells (Willingham *et al*, 1986) and in isolated transporting vesicles (Horio *et al*, 1988) suggested that P-gp could be an ATPase. Myosin possess strong ATPase activity and contains ATP binding sites (Strehler *et al*, 1986). C219 shows strong localisation patterns in human skeletal and cardiac muscle using immunohistochemical labelling and immunoblotting of the tissue (Wishart *et al*, 1990).

In rat liver extracts, a band of slightly faster mobility (~165 kD) compared to cultured human *mdr* cells (170 kD) was seen (Thiebaut *et al*, 1989). C219 localisation showed a bile canalicular pattern in rat liver as seen with MRK16 in human liver.

Sensitive immunocytochemical techniques have been developed to detect P-gp in cell lines with varying degrees of resistance using the C219 monoclonal antibody.

Immunocytochemical detection, with either alkaline phosphatase or peroxidase-antiperoxidase with silver gold intensification, gave a good

correlation with results obtained using immunoblot analysis (Friedlander *et al*, 1989). Such a technique detects P-gp in small numbers of cells that immunoblot analysis could miss, making it more suitable for clinical application. Epitope mapping of monoclonal antibodies to P-gp was performed by Georges *et al* (1990). He demonstrated that C219 recognises a highly conserved amino acid sequence found in all P-gp isoforms characterised to date. Other monoclonals such as C494 are gene specific and bind to a sequence present only in the class 1 isoform of hamster (pgp 1) and human (mdr 1). The monoclonal antibody C32 recognises a sequence conserved in hamster class 1 (pgp 1) and class 2 (pgp 2) isoforms but not in class 3 (pgp 3). These monoclonals were used to reveal differential expression and specific localisation of the three P-gp isoforms in hamster tissues by immunohistochemical staining and competition with epitope-specific peptides (Georges *et al*, 1990). Each P-gp isoform was found in a small, distinct group of differentiated cell types, and supports the notion that each P-gp isoform may have a different physiological role in normal cells. This approach may be particularly useful for the class 3 isoform (hamster pgp 3, human mdr 3 and mouse mdr 2) because it has not been possible to select *in vitro* for cells expressing high levels of this isoform. The expression of the class 1 isoform in brain capillary endothelium may reflect the involvement of P-gp in the function of the blood-brain barrier (Cordon-Cardo *et al*, 1989). This P-gp is also detected in the endothelium of small blood vessels in the testis, ovary, oviduct, uterus and vagina. This pattern of endothelial cell expression may indicate a physiological role for P-gp in regulating the entry of certain molecules into the central nervous system and other anatomic compartments, such as the testes. Bradley *et al* (1990) gave an alternative suggestion when pointing out that endothelial cells, hepatocytes and intestinal lining epithelial cells are all thought to secrete hormones and metabolites. Thus the P-gp class 1 isoform may function in specific secretory pathways that operate in these differentiated cells but not in similar cells in other sites. The pattern of expression of class 2 and 3 P-gp isoforms in specialised cells of the adrenal cortex, uterus and muscle suggests a normal function of these isoforms in the transport of normal metabolites. P-gp has been suggested to be involved in steroid transport in

the adrenal gland (Thiebaut *et al*, 1987). In hamster, as well as mouse, the class 2 isoform has been detected on the luminal surface of the lining epithelium of the gravid uterus (Bradley *et al*, 1990; Arceci *et al*, 1988). It is speculated that the expression of the class 2 isoform in uterine epithelium during pregnancy is also associated with specific aspects of steroid metabolism. The class 3 isoform (hamster pgp 3, human mdr 3 and mouse mdr 2) was the predominant form found in cardiac muscle fibres and a subset of striated muscle fibres (Bradley *et al*, 1990). Structural features of this isoform imply that it is a membrane transport protein.

Sugawara *et al* (1988) used MRK 16 to detect P-gp in human tissues and found strong staining in adult adrenal kidney and placental tissue. No P-gp was found in the immature adrenal and hence the expression may be developmentally regulated. Sugawara *et al* (1989) also reported that MRK 16 reacted with cortical adenomas of human adrenals secreting testosterone, aldosterone and corticosteroid, suggesting that these adenomas possess P-gp. MRK 16 did not react however with pheochromocytoma, non-functioning corticoid adenoma (adrenal derived hormones in the serum were unchanged). Therefore it was speculated that P-gp transports hormones or hormone metabolites through plasma membranes of adrenal corticoid cells.

Expression of P-gp has been examined in numerous clinical studies to determine whether or not it can be used as a useful marker for screening the mdr phenotype in tumours. In a study of childhood sarcoma, Chan *et al* (1989) elegantly demonstrated that the overexpression of P-gp influences the outcome of therapy. The probability of relapse-free survival was significantly different in patients with P-gp-positive versus negative tumours. Schlaifer *et al* (1990) warned that P-gp expression by macrophages may be responsible for overestimation of mdr content of tumour cells with Northern blot or immunoblot techniques of fresh tissue homogenates. Since mdr 1 expression in clinically refractory tumours untreated with chemotherapeutic drugs is difficult to detect using conventional assays, Noonan *et al* (1990) devised a sensitive, specific and quantitative protocol for measuring mdr 1 mRNA in clinical samples based on the polymerase chain reaction (PCR). Such a system requires much less tissue and ensures reliable detection of

mdr 1 gene expression in samples from cells with a low level of drug resistance.

## **AIMS**

At the outset of the project only C219 and MRK 16 were widely available as antibodies useful for the study of P-glycoprotein.

The initial aims of the project were to raise a useful polyclonal antibody (PA) or antibodies against the sequence of human mdr 1 in order to study the expression of the protein in cell lines and whole tissue and examine the physiological role of mdr.

### **3.2 CHOICE OF PEPTIDE SEQUENCES, IMMUNISATION AND RAISING THE ANTIBODIES**

In this study, performed previous to the Marquadt *et al* (1990) study using synthetic peptides to examine vincristine resistant HL/60 cells, five different peptide sequences were chosen. The sequence of the peptides and the regions to which these sequences correspond are given in Table 3.1. Peptides were synthesised, according to the deduced sequence of human P-gp (Chen *et al*, 1986), using the Marglin and Merrifield solid state method (Marglin and Merrifield, 1970) by Jonathan Rothbard, Imperial Cancer Research Fund, 44 Lincoln's Inn Field, London WC 2A 3PX.

The sequence of the human mdr 1 gene product is shown in Figure 3.1. Figure 3.2 shows the location of the peptide sequences in the postulated model of human mdr 1. Peptide 1-9 is the sequence of nine amino acids at the N-terminus of the mdr 1 gene product, which is predicted, from hydrophathy plots, to be cytoplasmic. Peptide 73-87 is upstream from three potential N-glycosylation sites, predicted to be in an extracytoplasmic domain. Potential transmembrane domains were determined for P-gp using the algorithm of Eisenberg *et al* (1984) and reported by Chen *et al* (1986)

**FIGURE 3.1**  
**PRIMARY STRUCTURE OF THE MDR 1 GENE PRODUCT AND**  
**ALIGNMENT OF THE N-TERMINAL AND C-TERMINAL HALVES**



The standard single-letter amino acid code is used. Colons indicate identical residues. Potential N-glycosylation sites (Asn-X-Ser/Thr) are underlined, the sites localized in the predicted extracytoplasmic region (see Figure 7) are underlined twice. Potential transmembrane segments (1-6 and 1a-6a), predicted by the algorithm of Eisenberg et al. (1984), are enclosed in thin boxes. Potential nucleotide-binding sites (NB-1 and NB-2) are enclosed in thick boxes.

Adapted from Chen *et al* (1986).



---

**TABLE 3.1**  
**PEPTIDES SYNTHESISED FROM HUMAN MDR 1 AMINO ACID**  
**SEQUENCE**

<b>ANTIBODY NO.</b>	<b>PEPTIDE SEQUENCE</b>	<b>P-GP AMINO ACIDS</b>
18/19	MDLEGDRNG	1-9
59/60	GEMTDIFANAGNLED	73-87
61/62	RALVRNPKIL	543-552
16/17	RIDDPETKRQN	741-751
20/21	VQAGTKRQ	1273-1280

---

One such domain includes residues 52-72. From such predictions, the peptide sequence of 73-87 should be extracytoplasmic and precede an N-glycosylation cluster. Peptide 543-552 spans ten amino acid residues in the predicted potential nucleotide-binding site, NB-2, as shown in Figure 3.2. Peptide 741-751 spans a region in the first extracytoplasmic loop in the second half of the protein. Peptide 1273-1280 represents the C-terminal end of the protein which is predicted to be located on the cytoplasmic side. Kartner *et al* (1985) have used antibodies against the C-terminal region of hamster P-gp to show that the C-terminus is located inside the cell.

The peptides chosen were aligned with mouse and hamster sequences to determine the extent of the homology between these and the human sequence. This is shown in Table 3.2. The sequence of human mdr 1 was reported by Chen *et al* (1986). Mouse mdr 1 and mdr 3 sequences were aligned by Devault and Gros (1990). The hamster gene family has been partially sequenced. Endicott *et al* (1987) reported the partial sequences of pgp 1 and pgp 2. Ng *et al* (1989) reported the partial sequence of pgp 3.

**TABLE 3.2**

**HOMOLOGY BETWEEN HUMAN, MOUSE AND HAMSTER SEQUENCES OVER THE PEPTIDE REGIONS**

		% HOMOLOGY TO HUM MDR 1
<b>PEPTIDE 1-9 (18/19)</b>		
HUM mdr 1	MDLEGDRNG	
MS mdr 1	MEFEENLKG	33
MS mdr 2	MDLEAARNG	77
MS mdr 3	MEL EEDLKG	55
<b>PEPTIDE 73-87 (59/60)</b>		
HUM mdr 1	GEMTDIFANAGNLED	
MS mdr 1	GNMTDSFTKASILPS	46
MS mdr 2	GEMTDKFVDNTGNFS	40
MS mdr 3	GDMTDSFASVGNVSK	53
<b>PEPTIDE 543-552(61/62)</b>		
HUM mdr 1	RALVRNPKIL	
MS mdr 1	RALVRNPKIL	100
MS mdr 2	RALVRNPKIL	100
MS mdr 3	RALVRNPKIL	100
HAM pgp1	RALVRQPHIL	80
HAM pgp2	RALVRQPHIL	80

**PEPTIDE 741-751 (16/17)**

HUM mdr 1	R I D D P E T K R Q N	
MS mdr 1	R D D D H E T K R Q N	82
MS mdr 2	P G D D - A V K Q Q K	36
MS mdr 3	N G G P P E T Q R Q N	55
HAM pgp 1	R N T D D E T K R H D	55
HAM pgp 2	R D D D P K T K Q Q N	73

**PEPTIDE 1273-1280 (20/21)**

HUM mdr 1	V S V Q A G T K R Q	
MS mdr 1	V - Q A G A K R S	70
MS mdr 2	V N I Q A G A K R S	50
MS mdr 3	V S V Q A G T Q N L	80
HAM pgp 1	V S V Q A G A K R	80
HAM pgp 2	V - Q A G A K R L	60

HUM	Human
MS	Mouse
HAM	Hamster

---

Synthetic peptides containing cysteine at the amino terminus were conjugated to thyroglobulin and also to bovine serum albumin (BSA) by Jonathon Rothbard, Imperial Cancer Research Fund, Lincolns Inn Fields. The thyroglobulin conjugates were used to immunise New Zealand White

rabbits (two rabbits per conjugate). The conjugated material (200µg) was emulsified with Freund's complete adjuvant and thereafter injected subcutaneously (sc) into the rabbits. Prior to this initial injection, pre-immune serum was obtained from each rabbit, stored at -20°C. This pre-immune serum was subsequently used as the negative control in ELISA assays to measure the antibody titres of the immunised animals. After three weeks, a second injection of conjugated material (100 µg) in incomplete adjuvant was made. At weeks six and eight, further boosts were given and the animals bled nine days after the last boost. Blood was removed from the animals one week following each boost to estimate the titre of the antibody. Antisera were tested for reactivity against synthetic peptides by using an ELISA assay procedure, as described in Section 2.6.1. The sera from all bleeds were collected and stored at -20°C until assayed.

### **3.3 MEASURING ANTIBODY TITRES USING THE ELISA ASSAY**

To estimate the titre of the antibodies and to assess the increase in titre following each boost, ELISA assays were performed. BSA-conjugated peptides were used in the assay and were adsorbed onto the microtitre plates overnight. Wells in the plate were also coated with the pre-immune serum as a negative control. By screening with the BSA- conjugate it was intended that the assay would measure the antibodies raised against the peptide sequences only. Table 3.3 shows the results obtained following each boost and the final titre of the antibody for each peptide and each rabbit immunised. The final antibody titres ranged from 1:6,400 to 1:102,400. Such titres were considered sufficient for using these antibodies to immunoblot for P-gp in mdr cell lines.

### **3.4 IMMUNOBLOTTING USING THE C219 MONOCLONAL ANTIBODY**

In order to assess whether the peptide antibodies raised against P-gp were valuable for use in immunoblotting, techniques for immunoblotting P-gp

in mdr cell lines and tissues were developed using the commercially available monoclonal antibody C219. The immunoblotting procedures for P-gp are described in Section 2.6.3

**TABLE 3.3**  
**PEPTIDE ANTIBODY TITRES**

PEPTIDE	RABBIT	1ST	TITRES		
			2ND	3RD	4TH (FINAL)
1-9	18	1:3,200	1:6,400	1:25,600	1:25,600
	19	1:3,200	1:12,800	1:12,800	1:12,800
73-87	59	1:3,200	1:6,400	1:6,400	1:6,400
	60	1:6,400	1:6,400	1:12,800	1:25,600
543-552	61	1:12,800	1:12,800	1:25,600	1:51,200
	62	1:12,800	1:51,200	1:51,200	1:51,200
741-751	16	1:12,800	1:25,600	1:25,600	1:102,400
	17	1:3,22	1:12,800	1:12,800	1:12,800
1273-1280	20	ND	1:51,200	1:51,200	1:102,400
	21	ND	1:51,200	1:51,200	1:102,400

ND not determined

Several cell lines were immunoblotted for P-gp using the Laemmli system without heating. Two of these cell lines were of human and two were of hamster origin. Wild type (sensitive) and derived (resistant) lines were matched. In the case of Messa, Dx-5, CHO-K1 and CHO-Adr<sup>R</sup>, a whole cell sonicate was electrophoresed and for the other cell lines a 100,000g fraction was examined. All mdr cell lines, except CH<sup>R</sup>C5, were grown continuously in the presence of the drug to which they were originally selected. The

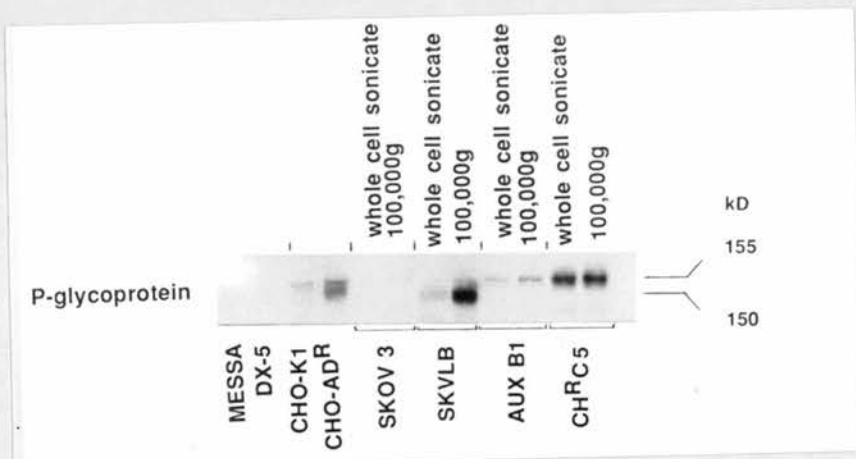
immunoblot of the various cell lines probed with C219 is shown in Figure 3.3. To compare the levels of P-gp detected in the sensitive cell line Aux B1 and its drug resistant counterpart CH<sup>R</sup>C5, the exposure time of the immunoblot was 30 days following Protein A-I<sup>125</sup> labelling. SKVLB (human), CH<sup>R</sup>C5 (hamster) and CHO-Ad<sup>R</sup> (hamster) cell lines demonstrated immunoreactivity with C219. Both fractions from the CH<sup>R</sup>C5 cell line cross-reacted strongly with C219 to give a band of 155 kD. This same protein was detected in both fractions of AuxB1 but at a greatly reduced level. This cell line pair has been widely used in the literature as a control for P-gp expression. Riordan and Ling (1979) reported the purification of P-gp from the colchicine resistant chinese hamster ovary cell line, CHRC5. The drug sensitive SKOV3 human cell line also displayed negligible levels of P-gp in the 100,000g fraction. SKVLB resistant cells show a greatly increased level of a 150 kD protein in the 100,000g fraction. CHO-K1 cells displayed a low level of the 155 kD P-gp and the CHO-Adr<sup>R</sup> cells showed a smeared lower band of ~150 - 155 kD. Both the 150 kD and the 155 kD proteins represent P-gp in different glycosylation states. In the case of SKVLB, P-gp has lost ~5 kD of carbohydrate residues. Greenberger *et al* (1988) reported the effect of heating P-gp samples; in CH<sup>R</sup>C5 cells, P-gp shifted from 155 kD to a broad smeared band of ~170 kD. He proposed that since both the Fairbank's system (SDS + urea) and the Laemmli system (SDS + boiling) should completely denature P-gp, in the absence of heat or urea the protein is probably incompletely unfolded and therefore migrates aberrantly.

Dox-5 failed to show P-gp protein expression using this system. However, the cell line was shown to be resistant to adriamycin, as shown in Chapter 4. The lack of a detectable protein using this immunoblotting technique is therefore probably due to the low level of P-gp expression in the mdr Dx-5 cell line.

---

**FIGURE 3.3**  
**EXPRESSION OF P-GP IN CELL LINES IMMUNOBLOTTED WITH C219**

Cell fractions were prepared, as described in Section 3.4, and Western blotted using the C219 monoclonal antibody.



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To assess the usefulness of the blotting technique on whole tissues, microsomes from mouse, human and Wistar rat liver were prepared and immunoblotted using the modified Fairbank's urea gel system.

Using the urea gel system, C219 was also shown to cross-react with normal mouse and rat liver. The MW of the P-gp detected in these tissues was smaller than the one detected in the CH<sup>R</sup>C5 cell line. The results are

shown in Figure 3.4. Silverman *et al* (1991) reported the sequencing of the first rat *mdr* gene. A full length rat cDNA clone was identified and the predicted protein was 1276 amino acids with a calculated Mr of 141 kD. The sequence was found to have high homology to mouse *mdr* 1. Mouse liver also contained a protein at ~60 kD that cross-reacted strongly with the C219 monoclonal antibody. This protein must contain the epitope VQAALD recognised by the C219 monoclonal antibody. Human liver showed very weak expression of P-gp although Fojo *et al* (1987) indicated that normal human liver contained substantial expression of P-gp. The low P-gp level may be due to sample degradation or variations in the expression of P-gp in different human liver samples.

Rat liver treated with dexamethasone, araclor, 2-acetylaminoflourene and untreated control were electrophoresed along with CH<sup>R</sup>C5 and AuxB1 to determine whether these treatments induced P-gp expression. Figure 3.5 shows the results and illustrates the effects of a range of xenobiotic treatments on rat P-gp expression. The treated rat liver showed increased levels of P-gp compared to control.

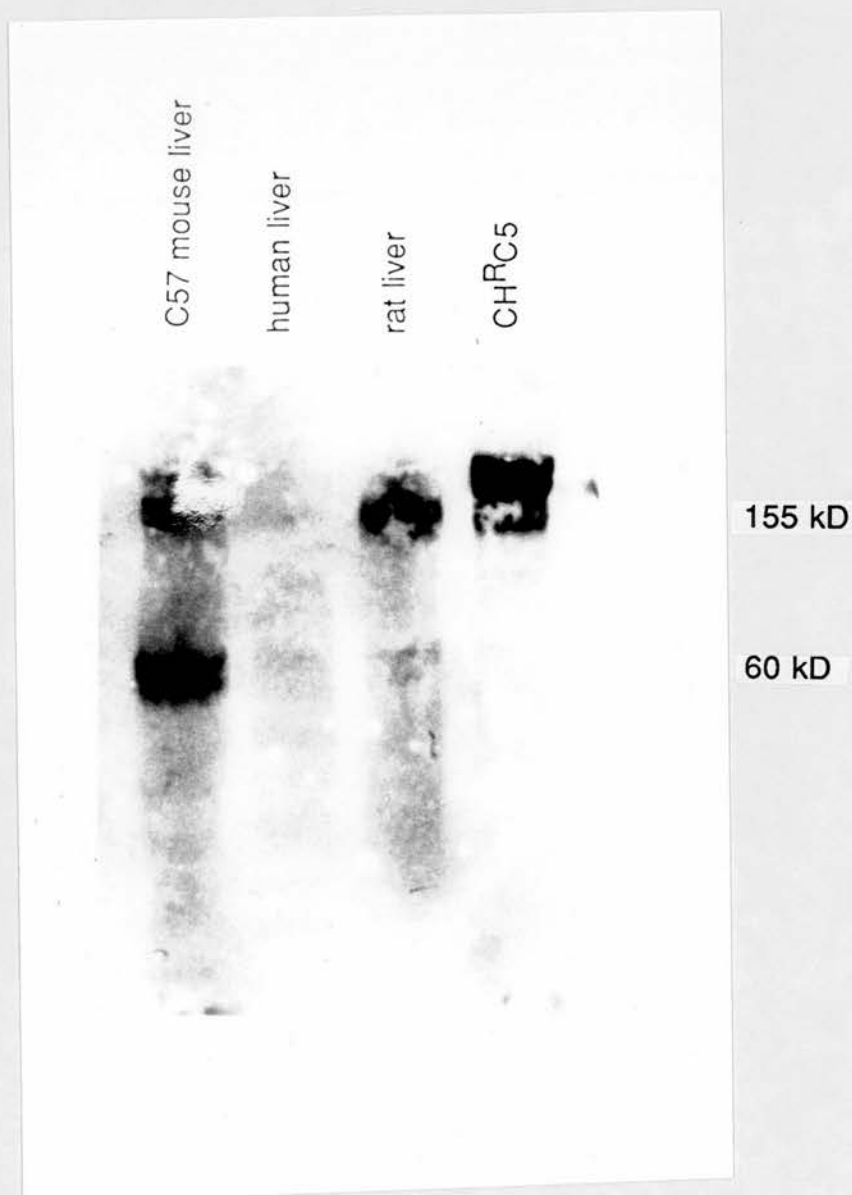
Having established the usefulness of the C219 monoclonal antibody in immunoblotting both cell lines and whole tissues, the reactivity of the polyclonal antibodies raised against peptide sequences of human *mdr* 1 was assessed.

### **3.5 IMMUNOBLOTTING USING PEPTIDE ANTIBODIES**

All the antibodies were assessed by immunoblotting. Both the Laemmli and urea gel system were employed. Antibodies were diluted from 1:50 to 1:1000 and C219 was used as the positive control antibody against the CHRC5 *mdr* cell line. From such immunoblotting studies, polyclonal antibody (PA) 21 and PA61 were considered to be useful. Polyclonal antibodies 21 and 61 had titres of 1:100,000 and 1:51,200 respectively. PA61 had 100% and 80% homology with mouse and hamster sequences respectively over the peptide region to which it was raised. PA21 had 70%, 50% and 80% homology with the mouse *mdr* 1, *mdr* 2 and *mdr* 3 primary amino acid sequences over the region to which it was raised. It also had

**FIGURE 3.4**  
**EXPRESSION OF P-GP IN MOUSE, RAT AND HUMAN LIVER**

Male C57Bl/6 mice, Wistar rat and human liver were prepared, as described in Section 2.5.3, and Western blotted (100 µg of each) using the C219 monoclonal antibody.



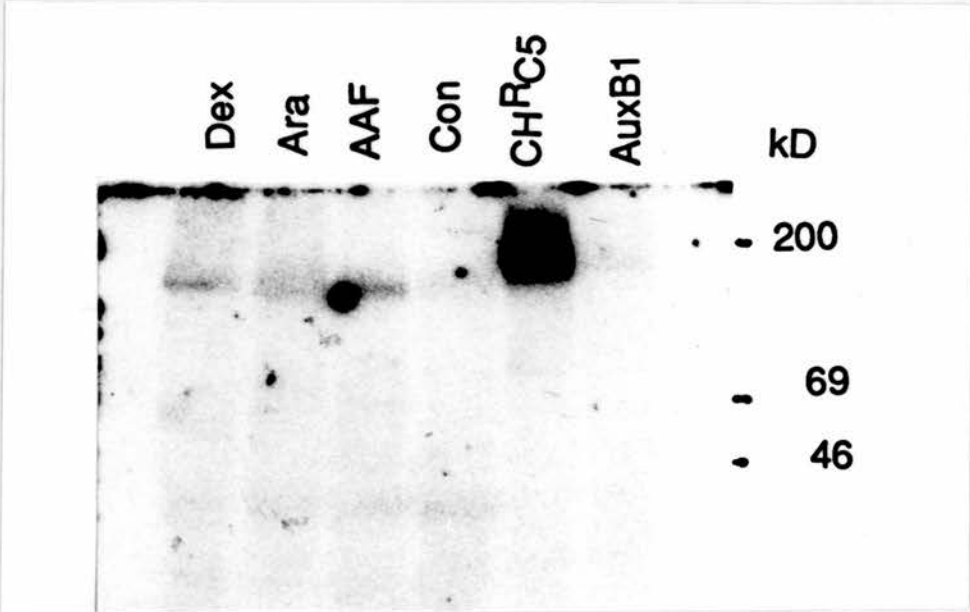
This represents one representative blot from one of several repeated experiments.

**FIGURE 3.5**  
**EXPRESSION OF P-GP IN DEXAMETHASONE, ARACLOR AND**  
**2-ACETYLAMINOFLUORENE-TREATED RATS**

Wistar rats were treated with dexamethasone (100 mg/kg, 3 dy), Araclor (500 mg/kg, 1dy) and 2-acetylaminofluorene (50 mg/kg, 4 dy). Control animals were treated with phosphate buffered saline.

Livers were removed following treatment, snap frozen and solubilised protein prepared from them, as described in Section 2.5.3 (Fairbank's method). 100 µg of each liver sample was electrophoresed along with the CH<sup>R</sup>C5 cell line and Western blotted using the C219 monoclonal antibody.

- Dex dexamethasone
- Ara arachlor
- AAF 2-acetylaminofluorene
- Con control



This represents the results of one experiment. Tissue was pooled from 4 rats in each treatment group.

80% and 60% homology to hamster pgp 1 and pgp 2 respectively over this region.

### 3.5.1 IMMUNOBLOTTING USING POLYCLONAL ANTIBODY 21

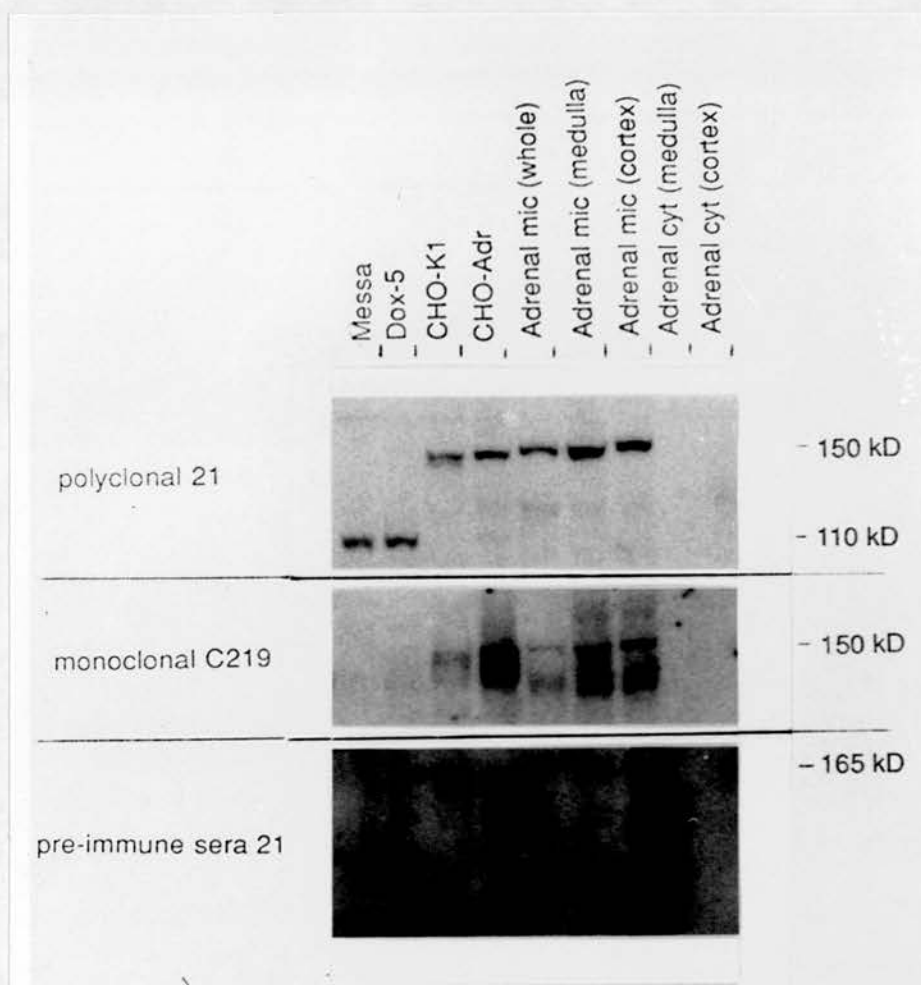
PA21 was raised against a ten amino acid peptide sequence which is highly conserved between human, mouse and hamster. The sequence is found at the C-terminal of all P-gp proteins thus far described. This region in the protein is cytoplasmic according to hydropathy plots (Chen *et al*, 1986) since it is near a long, relatively hydrophobic region.

Immunoblots were run using this antibody against cell lines and bovine adrenal microsomes. Figure 3.6 shows two immunoblots from an SDS PAGE gel that was run with two sets of identical samples and then cut in half. The samples were microsomal cell preparations from Messa, Dox-5, CHO-K1 and CHO-Adr<sup>R</sup> and adrenal microsomes from whole tissue, medulla and cortex.

The immunoblots were blotted with the C219 monoclonal antibody and PA21. The serum from the final bleed-out was used for immunoblotting to ensure a high titre. The antibody was used at a dilution of 1:100. A separate gel was run on the identical samples and blotted with the pre-immune serum from rabbit 21. C219 recognised P-gp in the hamster cell line CHO-Ad<sup>R</sup> more strongly than the low level of expression in CHO-K1. It also detected P-gp expression in all three adrenal microsome fractions. No obvious difference was apparent between the expression of P-gp in the cortex or medulla. The size of the P-gp detected using this electrophoresis and blotting system was 150 kD. A lower smeared band of 130-140 kD was also detected in the adrenal microsomes and CHOAd<sup>R</sup> cell line. No P-gp was detected in the cytosolic fractions of the adrenal cortex or medulla as

### FIGURE 3.6 IMMUNOBLOTTING CELL LINES AND ADRENAL MICROSOMES USING POLYCLONAL ANTISERUM 21 AND C219

Cell lines and adrenal fractions were prepared, as described in Section 2.5.3, and Western blotted (100  $\mu$ g of each) using the polyclonal antibody 21, monoclonal antibody C219 and the pre-immune serum 21.



The polyclonal antibody 21, C219 and pre-immune serum autorads were exposed for 16 hours, 11 days and 9 days respectively. The pre-immune serum blot was given a longer exposure than polyclonal antibody 21 to ensure that no cross-reactivity to P-gp had occurred.

expected. PA21 also recognised a protein of 150 kD in the adrenal microsomes but no such protein was detected as being present in the cytosolic fractions. CHO-Adr<sup>R</sup> cells showed a slightly higher level of expression of the protein than CHO-K1. Although both sets of samples had been treated identically, PA21 did not detect the lower smeared protein of between 130-140 kD. The antibody did however detect a protein of 110 kD represented at equivalent levels in both Messa and Dox-5 cell lines.

The autoradiograph of the immunoblot with the C219 monoclonal antibody required an exposure time of eleven days whereas the polyclonal antibody autoradiograph required only a 16 h exposure to achieve a reasonable level of detection. The consistent failure of antibodies raised against P-gp to detect P-gp expression in the mdr Dox-5 cell line suggests that very low levels of expression were present in the cell line.

The difference in the sensitivity of PA21 and C219 may be explained in two ways. Firstly, the difference may be due to one antibody being monoclonal and the other being polyclonal. Monoclonal antibodies recognise only one epitope in contrast to polyclonals which normally recognise several different epitopes represented in the protein sequence. Monoclonal antibody C219 recognises a six amino acid sequence which occurs both in the N-terminal and C-terminal half of the protein as shown in Figure 3.2. PA21 recognises a ten amino acid sequence which only occurs at the C-terminal end of the protein. From these facts it would be difficult to explain the difference in sensitivity of C219 and PA21 in terms of the number of epitopes recognised. A second explanation involves the antigenicity of the epitope and the ease with which it is recognised by the antibody. Since the C-terminal region is not masked by sugar residues, which may be true for external epitopes, the epitope site is exposed and easily recognised by an antibody raised against it.

The protein of 110 kD recognised by PA21 in the human cell line is unlikely to be involved in mdr since it is expressed to equivalent levels in both cell lines. The epitope recognised by PA21 must be present in these human cell lines.

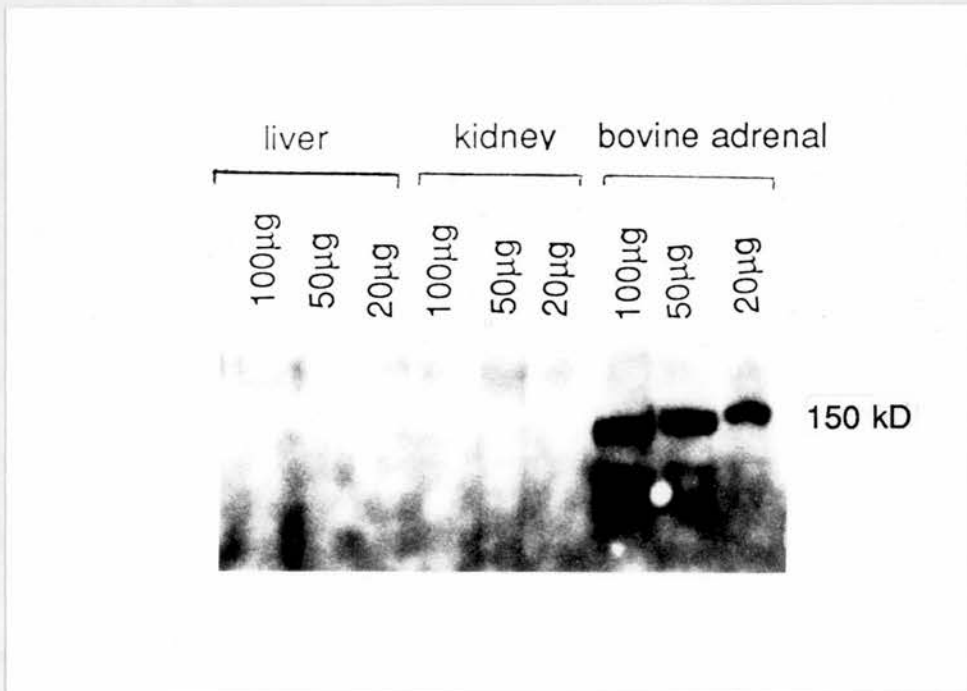
Figure 3.6 also shows the same samples blotted with pre-immune serum from rabbit 21. The immunoblot with this serum was exposed for nine

days. A faint band of 165 kD was detected in all the samples except the Messa sensitive cell line. Such a protein size or pattern of expression is not represented in the PA21 immunoblot. Therefore the protein recognised by PA21 is not recognised by the antibodies normally present in the rabbit before immunisation.

Using the Laemmli system, PA21 was found to cross-react well with bovine adrenal microsomes but failed to detect P-gp expression in normal rat liver or kidney even when 100 $\mu$ g of protein was immunoblotted as seen in Figure 3.7.

**FIGURE 3.7**  
**DETECTION OF P-GP IN BOVINE ADRENAL, RAT LIVER AND KIDNEY USING POLYCLONAL ANTIBODY 21**

Bovine adrenal, rat liver and kidney solubilised protein was prepared, as described in Section 2.5.3, and Western blotted (100  $\mu$ g, 50  $\mu$ g and 20  $\mu$ g of each) using the polyclonal antibody 21.



The detection of a protein in tissue fractions compared to cell fractions is always more difficult since the number of different cell types represented in the tissue microsomal fraction is much greater. Immunohistochemical methods are therefore usually employed to circumvent this problem.

A high level of P-gp was detected in bovine adrenal microsomes on loading between 100-20  $\mu$ g of protein. The strong detection of P-gp in this tissue indicates very high levels of expression compared to other tissues. Such a finding is in agreement with Croop *et al* (1989) who reported very high levels of expression of the *mdr 1* gene in mouse adrenal. The level of expression was 20 to 40 fold the level in the non *mdr* cell line NIH 3T3. Georges *et al* (1990) reported that *pgp 2* is predominantly expressed in the hamster adrenal gland. This expression may be involved in the transport of corticosteroids. The elevated levels of expression detected by PA21 in the bovine adrenal is therefore not surprising. The specificity of the polyclonal antibody for the protein recognised in bovine adrenal microsomes was tested for.

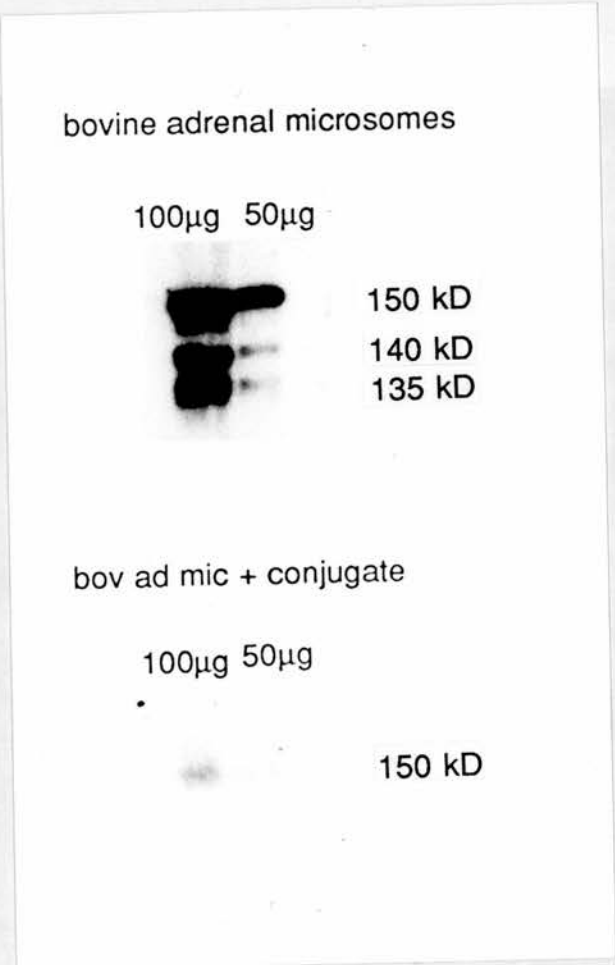
Figure 3.8 shows the results of an experiment in which 50  $\mu$ g and 20 $\mu$ g of bovine microsomal protein were run side by side and immunoblotted using PA21 at 1:500 dilution. The lower half of the figure shows an immunoblot with the same samples using PA21 at 1:500 dilution preincubated with an excess of BSA-conjugated peptide, to which peptide the antibody was raised. The incubation was at 37<sup>o</sup>C for 1 h with gentle agitation. The antibody solution was then spun down and the serum used as the 'competed-out antibody'. Peptide competition from the conjugate resulted in an antibody that recognised the protein in bovine adrenal microsomes at much reduced levels. This demonstrates the specificity of the peptide sequence in the antibody for the protein detected by PA21 in adrenal microsomes. Even at dilutions of 1:1000 and a protein loading of 20 $\mu$ g, PA21 still strongly detected P-gp.

### 3.5.2 IMMUNOBLOTTING USING POLYCLONAL ANTIBODY 61

PA61 was raised against amino acid residues 543-552 which are situated near the ATP-binding site in the N-terminal half of the protein. This

**FIGURE 3.8**  
**SPECIFICITY OF P-GP DETECTION IN BOVINE ADRENAL**  
**TISSUE BY POLYCLONAL ANTIBODY 21**

Adrenal fractions (100  $\mu$ g and 50  $\mu$ g of protein) were Western blotted using polyclonal antibody 21 and the 'competed out' polyclonal antibody 21, as described in Section 3.5.1. 1 mg of conjugate was used to compete out a 1:500 dilution of the polyclonal antibody.



region is highly conserved and located in the cytoplasm. The highest level of homology is observed in the nucleotide binding regions (Chen *et al*, 1986) with each region corresponding to two parts of the nucleotide-binding fold (Walker *et al*, 1982). These folds have been detected previously in bacterial transport proteins (Higgins *et al*, 1982).

PA61 recognised a protein in the CH<sup>R</sup>C5 cell line which was present at much lower levels in the Aux B1 drug sensitive parental line. This protein was exactly equivalent in size to P-gp, as shown in Figure 3.9. This figure shows the results of immunoblotting these cell lines with PA61 at a dilution of 1:2000 and 1:10,000. C219 recognises P-gp in the CH<sup>R</sup>C5 cell line at a dilution of 1:1000. The additional proteins recognised by PA61 are non-specific and are detected at equivalent levels in both the resistant and sensitive cells.

To assess whether or not PA61 recognises P-gp in other mdr cell lines, membrane preparations from CHO-Adr<sup>R</sup>, CHO-K1, SKVLB and SKOV were also prepared and immunoblotted. To determine the detectability of P-gp in mouse and rat liver, membrane fractions from these rodent tissues were extracted and immunoblotted alongside the cell lines. Figure 3.10 shows the immunoblot results obtained after probing the above samples with PA 61 and C219. P-gp (155kD) is detected most strongly in the CHRC5 cell line. The level of expression in AuxB1 is very low in comparison. SKVLB also had a raised level of P-gp (155kD) compared to it's sensitive parent SKOV. The cell lines CHO-Adr<sup>R</sup> and CHO-K1 showed very little difference in the expression of P-gp. Both mouse and rat liver expressed P-gp of a lower molecular weight of 150 kD. The C219 antibody detected P-gp in CH<sup>R</sup>C5 and SKVLB at the same molecular weight as PA61. A lower molecular weight form of P-gp was also detected in rat and mouse liver. A protein of 60 kD was also strongly detected in mouse liver by C219. Since the protein cross-reacts strongly with C219 but not PA 61, the protein must contain the epitope recognised by the monoclonal but not the one recognised by the polyclonal.

### FIGURE 3.9

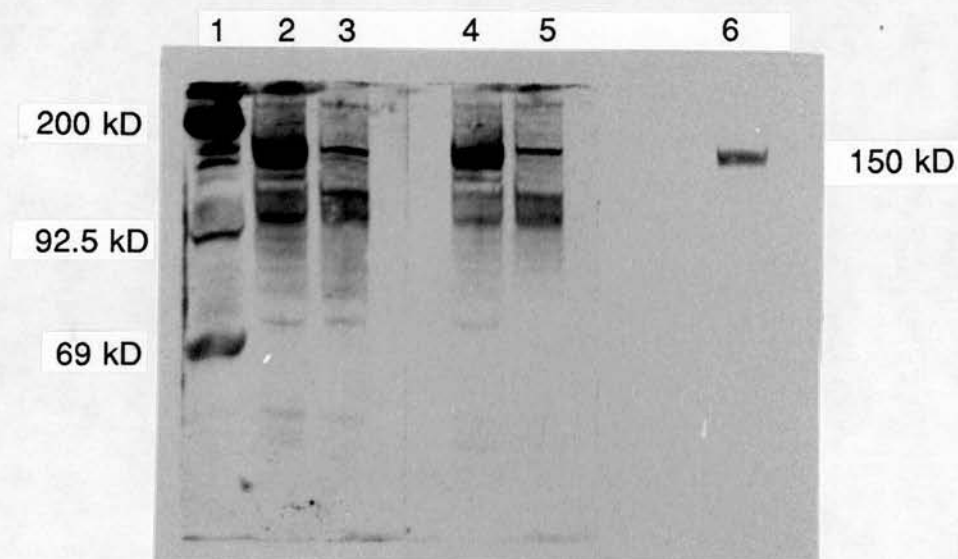
#### DETECTION OF P-GP IN MDR AND SENSITIVE CELLS BY C219 AND POLYCLONAL ANTIBODY 61

Cell lines were prepared, as described in Section 2.5.3, and Western blotted (100 µg each) using polyclonal antibody 61 and the monoclonal antibody C219.

Track no.	Sample
1	molecular weight markers
2	CHRC5
3	AuxB1
4	CHRC5
5	AuxB1
6	CHRC5

Tracks 2 /3 and 4/5 were blotted against polyclonal antibody 61 at a dilution of 1:2000 and 1:10,00 respectively.

Track 6 was blotted against monoclonal antibody C219 at a concentration of 0.1 mg/ml.

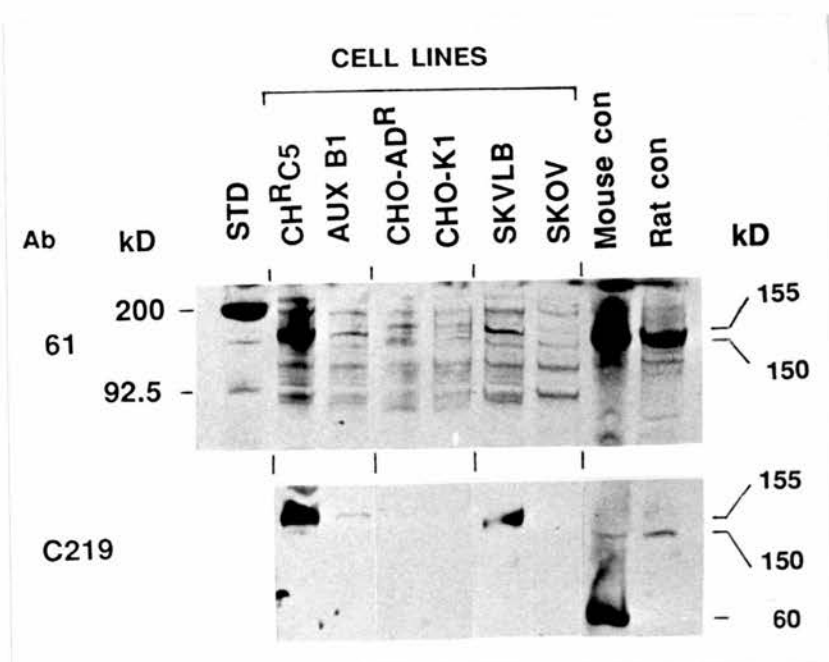


These results are representative of several experiments in which repeated cell line preparations were made and different antibody dilutions were used.

**FIGURE 3.10**  
**DETECTION OF P-GP IN CELL LINES AND RODENT TISSUES**  
**USING C219 AND POLYCLONAL ANTIBODY 61**

Cell lines, rat and mouse liver solubilised protein was prepared, as described in Section 2.5.3, and Western blotted (100 µg each) using the polyclonal antibody 61 and the monoclonal antibody C219.

STD                    standard  
 mouse con           mouse control (untreated)  
 rat con                rat control (untreated)



### 3.6 IMMUNOHISTOCHEMISTRY USING THE PEPTIDE ANTIBODIES

For testing the usefulness of the peptide antibodies the cell line CH<sup>R</sup>C5 and its sensitive parental cell line AuxB1 were used. P-gp in the CH<sup>R</sup>C5 cell line was shown to be recognised by PA61 following immunoblotting. All the peptide antibodies were tested including those that did not detect P-gp in the immunoblotting procedure. With immunohistochemistry the epitope recognised by the antibody is in a conformation which is normally adopted whereas the epitope is linearised to a great extent following immunoblotting. Epitope presentation can alter the extent to which the antibody recognises the antigen.

Cells were prepared from both cultured cell lines and spun onto glass slides to achieve an equal proportion of cells on each slide (50,000). The cells were fixed in acetone for four minutes and stored at -20°C until required. The cells were quickly thawed in acetone for one minute when immunohistochemistry was performed on them. The cells were washed twice in TBS for five minutes and incubated in normal rabbit serum (1:5) for ten minutes. The cells were then incubated in a 1:50 dilution of each polyclonal peptide antibody for 30 minutes at room temperature. Following this step the cells were washed in TBS and treated as outlined in Section 2.3.3 with biotinylated swine anti-rabbit second antibody. The rest of the procedure is also shown in this section.

#### 3.6.1 RESULTS OF PEPTIDE IMMUNOHISTOCHEMISTRY

Table 3.4 shows the results of the immunohistochemistry on the CH<sup>R</sup>C5 and AuxB1 cells.

None of the peptide antibodies stained CH<sup>R</sup>C5 more strongly than AuxB1. Both nuclear and cytoplasmic staining was evident. PA21 and PA61 did not specifically recognise P-gp in the plasma membrane of the CH<sup>R</sup>C5 cells but cross-reacted with other proteins present.

**TABLE 3.4**  
**PEPTIDE ANTIBODY IMMUNOHISTOCHEMISTRY RESULTS ON**  
**CH<sup>R</sup>C5 AND AUXB1 CELLS**

ANTIBODY	AUX	CH <sup>R</sup> C5	COMMENTS
16	+	+	staining is cytoplasmic
17	++	+	
18	+++	+	
19	++	+	nuclear staining in AuxB1
20	+	+	both nuclear staining
21	+/-	+	
59	++	++	
60	++	++	background staining in AuxB1
61	++	++	
62	++	+	

+ moderate staining  
 ++ strong

Using the same method to detect P-gp in the cells with the C219 monoclonal antibody resulted in both CH<sup>R</sup>C5 and AuxB1 being stained. The difference in intensity between the two cell lines was not apparent. The staining was however more plasma membrane specific.

### 3.7 SUMMARY OF RESULTS

Using hamster and human mdr cell lines and C219 as the positive control antibody, the peptide antibodies PA21 and PA61 were shown to cross-react with P-gp using the Laemmli and urea gel system for immunoblotting. The remaining peptide antibodies did not cross-react with P-gp following immunoblotting. Both PA21 and C219 detected P-gp in bovine adrenal microsomes at ~145 kD. PA61 and C219 detected P-gp in rat

and mouse liver.

The ability of PA61 to recognise P-gp in the chinese hamster mdr cell lines and both mouse and rat liver indicates that the peptide sequence against which the antibody was raised is highly conserved and the epitope is available for recognition by the antibody following immunoblotting. PA21 did not detect P-gp in either rat or mouse liver and this indicates that the homology between the peptide sequence in human, rat and mouse is not sufficient for antibody recognition or that mdr 1 is not expressed at sufficient levels for detection. However, the detection of P-gp in the chinese hamster ovary cell line CHO-Adr<sup>R</sup> infers that this may not fully explain the lack of detection since the hamster sequences have a similar level of homology to human mdr 1 as the mouse sequences. The difference in expression of P-gp between CH<sup>R</sup>C5 and AuxB1 is considerable. However, using immunohistochemistry no difference could be detected between these cell lines. Unlike C219 however, the polyclonal antibodies also cross-reacted with other proteins in the cell lines and whole tissue to varying extents. Such cross-reactivity would have to be eliminated by increasing the dilution of the antibody or pre-adsorbing it with pre-immune serum.

The specificity of C219 was considered to be much greater than either of the polyclonal antibodies raised. Polyclonal 61 has 100% homology with the mouse mdr 1, 2 and 3 gene sequences and therefore would not distinguish between them. Antibodies to detect specific mdr 1, 2 or 3 gene products will enable examination of their location and function. Since the antibodies raised were not specific enough to differentiate individual mdr 1, mdr 1, 2 and 3 gene products, it was decided to use Northern blotting to examine the levels of expression of each isoform in various models. Chapter 5 describes the use of such gene specific probes.

## CHAPTER 4

### P-GLYCOPROTEIN - AN EFFLUX PUMP FOR CARCINOGENS?

#### 4.1 INTRODUCTION

The physiological function of P-gp in normal tissues is unknown although its role as an efflux pump has been widely studied. Early work demonstrated that mdr cells were associated with a decrease in intracellular cytotoxic drug accumulation when compared to sensitive cells (Riehm and Biedler, 1971; Ling and Thompson, 1974). Such decreased accumulation has been generally ascribed to enhanced outward, rather than diminished inward, transport (Inaba *et al*, 1979). A number of investigators have found an energy-dependent mechanism responsible for the outward transport of all drugs to which cells display resistance. Depletion of ATP by removal of glucose or addition of metabolic inhibitors results in a reversal of the accumulation defect, whereas replacing glucose restores mdr (Dano, 1973). A number of radiolabelled drugs and photoactivatable drug analogs bind in a specific, saturable and energy-dependent manner to mdr plasma membranes but not to sensitive cell membranes (Cornwell *et al*, 1986a). Purified P-gp has been shown to possess ATPase activity (Hamada and Tsuruo, 1988). The deduced amino acid sequence of P-gp reveals that it bears striking homology to the ATP-binding domains of periplasmic transport proteins of bacteria such as the hyl B  $\alpha$ -hemolysin export pump (Higgins *et al*, 1982) as well as the yeast *Saccharomyces cerevisiae* STE6 gene product which exports hydrophobic lipopeptide pheromones (McGrath and Varshowsky, 1989). Unlike well-defined carrier molecules such as the  $\text{Ca}^{2+}$   $\text{Mg}^{2+}$  ATPase or  $\text{Na}^{+}\text{K}^{+}$  exchange pump, P-gp is less selective with regard to substrate.

Most experimentally induced mdr cell lines have been selected for at least 100-fold drug-resistance, although it has been argued that clinically

relevant drug resistance is in the order of two to ten fold (Yang, 1989). To understand the clinical importance of mdr, the normal levels of P-gp in a variety of human tissues have been studied.

Immunoblots have demonstrated the presence of P-gp in liver (Hitchins *et al*, 1987) and it has been localised to the apical biliary surface of hepatocytes (Thiebaut *et al*, 1987). The polarised expression of P-gp suggests a secretory role for the membrane glycoprotein. Endothelial cells of human capillary blood vessels also express P-gp, but cells from larger blood vessels and other tissue capillaries do not. This suggests that P-gp may exclude toxic compounds from the central nervous system and other sites (Cordon-Cardo *et al*, 1989; Thiebaut *et al*, 1989). P-gp almost certainly has a normal physiological function in human tissues related to secretion and protection of tissues from various naturally occurring toxins or commonly encountered xenobiotics.

The first report of pharmacological reversal of mdr came from Tsuruo *et al* (1981). He showed that verapamil, a calcium channel blocker, and the calmodulin (CaM) antagonist, trifluoperazine, potentiated the antiproliferative effect of vincristine *in vivo* and *in vitro*. Investigators have determined the magnitude of the effect of chemosensitisers by comparing IC<sub>50</sub> (the concentration of a compound which causes 50% reduction in viable cells) values for a cytotoxic drug in the absence and presence of a relatively non-toxic fixed concentration of reversing agent. A large amount of interest has been focused on devising clinical protocols incorporating chemosensitisers to circumvent resistance in humans (Skovsgaard *et al*, 1984; Ozols *et al*, 1987; Miller *et al*, 1988; Dalton *et al*, 1989; Gottesman and Pastan, 1989). The chemosensitisers can be grouped into various broad categories.

Calcium channel blockers, such as verapamil, have been studied for their effects on reversing mdr. Tsuruo *et al* (1981) showed that 2.2 - 6.6  $\mu$ M verapamil reversed the ~30 fold resistance to vincristine and seven fold resistance to vinblastine displayed by P388/VCR, a mdr murine leukemia cell line. Verapamil did not alter vincristine binding to tubulin, the cytotoxic target of vinca alkaloids (Owellen *et al*, 1974) but altered drug accumulation (Tsuruo *et al*, 1981). In certain studies, verapamil was found to be more

effective in reversing resistance to the selecting agents than the cross-resistant drugs (Beck *et al*, 1986; Ford *et al*, 1990). Bellamy *et al* (1988) used DNA alkaline elution to determine the number of DNA strand breaks in sensitive and resistant cell lines after exposure to doxorubicin. He found a decrease in the number of lesions in mdr cells which was elevated in the presence of verapamil. Since anthracycline cytotoxicity is through topoisomerase II-mediated DNA strand breaks (Ross *et al*, 1978) verapamil is believed to inhibit P-gp transport of doxorubicin, leading to increased intracellular doxorubicin concentrations and increased cellular toxicity. From studies using photoactivated verapamil analogues, it has been shown that verapamil inhibits the binding of many chemotherapeutic drugs and other chemosensitisers to P-gp (Safa *et al*, 1987). Although verapamil is an effective P-gp antagonist, it has toxic cardiovascular effects in humans at concentrations needed for such antagonism (Candell *et al*, 1979). Other less toxic compounds have therefore been examined. Structurally dissimilar calcium channel blockers, such as nicardipine, have been shown to be potent antagonists of mdr (Tsuruo *et al*, 1983).

Another group of anti-mdr agents are drugs which inhibit calmodulin-mediated processes. Tsuruo *et al* (1982) studied several drugs known to alter calcium homeostasis. Trifluoperazine, for example, was found to cause a 5-10 fold increase in vincristine and doxorubicin sensitivity in 20-40 fold resistant P388/VCR and P388/Adr cells respectively. Several studies have shown that CaM antagonists such as trifluoperazine modulate cell sensitivity to bleomycin through increased DNA damage and inhibition of repair (Chafouleas *et al*, 1984; Lazo *et al*, 1985). Skovsgaard (1980) tested the hypothesis that the drug transport pump would be competitively inhibited by an excess of a non-toxic analog of a chemotherapeutic drug. He examined the anthracycline analog N-acetyl-daunorubicin which lacks the ability to intercalate with DNA, is less cytotoxic than daunorubicin and can achieve higher cytoplasmic concentrations (Zunino *et al*, 1972). The compound was found to inhibit active daunomycin transport from mdr but not sensitive cells.

Steroids and hormonal analogues have also been examined for their effects on mdr reversal. Yang *et al* (1989) found that progesterone and deoxycorticosterone caused an increase in vinblastine accumulation and

some reversal of vinblastine resistance in mdr murine macrophages. Progesterone was the most potent steroid for inhibiting labelling of endometrial P-gp, suggesting that it may be a natural substrate for P-gp. The anti-oestrogens, for example tamoxifen, can partially overcome resistance in P388/Dox cells independent of their effect on oestrogen receptors (Foster *et al*, 1988).

Many compounds which circumvent mdr fall into a large group of compounds which share a broad structural similarity. They are amphipathic and lipophilic and have a heterocyclic ring separated from a cationic, amino group. These include quinidine (Tsuruo *et al*, 1984), the antibiotic erythromycin (Hofsli and Nissen-Meyer, 1989) and the anti-malarial quinacrine (Inaba and Maruyama, 1988).

Cyclosporins, as well as being immunosuppressants, have sensitising activity in mdr cells (Twentyman, 1988). However, cyclosporin A (CsA) has been shown to potentiate drug action *in vitro* and *in vivo* in both tumour and normal cells (Twentyman, 1988).

Cationic, amphipathic drugs, including most chemosensitisers, interact with polar lipids such as phosphatidylserine in the plasma membrane (Lullmann and Wehling, 1979). It has been suggested that membrane differences may alter drug accumulation in mdr cell lines. Chemosensitisers may perturb the mdr cell membrane fluidity and change cellular accumulation. Investigators have found increased membrane fluidity in mdr cells (Bhushan *et al*, 1989; Ramu *et al*, 1983). Evidence indicates that altered calcium physiology is not involved in mdr. Lee *et al* (1988) demonstrated that both drug sensitive and mdr cells lack voltage-gated calcium channels, unlike excitable tissues. Treatment of mdr cells to reduce or increase calcium levels had no influence on doxorubicin accumulation or cytotoxicity in mdr cells (Huet and Robert, 1988). The role of CaM in mdr is unknown. CaM regulates a variety of critical cellular functions including activation of plasma membrane calcium ATPase to stimulate protein kinases and phosphatases (Schulman and Greengard, 1978). Whether CaM-mediated phosphorylation may activate or regulate P-gp function in mdr is not known. Some evidence exists for the regulatory role of the calcium-dependent protein kinase C (PKC) in mdr. PKC has been found to enhance

cellular secretion in many tissues (Katakami *et al*, 1984). Weiss *et al* (1982) found that anthracyclines inhibit PKC and Fine *et al* (1988) showed that PKC activity was increased in MCF-7 mdr cells. Treatment of sensitive human KB cells with a phorbol ester activator of PKC caused a two-fold protection against the cytotoxicity of VP16, vincristine and mitoxantrone which could not be reversed with verapamil (Ferguson and Cheng, 1987). However, results of additional studies have been conflicting. Hait and DeRosa (1990) were unable to show significant changes in doxorubicin accumulation in HL-60 cells with low, normal or overexpressed activity of PKC. Specific inhibitors of PKC and cell lines resistant to these inhibitors will allow the hypothesis to be more rigourously tested.

Phorbol esters have been reported to increase P-gp phosphorylation (Hamada *et al*, 1987) which indicates that PKC may modulate the function of P-gp. However, insufficient data is available at present to draw any firm conclusions regarding the regulatory or modulatory role of PKC in P-gp associated mdr. The strongest body of evidence available is that body which suggests that chemosensitisers directly affect the function of P-gp. Cornwell *et al* (1986b) reported the irreversible binding of the photoactive analogs of vinblastine ( $[^3\text{H}]$  NABV and  $[^125\text{I}]$  NASV) to mdr membranes. P-gp was specifically photoaffinity labelled in mdr cell lines. Verapamil has been found to inhibit  $[^125\text{I}]$  NASV labelling of P-gp from KB-V1 cells (Cornwell *et al*, 1987). Chemosensitisers may compete for a common drug-binding site, for overlapping sites or for sites that cause allosteric changes which prevent the binding of other drugs. They may also inhibit P-gp by interacting with sites such as the ATPase or phosphorylation domains or act by altering the membrane environment to cause functional changes in P-gp.

## **AIMS**

The aim of this chapter was to determine whether P-gp functions as an efflux pump for xenobiotic substances and in particular carcinogens. It was proposed that P-gp may function as such in normal tissues. To test this hypothesis, the mdr cell line CH<sup>R</sup>C5 and it's drug sensitive counterpart

AuxB1 were used, which provided a model for P-gp expression and non-expression, respectively. The MTT assay system was employed to determine the extent to which carcinogens can reverse the mdr phenotype in CH<sup>R</sup>C5 cells when used at non-toxic levels. Inhibition of drug binding to the CH<sup>R</sup>C5 cells was measured to determine whether carcinogens act as substrates for the P-gp transport pump or whether these compounds must first be metabolised by the P450 drug metabolising system.

#### **4.2 RESISTANCE PROFILES OF MULTIDRUG RESISTANT CELLS USING THE MTT ASSAY**

Carmichael *et al* (1987a) reported the use of the MTT assay in screening cell lines for drug sensitivity. Good correlation was observed using the CHO-AuxB1 cell line and the pleiotropic drug-resistant mutant CH<sup>R</sup>C5 with similar degrees of relative resistance observed with both the MTT and clonogenic assays.

The MTT assay is dependent on the cellular reduction of 3,(4,5-dimethylthiazol-2-yl-2,5-diphenyl) tetrazolium bromide (MTT) by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be determined spectrophotometrically (Mosmann, 1985). The procedure for the assay is described in Section 2.12.1.

##### **(a) DETERMINING THE RELATIONSHIP BETWEEN CELL NUMBER AND MTT FORMAZAN CRYSTAL FORMATION (I.E. ABSORBANCE)**

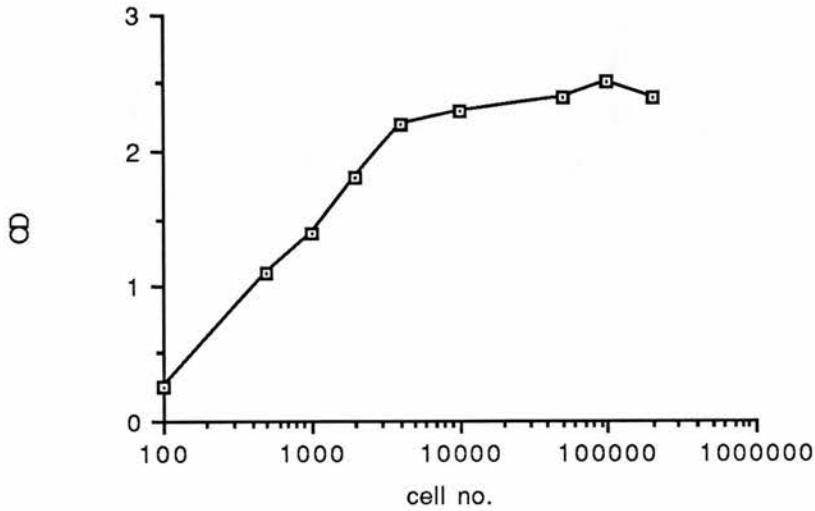
In order to determine the relationship between cell number and absorbance, increasing numbers of both CH<sup>R</sup>C5 and AuxB1 cells, ranging from 100 to 200,000 cells per well, were plated. MTT was added after four days and the plates incubated for a further four hours. A time period of four days was chosen since this incubation time allowed sufficient time for cell death to occur, but was short enough to obviate the need to re-feed the cells. The growth curves for both the AuxB1 and CH<sup>R</sup>C5 cell lines are shown in

FIGURE 4.1

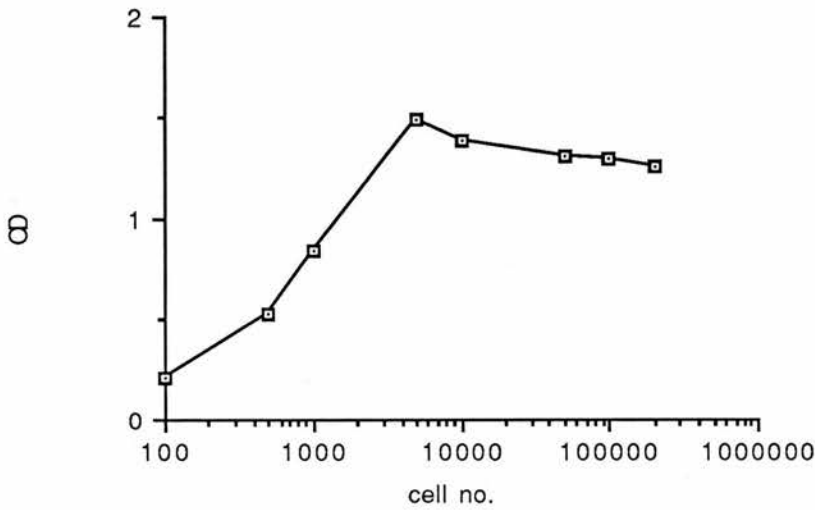
GROWTH CURVE OF AUXB1 AND CH<sup>R</sup>C5 CELLS

Each individual point represents the mean of 3 experiments (6 wells were read in each experiment).

(a) AuxB1 cells



(b) CH<sup>R</sup>C5 cells



Plates were set up with 100-200,000 AuxB or CH<sup>R</sup>C5 cells per well (6 wells per cell density) in a volume of 200  $\mu$ l. The cells were grown for a total time of 5 days followed by the addition of MTT (50  $\mu$ l of 2 mg/ml) and incubated for 4 hours until the crystals formed, the colour developed and plates read.

Figure 4.1. The relationship between cell number and absorbance was found to be linear between 100 to 5000 cells per well for CH<sup>R</sup>C5. However, at a seeding density of 5000 cells per well, the AuxB1 cells had reached confluency before the end of the assay period and the absorbance had almost reached a plateau. The absorbance values for CH<sup>R</sup>C5 are lower than AuxB1 for each time point, which is a reflection of the difference in doubling time for each cell line; reported to be 13-15h for AuxB1 and 22h for CH<sup>R</sup>C5 (Carmichael *et al*, 1987). In order to ensure that both cell lines were growing exponentially after 5 days in culture, all subsequent experiments employed a seeding density of 2500 cells per well for both CH<sup>R</sup>C5 and AuxB1.

(b) ASSESSMENT OF DRUG RESISTANCE IN THE CH<sup>R</sup>C5 CELL LINE COMPARED TO THE DRUG SENSITIVE AUXB1 CELL LINE

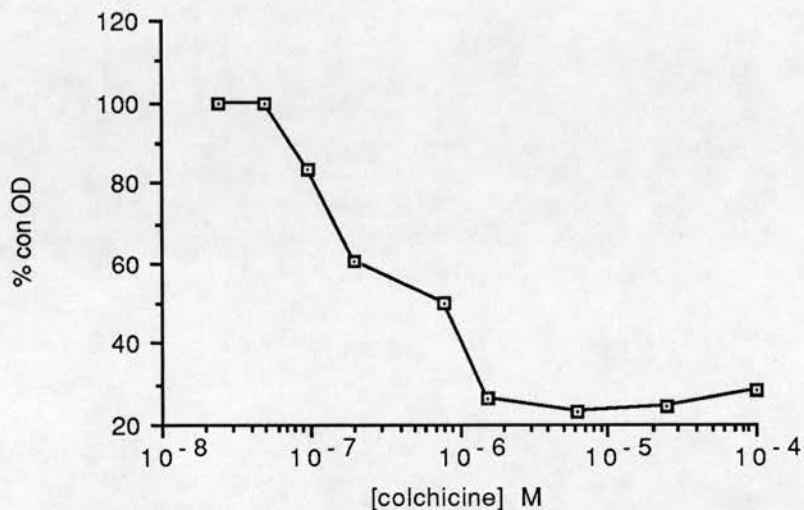
Chemosensitivity was assessed by continuous exposure of the cell lines to colchicine and doxorubicin. CH<sup>R</sup>C5 was made resistant to colchicine and has been shown to exhibit a cross-resistance pattern to several other drugs including adriamycin (Ling *et al*, 1983).

Equal numbers of AuxB1 and CH<sup>R</sup>C5 cells (2500 cells per well) were plated into each well in 180µl of culture medium to which 20µl of 10 x concentrated drug or serum free medium was added 24h later. Following incubation for 4 days, MTT solution (50µl of 2mg/ml) was added to each well and incubation continued for a further four hours. Following this incubation, DMSO (50µl) was added to each well to dissolve the formazan crystals which had formed. Absorbance values were read at 540nm. The results obtained with the two cell lines following colchicine and doxorubicin exposure are shown in Figures 4.2 and 4.3 and summarised in Table 4.1. These results show that CH<sup>R</sup>C5 is more resistant to colchicine and doxorubicin than the wild type AuxB1 sensitive cell line as expected. Colchicine is a drug which causes cytostatic effects on the cell lines which accounts for the lack of cell kill at high concentrations in both cell lines.

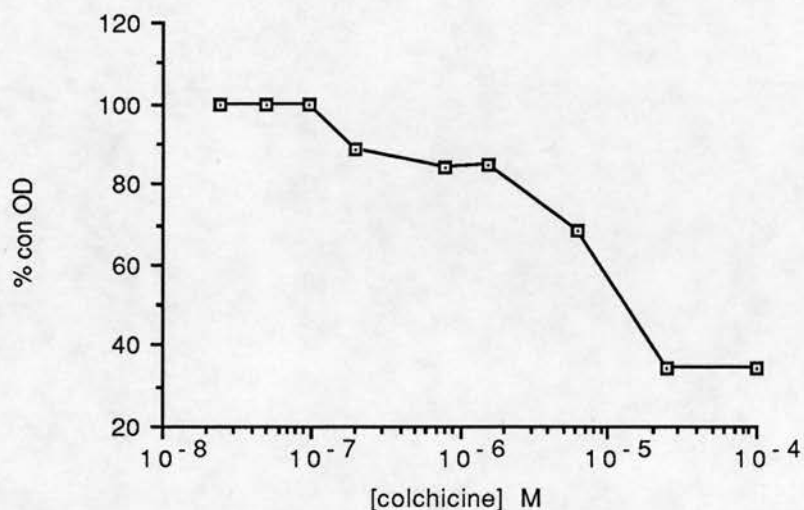
FIGURE 4.2

TOXICITY OF COLCHICINE TOWARDS CH<sup>R</sup>C5 AND AUXB1 CELLS

(a) Toxicity towards AuxB1 cells



(b) Toxicity towards CH<sup>R</sup>C5 cells



Plates were set up with 2500 CH<sup>R</sup>C5 or AuxB1 cells per well in a volume of 180  $\mu$ l. The cells were allowed to adhere overnight and then 20  $\mu$ l of 10 x stock colchicine in serum-free medium was added to give final concentrations in the range 0-100  $\mu$ M. The plates were then incubated for 4 more days followed by the addition of MTT (50  $\mu$ l of 2 mg/ml) and colour developed as described previously.

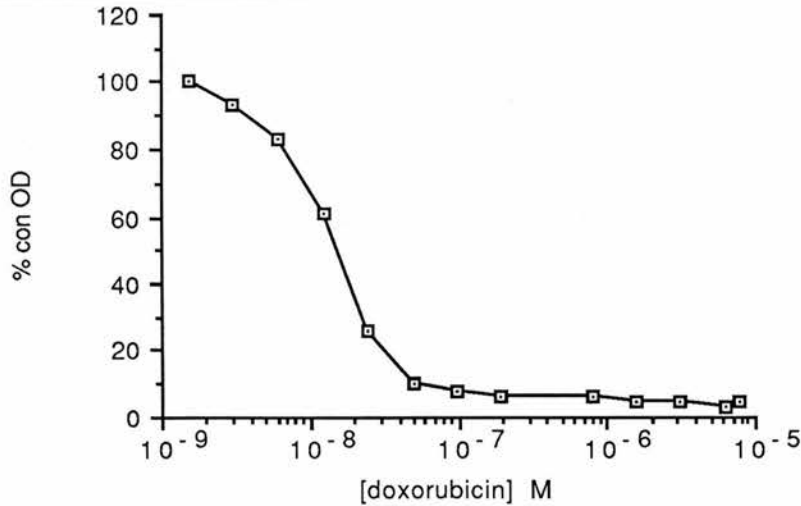
Each individual point represents the mean of 3 experiments (6 wells were read in each experiment).

**FIGURE 4.3**

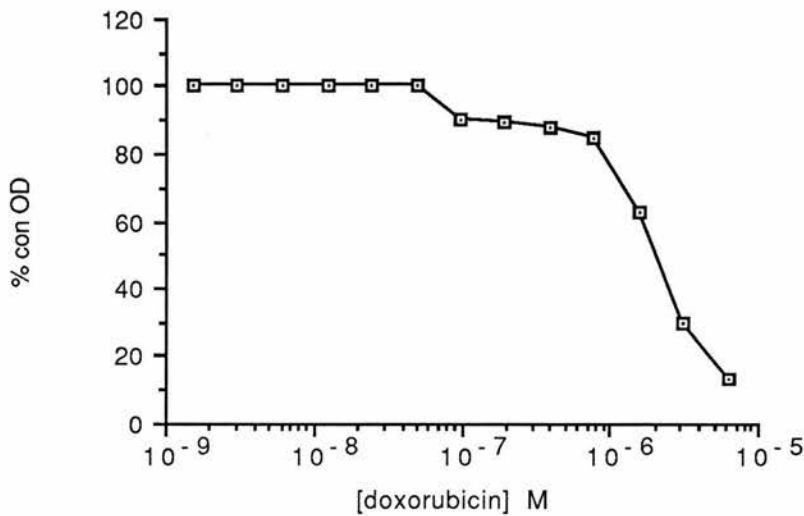
**TOXICITY OF DOXORUBICIN TOWARDS CH<sup>R</sup>C5 AND AUXB1 CELLS**

(a) Toxicity towards AuxB1 cells

Each individual point represents the mean of 3 experiments (6 wells were read in each experiment).



(b) Toxicity towards CH<sup>R</sup>C5 cells



Plates were set up with 2500 AuxB1 or CH<sup>R</sup>C5 cells per well in a volume of 180  $\mu$ l. The cells were allowed to adhere overnight and then 20  $\mu$ l of 10 x stock of doxorubicin in serum-free medium was added to give final concentrations in the range 0-50  $\mu$ M. The plates were then incubated for 4 days followed by the addition of MTT (50  $\mu$ l of 2 mg/ml) and colour developed as described previously.

doxorubicin, rapid changes in cell viability were observed even at high concentrations of the compound.

These results established that the cell lines demonstrated differential sensitivity to chemotherapeutic compounds and are suitable for use in the MTT assay.

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**TABLE 4.1**  
**TOXICITY OF DOXORUBICIN AND COLCHICINE TOWARDS**  
**CH<sup>R</sup>C5 AND AUXB1 CELLS**

DRUG	IC <sub>50</sub>		FOLD RESISTANCE
	CH <sup>R</sup> C5	AUXB1	
Doxorubicin	2 μM	0.015 μM	133
Colchicine	15 μM	0.8 μM	21

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#### 4.3 REVERSAL OF RESISTANCE WITH VERAPAMIL

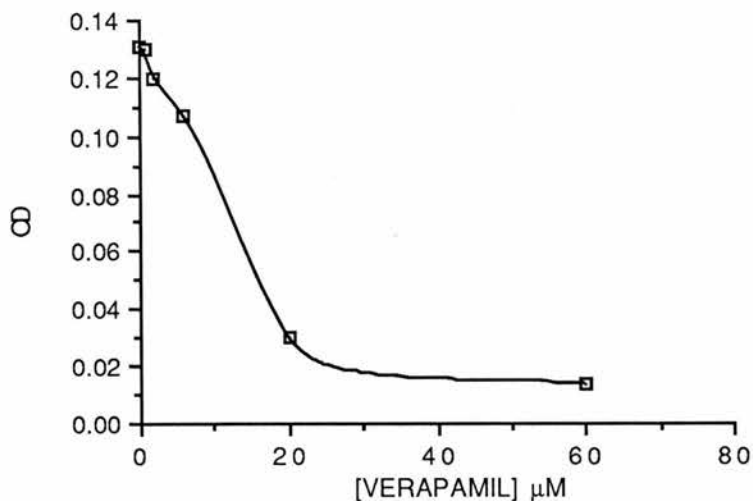
As discussed in the introduction to this chapter, verapamil can reverse the mdr phenotype in various cell lines. Eliason *et al* (1990) used the MTT assay to quantitate the modification by verapamil on P-gp in the mdr cell line KB5-5 and the parental line KB-3-1. Since l(-)-verapamil was shown to be approximately four times more active than the d-(+)-verapamil in modifying mdr (Eliason *et al*, 1990), l(-)-verapamil was used in this study.

In order to assess the cytotoxicity of verapamil itself, several concentrations of the compound, ranging from 60 to 0.6 μM, were tested on both resistant and sensitive cell lines. Both cell lines gave the same results. No difference in sensitivity to verapamil was observed throughout the range of verapamil concentrations used. The experiment was repeated, but incorporating 10<sup>-7</sup>M colchicine. CHRC5 cells were normally resistant to such a concentration of colchicine (Figure 4.2). The result is depicted in

**FIGURE 4.4**

**REVERSAL OF RESISTANCE TO COLCHICINE IN CH<sup>R</sup>C5 CELLS BY VERAPAMIL**

Each individual point represents the mean of 3 experiments (6 wells were read in each experiment).



Plates were set up with 2500 CH<sup>R</sup>C5 cells per well in a volume of 160  $\mu\text{l}$ . The cells were allowed to adhere overnight and then 20  $\mu\text{l}$  of 10 x stock colchicine in serum-free medium was added to give a final, non-toxic concentration of 0.1  $\mu\text{M}$ . Verapamil (20  $\mu\text{l}$  of 10 x) was also added to give final concentrations in the range 0-60  $\mu\text{M}$ . The plates were then incubated for a further 4 days followed by the addition of MTT (50  $\mu\text{l}$  of 2 mg/ml) and colour developed as described previously.

---

Figure 4.4. It was shown using this experiment that the calcium channel blocker verapamil can reverse the mdr phenotype in CH<sup>R</sup>C5 cells, which are normally resistant to colchicine. Since reversal was demonstrated using verapamil, a similar experiment was used with carcinogens to assess their ability to reverse the mdr phenotype and act as substrates for P-gp.

#### 4.4 CYTOTOXICITY OF CARCINOGENS ON DRUG RESISTANT CH<sup>R</sup>C5 AND DRUG SENSITIVE AUXB1 CELLS

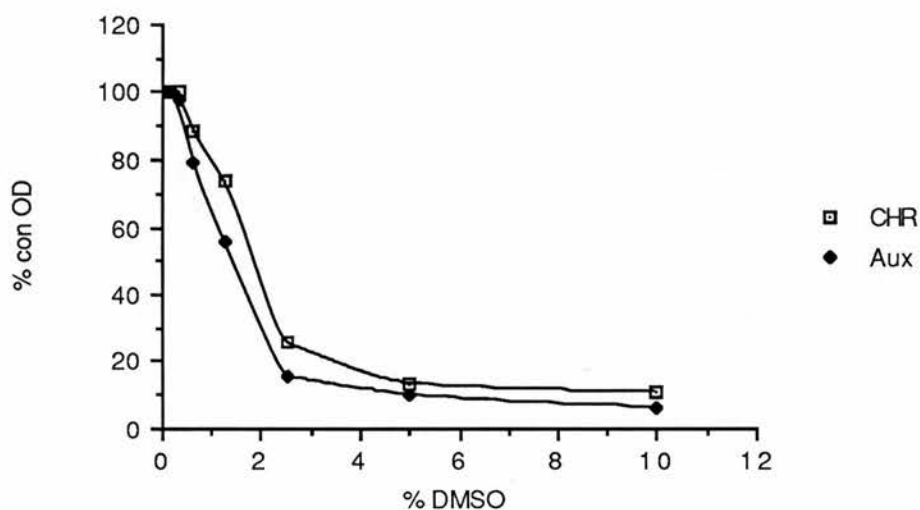
In order to ensure that the four carcinogens used in this study were not cytotoxic over the range of concentrations chosen for assessing their potential to compete for P-gp, the MTT assay was used to test their cytotoxicity. In each case, the control used was the solvent treatment alone i.e. serum free medium (20  $\mu$ l) or DMSO (20  $\mu$ l). Since DMSO was used as the solvent for some of the compounds, it was assessed for cytotoxicity towards CH<sup>R</sup>C5 and AuxB1 cells in order to ensure that it did not interfere with the assay in any way. The toxicity of DMSO towards CH<sup>R</sup>C5 and AuxB1 cells is shown in Figure 4.5. 0.25% DMSO was considered to be non-toxic to either cell line being used and this value was considered to be highest concentration of DMSO to which the cells could be exposed without altering the results of the assay. Compounds dissolved in DMSO were therefore diluted in serum-free medium to achieve such dilutions of DMSO before being added to the assay. The results are shown in Figures 4.6-4.8 and summarised in Table 4.2.

(a) 2-acetylaminofluorene: 2-AAF was slightly less cytotoxic to the CH<sup>R</sup>C5 cell line than the parental, drug sensitive AuxB1 cell line (Figure 4.6) with 50% reduction in the control O.D. at  $\sim$ 140  $\mu$ M for CH<sup>R</sup>C5 and  $\sim$ 125  $\mu$ M for AuxB1 cells (Figure 4.6).

(b) Benzo(a)pyrene: B(a)p was cytotoxic to both cell lines and caused 50% reduction in O.D. at a concentration of  $\sim$ 100  $\mu$ M for CH<sup>R</sup>C5 and 140  $\mu$ M for AuxB1 (Figure 4.7).

(c) Aflatoxin B<sub>1</sub>: AFB<sub>1</sub> was slightly cytotoxic to CH<sup>R</sup>C5 cells and AuxB1 cells. The IC<sub>50</sub> value for the CHRC5 cell line was  $\sim$ 60  $\mu$ M and 50  $\mu$ M for the AuxB1 cell line (Figure 4.8).

**FIGURE 4.5**  
**TOXICITY OF DIMETHYLSULPHOXIDE TOWARDS CH<sup>R</sup>C5 AND**  
**AUXB1 CELLS**



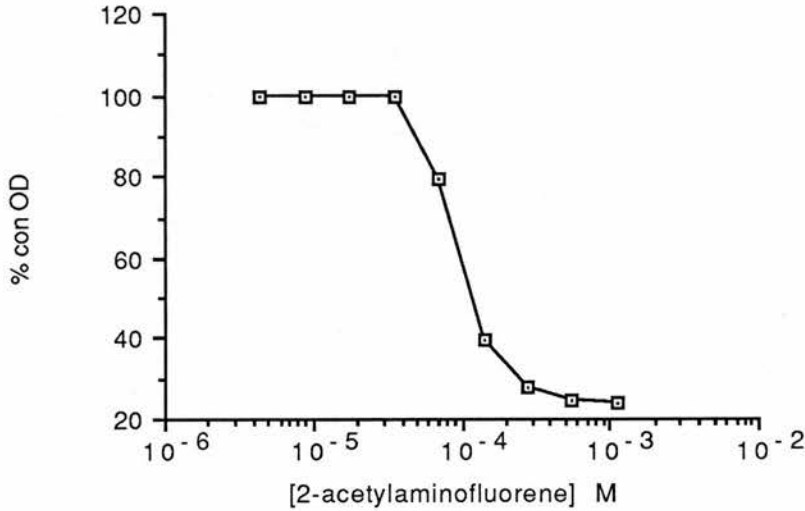
Plates were set up with 2500 AuxB1 and CH<sup>R</sup>C5 cells in a final volume of 180  $\mu$ l. The cells were allowed to adhere overnight and then 20  $\mu$ l of 10 x stock of DMSO in serum-free medium was added to give the final concentrations in the range 0-10%. The plates were then incubated for a further 4 days followed by the addition of MTT (50  $\mu$ l of 2 mg/ml) and colour developed.

Each individual point represents the mean of 3 experiments (6 wells were read in each experiment).

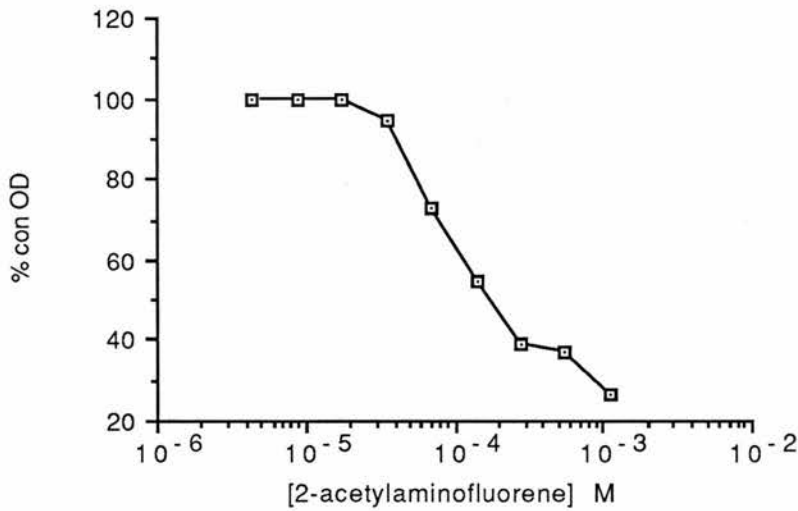
**FIGURE 4.6**

**TOXICITY OF 2-ACETYLAMINOFUORENE TOWARDS CH<sup>R</sup>C5 AND AUXB1 CELLS**

(a) Toxicity towards AuxB1 cells



(b) Toxicity towards CH<sup>R</sup>C5 cells



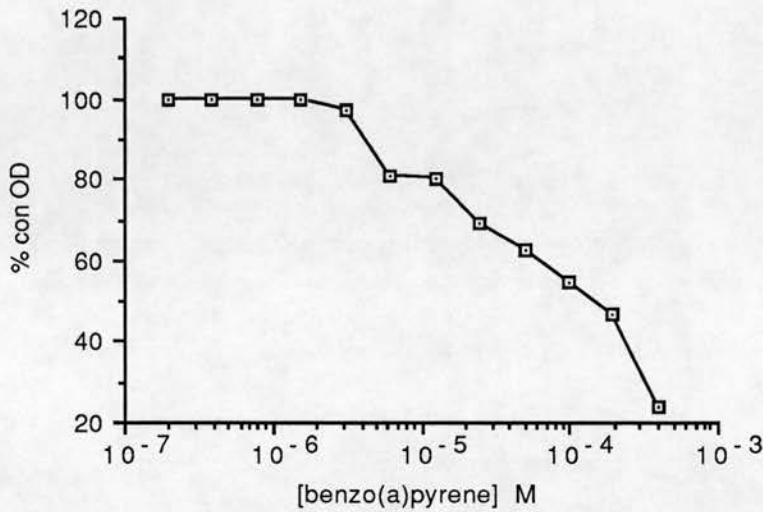
Plates were set up with 2500 AuxB1 or CH<sup>R</sup>C5 cells per well in a volume of 180  $\mu$ l. The cells were allowed to adhere overnight and then 20  $\mu$ l of 10 x stock 2-AAF in DMSO was added to give final concentrations in the range 0-1.1 mM. The plates were then incubated for 4 days followed by the addition of MTT (50  $\mu$ l of 2 mg/ml) and colour developed as described previously.

Each individual point represents the mean of 3 experiments (6 wells were read in each experiment).

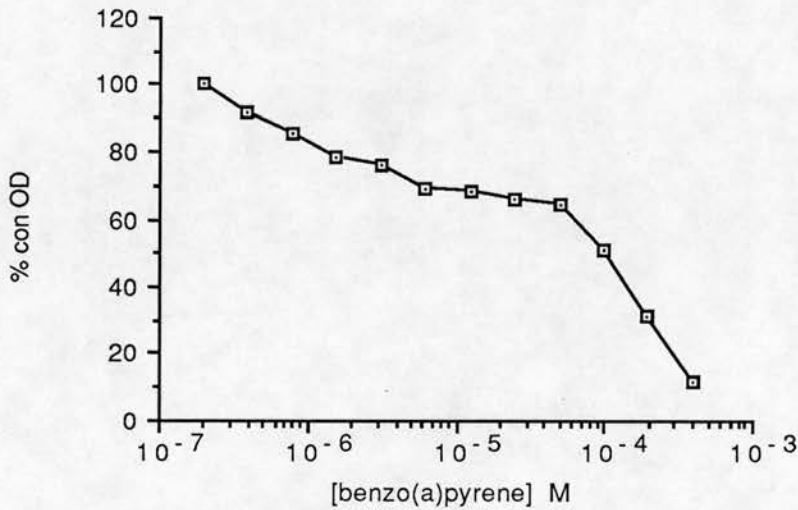
**FIGURE 4.7**

**TOXICITY OF BENZO(A)PYRENE TOWARDS CH<sup>R</sup>C5 AND AUXB1 CELLS**

(a) Toxicity towards AuxB1 cells



(b) Toxicity towards CH<sup>R</sup>C5 cells

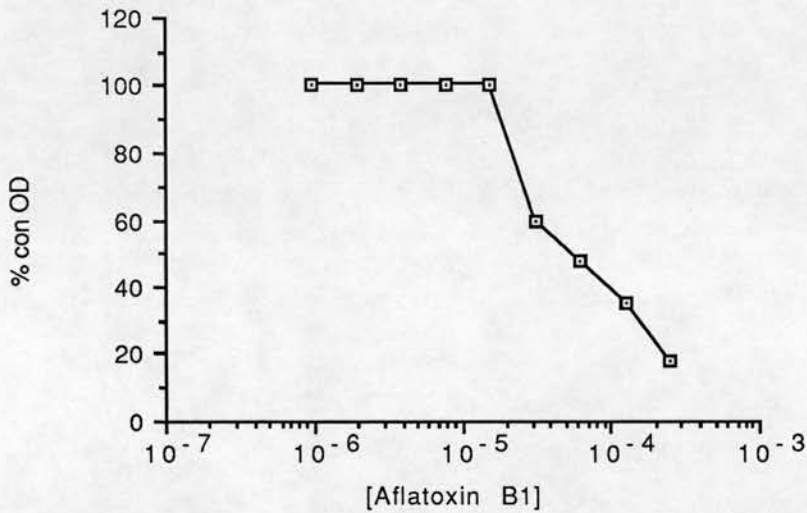


Plates were set up with 2500 AuxB1 or CH<sup>R</sup>C5 cells per well in a volume of 180  $\mu$ l. The cells were allowed to adhere overnight and then 20  $\mu$ l of 10 x stock of B(a)p dissolved in DMSO added to give final concentrations in the range 0-40 mM. The plates were incubated for a further 4 days and then MTT (50  $\mu$ l of 2 mg/ml) was added and the colour developed.

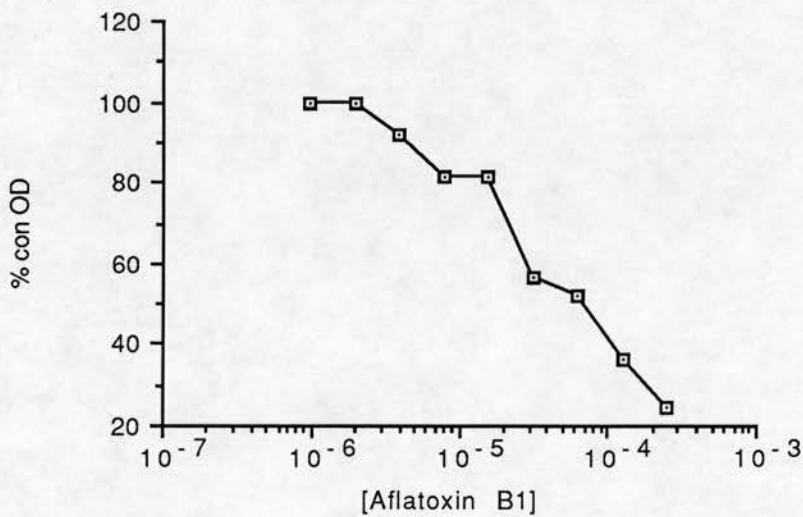
Each individual point represents the mean of 3 experiments (6 wells were read in each experiment).

**FIGURE 4.8**  
**TOXICITY OF AFLATOXIN B<sub>1</sub> TOWARDS CH<sup>R</sup>C5 AND AUXB1**  
**CELLS**

(a) Toxicity towards AuxB1 cells



(b) Toxicity towards CH<sup>R</sup>C5 cells



Plates were set up with 2500 AuxB1 or CH<sup>R</sup>C5 cells per well in a volume of 180  $\mu$ l. The cells were allowed to adhere overnight and then 20  $\mu$ l of 10 x stock AFB<sub>1</sub> in DMSO added to give final concentrations in the range 0-25 mM. The plates were incubated for a further 4 days and MTT (50  $\mu$ l of 2 mg/ml) added and the colour developed as described previously.

Each individual point represents the mean of 3 experiments (6 wells were read in each experiment).

---

**TABLE 4.2****TOXICITY OF CARCINOGENS TOWARD CH<sup>R</sup>C5 AND AUXB1 CELLS**

CARCINOGEN	IC <sub>50</sub>	
	CH <sup>R</sup> C5	AUXB <sub>1</sub>
2-Acetylaminofluorene	140 μM	125 μM
Benzo(a)pyrene	99 μM	141 μM
Aflatoxin B <sub>1</sub>	63 μM	50 μM

---

**4.5 EFFECT OF CARCINOGENS ON THE RESISTANCE PROFILE OF CH<sup>R</sup>C5 CELLS****(a) EFFECT OF 2-ACETYLAMINOFLUORENE ON COLCHICINE TOXICITY TOWARDS CH<sup>R</sup>C5 AND AUXB1 CELLS**

CH<sup>R</sup>C5 and AuxB1 cells were plated at 2500 cells per well in 160μl of medium and allowed to adhere overnight. 2-AAF (20μl) was then added to 48 wells of the 96-well assay plate to a final non-toxic concentration of 17.5 μM. Serum-free medium was added to the remaining wells. Colchicine (20 μl) was then added to all 96 wells of the plate in a concentration range from 0-0.1 mM. The final volume was therefore 200 μl. Triplicate plates were assayed for each cell line.

Figure 4.9 shows the results of the study. Co-incubation of the CH<sup>R</sup>C5 and AuxB1 cells with 2-AAF did not alter the cytotoxicity of colchicine toward the cells. As expected, colchicine was less toxic to the drug resistant cell line than the drug sensitive one. However, the presence of a non-toxic dose of the carcinogen 2-AAF did not alter the result. This indicates that 2-AAF does not compete for the plasma membrane protein, P-pg, of the drug resistant

CH<sup>R</sup>C5 cell line, at the concentration at which the carcinogen was tested.

(b) EFFECT OF BENZO(A)PYRENE ON COLCHICINE TOXICITY  
TOWARD CH<sup>R</sup>C5 AND AUXB1 CELLS

An experiment, such as described in Section 4.5.(a), was performed using benzo(a)pyrene (B(a)p), at a non-toxic dose in the presence of a range of cytotoxic colchicine concentrations. As before, both CH<sup>R</sup>C5 and AuxB1 cells were assessed to re-affirm the difference in cytotoxicity to colchicine between the two cell lines and the functionality of the P-gp pump.

Figure 4.10 shows the results of the experiment. As with 2-AAF, B(a)p did not alter the cytotoxicity of colchicine towards the cells. As expected, CH<sup>R</sup>C5 displayed greater resistance to colchicine than AuxB1 cells. B(a)p does not appear to compete for the binding of colchicine to the P-gp pump at the non-toxic concentrations at which it was used.

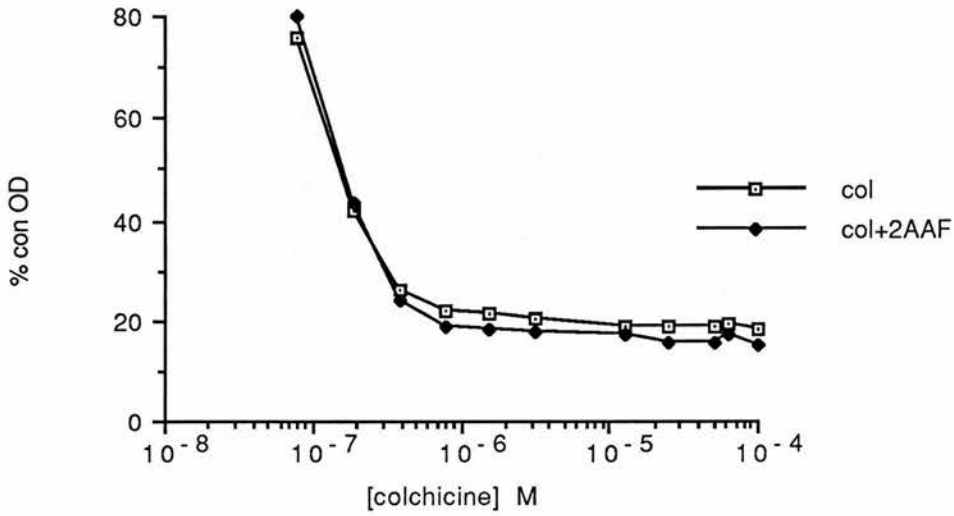
(c) EFFECT OF AFLATOXIN B<sub>1</sub> ON COLCHICINE CYTOTOXICITY  
TOWARD CH<sup>R</sup>C5 AND AUXB1 CELLS

An experiment, such as described in Section 4.5.(a), was conducted using AFB<sub>1</sub> at a non-toxic dose in the presence of colchicine at a variety of cytotoxic concentrations. Both CH<sup>R</sup>C5 and AuxB1 cells were assessed under these conditions.

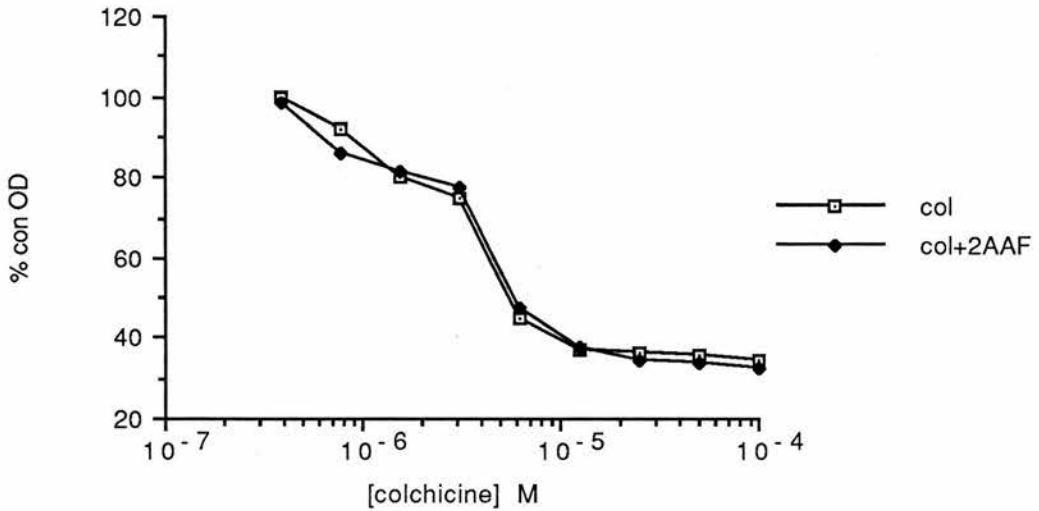
Figure 4.11 shows the results of the experiment. AFB<sub>1</sub> failed to alter the cytotoxicity caused by colchicine toward the CH<sup>R</sup>C5 cell line and hence does not appear to compete for the binding of P-gp. Table 4.3 summarises the results of Section 4.5.

**FIGURE 4.9 EFFECT OF TREATMENT WITH A NON-TOXIC DOSE OF 2-ACETYLAMINOFLUORENE ON THE CYTOTOXICITY OF COLCHICINE TOWARDS CH<sup>R</sup>C5 AND AUX B1 CELLS**

(a) Toxicity towards AuxB1 cells



(b) Toxicity towards CH<sup>R</sup>C5 cells



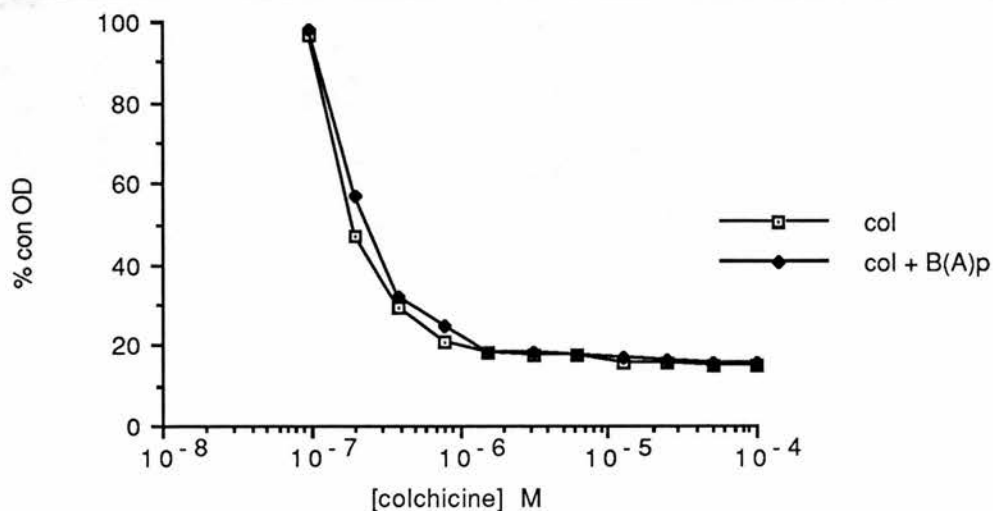
Plates were set up with 2500 AuxB1 or CH<sup>R</sup>C5 cells per well in a volume of 160  $\mu$ l. Cells adhered overnight and 20 $\mu$ l of 10 x stock colchicine added to give final concentrations in the range 0 - 0.1 mM. 2-AAF (20  $\mu$ l) was added to half the wells to give a final non-toxic concentration of 17.5  $\mu$ M. Serum free medium was added to the remaining wells. Plates were incubated for 4 days, MTT added and the colour developed and read.

Each individual point represents the mean of 3 experiments (6 wells were read in each experiment).

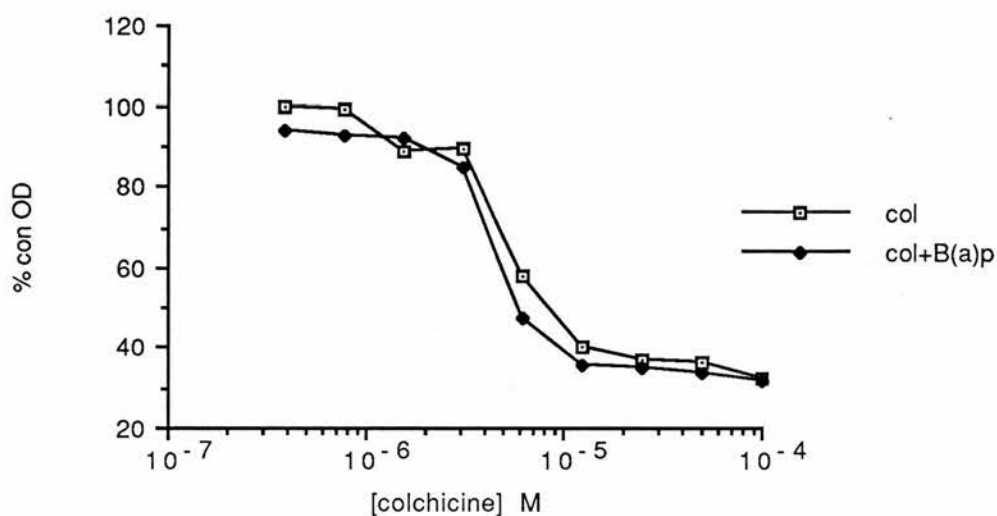
**FIGURE 4.10 EFFECT OF TREATMENT WITH A NON-TOXIC DOSE OF B(a)P ON THE CYTOTOXICITY OF COLCHICINE TOWARDS CH<sup>R</sup>C5 AND AUXB1 CELLS**

(a) Toxicity towards AuxB1 cells

Each individual point represents the mean of 3 experiments (6 wells were read in each experiment).



(b) Toxicity towards CH<sup>R</sup>C5 cells

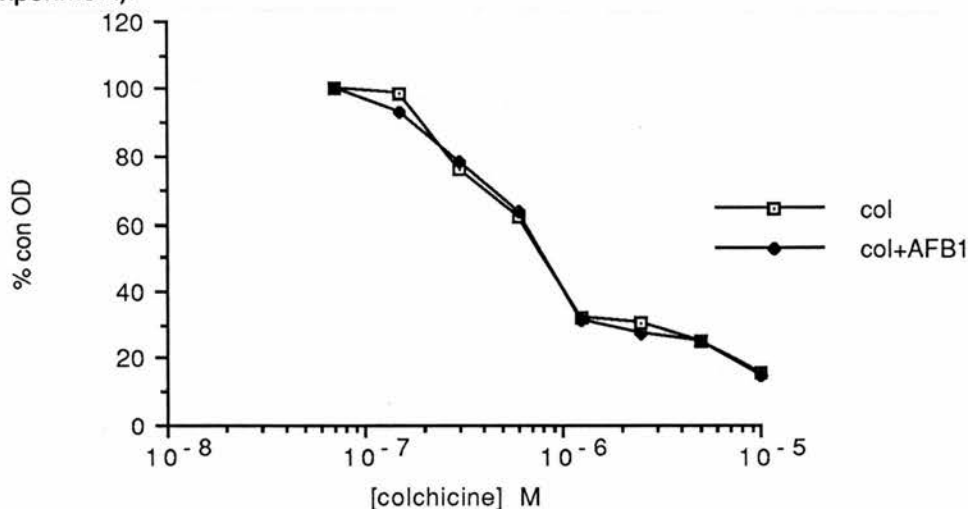


Plates were set up with 2500 AuxB1 or CH<sup>R</sup>C5 cells per well in a volume of 160  $\mu$ l. Cells adhered overnight and then 20  $\mu$ l of 10 x stock colchicine was added to give final concentrations in the range 0-0.1 mM. B(a)p (20  $\mu$ l) in DMSO was added to half the wells of the plate give a final, non-toxic concentration of  $\sim$ 4  $\mu$ M. Serum free medium (20 $\mu$ l) was added to the remaining wells. Plates were incubated MTT added and colour read.

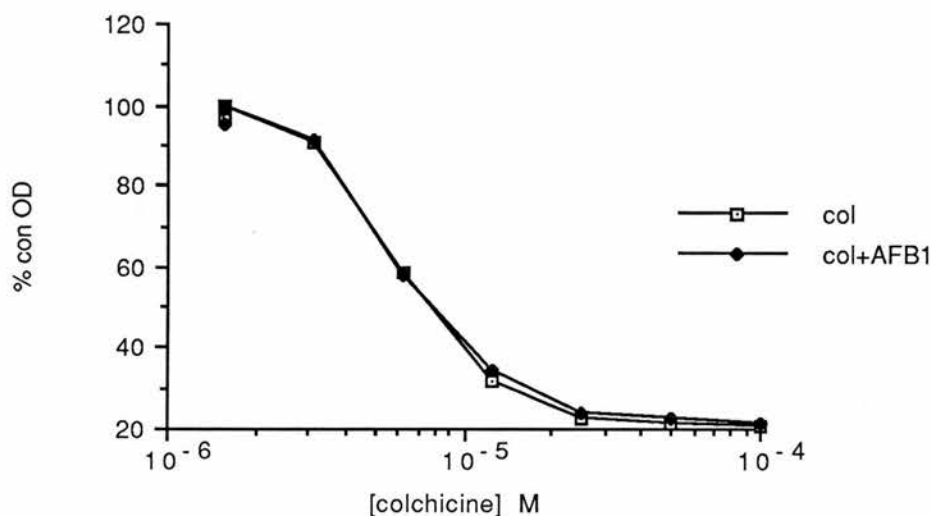
**FIGURE 4.11 EFFECT OF TREATMENT WITH A NON-TOXIC DOSE OF AFLATOXIN B<sub>1</sub> ON THE CYTOTOXICITY OF COLCHICINE TOWARDS CH<sup>R</sup>C5 AND AUXB1 CELLS**

(a) Toxicity towards AuxB1 cells

Each individual point represents the mean of 3 experiments (6 wells were read in each experiment).



(b) Toxicity towards CH<sup>R</sup>C5 cells



Plates were set up with 2500 AuxB1 or CH<sup>R</sup>C5 cells per well in a volume of 160  $\mu$ l. The cells were allowed to adhere overnight and then 20  $\mu$ l of 10 x stock of colchicine was added to give final concentrations in the range from 0-0.1  $\mu$ M. AFB1 (20  $\mu$ l) was also added to half the wells to a final, non-toxic concentration of 10  $\mu$ M. Serum free media was added to the remaining wells. Plates were incubated for a further 4 days, MTT added and the colour read.

---

**TABLE 4.3****TOXICITY OF COLCHICINE TOWARDS CH<sup>R</sup>C5 AND AUXB<sub>1</sub> CELLS IN THE PRESENCE OR ABSENCE OF CARCINOGENS**

CARCINOGEN	IC50 (μM)			
	CHRC5(+)	CHRC5(-)	AUXB1(+)	AUXB1(-)
2-AAF	30	30	0.95	0.95
B(a)p	50	20	0.85	0.9
AFB1	35	35	3.5	3.5

(+) plus carcinogen

(-) minus carcinogen

---

**4.6 SUMMARY OF RESULTS AND DISCUSSION**

In this section of the project, the MTT assay was characterised and used to assess the toxicity of chemotherapeutic agents and carcinogens on the CH<sup>R</sup>C5 and AuxB1 cell line. The mdr-reversing agent verapamil was also used in the assay to determine the reversibility of the mdr phenotype in CH<sup>R</sup>C5 cells. The assay was relatively quick and simple to perform compared to other clonogenic and dye exclusion techniques. Several compounds were assayed and their relative toxicity evaluated as IC<sub>50</sub> values. However, the assay does not distinguish between cytostatic and cytotoxic effects.

Experiments conducted in the absence of drug were designed to determine whether the cell number chosen for the assay meant that the cells were still in logarithmic growth at the end point of the assay. The O.D. obtained at the end of the assay also had to be high enough to give reproducible measurements of cytotoxicity.

The carcinogens tested caused little toxicity to either of the cell lines. It was aimed to establish whether carcinogens can act as substrates for P-gp

in CH<sup>R</sup>C5 cells and as such compete for the binding site on the protein and increase the cytotoxicity of the drug to which the cell line was made resistant. From the experiments conducted, it was found that this was not the case. The cytotoxic chemotherapeutic agents colchicine and doxorubicin did show differential toxicity toward AuxB1 and CH<sup>R</sup>C5 cells, with the drug sensitive line AuxB1 being much more sensitive to the effects of the drugs. Verapamil reversed the *mdr* phenotype of the CH<sup>R</sup>C5 cell line to some extent and thus demonstrated that known chemosensitising agents can be shown to cause such an effect using the MTT assay. Ford *et al* (1990) also reported a very small (4-fold) change in the resistance (400 fold) of MCF-7 Adr<sup>R</sup> cells to colchicine using verapamil. A 13-fold reversal of doxorubicin resistance (200-fold) and 33-fold reversal of vinblastine resistance (100-fold) in the same cell line was demonstrated. Fojo *et al* (1985) demonstrated that 20 µM verapamil completely reversed the 20- to 70-fold cross-resistance to doxorubicin, vinblastine and vincristine in colchicine selected KB human carcinoma cells but caused only a 60-fold reduction in the 220-fold resistance to colchicine.

The most likely reason for the lack of cytotoxicity of the carcinogens used in this study is the requirement for metabolic activation of drugs and carcinogens, by specific P450s, to cause toxicity. Such activation often results in the formation of highly reactive intermediates such as epoxides (Jollow and Smith, 1977). Various compounds undergo P450-dependent metabolic activation including the aromatic amine 2-AAF (McManus *et al*, 1984) and acetaminophen (Potter *et al*, 1973). The structures of the compounds used in this study are shown in Figure 4.1.2.

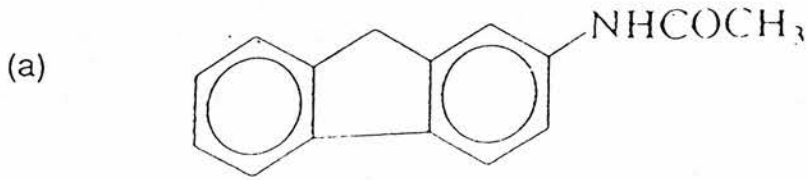
In the present study, the ovarian cell line employed is not known to express P450 enzymes and would not be capable therefore of metabolising the carcinogens examined to more toxic compounds.

Several reasons can be postulated for the lack of competition for P-gp between colchicine and the test carcinogens, apart from the explanations that P-gp may not function as an efflux pump for carcinogens and other xenobiotics or that the concentration of carcinogen employed in each case was insufficient to demonstrate a difference in toxicity levels.

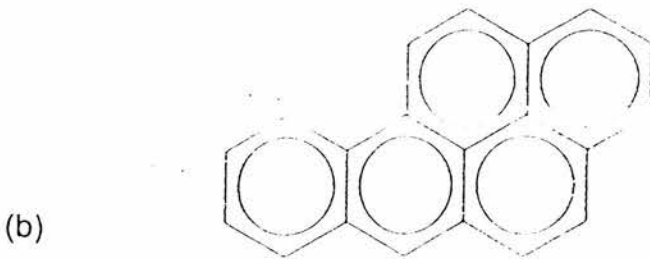
**FIGURE 4.12**

**STRUCTURE OF THREE COMPOUNDS WHICH UNDERGO P450-MEDIATED METABOLIC ACTIVATION**

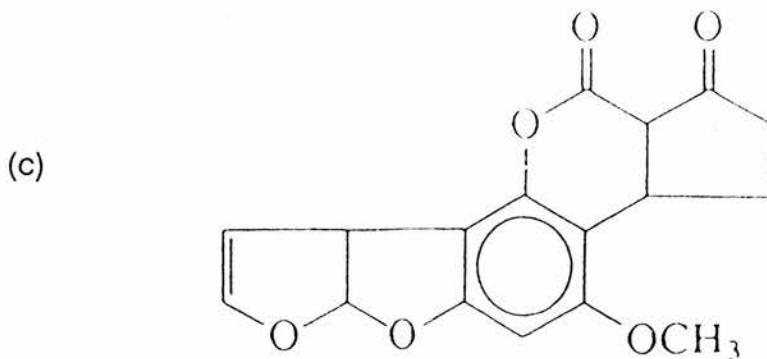
- (a) 2-acetylaminofluorene
- (b) benzo(a)pyrene
- (c) aflatoxin B<sub>1</sub>



*N*-2-Fluorenylacetylamine  
(2-Acetylaminofluorene)



Benzo[a]pyrene



Aflatoxin B<sub>1</sub>

Firstly, the carcinogens may have to be metabolically activated before acting as substrates for P-gp. To examine such a possibility would involve employing a drug resistant cell line known to express P450 enzymes capable of metabolising the carcinogen of interest. Such a cell line would probably be a hepatoma since liver tissue is known to express P450s at high levels *in vivo*. Alternatively an mdr cell line transfected in order to express P450 enzymes could be employed to metabolise the carcinogen of interest. The metabolites of the carcinogens could also be used directly in the assay to overcome the problem.

Secondly, colchicine may be a greatly preferred substrate for P-gp and would not allow the binding of other weaker substrates. By using several other chemotherapeutic drugs such as colcemid or vincristine this hypothesis could be tested.

Thirdly, the assay may lack the sensitivity required to detect small changes in the binding of colchicine to the P-gp transport pump. To improve the sensitivity of detection, azidopine photoaffinity labelling of P-gp could be utilised. The arylaside, 1,4 dihydropyridine, azidopine, is a calcium channel antagonist which photolabels calcium channels (Glossmann *et al*, 1987). [<sup>3</sup>H] azidopine can specifically photolabel P-gp. It has been demonstrated that binding is influenced by other calcium antagonists and drugs used to select resistant cells, for example vinblastine, colchicine and taxol (Yang *et al*, 1988). Such a system may demonstrate that carcinogens compete for P-gp labelling.

Since the study conducted does not conclusively prove the role of P-gp in xenobiotic efflux, further analysis will need to be undertaken to answer this question. Such a study would merit further consideration since the physiological role for P-gp in normal tissues remains unknown. By using the natural substrates for the P-gp pump, its activity in tumour cells could be manipulated to increase the response of refractory tumours to chemotherapy.

## CHAPTER 5

# EXOGENOUS REGULATION OF MDR GENES IN RAT AND MOUSE

### 5.1 INTRODUCTION

The hypothesis that exposure to chemical carcinogens led to the development of a cell population resistant to the cytotoxic effects of those carcinogens was first proposed by Haddow (1938) more than 50 years ago. Haddow showed that although polycyclic aromatic hydrocarbon (PAH) carcinogens were toxic and inhibited growth *in vivo*, the tumours induced by exposure to them were resistant to these effects (Haddow and Robinson, 1937). The inhibition of normal cell growth and stimulation of tumour cell growth has been reviewed (Vasilier and Guelstein, 1963; Melzer, 1980). Novicki *et al* (1985) noted that one acute effect of many hepatocarcinogens is inhibition of hepatocyte proliferation. Chronic administration results in altered hepatocytes which continue to grow despite the presence of the carcinogen.

Carcinogen-induced resistance to toxicity has been most extensively studied in rodent liver. The classical Solt-Farber model of chemical carcinogenesis in rat liver (Solt and Farber, 1976) involves treatment of rats with an initiating agent, for example diethylnitrosamine (DEN), followed by administration of 2-acetylaminofluorene (2-AAF) and partial hepatectomy. Hyperplastic nodules (preneoplastic lesions) may be isolated six to eight weeks after initiation with DEN, whereas neoplastic nodules (neoplastic lesions) representing primary hepatocellular carcinomas may be isolated six to eight months after initiation. Cells derived from the nodules appear cross-resistant to a wide variety of toxins (Carr, 1987).

Hepatocellular carcinoma (HCC) is a very common cancer and has a high rate of frequency in South East Asia and sub-Saharan Africa (Hutt, 1981). The resistance of HCC to ionizing radiation and chemotherapeutic agents has been studied (Falkson, 1975) and appears to have some

common similarities with the resistance of carcinogen altered rat hepatocytes to cytotoxicity, as discussed below.

Carr and Laishes (1981) showed that hepatocytes from rats treated with the hepatocarcinogen 2-AAF *in vivo* were resistant *in vitro* to the cytotoxic effects of many compounds, including chemotherapeutic agents such as adriamycin, compared to normal hepatocytes.

Batist *et al* (1986) isolated a doxorubicin resistant human breast cancer cell line (Dox<sup>R</sup> MCF7) that had developed the phenotype of *mdr*, and also displayed an increase in an anionic GST isozyme with high peroxidase activity. The induction of a similar anionic GST is also noted in rat hyperplastic liver nodules (Kitahara *et al*, 1984). Rat hyperplastic nodules (HNs) also contain lower levels of cytochrome P450 dependent enzymes, including aryl hydrocarbon hydroxylase (AHHase), aminopyrene N-demethylase and NADPH cytochrome c reductase relative to normal hepatocytes (Astrom, 1983; Cameron *et al*, 1976). Dox<sup>R</sup> MCF7 cells also displayed a marked decrease in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced AHHase activity and CYP1 gene transcription. Since AHHase is involved in the intracellular metabolism of the carcinogen benzo(a)pyrene, this change alters the sensitivity of the Dox<sup>R</sup> cells to the cytotoxic effects of this carcinogen (Cowan *et al*, 1986).

Thorgeirsson *et al* (1987) measured the expression of the *mdr* gene in normal rat liver cells, preneoplastic and neoplastic nodules and regenerating liver, since all these altered tissues had been shown to be resistant to a broad range of carcinogens. Levels of *mdr* mRNA were increased in both preneoplastic and neoplastic lesions. Expression of *mdr* was also dramatically increased 24-72 hours after partial hepatectomy in regenerating rat liver. This suggests that the expression of the *mdr* gene is regulated in liver and represents a component of the hepatic response to toxic insults.

More recently, Marino *et al* (1990) reported a study to determine whether the increased levels of *mdr* mRNA observed in regenerating rat liver was due to an increased rate of transcription or a post-translational event. Nuclear run-on assays were performed on nuclei from regenerating rat liver,

isolated 4 -72 h, after partial hepatectomy. Northern blot analysis showed that a greater than 20-fold increase in *mdr* mRNA levels had occurred but no increase was found in *mdr* gene transcription rates. This suggests that the observed increase in *mdr* mRNA levels may be due to a post-transcriptional event, for example message stabilisation.

It is unlikely that an increased expression of the *mdr* gene can totally explain the resistance to chemical carcinogens in early initiated cells since many of these carcinogens are compounds not usually effected by the *mdr* phenotype. These compounds do not appear to be transported by P-glycoprotein, for example *mdr* cells with high levels of P-gp are not more resistant to 2-AAF than their drug resistant counterpart (see chapter 4). Also if increased *mdr* following partial hepatectomy made liver cells resistant to 2-AAF then the Solt-Farber protocol would not result in liver nodules. Therefore other factors such as over-expression of GST may be contributing significantly to the development of the resistant phenotype, as suggested by Cowan *et al* (1986).

In this chapter, the effects of xenobiotics on *mdr* genes in mouse liver will be investigated. All three mouse *mdr* genes are expressed in the liver (Croop *et al*, 1989) but the changes in the expression levels of these particular genes following toxic insult have not been studied. The use of gene specific cDNA probes has allowed such a study of mouse *mdr* gene expression.

Burt and Thorgeirsson (1988) speculated that as a detoxification transport system, *mdr* 1 might be coordinately regulated in the liver, and other tissues, with such enzymes as UDP-glucuronyltransferase and cytochrome P450 isoforms. They demonstrated that treatment of rats *in vivo* with certain chemical carcinogens namely 2-AAF, isosafrole, phenothiazine and TCCD increases mRNA levels for *mdr* 1 and the CYP1 family.

Therefore, interaction of these compounds within the cell may generate an intracellular signal which results in alterations in RNA levels for several different detoxification systems.

## AIMS

The aims of this part of the study were to investigate the acute effects of a range of xenobiotic compounds, including both P450 and GST enzyme inducers, on hepatic *mdr* levels in both rat and mouse. The expression of *mdr* genes compared to P450 and GST genes were compared to determine whether or not similar regulatory mechanisms are operating following exposure to xenobiotics. Since the induction of gene expression is different in the rat and mouse, both rodent groups were examined.

In rats both mRNA and protein levels were studied, whereas in mice only mRNA levels were measured. Western blotting on mouse liver tissue resulted in cross-reactivity between a 60 kd protein and the C219 monoclonal antibody. Northern blotting was conducted using gene-specific cDNA probes in mouse but using a non-specific human cDNA clone in the rat. Therefore the expression of specific rat genes could not be determined.

## 5.2 XENOBIOTIC REGULATION OF MDR GENES IN RAT

### 5.2.1 TREATMENT OF WISTAR AND FISCHER RATS

Adult (~250g) male Wistar rats (2 per group) and Fischer 344 rats (4 per group) were treated with various xenobiotics.

The protocol for treating the Wistar rats is shown in Table 5.1. Figure 5.1 shows the structures of these compounds. The Fischer rats were treated with 3-MC and AFB<sub>1</sub> as for the Wistar rats except that two of the 3-MC treated rats were also treated for an extra day and then sacrificed 24 hours later. Two Fischer rats were dosed with 20 mg/kg of 2-AAF for 3 days and two rats for 4 days and then sacrificed 24 hours later respectively. The appropriate controls were included for all rat treatments. Both corn-oil and PBS were used to dissolve the compounds and therefore both corn-oil and PBS controls were done.

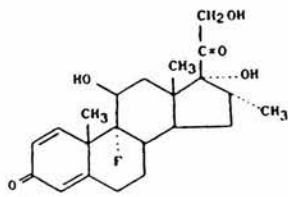
Most compounds were administered intraperitoneally. The exceptions were ethanol which was given in the drinking water, carbon tetrachloride, which was given sub-cutaneously, and 2-AAF which was administered

TABLE 5.1  
COMPOUNDS USED TO TREAT WISTAR RATS AND INDUCTION PROTOCOL

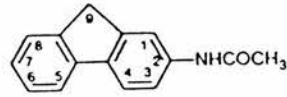
TREATMENT	ROUTE*	DOSE	DAYS	VECTOR
<u>P450 inducer</u>				
Corn oil/PBS	ip	-	3	-
Dexamethasone	ip	100 mg/kg	3	corn oil
Aroclor 1254	ip	500 mg/kg	1	corn oil
Ethanol	drinking	1%	10	
3-methylcholanthrene	ip	100 mg/kg	3	corn oil
Clofibric acid	ip	200 mg/kg	4	corn oil
$\alpha$ -Naphthaflavone	ip	80 mg/kg	3	corn oil
$\beta$ -Naphthaflavone	ip	80 mg/kg	3	corn oil
Pyrazole	ip	200 mg/kg	3	PBS
<u>Hepatotoxins</u>				
Carbon tetrachloride	sc	1:1 (0.3 ml)	3	corn oil
4-Acetamidophenol	ip	300 mg/kg	3	corn oil
Lead Nitrate	ip	0.1 mM/kg	3	PBS
<u>Carcinogens</u>				
Aflatoxin B1	ip	0.2 mg/kg	1	corn oil
4-Nitroquinoline oxide	ip	50 mg/kg	3	PBS
2-Acetylaminofluorene	ig	50 mg/kg	4	corn oil
Diethylnitrosamine	ip	200 mg/kg	3	PBS

\* ip intraperitoneal; ig intragastric; sc subcutaneous

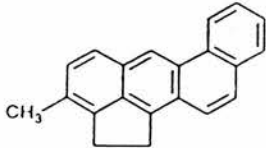
**FIGURE 5.1      STRUCTURES OF INDUCING COMPOUNDS**



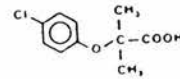
**Dexamethasone**



**2-acetylaminofluorene**



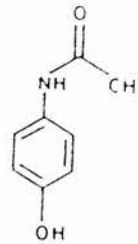
**3-methylcholanthrene**



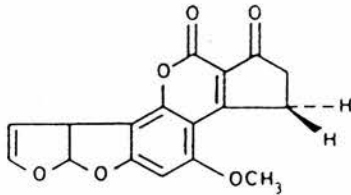
**clofibrate**



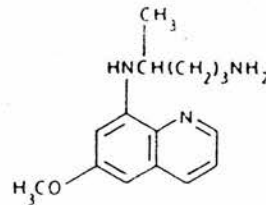
**pyrazole**



**4-acetaminophen**



**aflatoxin B<sub>1</sub>**



**4-nitroquinolenoxide**

intragastrically by means of a 100 mm plastic cannula, size 8 FG (International Market Supply, Cheshire) after grinding the compound with a mortar and pestle and resuspending it in corn-oil.

Both liver and kidney from Wistar rats were removed following treatment. Some was retained for histological examination and the remainder was washed briefly in PBS and frozen at  $-70^{\circ}\text{C}$  until RNA was prepared and cytosolic and microsomal protein fractionated. Only liver was removed from Fischer rats. No histological examination was performed on it.

#### 5.2.2 HISTOLOGICAL EXAMINATION OF 2-ACETYLAMINOFLUORENE-, AFLATOXIN B<sub>1</sub>- AND DIETHYLNITROSAMINE-TREATED WISTAR RAT LIVER AND KIDNEY

Liver and kidney were removed from Wistar rats following treatment with the compounds 2-AAF, AFB<sub>1</sub> and DEN. Pieces of these organs were washed briefly in PBS and prepared for histological examination as described in Section 2.3.

Both the liver and kidney tissues from all three treatments and the control were stained with hematoxylin and eosin (H/E). Such staining shows the morphology of the tissue. Glycogen distribution was also examined using the PAS method as described in Section 2.3.2.

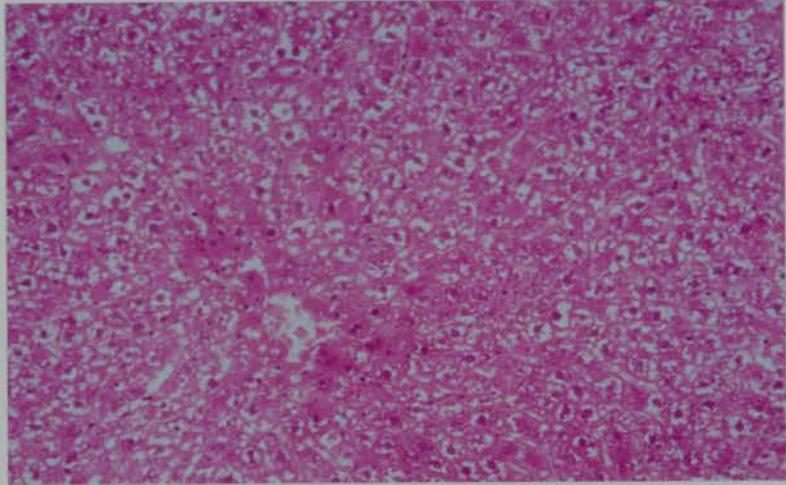
The results of the H/E staining is shown in Figure 5.2 and 5.3. Necrotic areas are clearly visible around the central vein and in zone 3 of the DEN treated liver but the 2-AAF and AFB<sub>1</sub> treated livers appear apparently normal in comparison. Such a result for a potent hepatotoxin such as DEN is expected. Figure 5.3 depicts the results of the treatments on the rat kidney. Some renal tissue damage is occurring for all three treatments but is much more readily apparent in the DEN treated kidney. Glycogen distribution in the treated livers is shown in Figure 5.4. Widespread glycogen storage, which is found in normal liver, is absent in the DEN treated liver. Following DEN treatment, glycogen storage is greatly diminished compared to 2-AAF and AFB<sub>1</sub> treated livers, which resemble the normal state of diffuse glycogen distribution.

**FIGURE 5.2**

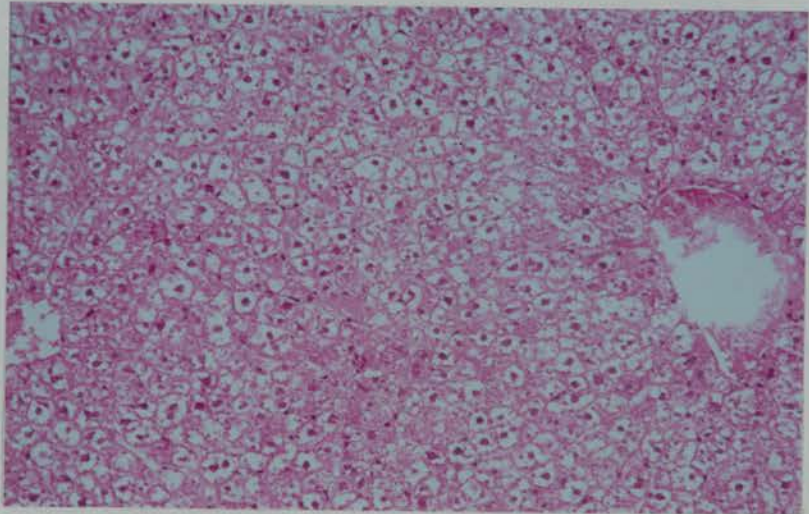
**H/E STAINING OF CONTROL AND TREATED WISTAR RAT LIVER**

Wistar rats were treated with 2-acetylaminofluorene, aflatoxin B1 and diethylnitrosamine, as described in Table 5.1, and the livers removed following treatment and sectioned. Hematoxylin and eosin staining was performed, as described in Section 5.2.2.

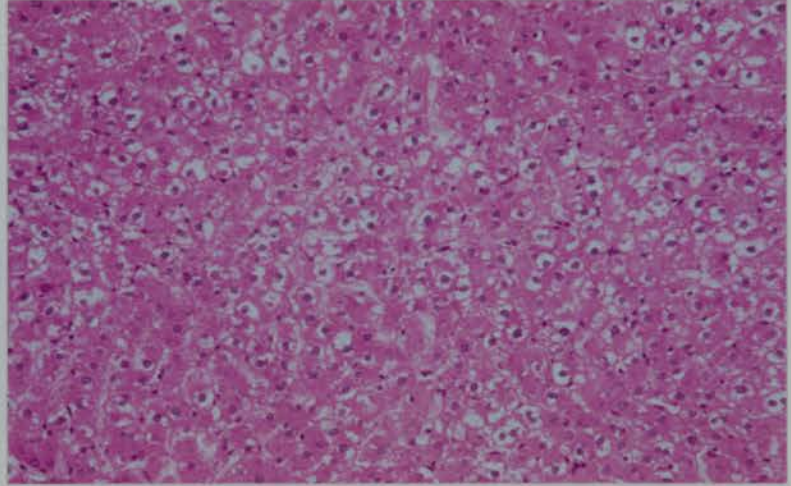
Control  
untreated  
(x 10 magnification)



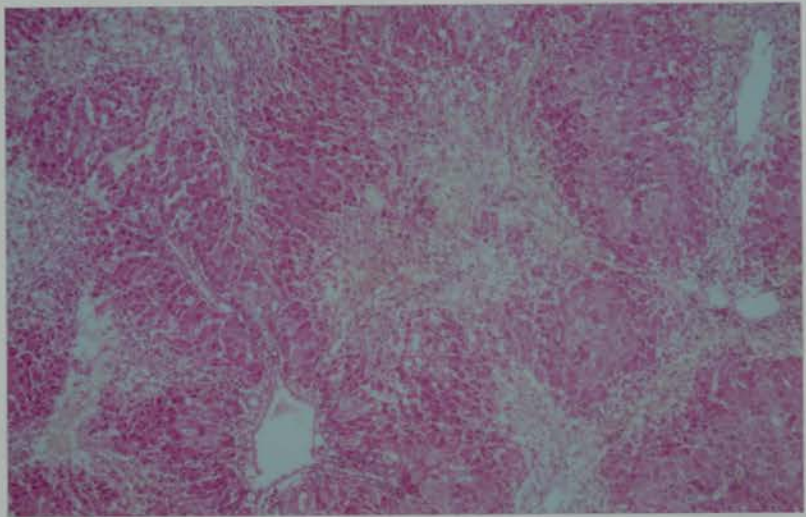
2-AAF treated  
(x 10 magnification)



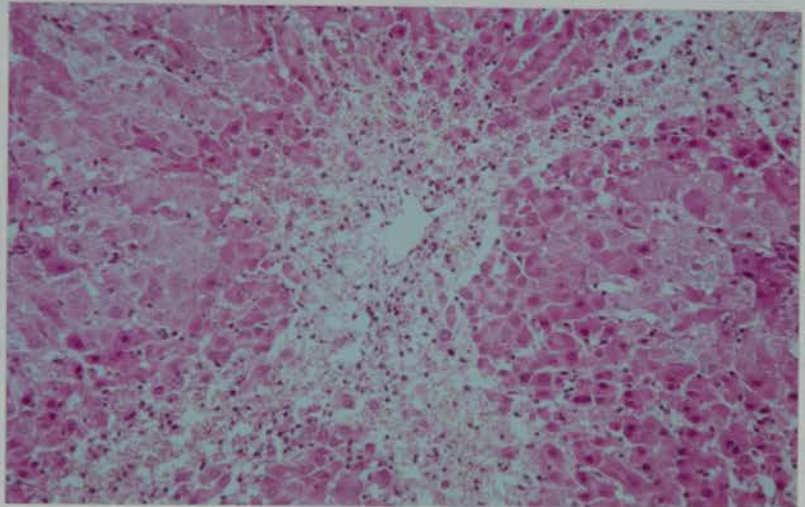
AFB<sub>1</sub> treated  
(x 10 magnification)



DEN treated  
(x 4 magnification)

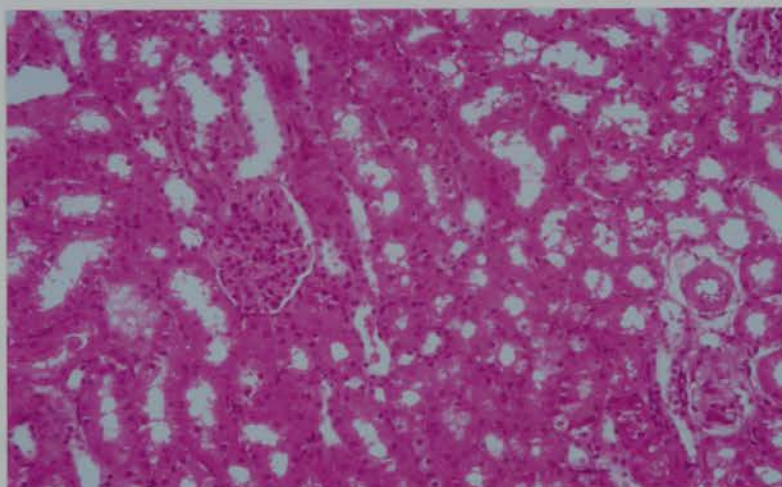


DEN treated  
(x 10 magnification)

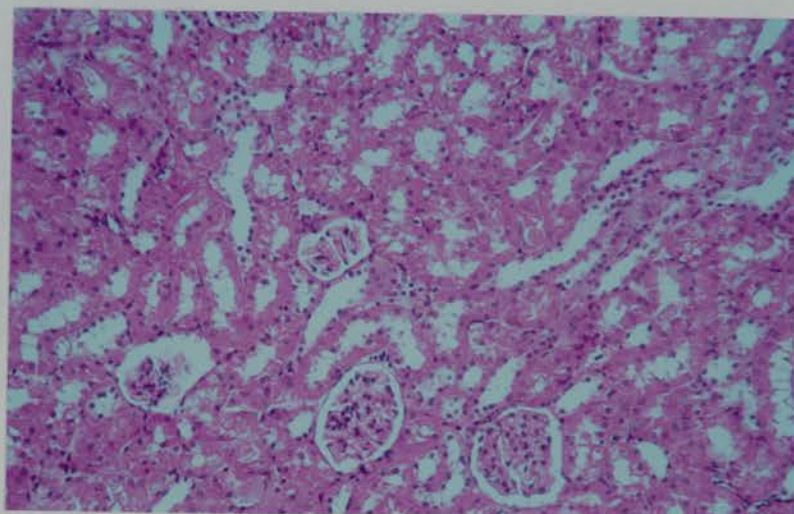


**FIGURE 5.3**  
**H/E STAINING OF CONTROL AND TREATED WISTAR RAT**  
**KIDNEY**

Wistar rats were treated with 2-acetylaminofluorene, aflatoxin B1 and diethylnitrosamine, as described in Table 5.1, and the kidneys removed following treatment and sectioned. Hematoxylin and eosin staining was performed, as described in Section 5.2.2.

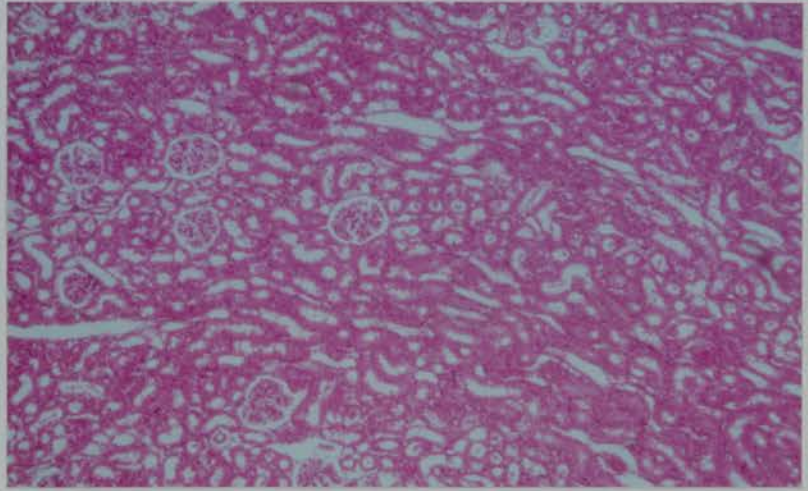


Control untreated  
(x 10 magnification)

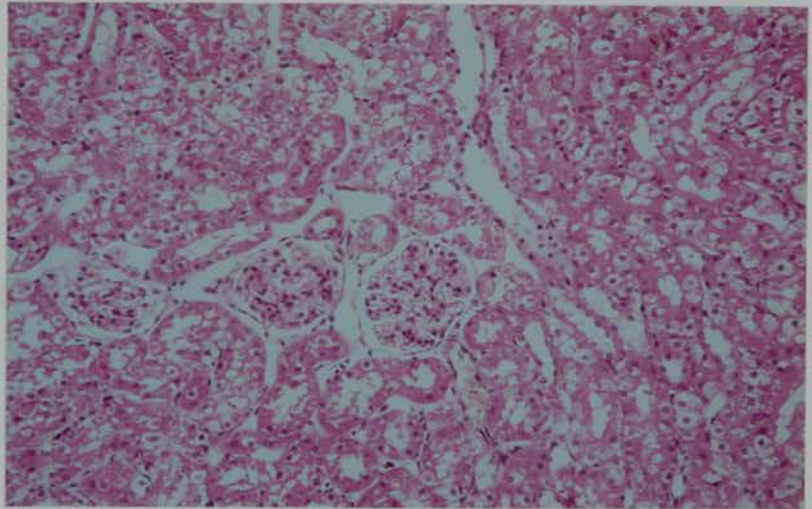


2-AAF untreated  
(x 10 magnification)

AFB<sub>1</sub> treated  
(x 4 magnification)

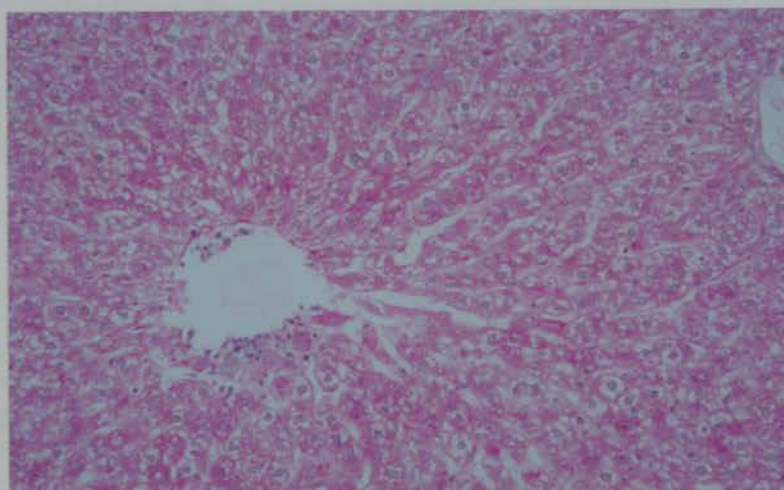


DEN treated  
(x 10 magnification)

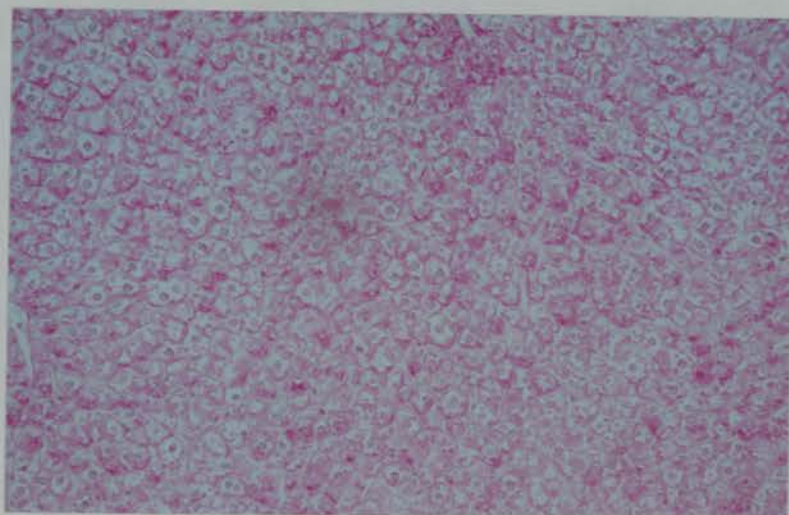


**FIGURE 5.4**  
**GLYCOGEN DISTRIBUTION IN CONTROL AND TREATED**  
**WISTAR RAT LIVER**

Wistar rats were treated with 2-acetylaminofluorene, aflatoxin B1 and diethylnitrosamine, as described in Table 5.1, and the livers removed following treatment and sectioned. Glycogen distribution was assessed using the Periodic Acid Schiff reaction, as described in Section 2.3.2.

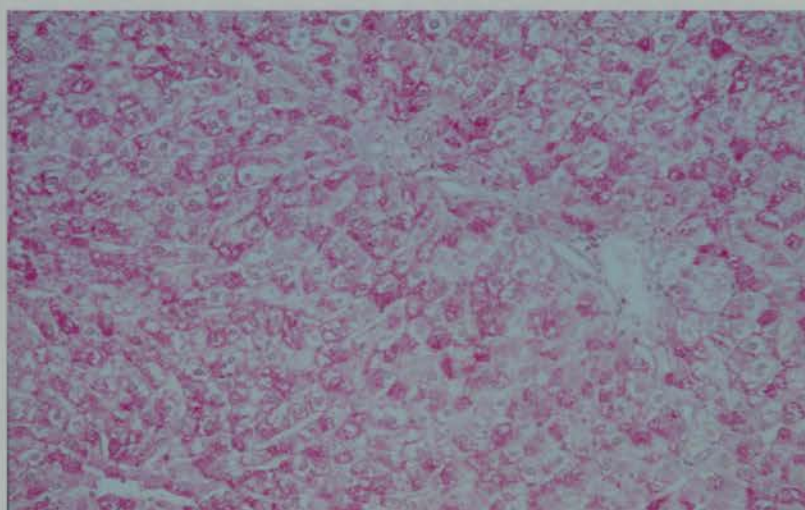


Control untreated  
(x 10 magnification)



2-AAF treated  
(x 10 magnification)

AFB<sub>1</sub> treated  
(x 10 magnification)



DEN treated  
(x 10 magnification)

### 5.2.3 ANALYSIS OF RAT MDR EXPRESSION FOLLOWING XENOBIOTIC TREATMENT

From each of the Wistar and Fischer rat treatment groups described, total RNA was extracted and microsomes prepared from pooled livers. Cytosolic fractions were also extracted from the Wistar rats. Actin controls were run on the RNA samples as well as comparing intensities of ethidium bromide staining to check for equal loading on Northern blots.

SDS PAGE gels were run on the cytosolic and microsomal fractions to check for equal loading. Immunoblotting was then performed using antibodies raised against P450 and GST proteins (Section 2.5.2). The monoclonal C219 was used to immunoblot P-glycoprotein (Section 2.5.3).

#### (a) NORTHERN BLOT ANALYSIS

Northern blots were performed on 15µg of each RNA sample. The human *mdr 1* gene cDNA probe, MDR5A, was used to detect the *mdr* transcript in treated rat livers. This clone encompasses the middle third of the human cDNA (Fojo *et al*, 1987) and has been previously used in Northern blot analysis against cultured rat cells treated with different chemotherapeutic drugs (Chin *et al*, 1990) and in rats following partial hepatectomy (Marino *et al*, 1990). Other cDNA probes such as the human cDNA probe pADR-1, isolated from Adr<sup>R</sup>MCF7 cells, have also been used to probe rat liver treated in various ways (Fairchild *et al*, 1987; Burt and Thorgeirsson, 1988).

The use of such probes was necessary since no rat probes were available at that time. Recently, clones have been identified which encode the rat homologue of the mouse *mdr 2* gene and a full length gene of 4253 bp which is 90% homologous to the mouse *mdr 1* gene.

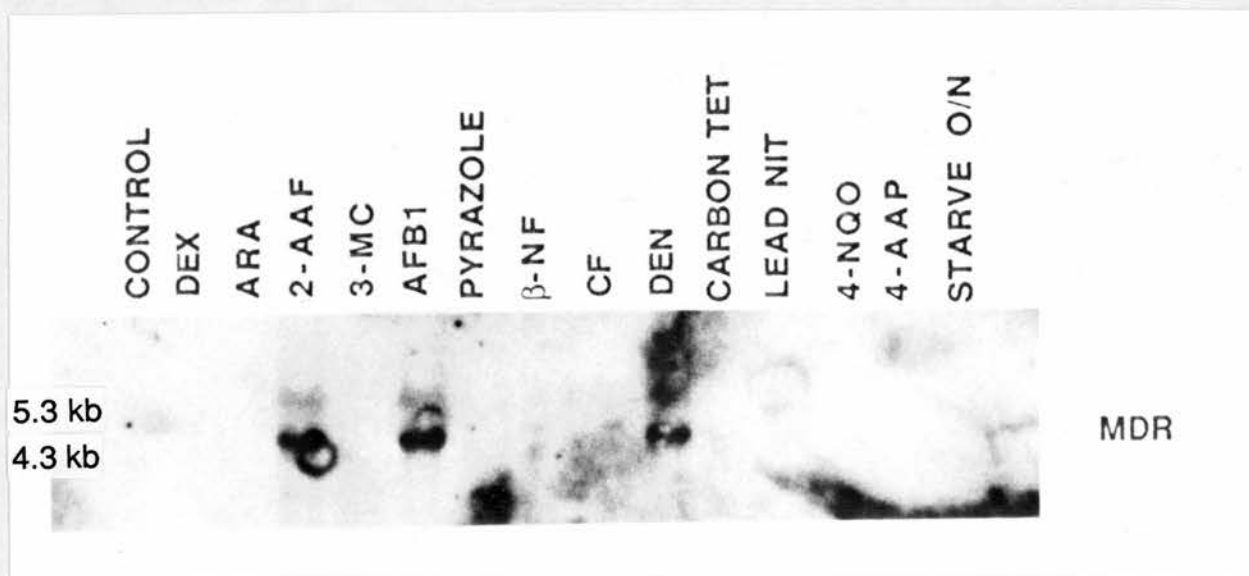
The MDR5A probe (hybridised at a final stringency of 2xSSC) detected two transcripts in the Wistar rat liver treated with 2-AAF, AFB<sub>1</sub> and DEN as shown in Figure 5.5. The transcript sizes were calculated to be approximately 5.3 and 4.3 kb. However, the 4.3 kb transcript was expressed at a much greater level than the 5.3 kb transcript. No other treatment induced

mdr mRNA levels above control, which was in fact undetectable using this system. Since it is known that a high degree of homology exists between the rodent mdr genes, as shown in Table 5.2, it could be speculated that these rat gene transcripts have equivalent mouse homologs which are also induced by similar compounds.

**FIGURE 5.5 EXPRESSION OF MDR 1 IN TREATED WISTAR RAT LIVER**

Wistar rats were treated, as described in Table 5.1, and the liver RNA (15 µg of each treated liver) probed with the mdr 1 human probe MDR5A, as described in Section 2.11.

DEX	dexamethasone	CF	clofibrate
ARA	arachlor	DEN	diethylnitrosamine
2-AAF	2-acetylaminofluorene	carbon tet	carbon tetrachloride
3-MC	3-methylcholanthrene	lead nit	lead nitrate
AFB1	aflatoxin B1	4-NQO	4-nitroquinoleneoxide
PYZ	pyrazole	4-AAP	4-acetaminophen
β-NF	β-naphthaflavone	stave o/n	starve overnight



Liver from each rat was kept separate and Northern blots were done using the livers from each individual treated rat. This blot represents the results obtained from one of the two rats from each treatment group. The results from the duplicates in each group were identical.

In all three cases where the induction of mdr in rat occurs, both transcripts

are induced together above control levels. This suggests a co-induction of both genes by a common regulatory element activated by such compounds.

**TABLE 5.2 PERCENTAGE IDENTITY OF RAT, HAMSTER, MOUSE AND HUMAN MDR SEQUENCES**

	HUM MDR 1	HUM MDR 2	MS MDR 3	MS MDR 1	MS MDR 2	HAM MDR 1a	HAM MDR 1b
RAT MDR 1b	79.1	71.5	82.5	90.4	69.6	78.1	80.4
HAM MDR 1b	75.7	47.7	76.8	83.4	77.9	82.0	
HAM MDR 1a	83.5	64.2	89.3	77.9	65.0		
MS MDR 2	71.1	86.1	71.1	69.4			
MS MDR 1	78.7	70.6	84.2				
MS MDR 3	82.2	71.6					
HUM MDR 2	74.9						

Adapted from Silverman *et al* (1991b)

Chin *et al* (1990) employed the same probe and conditions of



specificity of the probe for a particular gene has been proven.

Fischer rats treated with AFB<sub>1</sub> and 2-AAF also demonstrated raised hepatic mdr levels, as shown in Figure 5.6.

Again, two transcripts of 5.2 and 4.3 kb were co-induced, but the 4.3 kb transcript was induced to a much greater extent. 3-MC failed to cause any change to mdr levels in these rats. AFB<sub>1</sub> had a greater effect (~2-fold) in these Fischer rats than the Wistar rats. Induction of mdr by 2-AAF was maximal after 3 days of treatment as opposed to 4 days .

## (b) IMMUNOBLOTTING ANALYSIS

Microsomal fractions for immunoblotting were prepared from both Wistar and Fischer rat liver. C219 monoclonal antibody was used to detect P-glycoprotein in these samples. This antibody has been used in both immunoblotting of rat canalicular membrane vesicles (Thiebaut *et al*, 1987; Kamimoto *et al*, 1989) and in immunohistochemical detection of P-glycoprotein in rat hepatocellular carcinomas (Volm *et al*, 1990). The transcript size detected in the liver membrane vesicles was ~160 kD which was smaller than the 170 kD CH<sup>R</sup>C5 cell line P-gp standard, used in the study (Kamimoto *et al*, 1989).

The immunoreactivity of C219 with the rat membrane vesicles prepared from the Wistar rat livers, treated with different xenobiotics, is shown in Figure 5.7.

Table 5.3 shows the relative expression of P-gp, following each treatment, as defined by high, moderate or slight induction.

The molecular weight of the protein detected by C219 was calculated and found to be 150 kD. Dex also induced a lower protein of 60 kd not detected by any other treatment. The band of 150 kD on the immunoblot appeared to represent two reactive proteins migrating very closely together.

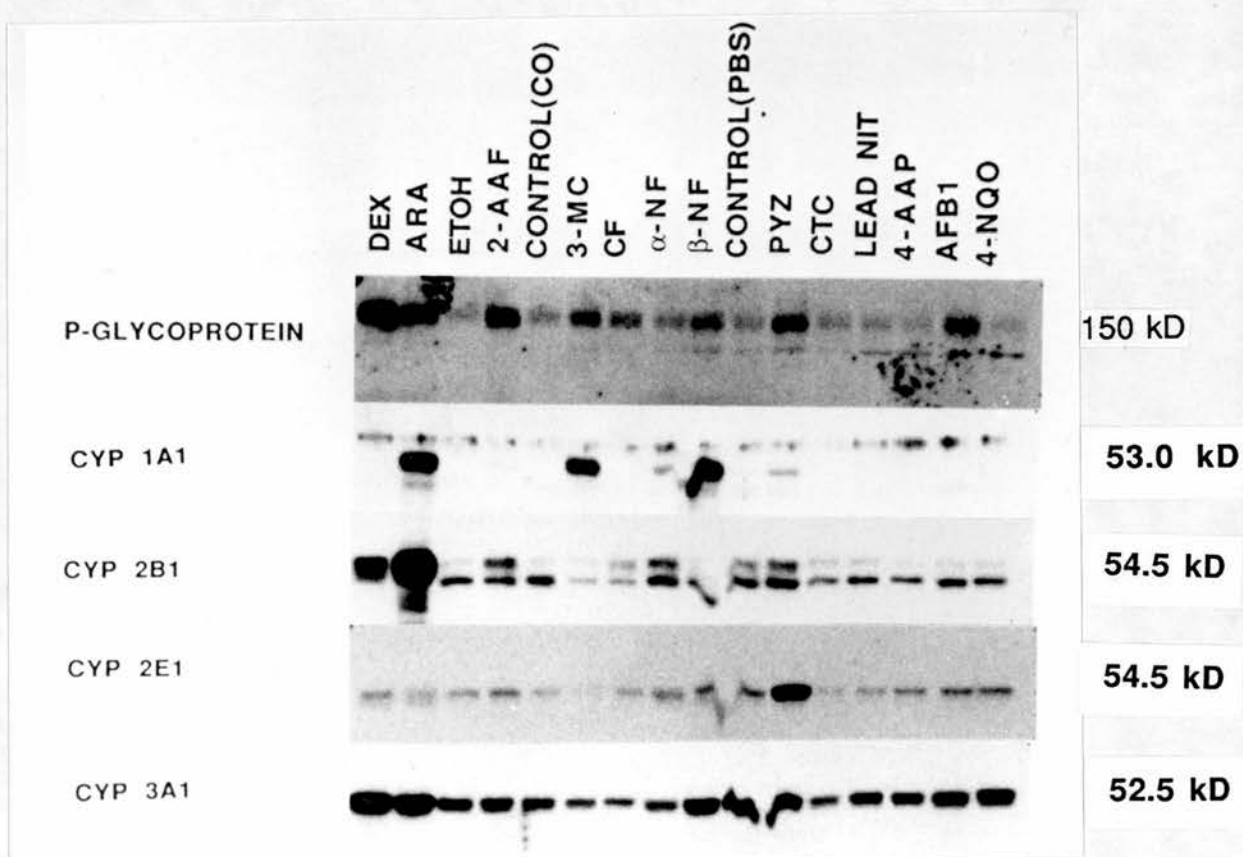
C219 does not distinguish between the various classes of P-gp and will therefore detect all the rat mdr transcript products, which contain the highly conserved epitope VQAALD.

From Figure 5.7, it can be seen that Dex, Ara, 2-AAF and pyrazole induced the expression of P-gp in Wistar rat liver to a high extent. 3-MC and

## FIGURE 5.7 EXPRESSION OF P-GLYCOPROTEIN AND CYP1A1, CYP2B1, CYP2E1 AND CYP3A1 IN WISTAR RAT LIVER TREATED WITH VARIOUS COMPOUNDS

Wistar rats were treated, as described in Table 5.1, and the liver microsomal protein was Western blotted using the monoclonal antibody C219 and cytochrome P450 polyclonal antibodies.

DEX	dexamethasone	PYZ	pyrazole
ARA	Araclor	CTC	carbon tetrachloride
ETOH	ethanol	LEAD NIT	lead nitrate
2-AAF	2-acetylaminofluorene	4-AAP	4-acetaminophen
CO	corn-oil	AFB1	aflatoxin B1
3-MC	3-methylcholanthrene	4-NQO	4-nitroquinoline N oxide
CF	clofibric acid		
$\alpha/\beta$ -NF	$\alpha/\beta$ -naphthoflavone		
PBS	phosphate buffered saline		



Livers from each treatment group (2 rats per group) were pooled. This blot represents the results obtained from one experiment.

**TABLE 5.3 INDUCTION OF P450 ENZYMES AND P-GLYCOPROTEIN IN WISTAR RAT LIVER**

Chem	P450 FAMILY				PGP
	1A1	2B1	2E	3A1	
Dex	NC	++	NC	+++	+++
Ara	+++	+++	NC	+++	+++
Eth	NC	NC	NC	NC	NC
2-AAF	NC	++	NC	NC	+++
3-MC	+++	NC	NC	-	++
Clo	NC	NC	NC	-	+
$\alpha$ -NF	NC	++	NC	NC	NC
$\beta$ -NF	+++	NC	NC	++	+
Pyz	+	+	+++	NC	+++
Car Tet	NC	NC	NC	-	NC
Lead Nit	NC	NC	NC	NC	NC
4-AAP	NC	NC	NC	NC	NC
AFB <sub>1</sub>	NC	NC	NC	+	++
4-NQO	NC	NC	NC	+	NC

+++ High induction  
 ++ Moderate  
 + Slight  
 - Suppression  
 NC No change

AFB<sub>1</sub> both induced P-gp to a moderate level. Clofibrate and  $\beta$ -naphthoflavone ( $\beta$ -NF) induced the expression of P-gp only slightly above control levels. Both corn-oil and PBS control rat liver cross-reacted with the C219 antibody to show the presence of P-gp in these samples occurred to the same extent, but were themselves not inducing the level of protein to any

extent. The induction of P-gp by certain compounds is occurring post-transcriptionally. Although 2-AAF and AFB<sub>1</sub> caused an increase in both mRNA and protein, the other P-gp inducers failed to increase the level of mRNA for the rat *mdr* genes.

### FIGURE 5.8 EXPRESSION OF P-GLYCOPROTEIN IN TREATED FISCHER RAT LIVER

Fischer rats were treated, as described in Section 5.2.1, and the liver microsomal protein was Western blotted using the monoclonal antibody C219.

3-MC 3-methylcholanthrene  
2-AAF 2-acetylaminofluorene  
AFB<sub>1</sub> aflatoxin B<sub>1</sub>

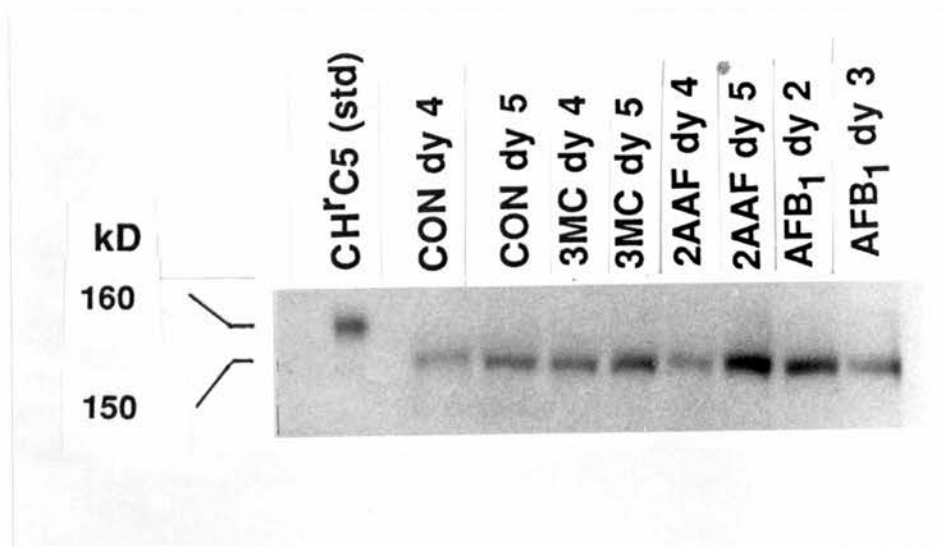


Figure 5.8 shows the result of blotting the Fischer rat liver microsomes treated with 3-MC, 2-AAF and AFB<sub>1</sub> with the C219 antibody.

A membrane fraction of CHRC5 (50 µg), the mdr chinese hamster cell line, was also examined to compare the relative sizes of the immunoreactive protein identified. The molecular weights of the proteins recognised by C219 against CHRC5 and the rat liver microsomes were 160 kD and 150 kD respectively.

Both 2-AAF, at day 5, and AFB<sub>1</sub>, at day 2, did induce P-gp expression significantly above controls.

Figure 5.6 shows that 3-MC did not cause an increase in P-gp mRNA in Fischer rat liver and this is reflected in the immunoblot results (Figure 5.8).

#### 5.2.4 ANALYSIS OF RAT CYTOCHROME P450 AND GST EXPRESSION FOLLOWING XENOBIOTIC TREATMENT

##### (a) NORTHERN BLOT ANALYSIS

The cDNA probes described in Section 2.7 were used to determine the mRNA levels in the Wistar and Fischer rat livers. The *CYP2B* and *CYP4A* cDNA clones used were of rat origin. *CYP2A* and *CYP3A* were human cDNA clones. Human *CYP2A7* displays 85% homology to the rat *CYP2A3*. The *CYP2C* cDNA is of mouse origin but is also highly homologous to the rat sequence.

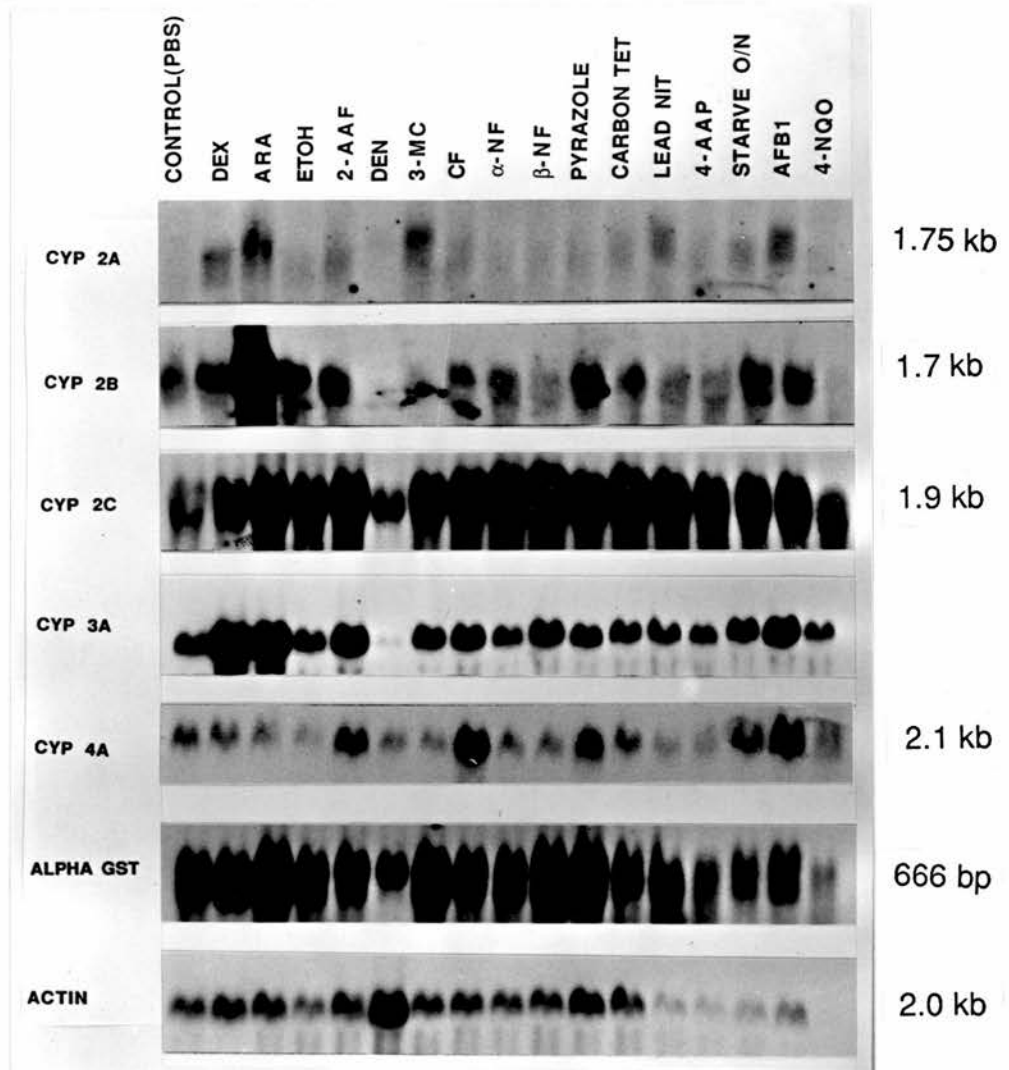
The effect of various xenobiotic treatments on hepatic cytochrome P450 and GSTα mRNA levels in Wistar rats is shown in Figure 5.9. Table 5.4 summarises the results of Figure 5.5 and 5.9.

The actin mRNA levels were assessed and DEN treatment appeared to increase actin levels substantially. The reason for this may be due to the necrotic effect of acute administration of DEN on the liver tissue. Liver regeneration can be induced by any acute treatment, surgical or chemical, that removes or kills hepatic parenchyma. Loss of parenchyma induces cell proliferation to restore the total mass of the liver (Michalopoulos, 1990). Several chemical treatments including DEN and carbon tetrachloride cause necrosis around the central zone of the hepatic lobule. mRNA levels for

**FIGURE 5.9 EXPRESSION OF CYTOCHROME P450s,  
 $\alpha$ -GST AND ACTIN mRNA IN TREATED WISTAR RAT LIVER.**

Wistar rats were treated as described in Table 5.1. RNA was extracted from each individual rat and Northern blotted using cDNA probes for CYP2A, CYP2B, CYP2C, CYP3A, CYP4A, GST  $\alpha$  and actin, as described in Section 2.11.

PBS	phosphate buffered saline	$\alpha/\beta$ NF	$\alpha/\beta$ naphthaflavone
DEX	dexamethasone	CARBON TET	carbon tetrachloride
ARA	Araclor	LEAD NIT	lead nitrate
EtOH	ethanol	4-AAP	4-acetaminophen
2-AAF	2-acetylaminofluorene	STARVE O/N	starve overnight
DEN	diethylnitrosamine	AFB <sub>1</sub>	aflatoxin B <sub>1</sub>
3-MC	3-methylcholanthrene	4-NQO	4-nitroquinolene oxide
CF	clofibrate		



Livers from each treatment group (2 rats per group) were pooled. This blot represents the results obtained from one experiment.

**TABLE 5.4****EFFECT OF XENOBIOTIC TREATMENT ON P450,  $\alpha$ -GST, MDR AND ACTIN HEPATIC mRNA LEVELS IN WISTAR RATS**

CHEMICAL	P450 families					GST- $\alpha$	MDR	ACTIN
	2A	2B	2C	3A	4A			
Dex	+	++	++	+++	NC	NC	NC	NC
Ara	+	+++	+++	+++	NC	++	NC	NC
Ethanol	NC	++	+++	NC	NC	NC	NC	NC
2-AAF	+	++	+++	++	+	NC	+++	NC
DEN	NC	-	NC	-	NC	-	+++	+++
3-MC	+	NC	+++	NC	NC	NC	NC	NC
Clofibrate	NC	NC	++	+	+++	++	NC	NC
$\alpha$ -NF	NC	NC	+++	NC	NC	NC	NC	NC
$\beta$ -NF	NC	NC	+++	+	NC	++	NC	NC
Pyrazole		++	+++		+	++	NC	+
Car. Tet.	NC	NC	+++	NC	NC	NC	NC	+
Lead Nitrate	+	NC	+++	NC	NC	NC	NC	NC
4-AAP	NC	NC	+++	NC	NC	-	NC	-
Starve o/n	+	++	+++	NC	NC	-	NC	-
AFB <sub>1</sub>	+	++	+++	++	++	-	+++	-
4-NQO		-	++	NC	NC	-	NC	-

+++ High induction  
 ++ Moderate  
 + Slight  
 - Suppression  
 NC No change

certain proteins such as actin,  $\alpha$ - and  $\beta$ -tubulin increase during liver regeneration 12 -72 hours after partial hepatectomy (Friedman *et al*, 1984). From microtome-cut sections of DEN treated Wistar rat liver it was observed

that such necrosis had in fact occurred in zone 3 and around the central vein, as shown in Section 5.2.2. However, 2-AAF and AFB<sub>1</sub> treated liver showed no such necrotic areas. No increase in actin mRNA was observed for 2-AAF or AFB<sub>1</sub> (Figure 5.9).

Messenger RNA encoding the major cytochrome P450 families in rat liver were determined, as shown in Figure 5.9, and found to be induced with various treatments. CYP2A1 and CYP2A2 are only just detectable in the liver (Matsuaga *et al*, 1988). CYP2A mRNA is induced by 3-MC as shown in Figure 5.9. Dex, Ara, 2-AAF, lead nitrate and AFB<sub>1</sub> also slightly induced the level of CYP2A mRNA. Whether such induction represents CYP2A1 or CYP2A2 is not clear from the probe used. Rat CYP2A3 is expressed only in rat lung but is 71% and 73% homologous to CYP2A1 and CYP2A2 gene expression cannot be determined from the CYP2A3 probe used.

The P450 2B sub-family mRNA was measured using the rat CYP2B1 full length cDNA clone. Rat CYP2B1 and CYP2B2 exhibit 97% amino acid homology (Atchison and Adesnik, 1983) and have similar substrate specificities. The two genes are coordinately regulated in liver by phenobarbital (PB) due to an increased gene transcription rate (Atchison and Adesnik, 1983). CYP2B2 is constitutively expressed whereas CYP2B1 is transcriptionally inactivated in untreated rats. CYP2B3 shares 77% homology to CYP2B1 and CYP2B2, and is constitutively expressed as a minor form in rat liver (Labbe *et al*, 1988). Figure 5.9 shows the induction of CYP2B by Ara to a high extent. Dex and ethanol also induce CYP2B to a moderate extent. 2-AAF, pyrazole, starvation overnight and AFB<sub>1</sub> induced CYP2B above control levels. DEN reduced CYP2B levels below control values.

CYP2C genes are in most cases constitutively expressed. Two members of the rat CYP2C family, designated CYP2C6 and CYP2C7 have been isolated and sequenced (Haniu *et al*, 1984) and have 75% amino acid similarity to each other. CYP2C11 is an adult male-specific form and is 70% similar to the sequence of rat CYP2C6 (Yoshioka *et al*, 1987). Rat CYP2C12 and rat CYP2C13 have also been described.

The complexity of the rat CYP2C sub-families means that it is not possible to exclude the possibility that the mouse Cyp2c probe used to blot

rat CYP2C RNA levels are detecting more than one member of the CYP2C sub-family.

A human cDNA CYP3A4 clone was used to probe the rat livers. Humans have at least four CYP3 genes all of which having 90% or greater similarity to each other. A cytochrome P450 inducible by the synthetic steroid, pregnenolone 16 $\alpha$ -carbonitrile (PCN) was purified from rat liver (Elshourbagy and Guzelian, 1980) and was identified as a cytochrome P450 sub-family 3 gene. Both rat *CYP3A1* and *CYP3A2* have now been isolated and share 90% nucleotide sequence identity. Rat *CYP3A1* is inducible by glucocorticoids such as Dex due to increased transcription rates (Simmons et al, 1987) but the regulation is distinct from glucocorticoid regulated genes such as tyrosine aminotransferase (Shuetz and Guzelian, 1984), since CYP3A1 induction requires approximately 10 fold higher levels of steroid to induce it.

Rat CYP3A was induced to a high extent by Dex and Ara. It was also induced by 2-AAF and AFB<sub>1</sub> to a moderate extent. DEN caused suppression of mRNA levels for CYP3A.

CYP4A mRNA levels are increased in clofibrate- and aflatoxin B<sub>1</sub>-treated rat livers. Clofibrate activation occurs within one hour of administration (Hardwick et al, 1987).

A wide range of chemicals, both natural and synthetic induce GST in the rat, including PAHs (Igarishi *et al*, 1987). GST  $\alpha$  (rat subunits 1 or 2) was induced by Ara, 3-MC,  $\beta$ -NF, pyrazole and substantially suppressed by DEN.

## (b) IMMUNOBLOT ANALYSIS

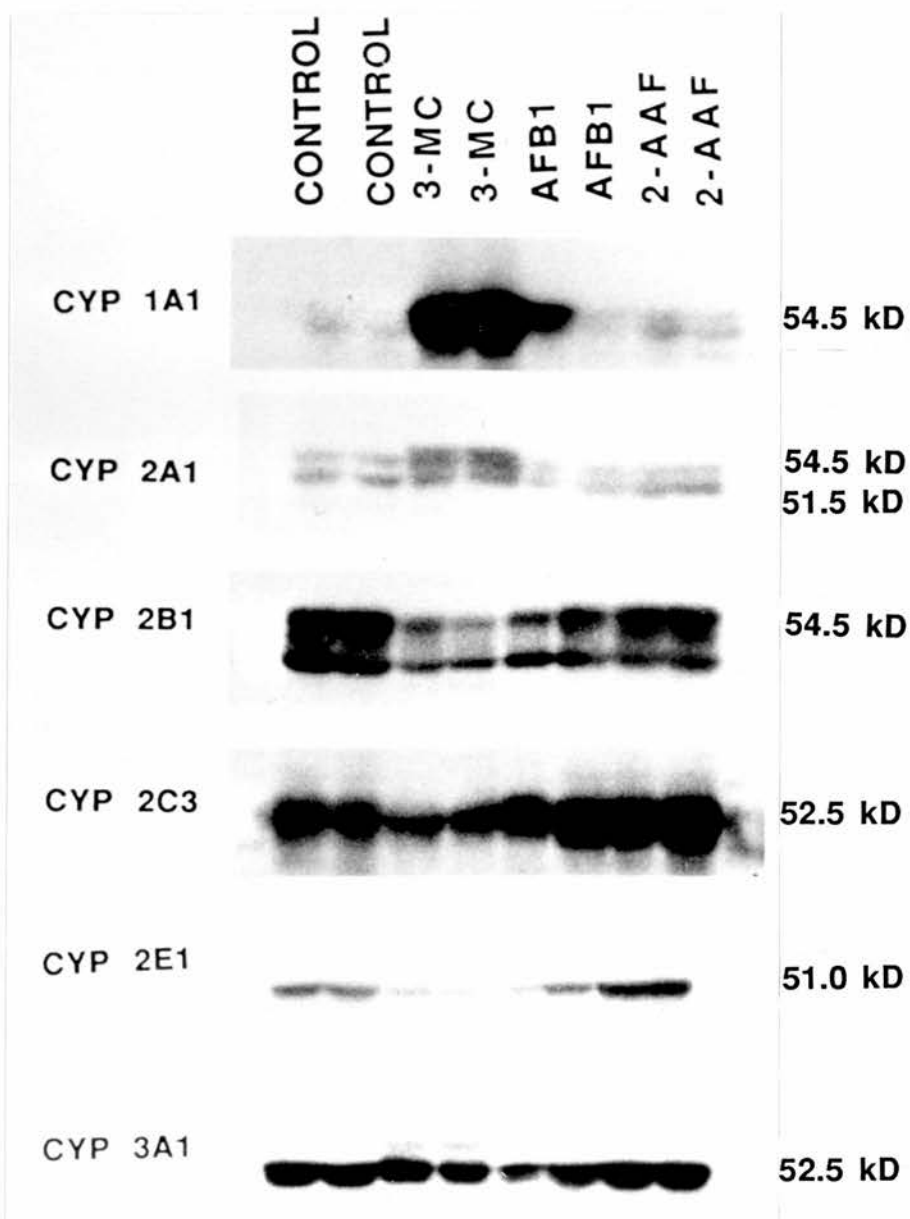
The results of the cytochrome P450 analysis for the Wistar rats is shown in Figure 5.7 and summarised in Table 5.3.

CYP1A1 is induced by Ara, 3-MC and  $\beta$ -NF. CYP2B1 protein is induced by Ara to the greatest extent but also by Dex, 2-AAF,  $\alpha$ -naphthoflavone ( $\alpha$ -NF) and pyrazole.  $\alpha$ -NF failed to cause an increase in the mRNA level of CYP2B (Figure 5.9), therefore the increase in CYP2B1 protein with  $\alpha$ -NF seems to be due to protein stabilisation or increased

**FIGURE 5.10 EXPRESSION OF VARIOUS P450s IN TREATED FISCHER RAT LIVER**

Fischer rats were treated, as described in Section 5.2.1, and the microsomal protein was Western blotted using polyclonal antibodies against cytochrome P450 isozymes as described in Section 2.6.2.

3-MC 3-methylcholanthrene  
 AFB1 aflatoxin B1  
 2-AAF 2-acetylaminofluorene



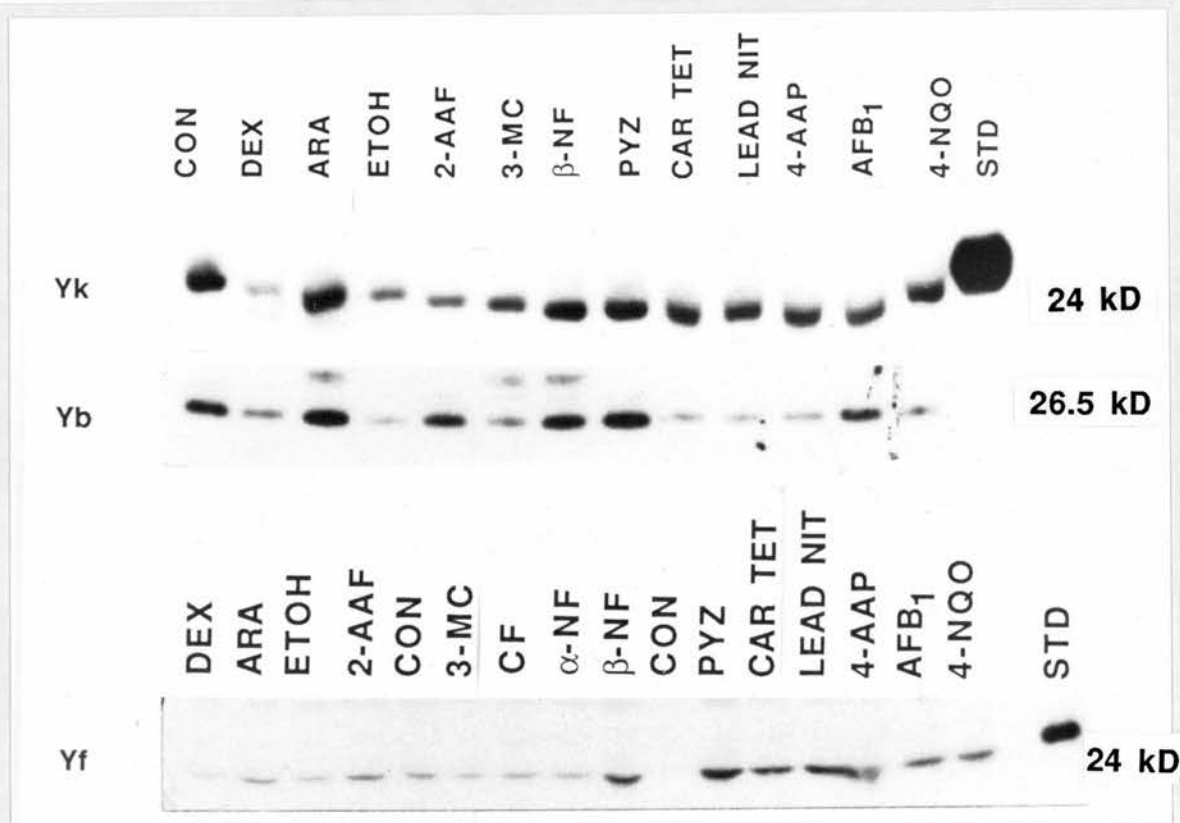
Livers from each treatment group (2 rats per group) were pooled. This blot represents the results obtained from one experiment.

**FIGURE 5.11 EXPRESSION OF Yk, Yb and Yf GST IN TREATED WISTAR RAT LIVER**

Wistar rats were treated, as described in Table 5.1, and the cytosolic protein was Western blotted using polyclonal antibodies against the glutathione S-transferases, as described in Section 2.6.2.

CON	control	CAR TET	carbon tetrachloride
DEX	dexamethasone	LEAD NIT	lead nitrate
ARA	Araclor	4-AAP	4-acetaminophen
ETOH	ethanol	AFB1	aflatoxin B1
2-AAF	2-acetylaminofluorene	4-NQO	4-nitroquinoleneoxide
3-MC	3-methylcholanthrene	STD	standard
$\beta$ -NF	$\beta$ -naphthoflavone		
PYZ	pyrazole		

The standards are purified proteins for each respective isozyme.



Livers from each treatment group (2 rats per group) were pooled. This blot represents the results obtained from one experiment.

transcription efficiency. CYP2E1 protein is induced by pyrazole but appears to be completely unaffected by dosing the rats with 1% ethanol for 10 days although the cytochrome is known to be inducible by such a regimen (Koop *et al*, 1982).

CYP3A1 induction by Dex, Ara,  $\beta$ -NF and AFB<sub>1</sub> at the mRNA level is also reflected at the protein level. However, the increased mRNA in 2-AAF treated rat is not reflected in the protein analysis, suggesting that increased translation of the message is not occurring.

Figure 5.10 shows the results of the P450 immunoblotting following treatment of adult male Fischer rats with 3-MC, 2-AAF and AFB<sub>1</sub>. 3-MC clearly induced CYP1A1 and CYP2A1 but suppressed CYP2B1, CYP2C3 and CYP2E1 in the Fischer rat liver. AFB<sub>1</sub> and 2-AAF induced CYP2C3 moderately.

Wistar rat cytosolic protein was immunoblotted with GST antibodies against the Yk, Yb and Yf subunits. As shown in Figure 5.11, Wistar rat Yk ( $\alpha$ - class) GST is suppressed by acute administration of Dex, ethanol, 2-AAF and 3-MC. Rat Yb ( $\mu$ -class) is suppressed by acute administration of Dex, ethanol, 3-MC, carbon tetrachloride, 4-AAP and 4-NQO. In contrast the negligible level of rat Yf ( $\pi$ -class) GST observed in control liver are induced to some extent by all the treatment compounds.

Although it has been shown that hepatic mdr levels in rodent tissue can be regulated, the specific genes affected or their role in drug resistance is not yet known.

In order to examine a more well defined rodent model, it was decided to examine the regulation of the hepatic mdr genes of mouse. All three gene sequences have been published and the involvement of these genes in the MDR phenotype has been elucidated.

### **5.3 XENOBIOTIC REGULATION OF MDR GENES IN MOUSE**

In order to study mdr gene expression in mouse liver and other tissues, cDNA probes specific for each gene was necessary. To establish the specificity of the cDNA probes being used, it was decided to probe liver and

pregnant uterus (day 16 of gestation) of C57BL/6 mice.

The *mdr 1* mouse gene is known to be localised to the luminal surface of the secretory epithelium of the uterus during pregnancy (Arceci *et al*, 1988). At day 16 of gestation, the mRNA is expressed at it's highest level. *Mdr 2* and *3* are not detected in this tissue.

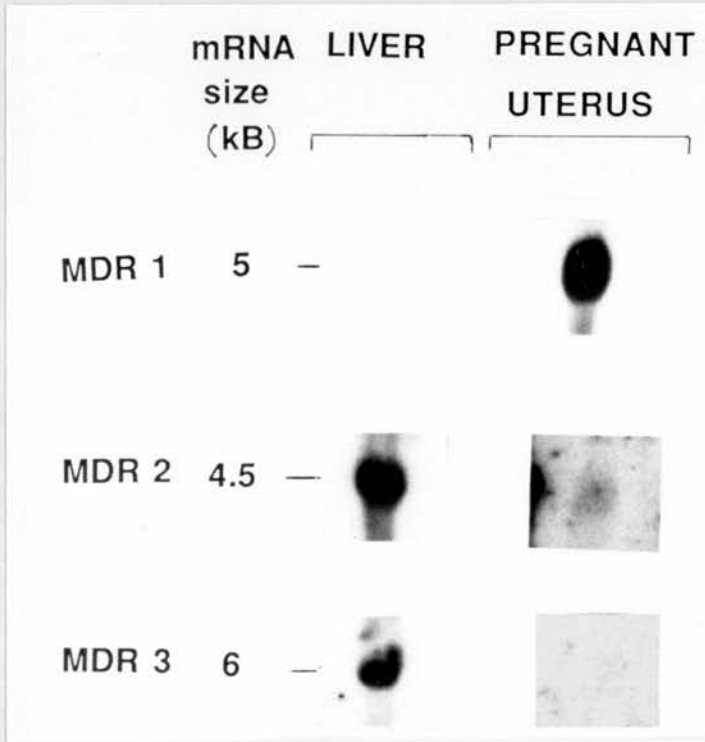
Croop *et al* (1989) reported the high level of expression of *mdr 2* in normal mouse liver with lower levels of *mdr 3* also expressed in this tissue. *Mdr 1*, *2* and *3* mRNA species are 5, 4.5 and 6 kb respectively. The distinct pattern of tissue-specific expression is shown in Table 5.5.

The actual cDNA probes used in this study are shorter than those used by Croop, although they cover the same region. The *mdr* probe used by Croop was a 424bp fragment of  $\lambda$ DR11 whereas a 165 bp fragment of  $\lambda$ DR11 was used in this study. The *mdr 3* probe used by Croop was 373 bp as opposed to 121 bp used in this study. The *mdr 2* cDNA probe was the same length as that used by Croop. The shorter probes distinguish between the three genes to a greater extent and have been used in hybridisation experiments to study the sub-chromosomal organisation of the mouse *mdr* gene complex and identify the degree of specific gene amplification and expression in a series of *mdr* cell lines (Raymond *et al*, 1990).

The specificity of the probes is shown in Figure 5.12.

**FIGURE 5.12      EXPRESSION OF MDR 1, MDR 2 AND MDR 3 IN LIVER AND PREGNANT UTERUS**

RNA (15  $\mu$ g) was prepared from C57BL/6 mouse liver and pregnant uterus (day 16 of gestation) and Northern blotted against *mdr 1*, *mdr 2* and *mdr 3* specific cDNAs, as described in Section 2.11.



**TABLE 5.5**  
**EXPRESSION OF MDR RNA IN VARIOUS MOUSE TISSUES**

TISSUE	mdr 1	mdr 2	mdr 3
Pregnant uterus	+++++	-	-
Uterus	-	-	-
Adrenal	++++	++	-
Kidney	++	-	+
Liver	+	++	+
Muscle	+	++	+
Spleen	+	++	+
Heart	++	++	++
Brain	+	-	++
Lung	+	-	++
Intestine	-	-	+++
Spinal cord	-	-	++
Testes	-	-	++
Stomach	-	-	+
Placenta	+++	-	-

Symbols represent expression compared with that of the 3T3 cell line.

- + less than one half of the level of expression
- ++ 1-10 fold
- +++ 10-20 fold
- ++++ 20-40 fold
- +++++ greater than 40 fold
- no detectable levels

Adapted from Croop *et al* (1989).

### 5.3.1 TREATMENT OF C57BL/6 AND DBA 2/N MICE WITH PB, DEX AND TCPOBOP

8 week old male and female mice of the C57BL/6 and DBA2/N strains (4 per group) were treated with the xenobiotic compounds phenobarbital (PB), dexamethasone (Dex) and TCPOBOP in order to determine whether known P450 inducers also affected *mdr* gene expression.

PB (in PBS) and Dex (in corn-oil) were administered i.p. at 80 mg/kg for three days and 100 mg/kg for four days respectively. Mice were sacrificed 24h after the final dose of treatment. One i.p. injection of TCPOBOP (3 mg/kg, in corn-oil) was administered and the animals killed four days after treatment. Control animals received corn-oil.

Following sacrifice, the livers were removed, rinsed in cold PBS and frozen rapidly on dry-ice before storage at -70°C.

The effect of TCPOBOP in DBA2/N male and female mice was studied over a period of 12 weeks following a single injection of the compound, as described above. Two mice of each sex were sacrificed at 4, 8 and 12 weeks following treatment.

### 5.3.2 ANALYSIS OF MOUSE MDR CONTENT FOLLOWING XENOBIOTIC TREATMENT AND COMPARISON WITH CYTOCHROME P450 AND GST $\pi$ EXPRESSION

#### (a) NORTHERN BLOT ANALYSIS

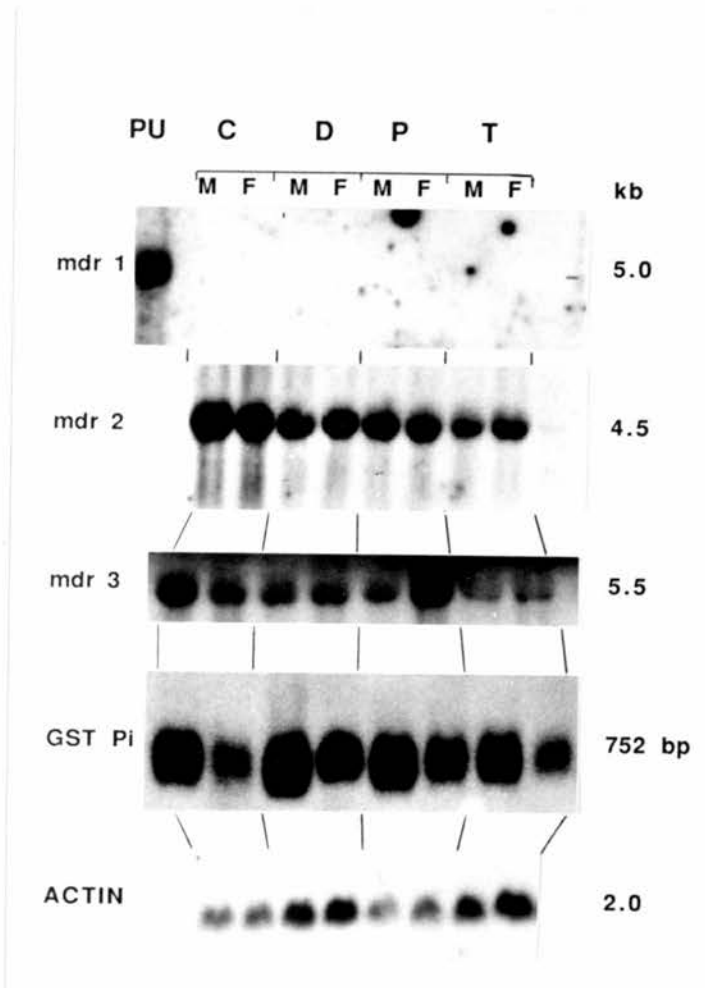
Northern blots were performed on 15 $\mu$ g of hepatic RNA from the mice treated as described in Section 5.3.1. The RNA was probed with the mouse *mdr* 1, 2 and 3 genes previously described. The C57BL/6 mouse RNA was also examined for *Cyp2b* and GST  $\pi$  mRNA expression.

The RNA samples were checked for equal loadings by staining with ethidium bromide and probing with actin. The results of the analysis following Dex, PB and TCPOBOP treatment of DBA2/N and C57BL/6 mouse liver are shown in Figures 5.13(a) and 5.14(a) respectively.

**FIGURE 5.13 (a) MDR 1, 2, 3 , GST Pi AND ACTIN  
mRNA LEVELS IN DBA2/N TREATED MICE**

DBA2/N mice were treated, as described in Section 5.3.1, and the liver RNA (15 µg per sample) was Northern blotted using *mdr 1*, *mdr 2*, *mdr 3*, glutathione S-transferase  $\pi$  and actin cDNAs, as described in Section 2.11.

PU	pregnant uterus	M	male
C	control	F	female
D	dexamethasone- treated		
P	phenobarbital- treated		
T	TCPOBOP- treated		

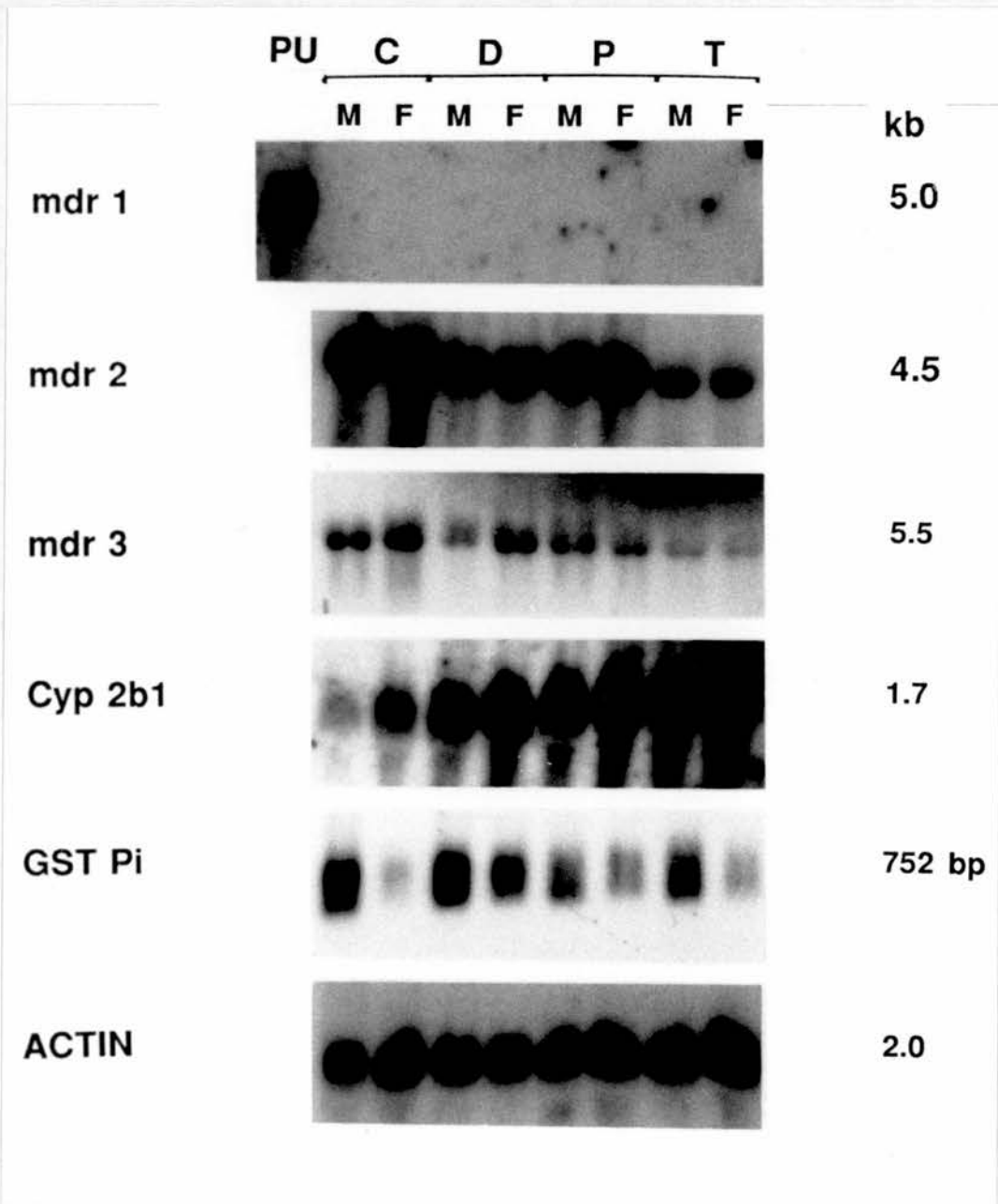


Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

**FIGURE 5.14 (a) MDR 1, 2, 3, Cyp2b1, GST Pi AND ACTIN mRNA LEVELS IN C57BL/6 TREATED MICE**

C57BL/6 mice were treated, as described in Section 5.3.1, and the liver RNA was Northern blotted using *mdr 1*, *mdr 2*, *mdr 3*, Cyp2b1, glutathione S-transferase  $\pi$  and actin cDNAs, as described in Section 2.11.

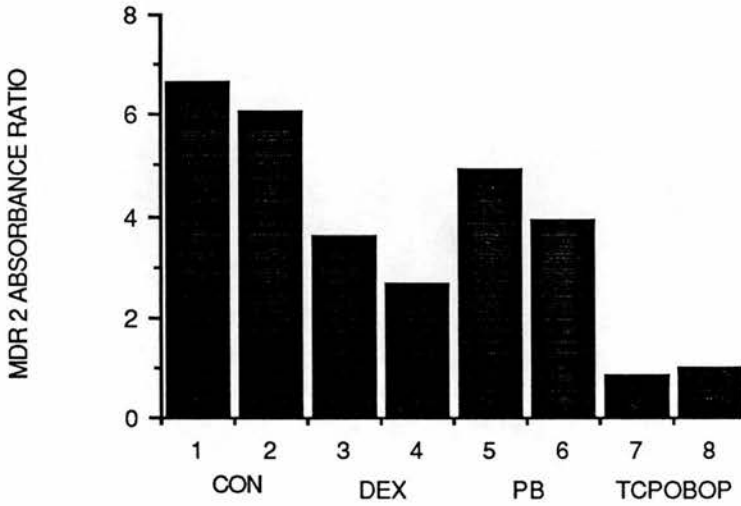
PU	pregnant uterus	T	TCPOBOP-treated
C	control	M	male
D	dexamethasone-treated	F	female
P	phenobarbital-treated		



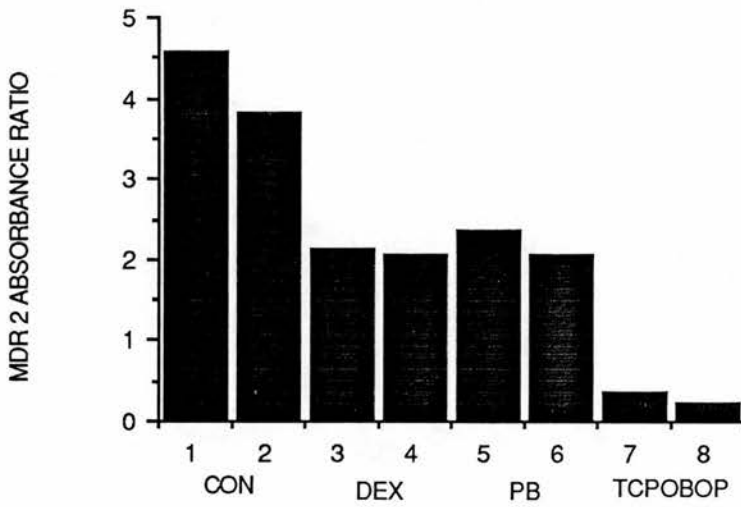
Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

**MDR 2 ABSORBANCE RATIO FOR DEXAMETHASONE-, PHENOBARBITAL- AND TCPOBOP-TREATED MICE.**

**FIGURE 5.13 (b) DBA2/N TREATED MICE**



**FIGURE 5.14 (b) C57BL/6 TREATED MICE**



Mdr 2 absorbance ratio: Ratio of the integrals of the optical densities of hybridisation signals on Northern blots probed with mdr 2 cDNA compared to actin cDNA.

1, 3, 5 and 7 represent male mice  
 2, 4, 6 and 8 represent female mice

con                    control  
 dex                    dexamethasone  
 PB                    phenobarbital  
 TCPOBOP            1,4-bis [2-(3,5-dichloropyridyloxy)] benzene

**CALCULATION OF THE ABSORBANCE RATIO (MDR 2 COMPARED TO ACTIN) FOR DBA2/N MICE**

**TABLE 5.6**

**DBA2/N MICE - MDR 2 LEVELS NORMALISED TO ACTIN**

SEX	TREATMENT	ACTIN	MDR 2	ABSORBANCE RATIO
M	CON	2471	23044	9.33
F	CON	3065	18668	6.09
M	DEX	4068	14757	3.63
F	DEX	5400	14430	2.67
M	PB	2783	13666	4.91
F	PB	3510	13838	3.94
M	TCPOBOP	8750	7384	0.84
F	TCPOBOP	10188	10142	0.99

**CALCULATION OF THE ABSORBANCE RATIO (MDR 2**

**TABLE 5.7 COMPARED TO ACTIN) FOR C57BL/6 MICE**

**C57BL/6 MICE - MDR 2 LEVELS NORMALISED TO ACTIN**

SEX	TREATMENT	ACTIN	MDR 2	ABSORBANCE RATIO
M	CON	4038	18573	4.59
F	CON	4819	18389	3.82
M	DEX	5088	10817	2.13
F	DEX	5324	11017	2.07
M	PB	6164	14452	2.34
F	PB	8279	17049	2.06
M	TCPOBOP	6476	2293	0.35
F	TCPOBOP	9184	2111	0.23

The values in columns 1 and 2 represent the integrals of peak areas derived from densitometry readings, of one representative autoradiograph, using a Joyce Loebel scanner.

Semi-quantitation of the mRNA levels for *mdr 2* were obtained by scanning the blots using a densitometer. The autoradiographs used for scanning were non-saturated. Tables 5.6 and 5.7 show the calculations for the absorbance ratio of *mdr 2* compared to actin, for both strains of mice following the various treatments. The normalised results for DBA2/N and C57BL/6 are shown as histograms in Figures 5.13(b) and 5.14(b) respectively.

The RNA from both mouse strains had slightly variable actin levels. However, after taking these levels into account, the overall results do not alter.

*Mdr 1* could not be detected in control animals of either strain, but was strongly detected in C57BL/6 pregnant uterine tissue as a mRNA transcript of 5 kb.

*Mdr 2* mRNA was detected at moderate levels in both strains of mice. Control levels between male and female mice did not vary significantly indicating no sexual difference in *mdr 2* hepatic expression.

Treatment of both mouse strains with PB and Dex resulted in a slight suppression of *mdr 2* mRNA levels. However, treatment of the mice with a single dose of TCPOBOP (75µg for a 25g mouse) caused a dramatic suppression of *mdr 2* levels in DBA2/N and C57BL/6 mice.

*Mdr 3* mRNA levels were also measured in these samples and the results were comparable to those for *mdr 2*. TCPOBOP again caused substantial suppression of mRNA levels. Dex and PB also caused slight suppression of mRNA in both strains of mice.

The influence of Dex, PB and TCPOBOP on GST  $\pi$  levels was also assessed in these samples. Male and female control levels were significantly different in both strains with females being much lower than males. In male mice, the  $\pi$  class GST is known to dominate (McLellan and Hayes, 1987) and expression has been demonstrated to be testosterone-dependent. Male levels were not induced by Dex, PB and TCPOBOP. Female mRNA levels were increased by Dex and PB. TCPOBOP caused no increase in GST  $\pi$  mRNA levels in either species of mouse.

All three treatments induced the level of mouse *Cyp 2b* mRNA in C57BL/6 mice (Figure 5.14 (a)). Female mRNA levels were higher than male levels in each case. TCPOBOP induced both male and female mRNA levels

to the greatest extent, although female levels were still greater than male. Figure 5.15 (a) shows the Northern blot of DBA2/N mice sampled over a 12 week period, following a single i.p. injection of TCPOBOP.

Mdr 2, actin and Cyp2c mRNA levels were measured after 4 days, 4, 8 and 12 weeks. Actin levels were essentially equal for all time points. Control male and female mdr 2 mRNA levels were similar to previous DBA2/N controls, whereas mRNA levels for both male and female mice were dramatically suppressed after 4 days following TCPOBOP administration. Levels remained low even up to 12 weeks. In contrast, Cyp2c1 mRNA levels rose above control at 4 days and remained high up to 12 weeks. At 12 weeks, both male and female Cyp2c1 mRNA levels were starting to fall back to control but were still elevated.

Figure 5.15 (b) shows the semi-quantitation of the mdr 2 mRNA levels after long-term TCPOBOP exposure, after normalising the absorbance ratio of mdr 2 to actin as calculated in Table 5.8.

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**TABLE 5.8**  
**LONG-TERM TCPOBOP - MDR 2 LEVELS NORMALISED TO ACTIN**

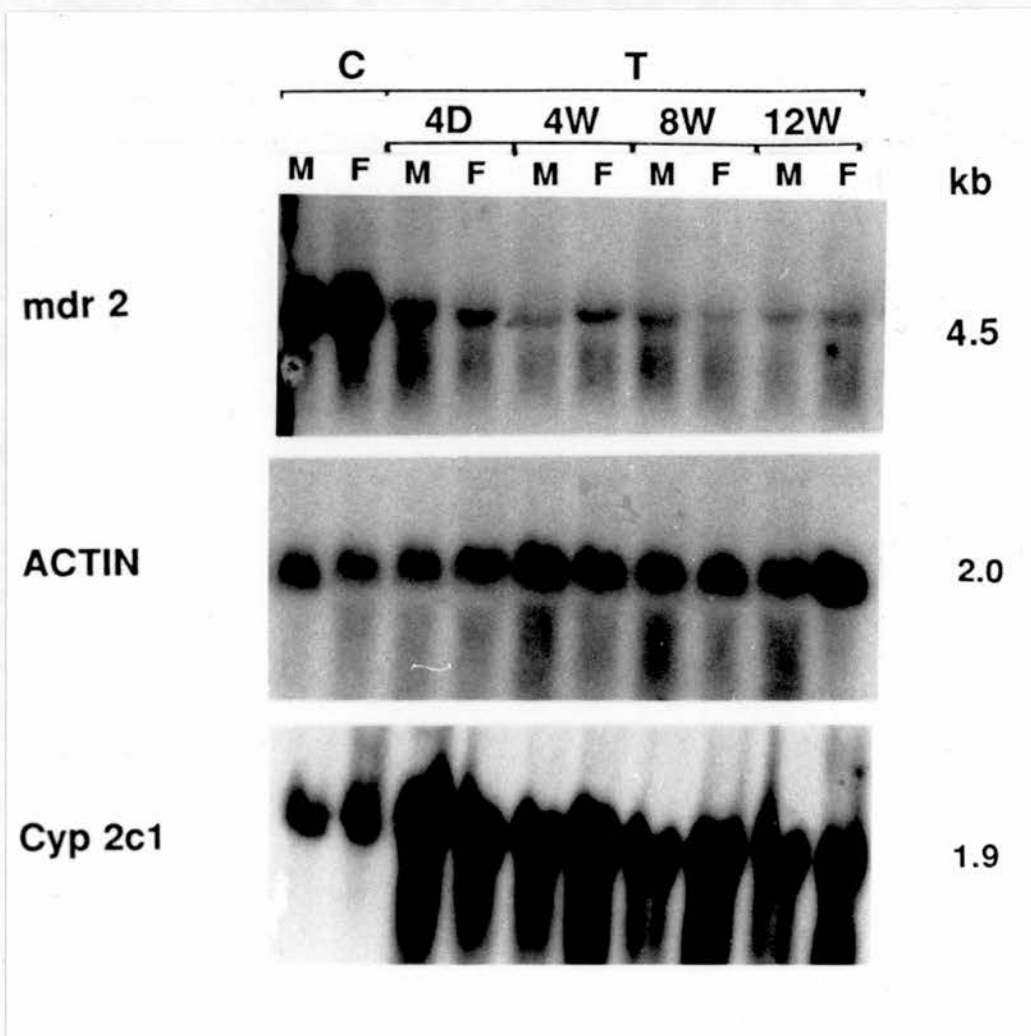
SEX	TREATMENT	ACTIN	MDR 2	ABSORB RATIO
M	CON	4272	15314	3.58
F	CON	4387	13107	2.99
M	4 DY TCPOBOP	3242	1697	0.52
F	4 DY TCPOBOP	4012	1443	0.36
M	4 WK TCPOBOP	4785	297	0.06
F	4 WK TCPOBOP	7769	1188	0.15
M	8 WK TCPOBOP	5998	933	0.10
F	8 WK TCPOBOP	6811	290	0.04
M	12 WK TCPOBOP	6298	340	0.05
F	12 WK TCPOBOP	6811	559	0.07

---

**FIGURE 5.15 (a) EXPRESSION OF MDR 2, ACTIN AND Cyp2c1 AT 4 DAYS, 4, 8 AND 12 WEEKS FOLLOWING A SINGLE INJECTION OF TCPOBOP**

DBA2/N male and female mice were treated with a single injection (3 mg/kg) of TCPOBOP. RNA was extracted from the mouse livers at various time points and Northern blotted using *mdr 2*, *actin* and *CYP2C1* cDNAs.

C	control	4 D	4 days following treatment
T	TCPOBOP treated	4 W	4 weeks following treatment
M	male	8 W	8 weeks following treatment
F	female	12 W	12 weeks following treatment

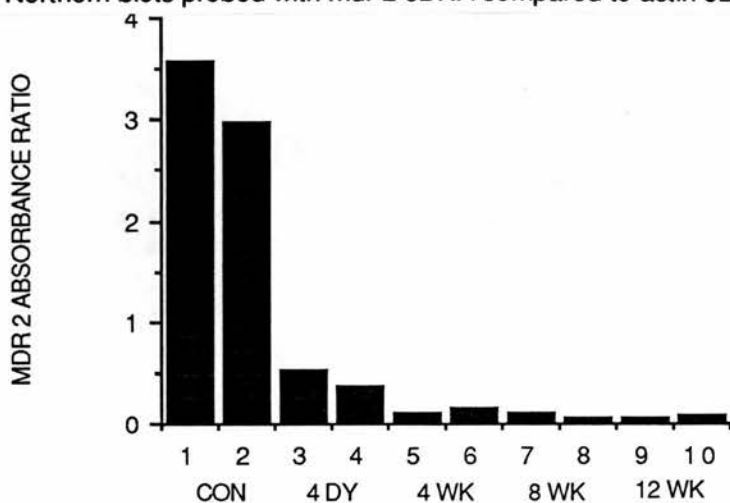


Livers from each treatment group (2 mice per group) were pooled. This blot represents the results obtained from one experiment.

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**FIGURE 5.15 (b) SUPPRESSION OF MDR 2 LEVELS UP TO 12 WEEKS WITH TCPOBOP**

Mdr 2 absorbance ratio: Ratio of the integrals of the optical densities of hybridisation signals on Northern blots probed with mdr 2 cDNA compared to actin cDNA.



1, 3, 5 and 7 represent male mice

2, 4, 6 and 8 represent female mice

con	control
4 dy	4 days following 3 mg/kg TCPOBOP
4/8/12 wk	4/8/12 weeks following treatment

---

**(b) IMMUNOBLOTTING ANALYSIS**

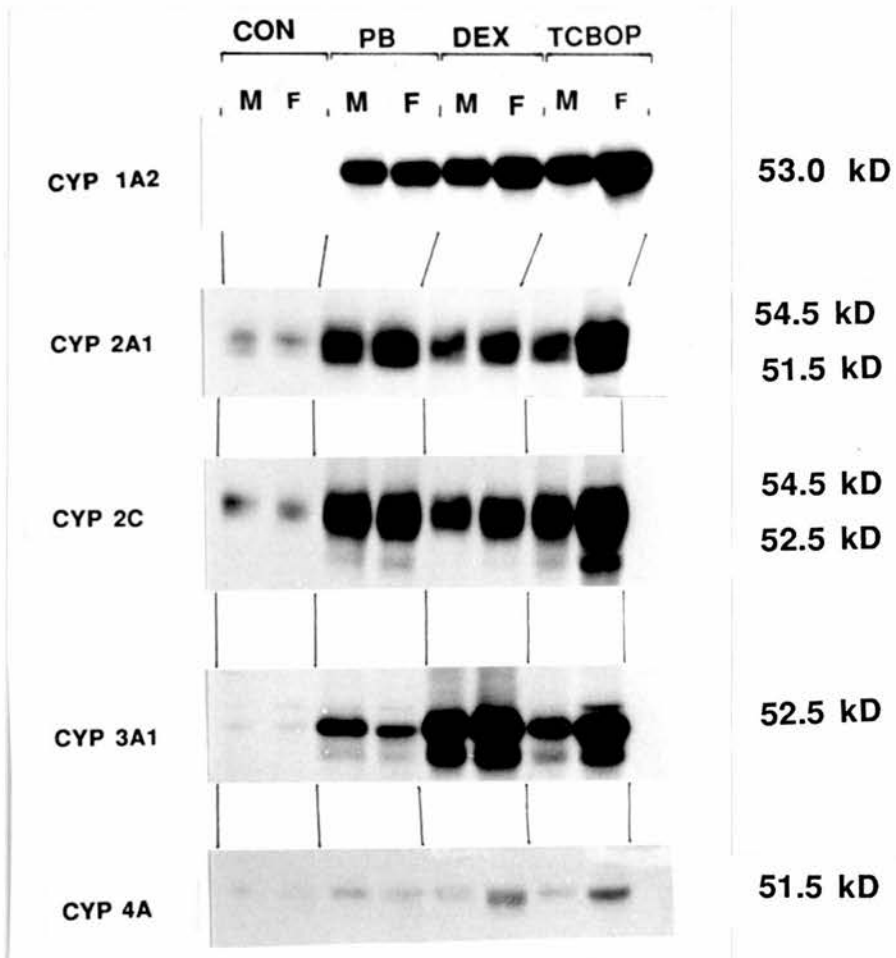
Microsomes were prepared from male and female mouse Dex, PB and TCPOBOP treated liver as well as control. Microsomal protein (15  $\mu$ g) was immuno-blotted with antibodies against Cyp1a, Cyp2a, Cyp2c, Cyp3a and Cyp4a. The analysis was performed to verify that the compounds had induced the various cytochrome P450 enzymes as expected and also to determine whether or not P-glycoprotein is coordinately regulated along with them. The results are shown in Figure 5.16.

The control male and female liver expressed very low levels of all cytochromes compared to treated animals; Cyp2a1 and Cyp2c showed the highest control levels. PB induced all the cytochrome P450 subfamilies, but induced male levels to a greater extent than female for Cyp3a1 and Cyp4a, whereas Cyp1a2, Cyp2a1 and Cyp2c were induced to an equivalent extent

**FIGURE 5.16 EXPRESSION OF VARIOUS CYTOCHROME P450s IN DBA2/N MICE TREATED WITH PB, DEX AND TCPOBOP**

DBA2/N mice were treated, as described in Section 5.3.1, and microsomal liver protein Western blotted using polyclonal antibodies against cytochrome P450 isozymes, as described in Section 2.6.2.

M	male	F	female
con	control	PB	phenobarbital
dex	dexamethasone		
TCBOP	1,4-bis [2-(3,5-dichloropyridyloxy)] benzene		



Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

by PB in males and females.

Dex, a synthetic glucocorticoid, induced all the cytochrome P450s examined in the male and female livers with Cyp3a1 being induced to the greatest extent.

TCPOBOP also induced females to a greater extent than males. This difference was apparent in all five gene families analysed.

Dex and TCPOBOP showed a similar pattern of induction but TCPOBOP induced female Cyp2a1 and Cyp2c to a greater extent than Dex whereas dex induced Cyp3a1 to a slightly greater extent. Dex induced male liver Cyp3a1 levels to a great extent whereas TCPOBOP did not. Both dex and TCPOBOP induced Cyp4a to the same high level in female and to a lower level in males.

TCPOBOP itself is highly hydrophobic and accumulates primarily in adipose tissue and to a lesser extent in the liver (Poland *et al*, 1980). It is metabolised slowly and hence exerts an effect over a long period of time during which time it is released from the fatty tissue of the animal.

### 5.3.3 TREATMENT OF C57/ICRF AND DBA2/N MICE WITH OTHER P450 SUBSTRATES

8 week old C57BL6 mice (4 per group) were treated with the compounds shown in Table 5.9. All animals were sacrificed by cervical dislocation the day after their last dose of treatment, except mice treated with TCPOBOP which were left for four days after a single dose of the compound. Control mice were administered with corn-oil.

Immediately following sacrifice, the livers and kidneys were removed, washed briefly in PBS and snap-frozen on card-ice. The tissues were then stored frozen at -70°C until RNA, microsomal and cytosolic protein was prepared from them.

DBA2/N male mice (4 per group) were also treated with 3-MC and PB, using the same protocol as shown in Table 5.9. Control mice were again treated with corn-oil.

**TABLE 5.9**  
**COMPOUNDS AND INDUCTION PROTOCOL USED FOR MOUSE**  
**TREATMENTS (SECTION 5.3.3)**

COMPOUND	VECTOR	ROUTE	DOSE	DAYS
Control	corn-oil	ip	-	3
Dexamethasone	corn-oil	ip	100mg/kg	3
3-Methylcholanthrene	corn-oil	ip	200 mg/kg	3
Pyrazole	PBS	ip	200 mg/kg	3
Diethylnitrosamine	PBS	ip	200 mg/kg	3
$\beta$ -Naphthoflavone	corn-oil	ip	80 mg/kg	3
Phenobarbitone	PBS	ip	80 mg/kg	3
2-Acetylaminofluorene	corn-oil	ig	50 mg/kg	4
TCPOBOP	corn-oil	ip	3 mg/kg	1
Aflatoxin B <sub>1</sub>	corn-oil	ip	0.2 mg/kg	1

PBS phosphate buffered saline

ip intraperitoneal

ig intragastric

- not applicable

#### 5.3.4 ANALYSIS OF MDR, CYTOCHROME P450 AND GST EXPRESSION FOLLOWING TREATMENT WITH A RANGE OF P450 SUBSTRATES

From each treatment group described, total RNA was extracted from both liver and kidney. RNA loading for Northern blots was assessed by ethidium bromide staining and probing with actin.

Both cytosolic and microsomal fractions were also extracted from the

mouse livers. 12% SDS PAGE gels were run on these fractions to check for equal protein loading. Cytosolic and microsomal proteins were electrophoresed using 12% and 9% gels respectively, and immunoblotted for GST and cytochrome P450 isozymes.

#### (a) NORTHERN BLOT ANALYSIS

Mdr 1, 2 and GST  $\pi$  mRNA levels were assessed in each kidney and liver sample (Figures 5.17, 5.18 respectively) using the probes previously described in Section 2.7.

Actin levels were high in both the liver and kidney of mice treated with DEN. PB and TCPOBOP increased actin levels slightly in mouse liver. Dex, PB and TCPOBOP increased actin levels in C57BL/6 mouse kidney. PB is known to cause massive proliferation of liver endoplasmic reticulum membrane (Remmer and Merker, 1963). TCPOBOP has similar effects on liver proliferation (Poland *et al*, 1980). Therefore, it is not surprising that these compounds affect cell cycle regulated genes such as actin. DEN causes increases in actin levels probably due to its necrotic effects on liver tissue, as discussed in Section 5.2.4(a).

Interestingly, PB did not cause an increase in the actin levels of DBA mice treated with the compound.

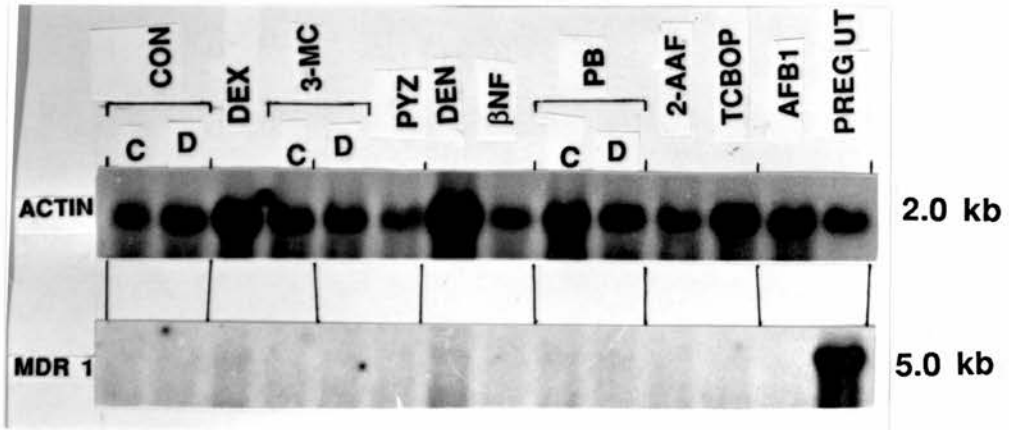
Mdr 1 mRNA levels were measured in both kidney and liver. Kidney has been previously reported to express mdr 1 to a moderate level (Croop *et al*, 1989) using a specific 424 bp probe. Kidney mRNA levels in Croop's study were much smaller than those detected in the pregnant uterus tissue, which shows the highest expression of the mdr 1 gene.

Figure 5.17 shows the results obtained after probing the kidney RNA from normal and treated mice with a 165 bp mdr 1 specific probe. Mdr1 mRNA in pregnant uterus control hybridised strongly whereas mdr 1 could only be detected in the DEN treated mouse kidney RNA sample. Figure 5.19 shows a longer exposure of the DEN treated kidney mdr 1 mRNA expression level. This experiment was repeated and blots washed at both 0.2 x and 2 x SSC to determine whether or not stringency of washing would effect the result. However, even at a lower stringency, the result was still negative for

**FIGURE 5.17 LEVELS OF EXPRESSION OF ACTIN AND MDR 1 mRNA IN C57BL/6 MOUSE KIDNEY TREATED WITH VARIOUS COMPOUNDS**

C57BL/6 male mice were treated, as described in Section 5.3.3, and kidney RNA (15 µg) was Northern blotted using actin and mdr 1 cDNAs.

C	C57BL/6	PB	phenobarbital
D	DBA2/N	2-AAF	2-acetylaminofluorene
CON	control	TCBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
DEX	dexamethasone	AFB1	aflatoxin B <sub>1</sub>
3-MC	3-methylcholanthrene	PREG UT	pregnant uterus
PYZ	pyrazole		
DEN	diethylnitrosamine		
β-NF	β-naphthoflavone		

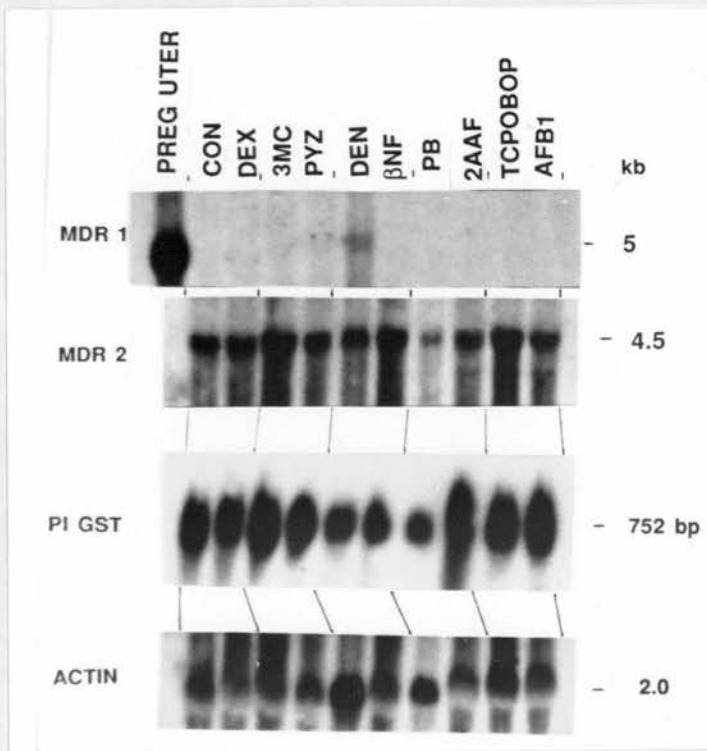


Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

**FIGURE 5.18 LEVELS OF EXPRESSION OF MDR 1, MDR 2, GST Pi AND ACTIN mRNA IN C57BL MOUSE LIVER TREATED WITH VARIOUS COMPOUNDS**

C57BL/6 male mice were treated, as described in Section 5.3.3, and liver RNA (15 µg) was Northern blotted using *mdr 1*, *mdr 2*, glutathione S-transferase  $\pi$  and actin cDNAs.

PREG UTER	pregnant uterus	$\beta$ -NF	$\beta$ -naphthoflavone
CON	control	PB	phenobarbital
DEX	dexamethasone	2-AAF	2- acetylaminofluorene
3-MC	3-methylcholanthrene	AFB1	aflatoxin B1
PYZ	pyrazole		
DEN	diethylnitrosamine		
TCBOP	1,4-bis[2-(3,5- dichloropyridyloxy)]benzene		



Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

all other kidney samples. The samples appeared intact on ethidium stained gels and after probing with actin. It was concluded that the kidney tissue from these particular C57BL/6 mice contained extremely low levels of *mdr 1* which could not be detected using such a system of Northern blotting. Extraction of mRNA instead of total RNA from the kidney samples, or using a longer probe might allow for better detection of the gene transcript.

*Mdr 1* could not be detected in normal C57BL/6 mouse liver as shown in Figure 5.18.

DEN treated mice did however induce the expression of *mdr 1* in the liver. Figure 5.19 shows the relative levels of expression of *mdr 1* in the DEN treated liver and kidney tissues are equivalent. Such induction may be similar to the increase in *mdr* genes seen in rat liver treated with DEN and may be due to the necrotic effects of DEN in the tissue.

PB treated mice demonstrated decreased *mdr 2* transcript levels. Following actin adjustment, Dex, 3-MC, pyrazole, DEN, TCPOBOP, 2-AAF,  $\beta$ -NF and AFB<sub>1</sub> treated C57BL/6 mouse livers had equivalent levels of *mdr 2* mRNA as control.

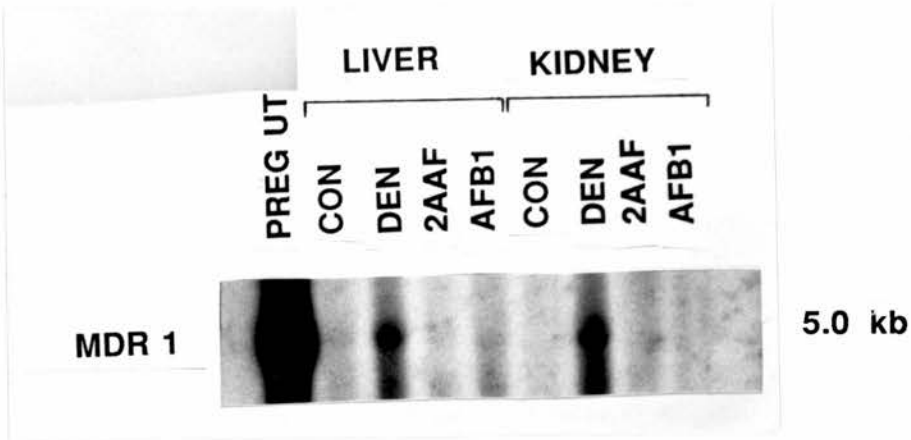
Burt and Thorgeirsson (1988) showed that both 2-AAF and AFB<sub>1</sub> induced the expression of *mdr* in C57BL/6 mouse liver, using the human *mdr* cDNA probe, pADR-1. No *mdr* transcript was detected in the normal mouse liver control. In this study, a 4.5 kb transcript was detected. Croop *et al* (1989) described *mdr 2* mRNA in mouse liver as being 4.5 kb. The *mdr 2* transcript was found to be the major *mdr* RNA species in normal mouse liver. From these studies, it is uncertain which *mdr* transcript was induced by 2-AAF and AFB<sub>1</sub> by Burt and Thorgeirsson (1988) since the probe was not gene specific. However, their results for control mice do not suggest that the transcript was *mdr 2*.

Figure 5.20 contrasts the results found with C57BL/6 and DBA2/N mice following treatment with 3-MC and PB. The actin levels following treatment with PB were elevated in C57BL/6 mouse liver whereas no such increase was found in the DBA2/N liver. All other actin mRNA levels were approximately equivalent. Control levels for C57BL/6 and DBA2/N for *mdr 2* mRNA were equivalent. PB treatment caused no decrease in *mdr 2* mRNA levels in DBA2/N mouse liver whereas C57BL liver showed a decline in the

**FIGURE 5.19 LEVELS OF MDR 1 EXPRESSION IN MALE C57BL/6 LIVER AND KIDNEY TREATED WITH DIETHYLNITROSAMINE, 2-ACETYLAMINOFLUORENE AND AFLATOXIN B<sub>1</sub>**

Male C57BL/6 mice were treated, as described in Section 5.33, and liver RNA was prepared from both liver and kidney. Northern blotting (15 µg samples) was performed using the *mdr 1* cDNA, as outlined in Section 2.11.

PREG UT      pregnant uterus  
 CON          control  
 DEN          diethylnitrosamine  
 2-AAF        2-acetylaminofluorene  
 AFB<sub>1</sub>        aflatoxin B<sub>1</sub>



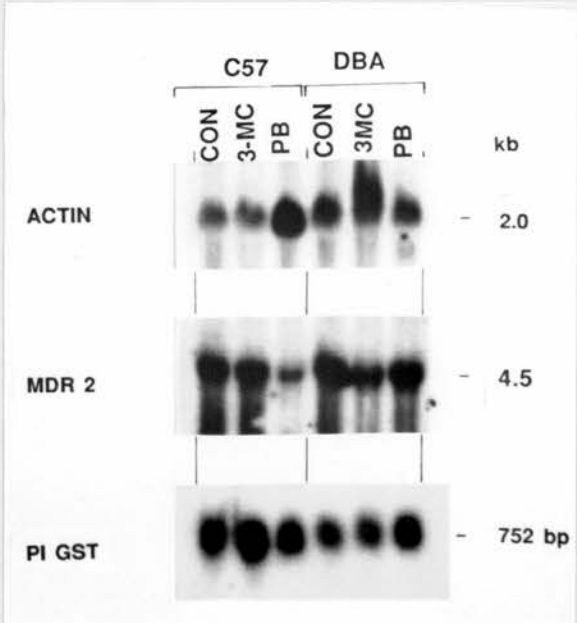
Livers and kidneys from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

**FIGURE 5.20      LEVELS OF ACTIN, MDR 2 AND GST Pi mRNA  
IN C57BL/6 AND DBA2/N MOUSE LIVER  
TREATED WITH 3-MC AND PB**

Male C57BL/6 and DBA2/N mice were treated, as described in Section 5.3.3. Mice of both species, treated with 3-methylcholanthrene and phenobarbital, were Northern blotted (15 µg per sample) using actin mdr 2 and glutathione S-transferase cDNA probes, as described in Section 2.11.

- CON    control
- 3-MC   3-methylcholanthrene treated
- PB     phenobarbital treated

Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.



expression of this transcript. 3-MC caused a slight decline in the level of *mdr 2* mRNA for DBA2/N mouse liver, whereas no change occurred in the C57BL/6 mouse. GST  $\pi$  mRNA levels were also measured in these mouse strains.

The level of GST  $\pi$  mRNA was higher in C57BL mice than DBA2/N mice. PB induced GST  $\pi$  mRNA slightly in DBA2/N mice.

Figure 5.18 also shows the results of a range of treatments on GST  $\pi$  mRNA levels.

DEN,  $\beta$ -NF and PB caused a slight decrease in the mRNA levels in C57BL6 mice; all other GST  $\pi$  levels were unaffected by the treatments.

## (b) IMMUNOBLOTTING ANALYSIS

Immunoblotting of microsomal protein (15 $\mu$ g) and cytosolic protein (30 $\mu$ g) was performed on the mouse livers using cytochrome P450 and GST antibodies respectively. Rat CYP4A1 and CYP4A2/3 specific monoclonals were also available for use. CYP4A1 shares ~65% sequence similarity with CYP4A2 and CYP4A3. Polyclonal antibodies used against CYP4A do not differentiate between CYP4A1 (51.5 kD), CYP4A2 (51.1 kD) and CYP4A3 (52 kD).

Monoclonal antibodies were raised against two different CYP4A preparations from clofibrate treated male Sprague-Dawley rat liver. Immunoglobulins purified from the clones were characterised by isoelectric focusing and immunoblotting and were found to cross-react with the antigens used for immunisation (Henderson *et al*, 1991).

The results of the treatments on both C57BL/6 and DBA2/N mouse liver cytochrome P450 protein levels is shown in Figure 5.21 and summarised in Table 5.10.

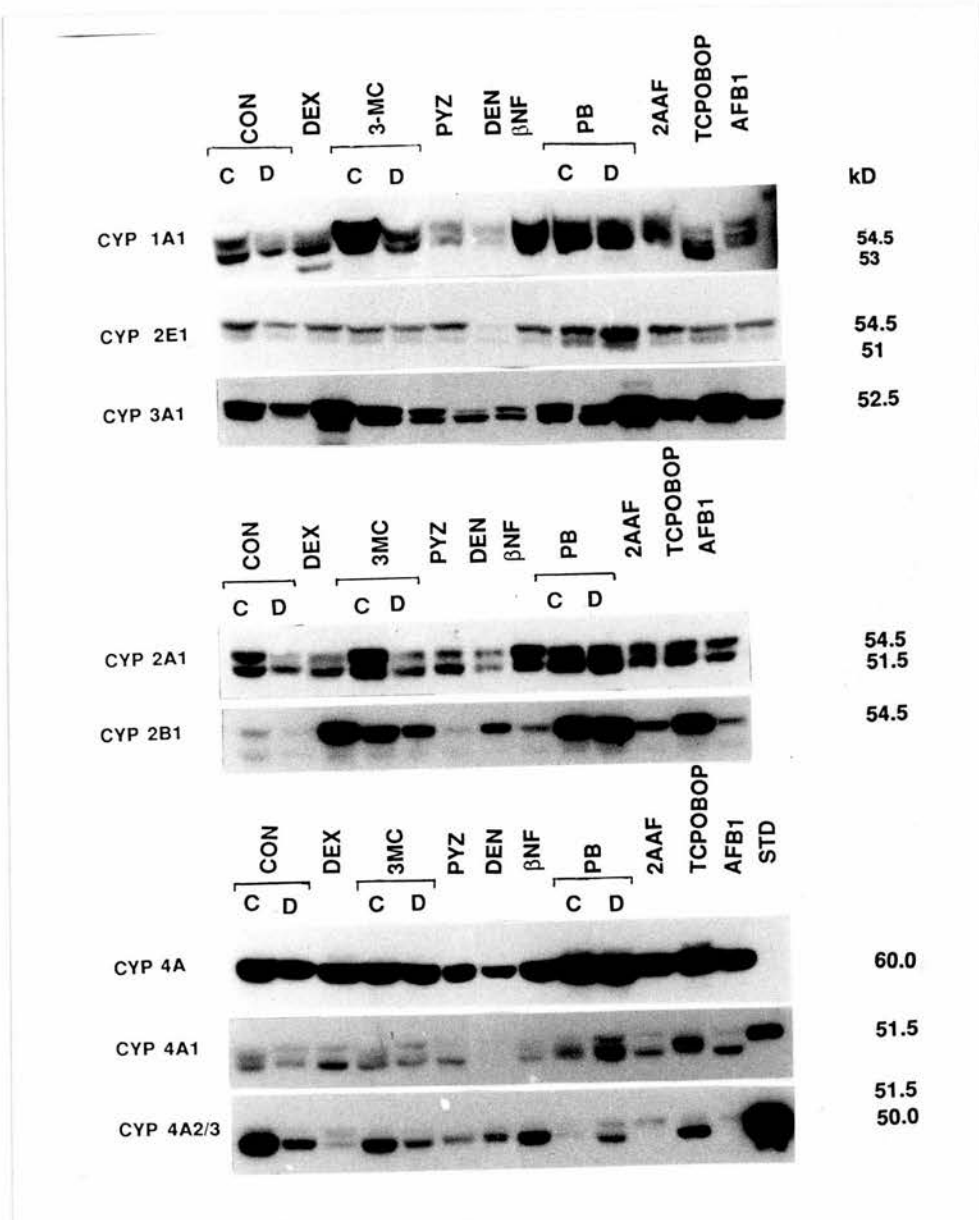
The control level of C57BL/6 protein was found to be higher than DBA2/N for each cytochrome P450 family examined. Both intra- and inter-species variations in the activities and enzyme expression for the P450s has been found (Gonzalez *et al*, 1989).

C57BL/6 and DBA2/N mouse livers show a difference in response to the CYP1A1 inducer 3-MC, which indicates that the C57BL6 mouse strain

**FIGURE 5.21 LEVELS OF EXPRESSION OF VARIOUS CYTOCHROME P450s IN C57BL/6 MICE FOLLOWING TREATMENT WITH INDUCING COMPOUNDS**

Mice were treated, as described in Section 5.3.3, and liver microsomes prepared following treatment. Microsomes were Western blotted (15 µg) using P450 polyclonal antibodies, as outlined in Section 2.6.2.

CON	control	PYZ	pyrazole	PB	phenobarbital
DEX	dexamethasone	DEN	diethylnitrosamine	2-AAF	2-acetylaminofluorene
3-MC	3-methylcholanthrene	β-NF	β-naphthoflavone	AFB <sub>1</sub>	aflatoxin B <sub>1</sub>
TCBOP	1, 4-bis [2-(3,5-dichloropyridyloxy)]	benzene		STD	standard
C	C57/6	D	DBA2/N		



Livers from each mouse strain (4 mice per strain) were pooled. This blot represents the results obtained from one experiment.

TABLE 5.10  
 EXPRESSION LEVELS OF P450 AND GST ISOZYMES IN MALE C57BL/6 MOUSE LIVER

CHEM	DOSE MG/KG (DAYS)	P450 FAMILY										GST					
		1a1	2a1	2b1	2e1	3a1	4a1	4a2/3	Ya1	Ya2	Ya3	Yb1	Yf				
Dex	100 (3)	NC	NC	+++	NC	++	NC	-	NC	++	NC	-	NC	NC	NC	NC	NC
3MC	200 (3)	+++	+++	++	NC	+	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
				(++)											(+)		(+)
Pyz	200 (3)	NC	NC	NC	NC	-	-	-	+++	NC	NC	NC	NC	NC	NC	NC	NC
DEN	200 (3)	-	-	+	-	-	-	-	NC	NC	NC	NC	NC	NC	NC	-	NC
BNF	8(3)	+++	++	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
PB	80(3)	++	++	+++	NC	NC	++	-	NC	NC	NC	NC	NC	NC	NC	NC	-
		(++)	(++)	(+++)	(++)	(+++)	(+++)	(++)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
2AAF	50 (4)	NC	NC	+	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
TCPOBOP	3 (3)	NC	NC	+++	NC	+++	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
AFB1	0.2 (1)	NC	NC	+	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC

++++ high induction ++ moderate + slight - suppression ( ) DBA2/N mice NC no change

has an intact Ah receptor whereas the 'non-responsive' DBA2/N mouse has a much lower number of cytosolic Ah receptors, a qualitative defect in receptor ligand binding or both (Okey *et al*, 1980). C57BL/6 mice showed a high induction of Cyp1a-1 whereas DBA2/N showed no such elevation in Cyp1a-1 protein levels.  $\beta$ -NF also induced C57BL/6 Cyp1a-1 protein to a high level. Both 3-MC and  $\beta$ -NF operate via the Ah locus (for a review see Eisen *et al*, 1983). PB induced both strains to a moderate extent. This compound does not operate via the Ah locus but all PB-like compounds are hypothesised to bind a common receptor.

DEN suppressed the expression of all the cytochrome P450 proteins examined in C57BL/6 mouse liver, with the notable exception of Cyp2b.

Cyp2a was highly induced by 3-MC and moderately by  $\beta$ -NF. PB also induced Cyp2a in both mouse species to a moderate extent.

Both Dex and PB treated livers showed highly increased Cyp2b protein. Dex is known to stabilise the CYP2B1 protein in rat (Simmons *et al*, 1987). TCPOBOP, the PB-like ligand, also highly induced Cyp2b protein levels. 2-AAF and AFB<sub>1</sub> are known to be weak inducers of cytochrome P450 gene families 1 and 2 (Astrom *et al*, 1986; Ishii *et al*, 1986). These compounds induced Cyp2b slightly above control protein levels.

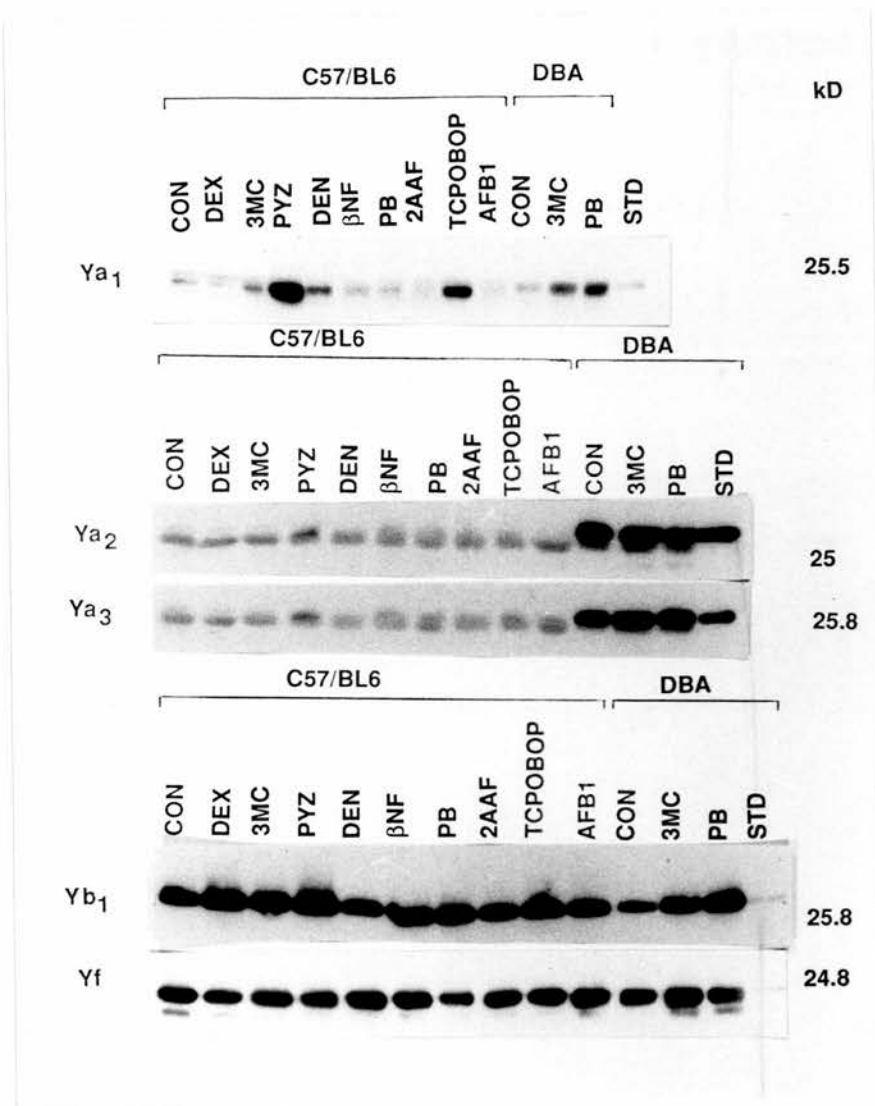
Cyp3a was moderately induced by Dex in this study. This has been described previously by other investigators for the rat (Simmons *et al*, 1987). PB increased DBA2/N Cyp3a expression levels to a much greater extent than C57BL/6. TCPOBOP showed elevated protein levels to a similar extent as Dex treatment in C57BL/6 mice and PB treatment in DBA2/N mice. In mouse, TCPOBOP and Dex both induce Cyp2b and Cyp3a to similar extents. PB also induces Cyp2b but fails to affect Cyp3a in C57BL/6 mice. Both species of PB treated mice showed increased protein levels for the Cyp4a family using the polyclonal antibody. However, using monoclonal antibodies it was shown that the mouse orthologue of the rat CYP4A1 was induced to a greater level than the mouse orthologue of the rat CYP4A2/3. DEN decreased the levels of the mouse orthologues of the rat CYP4A1 and CYP4A2/3 in C57BL/6 mice.

In addition to measuring the P450 protein levels, GST Ya<sub>1</sub>, Ya<sub>2</sub>, Ya<sub>3</sub>, Yb<sub>1</sub> and Yf protein levels were assessed, as shown in Figure 5.22 and

**FIGURE 5.22 INDUCIBILITY OF GST ISOZYMES IN C57BL/6 AND DBA2/N MICE BY VARIOUS COMPOUNDS**

Mice were treated, as described in Section 5.3.3, and liver cytosols prepared following treatment. Microsomes were Western blotted (20 µg for Ya<sub>1</sub>, Ya<sub>2</sub> and Ya<sub>3</sub>; 5 µg for Yb and Yf) using glutathione S-transferase polyclonal antibodies, as outlined in Section 2.6.2.

CON	control	DEN	diethylnitrosamine	AFB <sub>1</sub>	aflatoxin B <sub>1</sub>
DEX	dexamethasone	β-NF	β-naphthoflavone	STD	standard
3-MC	3-methylcholanthrene	PB	phenobarbital	C	C57/6
PYZ	pyrazole	2-AAF	2-acetylaminofluorene	D	DBA2/N
TCPOBOP	1, 4-bis [2-(3,5-dichloropyridyloxy)] benzene				



Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

summarised in Table 5.10.

Both Ya<sub>2</sub> and Ya<sub>3</sub> protein subunits were present at much higher levels in DBA2/N mice compared to C57BL/6. No treatment caused induction in either of these subunits. Ya<sub>1</sub> control levels were equivalent in C57BL/6 and DBA2/N mice. 3-MC and PB caused an increase in the DBA2/N Ya<sub>1</sub> subunit protein. Pyrazole and, to a lesser extent, TCPOBOP treatment of C57BL/6 mice elevated Ya<sub>1</sub> protein levels. GST Yb<sub>1</sub> C57BL/6 protein levels remained unchanged following various treatments, whereas 3-MC and PB treated DBA2/N mice exhibited elevated levels of Yb<sub>1</sub> and Yf GST.

## **5.4 ANTICANCER DRUGS AND CYP3A SUBSTRATES AS REGULATORS OF MDR GENES IN MICE**

### **5.4.1 TREATMENT OF C57BL/6 MICE WITH ANTI-CANCER DRUGS AND CYP3A SUBSTRATES**

8 week old male C57BL mice (4 per group) were treated with the compounds listed in Table 5.11. Figure 5.23 shows the structures of these compounds and also includes the structure of butylated hydroxyanisole (BHA).

All compounds were administered intraperitoneally except progesterone which was given sub-cutaneously. Control mice were injected with corn-oil.

All mice were sacrificed 24h following the last dose of treatment. Livers, lungs and kidneys were removed, washed briefly in PBS and snap-frozen on card-ice. Tissues were stored at -70°C until required. RNA was extracted from the liver and kidney. Microsomal and cytosolic protein was fractionated from liver, lung and kidney.

Liver RNA samples were probed using mdr 1, mdr 2, GST  $\pi$  and actin cDNAs. Kidney RNA was probed with mdr 1. Samples were assessed for equal loading using ethidium bromide staining and actin level measurements. Cytosolic and microsomal fractions from liver, lung and kidney tissues were immunoblotted against GST and cytochrome P450 antibodies respectively.

The compounds outlined in Table 5.11 can be broadly divided into two groups. Triacetyloleandomycin (TAO), rifampicin (RIF), erythromycin (ERY), clotrimazole (CLOT) and progesterone (PROG) are all Cyp3a substrates whereas adriamycin (ADR), etoposide (VP-16), vincristine (VINC) and vinblastine (VINB) are all used as anti-cancer drugs in clinical medicine.

It is likely that multiple Cyp3a proteins are expressed in liver, as suggested by cDNA cloning (Beaune *et al*, 1986a; Gonzalez *et al*, 1988; Molowa *et al*, 1986). CYP3A is expressed in human fetal liver to significant levels (Kitada *et al*, 1985). CYP3 expression varies in human liver which

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**TABLE 5.11  
COMPOUNDS USED IN SECTION 5.4.1 AND INDUCTION  
PROTOCOL**

COMPOUNDS	VECTOR	ROUTE	DOSE	DAYS
Control	corn-oil	ip		3
Triacetyloleanomycin	corn-oil	ip	500 mg/kg	3
Rifampicin	corn-oil	ip	200 mg/kg	3
Erythromycin	corn-oil	ip	500 mg/kg	3
Clotrimazole	corn-oil	ip	50 mg/kg	3
Adriamycin	PBS	ip	4 mg/kg	3
VP-16 (Etoposide)	corn-oil	ip	12 mg/kg	3
Vincristine	corn-oil	ip	0.3 mg/kg	3
Vinblastine	corn-oil	ip	3 mg/kg	3
Progesterone	corn-oil	sc	1 mg/kg	11

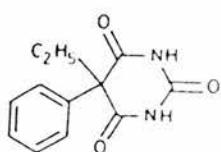
ip intraperitoneal

sc sub-cutaneous

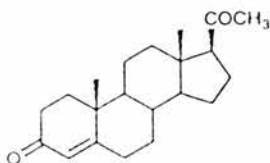
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could have important clinical implications since CYP3A metabolises a variety of drugs including the calcium channel blocker nifedipine (Gonzalez

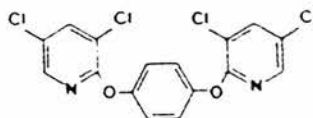
**FIGURE 5.23 STRUCTURES OF THE P450 SUBSTRATES AND ANTI-CANCER DRUGS USED TO TREAT C57BL/6 MICE**



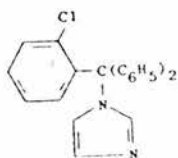
**phenobarbitone**



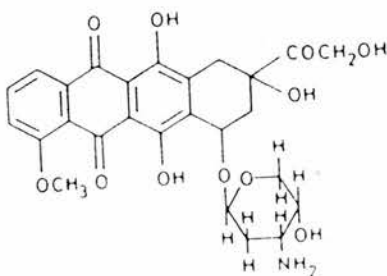
**progesterone**



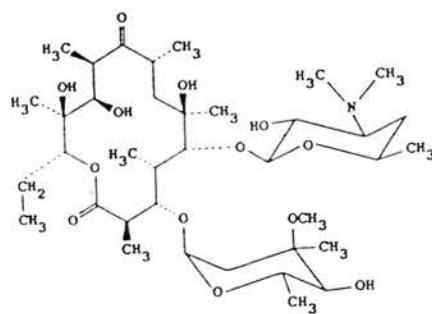
**TCPOBOP**



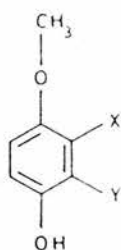
**clotrimazole**



**adriamycin**

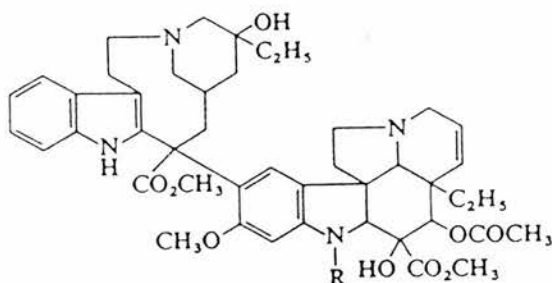


**erythromycin A**



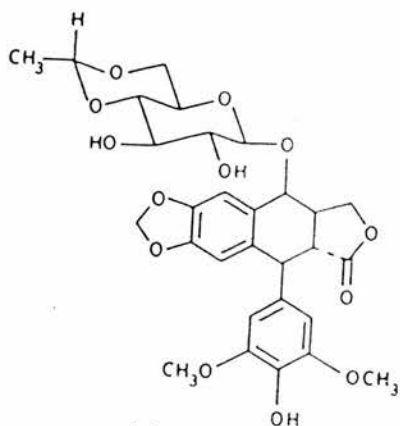
Y = H, X = C(CH<sub>3</sub>)<sub>3</sub> 2-t-butyl-4-hydroxyanisole  
 X = H, Y = C(CH<sub>3</sub>)<sub>3</sub> 3-t-butyl-4-hydroxyanisole

**butylated hydroxyanisole**

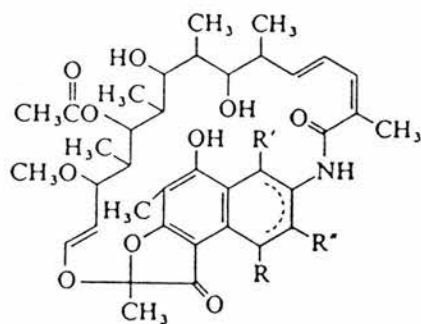


Vinblastine, R = -CH<sub>3</sub>  
 Vincristine, R = -CHO

**vincristine/vinblastine**



**etoposide**



R = R' = -OH, R'' = -CH=N-N(CH<sub>3</sub>)<sub>2</sub>

**rifampicin**

*et al*, 1988), progesterone (Schwab *et al*, 1988), the immunosuppressant cyclosporin (Kronbach *et al*, 1988) and the antibiotic erythromycin (Watkins *et al*, 1985). It is extremely interesting to note that all of these CYP3A substrates are also substrates for P-gp.

TAO is a macrolide antibiotic and an inducer of Cyp3a1 (Wrighton *et al*, 1985). The TAO metabolite binds strongly to the reduced iron of the haem prosthetic group of this cytochrome resulting in the formation of a persistent metabolic intermediate complex (Pessayre *et al*, 1981). The rabbit CYP3A6 mRNA is stabilised by TAO (Dalet *et al*, 1986) as are the rat CYP3A1 and CYP3A2 proteins (Watkins *et al*, 1986). Dex however transcriptionally activates the rat *CYP3A1* gene (Simmons *et al*, 1987).

CYP3A6 catalyses erythromycin N-demethylation and is induced significantly by rifampicin (Murray and Reddy, 1990). Clotrimazole can also induce the expression of CYP3A1.

#### 5.4.2 ANALYSIS OF MOUSE MDR, CYTOCHROME P450 AND GST EXPRESSION FOLLOWING TREATMENT WITH ANTI-CANCER DRUGS AND CYP3A SUBSTRATES

Treated mouse liver RNA samples (15µg), including a C57BL/6 mouse pregnant uterus control for *mdr 1*, were blotted. Liver, kidney and lung microsomes were immunoblotted using polyclonal antibodies raised against rat CYP2A1, CYP2B1, CYP2C3, CYP2E1, CYP3A1 and CYP4A. Monoclonal antibodies raised against rat CYP4A1 and CYP 4A2/A3 were also used to immunoblot the microsomal samples. Liver, kidney and lung cytosolic protein was immunoblotted using polyclonal antibodies against Ya<sub>1</sub>, Ya<sub>2</sub>, Ya<sub>3</sub>, Yb<sub>1</sub> and Yf GST subunit proteins.

Yf is the most highly expressed GST subunit in male mouse liver and Yb<sub>1</sub> is also highly expressed.

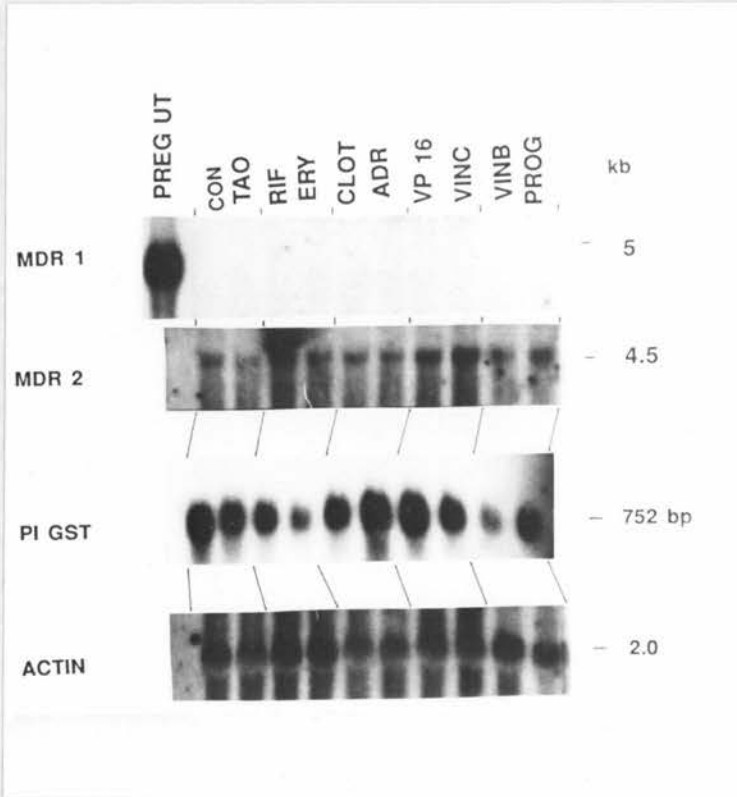
##### (a) NORTHERN BLOT ANALYSIS

The results obtained following Northern analysis on the liver RNA samples of treated mouse livers is shown in Figure 5.24.

**FIGURE 5.24 LEVELS OF EXPRESSION OF MDR 1, MDR 2, GLUTATHIONE S-TRANSFERASE Pi AND ACTIN mRNA IN MALE C57BL/6 LIVER FOLLOWING TREATMENT WITH ANTI-CANCER DRUGS AND CYP3A SUBSTRATES**

Mice were treated, as described in Section 5.4.1, and RNA was prepared from the liver following treatment. Northern blotting (15 µg of each sample) was performed, as described in Section 2.11, using *mdr 1*, *mdr 2*, glutathione S-transferase Pi and actin cDNA probes.

PREG UT	Pregnant uterus	ADR	adriamycin
CON	control	VP 16	etoposide
TAO	triacetyloleandomycin	VINC	vincristine
RIF	rifampicin	VINB	vinblastine
ERY	erythromycin	PROG	progesterone
CLOT	clotrimazole		



Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

Actin levels were approximately equivalent in all the samples. The mdr 1 cDNA detected a strongly hybridising 5 kb transcript in the pregnant uterus control but detected no other transcript in control or treated mice.

The mdr 2 cDNA probe strongly detected a 4.5 kb transcript in both control and treated mice. Rifampicin, etoposide (VP-16) and vincristine substantially induced mdr 2 mRNA levels to above control levels and progesterone increased mdr 2 mRNA slightly. No other Cyp3a substrates or anti-cancer drugs examined in this study altered mdr 2 mRNA levels. GST  $\pi$  mRNA levels were suppressed in both erythromycin and vinblastine treated mice. Vincristine treated mice had normal GST  $\pi$  expression. The difference in the expression of mdr 2 and GST  $\pi$  in vinblastine and vincristine treated mice was somewhat surprising since these compounds are remarkable similar in structure (Figure 5.23). Progesterone only slightly induced mdr 2 mRNA levels although it is a known substrate for rat CYP3A and mdr 1 (Waxman *et al*, 1988; Yang *et al*, 1989).

From the results shown in Figure 5.24, it is evident that progesterone does not induce the expression of mdr 1 mRNA in mouse liver although it has been shown to induce the gene to high levels in mouse uterus.

VP-16 is a DNA topo II inhibitor and causes an increase in mdr 2 expression. Since topo II is a common drug target, down-regulation in expression of topo II and a concomitant rise in mdr expression could protect the cell against the toxic effects of these drugs. Chin *et al* (1990) found a coordinated response of mdr and topo II was found in rat and mouse cell lines challenged with several anti-tumour drugs. Agents that caused an elevation of mdr RNA levels promoted a rapid decline in topo II RNA levels.

The lack of regulation of mdr 2 mRNA levels by triacetyloleandomycin, erythromycin and clotrimazole indicates that not all Cyp3a substrates act as regulators of mdr 2 mRNA expression.

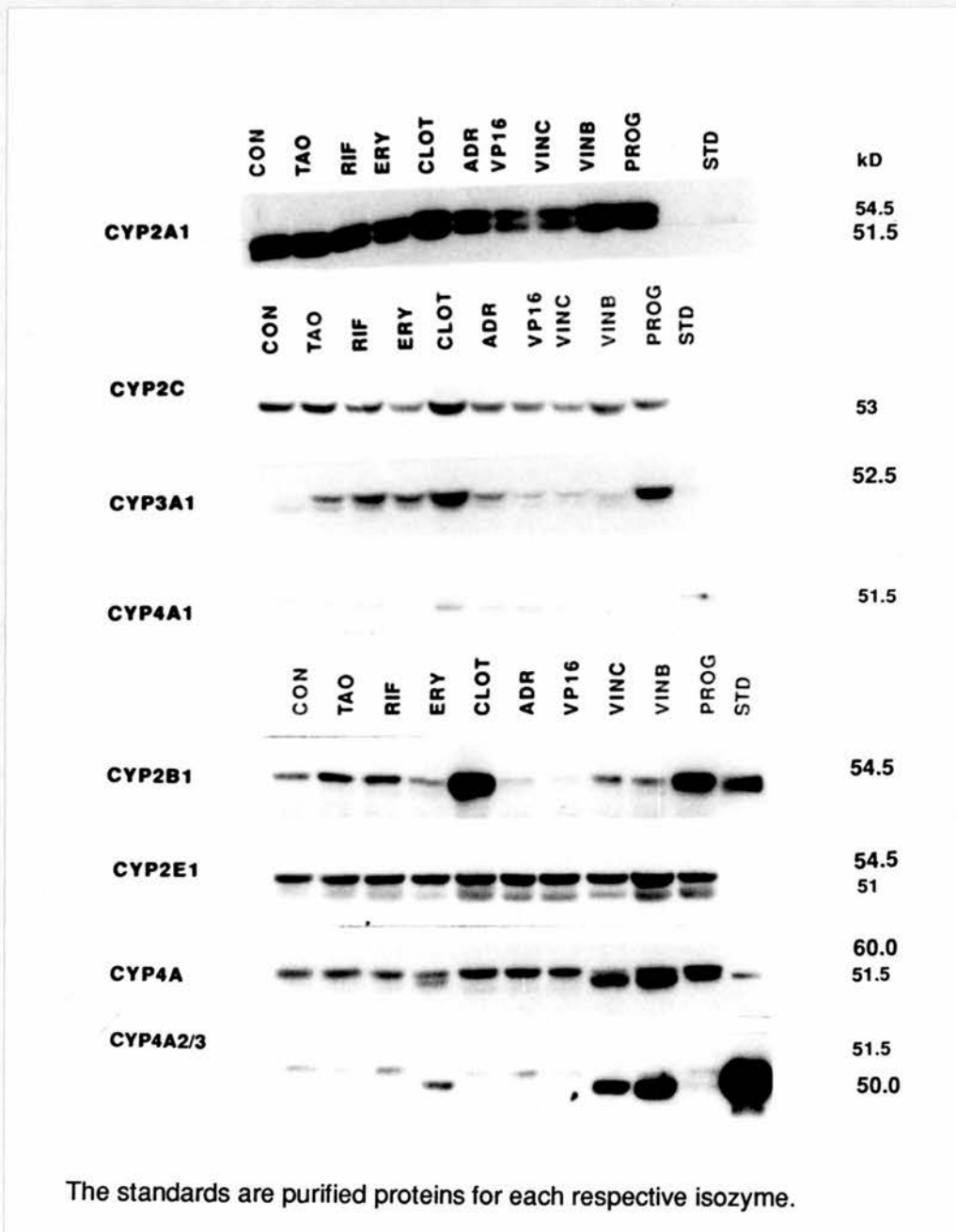
## (b) IMMUNOBLOTTING ANALYSIS

To assess the induction characteristics of the compounds used in this study, the microsomes and cytosols prepared from liver were probed with cytochrome P450 and GST antibodies respectively.

## FIGURE 5.25 INDUCTION OF CYTOCHROME P450 ISOZYMES IN C57BL/6 MOUSE LIVER FOLLOWING TREATMENT WITH VARIOUS COMPOUNDS

Male C57BL/6 mice were treated, as described in Section 5.4.1, and liver microsomes were prepared. Western blotting (30  $\mu$ g per sample) was performed, as described in Section 2.6.2, using P450 polyclonal antibodies.

CON	control	ADR	adriamycin
TAO	triacetyloleandomycin	VP 16	etoposide
RIF	rifampicin	VINC	vincristine
ERY	erythromycin	VINB	vinblastine
CLOT	clotrimazole	PROG	progesterone



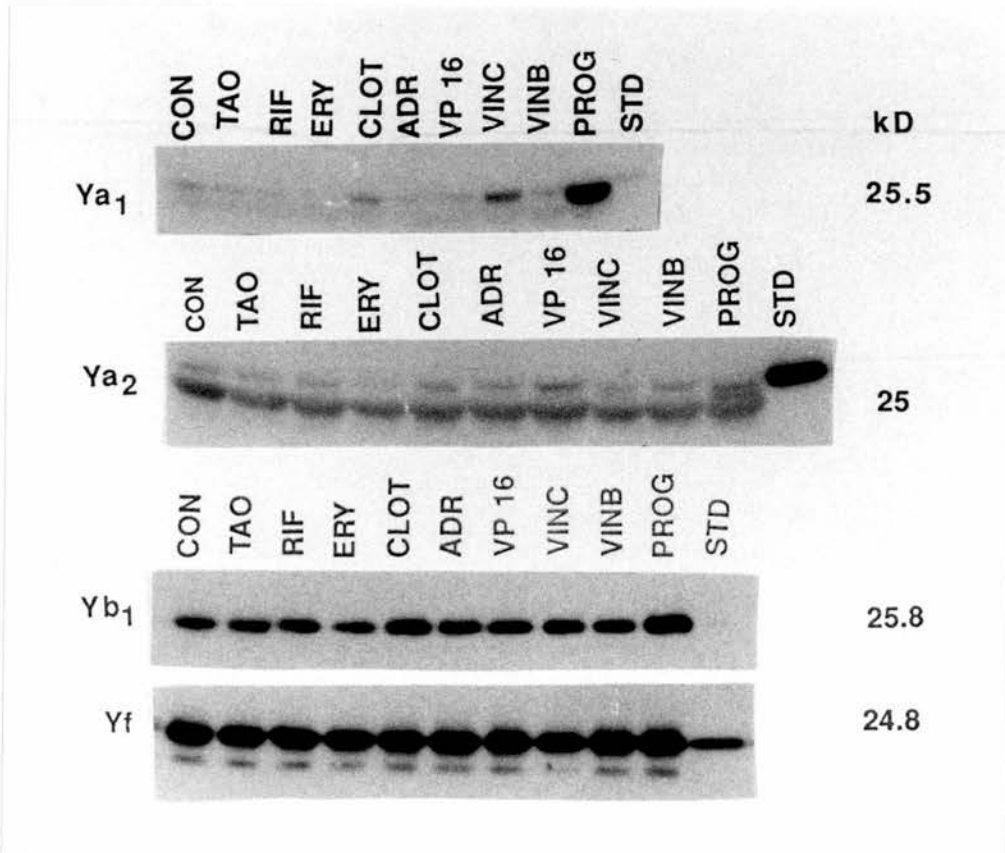
Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

**FIGURE 5.26 INDUCTION OF GST ISOZYMES IN C57BL/6 MOUSE LIVER FOLLOWING TREATMENT WITH VARIOUS COMPOUNDS**

Male C57BL/6 mice were treated, as described in Section 5.4.1, and liver cytosols were prepared. Western blotting (20 µg for Ya<sub>1</sub> and Ya<sub>2</sub>; 5 µg for Yb<sub>1</sub> and Yf) was performed, as described in Section 2.6.2, using glutathione S-transferase polyclonal antibodies.

CON	control	ADR	adriamycin
TAO	triacetyloleandomycin	VP 16	etoposide
RIF	rifampicin	VINC	vincristine
ERY	erythromycin	VINB	vinblastine
CLOT	clotrimazole	PROG	progesterone

The standards are purified proteins for each respective isozyme.



Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

As shown in Figure 5.25, triacetyloleandomycin, rifampicin, erythromycin, clotrimazole and progesterone all induced the mouse orthologue of rat CYP3A1 above control levels,. The anti-cancer drugs did not induce CYP3A1. Clotrimazole and progesterone induced the mouse orthologue of rat CYP2A1, CYP2B1 and CYP4A above control levels. Vincristine and vinblastine varied slightly in their induction pattern. Vinblastine induced the mouse orthologue of rat CYP2A1, CYP2E1 and CYP4A2/A3, whereas vincristine only induced CYP4A2/A3. Metabolism of these compounds would therefore appear to vary in mouse liver and hence differences in the regulation of *mdr* genes by these compounds might be expected. As a general observation, the anti-cancer drugs induced the mouse orthologue of rat CYP4A most significantly in mouse liver. The results of the GST immunoblotting is shown in Figure 5.26.

Progesterone caused dramatic induction of  $Y_{a_1}$  GST mRNA levels as well as a moderate increase in the  $Y_{b_1}$  GST mRNA. The other compounds had little effect on GST mRNA levels in the mouse liver.

To assess the effects of these compounds in extra-hepatic tissue, both kidney and lung tissues from the treated mice were probed with the P450 and GST antibodies.

As shown in Figure 5.27, mouse kidney orthologues of rat CYP2B1, CYP4A1 and GST isozymes  $Y_{a_1}$ ,  $Y_{a_3}$ ,  $Y_f$  and  $Y_{b_1}$  were largely unaffected by these compounds apart from the slight induction of CYP2B1 by rifampicin and clotrimazole. The CYP4A1 orthologue did not appear to be induced by the anti-cancer drugs to any extent in mouse kidney.

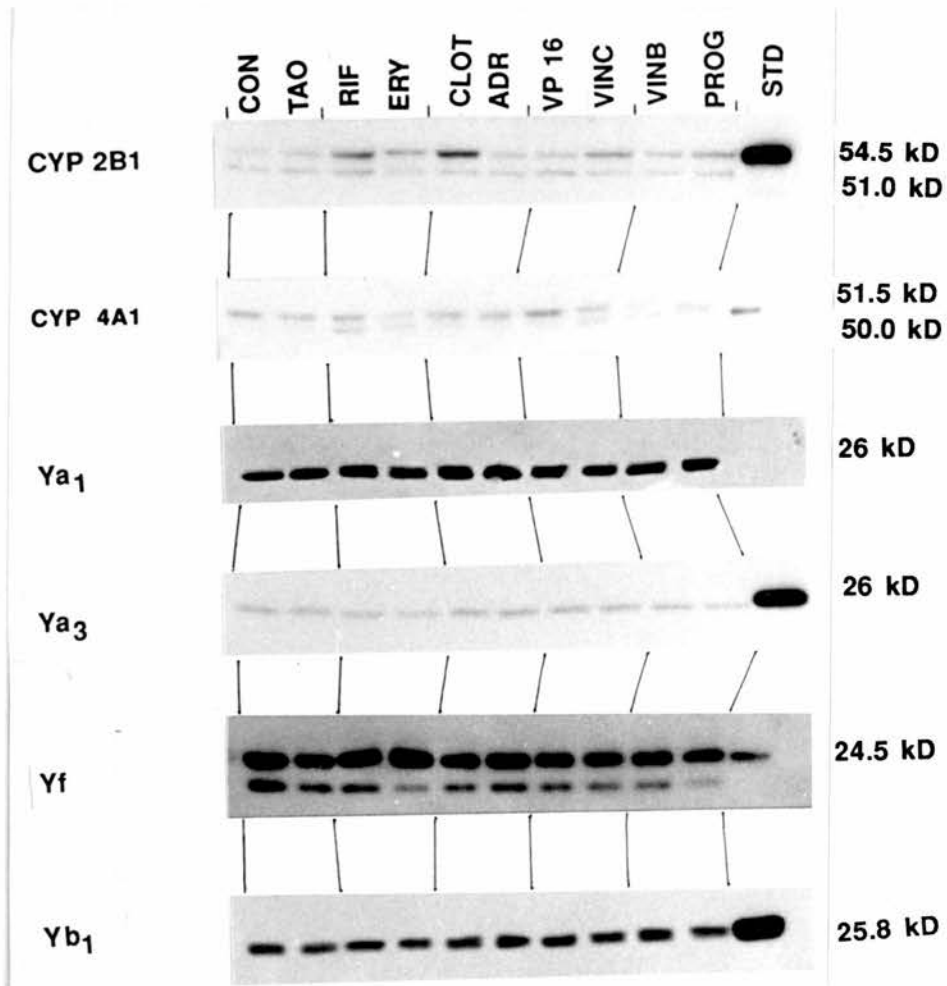
All isozymes measured remained unchanged by treatment in mouse lung (Figure 5.28).

Table 5.12 summarises the immunoblotting results for the P450 and GST protein expression in mouse liver.

**FIGURE 5.27 INDUCTION OF CYTOCHROME P450s AND GST ISOZYMES IN MALE C57BL MOUSE KIDNEY FOLLOWING TREATMENT WITH VARIOUS COMPOUNDS**

Male C57BL/6 mice were treated, as described in Section 5.4.1, and both microsomes and cytosols were prepared from the kidney. Western blotting (30  $\mu$ g of microsomal and cytosolic protein) was performed, as described in Section 2.6.2, using P450 and glutathione S-transferase polyclonal antibodies.

CON	control	ADR	adriamycin
TAO	triacetyloleandomycin	VP 16	etoposide
RIF	rifampicin	VINC	vincristine
ERY	erythromycin	VINB	vinblastine
CLOT	clotrimazole	PROG	progesterone

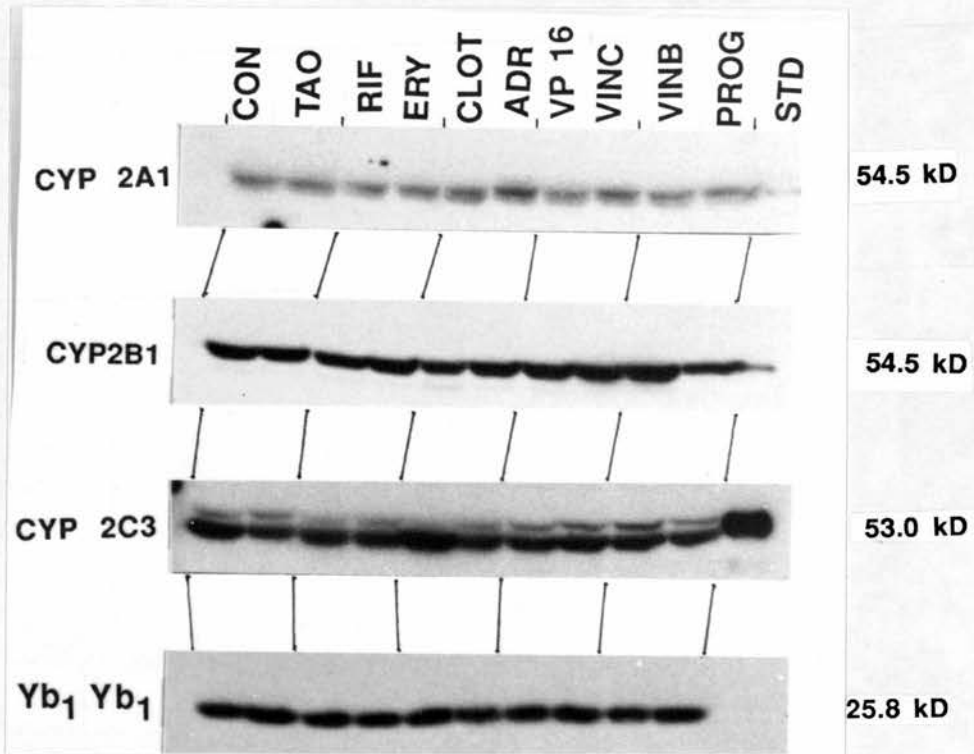


Kidneys from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

**FIGURE 5.28 INDUCTION OF CYTOCHROME P450s AND GST ISOZYMES IN MALE C57BL/6 MOUSE LUNG FOLLOWING TREATMENT WITH VARIOUS COMPOUNDS**

Male C57BL/6 mice were treated, as described in Section 5.4.1, and both microsomes and cytosols were prepared from the lungs. Western blotting (50 µg of microsomal and cytosolic protein) was performed, as described in Section 2.6.2, using P450 and glutathione S-transferase polyclonal antibodies.

CON	control	ADR	adriamycin
TAO	triacetyloleandomycin	VP 16	etoposide
RIF	rifampicin	VINC	vincristine
ERY	erythromycin	VINB	vinblastine
CLOT	clotrimazole	PROG	progesterone



Lungs from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

**TABLE 5.12  
EXPRESSION LEVELS OF P450 AND GST ENZYMES IN C57BL/6-TREATED MOUSE LIVER**

CHEM	DOSE MG/KG (DAYS)	P450 FAMILY						GST				
		2a1	2b1	2c3	2e1	3a1	4a1	4a2/3	Ya1	Ya2	Yb1	Yf
TAO	500 (3)	NC	NC	NC	NC	++	NC	NC	NC	NC	NC	NC
RIF	200 (3)	NC	NC	NC	NC	++	NC	NC	NC	NC	NC	NC
ERY	500 (3)	NC	NC	NC	NC	++	NC	NC	NC	NC	NC	NC
CLOT	50 (3)	++	+++	+	NC	+++	++	NC	NC	NC	NC	NC
ADR	4 (3)	NC	NC	NC	NC	NC	++	NC	NC	NC	NC	NC
VP16	12 (3)	NC	NC	NC	NC	NC	++	NC	NC	NC	NC	NC
VINC	0.3 (3)	NC	NC	NC	NC	NC	++	+++	+	NC	NC	+
VINB	3 (3)	+	NC	NC	++	NC	+++	+++	NC	NC	NC	NC
PROG	1 (11)	++	+++	NC	NC	+++	+++	NC	+++	NC	+	NC

+++ High induction    ++ Moderate    + Slight    NC no change

## 5.5 EFFECT OF BHA ON MDR EXPRESSION IN MOUSE LIVER

### 5.5.1 TREATMENT OF BALB/C MICE WITH BUTYLATED HYDROXYANISOLE (BHA)

8 week old female Balb/c mice were fed the monofunctional anti-carcinogen BHA (7.5 g/kg) for 14 days, as described by Pearson *et al* (1988). Following treatment, both RNA and cytosolic protein were prepared from the snap-frozen liver tissue. Control mice were fed a standard laboratory chow diet. Untreated DBA2/N mouse RNA was included in the Northern blot analysis to act as a control for the *mdr 2* probe.

BHA is a phenolic anti-oxidant and is known to activate the transcription of the GST Ya subunit gene, through the antioxidant responsive element (ARE) (Rushmore and Pickett, 1990).

### 5.5.2 ANALYSIS OF MDR 2 AND GST EXPRESSION IN MOUSE LIVER

#### (a) NORTHERN BLOT ANALYSIS

RNA (15 $\mu$ g) from Balb/c control and BHA treated mouse liver as well as control DBA2/N mouse liver was probed with actin and *mdr 2* cDNAs. RNA amounts were standardised using ethidium bromide staining.

As shown in Figure 5.29, actin levels were found to be approximately equivalent, although the BHA treated mice were slightly higher.

After adjustment for this slight discrepancy in actin levels, *mdr 2* mRNA for BHA treated Balb/c mice liver was still found to be induced two fold over the control.

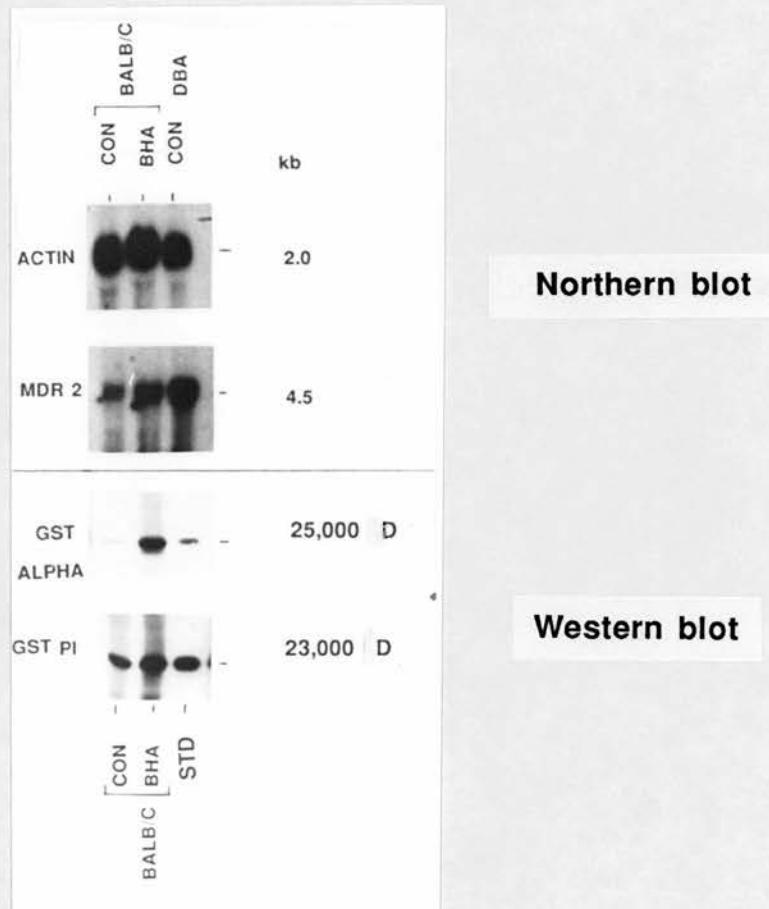
Control DBA2/N mice had far greater expression of *mdr 2* mRNA than control Balb/c mice. Such a difference will be further discussed in Chapter 6.

GST  $\alpha$  protein levels in BHA treated mice were much higher than controls as expected. GST  $\pi$  protein levels were also slightly increased by BHA treatment.

**FIGURE 5.29 LEVELS OF ACTIN AND MDR 2 mRNA AND ALPHA AND PI GST ISOZYME EXPRESSION IN BHA TREATED BALB/C MICE**

Male C57BL/6 mice were treated, as described in Section 5.5.1, and hepatic RNA and cytosolic protein prepared following sacrifice. Northern blotting (15 µg RNA per sample) was performed, as outlined in Section 2.11, using *mdr 2* and actin cDNA probes. Western blotting (20 µg of protein for alpha; 5 µg of protein for pi) was performed, as outlined in Section 2.6.2, using glutathione S-transferase polyclonal antibodies.

- CON control
- BHA butylated hydroxyanisole
- STD standard



Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

## 5.6 SUMMARY OF RESULTS AND DISCUSSION

From previous studies on rat and mouse, it appeared that both rodent groups respond to xenobiotics by inducing the expression of *mdr* genes (Burt and Thorgeirsson, 1988).

The compounds chosen to study the inducibility of *mdr* levels in rat, as shown in Table 5.1, were grouped into P450 inducers, hepatotoxins and carcinogens. The compounds do not strictly adhere to these groupings, for example 2-AAF is both a hepatotoxin and hepatocarcinogen. Much is already known about the effects of these compounds on P450 and GST expression and their mechanism of action.

3-MC is a classical inducer of a major xenobiotic regulated P450 family. Among the enzymes actively induced by 3-MC, is aryl hydrocarbon hydroxylase (AHH) activity (Conney *et al*, 1956). The mechanism of 3-MC induction of AHH activity was found to be under the control of the Ah locus or TCDD receptor. Poland *et al* (1986) synthesised a specific photoaffinity ligand that binds to the TCDD receptor and showed that it has a molecular mass of 95-126 kD (Poland and Glover, 1987). An extensive review of this receptor has been published by Whitlock (1987).

The regulatory pathway by which 3-MC and other polyaromatic hydrocarbons (PAHs) affect the expression of specific genes has been well documented (Eisen *et al*, 1983). A possible model for regulation of CYP1A1 gene expression by the Ah locus might be as follows. PAHs form a ligand complex with the Ah receptor which is translocated to the nucleus and interacts with the xenobiotic responsive element (XRE) leading to transcriptional activation of the P450 gene. The XRE core sequence,

5' - T - GCGTG - 3'

3' - A - CGCAC - 5'

is found in multiple copies of the 5' flanking region of the CYP1A1 structural gene (Whitlock, 1987). For the GST Ya gene, two regions of the 5' flanking sequence have regulatory elements involved in the regulation of gene expression by poly-aromatic compounds (Rushmore *et al*, 1990; Paulson *et*

al, 1990). One of the regions is identical to the consensus sequence shown above but the second region has no identity to such a sequence and is responsive to  $\beta$ -NF and 3-MC as well as contributing to the maximal basal level of expression of the Ya subunit gene. This element is also responsive to phenolic anti-oxidants in cells that lack functional Ah receptors, and is known as the antioxidant responsive element (ARE). However, response of this element to  $\beta$ -NF requires a functional Ah receptor and functional CYP1A1 protein (Rushmore and Pickett, 1990).

Dexamethasone induces the steroid inducible CYP3A1 gene in a non-classical glucocorticoid manner. Either such activation depends on other factors or differences exist between classical glucocorticoid-regulated genes and CYP3A1 in their affinity for receptor ligand binding to cis-acting DNA control elements.

Araclor induces cytochromes in a manner similar to PB. PB induces CYP2B1, CYP2B2 and also CYP3A1. Data regarding the specific PB receptors or cis-acting DNA sequence elements in this induction process are lacking. The PB-like ligand, TCPOBOP, is 660 fold more potent than PB as an inducer in mice but is ineffective in rats (Poland et al, 1981).

Such P450 inducing agents as discussed above were studied to examine their effects on rat *mdr* levels in order to examine whether common induction of any P450 or GST genes occurs in association with the *mdr* genes. Burt and Thorgeirsson (1988) suggested that induction of rat *mdr* genes and P450 1 and 2 gene families by these compounds represent a coordinated response to harmful xenobiotics in the rat liver regulated through the Ah locus. Compounds known to regulate expression via the Ah locus, were administered to rats in order to clarify this hypothesis.

3-methylcholanthrene and  $\beta$ -NF both failed to induce the levels of Wistar rat *mdr* mRNA. Also in Fischer rats, 3-MC caused no increase in *mdr* mRNA above control values. In both strains of rat, 2-AAF and AFB<sub>1</sub> did however induce *mdr* mRNA. 2-AAF induction has not been shown to be mediated via the cytoplasmic Ah receptor. Receptor mediation has been suggested as a mechanism for AFB<sub>1</sub> induction but convincing results to prove such a mechanism are still lacking.

From these results, it is doubtful whether the Ah receptor is involved

in *mdr* induction. However, a receptor or class of receptors, similar to the Ah receptor, may regulate xenobiotic mediated induction of *mdr* expression. Such receptors may activate genes in order to protect the cell from xenobiotic insult.

The induction of rat *mdr* by 2-AAF is known to occur by increased gene transcription in rats exposed to the compound *in vivo* from studies done using nuclear run-on assays.

Whether or not AFB<sub>1</sub> and DEN cause increased transcription or stabilisation of the message is not known.

Marino *et al* (1990) reported that increased expression of the *mdr* gene in regenerating rat liver appears to be regulated by a post-transcriptional event such as message stabilisation. The *mdr* 1 gene is normally a rapidly turned over message, with a half life of 30-60 minutes (Chin *et al*, 1990). The 3' non-coding region of the gene contains the Shaw and Kamen motif ATTTA, is AT rich and is capable of forming stem and loop structures.

Therefore both increased transcription and message stabilisation may contribute to the increase in *mdr* 1 mRNA in treated rat livers.

Both 2-AAF and AFB<sub>1</sub> cause an increase in P-gp protein as assayed using the C219 monoclonal antibody.

C219 reacts with an epitope on the cytoplasmic face of P-gp (Riordan *et al*, 1985). It recognises a region which is highly conserved in all P-gps analysed and represents a universal probe for the protein (Kartner *et al*, 1985). This antibody has been used extensively in studies designed to detect the localisation of P-gp in different tissues in order to elucidate its normal function. Tobe *et al* (1990) used C219 to assay rat renal brush border membranes (BBM) and basolateral membranes and glomeruli by immunoblotting. He found that the apparent molecular weight of P-gp in CHO cells was 170 kD, whereas C219 bound to a protein of 155 kD in BBM. In the immunoblots shown in Figure 5.7 and Figure 5.8 the Mr of the protein detected by C219 in rat liver is 150 kD.

Dex, pyrazole and Ara also strongly induced the expression of P-gp in rat liver. However, these compounds did not increase the level of *mdr* transcription as measured by Northern blotting. This suggests that the RNA

from rat livers treated with Dex, Ara and pyrazole is more efficiently translated and/or the protein translated is more stable.

Many examples exist whereby challenging hepatic enzymes with various chemicals results in increased protein but decreased mRNA levels compared to untreated animals. Rats treated with pyridine display induced expression of CYP2E protein but a decline in CYP2E mRNA expression (Kim and Novak, 1990). This also occurs in rabbits treated with acetone (Porter et al, 1989). The reason for this is thought to be due to increased translatability of the message caused by transfer of RNA to larger polysomes.

In the mouse, DEN induced *mdr 1* in the liver and kidney (Figure 5.19), whereas it suppressed all cytochrome P450 families at the protein level with the exception of *Cyp2b1* (Figure 5.9).

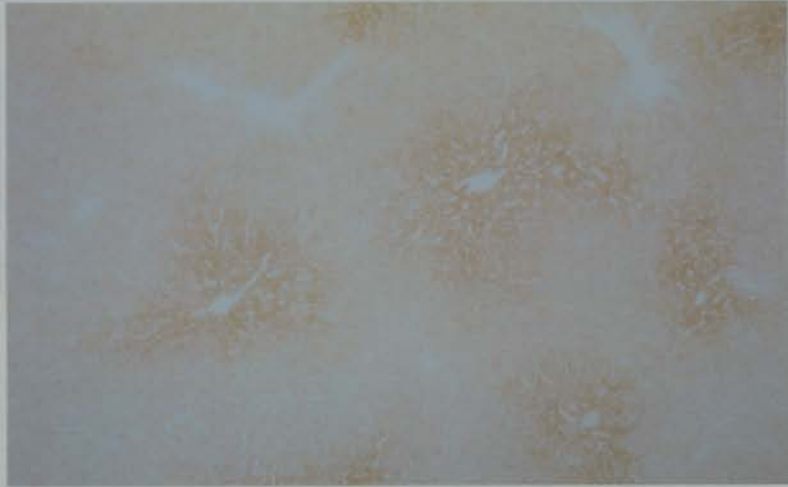
The damaging effects of DEN on rat liver and kidney was assessed (Figure 5.2 - 5.4). The necrotic areas around the central vein in liver and in the kidney tissue indicated extensive and acute damage to the tissues. Actin levels were also induced to a high extent in rat (Figure 5.9) and mouse (Figure 5.17).

Short-term exposure of the liver to a high dose of DEN causes a decrease of P450 enzymes and an increase in *mdr* genes in a very short period of time. P450 isozymes decrease due to a decline in the number of hepatocytes which express P450 enzymes to a high extent, with the development of necrotic areas around the central vein. P450 and GST expression is normally found in these cells (Figure 5.30). Such zonal expression of P450s is decreased in DEN treated liver compared to normal liver as shown in Figure 5.30. In contrast, P-gp expression is induced in the DEN treated rat liver. The hepatotoxic nature of the compounds capable of

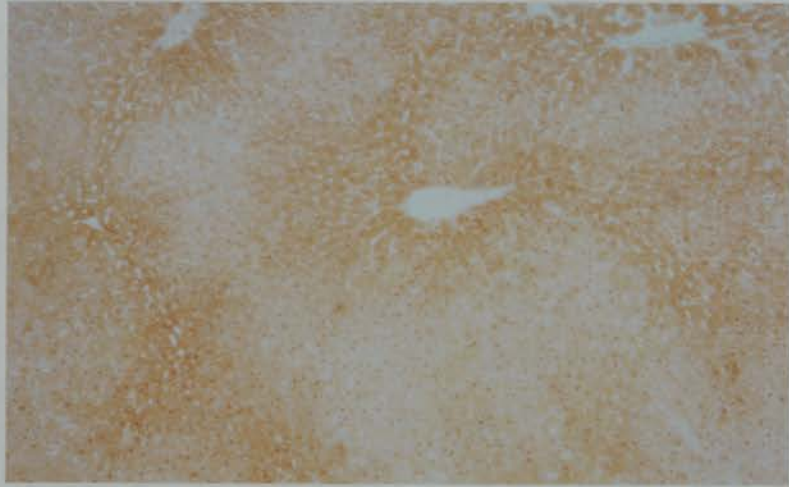
**FIGURE 5.30 EXPRESSION OF P450 AND GLUTATHIONE S-TRANSFERASES IN NORMAL AND DEN TREATED RAT LIVER**

Male Wistar rat was treated with diethylnitrosamine, as described in Section 5.3.3. Control rats were treated with corn-oil. Following treatment the livers were removed and prepared for histological staining, as outlined in Section 2.3.3., using P450 and glutathione S-transferase polyclonal antibodies.

Control  
(x 4 magnification)  
GST  $\alpha$



Control  
(x 4 magnification)  
CYP4A



DEN treated  
(x 10 magnification)  
CYP4A



inducing *mdr 1* expression appears to be significant. DEN may initiate regenerative changes and thus induce *mdr 1*. Liver regeneration is well known to cause increases in *mdr* genes in the rat (Marino *et al*, 1990).

The cell population which is non-necrotic in the DEN treated liver may be rapidly dividing. The increase in actin levels following DEN treatment would suggest such a state. Rapid division of cells often results in a less well differentiated phenotype. This may also partly explain the loss of P450 expression in the liver tissue treated with DEN.

From the studies on xenobiotic induction in the rat, several conclusions were reached.

1. Hepatocarcinogens such as 2-AAF, AFB<sub>1</sub> and DEN quickly induced *mdr* mRNA levels, whereas other well known P450 inducers such as 3-MC and Dex failed to do so.
2. Compounds known to operate via the Ah locus, for example 3-MC and  $\beta$ -NF, did not induce *mdr* mRNA levels. This suggests that *mdr* induction is not operating through this mechanism. The possibility of the induction operating via another receptor however is not excluded.
3. The pattern of *mdr* induction does not mimic any particular P450 family and is therefore presumed to be part of the general response to xenobiotic insult shown by P450 and GST isozymes.

TCPOBOP causes dramatic increases in the expression of all the cytochrome P450 proteins in mouse liver. The mechanism of action of TCPOBOP is unknown, but for any drug-induced response the presence of a potent agonist suggest that the effect may be mediated through a receptor. Some evidence for such a receptor exists (Kende *et al*, 1985; Kelley *et al*, 1985).

The suppression of *mdr 2* and *3* mRNA levels by TCPOBOP may represent an important and effective route by which P-gp expression levels in tumours may be regulated to allow for effective chemotherapy. Certain anti-cancer drugs such as VP-16 and vincristine increase *mdr 2* mRNA levels in mouse liver. Both compounds induced the mouse orthologue of rat CYP4A to a moderate extent. It can be concluded that *mdr 2* expression is effected by some anti-cancer drugs in mouse liver.

Cyp3a family substrates in general do not appear capable of

influencing the level of expression of *mdr 2* mRNA in mouse liver. However, rifampicin and to a small extent progesterone increased the level of *mdr 2* mRNA above those of control.

Progesterone induced many P450 families including the mouse orthologue of rat CYP2A1, CYP2B1, CYP3A1 and CYP4A. It also induced the expression of GST Ya<sub>1</sub> to high levels. Progesterone and deoxycorticosterone cause an increase in labelled vinblastine accumulation and a modest reversal of vinblastine resistance in *mdr J7 VI-1* murine macrophages (Yang *et al*, 1989). Progesterone also inhibits azidopine photoaffinity labelling of endometrial P-gp (Naito *et al*, 1989). These studies indicate that progesterone may be a substrate for the *mdr 1* gene product, P-gp. The regulation of *mdr 1* mRNA by progesterone suggested the possibility that steroid hormones may also be substrates for *mdr 2*. However, progesterone only slightly elevated *mdr 2* mRNA levels in mouse liver. Progesterone also had no effect on *mdr 1* mRNA levels in mouse liver. Therefore, progesterone induction appears to be *mdr 1* and uterus specific.

Butylated hydroxyanisole increased *mdr 2* levels by 3 fold in female Balb/c mice. BHA is known to induce hepatic mRNAs that code for  $\alpha$ ,  $\mu$  and  $\pi$  class GST by 50, 15 and 2-5 fold respectively (Pearson *et al*, 1988). An element known as the ARE has been shown to be required for the activation of the Ya subunit gene, in the rat, by phenolic anti-oxidants (Rushmore and Pickett, 1990). The induction of the *mdr 2* gene by this compound may be due to a similar type of control mechanism.

To examine the transcriptional regulation of the *mdr 2* gene, it will be necessary to characterise the 5' upstream region to identify regulatory elements. The mechanism by which TCPOBOP down-regulates the expression of *mdr 2* and 3 in C57BL/6 and DBA2/N mice may then be elucidated.

## CHAPTER 6

# ENDOGENOUS REGULATION OF MULTIDRUG RESISTANCE GENES IN MOUSE

### 6.1 INTRODUCTION

The endocrine system and the nervous system control adjustments in the body to enable it to adapt to environmental changes. The endocrine system operates through hormones which circulate in the blood and modify the activity of target organs. Some hormones act on several tissues rather than on a specific target tissue, for example, growth hormone (GH), thyroxine and insulin, which implies that the receptors for these hormones are widespread. Other hormones act on one tissue, for example, thyrotrophin, adrenocorticotrophin (ACTH) and gonadotrophins are secreted by the anterior pituitary and have specific target tissues, namely the thyroid gland, adrenal cortex and gonads respectively.

Some of the principle endocrine glands and the hormones they produce are shown in Table 6.1.

Regulation of hormonal release by the anterior pituitary is complex. Modulation of pituitary secretion by hormones secreted from the target endocrine gland is known as "direct negative feedback". Feedback control can occur via the hypothalamus and may be positive or negative. "Short-loop" feedback also occurs, in which the pituitary hormone regulates its own secretion.

#### GROWTH HORMONE

Growth hormone (somatotropin, GH) is a protein hormone produced in somatotrophs in the pituitary gland. GH has many different metabolic effects and stimulates the synthesis of proteins and nucleic acids in a wide range of tissues. Predominant among these is somatomedin C/insulin-like growth factor (SmC/IGF-1) which is stimulated in many tissues, especially

the liver (D'Ercole *et al*, 1984, McConaghey and Sledge, 1970) and probably mediates the actions of GH. Specific binding sites for GH are found in high concentrations in the liver and have been studied in many species

**TABLE 6.1**  
**PRINCIPLE ENDOCRINE GLANDS AND THE HORMONES**  
**PRODUCED**

<b>GLAND</b>	<b>HORMONE</b>
Hypothalamus median eminence	Thyroid releasing hormone Somatostatin Gonadotrophin releasing hormone Corticotrophin releasing factor Growth hormone releasing hormone Prolactin inhibiting factor (Dopamine)
Anterior Pituitary	Thyroid stimulating hormone Luteinising hormone Follicle stimulating hormone Prolactin Adrencorticotrophin
Posterior Pituitary	Vasopressin (antidiuretic hormone) Oxytocin
Thyroid	Thyroxine and triiodothyronine Calcitonin
Parathyroid	Parathyroid hormone
Adrenal cortex	Aldosterone and cortisol
Adrenal medulla	Adrenaline and noradrenaline

Adapted from Gould *et al* (1982).

and cultured cell lines (Wallis, 1980).

The nature of somatomedins is now well understood. Somatomedin C stimulates cartilage growth *in vitro*, DNA synthesis and proliferation. It acts as a growth factor for many cells in culture and appears to synergise with platelet derived growth factor (PDGF) to promote growth (Stiles *et al*, 1979).

In the hypophysectomised rat, circulating levels of somatomedin C are lowered to 5 - 10% compared to those in the intact animal (Maes *et al*, 1986; D'Ercole *et al*, 1984). Greater than 50% of circulating somatomedin C in the rat is produced by the liver (D'Ercole *et al*, 1984) and this is a major target organ for the effects of GH on the production of the polypeptide.

GH also stimulates somatomedin C levels in a variety of other species, including mouse and man (Copeland *et al*, 1980). In man and other species, shortage of GH due to pituitary deficiency lowers somatomedin levels. From studies done by Mathews *et al* (1986) on the GH deficient (lit/lit) mouse and Roberts *et al* (1986) on hypophysectomised rat, it was concluded that GH causes a transcriptional increase in somatomedin C in the liver.

### THYROTROPHIN

Thyrotrophin releasing hormone produced by the hypothalamus stimulates the synthesis and release of thyrotrophin by the pituitary thyrotrophs. Thyrotrophin or thyroid stimulating hormone (TSH) is bound to a specific receptor on the thyroid follicular cell surface and activates release of the thyroid hormones, thyroxine (T<sub>4</sub>) and triiodothyronine (T<sub>3</sub>).

With hypothyroidism, the concentrations of T<sub>4</sub> and T<sub>3</sub> are diminished.

### ADRENOCORTICOTROPIC HORMONE (ACTH)

ACTH is a 39 amino acid peptide synthesised and secreted by the anterior pituitary and acts on several target tissues including the adrenal cortex where it stimulates the synthesis of steroids. ACTH interacts with a receptor in the adrenocortical plasma membrane and activates cAMP which in turn activates a protein kinase within the cortical cells (Schimmer, 1980; Bost and Black, 1986).

The short-term action of ACTH on the adrenocortical cell is the stimulation of the conversion of cholesterol to glucocorticoid, mineralocorticoid or androgen precursor steroids and is catalysed by enzymes in the endoplasmic reticulum and mitochondria (Hornsby, 1988).

As well as short-term effects, ACTH also exerts long-term effects on the synthesis of enzymes involved in steroidogenesis. All the cytochrome P450 enzymes involved are inducible by ACTH, acting via cAMP (Waterman and Simpson, 1985; Zuber *et al*, 1986). The mode by which ACTH causes such induction is not fully understood. John *et al* (1986) showed using nuclear run-off experiments that increased transcription of steroid enzyme genes accounts for increased protein.

### REGULATION OF XENOBIOTIC METABOLISING ENZYMES BY HORMONES

A major mechanism by which the organism regulates xenobiotic metabolism is hormonal control. The control is directed mainly at the liver with most of the pituitary regulated hormones (adrenal, thyroid and sex hormones) involved (for a review see Skett, 1987).

The expression of constitutively expressed cytochrome P450 genes is regulated by hormones in various ways. During development, P450 gene expression is activated. Immediately following birth in rats, CYP2E1, CYP2D1 and CYP2D2 mRNA levels are elevated (Gonzalez *et al*, 1987; Song *et al*, 1986). Changes in the methylation state of the CYP2E1 gene correlates with transcriptional activation (Umeno *et al*, 1988).

The role of pituitary hormones in the regulation of sex-specific P450s has been thoroughly investigated. In rats, pituitary growth hormone secretion in male and female varies, with male rats having a pulsatile pattern of secretion whereas females have a more constant secretory pattern. These patterns are determined by neonatal androgen exposure and adult testosterone levels (Jansson *et al*, 1985). CYP2C11 expression in males is dependent on pulsatile growth hormone secretion and hypophysectomy reduces its expression (Kato *et al*, 1986). Growth hormone secretion is also involved in the regulation of the female specific CYP2C12 (Mode *et al*,

1987). Mice have also been investigated for developmental and sex-specific regulation of P450 enzymes. A female specific testosterone 16 $\alpha$ -hydroxylase in the Cyp2b subfamily has been characterised (Noshiro and Negishi, 1988).

Certain male and female P450 forms are suppressed on reaching puberty. The expression of CYP3A2 mRNA in female rats decreases and leads to loss of testosterone 6 $\beta$ -hydroxylase expression at puberty (Gonzalez *et al*, 1986a). The developmental decrease was found to be due to the continuous levels of GH found in adult females (Kato *et al*, 1986). Waxman (1988b) also suggested that such suppression could be the result of decreased circulating corticosteroid. The actions of GH on hepatic metabolism appear to be inextricably intertwined with those of other endocrine systems notably the thyroid gland and adrenal cortex (Skett, 1987).

Inducibility of genes is also developmentally regulated. TCCD induces the expression of the mouse *Cyp1a1* gene at 10 days of gestation whereas *Cyp1a2* is not activated until after birth (Ikeda *et al*, 1983). In rat, both *CYP1A1* and *CYP1A2* induction occurs one week after birth. Therefore, a species specific difference in developmental control occurs one week after birth (Giachelli and Omiecinski, 1987). In general, most P450 enzymes are not expressed or induced prior to birth.

Cytochrome P450 expression is also tissue specific. Most P450 genes are expressed in hepatocytes, but more specialised steroid hydroxylases, for the biosynthesis of aldosterone and cortisol are found in such tissues as the adrenal gland, ovary and testis. Extrahepatic expression is considerably lower than in the liver.

Changes in GST gene expression during development has been studied most closely in the liver. Before birth, overall GST activity is low whereas after birth the levels increase. At maturity the  $\alpha$  family is more highly expressed in the female and the  $\mu$  family is more abundant in the male (Igarashi *et al*, 1987). In mouse, the Yf ( $\pi$ ) subunit is expressed in male liver at levels approximately 10 fold higher than in the female and constitutes ~70% of the total hepatic GST content whereas in the female it represents only a minor form (McLellan and Hayes, 1987). In rat, GST  $\pi$  occurs at high

concentrations in preneoplastic nodules in both males and females (Kitahara *et al*, 1984). Very little is known about developmental changes in mouse GST isozymes apart from male hormone-dependent expression of the  $\pi$  class in liver (McLellan and Hayes, 1987). Variations in the CDNB-dependent GST activity in female mice have been shown to vary with age in liver, lung and intestinal tissue (Fujita *et al*, 1985).

In rat, growth hormone was found to be most active of the pituitary hormones in influencing steroid metabolism (Wilson, 1976). Growth hormone was discovered to be the factor responsible for the pattern of drug and steroid metabolism in males and females (Mode *et al*, 1983). Administration of oestrogens to male rats feminises the pattern of GH secretion whereas androgens masculinise the GH pattern in female rats (Mode *et al*, 1982).

Many hepatic proteins are sexually differentiated including epidermal growth factor (Ekberg *et al*, 1989), insulin-like growth factor 1 (Maitler *et al*, 1989), prolactin and oestrogen (Norstedt, 1982) and are under control of the GH secretory pattern. In the mouse, a similar system operates. The production of the mouse major urinary proteins (MUPS) and metabolism of hexobarbital is regulated by the pattern of GH release (Macleod and Shapiro, 1989).

The role of thyroid hormones in the control of hepatic drug metabolism was reviewed by Skett and Weir (1983) and Leaky *et al* (1982). The overall conclusions are rather confusing but thyroidectomy does result in substrate- and sex-specific effects on cytochrome P450 drug metabolism. The role of the thyroid gland on Phase II metabolism has also been studied. Moscioni and Gartner (1983) did show however that a thyrotoxic dose of T<sub>3</sub> could decrease bilirubin gluconyl-transferase and increase p-nitrophenol gluuronyl tranferase activity in the rat. Decreased glutathione tranferase activity towards CDNB and decreased levels of glutathione transferase A (3-3), AA (2-2) and L (1-1) were found after thyroid hormone treatment in the rat (Beckett *et al*, 1986; Beckett *et al*, 1988).

## **AIMS**

From studies on the endogenous regulation of both cytochrome P450 and GST drug metabolising hepatic isozymes, it was decided to examine such regulation for the *mdr* genes in mouse. In light of the findings for other hepatic enzyme systems, the possible role of the pituitary was investigated.

The first line of consideration was to examine the effects of removing the pituitary by hypophysectomy and thereby ablating the effects of all pituitary hormones. Subsequent studies relied on the use of animal models to examine the effects of deleting specific pituitary hormones on the expression levels of the hepatic *mdr* genes. These various models are discussed below.

### **HYPOPHYSECTOMY**

Hypophysectomy involves the removal of the pituitary gland, which is the main regulator of the endocrine system. The hypothalamus releases various factors which control the function of the pituitary. Removal of the gland leads to the functional paralysis of the adrenal, thyroid and gonads. Many diverse processes are governed by the anterior pituitary and removal of such a gland is an extremely crude way of examining hormonal control but can provide useful information as an initial study.

However, to determine which hormones are acting directly to regulate specific effects and which are acting permissively, a more subtle approach must be taken.

### **"LITTLE MOUSE" MODEL**

Mutant mouse strains are commonly used to examine endocrine-related phenomena. The "little" mouse mutant was derived from the C57BL/6 strain and has a specific autosomal recessive defect in GH synthesis and secretion. Growth hormone releasing hormone (GHRH) is secreted from the hypothalamus and stimulates GH synthesis and secretion from pituitary somatotrophs via a specific receptor on the surface of these cells. In the

mutant (lit/lit) mouse, GH is neither synthesised nor released in response to GHRH, due to defective cell receptors (Clark and Robinson, 1985; Jansson *et al*, 1986). Homozygous mice are ~60% of the size of normal mice. Pituitary GH content and GH mRNA levels in "little mice" was measured to be 4-8% of normal levels (Cheng *et al*, 1983; Clark and Robinson, 1985). Other pituitary functions are however normal which makes it an excellent model for the study of GH regulation in gene expression.

### TRANSGENIC HYPOTHYROID MOUSE MODEL

In the anterior pituitary, many hormones including thyrotropin, are produced by different specialised secretory cells (see Section 6.1). Since cells with different specialised functions are often physically close to and interspersed with each other, it is almost impossible to produce an experimental animal with a single hormone deficiency using surgery. In order to study the involvement of thyroxine in the regulation of *mdr* genes in the liver, a model in which to explore complete thyroid deprivation was required.

The thyroid gland contains two known types of secretory cell; follicle cells which produce thyroxine and C-cells which secrete calcitonin. The gland is in close contact with the parathyroid gland which secretes parathyroid hormone (PTH). The C-cells lie in clusters between the thyroid follicles. Surgical removal of the thyroid results in the loss of the parathyroid and the thyroid C-cells as well as the follicle cells. Drugs which have been used to inhibit thyroxine production, such as methimazole and propylthiouracil, do not consistently inhibit thyroxine and have toxic side-effects in some cases. However, genetically hypothyroid mice retain significant levels (10%) of circulating thyroxine (Stein *et al*, 1989).

A mouse model employing the technique of transgenic ablation was utilised in this study. The model was kindly provided by Dr. R. Al-Shawi, University of Edinburgh. The basis of such a technique has been well established. Behringer *et al* (1988) targeted the expression of diphtheria toxin A-protein to the pituitary with the GH promoter which resulted in the specific destruction of the pituitary somatotrophs and lactotrophs. An alternative

approach was examined by Borrelli *et al* (1989) and is based on the properties of herpes virus thymidine kinase (HSV1-TK). The expression of the herpes virus gene (HSV-tk) was targeted to the pituitary by means of the GH promoter. Following treatment of the transgenic mice with 1-(2-deoxy-2-fluoro- $\beta$ -D-arabinofuranosyl)-5-iodouracil (FIAU), the somatotrophs and lactotrophs of the pituitary were completely and selectively ablated. Removal of FIAU resulted in regeneration of these cells.

FIAU and Ganciclovir (9-[1,3-dihydroxy-2-propoxy(methyl)]-guanine; DHPG) are phosphorylated by HSV1-TK but not by cellular nucleoside kinases. These compounds are lethally toxic when phosphorylated.

In the model used in this study (Wallace *et al*, 1991), transgenic mice were examined in which HSV-TK expression was targeted to the thyroid follicle cells by coupling the HSV-tk gene to the bovine thyroglobulin promoter (deMartynoff *et al*, 1987). Expression was largely confined to the thyroid follicle cells. Female mice were used since transgenic HSV1-tk reporter genes are expressed in the testis irregardless of the promoter used, resulting in male sterility (Al-Shawi *et al*, 1988).

Mature female mice were infused with DHPG which resulted in complete loss of thyroxine production. The thyroid glands regressed and follicle cells were destroyed. Serum levels of circulating thyroxine fell below the limit of detection within 14 days while the level of PTH was unaffected and calcitonin levels were reduced by less than 50%. Following withdrawal of DHPG, thyroxine levels remained negligible for at least 60 days. The production of mouse major urinary protein (MUP), known to require thyroxine (Knopf *et al*, 1983) ceased in the hypothyroid mice but was restored by thyroxine administration (Wallace *et al*, 1991).

As part of the thesis aims, developmental regulation of expression was briefly examined in order to assess whether or not *mdr* genes are expressed in a similar manner to P450 and GST hepatic enzymes both pre- and post-natally.

Finally, four rodent species were compared to determine the extent of variation of *mdr 2* gene expression.

## 6.2 PITUITARY REGULATION

### 6.2.1 HYPOPHYSECTOMISED MOUSE MODEL - ANIMAL TREATMENT

Male and female C57BL/6 mice were obtained from Charles River Laboratories, Willmington, Massachusetts, USA. These animals were hypophysectomised at eight weeks of age. Control animals were sham-operated. Mice were allowed to recover from the operation for two weeks before treatment. Animals (four per group) were treated with phenobarbital (80 mg/kg, 3 dy, i.p.) and dexamethasone (100 mg/kg, 3 dy, i.p.). Control animals received corn-oil. Animals were sacrificed by cervical dislocation 24 hr after the final treatment. The liver was removed immediately after sacrifice, frozen on dry ice and stored at  $-70^{\circ}\text{C}$ .

Changes in body weight and measurements of triiodothyronine ( $T_3$ ) levels were used to measure the success of the operative procedure. Completely hypophysectomised mice have a stable or gradually decreasing body weight whereas incompletely hypophysectomised animals have an increase in body weight.  $T_3$  decline is also indicative of successful surgery. These measurements had been previously recorded and it was concluded that the hypophysectomy procedure had been successful on these animals (Dolan, 1990).

### 6.2.2 ANALYSIS OF MDR AND GST $\pi$ GENE EXPRESSION FOLLOWING HYPOPHYSECTOMY

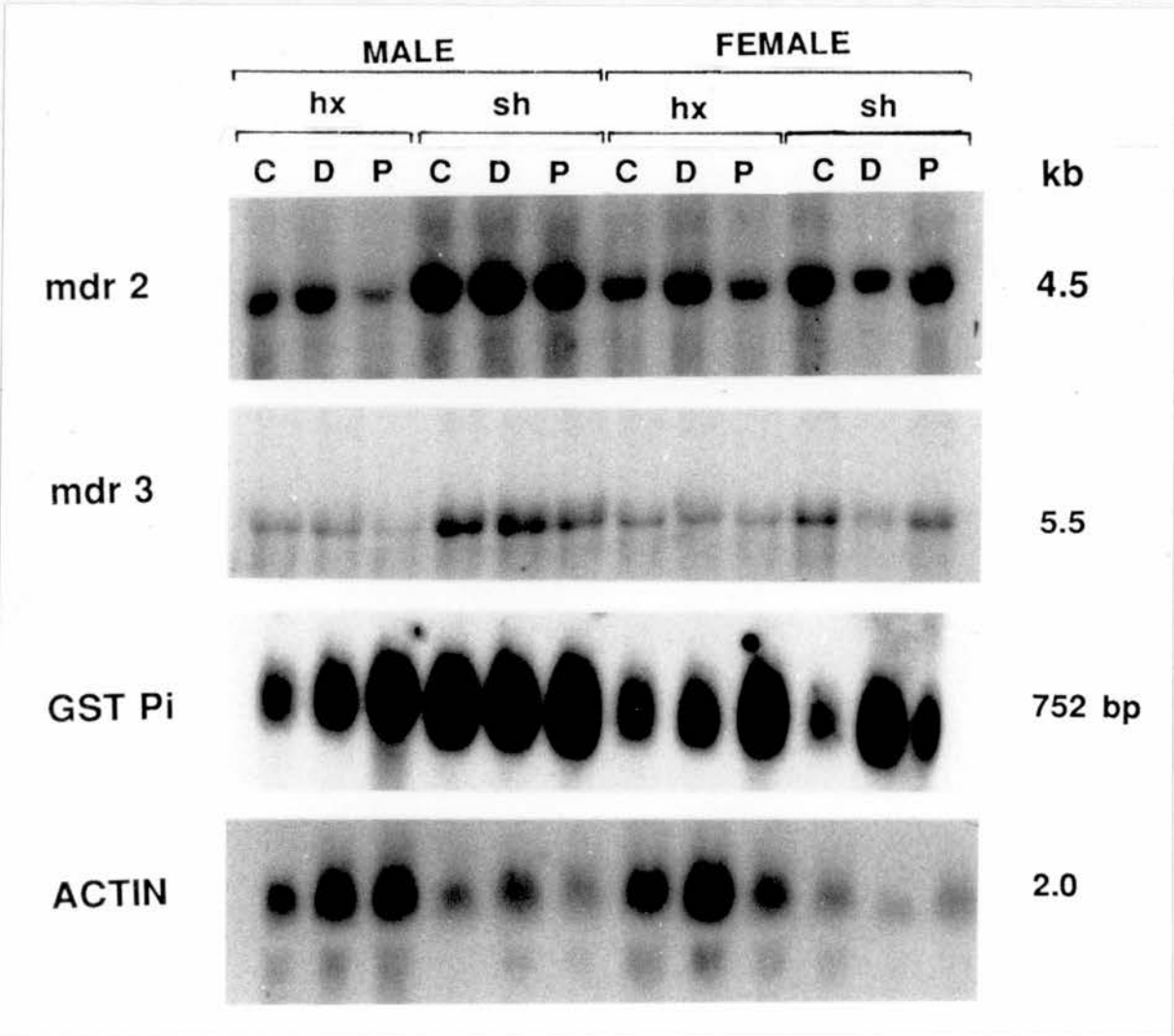
RNA was prepared from male and female hypophysectomised (hypox) and sham-operated (sham) C57BL/6 male and female control, dexamethasone (100 mg/kg, 3 dy) and phenobarbital (80 mg/kg, 3 dy) treated. Dexamethasone and phenobarbital treated mice had been previously used to study the expression of GST Pi following hypophysectomy. The known results of such treatment acted as an internal control for the experiment. RNA (15 $\mu\text{g}$ ) from each treatment was probed with mdr 2, mdr 3, GST  $\pi$  and actin (Figure 6.1).

The actin levels for the hypox animals were consistently higher than

**FIGURE 6.1 LEVELS OF GST Pi, ACTIN, MDR 2 AND MDR 3 IN SHAM AND HYPOX MALE AND FEMALE C57BL/6 MICE TREATED WITH DEXAMETHASONE AND PHENOBARBITAL**

Male and female mice were treated, as described in Section 6.2.2, and RNA was prepared from the liver following treatment. Northern blotting (15 µg per sample) was performed, as described in Section 2.11, using glutathione S-transferase Pi, actin, mdr 2 and mdr 3 cDNA probes.

- hx hypophysectomised
- sh sham operated
- C control
- D dexamethasone treated
- P phenobarbital treated



Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

the sham operated animals, although the ethidium bromide staining of the ribosomal RNA was equivalent. Actin may be regulated to some extent by pituitary factors which are removed after hypophysectomy.

Following hypophysectomy a sharp decline in the levels of GST  $\pi$  in the male mouse occurs. In the female, a slight elevation in hepatic Yf was noted after removal of the pituitary. Treatment with the compounds phenobarbital and dexamethasone had little effect on sham male mice but induced Yf mRNA levels in female mice substantially.

Dexamethasone and phenobarbital may fail to induce Yf levels in male mice due to the already high levels of expression seen in untreated animals. Following hypophysectomy, the male control level falls due to removal of the positive regulation by GH (McLellan and Hayes, 1987). Dexamethasone and phenobarbital are then observed to induce Yf levels above those of the greatly suppressed control levels. Phenobarbital induces mRNA levels to a greater extent than dexamethasone. In female mice by contrast, the control levels rise following hypophysectomy probably due to release of suppression by GH and perhaps other hormones. The induction of Yf levels by phenobarbital and dexamethasone appears to be maintained following hypophysectomy, with phenobarbital inducing Yf mRNA levels to a greater extent than dexamethasone.

The *mdr 2* and *mdr 3* mRNA levels show a similar pattern following hypophysectomy and xenobiotic treatment. In both male and female mice, the levels of *mdr 2* and *mdr 3* mRNA are decreased in hypophysectomised mice compared to sham-operated mRNA levels. The actin levels for sham-operated dexamethasone treated females are low in comparison to the sham-operated control and phenobarbitone treated female mice. Following adjustment for actin, control, dexamethasone and phenobarbital treated sham-operated female mice samples have similar *mdr 2* and *mdr 3* levels of expression, as the sham-operated male mice. Following hypophysectomy, male control, phenobarbital and dexamethasone treated mice *mdr 2* and *mdr 3* mRNA levels are all significantly reduced. Phenobarbital treated mice show greatly reduced levels of both *mdr 2* and *mdr 3* mRNA expression. Such a result may indicate that the suppression caused by phenobarbital in hypophysectomised male C57BL mice is normally masked by the pituitary

factor(s) maintaining the levels of *mdr 2* and *mdr 3* expression observed in the control male animals. Following hypophysectomy, control, dexamethasone and phenobarbital treated female mice mRNA *mdr 2* and *mdr 3* levels were again significantly reduced in agreement with the male pattern. However, phenobarbital does not have the same effect in suppressing *mdr 2* and *mdr 3* levels in hypox female animals as it does in hypox male mice. Therefore the effect of phenobarbital on *mdr 2* and *mdr 3* expression appears to be male specific. This finding does however require to be verified by further treatment of more animals.

### 6.2.3 "LITTLE MOUSE" MODEL - ANIMAL TREATMENT

Male and female "little" mice (*lit/lit*) and controls (+/+) were obtained from Charles Rivers Laboratories, Willmington, USA at the age of eight weeks. The mice were acclimatised for two weeks before treatment commenced. Phenobarbital (80 mg/kg, 3 dy) and dexamethasone (100 mg/kg, 4 dy) were administered to the mutant and normal mice. The animals were sacrificed 4 days following the start of treatment. Livers were removed, snap-frozen and stored at -70°C until required.

### 6.2.4 ANALYSIS OF MULTI-DRUG RESISTANCE GENE EXPRESSION IN "LITTLE MICE"

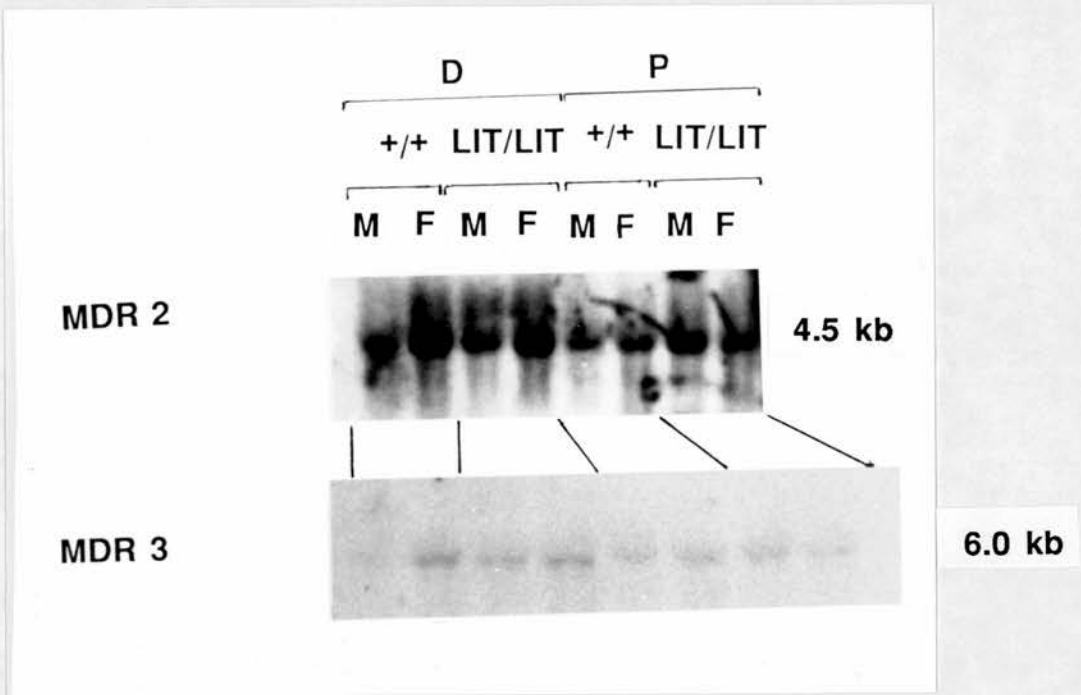
Northern blot analysis was performed on the above animals using RNA (15µg) extracted from the liver. Both *mdr 2* and *mdr 3* levels were examined.

As shown in Figure 6.2, no difference was observed between male +/+ (control) and male *lit/lit* (GH-deficient) treated with dexamethasone. Female +/+ and *lit/lit* treated with dexamethasone and phenobarbital showed equivalent mRNA levels. A slight increase was observed in *lit/lit* male mice compared to +/+ male mice treated with phenobarbital. Since hypophysectomy caused a marked decrease in the expression of *mdr 2* and *mdr 3* mRNA in phenobarbital treated male mice, such a result does not agree with the hypothesis that growth hormone is suppressing *mdr 2* mRNA levels

**FIGURE 6.2      LEVELS OF EXPRESSION OF MDR 2 AND MDR 3 IN +/+ AND LIT/LIT MICE**

Male and female +/+ and lit/lit mice were treated, as described in Section 6.2.3, and RNA prepared from the liver following treatment. Northern blotting (15 µg per sample) was performed, as described in Section 2.11, using mdr 2 and mdr 3 cDNA probes.

+/+	growth hormone sufficient	D	dexamethasone treated
lit/lit	growth hormone deficient	P	phenobarbital treated
M	male	F	female



Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

in these mice. These observations may be due to a slight variation in actin levels. Mdr 3 mRNA levels were much lower than those seen for mdr 2, but were equivalent between +/+ and lit/lit mice.

#### 6.2.5 TRANSGENIC HYPOTHYROID MOUSE MODEL - ANIMAL TREATMENT

Transgenic mouse lines were established by injecting the HSV-tk thyroglobulin construct into either pronucleus of C57BL/6 x CBA/CaF<sub>2</sub> embryos (Al-Shawi, 1988). Transgenic offspring were identified and four lines were established. Transgenic female mice, 12-18 weeks of age, were implanted with mini-pumps to administer DHPG (4.5 mg/dy for 14 days). Control animals were given saline.

#### 6.2.6 ANALYSIS OF MDR GENE EXPRESSION IN HYPOTHYROID MICE

RNA was prepared from both hypothyroid and control mice and probed with actin, mdr 2 and GST  $\alpha$ .

The results, shown in Figure 6.3, demonstrate that the actin levels between control and hypothyroid mice were equivalent. Mdr 2 mRNA levels were also unchanged between hypothyroid and normal mice, which indicated the lack of involvement of thyroid hormones in the pituitary regulation of hepatic mdr 2 mRNA levels. Interestingly, the expression of the  $\alpha$  class GST increased in the hypothyroid animal compared to control. The levels of Ya<sub>1</sub> has been shown to increase in hypox mouse liver (Dolan, 1990). Such an increase may be due, in part, to diminished levels of thyroid hormone affecting the transcriptional expression of the gene.

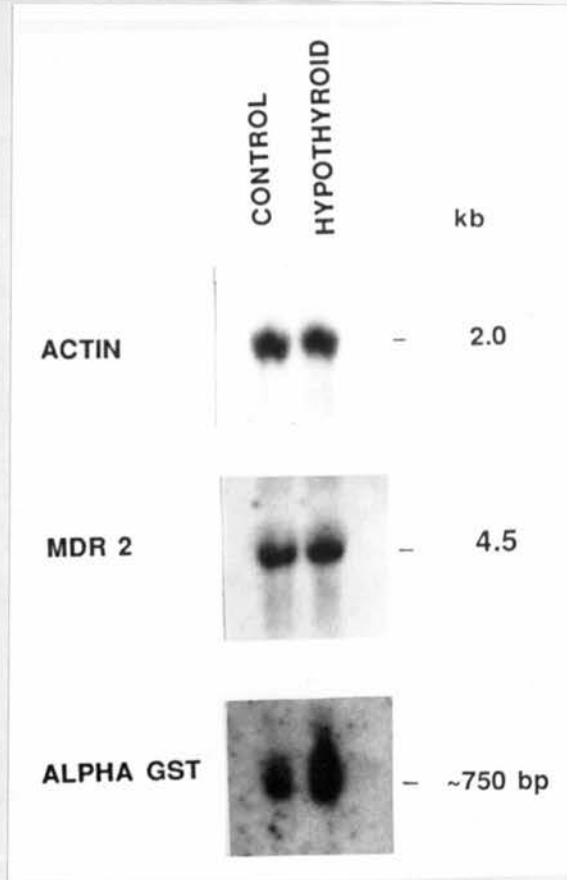
#### 6.2.7 REGULATION OF ADRENOCORTICOTROPIC HORMONE BY DEXAMETHASONE AND TCPOBOP - ANIMAL TREATMENT

In order to establish whether or not adrenal hormones are involved in the regulation of hepatic mdr 2 and mdr 3 gene expression, adult male C57BL/6 mice (4 per group) were treated with dexamethasone (200 mg/kg,

**FIGURE 6.3      LEVELS OF ACTIN, MDR 2 AND GST- $\alpha$  IN  
HYPOTHYROID COMPARED TO NORMAL MICE**

Transgenic mice were obtained, as described in Section 6.2.5, and RNA was prepared from the livers of hypothyroid animals and control animals. Northern blotting was performed, as described in Section 2.11, using actin, mdr 2 and glutathione S-transferase alpha cDNA probes.

Livers from each treatment group (4 mice per group) were pooled. This blot represents the results obtained from one experiment.



3 dy) and TCPOBOP (3 mg/kg, 1 dy). Control animals (6 mice) were given injections (0.2 ml, 3 dy ) of corn-oil.

The mice were sacrificed 4 days following the start of treatment. Cardiac puncture was performed on each animal to collect the serum for measurement of corticosterone levels. Livers were also collected from these animals and frozen at -70°C. RNA was prepared from the tissue to assess whether or not the treatments had effected *mdr* expression levels.

#### 6.2.8 MEASUREMENT OF CORTICOSTERONE LEVELS IN DEXAMETHASONE AND TCPOBOP TREATED MICE

Corticosterone levels were measured in the three groups of mice described above, as outlined in Section 2.13.2.

The standard curve data and graph for the assay is shown in Appendix 2. From the assay, the percentage binding for the control, dexamethasone and TCPOBOP treated mice was found to be 39.4%, 41.1% and 36.6% respectively. From the standard curve, the corticosterone levels were calculated as 380 nmol/l (control), 200 nmol/l (dexamethasone treated) and 660 nmol/l (TCPOBOP-treated).

The control value was as expected. Normal rat corticosterone levels vary between 100-300 nmol/l. Dexamethasone treatment decreased normal corticosterone levels by ~50% and TCPOBOP increased levels by ~70%.

Dexamethasone is a known synthetic glucocorticoid and would be expected to exert negative feedback on ACTH release and thereby reduce plasma glucocorticoid concentrations.

In this study, dexamethasone administered for a period of 3 days did suppress corticosterone levels but the response was not large.

TCPOBOP has been shown to be a potent inducer of mouse microsomal monooxygenase activity (Poland *et al*, 1980). The effects of TCPOBOP in mouse hepatocarcinogenesis have also been well studied. The compound was shown to be a hepatic neoplasm promoter when administered at 3 mg/kg, once a week for 5 months after an initiating dose of diethylnitrosamine; hepatic enzymes involved in drug metabolism were also shown to be induced by TCPOBOP (Dragini *et al*, 1985, 1987).

In this study, TCPOBOP was shown to cause ~70% increase in corticosterone levels. The pathway in the adrenal cortex leading from cholesterol to glucocorticoids (corticosterone in the case of mice) is known to involve several different forms of cytochrome P450 enzymes. Such P450 enzymes in the adrenal may be induced by TCPOBOP to cause an increase in overall corticosterone production. This hypothesis is an interesting one and requires further examination, but is outwith the scope of this study.

#### 6.2.9 ANALYSIS OF MDR GENE EXPRESSION IN ANIMALS WITH ELEVATED AND DEPLETED CORTICOSTERONE LEVELS

Animals were treated as described in Section 6.2.7. Following sacrifice, livers were removed and RNA was prepared from the three groups. RNA (15µg) was probed with *mdr 2* and actin.

As shown in Figure 6.4, the actin level for dexamethasone-treated mice were slightly raised above control levels. Following visual adjustment for actin, it was concluded that TCPOBOP significantly decreased *mdr 2* levels compared to the control value whereas dexamethasone caused only a slight decrease to *mdr 2* mRNA levels. The effects of dexamethasone on corticosterone levels were not considered great enough to be significant in terms of causing changes to hepatic enzymes. The effect of TCPOBOP on C57BL6 mice *mdr 2* mRNA expression in this study was significant and the corticosterone levels were increased substantially.

The effect of dexamethasone treatment following hypophysectomy on *mdr 2* and *mdr 3* mRNA expression was negligible (see Figure 6.1). The effects of corticosterone administration on hepatic drug metabolism *in vivo* are manifested in hypophysectomised rats (Bousquet *et al*, 1965). Therefore adrenocortical steroids can exert effects on hepatic microsomal enzymes independent of the pituitary gland.

The changes in corticosterone levels by TCPOBOP treatment are interesting in light of the decrease in *mdr 2* expression as a consequence of such treatment.

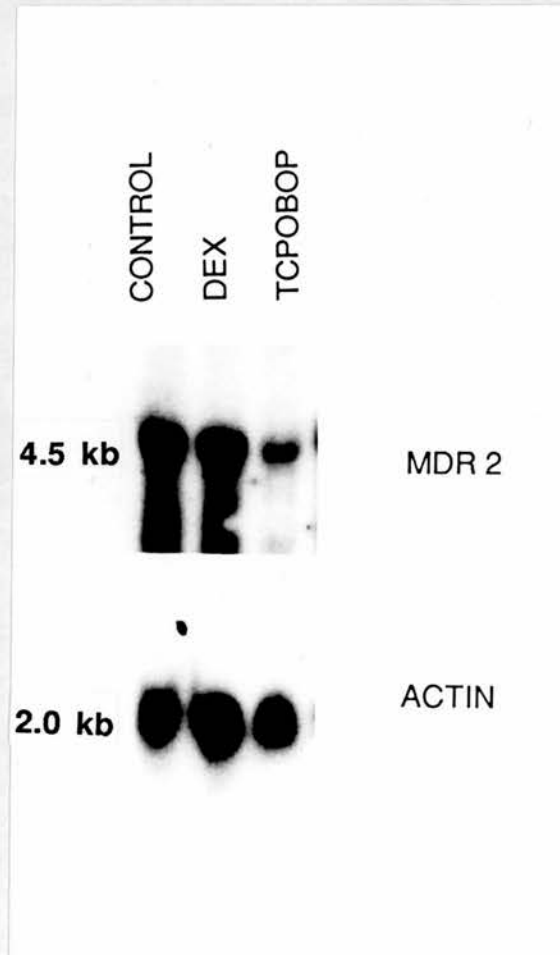
### 6.3 DEVELOPMENTAL REGULATION

Since many hepatic enzymes are developmentally regulated, including cytochrome P450s and GSTs (see Section 6.1), it was of interest to determine whether or not the *mdr* genes were likewise regulated.

#### FIGURE 6.4 LEVELS OF MDR 2 AND ACTIN IN DEXAMETHASONE- AND TCPOBOP-TREATED C57BL/6 MICE

Mice were treated with dexamethasone and TCPOBOP, as described in Section 6.2.7, and RNA prepared from the liver following treatment. Northern blotting was performed, as described in Section 2.11, using *mdr 2* and actin cDNA probes.

DEX                    dexamethasone  
TCPOBOP            1,4-bis [2-(3,5-dichloropyridyloxy)] benzene



Livers from each treatment group (4 mice for dexamethasone and TCPOBOP and 6 mice for control) were pooled. This blot represents the results obtained from one experiment.

In order to examine expression both before birth and pre- and post-pubertally, a time course from 15 days of gestation to 8 week old adult mouse was examined.

### 6.3.1 TIME COURSE OF EXPRESSION - ANIMAL TREATMENT

Untreated C57BL/6 male mice (2 per group) were sampled at 1, 2, 4, 6 and 8 weeks of age. Liver at day 15 of gestation was also obtained from fetal mice (4 animals pooled).

All livers were removed quickly following sacrifice, washed in PBS and snap-frozen on card-ice. Tissue was stored at  $-70^{\circ}\text{C}$  until RNA was prepared from it.

### 6.3.2 ANALYSIS OF MDR GENE EXPRESSION IN EMBRYONIC AND ADULT MOUSE LIVER

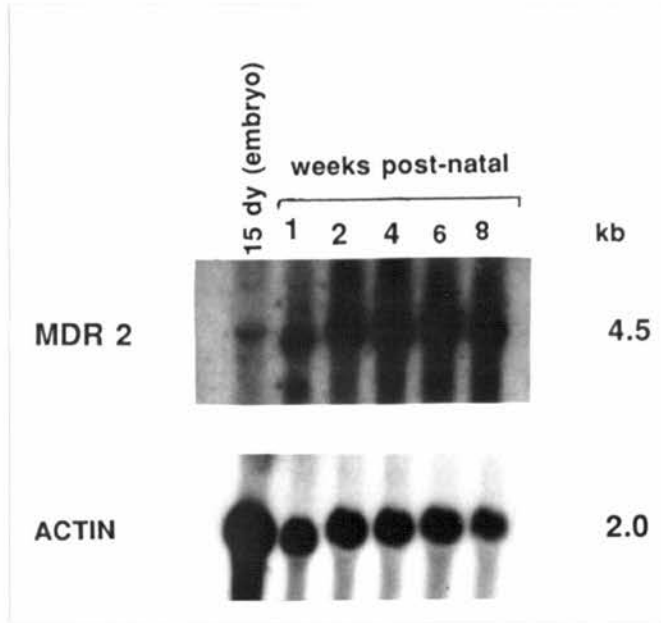
Hepatic RNA was extracted from mice at all time points and RNA (15 $\mu\text{g}$ ) was examined with actin and *mdr 2* cDNA probes. Figure 6.5 shows the results of such analysis on *mdr 2* expression during development.

In the 15 day fetal liver, *mdr 2* mRNA levels are just detectable but very low. Following birth, the levels of *mdr 2* begin to increase and at 2 weeks the *mdr 2* mRNA reaches maximal levels. Figure 6.5 also shows the actin measurements for the same blot. The levels were equivalent for the RNA from 1-8 weeks inclusive. The 15 day fetal liver RNA had been overloaded compared to the other liver samples. Following adjustment for actin, the *mdr 2* levels in the liver appear to be negligible before birth and rise rapidly post-natally.

MacFarland *et al* (1991) investigated the levels of expression of *mdr 1* in fetal tissue. In the fetal brain and testis, the polyclonal antibody MRK16, specific for *mdr 1*, gave strong staining. In the testis the strongest staining was in the interstitial cells (Leydig cells) which actively secrete testosterone. In the brain the cell surface of the Ependymal cells, which line the central canal and ventricular surface between the brain cells and cerebrospinal fluid were stained most strongly. In liver *mdr 1* staining was found on the microvilli

**FIGURE 6.5      LEVELS OF MDR 2 AND ACTIN IN 15 DAY EMBRYO AND 1-8 WEEK OLD C57BL/6 MICE**

Male C57BL/6 mice were sampled at various ages, as described in Section 6.3.1, and RNA was prepared from the liver. Northern blotting (15 µg per sample) was performed, as described in Section 2.11, using *mdr 2* and actin cDNA probes.



Livers from each time point (2 mice per group) and from embryonic liver (4 mice per group) were pooled. This blot represents the results obtained from one experiment.

of the bile canaliculi and in cytosolic granules in some sinusoidal cells. However, the P-gp staining was much weaker in the liver than in other tissues such as the brain. The levels of the mdr 2 gene product has not been examined using immunohistochemistry since a peptide specific antibody is not yet available.

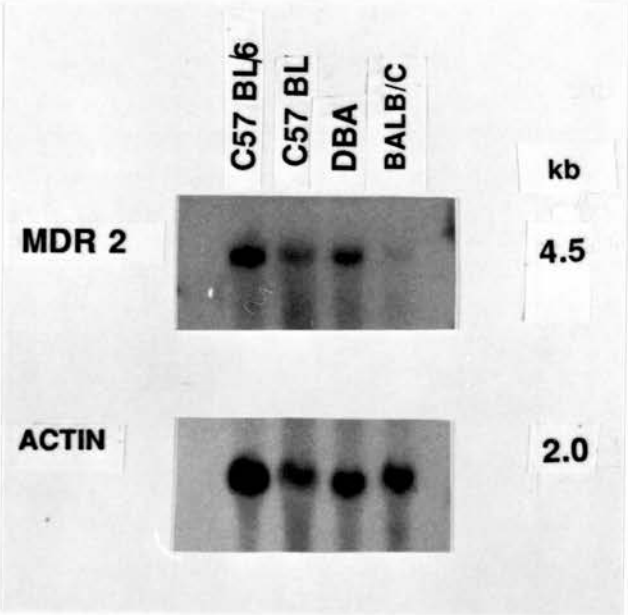
#### **6.4 VARIATION IN RODENT MDR GENE EXPRESSION**

In order to assess whether variation in expression of the mdr 2 gene occurs between different strains of mice, C57BL/6, C57BL (ICRF strain), DBA2/N and Balb/c mouse liver was examined. Mdr 2 and actin mRNA levels were measured. All animals (4 per strain) were adult when sacrificed. Livers were collected, snap-frozen and stored at -70°C until RNA was prepared from it.

Although the actin levels varied, with C57BL/6 being greater than the rest, it was still evident that the Balb/c mice showed negligible levels of mdr 2 gene expression compared to other strains examined (Figure 6.6).

**FIGURE 6.6      LEVELS OF MDR 2    IN C57BL/6 (CHARLES RIVER STOCK), C57BL (ICRF STOCK), DBA2/N AND BALB/C MICE**

Male mice from different strains were obtained, as described in Section 6.4, and RNA was prepared from the liver. Northern blotting was performed, as outlined in Section 2.11, using *mdr 2* and actin cDNA probes.



Livers from each mouse strain (4 mice per strain) were pooled. This blot represents the results obtained from one experiment.

## 6.5 SUMMARY OF RESULTS AND DISCUSSION

From the results in this chapter, it was concluded that *mdr 2* and *mdr 3* gene expression in mouse liver is controlled by the pituitary.

Removal of the pituitary by hypophysectomy resulted in a decrease in *mdr 2* and *mdr 3* gene expression in both male and female animals (Figure 6.1).

However, from analysis of animal models with depleted growth hormone (Figure 6.2) and thyroid hormone (Figure 6.3), neither of these hormones appeared to effect *mdr* expression when their release was impaired. The growth hormone model has previously been used to show the involvement of this hormone in GST expression and the effects of GH depletion was assessed by measuring weight gain and  $T_3$  levels in these animals (Dolan, 1990).

The thyroid hormone model was confirmed as being valid for the study of hypothyroid mice by measurement of MUP and  $T_3/T_4$  levels. Histological examination of follicular cell was also performed. All these parameters confirmed the hypothyroid state of the animals (Wallace *et al*, 1991).

Adrenal hormone status was also examined by using compounds which elevated and depleted corticosterone levels. Depletion of corticosterone levels by dexamethasone was not considered to be substantial enough to mimic the effects of adrenalectomy. Further studies in this regard would require removal of the adrenal gland to ensure complete down-regulation of corticosterone production. However, the effects of glucocorticoids on hepatic *mdr 2* and *mdr 3* expression is dubious since dexamethasone given to mice following hypophysectomy did not cause an elevation in *mdr 2* or *mdr 3* mRNA levels (Figure 6.1). TCPOBOP administration caused an elevation in corticosterone levels and a significant decrease in *mdr 2* gene expression in C57BL/6 mice. The findings from this preliminary investigation would merit further experimentation. This increase in corticosterone is the opposite effect to that seen following pituitary removal and hence does not explain the effect seen with hypophysectomy.

The regulation of *mdr 2* and *mdr 3* gene expression by pituitary

hormones other than GH, thyroid hormone and adrenal hormones seems unlikely since no male/female sex difference in *mdr* expression exists in normal untreated mice. Pituitary hormones such as FSH and LH are known to affect the gonads. Prolactin stimulates milk production and breast development in females and facilitates reproductive function in males.

The models used in this study do not allow for interaction between different types of hormones in a regulated manner. However it is known that certain hormones act synergistically, for example GH and thyroid hormone. Knopf *et al* (1983) reported that transcription of the MUP genes in mouse liver depends on both thyroxine and GH, which appear to act synergistically. Thyroxine stimulates GH gene transcription (Evans *et al*, 1982). Thyroxine deprivation influences MUP expression directly and indirectly through GH. GH deprivation reduces growth by up to 50% for up to at least eight and a half weeks after birth due to GH deficiency and partly due to consequential IGF-I deficiency (Behringer *et al*, 1990). There are many interactions between hormones and it is difficult *in vivo* to dissect out the individual effects of the various hormones. Some hormones, for example glucocorticoids, are permissive agents for the actions of other hormones (Skett *et al*, 1987).

Another approach to the study of hormonal control is by supplementing hormones to hypophysectomised animals. However, such supplementation must mimic the normal pattern of hormone secretion. The use of osmotic mini-pumps can be employed to overcome this problem. Such a system can be readily adapted to allow two (or more) hormones to be administered simultaneously. Using this approach, the interaction between different hormones could be used to assess their role in the regulation of *mdr* gene expression in the liver.

The examination of *mdr 2* gene expression in the liver showed that the expression was very low before birth; was maximal at two weeks following birth and thereafter remained unchanged. In mice, puberty occurs at ~4 - 6 weeks of age and therefore *mdr 2* gene expression is not affected by changes in sex hormone expression occurring at puberty.

Such elevation in expression following birth is commonly observed for many hepatic enzymes and may be due to an increase in xenobiotic

metabolism which occurs concomitantly with birth.

The differences in mdr 2 mRNA expression noted between the Balb/c mouse strain and other strains examined was of interest. Differences in the expression of mdr 2 transcripts was also noted in a study by Teeter *et al* (1990) in which he noted that individual variations in the levels of mdr gene expression within the same strain was apparent and especially true for mdr 2. Whether the low level of mdr 2 gene expression in the Balb/c mouse strain is due to strain variation or individual variation is not known, since these observations are still preliminary ones, made on a small group of animals.

Further studies are required to determine whether the variation in mdr 2 gene expression in different mice is genetically determined. Animal models with different levels of mdr 2 gene expression may be useful in studies on the physiological role of the mdr 2 gene product.

## CHAPTER 7      OVERALL CONCLUSIONS OF THE THESIS AND DISCUSSION

Considerable evidence has accumulated to indicate that P-gp plays a central role in both intrinsic and acquired resistance to cancer chemotherapy. Inhibition of P-gp may aid in overcoming such resistance. An attractive approach to inhibit the function of P-gp is to modulate or suppress P-gp at the transcriptional level. It was presumed that P-gp, like enzymes such as cytochrome P450s may be part of an adaptive response system against environmental toxins. Therefore in an analagous way to the P450 system, P-gp may be subject to regulation by foreign chemicals and hormones. To circumvent the resistance conferred by P-gp, an understanding of how it is regulated is of obvious importance.

The major aim of the work presented within this thesis was to determine the factors which contribute to the regulation of expression of rodent *mdr* genes. The findings presented strongly suggests that both xenobiotic and hormonal controls are involved in such regulation.

In conjunction with *in vivo* xenobiotic regulation studies in rat and mouse, *in vitro* studies using established cell lines of hamster origin were performed to investigate whether or not carcinogens act as substrates for P-gp without prior metabolism by cytochrome P450 enzymes. It was concluded that unmetabolised carcinogens failed to act as substrates for P-gp in cell lines which express the protein to high levels.

### 7.1 XENOBIOTIC REGULATION

At the outset of the project, both C219 and MRK16 monoclonal antibodies were available to study the regulation of *mdr* proteins. However, both antibodies were of limited usefulness for different reasons. Monoclonal antibody C219 was considered to cross-react with proteins, other than P-gp, which were not involved in drug efflux. Thiebaut *et al* (1989) concluded that the antibody cross-reacted with muscle myosin. This conclusion was reiterated by Wishart *et al* (1990) following immunohistochemical studies using C219 on frozen sections of skeletal and granulation tissue, known to

contain myosin. However, more recent findings are not consistent with this proposal. Bradley *et al* (1990) demonstrated the specific expression of the class III hamster P-gp isoform, homologous to mouse *mdr 2*, in cardiac muscle fibres and a subset of striated muscle fibres using C219. To prove that specific staining was achieved, the antibody was competed with excess of the C219-epitope peptide; staining which was non-specific was not removed by co-incubation with the peptide. This study demonstrated that C219 did not cross-react non-specifically with myosin as previously reported.

Monoclonal antibody MRK16, generated against human myelogenous leukemia K-562 cells resistant to adriamycin (K562-Adm), was found to be species specific (Hamada and Tsuruo, 1986). Such an antibody was therefore of no use in the investigation of rodent P-gp expression.

In order to study the expression of P-gp in rodent liver, it was decided to raise polyclonal antibodies for use in Western blotting and immunohistochemistry. The method chosen for raising antibodies was to immunise rabbits with peptide conjugates. The peptides represented 5 sequences of between 5 and 15 amino acids in length covering different parts of the human *mdr 1* gene (Chen *et al*, 1986). This strategy was employed in an effort to raise antisera against specific regions of P-gp which were considered to be highly conserved, extracytoplasmic or potentially immunogenic. Such a strategy for raising antibodies was also adopted and reported subsequently by Marquadt *et al* (1990) and Tanaka *et al* (1990). Marquadt *et al* (1990) prepared antisera against peptides that corresponded to the deduced sequence of human *mdr 1* and found these to be highly reactive with P-gp contained in HL60 cells, isolated for resistance to vincristine. Tanaka *et al* (1990) generated antibodies against the amino- and carboxy-terminal halves of P-gp using recombinant protein fragments produced in *E. Coli*. These antibodies specifically immunoprecipitated <sup>3</sup>H-azidopine-labelled P-gp from *mdr* KB-C1 cells.

This type of strategy for raising antibodies differs from the ones employed by Kartner *et al* (1985) and Hamada and Tsuruo (1986). Kartner raised the C219 monoclonal antibody by immunizing mice with purified

plasma membranes from CH<sup>R</sup>B30 and CEM VLB500 P-gp expressing cell lines. In contrast, Hamada and Tsuruo immunized mice with intact K-562/ADM cells to raise the MRK16 antibody.

In the present study 2 antibodies of high titre were raised following immunisation of rabbits with mdr peptide conjugates. Both these antibodies cross-reacted with P-gp in mdr hamster and human cell lines. Polyclonal antibody 21 detected P-gp in bovine adrenal medulla and adrenal cortex microsomes to the same level found in the CHO colchicine resistant cell line CH<sup>R</sup>C5. Immunoreactivity was shown to be specific by competing the PA21 antibody with an excess of the PA21 epitope peptide; staining that remained following co-incubation with excess peptide was considered non-specific. Similar high levels of P-gp expression have been reported in the hamster adrenal cortex (Bradley *et al*, 1990) and the mouse adrenal gland (Croop *et al*, 1989) as those found in bovine adrenal in the present study. Due to the high levels of expression found in bovine adrenal and the suggested role of P-gp in steroid transport (Thiebaut *et al*, 1987), primary bovine adrenal cell culture would be a potentially useful model to study the regulation of P-gp expression. The relatively large size of the adrenal in this mammalian species would facilitate these kinds of study using primary cell culture.

Polyclonal antibody 21 failed to detect P-gp in normal rat liver and kidney microsomes. Since the sequence of the rat P-gp protein has not yet been published, it is not known whether the epitope (1273-1280 of human mdr 1) against which the peptide antibody was raised is conserved in this rodent species. Low sequence homology in this region of the mdr protein may account for the lack of cross-reactivity in liver and kidney. In contrast, PA61 detected P-gp in normal rat and mouse liver as well as the mdr hamster cell line CH<sup>R</sup>C5. This antibody was raised against a peptide region which mapped to the putative ATP binding site of human mdr 1. From the known mouse and hamster sequences, homology to the human mdr 1 sequence was compared and found to be between 80-100%. This probably explains the strong inter-species cross-reactivity shown by PA61.

Unfortunately, neither PA21 nor PA61 were effective in detecting differences between P-gp expression levels in the mdr cell line CH<sup>R</sup>C5 and

its parental sensitive cell line AuxB1 in immunohistological studies. Both the antibodies cross-reacted with other proteins present in the membrane and cytosol. This is not unexpected since the antibodies were raised against short peptide sequences which could potentially cross-react with many proteins. Antibodies raised against the entire purified P-gp would have an advantage in this respect. Pre-adsorption of the antibodies with pre-immune serum may have increased the specificity of the two antibodies. In retrospect, it would have been more useful to raise antibodies specific for particular rodent proteins, for example the products of mouse *mdr* 1, 2 or 3. Mouse liver is known to express the products of *mdr* 1, 2 and 3 with *mdr* 2 expression being highest. Gene specific antibodies would have been more useful in differentiating between the gene products in liver tissue, and would have allowed an examination of the factors regulating the expression of particular *mdr* gene products at the protein level.

The expression of 3 hamster P-gp isoforms have been studied in normal hamster tissues by immunohistochemical analysis using isoform-specific monoclonal antibodies (Bradley *et al*, 1990). It was shown that each isoform is expressed in small distinct cell groups and is tightly regulated, in some cases by hormonal control. These distinct patterns of expression of P-gp has relevance to chemotherapy. An understanding of the expression of distinct isoforms in normal cells may elude to the basis of P-gp expression in malignant cells.

Since it was already established that certain well-known hepatocarcinogens induced the mRNA levels of P-gp in rat liver (Burt and Thorgeirsson, 1988), studies were conducted to examine the possibility of carcinogens acting as substrates for P-gp. It has been previously established, from examination of rodent cytochrome P450 drug metabolising enzymes, that compounds which act as inducers of different cytochrome P450 isozymes also act as substrates for those enzymes (see review of Gonzalez, 1989). Co-induction of P-gp and cytochrome P450 1 and 2 gene families, representing a coordinated response of rats against harmful xenobiotics, has been suggested by other studies (Burt and Thorgeirsson, 1988). On the basis of potential overlapping regulation of cytochrome P450s and *mdr* genes, the possibility of carcinogens acting as P-gp substrates was studied.

The *mdr* cell line CH<sup>R</sup>C5 and its parental cell line AuxB1 were used in a tissue culture assay to determine whether or not carcinogens could compete with P-gp for efflux of colchicine, a well known P-gp substrate. The carcinogens studied included 2 acetylaminofluorene and aflatoxin B1. These have previously been shown to induce P-gp expression in rats *in vivo* (Burt and Thorgeirsson, 1988). These compounds are also known to be metabolised by and weak inducers of cytochrome P450 gene families 1 and 2 (Astrom *et al*, 1986; Ishii *et al*, 1986). Benzo(a)pyrene, also studied as a potential substrate for P-gp, is also actively metabolised by cytochrome P450 1A1 (Goldstein *et al*, 1982).

Both cell lines were grown exponentially for 4 days in the presence of a non-toxic dose of carcinogen and a range of colchicine concentrations. The cell lines showed a differential response to colchicine; CH<sup>R</sup>C5 cells were 21 fold more resistant than AuxB1 cells.

The assay was designed to show whether or not the unmetabolised carcinogens could compete with colchicine for P-gp efflux and hence increase the toxic effect of colchicine in the P-gp expressing cell line. However, the compounds used in the assay failed to increase the toxicity of colchicine towards the P-gp expressing cell line.

It was concluded from the study that unmetabolised carcinogens could not compete for P-gp efflux and hence do not act as substrates for P-gp. The results from this work may be due to the lack of phase I metabolism in the immortalised *mdr* cell line. The requirement for cytochrome P450 metabolism of the carcinogens in order to interact with P-gp correlates well with the hypothesis proposed by Burt and Thorgeirsson (1988) which suggested the coordinated response of *mdr* and cytochrome P450 families 1 and 2 against xenobiotics. Such a coordinate response may be due to the requirement for P450 metabolism of compounds before they can act as P-gp inducers.

Xenobiotic regulation of P-gp was studied *in vivo* using rodent species. Two rat strains, Wistar and Fischer, and two mouse strains, DBA2/N and C57BL/6, were examined. Regulation of *mdr* gene expression by xenobiotics in these rodent species were shown to vary considerably. However, inter-strain variation was negligible.

In order to determine the type of compounds capable of inducing *mdr* expression, rats were treated with a range of compounds known to be inducers of specific cytochrome P450 isozymes, hepatotoxins and carcinogens. Following treatment of the animals using standard dosing regimes the expression of the inducible cytochrome P450 enzymes was examined at both the mRNA and protein level. The compounds were found to induce specific cytochrome P450 enzymes, as reported in the literature (for a review see Gonzalez, 1989). At the mRNA level, the carcinogens 2-acetylaminofluorene (2-AAF), diethylnitrosamine (DEN) and aflatoxin B1 (AFB1) induced the expression of rat *mdr*. It has been previously proposed that *mdr* genes may respond to the same set of inducers as cytochrome P450s 1 and 2. Since polyaromatic hydrocarbons are known to affect gene expression via the cytoplasmic aromatic hydrocarbon (Ah) receptor (Eisen *et al*, 1983), it was suggested that various ligands, such as AFB1, on binding the Ah receptor, may result in induction of cytochrome P450 1 or *mdr* (Burt and Thorgeirsson, 1988). The results from the present studies disagree with such a hypothesis. 3-methylcholanthrene and  $\beta$ -naphthoflavone, both known to potently induce CYP1A1 via the Ah locus, failed to induce *mdr* mRNA levels whereas 2-AAF and AFB1, which are weak inducers of CYP1A1, successfully induced *mdr* mRNA. These results strongly suggest that the regulation of *mdr* is not via the Ah locus. The original hypothesis was subsequently retracted following further studies conducted in an isolated hepatocyte system using the very potent cytochrome P450 1A inducer TCDD (Thorgeirsson *et al*, 1991). TCDD is the definitive compound for the Ah receptor and it was expected to be the most potent inducer of *mdr*. At concentrations of TCDD found to cause maximal induction of CYP1A, induction of *mdr* expression was very poor, therefore the proposed involvement of the Ah receptor in *mdr* regulation was disproved. However, an alternative receptor (*mdr*-R) sharing overlapping substrate specificity with the Ah receptor and involved in xenobiotic induction of *mdr* expression was hypothesised (Thorgeirsson *et al*, 1991). Experiments conducted to show that inhibition of protein synthesis in primary rat hepatocytes induced *mdr* mRNA levels also suggested the involvement of a distinct trans-acting regulatory protein (Thorgeirsson *et al*, 1991). If such a receptor for *mdr*

induction exists, antagonists for this receptor could potentially overcome resistance.

Results of *mdr* induction in the rat differed from the mRNA level to the protein level. 2-AAF and AFB1 induced both *mdr* mRNA and protein levels. However some compounds which did not induce *mdr* mRNA, such as dexamethasone, arachlor and pyrazole, did induce *mdr* protein levels. These findings suggest that the regulation of *mdr* by xenobiotics may occur by both transcriptional and post-transcriptional mechanisms depending on the compound. AFB1, for example, may induce *mdr* protein levels by increasing transcription rates or improving message stability whereas dexamethasone may induce *mdr* protein levels by increasing the translatability of the *mdr* message. In future, to assess whether or not mRNA levels are induced by increased transcription rates or increased message stability, nuclear run-off assays could be done.

Diethylnitrosamine decreased the mRNA expression of cytochrome P450 enzymes but induced *mdr* mRNA levels. On this basis, the induction of *mdr* mRNA levels by diethylnitrosamine appeared to differ from that of 2-AAF and AFB1. DEN caused widespread necrosis in the liver leading to a decrease in cytochrome P450 expression. However, at the start of animal dosing, DEN may have initially induced cytochrome P450 enzymes, in a manner similar to 2-AAF and AFB1. Therefore, it cannot be concluded whether or not DEN induces *mdr* mRNA by the same or different mechanisms as 2AAF and AFB1. By dosing rats with DEN at a less toxic level it could be determined whether or not cytochrome P450 and *mdr* expression is co-ordinated following administration of DEN.

The regulation of *mdr* mRNA levels by cytotoxic agents was recently studied in rodent cells (Chin *et al*, 1990). It was found that *mdr* levels in immortalised rodent cells increased substantially following exposure of these cells *in vivo* to chemotherapeutic agents such as adriamycin, duanomycin, m-AMSA and mitoxantrone. From nuclear run-off experiments, the increase in mRNA was suggested to be due to both transcription and post-transcriptional mechanisms. This was concomitant with a down-regulation of topoisomerase II. This type of concerted response was hypothesised to be a mechanism by which cells could protect themselves

against the toxic effects of these drugs.

In this thesis, the overall findings of *mdr* regulation by xenobiotics in rats were as follows:

1. Hepatocarcinogens (for example 2-AAF, DEN and AFB1) induced two rat *mdr* genes (5.3 kb and 4.3 kb). The identification of these genes is unknown, although a recent report regarding the cloning and sequencing of the first rat *mdr* cDNA suggests that the 4.3 kb transcript may represent the homologue of the mouse *mdr* 1 gene (Silverman *et al*, 1991).
2. P450 inducers known to operate via the Ah locus failed to induce *mdr* mRNA levels. This mechanism of *mdr* regulation was therefore refuted. An alternative receptor with overlapping substrate specificity, as suggested by Thorgeirsson *et al* (1991), is a viable alternative hypothesis.
3. The induction of *mdr* mRNA and protein in the rat does not appear to mimic the induction of a specific cytochrome P450 gene family or families as previously proposed (Burt and Thorgeirsson, 1988).

Xenobiotic regulation of *mdr* induction in mouse was also studied in the course of the thesis. Gene-specific probes for mouse *mdr* 1, 2 and 3, were reported to correspond to discrete regions showing low sequence homology among the 3 genes (Raymond *et al*, 1990). It has previously been established that mouse *mdr* 1 cDNA can convey the *mdr* phenotype when expressed in the drug-sensitive Chinese Hamster LR 73 cells (Gros *et al*, 1986). However, *mdr* 2 mouse cDNA failed to confer drug resistance following similar transfection experiments (Gros *et al*, 1988). Using these gene specific clones, it was shown that *mdr* expression was associated with the independent expression of *mdr* 1, *mdr* 3 but not *mdr* 2 (Raymond *et al*, 1990). The characterisation of the mouse genes has also involved studies into the tissue-specific expression of these genes in normal mouse tissues (Croop *et al*, 1989). It was found that the expression level of each mouse

mdr gene was dramatically different from tissue to tissue. Specific mRNA transcript sizes of 4.5, 5 and 6 kb for mouse mdr 1, 2 and 3 respectively were detected. Mouse liver was shown to express all 3 genes to some extent, although mdr 2 was identified as the major mdr RNA species in the tissue. Kidney was found to express mdr 1 more strongly than liver.

Since the liver is the major organ of detoxification it was decided to study the expression of mouse mdr genes in this tissue. Foreign compounds are known to both induce and suppress cytochrome P450 gene expression. In order to determine whether or not this was the case for the mouse mdr genes, mice were treated with a wide range of compounds including known modulators of cytochrome P450 as well as chemotherapeutic drugs and carcinogens. Most of the compounds, including the anti-cancer drugs, did not alter the expression of either mouse mdr 2 or 3. Similar to the findings in rat, the polycyclic aromatic hydrocarbons known to operate via the Ah locus, for example 3-methylcholanthrene, had no effect on mouse mdr expression. Of the compounds tested, DEN alone induced mdr 1 mRNA levels and mdr 2 mRNA was induced by vincristine (~2 fold), etoposide (~2 fold), progesterone (~2 fold) and the phenolic antioxidant butylated hydroxyanisole (BHA) (~3 fold). A profound effect on hepatic mdr expression was seen with the phenobarbital-like inducing agent 1,4 bis [2-(3-dichloropyridyloxy)] benzene (TCPOBOP). TCPOBOP is a herbicide contaminant and is known to be an extremely potent modulator of gene expression (Poland *et al*, 1980). Phenobarbital and dexamethasone had similar but less marked effects. These 3 agents had the effect of suppressing the expression of mdr 2 and mdr 3 mRNA in mouse liver. TCPOBOP suppressed the level of mdr 2 well below that of control animals.

To determine the duration of the effect of the TCPOBOP response, mice were sampled for up to 3 months following a single injection (75µg) of TCPOBOP and the effects on cytochrome P450 and mdr expression were studied. Cytochrome P450 enzyme levels, in all the xenobiotic inducible cytochrome P450 families, were still highly induced following this period. This effect has been previously reported and is explained by the fact that TCPOBOP is not metabolised or excreted but stored in adipose tissue (Poland *et al*, 1981).

The mechanism by which TCPOBOP is effective is unknown at present. Since dexamethasone can also induce the expression of similar cytochrome P450s as TCPOBOP, it was suggested that the effects may involve the glucocorticoid receptor. This has now been shown not to be the case (C. R. Wolf, personal communication). However, this does not exclude the possibility that TCPOBOP may be a ligand for a hitherto unidentified receptor. Nebert (1990) proposed that chemicals, such as TCPOBOP, that can activate particular cytochrome P450s are ligands for receptors in the nuclear receptor super-family.

The effects of TCPOBOP on gene expression in mice are the opposite of those associated with the development of drug resistance in rat liver preneoplastic lesions (Sato, 1989) and in a variety of drug resistant cell lines (Cowan *et al*, 1986). Under these circumstances, cytochrome P450 gene expression is suppressed whereas *mdr* is induced. It may be possible to use TCPOBOP to reverse the expression of *mdr* genes and induce cytochrome P450 gene expression in tumour cells. Following treatment of tumourous tissue with an anticancer drug, treatment with TCPOBOP may increase the metabolism of anti-cancer compounds by increasing cytochrome P450 expression but decreasing the efflux of the drug from the tumour cells by suppressing *mdr* expression, and hence improve the usefulness of treatment. To study whether or not TCPOBOP can induce cytochrome P450 genes and suppress *mdr* gene expression in tumours, it will be necessary to treat human tumour xenografts in nude mice with the compound and measure both gene and protein expression.

The mechanism by which TCPOBOP can regulate gene expression is obviously of great interest and importance. Studying and comparing the regulatory elements of the genes which the compound can induce or suppress may determine the important elements involved in such regulation. The regulation of *mdr 1* gene expression in the mouse has been examined by Raymond and Gros (1990). They concluded that the regulation of expression of *mdr 1* may be conferred through interaction of a combination of positive and negative cis-acting factors with regulatory trans-acting factors. It was theorised that negative trans-acting factors could suppress the basal transcription of cells in which *mdr 1* is not expressed.

In a similar manner, TCPOBOP may be able to effect the expression of trans-acting factors involved in *mdr* gene regulation and cause gene transcription to be suppressed. Alternatively, TCPOBOP may be able to alter the methylation state of the *mdr* gene and in this way suppress transcription of mRNA.

In future, to determine the regulatory elements involved in TCPOBOP regulation of the *mdr* 2 and 3 genes, chimeric genes containing 5' deletions in the *mdr* 2 or *mdr* 3 promotor region could be constructed. These constructs, fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene (Gorman *et al*, 1982), could be transfected into cells and used in transient expression assays. This system could measure the level of gene expression in the constructs and allow an assessment of the elements involved in basal and TCPOBOP inducible expression of the mouse *mdr* 2 and *mdr* 3 genes.

## **7.2 HORMONAL REGULATION**

Studies conducted into the regulation of the *mdr* genes were based on the premise that the products of these genes, like cytochrome P450 enzymes, may be part of an adaptive response to toxic insult. Therefore, as found with the cytochrome P450 system, P-gp may be regulated by hormones (Skett,1987). Hormonal control of both phase I (cytochrome P450) and phase II (glutathione S-transferase) drug metabolising enzymes has been previously studied.

In the present study, it was found that both *mdr* 2 and *mdr* 3 gene expression is regulated by pituitary hormones. However, such regulation was considered to be complex since depletion of growth hormone, thyroid hormone and changes in the status of adrenal hormones failed to influence the expression of these genes. No significant differences between male and female *mdr* gene expression was detected. The substantial increase in *mdr* 2 expression in the liver following birth tends to suggest that *mdr* 2 may play a significant role in the adaptive response of an organism to toxic compound exposure which increases at this time. Cytochrome P450s and other drug

metabolising enzymes are known to be elevated in response to increased xenobiotic metabolism during development (Gonzalez *et al*, 1987).

### 7.3 CONCLUDING REMARKS

The original premise on which the studies in this thesis were based was that P-gp, like enzymes such as cytochrome P450 dependent monooxygenases, may be part of an adaptive response system against environmental toxins. By analogy to cytochrome P450s, it was proposed that P-gp may be subject to regulation by hormones and foreign chemicals.

From the main findings of the thesis it can be concluded that xenobiotic compounds can both induce and suppress the expression of *mdr* genes in the rodent species.

In the rat, compounds known to be weak inducers of the cytochrome P450 1 gene family were shown to induce rat hepatic *mdr* expression. However, the involvement of the Ah locus in the regulation of such expression, as suggested by Burt and Thorgeirsson (1988), was disproved, in agreement with subsequent literature (Thorgeirsson *et al*, 1991). The hepatotoxic compound DEN induced rat *mdr* expression liver.

From these findings it was concluded that *mdr* and cytochrome P450 genes may be co-regulated and that regulation of gene expression may operate via common regulatory elements. A model for xenobiotic induced regulation of *mdr* gene expression in rat was suggested by Thorgeirsson *et al* (1991) and is suitable for explaining the results obtained in this thesis with regard to induction of rat *mdr* with foreign compounds.

The model proposes that a receptor (*mdr-R*) is involved in the induction of *mdr* gene expression and shares overlapping substrate specificity with the Ah locus. The model also suggests the presence of a trans-acting regulatory protein involved in the regulation of *mdr* gene expression, distinct from the proposed receptor.

The compounds shown to induce *mdr* in the rat (2-AAF, AFB1 and DEN) may complex with the proposed receptor and induce *mdr* expression as well as cytochrome P450 gene expression. Due to the high dose of DEN

used to treat the rats in the present study, induction of cytochrome P450 cannot be measured following DEN treatment due to the necrosis of cells normally expressing cytochrome P450 genes to high levels.

In the mouse, the phenobarbital-like cytochrome P450 inducing compound TCPOBOP induced cytochrome P450 gene expression to high and sustainable levels with concomitant decreases in *mdr 2* and *mdr 3* gene expression. It is proposed that a receptor for TCPOBOP may be able to form a substrate/receptor complex which can induce cytochrome P450 genes but suppress *mdr* gene expression. Similar inducers such as phenobarbital and dexamethasone, which are known to induce similar cytochrome P450 enzymes, also suppressed the expression of *mdr* genes.

Various experimental findings support a receptor mechanism for TCPOBOP induction. Poland *et al* (1980, 1981) identified TCPOBOP as a potent agonist exhibiting a graded response which was tissue and species specific. Furthermore, Kelley *et al* (1985) conducted studies into the induction of cytochrome P450s in mice by ten structural analogues of TCPOBOP and found that these analogues had distinct structure-dependent potencies. These findings showed that any changes in the pyridyloxy or benzene ring of the compound affected enzyme induction activity. These studies provide evidence for a receptor-mediated response although such a receptor has not yet been identified.

These data regarding the induction and suppression of *mdr* gene expression in the rat and mouse respectively, via cytochrome P450 inducing agents, show that distinct rodent species display differences regarding the regulation of *mdr* gene expression. In the rat, regulation of *mdr* gene expression appears to be via a receptor similar to the product of the Ah locus. Compounds which can induce *mdr* gene expression appear to belong to cytochrome P450 inducers which operate via such a receptor. However, in the mouse *mdr* mRNA levels are suppressed by phenobarbital-like compounds. These cytochrome P450 inducers are considered to operate via a phenobarbital-like receptor. *Mdr* mRNA levels in the mouse are induced by butylated hydroxyanisole (BHA) and some chemotherapeutic agents. BHA is a phenolic antioxidant and known to induce glutathione S-transferase gene expression via xenobiotic regulatory sequences (XREs). Mouse *mdr* gene

expression has also been shown to be positively regulated by pituitary hormones. Regulation of mouse *mdr* genes appears to be somewhat complex and may involve both positive and negative regulatory elements.

Further studies into the regulatory elements contained in the promoters of the mouse *mdr* genes should shed light on the mechanisms involved in both xenobiotic and hormonal regulation.

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## APPENDIX 1

### MATERIAL SOURCES

Aldrich Fine Chemicals Ltd., The Old Brickyard, New Road, Gillingham, Dorset, SP8 4JL	2-acetoaminoflourene, adriamycin, benzo(a)pyrene vinblastine sulphate, vincristine.
Amersham International PLC, UK Sales Office, Lincoln Place, Green End, Aylesbury, Buckinghamshire	$\alpha^{32}\text{P}$ dCTP, Hybond N membrane, $^{125}\text{I}$ - Protein A, Western Blotting ECL detection system
Andermann and Co. Ltd., Laboratory Supplies Division, 145, London Road, Kingston-upon-Thames, Surrey, KT2 6NH	0.45uM pore size nitrocellulose sheets
BDH, Macfarlane Robson Ltd., Burnfield Avenue, Thornliebank, Glasgow, G46 7TP.	acetic acid, ammonium formate, boric acid, bromophenol blue, CDTA, carbon tetrachloride, chloroform, Denhardt's, dimethyl sulphoxide, ethanol, formaldehyde, glycine, hydrogen peroxide, magnesium chloride, methanol, N N' - methylenebisacrylamide, NNN N'-tetramethylethylenediamine (TEMED), orthophosphoric

acid, phenobarbital,  
phenol, poly-acrylamide, KCl, sodium  
acetate, sodium chloride, sodium citrate,  
disodium hydrogen phosphate, sodium  
dodecyl sulphate, sodium dihydrogen  
orthophosphate, sodium azide,  
sodium hydroxide, sodium pyro-  
phosphate, sucrose, xylene cyanol.

Biorad Laboratories Ltd,  
Caxton Way, Watford Business  
Park, Watford, Hertfordshire,  
WD1 8RP.

ammonium persulphate, 4 chloro-1  
naphthol, ethidium

Boehringer Mannheim,  
Boehringer-Mannheim House,  
Bell Lane, Lewes, East Sussex,  
BN7 1LG

dATP, dGTP, dTTP, Klenow fragment,  
RNase A.

Bristol Myers

Etoposide (Vepesid 16)

CIS Ltd., Unit 5,  
Lincoln Park Business Centre,  
Lincoln Road,  
High Wycombe, Bucks.,  
HP12 3RD

C219 monoclonal antibody

DAKO Ltd,  
16 Manor Courtyard,  
Hughenden Avenue,  
High Wycombe, Bucks.,  
HP13 5RE

ABC complex

Difco Ltd., PO B0x 14B, Central Avenue, West Molesey, Surrey.	Agar, bacto-tryptone, yeast extract, trypsin
Fisons, Gallenkamp, Braeview Place, Nerston, East Kilbride, Glasgow, G74 3XJ	2-mercaptoethanol, glycerol
Gibco-BRL Ltd., PO Box 35, Trident House, Renfrew Road, Paisley, PA3 4EF	Restriction enzymes, low melting- point agarose, formamide, guanidine-HCl, $\alpha$ -MEM, RPMI, new- born calf serum, penicillin/strepto- mycin, urea, RNA-ladder, fetal calf serum
Int. Market Supply, Dane Mill, Broadhurst Lane, Cheshire, CW12 1LA	plastic feeding cannula
Koch Chemicals Ltd., 2, Marshgate Drive, Hertford, Hertfordshire	dipotassium hydrogen orthophosphate, potassium dihydrogen orthophosphate
Kodak Ltd., Box 33, Swallowdale Lane, Hemel Hempstead, Hertfordshire, HP2 7EU	Kodak X-Omat AR-5 film
Northumbria Biologicals Ltd.,	DMEM

South Nelson Industrial Estate,  
Cramlington, Northumberland,  
NE23 9HL

Oxoid, Wade Road,  
Basingstoke, Hampshire.

phosphate buffered saline

Pharmacia , Pharmacia House,  
Midsummer Boulevard,  
Milton Keynes, MK9 3HP

Dextran Sulphate, hexadeoxyribo-  
nucleotides.

Scottish Antibody Production  
Unit, Glasgow and West  
Scotland Blood Transfusion  
Service, Law Hospital,  
Carluke, Lanarkshire, ML8 5ES

HRP anti-rabbit IgG, HRP anti-mouse  
IgG

Sigma Chemical Co. Ltd.,  
Fancy Road, Poole,  
Dorset, BH17 7NH

4 acetamidophenol, Aflatoxin B<sub>1</sub>,  
bovine serum albumin, 1, 2-  
benzanthracene, clofibrac acid,  
clotrimazole, coomassie  
brilliant blue R, dexamethasone,  
diethyl pyrocarbonate, erythromycin,  
ethylenediaminetetra acetic acid,  
L-glutamine, isoamyl alcohol, lead  
nitrate, lithium chloride, lysozyme,  
3-methyl colanthrene, MOPS, MTT,  
N, N'-methylene-bis-acrylamide,  
molecular weight rainbow markers,

$\alpha$ -naphthoflavone,  $\beta$ -naphthoflavone,

4-nitroquinolene N-oxide, n-Nitroso-  
diethylamine, phenol red, PMSF,  
proteinase-K, potassium acetate,  
PVP, progesterone, pyrazole,  
rifampicin, tetracycline, TAO, Tris-  
HCl, Tween 20, Type II agarose,  
verapamil.

Whatman, Mackay and Lynn  
Ltd., 2, West Bryson Road,  
Edinburgh, EH11 1EH

3 MM paper

## APPENDIX 2 CORTICOSTERONE STANDARD CURVE DATA AND GRAPH

[Corticosterone] nmol/L (standards/samples)	unbound counts (mean of 3)	bound counts (mean of 3)	% binding (mean of 3)
Total	5218		
2.25	2957	2261	43.3
4.5	2989	2229	42.7
9	3027	2191	42.0
36	3133	2085	39.3
72	3335	1883	36.1
144	3483	1735	33.2
285	3764	1454	27.8
576	4186	1032	19.8
1152	4439	779	14.9
control	3163	2056	39.4
dexamethasone	3069	2149	41.1
TCPOBOP	3307	1911	36.6

From standard curve,

control	<b>39.4 %</b> = 380 nmol/L
dexamethasone	41.1% = 200 nmol/L
TCPOBOP	36.6% = 660 nmol/L

