

**Xenobiotic regulation of cytochrome P450
gene expression**

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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Abbreviations

2-AAF	2-acetylaminofluorene
ACTH	adrenocorticotropic hormone
AHH	aromatic hydrocarbon hydroxylase
AFB ₁	aflatoxin B ₁
Ah	Aromatic hydrocarbon
ALVS	5-aminolevulinate synthase
APS	ammonium persulphate
Arnt	Aromatic hydrocarbon nuclear translocator
BA	benzanthracene
B(a)P	benzo(a)pyrene
BCIP	5-bromo-4-chloro indolyl phosphate
BME	Eagle's basal medium
β-NF	β-naphthoflavone
BSA	bovine serum albumin
BTE	basic transcription element
CAH	idiopathic autoimmune chronic active hepatitis
cAMP	cyclic adenosine monophosphate
CAT	cloramphenicol acetyl transferase
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
cDNA	complementary DNA
CO	carbon monoxide
Coh	coumarin hydroxylase
DEN	diethylnitrosamine
depc	diethylpyrocarbonate
Dex	dexamethasone
DF	dilution factor
DMEM	Dulbecco's modification of Eagle's medium
DMF	dimethylformamide
DMH	dimethylhydrazine
DMSO	dimethylsulphoxide
DNS	denaturing solution
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diammine tetraacetic acid

ER	endoplasmic reticulum
ER	oestrogen receptor
ESR	electron spin resonance
EtBr	ethidium bromide
FAD	flavin adenine dinucleotide
FCS	foetal calf serum
FMN	flavin mononucleotide
FSH	follicle stimulating hormone
GRE	glucocorticoid responsive element
GST	glutathione S-transferase
H & E	haematoxylin and eosin
HRP	horseradish peroxidase
HSP	heat shock protein
Ifn	interferon
IGF-1	insulin-like growth factor 1
IgG	immunoglobulin G
IL	interleukin
Hx	hypophysectomised
kb	kilobase
kD	kilodalton
Kd	dissociation constant
LH	luteinising hormone
LKM	liver-kidney microsomal antibody
LTR	long terminal repeat
3-MC	3-methylcholanthrene
MDR	multidrug resistance
MMTV	murine mammary tumour virus
MOPS	morpholinepropane sulphonic acid
MRA	morpholino anthracycline (doxorubicin)
MRA-CN	cyanomorpholino anthracycline (doxorubicin)
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
M _r	molecular weight
MT	metallothionein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MUPS	major urinary proteins
NADPH	β-nicotinamide adenine dinucleotide phosphate (reduced form)

NaPPi	sodium pyrophosphate
NBT	nitroblue tetrazolium
NDEA	N-nitrosodiethylamine
NDMA	N-nitrosodimethylamine
OD	optical density
OLB	oligo labelling buffer
P450	cytochrome P450
PAH	polycyclic aromatic hydrocarbon
PB	phenobarbital
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PCN	pregnenolone 16- α carbonitrile
pcna	proliferating cell nuclear antigen
PPAR	peroxisome proliferator activated receptor
P/S	penicillin/streptomycin
PTH	parathyroid hormone
PVP	polyvinyl pyrrolidone
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERRS	surface enhanced resonance Raman spectroscopy
Sh	sham-operated
SSC	150mM sodium chloride, 15mM sodium citrate
TA	tienilic acid
TACE	chlorotrianisene
TBE	tris-boric acid-EDTA
TBST	tris buffered saline with 0.5% Tween 20
TCDD	tetrachlorodibenzo-p-dioxin
TCPOBOP	1,4 bis 2-(3,5 dichloropyridyloxy) benzene
TE	tris-EDTA
TEMED	N,N,N'N'-tetramethyl ethylene diamine
TLC	thin layer chromatography
TNF	tumour necrosis factor
Topo II	topoisomerase II
XRE	xenobiotic response element

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Publications arising from research

1. 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene - An extremely potent modulator of murine hepatic cytochrome P450 expression

Gillian Smith, Colin J. Henderson, Malcolm G. Parker, Roger White, Remi G. Bars & C. Roland Wolf

Biochemical Journal, in press, 1992

2. Human tumour xenografts as an *in vivo* model to study the regulation of human cytochrome P450 genes

Gillian Smith, David J. Harrison, Nick East, Frances Rae, Helga Wolf & C. Roland Wolf

Submitted to Cancer Research, 1992

3. Molecular genetics of the human cytochrome P450-dependent monooxygenases

C.R. Wolf, C.A.D. Smith, G. Smith, A.C. Gough, S. Bryant, N.K. Spurr & D.J. Harrison
Proceedings of the Sendai International Symposium on Recent Advances in Cancer Research

4. Production of cytochrome P450 reductase yeast-rat hybrid proteins in *Saccharomyces cerevisiae*

H. Frances J. Bligh, C. Roland Wolf, Gillian Smith & Jean D. Beggs

Gene, **110**, 33-39, 1992

5. Regulation of multi-drug resistance gene expression in the mouse liver

Alison L. Russell, Colin J. Henderson, Gillian Smith & C. Roland Wolf

Submitted to Cancer Research, 1992

Abstract

Cytochromes P450 (P450) are a ubiquitous class of monooxygenases located in the endoplasmic reticulum of mammalian cells. These enzymes catalyse the Phase I oxidative metabolism of a wide range of structurally diverse chemicals resulting in increased hydrophilicity and excretion. Certain chemicals are, however, metabolically activated by cytochrome P450, leading to the formation of cytotoxic and/or carcinogenic metabolites. This has been exploited in the design of many prodrugs, including anti-tumour agents, which are inactive as administered but become active in vivo following metabolism by one or more of the P450 isozymes. The regulation of P450 gene expression has been well documented in experimental animals, but at present there is very little information available about the regulation of human P450 genes, particularly in extra-hepatic tissues.

Regulation of P450 expression by a range of xenobiotics, known to have profound effects on the expression of rodent P450 genes, has been studied in a mouse model and in cultured cells. Of particular interest were the potent and pleiotropic effects on murine P450 expression of TCPOBOP (1,4 bis [2,(3,5-dichloropyridyloxy)] benzene), which showed marked tissue and species specificity in its inductive effects in rodents. A model was developed, using human tumours grown as xenografts in immune deficient mice, in which the in vivo regulation of human P450 genes could be examined. TCPOBOP was shown to be equally effective at influencing human P450 gene expression and, in most cases, the patterns of gene regulation observed in experimental animals were also seen in the human tumours. These studies suggest that modulation of intra-tumour P450 levels by agents such as TCPOBOP may lead to enhanced metabolism of anticancer drugs such as cyclophosphamide, which require P450-mediated activation in order to exert their anti-tumour effects. Preliminary results with cyclophosphamide and TCPOBOP indicated that this was indeed the case. The cell line Hepa 1, which did not respond to P450 inducing agents in vitro, became responsive to the same inducing agents when grown in vivo as a solid tumour xenograft. This therefore provides an extremely powerful model for studying the in vivo regulation of human P450 genes, not only by xenobiotics, but by hormonal or metabolic factors which are difficult to assay in vitro.

Chapter 1: Introduction

1.1 Introductory Remarks

In 1775, a British surgeon, Sir Percival Pott, proposed that a link existed between the incidence of scrotum cancer in chimney sweeps and their exposure to soot. It was not until the 1930s, however, that polycyclic aromatic hydrocarbons such as dibenz[*a,h*]anthracene and benzo[*a*]pyrene were identified as the carcinogenic agents in coal tar. Subsequent epidemiological studies have shown that many ubiquitous environmental pollutants are potent carcinogens. Indeed, environmental chemicals have been strongly implicated as the causative agent in many types of cancer, and it has been proposed that up to 80% of all cancer incidence is related to carcinogen exposure (Doll & Peto, 1980).

Although many of these compounds are inherently cytotoxic and/or carcinogenic, others require metabolic activation before exerting their carcinogenic effects. A superfamily of inducible microsomal monooxygenases, the cytochromes P450 (P450) have been shown to play a central role in this metabolic activation. Both inter-individual variation and genetic polymorphism in the expression of certain of these P450 enzymes has been linked to cancer incidence. Cytochromes P450 metabolise a wide variety of structurally diverse chemicals, several of which have an important endogenous role. In many cases, P450 metabolism results in the introduction of a hydrophilic moiety into an otherwise hydrophobic molecule, thus facilitating its excretion from the body. Other substrates, however, are metabolically activated by P450 enzymes leading to increased cytotoxicity or carcinogenicity. This property is exploited in the metabolism of many anticancer drugs which are inactive as administered, but become active as anti-tumour agents following activation by one or more of the P450 isozymes.

Understanding the factors which regulate the expression of cytochrome P450 genes is therefore of central importance. A number of model systems have been established which have provided valuable information about how P450 genes are regulated in experimental animals, but very little information is currently available about the regulation of cytochrome P450 genes in man, particularly in extra-hepatic tissues. The experiments described within this thesis are designed to address this question and, additionally, to determine to what extent the mechanisms of regulation observed in rodent and cell culture models are applicable to the regulation of human P450 genes.

1.2 Introduction to Drug Metabolism

Almost any foreign chemical entering the body, whether it be a clinical drug or environmental toxin, is metabolised by one or more of the drug metabolising enzymes. These enzyme systems, which also have an important endogenous role in maintaining cellular homeostasis, generally have wide substrate specificities, such that each can metabolise a variety of structurally diverse chemicals. The enzymes which are involved in drug metabolism, the drug metabolising enzymes, are subdivided into two classes - Phase I and Phase II.

Phase I enzymes catalyse functionalisation reactions where, in most cases, a reactive centre is created in the substrate such that it is can participate in subsequent Phase II metabolism. Phase II reactions, in contrast, are conjugative, involving the addition of a hydrophilic moiety to the molecule to facilitate its excretion from the body.

Phase I reactions: Oxidation, reduction, hydrolysis and hydration are all examples of Phase I reactions, mediated by a number of enzyme systems including alcohol dehydrogenase and xanthine oxidase. One, if not the most important of this class of enzymes, however, is the microsomal mixed function oxidase, cytochrome P450 (P450). This ubiquitous class of enzymes plays a central role in the oxidative metabolism of structurally diverse xenobiotics and also has an important endogenous role in the synthesis and/or degradation of many important biosynthetic intermediates. P450 structure and function will be discussed in Section 1.3.1, while the regulation of cytochrome P450 expression in a number of model systems will form the basis of Chapters 3, 4 and 5. In most cases, the products of these Phase I reactions contain a chemically reactive functional group e.g. -OH, -NH₂, -SH, -COOH, which forms the reactive centre for interaction with Phase II enzymes.

Phase II reactions: Again, several distinct enzyme systems are responsible for the Phase II metabolism of a structurally diverse range of chemicals, resulting in the generation of water soluble products which are excreted in bile or urine. Examples of common Phase II reactions are glucuronidation, catalysed by the UDP-glucuronosyltransferases and glutathione conjugation, catalysed by the glutathione S-transferases. Although xenobiotic regulation of Phase II metabolism will not be discussed in detail in this thesis, it is important to realise that many of the compounds e.g. phenobarbital and 3-methylcholanthrene, which are powerful modulators of cytochrome P450 expression can also influence the expression of

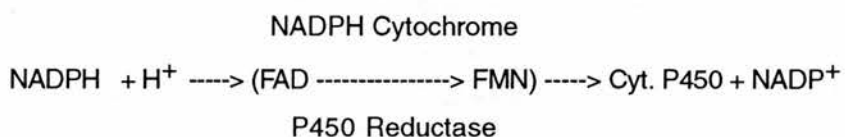
Phase II enzymes such as the glutathione S-transferases. Thus, the effect of a particular xenobiotic can not be considered simply in terms of its Phase I metabolism. The ultimate disposition and action of a particular compound within the cell is therefore a balance between its affinity for several distinct enzyme systems which are active in its metabolism. Similarly, xenobiotic-mediated induction of a specific isozyme e.g. a hepatic P450, will alter the overall enzyme profile of the cell, and may thus affect cellular homeostasis.

1.3 The Cytochrome P450 mono-oxygenase superfamily

1.3.1 Introduction to cytochrome P450 - structure and catalytic function

Cytochrome P450 is the generic name for a superfamily of haem-containing monooxygenases located, like many other Phase I enzymes, in the endoplasmic reticulum of mammalian cells. P450s are monomeric enzymes, with molecular weights in the range 45-55 kD, which function as the terminal oxidases (electron acceptors) in an electron transport chain, supported by the flavoprotein NADPH-cytochrome P450 reductase. The name cytochrome P450 arises from the spectral absorbance maximum obtained at 450nm when the protein is reduced and complexed with carbon monoxide (CO). P450 structure, function and regulation has recently been extensively reviewed (Gonzalez, 1990; Gonzalez & Nebert, 1990; Okey, 1990).

NADPH-cytochrome P450 reductase is a 78kD monomeric flavoprotein which contains one mole of flavin adenine dinucleotide (FAD) and one mole of flavin mononucleotide (FMN) per mole of apoprotein. The role of NADPH-cytochrome P450 reductase is to transfer reducing equivalents (electrons) from NADPH to cytochrome P450 according to the following equation:



From the stoichiometry of the above equation, it can be seen that the left hand side (NADPH + H⁺) is a 2 electron donor, while the right hand side (Cytochrome P450) is a one electron acceptor. Although it has not been formally proven, it is hypothesised that NADPH-P450 reductase accepts both electrons and then transfers them sequentially to cytochrome P450. By measuring the relative redox potentials, it appears to be more energetically favourable (ΔE^0 is more positive) for FAD to act

as the electron acceptor from (NADPH + H⁺), and FMN to transfer electrons to cytochrome P450.

Cytochrome P450 is present in a large (10-25 fold) molar excess over reductase (Estabrook et al, 1971; Peterson et al, 1976). The functional interaction between P450 and reductase, however, appears to exist as a 1 :1 ratio (Miwa et al, 1979; Miwa & Lu, 1984). Therefore, only a fraction of the total microsomal P450 can exist as a functional complex at any one time. Accessibility to reductase is therefore rate-limiting, particularly in cases where the levels of P450 apoprotein are significantly increased by exposure to a drug or environmental pollutant. Although, in certain cases, the level of reductase is also increased on exposure to these agents, the fold increase is usually significantly lower than for the corresponding P450 apoprotein.

The nature of the interaction between P450 and reductase is not yet fully understood. Two possible mechanisms have, however, been proposed (Miwa & Lu, 1984; Wagner et al, 1984):

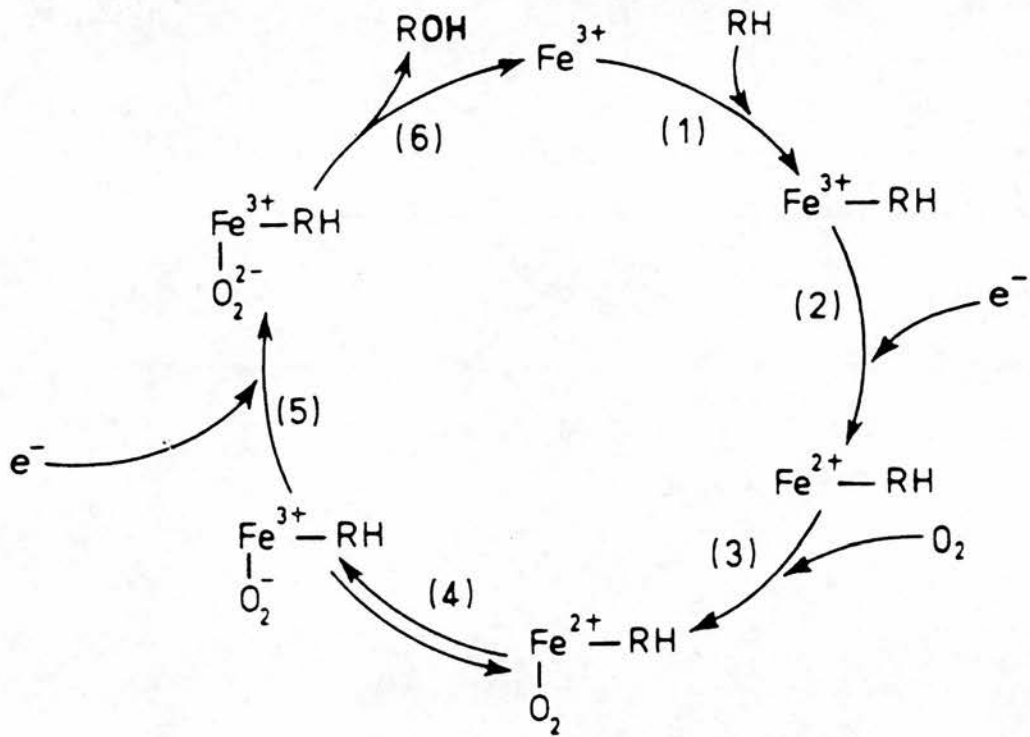
- (a) P450 and reductase form a stable, catalytically active complex
- (b) Transient complexes are formed as a result of random collisions occurring from lateral diffusion within the microsomal membrane

Although the exact mechanism of interaction has not yet been elucidated, and it has therefore not been demonstrated to what extent the mechanism of interaction is conserved between different P450 isozymes, recent evidence suggests that there is an electrostatic interaction between P450 and reductase which mediates electron transfer (Bernhardt et al, 1984, 1988). Based on the crystal structure of P450cam, the soluble camphor hydroxylase, isolated from *Pseudomonas putida*, it has been suggested that specific ionic amino acids such as lysine and arginine interact with putidaredoxin, the bacterial equivalent of NADPH-cytochrome P450 reductase. Similarly, Shimizu et al (1991) demonstrated that specific lysine and arginine residues were involved in forming an electron transfer complex in the interaction of rat CYP1A1 with reductase. Shen & Strobel (1992) showed that modification of these lysine residues with acetic anhydride lead to a 95% decrease in 7-ethoxycoumarin hydroxylation in a reconstituted CYP1A1/reductase complex. Spectrophotocatalytic analysis indicated that this did not occur as a result of major conformational change.

P450 heme is present as a prosthetic group, ferric photoporphyrin IX, which is non-covalently bound to the apoprotein. Cyclic oxidation/reduction of the

heme iron is central to the activity of the enzyme. Although the catalytic cycle is not yet fully understood, a simplified reaction scheme is illustrated in Figure 1.3.1.

Figure 1.3.1 Catalytic cycle for P450 oxidation



The overall stoichiometry of the reactions catalysed by cytochrome P450 can be represented by:



where RH is substrate and ROH is oxidised substrate.

Step (1) in Figure 1.3.1 illustrates substrate binding to ferric iron. Substrate binding occurs at the opposite side of the porphyrin ring to the cysteine sulphur.

Step (2): First electron reduction of ferric to ferrous iron.



The electron is derived from [NADPH + H⁺], and is transferred to cytochrome P450 by NADPH-cytochrome P450 reductase. This electron transport system is modified in mitochondrial P450s, such as those found in the adrenal gland - in this system a non-heme protein, adrenodoxin and a flavoprotein, NADPH-adrenodoxin reductase mediate the transfer of electrons from NADPH to reductase (Lambeth et al, 1980)

Step (3): Binding of molecular oxygen. This has not been well characterised to date in mammalian systems, but the intermediates shown have been identified spectrophotometrically in the adrenal mitochondria and bacterial P450cam.

Steps (4) and (5) are hypothetical electron re-arrangement reactions. The intermediates in this proposed pathway are highly reactive and have not yet been isolated and characterised. A second electron is transferred to the cycle from NADPH cytochrome P450 reductase at this stage.

Step (6): In this step, one atom of molecular oxygen is inserted into the carbon chain of the substrate to produce the oxidised reaction product. The precise reaction mechanism for this step is unknown, but is thought to proceed via an unstable hydroxylated intermediate.

The derivation of the catalytic cycle for cytochrome P450 is based on studies of P450cam (CYP101A), the soluble camphor hydroxylase isolated from *Pseudomonas putida* (Poulos et al, 1985). Although there is less than 30% amino acid sequence identity between P450cam and the mammalian P450s, there is considerable evidence that the structure of P450cam can be used as a model for the mammalian enzymes (Nelson & Strobel, 1989). At present, P450cam is the only P450 for which a crystal structure has been determined (Poulos et al, 1985,

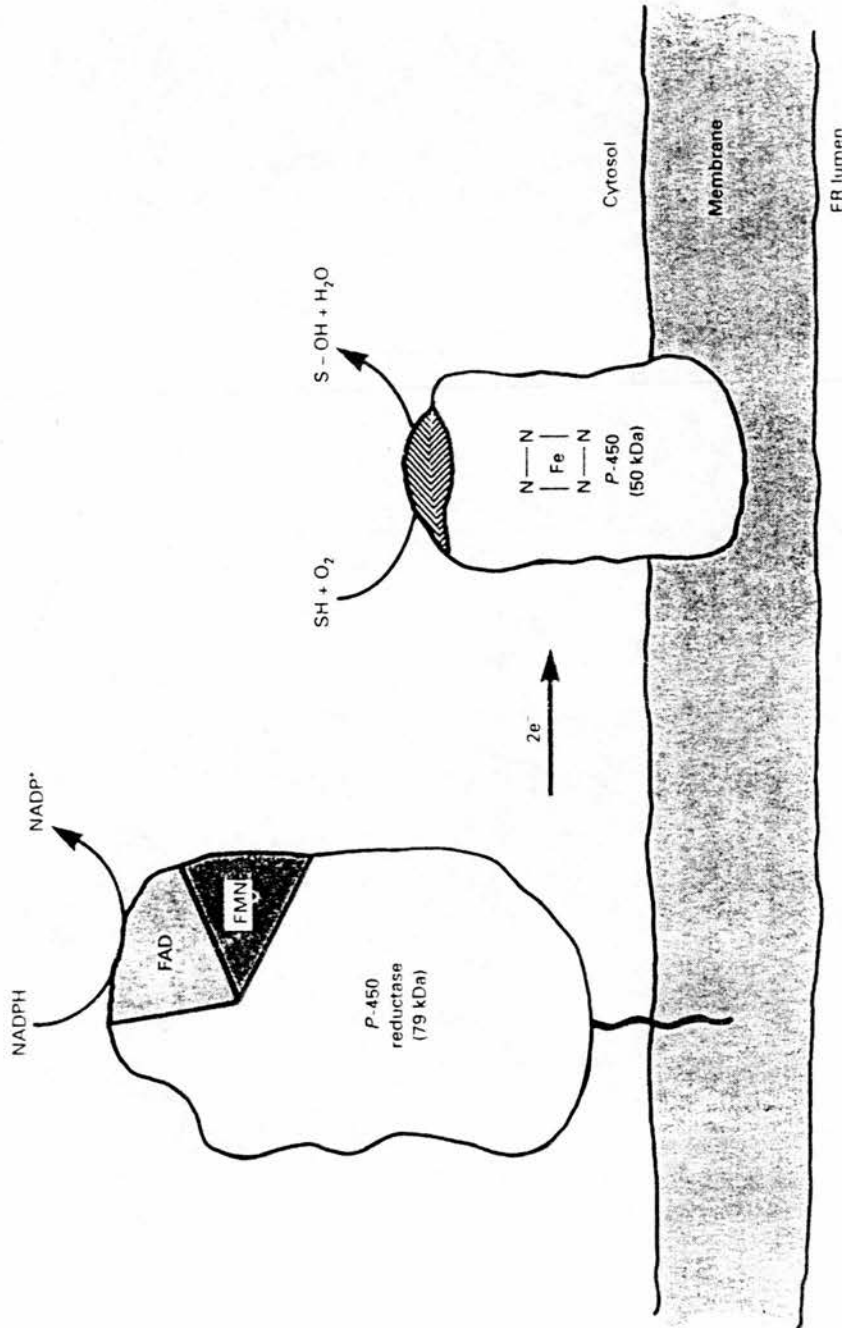
1987). There is, however, a high degree of homology in the active site region between P450cam and many of the eucaryotic isozymes, which suggests that the mechanisms of oxygen and substrate binding and oxygen activation will be conserved.

Site-directed mutagenesis of individual mammalian isozymes has revealed several "key residues" which are important for catalytic function or the control of substrate specificity (reviewed by Johnson *et al.*, 1992). The position of these residues has been related to the crystal structure of P450cam, and, more relevantly, will be compared to structures of the mammalian enzymes as they become available. Several amino acid residues have been thus identified (Aoyama *et al.*, 1989; Lindberg & Negishi, 1989; Kronbach *et al.*, 1991). These studies suggest that P450 structures can selectively accommodate genetic change such that substrate metabolism is altered but the fundamental capacity of the enzyme to reduce molecular oxygen is unaffected. Johnson (1992) proposed the following model to account for this: Many of the "key residues" identified to date are found in loop regions in the structure of P450cam (Poulos, 1989) and are likely to be similarly placed in the mammalian enzymes. These regions bind and orientate the substrate with respect to the heme plane. Thus, alteration in the amino acid composition of this region can dramatically influence the substrate specificity and thus the catalytic function of the enzyme. The overall membrane topology of the enzyme and oxygen binding to the heme iron are unaffected by changes in these loop regions. In agreement with this, Gotoh (1992) aligned the sequences of several CYP2 proteins with P450cam and concluded that sequence diversification occurred primarily in substrate recognition regions.

The membrane topology of mammalian P450s has been studied by a number of techniques including computer modelling and site-directed mutagenesis (Figure 1.3.1.2). The findings of several groups have been recently reviewed (Black, 1992), and suggest that all mammalian P450s may be similarly constructed. Microsomal P450s are bound to the endoplasmic reticulum (ER) by one or two transmembrane peptides at the NH₂-terminal end. The active site, which is located in the cytoplasmic domain of the protein may have further peripheral membrane contacts. The heme-porphyrin plane lies between perpendicular and parallel to the plane of the endoplasmic reticulum. Purified P450s are amphipathic and exist as micellar aggregates of approximately six monomers. Signal peptides control the insertion of membrane proteins into the ER (Wickner & Lodish, 1985). P450s are inserted into the ER by an NH₂-terminal signal peptide which is not cleaved on insertion, but acts as anchor in the membrane. Modification of the NH₂-terminal

amino acids has been shown to lead to the complete translocation of the P450 across the ER membrane.

Figure 1.3.1.2 Orientation of P450 and NADPH-cytochrome P450 reductase in the endoplasmic reticulum



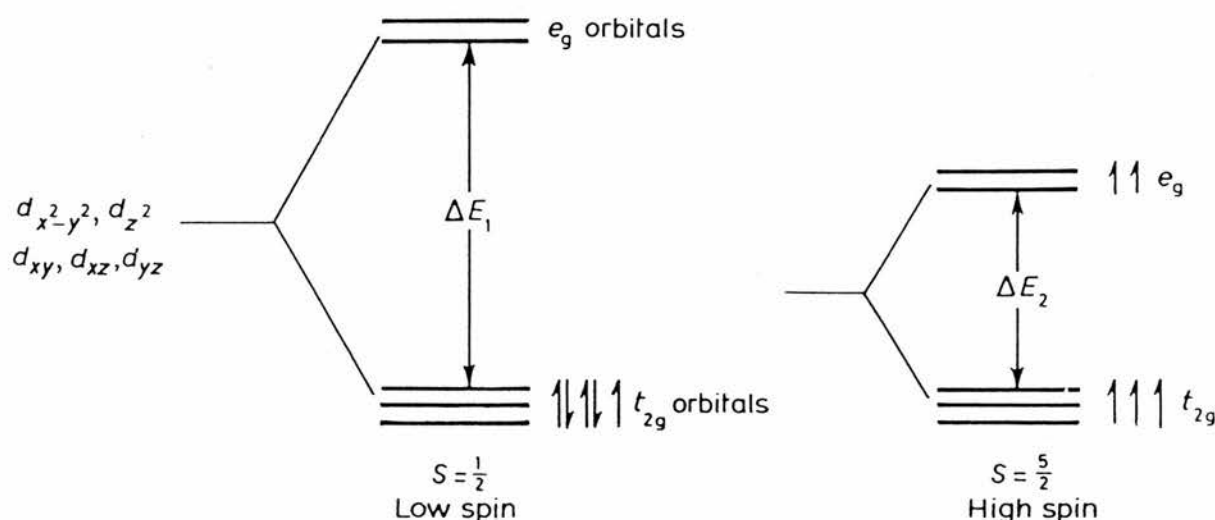
Redrawn from Waxman & Azaroff, 1992

1.3.2 Spectrophotometric analysis of P450 - substrate interaction

The heme iron in cytochrome P450 is bound by four equatorial ligands, the pyrrole nitrogens of photoporphyrin IX, and an additional one or two axial ligands which lie normal to the porphyrin plane. These axial ligand(s) are amino acids in the heme binding pocket of the apoprotein, one of which is the thiol of a cysteine residue in the -COOH terminus of the apocytochrome which has been shown to be highly conserved in all P450 sequences (Poulos *et al.*, 1987). Thus, the incorporation of heme to form a catalytically active P450 can result in two different co-ordination spheres around the central iron - (a) where the iron is bound to only one axial ligand, the conserved cysteine residue, and the sixth d-orbital is occupied by a lone pair of electrons; or (b) where the iron is bound by two axial ligands, the conserved cysteine and another amino acid. The particular co-ordination adopted can be rationalised by ligand field theory.

Free (uncomplexed) ferric iron has 5 degenerate d orbitals which each contain a single unpaired electron. When the iron is surrounded by a ligand co-ordination sphere, however, the energy levels of the d orbitals are influenced by differences in the electronic environment and lose their degeneracy, resulting in the creation of two distinct energy levels, designated t_{2g} and e_g . Two unique distributions of the 5 d-electrons are therefore possible (Figure 1.3.2):

Figure 1.3.2 High and low spin complexes of ferric iron



In (a), the low-spin state, maximum electron pairing occurs, the repulsion between electrons in an electron pair is less than the ligand field energy (ΔE) and it is therefore more energetically favourable for the electrons to exist as pairs wherever possible. In (b), the high spin state, however, the ligand field splitting energy is lower and less than the electron-electron repulsion energy. The electrons are therefore found unpaired, each occupying a unique orbital. ΔE , the ligand (crystal) field splitting energy, is a function of the nature of the ligands as each ligand interacts differently with the d orbital electrons of the heme iron. Whether the complex adopts a high or a low spin configuration is therefore a function of ΔE and is determined by the nature of the ligands. As the four equatorial nitrogens are invariant, the overall stereochemistry of the complex is dependent on the nature of the axial ligand(s).

No quantitative assessment has yet been made of the relationship between the spin state and geometric configuration of metal-porphyrin complexes such as cytochrome P450. In most cases, however, 5 co-ordinate models have been observed to be high spin, with an out of plane heme iron with a lone pair of electrons in place of the sixth axial ligand. Six co-ordinate complexes are generally low spin, with the heme iron lying in the equatorial plane. Most free P450s i.e. not bound by substrate, exist in a low spin 6 co-ordinate stereochemistry, with a characteristic absorption band at around 420nm. Substrate binding can occur in one of two ways:

(a) Type I substrates e.g. benzphetamine, caffeine, testosterone

These bind to the apoprotein, thus altering the conformation of the heme binding site. This results in conversion from low to high spin configuration, accompanied by a Type 1 spectral change with a minimum at 420nm and maximum at 390nm. Any change in the intensity of the absorbance is directly proportional to ΔE , the ligand field splitting energy.

(b) Type II substrates e.g. the nitrogenous bases aniline and pyridine, ligate directly to the heme iron, replacing one of the axial ligands and resulting in a 6 co-ordinate low spin complex (Absorbance maximum at 420nm).

Thus, an estimate of the extent of ligand binding and the nature of the bound ligand can be made spectrophotometrically. A further spectrophotochemical technique, Surface Enhanced Resonance Raman Spectroscopy (SERRS) has also been used to determine the spin state of the heme iron in a series of P450s, stabilised by absorption onto a silver colloid (Kelly *et al*, 1987; Rospendowski *et al*, 1991). This sensitive technique requires only a small amount of sample and can be used to

determine the specific oxidation and spin state of the heme iron. In addition, it can be used to differentiate between P450 and P420, the biologically inactive form of the enzyme. Wolf *et al* (1988), using this technique, demonstrated that the differences in catalytic activity between rat CYP2B1 and CYP2B2 were related to differences in the heme environment of the proteins. In addition, these workers found that changes in the ionic strength of the solvent affected P450/reductase coupling, suggesting that an electrostatic interaction between the proteins occurs (see Section 1.3.1).

Substrate binding can also affect the redox biochemistry of the electron transport chain. It has been demonstrated that the ligand induced transition from low to high spin in both bacterial and mammalian P450s is accompanied by an increase in the mid-point redox potential of the haemoprotein (E°) (Fischer & Sligar, 1983). This in turn leads to an increasingly negative value of ΔG° for the reaction, facilitating the transfer of the first electron from NADPH-cytochrome P450 reductase to cytochrome P450. Therefore, the reduction of ferric to ferrous iron is more thermodynamically favourable in the presence of substrate. Fischer & Sligar also demonstrated that a linear free energy relationship exists between redox potential and spin state, and proposed that both redox potential and spin state are correlated with the extent of hydration at the active site. In the absence of substrate, the substrate binding pocket is filled with solvent molecules, with an additional molecule of solvent co-ordinated to the heme iron. This highly symmetrical coordination complex is low spin. The presence of solvent also acts to stabilise Fe^{3+} rather than Fe^{2+} , as the more highly charged iron has a better electrostatic interaction with the porphyrin core. In addition, Eyer & Backes (1992) showed that the rate of transfer of the first electron was directly proportional to the rate of functional reductase/P450 complex formation and that this was generally applicable to P450 catalysis i.e. the mechanism is not isozyme specific.

1.3.3 Nomenclature and evolution of P450 genes

1.3.3.1 Nomenclature

More than 150 different P450 genes had been characterised by the end of 1990 - these had been isolated from a total of 23 different eukaryotic and 6 prokaryotic species. It therefore became necessary to devise a systematic nomenclature system which could identify each gene uniquely. Until the advent of this nomenclature system, individual genes had been given trivial names by the labs in which they had been characterised, leading to a great deal of confusion in the

literature, as the same gene product often had several trivial names. Nebert et al (1987, 1989, 1991) proposed a nomenclature system based on the evolution of the P450 superfamily, the main proposals of which are outlined below:

1. All P450 genes have the root symbol *CYP* (*Cyp* for mouse genes) to represent Cytochrome P450.
2. The root symbol is followed by an arabic number to denote the gene family and a letter to denote the subfamily. Protein sequences within the same gene family have >40% amino acid homology. Twenty seven distinct gene families have been described to date, ten of which are mammalian. There are 18 distinct mammalian subfamilies which exhibit >60% sequence identity.
3. An Arabic number is used to denote an individual gene within a particular subfamily. A hyphen precedes this final number in mouse genes. The number of a particular gene within a subfamily is assigned according to the order in which the genes were isolated e.g. CYP2A1 is a rat gene, while CYP2A6 is human.
4. The human nomenclature is to be used for all species except mouse.
5. *Genes and cDNAs* are to be written in *italic script* - mRNAs and proteins in non-italics.

Examples : *Cyp2a-4* Family 2A, gene 4, cDNA, mouse
Cyp2a-5 Family 2A, gene 5, protein, mouse
CYP2B1 Family 2B, gene 1, mRNA, rat
CYP2B6 Family 2B, gene 6, gene, human

This nomenclature system is based on the assumption that each P450 protein is the product of a separate gene. To date, two exceptions to this have been reported, where alternative splicing of the mRNA coding for these genes lead to the production of functional proteins with different catalytic activities to the parent genes (Lacroix et al, 1990; Lephart et al, 1990).

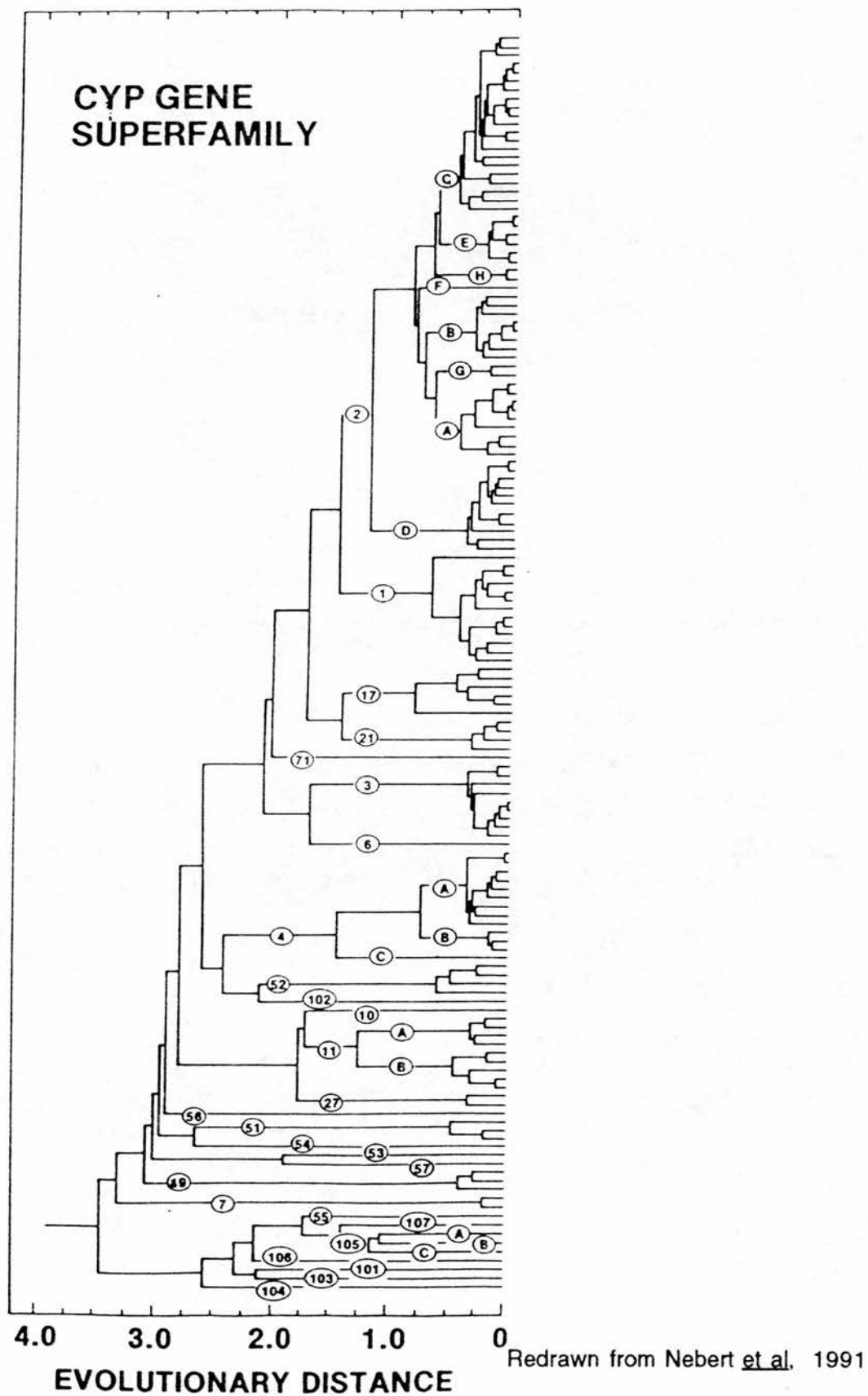
1.3.3.2 Evolution

Cytochrome P450 genes are thought to have been present in the earliest organisms. The large number of P450s which have been characterised to date are thought to have evolved from a single gene and attempts have been made to correlate the divergence of P450 sequence with species diversification, based on paleontological evidence. From these studies and alignments of known P450

sequences, a phylogenetic tree has been constructed for P450 evolution (Figure 1.3.3.2). Although this is a useful model, it is not a quantitative representation of the evolutionary pathway. Such a model assumes that the rate of gene divergence is linear, which has been shown to be inaccurate for P450 gene divergence (Nebert & Gonzalez, 1987), and it does not allow for gene conversion events. Genes from within the same subfamily have all been found to lie within the same gene cluster (Matsunaga *et al*, 1990). In addition, the intron-exon organisation is unique to each gene family, with the intron-exon boundaries at the same position for members within the same family (Gonzalez *et al*, 1985; Zaphiropoulos *et al*, 1990).

Following the first divergence between microsomal and mitochondrial species (approximately 1.5×10^9 years ago), the number of microsomal proteins has increased greatly. This is thought to have occurred in response to environmental challenge and may have coincided with vertebrate adaptation to terrestrial life. Mitochondrial P450s are primarily involved in endogenous metabolism, catalysing reactions such as steroid oxidations, and thus have diversified to a lesser extent. In general, P450s which are involved in xenobiotic metabolism, the "drug metabolising P450s", are found within families CYP1, CYP2 and CYP3. The greatest structural diversity is seen in the CYP2 subfamily, which plays a central role in xenobiotic metabolism.

Figure 1.3.3.2 Evolution of cytochrome P450 genes



1.3.4 Regulation of P450 expression

The expression of P450 genes can be regulated at a number of levels. The expression of many isozymes is dramatically influenced by the administration of foreign compounds - these chemicals often increase the expression of the particular isozyme(s) which are active in their metabolism. Xenobiotic regulation of P450 expression will be discussed in Section 1.3.4.2. Constitutive expression is often under endocrine control, while the expression of several P450 genes is sexually differentiated and tissue and species specific.

1.3.4.1 Constitutive expression

Most constitutively expressed P450s become transcriptionally active after birth. Unlike most experimental animals, however, human fetuses have detectable levels of P450 protein (Kitamura et al, 1992). Four proteins have been purified from human fetal liver - P450 HFLa, P450HFLb, P450HFLc and P450HFLd. N-terminal sequencing of the purified proteins suggested that they were members of the CYP3A subfamily. P450 HFLa has subsequently been classified CYP3A7 and the CYP3A7 cDNA has been shown to be highly homologous to the cDNA for CYP3A4, isolated from a cDNA library prepared from adult human liver. As trans-placental induction of P450 activity has been demonstrated in mice and rats (Vodicnik et al, 1980; Tagaki et al, 1976), it seems likely that these human fetal P450s will be regulated in a similar manner, and will therefore be capable of activating carcinogens in situ, thus initiating genetic damage. In support of this, it has been demonstrated that CYP3A7 is active in the metabolism of Aflatoxin B1 in an in vitro system (Kitamura et al, 1992). Similarly, in a recent study, Murray et al, (1992) identified a CYP1A protein in both adult and fetal human liver. Immunohistochemical analysis demonstrated that expression of this protein was localised predominantly to hepatocytes, and while expression in human liver was very heterogeneous, the distribution in fetal liver was more uniform.

Most studies on the ontogenic development of P450 expression have been carried out in rodent models, and have shown that many P450 genes are differentially regulated, both in the time of their transcriptional activation and the tissue specificity of their expression. The expression of CYP2E1 in the rat is transcriptionally activated immediately after birth (Song et al, 1986), while other genes, such as CYP2C6 and CYP2C7, are not activated until puberty (Zaphiropoulos et al, 1989). Developmental regulation of rat CYP2E1 expression has been correlated with the demethylation of specific cytosine residues in the 5' region of the

gene (Umeno et al, 1988). The expression of CYP2A1 is lost in male rats at puberty (Matsunaga et al, 1988), while females lose the expression of CYP3A2 (Gonzalez et al, 1986). This has been related to differences in serum growth hormone concentrations (Zaphiropoulos et al, 1989). In addition, it has been shown that the expression of several P450 genes is regulated by binding to a series of transcription factors (Johnson, 1990). Yano et al (1992) proposed that the transcriptional activation of P450 gene expression is directly related to the expression of these transcription factors, some of which are expressed at different stages of development.

Hormonal regulation of P450 gene expression has recently been comprehensively reviewed (Lund et al, 1991). P450 expression in liver, lung, kidney, adrenals, gonads, prostate and kidney is under endocrine control. Although P450 expression in these extra-hepatic tissues is significantly lower than in the liver, in situ formation of reactive metabolites may lead to tissue specific sensitivity to a particular drug or toxin, leading to enhanced cytotoxicity or adverse side effects. The influence of particular hormones is tissue specific. For example, the peptide hormones adrenocorticotrophic hormone (ACTH), luteinising hormone (LH), follicle-stimulating hormone (FSH) and parathyroid hormone (PTH) influence P450 expression in the adrenal gonads and kidney, while P450 expression in the prostate is androgen dependent. In the liver, the major steroid hormone-metabolising P450s are regulated by growth hormone (Gustafsson, 1978; Colby, 1980). Many of the P450s which are regulated by hormones have an important endogenous function - these are summarised in Table 1.4.

Most of the studies on the influence of hormones on P450 expression have been carried out in the rat, where pronounced a pronounced sexual differentiation in the hepatic metabolism of both endogenous compounds and xenobiotics has been observed. For example, CYP2C7 and CYP2C12 are female specific, while CYP2C11 and CYP2C13 are expressed only in male rats (Zaphiropoulos et al, 1988; Gonzalez et al, 1986). This has been attributed to differences between the sexes in androgen exposure, both neonatally and at later stages of development. These androgen-mediated effects on hepatic P450 expression have been shown to be mediated by growth hormone, via the hypothalamic-pituitary axis (Gustafsson et al, 1980). The pattern of growth hormone secretion has been shown to vary dramatically between males and females (Eden, 1979). Hypophysectomy was found to eliminate the sexual differentiation in the expression of these hepatic P450s. The administration of physiologically relevant doses of exogenous growth hormone to hypophysectomised

rats resulted in the re-establishment of sexually differentiated P450 expression and hence steroid metabolism (Morgan et al, 1985). The effects of growth hormone are thought to be exerted at the level of transcription.

Hepatic gene expression can also be influenced by thyroid and glucocorticoid hormones (Waxman, 1990) and insulin (Yamazoe et al, 1989). Insulin is thought to affect P450 gene expression indirectly - growth hormone secretion in the pituitary is suppressed in the absence of insulin (Tannenbaum, 1981). Growth hormone is thought to exert its growth-promoting effects via insulin-like growth factor I (IGF-I) (Daughaday, 1989). In a recent study (Ram & Waxman, 1992), thyroid hormone has also been shown to regulate the expression of NADPH-cytochrome P450 reductase.

Similar mechanisms of gene regulation have been shown to exist in the mouse (Macleod & Shapiro, 1989). Henderson et al (1990) demonstrated that P450 expression in mouse kidney is sexually differentiated, mediated by testosterone and regulated at the level of transcription. A companion study (Henderson & Wolf, 1991) showed that the effects of testosterone are exerted through the androgen receptor.

Although the effects of hormonal regulation on human P450 gene expression have not yet been fully characterised, it seems likely that the effects will be as complex, if not more complex than those observed in experimental animals. This is likely to have profound toxicological consequences and may alter the susceptibility of tissues which are sensitive to the effects of hormones to environmental insult or drug treatment.

P450 gene expression can also be regulated post-transcriptionally. Freeman et al (1992) demonstrated that induction of Cyp2e-1 protein expression by acetone treatment was not accompanied by an increase in the expression of the corresponding mRNA. Indeed, the levels of mRNA were significantly decreased. This phenomenon has also been observed in ethanol (Song et al, 1986) and pyridine (Kim & Novak, 1990) treated rats and ethanol treated rabbits (Porter et al, 1989). The increase in Cyp2e-1 protein must therefore arise as a consequence of increased mRNA translation or, more likely, by post-translational protein stabilisation. Similarly, the regulation of CYP1A2 expression by TCDD and CYP2B1/2 by dexamethasone have been shown to occur by mRNA stabilisation (Kimura et al, 1986; Simmons et al, 1987). Hormonal regulation of CYP2E1 expression in the rat has also been reported (Johansson et al, 1989), evidenced by alterations in the phosphorylation status of the protein.

Post-translational modification by phosphorylation has been shown to be an additional mechanism by which several P450 genes are regulated. *In vivo* studies have shown that many hepatic P450 genes can be phosphorylated by purified protein kinases (Pyerin *et al*, 1987) and the phosphorylation status of several P450 genes has also been studied in isolated hepatocytes and *in vivo* (Koch & Waxman, 1989; Bartlomowicz *et al*, 1989). Phosphorylation *in vitro* can be increased by raising the intracellular levels of cAMP by the addition of hormones such as glucagon which control the regulation of intracellular adenylate cyclase. Phosphorylation status has been implicated in the regulation of P450 activity and may also be important in controlling P450 degradation (Eliasson *et al*, 1990). In all cases studied to date, phosphorylation of P450 genes lead to a decrease in activity. This was reflected in the studies of Oesch *et al*, who reported that the rate of generation of cytotoxic metabolites from compounds such as cyclophosphamide was significantly reduced when the phosphorylation status of CYP2B1 was increased. Similarly, CYP1A2, which is involved in the metabolic activation of aromatic amines e.g. 2-aminofluorene, was phosphorylated *in vitro* by purified protein kinases. Inhibition of dephosphorylation by okadaic acid lead to decreased mutagenicity of all the aromatic amines tested. Polyacrylamide gels showing native and phosphorylated P450s revealed the presence of a higher molecular weight band in the phosphorylated samples, which may represent a complex with ubiquitin, thus targetting the proteins for degradation.

The mechanism for P450 degradation has not been fully elucidated, but it is known that P450 turnover obeys first order kinetics (Parkinson *et al*, 1983; Watkins *et al*, 1986). The half life ($t_{1/2}$) of individual isozymes can therefore be obtained from calculating the first order rate constant for the reaction. Individual P450 half lives vary widely e.g rat CYP1A2 = 10 hours, while CYP2C6 and CYP2C11 = 20 hours (Shiraki & Guengerich, 1984), and are, in general, significantly shorter than the $t_{1/2}$ for reductase and cytochrome b_5 (Correia, 1991). Induced P450s often have markedly longer half lives due to substrate-induced protein stabilisation, but these values do not necessarily reflect the true half life of the proteins.

P450 heme has a shorter $t_{1/2}$ than the corresponding apocytochromes (Correia, 1991). Dynamic exchange of heme between apoproteins has been shown to occur and this greater lability has been proposed to account for the shorter half life (Sadano & Omura, 1983).

Although individual P450s have different half lives, it is likely that there is

a degradation pathway or pathways which is common to all isozymes. The variation in $t_{1/2}$ between isozymes may well represent different binding affinities for the enzymes within this degradative pathway. Although the mechanism of degradation of the apoprotein has not yet been elucidated, heme catabolism is relatively well characterised. Labelling studies in rats given 5 - [^{14}C] amino-laevulinic acid (ALA) showed that microsomal heme is converted to bilirubin and carbon monoxide (CO) by heme oxygenase (Landaw *et al*, 1970). This microsomal enzyme ligates heme and catalyses the breakdown of the tetrapyrrole ring to yield CO, biliverdin IX and iron (Tenhunen, 1969). Biliverdin is then converted to bilirubin by the cytoplasmic biliverdin reductase, followed by bilirubin conjugation with glucuronic acid and elimination. Maines & Kappas (1977) proposed that heme oxygenase activity is rate limiting in the degradation of hepatic P450 heme. Bissell & Guzelian (1980), however, demonstrated that heme oxidase mediated degradation of heme to biliverdin accounted for only 60-70% of P450 heme in rat liver homogenates, implying that an alternative pathway(s) exists. This has not, as yet, been characterised, and is not clear whether heme catabolism is regulated in humans in a similar manner to rodents.

A recent study (Celier & Cresteil, 1991) demonstrated that administration of 3-MC to congenitally jaundiced Gunn rats did not lead to an increase in hepatic P450 protein, due to decreased heme availability as determined by tryptophan pyrrolase activity. Heme oxygenase activity was also significantly lower in these rats, suggesting that it was not responsible for the decrease in available heme. In order for new catalytically active P450 to be synthesised following xenobiotic administration, an endogenous heme pool must exist. The rate limiting enzyme for heme biosynthesis, 5-aminolevulinic acid synthase (ALVS) is inducible by both phenobarbital and TCDD (Poland & Glover, 1973), which are also powerful regulators of P450 expression (Guengerich, 1987). It has been proposed, however, that these agents act indirectly to decrease the amount of available heme by increasing the levels of P450 apoprotein to a greater extent than they induce ALVS activity. Heme from the intracellular heme pool is therefore taken up at a rate which exceeds its synthesis.

1.3.4.2 Xenobiotic regulation of P450 gene expression

Regulation of P450 gene expression by foreign compounds has been studied extensively in a number of model systems. Xenobiotic regulation of P450 expression in mouse and human tissues and in a cell culture model will form the basis of Chapters 3, 4 and 5.

Exposure to a wide variety of structurally diverse xenobiotics results in increased expression of the P450 isozymes which are active in their metabolism (Conney, 1982; Okey, 1990). Although there is some overlap in substrate specificity between isozymes, in general, different classes of chemical are metabolised by distinct P450s. Certain chemicals which contain a variety of functional groups e.g. Aflatoxin B₁ can be metabolised by several distinct P450 isozymes, often resulting in the formation of regio- and stereospecific reaction products.

Regulation of the expression of CYP1A genes has been most fully characterised to date. CYP1A genes are regulated at the level of transcription on exposure to polycyclic aromatic hydrocarbons (PAHs) (Whitlock, 1990). Recent work in a cell culture model has considerably advanced our knowledge of the mechanism of this transcriptional regulation (Reyes *et al*, 1992). It has been known for some time that PAHs such as TCDD and aromatic amines interact with and bind to a cytosolic protein, the Ah receptor. The Ah receptor has recently been purified from mouse liver (Poland *et al*, 1991) and the corresponding cDNA cloned and characterised. The ligand-receptor complex is then translocated to the nucleus where it binds DNA at an enhancer sequence upstream of the transcription start site leading to transcriptional activation of the CYP1A gene(s) (Fujisawa-Sehara *et al*, 1987, 1988). The mouse hepatoma cell line, Hepa-1 has been shown to contain a functional Ah receptor. In a recent study, Hankinson *et al* (1991) isolated a series of mutant clones from this cell line which were defective in some aspect of the ligand activated transcription process. Mutants were selected on the basis of their resistance to benzo(a)pyrene. Four unique mutant cell lines were thus isolated:

(A) contained an inactivating mutation in the Cyp1a1 structural gene;

(B) mutants had greatly reduced levels of the Ah receptor;

In (C) mutants the receptor could not translocate to the nucleus and

(D) combined the mutations in (B) and (C).

Further studies of the (C) mutant lead to the identification of the cDNA encoding a gene responsible for translocation of the receptor to the nucleus, the Ah receptor nuclear translocator or Arnt gene (Hoffmann *et al*, 1991). The deduced amino acid sequence of the protein encoded by this cDNA contains a basic helix-loop-helix domain, a common motif in proteins which bind DNA. It has been proposed that the Ah receptor and the Arnt gene bind DNA as a heterodimeric complex, almost certainly in the 5' upstream region of Cyp1a genes, termed the xenobiotic regulatory element or XRE (Reyes *et al*, 1992).

A similar regulatory mechanism is thought to exist for hormone responsive P450s, such as those in the CYP3A family. In this case, the hormone e.g. the synthetic glucocorticoid dexamethasone, interacts with a glucocorticoid responsive element (GRE) in the upstream region of the gene which initiates or leads to enhanced transcription. CYP4A genes are also thought to be regulated by binding to an intracellular receptor. CYP4A gene expression is inducible by a range of structurally dissimilar compounds termed peroxisome proliferators. These compounds, which include drugs such as nafenopin and clofibric acid, cause marked peroxisome proliferation in several species. The cDNA for a receptor which is responsive to these compounds, the peroxisome proliferator activated receptor (PPAR), has recently been cloned (Issemann & Green, 1990). This receptor is a member of a superfamily which also contains receptors for steroids, thyroid hormones and retinoic acid. Fatty acids, which are also metabolised by CYP4A genes, are likely to be endogenous ligands for the PPAR (Gottlicher *et al.*, 1992).

Although CYP2 genes are centrally involved in the metabolism of many structurally dissimilar chemicals, little is known about their mechanism of regulation. The regulation of CYP2B gene expression has been studied in the most detail. CYP2B gene expression is regulated by barbiturates such as phenobarbital (Guengerich, 1987). A 17 base pair sequence has been identified in the 5' flanking regions of CYP2B genes which is thought to be responsible for transcriptional activation by phenobarbital and which is conserved between rat CYP2B1, CYP2B2 and the barbiturate-inducible genes in the bacteria *Bacillus megaterium*. In the absence of bound barbiturate, a repressor is bound to a 24 base pair sequence which overlaps the phenobarbital binding sequence. This repressor is thought to be displaced on barbiturate binding, leading to transcriptional activation of the gene (He & Fulco, 1991).

Many of these xenobiotic-responsive genes also have important endogenous functions e.g. CYP4A proteins are involved in the ω - and ω -1 hydroxylation of fatty acids. It is therefore important to realise that exposure to many xenobiotic regulators can lead to altered expression of the genes which are involved in the regulation of many important biosynthetic processes within the cell. The P450-mediated metabolism of a particular drug or environmental pollutant can therefore lead, in addition to increased inherent cytotoxicity, to altered cellular homeostasis.

1.4 Endogenous role of cytochrome P450

In addition to their role in xenobiotic metabolism, P450 enzymes are also

active in the metabolism and biosynthesis of many endogenous compounds such as steroid hormones, fatty acids, prostaglandins and vitamins. Individual P450 isozymes exhibit a high degree of regio- and stereoselectivity in the metabolism of many of these endogenous compounds. This is illustrated by the P450-mediated metabolism of testosterone and cholesterol, the metabolism of which leads to the production of sex hormones, glucocorticoids and mineralocorticoids. Indeed, analysis of the regio- and stereospecific hydroxylation products of the steroid nucleus has been shown to be a convenient method of determining the relative concentrations of individual P450 isozymes present in a complex mixture such as rat liver microsomes (Wood et al, 1983; Waxman, 1988). The major biosynthetic reactions catalysed by P450 are summarised in Table 1.4.

Vitamin D3 (cholecalciferol) is responsible for regulating cellular calcium and phosphorus homeostasis. The vitamin is inactive as administered, however, and undergoes oxidative metabolism to the active 1,25-dihydroxyvitamin D3, catalysed by an initial P450-mediated hydroxylation in the liver to a product which is further hydroxylated by a P450 in the kidney mitochondria. In addition, further P450 mediated reactions can metabolise the pro-vitamin to different hydroxylation products which are catalytically inactive (DeLuca, 1982; Henry & Norman, 1984). Vitamin A (retinol) is also metabolised by a P450 mediated oxidation to retinoic acid which itself undergoes further oxidative metabolism. Rat liver microsomes have been shown to be capable of oxidising both retinol and retinoic acid, and it has been demonstrated that these reactions can also be performed by purified CYP2B1 and CYP2C7 in a reconstituted system (Leo et al, 1984, 1985). Vitamins E (α -tocopherol) and B2 (riboflavin) are also thought to be metabolised by P450 enzymes, although the individual isozymes responsible for their metabolism have not yet been determined.

Arachadonic acid is metabolised by cytochrome P450 to a number of products (Karara et al, 1989), including epoxyeicosatetraenoic acids which regulate insulin and glucagon release from the pancreas (Karara et al, 1989; Capdevila et al, 1990), and hydroxyeicosatetraenoic acids which can act as templates for prostaglandin and leukotriene biosynthesis. CYP4A isozymes are responsible for the metabolism of arachadonic acid to various ω - and ω -1 hydroxylated products (Capdevila et al, 1981). Many individual prostaglandins, including PGA_1 , PGA_2 , PGE_1 and PGE_2 are further metabolised by P450s in the liver, kidney and lung to yield ω - and ω -1 hydroxylated products. ω -oxidation of prostaglandins is the major metabolic route leading to excretion in the urine (Pace-Asciak & Edwards, 1980). Arachadonic acid

can also be metabolised to prostacyclin and thromboxane, which function as cellular mediators of platelet aggregation and thrombosis, respectively. Although the enzymes involved in these biosynthetic pathways have not been formally characterised, partial purification and spectrochemical studies have shown that they are catalysed by P450.

Table 1.4 Biosynthetic processes catalysed by cytochrome P450

REACTION	P450
STEROL SYNTHESIS	
Lanosterol -> 4,4-Dimethyl- Δ 8,14,24-cholestatrienol -----> Cholesterol	CYP51 (P450 _{14DM})
24-Methylenedihydrolanosterol	
4,4-Dimethyl- Δ 8,14,24(28)-ergostatrienol-----> Ergosterol	
STEROID HORMONE SYNTHESIS	
Cholesterol -> Pregnenolone	CYP11A1 (P450 _{scc})
Pregnenolone -> 17 α -Hydroxypregnenolone	CYP17
Progesterone -> 17 α -Hydroxyprogesterone	(P450 _{17α-C17,20})
17 α -Hydroxy-20-dihydropregnenolone -> DHEA	
17 α -Hydroxy-20-dihydroprogesterone -> Androstenedione	
Androstenedione -> Estrone	CYP19 (P450 _{AROM})
Testosterone -> Estradiol	
11-Deoxycortisol -> Cortisol	CYP11B1 (P450 _{11β})
PROSTAGLANDIN METABOLISM	
PG _{H2} -> Prostacyclin (PGI ₂)	P450 _{PGI2}
PG _{H2} -> Thromboxane A ₂ (TxA ₂)	P450 _{TxA2}
LEUKOTRIENE METABOLISM	
LT _{B4} -> 20-Hydroxy-LT _{B4}	P450 _{LTB4}
VITAMIN A METABOLISM	
Retinoic acid -> 4-Hydroxyretinoic acid	P450 _{4-RA}

Abbreviations: 14DM: 14 α -demethylase; scc: side chain cleavage; 17 α : 17 α -hydroxylase; C17,20: 17,20-lyase; DHEA: dehydroepiandrosterone; AROM: aromatase; 11- β : 11 β -hydroxylase; PGH₂: prostaglandin endoperoxide; LTB₄: 5(S),12(R)-dihydroxy-6-*cis*-8,10-*trans*-14-*cis*-eicosatetraenoic acid; RA: retinoic acid

1.5 Xenobiotic metabolism by cytochrome P450

A wide variety of foreign compounds (xenobiotics) are metabolised by the P450 system. For ease of discussion these will be divided into two sections:

- 1) Environmental pollutants, chemical toxins and carcinogens
- 2) Drugs in common clinical use, including anticancer drugs

1.5.1 Xenobiotic metabolism

The diversity of P450 has been proposed to have occurred as an adaptive response to environmental challenge. The so called "drug metabolising" P450s i.e. those within the CYP1, CYP2 and CYP3 families, are thought to have appeared at more advanced stages of evolution to protect against toxins in the environment (Nebert, 1979). This adaptive response is analogous to the response of the immune system when challenged by a foreign compound. It has been proposed that an organism encountering an environmental toxin can activate or induce the synthesis of a P450 which is active in its metabolism. This is illustrated by the P450-mediated production of phytoalexins by plants. These compounds are not toxic to the plants and are not necessary for their survival, but are sufficiently toxic to animals and birds that the plant is considered inedible. This selective toxicity has been successfully exploited in the design of many common therapeutic agents which are structural and/or functional analogues of natural products. TCPOBOP, a potent and pleiotropic inducer of P450 activity, the effects of which will be described in later chapters, was isolated as a contaminant in the preparation of a commercially available herbicide.

Thus, a variety of P450 enzymes exist which are active in the metabolism of a wide range of structurally diverse chemicals. Significant inter-species variation in the metabolism of many chemicals exists, however. This is illustrated by the metabolism of Aflatoxin B₁.

Aflatoxin B₁ (AFB₁) is a mycotoxin produced by various species of the mould *Aspergillus*. Metabolism of AFB₁ by cytochrome P450 results in the generation of a highly carcinogenic metabolite, Aflatoxin 8,9-oxide (Swenson *et al.*, 1975, 1977). Exposure to AFB₁ has been linked with the aetiology of liver cancer, particularly in regions of Asia and Africa where exposure to the toxin is common (Harris & Sun, 1984). A recent study (Forrester *et al.*, 1990) demonstrated that several distinct P450 isozymes were capable of metabolically activating this compound. While CYP3A proteins were the most active in the conversion of AFB₁ to mutagenic products in human liver microsomes, CYP2C, CYP1A2 and CYP2A1 proteins were

also capable of metabolic activation. Significant inter-individual variation in the metabolism of AFB₁ was found to correlate with variation in the expression of CYP3A protein(s). The metabolism of AFB₁ in rodent models was significantly different, however. In the rat, metabolism by CYP3A was not as dominant as in the human, and CYP2B and CYP4B proteins were also catalytically active (Robertson *et al*, 1981; Kawajiri *et al*, 1980).

Further examples of compounds which are metabolised by P450 isozymes in a species specific manner are given in are given in Chapters 3 and 5.

1.5.2 Clinical applications

P450 enzymes have been shown to be involved in the metabolism of some of the earliest drugs, many of which were administered before their metabolic pathways had been determined. Liu (1991) described a range of Chinese medicinal herbs including the *Fructus Shizandrae* derivatives, dibenzo(a,c)cyclooctenes isolated from the fruit kernels of the plant *Schizandra Bail*. Isoquinoline alkaloids extracted from the herb *Corydalis bungeane Diels* and the amide alkaloids *Clausena lansium*, found in fruit tree leaves, have also been shown to undergo P450-mediated metabolism. These have been and still are used to treat a variety of disorders including viral and idiopathic hepatitis. Moreover, P450 enzymes catalyse the metabolism of a variety of drugs which are currently used in clinical practise. For some of these drugs, the P450 isozyme responsible for their metabolism and the metabolic process(es) involved have been characterised - examples of these are given in Figure 1.5.2. Other drugs, such as cimetidine, can inhibit P450 function.

Confident identification of the enzymes involved in the metabolism of a particular drug in man is often difficult, due to factors such as drug/drug interactions, effects of diet and ingestion of environmental toxins, all of which can influence P450 expression. An additional complication is that the previous medication history for a particular individual is not always known.

Table 1.5.2 Human P450 isozymes involved in the metabolism of clinically important drugs

P450	Drug	Reaction type
CYP 1A2	Phenacetin	O-deethylation
	Caffeine	N-demethylation
	Theophylline	N-demethylation
	Acetaminophen	
CYP 2C	Mephenytoin	Hydroxylation
	Hexobarbital	Hydroxylation
	Diazepam	Hydroxylation
	Tolbutamide	Hydroxylation
CYP 2D6	Debrisoquin	Hydroxylation
	Dextromethorphan	O-demethylation
	Amitriptyline	Demethylation
	Codeine	O-demethylation
CYP 2E1	Acetaminophen	
	Ethanol	
CYP 3A	Erythromycin	N-demethylation
	Nifedipine	Ring oxidation
	Cyclosporine	Hydroxylation, N-demethylation

Adapted from Watkins, 1990

Acetaminophen, a widely used analgesic and antipyretic is known to cause centrilobular hepatic necrosis, both in experimental animals and man (Prescott *et al.*, 1971). The parent compound is metabolised by CYP1A2 and CYP2E1 (Morgan *et al.*, 1983; Raucy *et al.*, 1989) to the hepatotoxin NAPQI (N-acetyl-p-benzoquinimine) which binds to and depletes cellular glutathione and covalently binds to cellular macromolecules (Hinson *et al.*, 1981). Paracetamol (4-hydroxy acetanilide) is similarly metabolised. Acetaminophen can be metabolised by both adult and fetal livers. Chronic alcoholics who have greatly induced levels of CYP2E1 show toxicity to acetaminophen at much lower doses, although this may also be due in part to reduced glutathione availability. Acetaminophen overdose is treated by the

administration of N-acetyl cysteine to raise the level of circulating glutathione and aid elimination.

Cimetidine (Tagamet) administration leads to the inhibition of P450 activity and, as such, can affect the metabolism of other co-administered drugs (Knodell et al, 1991). This is exemplified in the reduced clearance of theophylline, diazepam and alcohol in patients on cimetidine therapy (Reitberg et al, 1981; Klotz & Reimann, 1980; Nelson et al, 1985). There is considerable inter-individual variability in the extent to which the clearance of these drugs is affected - whether this is simply a function of individuality in the expression of the particular P450 isozymes responsible for their metabolism has not been established. Inhibition is thought to occur by the imidazole ring of cimetidine interacting with P450 heme (Knodell et al, 1982), with steric interactions with the amino acids in the heme binding site determining binding affinity. The extent of inhibition is therefore different for individual P450 isozymes. CYP2C gene expression is largely unaffected and there is correspondingly only a very slight inhibition of tolbutamide and hexobarbital hydroxylation. CYP1A2 and CYP2E1 activities show moderate inhibition but, in contrast, cimetidine binds with high affinity to the heme in CYP3A4 and CYP2D6 resulting in almost complete inhibition of the oxidation of nifedipine. The concentration of drug needed to inhibit P450 in isolated microsomes (mM) is, however, usually much higher than circulating blood levels in patients on cimetidine therapy. In a similar manner to cimetidine, 8-methylpsoralen, used in the treatment of psoriasis, binds to CYP1A2 resulting in its activation. Patients receiving this medication therefore have an increased ability to metabolise and eliminate caffeine (Mays et al, 1987; Tinel et al, 1987).

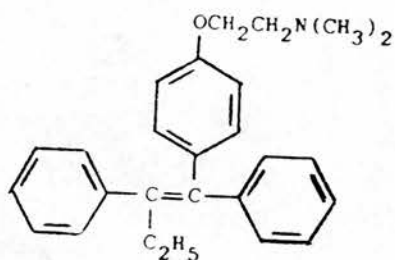
In many cases, two or more drugs are in competition for metabolism by the same P450 isozyme. The extent of metabolism of a particular drug is therefore dependent on its lipophilicity and the binding affinity of the drug for the P450 isozyme active in its metabolism. In addition, certain drugs can be metabolised to different products by different P450s, and the overall metabolic outcome is therefore dependent on the relative binding affinities for each isozyme. Such interactions often leads to the impaired metabolism of one drug, while the metabolism of another is increased. This is exemplified in the co-administration of cyclosporin and erythromycin (Ptachcinski et al, 1985), both of which are metabolised by hepatic CYP3A enzyme(s). Cyclosporin metabolism is inhibited by the erythromycin administration, leading to cyclosporin toxicity. Similarly, rifampicin administration can lead to failure of oestrogen based contraceptives

(Guengerich, 1989). The situation is further complicated by the demonstration that certain compounds which are potent inhibitors of one P450 isozyme can significantly increase the metabolism of another. For example, quinidine inactivates CYP2D6 (Leeman et al, 1986), but is itself metabolised by CYP3A (Guengerich et al, 1986).

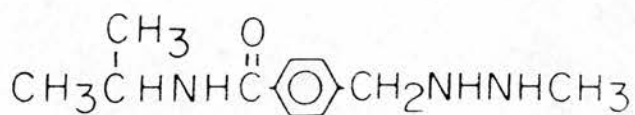
1.5.3 Anticancer drug metabolism

Many of the anticancer drugs in current use are subject to metabolism by one or more of the P450 isozymes (Le Blanc & Waxman, 1989). These include CCNU (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea), which acts by inducing DNA strand breakage (May et al, 1974) and procarbazine (N-isopropyl- α -(2-methylhydrazino)-p-toluamide) (Lee & Lucier, 1976) which inhibits transcription by DNA methylation. The epipodophyllotoxin etoposide (VP-16) is also activated by a P450 mediated process (Relling et al, 1992). Etoposide is thought to stabilise complex formation between DNA and topoisomerase II (Topo II), inhibiting the process of DNA repair and resulting in the formation of strand breaks. The chemical structures of these drugs and of cyclophosphamide, tamoxifen and the morpholinodoxorubicins, the metabolism of which is described below, are given in Figure 1.5.3.

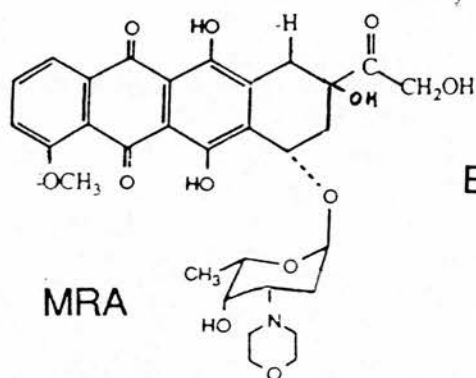
Figure 1.5.3 Anti-cancer drugs which undergo P450-mediated metabolism



Tamoxifen

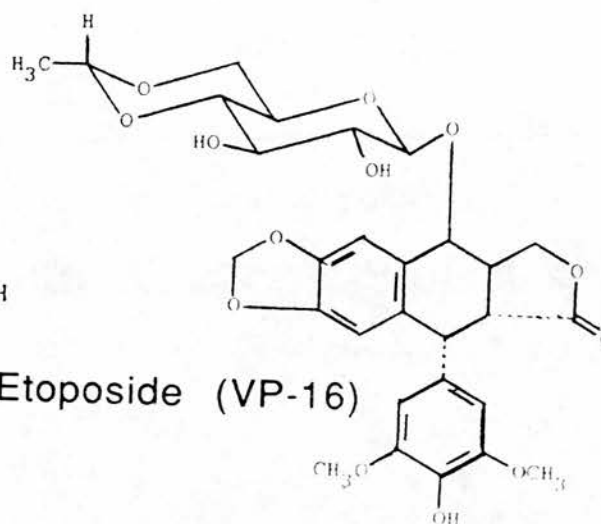


Procarbazine

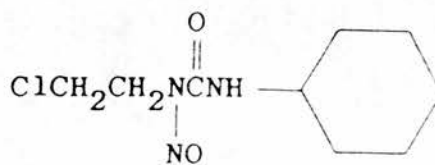


Etoposide (VP-16)

MRA



Cyclophosphamide

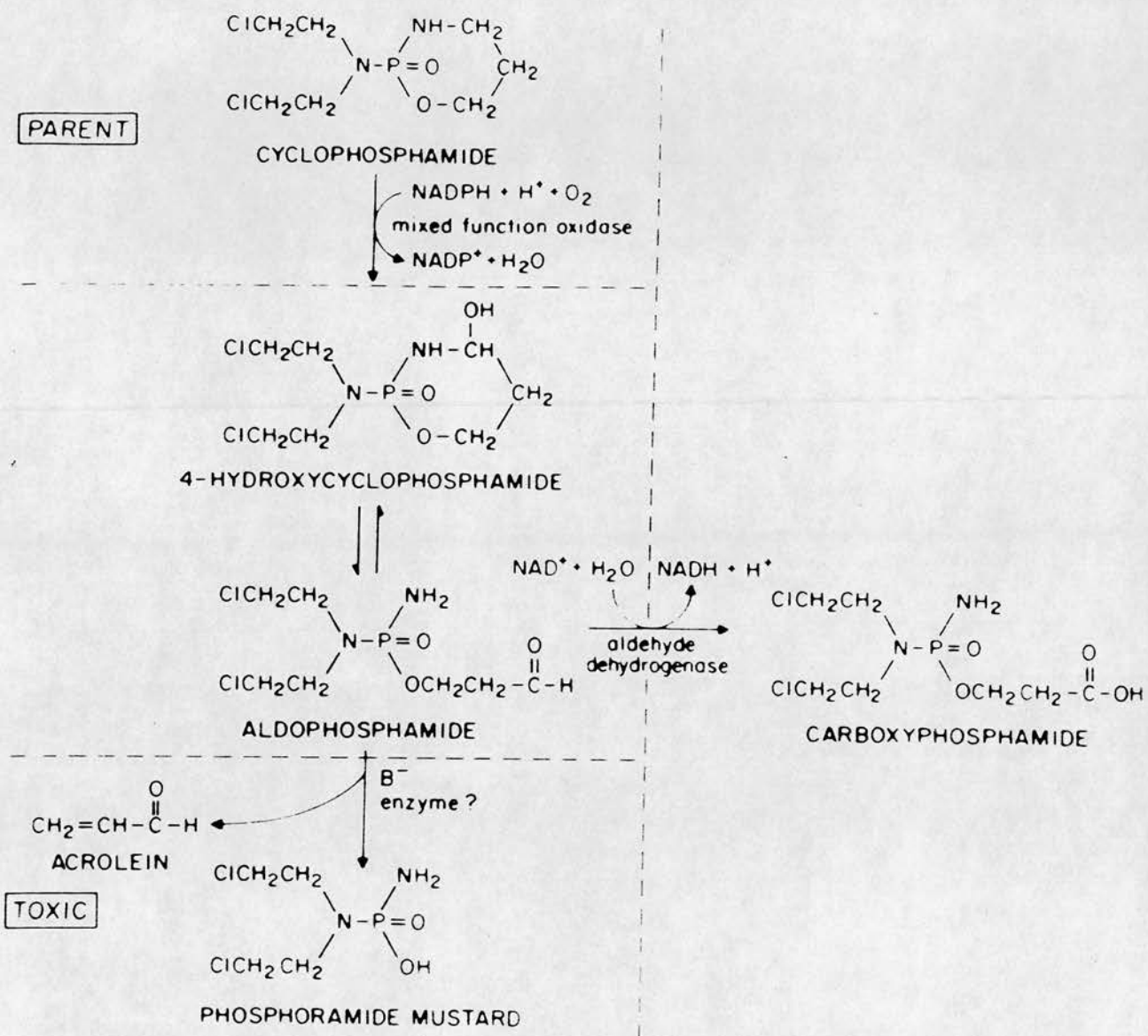


CCNU

More recent studies have identified the P450 isozymes which catalyse the activation and/or detoxification of cyclophosphamide, tamoxifen and the morpholinodoxorubicins. This raises the possibility that increasing the expression of these P450s could lead to increased drug metabolism and hence greater efficacy.

Synthesis of the immunosuppressive bifunctional alkylating agent cyclophosphamide was first reported by Arnold & Bourseaux in 1958. Cyclophosphamide toxicity is thought to be mediated by the formation of reactive carbonium ions which alkylate guanine residues, leading to the formation of DNA cross links (Calabresi & Parks, 1985). The parent compound is inactive as administered and has been shown to undergo P450 mediated metabolism to generate two reactive products, phosphoramidate mustard and acrolein. In support of this, it was demonstrated that activation was NADPH and O₂ dependent, was inhibited by the non-specific P450 inhibitor SKF525A and induced by phenobarbital (Brock & Hohorst, 1963,1967; Brock, 1967). A simplified reaction scheme for cyclophosphamide metabolism is shown in Figure 1.5.4. Several further metabolites are also formed which are not described in this reaction scheme - these do not have cytotoxic properties.

Figure 1.5.4 Cyclophosphamide metabolism



Adapted from Prough & Powis, 1987

Step (1): The first step is a P450-mediated hydroxylation, catalysed by CYP2B proteins. Several independent lines of evidence support the identification of a protein(s) from the CYP2B family in catalysing this reaction. Pretreatment of rats with phenobarbital resulted in an increased rate of cyclophosphamide metabolism (Sladek, 1972), while CYP2B1 mRNA was detected in HepG2 cells which metabolically activated the drug (Dearfield et al, 1986). Additional evidence was provided by Black et al (1989) who demonstrated that expression of a full-length rat CYP2B1 cDNA in *Saccharomyces cerevisiae* resulted in an increased mutation frequency on exposure to cyclophosphamide.

Step (2): 4-hydroxycyclophosphamide exists in tautomeric equilibrium with aldophosphamide, neither of which have any cytotoxic activity. Aldophosphamide can then be further metabolised in one of two ways:

Step (3): Hydrolysis to phosphoramidate mustard and acrolein, both of which metabolites are cytotoxic or;

Step (4): Oxidation to carboxyphosphamide, a non-toxic metabolite, catalysed by either aldehyde oxidase or NAD-dependent aldehyde dehydrogenase.

The major cytotoxic metabolites, phosphoramidate mustard and acrolein exert their cytotoxic effects in different ways: the major cytotoxic effects arise from DNA cross-linking, initiated by the aziridinium ion of the phosphoramidate mustard. Acrolein, in contrast, produces single DNA strand breaks rather than inter-strand cross links (Erickson et al, 1980; Cairney et al, 1984). The clinical use of cyclophosphamide is often compromised by its toxicity to the bladder, which usually manifests as haemorrhagic cystitis. Acrolein has been identified as the primary causative urotoxic agent (Brock et al, 1981; Sladek et al, 1982).

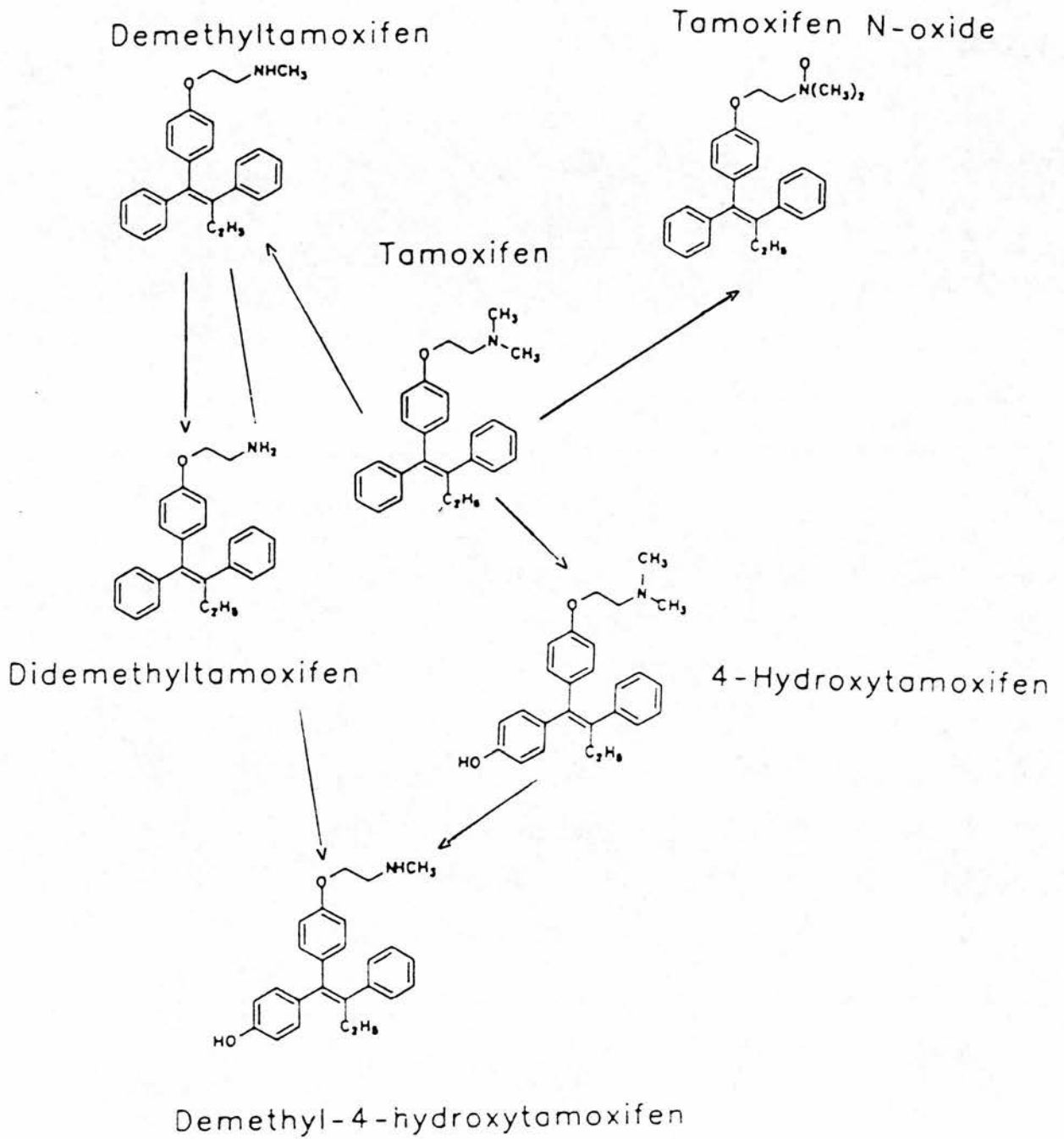
In 1896, Beatson demonstrated that an "ovarian product" promoted tumour growth in the breast, providing early evidence for the involvement of oestrogens in the aetiology of breast cancer. More recent studies have shown that approximately one third of breast tumours are oestrogen dependent (Lippmann & Dickson, 1989), and there is evidence linking oestrogen-based contraceptives to the initiation of both benign and malignant tumours (Fornander et al, 1989). Tamoxifen, (Z-1-[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-butene), which has become the most widely used drug in the clinical management of breast cancer, has been shown to act by binding to the oestrogen receptor (ER) where it can mimic the effects of endogenous oestrogen. The compound has different effects in different species,

however. In the mouse, it acts as a full oestrogen agonist, while in the rat it can act as a partial agonist or partial antagonist. In humans, tamoxifen is a full oestrogen antagonist (Jordan & Robinson, 1987) and as such has become a powerful drug in the treatment of hormone-dependent breast cancer. Binding of oestradiol and tamoxifen to the ER is competitive. This was confirmed by the studies of Lino *et al* (1991) which showed that tamoxifen inhibited the oestrogen dependent tumour growth of MCF7 cells grown as a solid tumour xenograft in athymic nude mice while the addition of exogenous oestradiol reversed tamoxifen induced growth inhibition.

Tamoxifen bound to the ER blocks cell division and the cell cycle is interrupted at the G₁/S interface (Osborne *et al*, 1983; Sutherland *et al*, 1983). The effects of the drug are cytostatic rather than cytotoxic (Jordan, 1983) and it is therefore necessary to administer the drug continuously to maintain therapeutic serum concentrations. For therapy to succeed, it is also a prerequisite that the tumour remains hormone dependent (Jordan, 1990). The drug is currently undergoing trials for prophylaxis in women who are considered to be at high risk of developing breast cancer (Powles *et al*, 1990).

Although five tamoxifen metabolites have been detected in human serum (Jordan, 1982; Kemp *et al*, 1983), the two major forms are N-desmethyl tamoxifen and 4-hydroxy tamoxifen (Figure 1.5.5).

Figure 1.5.5 Tamoxifen metabolism



N-desmethyl tamoxifen is the major metabolite in plasma, while 4-hydroxy tamoxifen is the most potent anti-oestrogen and binds the ER with the greatest affinity. The involvement of cytochrome P450 in the metabolism of tamoxifen was first demonstrated by Mani & Kupfer (1991), who showed that tamoxifen was metabolised to a reactive intermediate in human liver microsomes which bound irreversibly to microsomal proteins. This process was NADPH and O₂ dependent and was inhibited by CO and antibodies to NADPH-cytochrome P450 reductase. In addition, binding was increased by pretreatment of the microsomes with phenobarbital. Similar results were obtained for the compound chlorotrianisene (TACE) which can also act as both an oestrogen agonist and antagonist. Previous studies had shown that TACE is metabolised by P450 (Jeudes *et al*, 1987; Jeudes & Kupfer, 1990). Jacolot *et al* (1991) demonstrated that a protein(s) from the CYP3A family was responsible for the metabolism of tamoxifen to N-desmethyltamoxifen in human liver microsomes. Whether the drug can also be metabolised within breast tissue and which P450 is involved in the *in situ* metabolism has not yet been determined. It is also not known which P450 is responsible for metabolism to 4-hydroxy tamoxifen, the most active form. Plasma concentrations of tamoxifen metabolites were shown to vary widely between individual patients. This may be due to inter-individual variation in the expression of the drug metabolising enzymes which are active in its metabolism.

Both tamoxifen and N-desmethyl tamoxifen are potent inhibitors of protein kinase C (O'Brian *et al*, 1985,1986, 1988), which may be one mechanism by which these compounds inhibit tumour cell proliferation. Tamoxifen has also been shown to bind calmodulin which is involved in DNA synthesis. Inhibition of calmodulin activity may therefore inhibit DNA synthesis, leading in turn to tumour growth inhibition (Lam, 1984). Turner *et al* (1991) proposed that tamoxifen cytotoxicity was mediated by a reactive free radical intermediate(s). Attempts were made to isolate this intermediate by spin trapping, using nitrones to trap the radical as the corresponding nitroxide, detected by electron spin resonance (ESR). Intracellular production of superoxide radicals was indeed detected, but there was no evidence for the production of hydroxyl radicals.

As with many other anticancer drugs, prolonged tamoxifen therapy can lead to the development of resistance. A number of mechanisms have been proposed to account for this: Loss of oestrogen receptors or selection of hormone independent clones may be responsible, as may alteration in the steady state serum levels of drug, leading to decreased cytostatic action; A correlation has also been found between

the isomerisation of *trans*-4-hydroxy tamoxifen to the *cis* -4-hydroxy form and decreased antitumour effects (Osborne et al, 1992). The *trans* isomer, although a minor metabolite, has been shown to bind to the ER with a greater affinity than tamoxifen itself and acts as an oestrogen antagonist. As such, it has been proposed to be the major active form of the drug. In contrast, the *cis* isomer has reduced affinity for the ER and acts as a full oestrogen agonist. A high concentration of *cis*-4-hydroxy tamoxifen has been observed in patients who have developed resistance to tamoxifen therapy.

The morpholino and cyanomorpholino-doxorubicin analogues (MRAs) are intensely potent anthracyclines which do not have the same cardiotoxic side effects as their parent compound, adriamycin (Acton et al, 1984). The clinical effectiveness of adriamycin is often significantly impaired by the development of drug resistance (Batist et al, 1986). Neither MRA or MRA-CN show cross-resistance in cell lines which are resistant to adriamycin (Streeter et al, 1985). While adriamycin participates in redox cycling, the morpholino derivatives exert their anti-tumour effects by different mechanisms - MRA initiates DNA strand breakage, while MRA-CN causes DNA cross-linking (Scudder et al, 1988; Jesson et al, 1987). Lau et al (1989) demonstrated that MRA cytotoxicity could be potentiated up to 100-fold in the presence of human liver microsomes and NADPH and inhibited by carbon monoxide and SKF-525A, thus implying P450 involvement in the metabolic activation of these compounds. A recent *in vitro* study (Lewis et al, 1992) demonstrated that CYP3A isozymes (CYP3A3 and/or CYP3A4) were capable of metabolically activating these drugs.

NADPH-cytochrome P450 reductase also plays a role in the activation of anti-tumour agents. This is of particular importance in the metabolism of anthracycline antibiotics such as adriamycin. The anti-neoplastic properties of this agent have been shown to depend on the one electron reduction of the parent compound to the corresponding semiquinone free radical (Sinha, 1980; Basra et al, 1985). This reactive species can either bind DNA (Sinha, 1980) or protein (Ghezzi et al, 1981) directly or undergo redox cycling, leading to the production of superoxide radicals (Sinha, 1989). A recent study (Bartoszek & Wolf, 1992) using the breast carcinoma cell line MCF-7, demonstrated that the cytotoxicity of adriamycin was significantly increased by the addition of exogenous NADPH and NADPH-cytochrome P450 reductase. Neither of these agents was toxic in its own right.

1.5.4 The role of P450 in autoimmune disease

The inflammatory liver disease autoimmune chronic active hepatitis is thought to be caused by loss of immunological tolerance against liver tissue and, as such, responds well to immunosuppressive therapy. Administration of the diuretic tienilic acid lead to the onset of hepatitis, accompanied by a high serum concentration of antibodies which react with liver and kidney microsomes (LKM-2). The antigen recognised by these antibodies was found to be a CYP2C protein which is involved in the metabolism of tienilic acid (TA). It has been proposed that CYP2C9 metabolism of TA leads to the formation of an active metabolite which binds to the P450 resulting in altered antigenicity. This modified protein is no longer recognised by the immune system which causes autoantibodies to be raised against it (Homberg *et al*, 1984; Beaune *et al*, 1987).

Two further P450 proteins have been similarly identified as human autoantigens. These are recognised by the so-called liver-kidney-microsomal antibodies (LKMs) in sera from patients with drug-induced or idiopathic chronic immune hepatitis. These are summarised in Table 1.5.4.

Table 1.5.4 Microsomal autoantigens in liver disease

Mol. wt.	Nomenclature	Associated P450	Disease
50	LKM-1	CYP2D6	autoimmune CAH
50	LKM-2	CYP2C9	drug-induced hepatitis (tienilic acid)
?	LKM-3	?	chronic hepatitis D
50	-	CYP1A2	autoimmune CAH, dihydralazine hepatitis
55	-	?	autoimmune CAH
64	-	?	autoimmune CAH

CAH: idiopathic autoimmune chronic active hepatitis

Adapted from Manns, 1991

LKM-1 autoantibodies characterise autoimmune hepatitis type II and recognise CYP2D6 protein. Not all patients expressing these antibodies have received drugs which are known CYP2D6 substrates, however. It has been proposed that the autoimmune response is initiated by compounds in the diet or environment (Zanger *et al*, 1988; Manns *et al*, 1989).

Oxidative metabolism of the anaesthetic halothane can also lead to an adverse immunological response. Halothane (1-bromo-1-chloro-2,2,2-trifluoroethane) is metabolised by cytochrome P450 to an unstable halohydrin intermediate which goes on to form trifluoroacetyl chloride or bromide (Gandolfi et al, 1980). This is then hydrolysed to yield trifluoroacetic acid which acetylates hepatic proteins. These acetylated proteins are not recognised by the immune system, which mounts an immunological response leading to liver damage, manifested as halothane hepatitis (Poul & Gillette, 1982). The P450 isozyme responsible for halothane metabolism has not yet been identified, but a trifluoroacetyl adduct of halothane bound to a 54kD P450 protein has been detected in rat hepatocytes (Satoh et al, 1985).

1.6 Aims of thesis

Cytochrome P450 enzymes are therefore, in addition to their endogenous role, centrally involved in the metabolism of many environmental toxins and commonly prescribed drugs. At present, however, there is very little information available about the regulation and expression of these genes in human tissues. P450 expression has been extensively studied in experimental animals, but significant inter-species variation has been observed in the metabolism of many chemicals, and it is therefore not clear which species best represents P450 regulation in man. Cultured cells have also been used in the study of cytochrome P450 expression, but there are often inherent limitations imposed by the in vitro culture environment which are not observed in vivo.

The aims of this thesis were therefore three-fold:

- 1) To study the xenobiotic regulation of cytochrome P450 gene expression in a number of model systems - in particular, to characterise the effects of TCPOBOP (1,4-bis 2-(3,5-dichloropyridyloxy)benzene)), an extremely potent modulator of P450 expression, which had previously been shown to be both tissue and species specific in its inductive effects.
- 2) To develop a model in which the expression and regulation of human P450 genes could be investigated, and to determine if this could be exploited to therapeutic benefit.
- 3) To determine the response of human tumour cells to a range of xenobiotic modulators and to determine to what extent the response of the human tumours was representative of the response obtained in experimental animals and cultured cells.

Chapter 2: Materials and Methods

2.1 Chemicals

Unless otherwise indicated, all chemicals were purchased from either Sigma Chemical Co. Ltd., Poole, Dorset, or BDH Ltd., Burnfield Avenue, Thornliebank, Glasgow, and were of analytical grade or better. Cell culture reagents were obtained from Gibco, Ltd., PO Box 35, 3 Washington Road, Paisley. A complete list of the chemical reagents used and their respective suppliers is given in Appendix 1. TCPOBOP was synthesised according to a modified version of the method of Poland *et al* (1980). Two molar equivalents of 2,3,5-trichloropyridine were added to 1 molar equivalent of hydroquinone in DMSO. The solution was heated to 65°C under reflux for 30 minutes before the addition of 30mM NaOH to make a 50% aqueous solution. After a further 2 hours at 110°C, the solution was cooled to room temperature and the solid product collected by the addition of 10% NaOH and subsequent filtration. The product was washed with dH₂O to remove any aqueous impurities and re-crystallised from carbon tetrachloride before analysis by nuclear magnetic resonance spectroscopy and mass spectrometry to confirm the chemical structure and molecular mass.

2.2 Animal experiments

2.2.1 Rodent inductions

All the experiments described within this section, with the exception of part (c) below, were carried out in the Faculty of Medicine Animal Area, Department of Biochemistry, Edinburgh University.

Male and female C57/BL6 and DBA/2 mice (average weight 20g) and Wistar rats (average weight 250g) were obtained from Banton & Kingman, Hull, UK. The animals were allowed to acclimatise for at least 7 days before treatment in a controlled environment with a 12hr light/dark cycle, and were fed *ad libitum* on standard rodent laboratory chow. These animals were then treated according to the following experimental protocols:

(a) Administration of dexamethasone, phenobarbital and TCPOBOP to DBA/2 mice: Characterisation of the extent of P450 induction

Male and female DBA/2 mice (10 of each sex per treatment group) were treated intra-peritoneally as described below:



Control, corn oil, 3 injections
Dexamethasone, 100mg/kg in corn oil, 4 injections
Phenobarbital, 80mg/kg in PBS, 3 injections
TCPOBOP, 3mg/kg in corn oil, SINGLE INJECTION

Injections were administered at intervals of 24 hours, in 0.2mls vehicle per mouse. 24 hours following the final injection, or on Day 4 for the TCPOBOP-treated group, the animals were sacrificed by cervical dislocation and their livers removed, rinsed in phosphate buffered saline (PBS) and snap-frozen on cardice. Organs from within the same treatment group were pooled. Tissue was stored at -70°C prior to processing.

(b) Duration of the inductive effect

Male and female DBA/2 mice (6 of each sex per treatment group) were given a single intra-peritoneal injection of TCPOBOP (3mg/kg) on Day 1 and were sacrificed by cervical dislocation on Day 4 and at 2, 4, 8 and 12 weeks respectively. Livers were removed and snap-frozen as previously described. Control animals received injection vehicle (corn oil) only, and were sacrificed on Day 4.

(c) Long term effects of TCPOBOP

This experiment was carried out at the Imperial Cancer Research Fund Clare Hall Laboratories, Blanche Lane, South Mimms, Potters Bar, Herts. EN6 3LR. DBA/2 mice (3 of each sex per treatment group) were treated as described in part (c) above, except that mice were sacrificed by cervical dislocation on Day 4 and at 3, 4, 6, 8, 10 and 12 months following a single intra-peritoneal injection of TCPOBOP (3mg/kg).

(d) Induction in hypophysectomised animals

C57BL/6 mice were obtained from Charles Rivers Laboratories, Willmington, Massachusetts, USA, where para-pharyngeal hypophysectomy and sham-operations were carried out. The extent of hypophysectomy was assessed by monitoring weight gain of the hypophysectomised animals relative to the sham-operated controls. Both groups were then treated with a range of P450 inducing agents as described in part (a) above.

(e) TCPOBOP administration to Wistar rats

Male and female Wistar rats (average weight 250g, 3 of each sex per treatment group) were administered a single IP injection of TCPOBOP (3mg/kg). Control animals received injection vehicle (corn oil) only. Three days following injection, the rats were sacrificed and their livers removed, rinsed in PBS and snap-frozen on cardice. Tissue was stored at -70°C prior to processing.

2.2.2 Induction experiments in mice bearing human tumours as xenografts

These experiments were conducted in the ICRF Clare Hall Laboratories. Animal maintenance and treatment was carried out by Nick East and Hazel Holdsworth, under the direction of Mr. D. Watling.

2.2.2.1 Mice and Tumours

The tumours NCH (infiltrating ductal carcinoma of the breast) and GFH (adenocarcinoma of colon) were maintained as xenografts growing sub-cutaneously in the flanks of 6-12 week old specific pathogen free Nu-Nu mice of mixed genetic background and were passaged as required. These animals were maintained as previously described (Malik et al, 1989). The tumours were established from primary untreated human tumours without prior tissue culture. These animals were then treated according to the following experimental protocols:

(a) Assessment of tumour P450 levels

Human breast and colon tumours were established bilaterally as xenografts by sub-cutaneous injection of tumour mash in each flank of a panel of immune-deficient mice and allowed to grow until they had reached 1cm in diameter. The tumour-bearing mice were then assigned at random into groups (3 mice per treatment group) and treated intra-peritoneally with a range of P450 inducing agents as follows:

Control , corn oil, 3 injections
β-naphthoflavone, 80mg/kg, 3 injections in corn oil
3-methylcholanthrene, 100mg/kg, 3 injections in corn oil
Clofibrac acid, 200mg/kg, 4 injections in corn oil
TCPOBOP, 3mg/kg, SINGLE INJECTION in corn oil
Dexamethasone, 100mg/kg, 4 injections in corn oil
Phenobarbital, 80mg/kg, 3 injections in PBS
Araclor 1254, 500mg/kg, SINGLE INJECTION in corn oil

All injections were made in 0.2mls vehicle, and at intervals of 24 hours. Animals were sacrificed 24 hours following the final injection, with the exception of the TCPOBOP and Araclor 1254 treated groups, which were sacrificed on Day 4 and Day 8 respectively. Livers and tumours were removed, rinsed in PBS, snap-frozen and stored at -70°C prior to processing. Tissue from animals within the same treatment group was pooled, although left and right flank tumours were kept separate for protein and RNA analysis respectively.

(b) Increased dose of TCPOBOP

Mice bearing human breast and colon tumours as xenografts were established as described in (a) above, and treated intra-peritoneally as follows:

Stock control, NO TUMOUR, corn oil, 3 injections
Control, corn oil, 3 injections
3-methylcholanthrene, 100mg/kg, 3 injections in corn oil
TCPOBOP 1, 3mg/kg, SINGLE INJECTION in corn oil
TCPOBOP 2, 15 mg/kg, SINGLE INJECTION in corn oil

Following treatment, livers and tumours were removed and processed as previously described. An untreated, unxenografted group of mice (stock control) were included in this study to determine whether the xenografting process itself had any effect on cytochrome P450 levels.

(c) Colon dose-response

Mice bearing human colon tumour xenografts, as described in (a) above, were treated intra-peritoneally with TCPOBOP in a range of concentrations as detailed below :

Control , corn oil, single injection
TCPOBOP 1, 1mg/kg, SINGLE INJECTION in corn oil
TCPOBOP 2, 3mg/kg, SINGLE INJECTION in corn oil
TCPOBOP 3, 10mg/kg, SINGLE INJECTION in corn oil
TCPOBOP 4, 15mg/kg, SINGLE INJECTION in corn oil

Livers and tumours were removed on Day 4 following treatment and processed as previously described.

(d) TCPOBOP and cyclophosphamide administration

This experiment was designed to determine whether prior administration of TCPOBOP to mice bearing human breast or colon tumours as xenografts would alter their response to subsequent cyclophosphamide administration. Mice were implanted with human breast and colon xenografts as described previously. The tumours were allowed to grow until an average diameter of 0.2cm was achieved, when the mice were assigned at random into groups and treated intra-peritoneally as follows :

Group 1: Control, corn oil, single injection

Group 2: TCPOBOP, 3mg/kg in corn oil, SINGLE INJECTION on Day 1

Group 3: Cyclophosphamide, 0.25mg/mouse in corn oil, 3 injections on Day 6, 13 and 20

Group 4: TCPOBOP, 3mg/kg in corn oil, SINGLE INJECTION on Day 1, followed by Cyclophosphamide, 0.25mg/mouse in corn oil, 3 injections on Day 6, 13 and 20

Tumour surface area was measured (the product of the two largest tumour diameters, measured at right angles using callipers) on TCPOBOP administration, and at 7 day intervals thereafter. At the end of the experiment, animals were sacrificed, livers and tumours removed and processed as previously described.

(e) The effect of TCPOBOP on tumour regression

Human colon tumours were established as xenografts as described in (d) above and allowed to grow to approximately 0.2cm in diameter. Mice were then treated intra-peritoneally with TCPOBOP at a range of concentrations as described below to assess the effect of this compound on tumour growth.

Control , corn oil, single injection
TCPOBOP 1, 1mg/kg, SINGLE INJECTION in corn oil
TCPOBOP 2, 3mg/kg, SINGLE INJECTION in corn oil
TCPOBOP 3, 15mg/kg, SINGLE INJECTION in corn oil
TCPOBOP 4, 30mg/kg, SINGLE INJECTION in corn oil
TCPOBOP 5, 60mg/kg, SINGLE INJECTION in corn oil

Tumour growth was monitored on a daily basis, as long as any tumour persisted. Tissue was collected at the end of the experiment as previously described.

(f) Generation of xenograft tumours from the cell line Hepa-1

10^8 cells were implanted into each flank of Nu-Nu mice and the resulting solid tumours were allowed to grow until they had reached 1cm in diameter. The tumour bearing mice were then treated intra-peritoneally as follows:

Control, corn oil, 3 injections
3-methylcholanthrene, 100mg/kg, 3 injections in corn oil
Phenobarbital, 80mg/kg, 3 injections in PBS
TCPOBOP, 3mg/kg, single injection in corn oil

Livers and tumours were removed on Day 4 following treatment and processed as previously described.

2.3 Human tissue samples

2.3.1 Sources of material

Samples of normal colonic mucosa and colon tumours were obtained from patients undergoing surgery at the Royal Infirmary or Western General Hospital, Edinburgh. Identification of normal and tumour tissues was made histopathologically. Samples were snap-frozen as soon as was practical following resection and were stored at -70°C prior to processing. The available clinical data on these samples is summarised in Table 2.3.

Table 2.3 Clinical data on human colon tumour samples

Sample	Patient	Location	Size	Duke's status*
1	91/4328	hepatic flexive colon	11 x 7 cm	B
2	91/4818	rectum	4 x 3.5 cm	B
3	91/7239	ascending colon	13 x 6 cm	B
4	91/7283	sigmoid colon	3 x 3 cm	C
5	91/7952	sigmoid colon	4 x 3.5 cm	C

* The most commonly used system for classifying colon tumours and assessing their prognosis is that of Duke, summarised in Monfardini *et al.*, (1987).

2.4 Analysis of rodent and human tissue samples

2.4.1 Subcellular fractionation

Microsomal and cytosolic fractions were prepared from tissue samples as described by Meehan *et al.* (1988a). Tissue samples (~1g) were thawed rapidly at 37°C, scissor minced and homogenised (Silverson Laboratory Mixer Emulsifier) at 4°C in 3 volumes of 10mM KHPO₄, pH 7.4, containing 1.15% (w/v) KCl and 0.1mM EDTA. The homogenate was centrifuged at 11000rpm for 20 minutes (Du Pont Sorvall RC5B Refrigerated Superspeed Centrifuge, SS-34 rotor) to remove cell debris, the pellet discarded and the resulting supernatant transferred to ultracentrifuge tubes and spun at 45000rpm (Du Pont Sorvall OTD65B Ultracentrifuge, TFT 45.6 rotor) for 80 minutes to separate the microsomal and cytosolic fractions. The supernatant (cytosolic fraction) was removed and stored at -40°C for future analysis. The microsomal pellet was washed by resuspension in phosphate buffer using a glass-teflon hand homogeniser and re-centrifuged at 45000rpm for 1 hour. The final pellet was resuspended in 0.25M sucrose/10mM phosphate, pH 7.4 to a protein concentration of 10-20mg/ml and stored at -40°C until required. Whenever possible, several aliquots of each microsomal suspension was prepared, to minimise the number of freeze-thaw cycles to which each was subjected.

2.4.2 Preparation of RNA

All tips, eppendorfs and tubes used in the preparation of RNA were soaked overnight in dH₂O containing 0.1% diethylpyrocarbonate (depc-dH₂O) and

autoclaved before use to ensure sterility and to eliminate any contaminating RNAases. All glassware was rinsed in depc-dH₂O before use.

RNA was isolated from tissue samples according to the method of Cox (1986). 1g of frozen tissue was placed directly in 20mls of ice-cold 8M guanidinium hydrochloride in a 30ml depc-treated polypropylene tube, scissor minced and homogenised. The resulting homogenate was centrifuged (RC5B, SS-34 rotor, 9000rpm, 5 minutes, 4°C) to remove cell debris and the supernatant transferred to a fresh tube, mixed with 0.5 volumes (10mls) of cold absolute alcohol and placed at -20°C for 45 minutes to precipitate nucleic acids. Following centrifugation (9000rpm, 10minutes, 4°C), the pellet was resuspended using a glass-teflon homogeniser in 20mls of 6M guanidinium hydrochloride and ethanol-precipitated as before. This 6M precipitation step was repeated once more and the resulting pellet dissolved in 1ml of depc-dH₂O. 0.5 volumes of 7.5M ammonium acetate and 2.5 volumes of cold absolute alcohol were added and the tubes placed at -20°C for > 2 hours to selectively precipitate RNA (DNA remains in solution under these conditions), which was collected by centrifugation (9000rpm, 10 minutes, 4°C). The purity of the RNA was further increased by 2-3 re-precipitation steps with 0.1 volumes 2M sodium acetate, pH5.2 and 2.5 volumes cold absolute alcohol. The final pellet was resuspended in a minimum volume of depc-dH₂O and stored at -70°C until required. The purity and [RNA] of the sample was determined as described in section 2.6.1

2.5 Protein analysis

2.5.1 Protein estimation

Protein concentration was determined spectrophotometrically according to Lowry et al (1951), using serial dilutions of bovine serum albumin (BSA) to generate a standard curve each time the assay was performed. The absorbances obtained at 600nm were effectively linear over the range 0-200µg/ml, and all samples were therefore diluted (0.1M NaOH) to lie within this range.

2.5.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). All protein samples were analysed by SDS-PAGE prior to immunoblotting to demonstrate equivalence of loading and to

ensure that the samples were not degraded. Samples were prepared for electrophoresis by dilution in distilled deionised water (dH_2O) to a concentration of 3mg/ml of total protein, before the addition of an equal volume of "boiling mix" (0.05M Tris-HCl pH8, 2% (w/v) SDS, 5% (v/v) β -mercaptoethanol, 10% (v/v) glycerol, 0.005% (w/v) bromophenol blue) to give a final protein concentration of 1.5mg/ml. Proteins were denatured by heating to 100°C for 5 minutes. For Coomassie Brilliant Blue stained gels, $15\mu\text{g}$ of total cellular protein (or microsomal or cytosolic fractions as appropriate) was loaded per track. Molecular weight markers containing alpha-lactalbumin ($M_r=14,200$), soybean trypsin inhibitor ($M_r=20,100$), trypsinogen ($M_r=24,000$), bovine erythrocyte carbonic anhydrase ($M_r=29,000$), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase ($M_r=36,000$), chicken egg albumin ($M_r=45,000$) and bovine serum albumin ($M_r=66,000$) were prepared according to the manufacturer's instructions and were loaded at $10\mu\text{l}$ per track. SDS-PAGE gels (0.15cm) were cast using Biorad Protean II electrophoresis apparatus. Separating gel composition was dependent on the particular proteins of interest - P450 proteins were analysed using 9% separating gels. The composition of the separating gel is given below :

9%: 8.0mls 40% (w/v) acrylamide, 5.5mls 2% (w/v) bis-acrylamide, 9.25mls separating gel buffer, 12.3mls dH_2O , 2mls 1% (w/v) APS, $20\mu\text{l}$ TEMED; where TEMED (N,N,N',N'-tetra-methyl ethylenediamine) and APS (ammonium persulphate) function as cross-linking agents; separating gel buffer (1.5M Tris-HCl, 0.5%w/v SDS, pH8.8)

Gels were poured to a height of 12cm and immediately overlaid with a 1:1 mixture of isobutanol and water. The rapid separation of this mixture into organic and aqueous phases resulted in an even boundary forming at the interface with the gel. The presence of this overlay also served to prevent evaporation of the gel components during polymerisation. Once set, the butanol overlay was removed and the gel rinsed several times with dH_2O , before a stacking gel (4.5% (w/v) acrylamide: 1.1ml (w/v) 40% acrylamide, 0.7ml (w/v) 2% bisacrylamide, 2.5mls stacking gel buffer (0.5M Tris-HCl, 0.5% (w/v) SDS, pH 8.8), 5.4mls dH_2O , 0.3mls 1% (w/v) APS, $10\mu\text{l}$ TEMED) was poured to the top of the plates and a well-forming comb (15 tracks) inserted. The same percentage stacking gel was poured in every case, irrespective of the composition of the separating gel. When the stacking gel had set, the plate assemblies were securely attached to the central cooling reservoir of the Protean II apparatus and the positions of the wells clearly marked. The top buffer chamber was filled with freshly prepared electrode buffer (0.05M Tris-HCl pH

8.3, 0.05M glycine & 0.1% SDS), and the combs carefully removed. Samples were loaded using a Hamilton syringe, before the entire apparatus was placed in a gel tank containing 3 litres of electrode buffer. Gels were run (50mA/gel stacking, 20mA/gel separating) with cold water cooling and with continuous mixing of the electrode buffer to minimise any build-up of buffer gradients. When the dye front was approximately 1cm from the bottom of the plates, electrophoresis was stopped, the gels removed from the glass plates, stained for 1 hour in Coomassie Brilliant Blue (0.25% (w/v) Coomassie Brilliant Blue, 45.5% (v/v) methanol, 9% (v/v) acetic acid) and destained (10% (v/v) methanol, 9% (v/v) acetic acid) to visualise the protein bands.

2.5.3 Immunoblotting

Immunoblotting (Western blotting) was performed essentially as described by Towbin et al (1979), with modifications according to Lewis et al (1988). Proteins were separated on SDS-PAGE, with the amount of protein loaded dependent on the tissue or cell type being studied. Following electrophoresis, gels were removed from the glass plates, the stacking gel removed and discarded and the remaining gel portion marked uniquely for orientation. A gel/membrane "sandwich" was then assembled as follows: A large basin was filled with transfer buffer (20mM sodium orthophosphate.12H₂O, 20% (v/v) methanol), into which was placed a plastic cassette containing a nylon "Scotchbrite" pad, a sheet of 3mm filter paper cut to a size slightly larger than the gel and a sheet of nitrocellulose (pore size 0.45 µm) cut to a similar size. The gel was placed on top of the nitrocellulose filter, covered with another sheet of filter paper, a further nylon pad and the cassette closed, thus clamping the gel sandwich firmly together. Each gel was assembled in a similar manner, and the cassette(s) placed into a Biorad transblot apparatus containing freshly prepared transfer buffer. Proteins were then transferred electrophoretically (250mA, 16 hours) from the gel to the nitrocellulose sheet. Following transfer, the gel sandwiches were disassembled and the nitrocellulose filter cut to an exact replica of the gel. Any excess gel and nitrocellulose were discarded at this stage. The nitrocellulose filter was washed several times in TBST (50mM Tris-HCl, pH7.9, 0.15M NaCl, 0.05% (v/v) Tween 20) to eliminate any trace of transfer buffer and non-specific antigen binding blocked by incubation in a solution of low-fat dried milk powder (3% (w/v) Marvel in TBST, 1 hour). The filter was then washed thoroughly and incubated with the specific antisera of interest (diluted 1:500 in TBST, 1 hour). Excess unbound antigen was removed by washing

the filter several times in TBST before incubation with an HRP-labelled secondary antibody (donkey anti-rabbit IgG-HRP for polyclonal primary antisera or donkey anti-mouse IgG-HRP for monoclonal primaries, diluted 1:1000 in TBST, 1 hour). After visualisation of the immunoreactive polypeptides using freshly prepared 4-chloro 1-naphthol as substrate (0.6g/l 4-chloro-1-naphthol, 20% (v/v) methanol in TBS, 0.04% (v/v) hydrogen peroxide) the signal was enhanced with ^{125}I -protein A (50 μl or 0.19MBq in 50mls TBST) (Amersham International, plc) and subsequent autoradiography (Kodak X-Omat AR5 X-ray film) with intensifying screens at -70°C . Differing exposure times were used in order to optimise the autoradiographic signal from each blot. When the level of expression of the proteins of interest was particularly low, enhanced chemiluminescent detection (ECL) was used (Amersham International, plc). This technique relies on the generation of an unstable photodynamic complex on reaction of H_2O_2 , HRP-labelled secondary antibody and the cyclic diacylhydrazine luminol. Dissociation of this complex results in luminol returning to its ground state with associated light emission. The amount of light emitted is directly proportional to the amount of immobilised protein bound to the HRP-labelled secondary antibody.

In order to determine the relative levels of protein expression, an appropriate exposure of each autoradiograph was scanned using a Joyce-Loebl scanning densitometer. Linearity of the method was established by generating a standard curve from a series of microsomal standards loaded in serial dilutions.

2.5.4 Mini-kits

When a limited number of samples was to be analysed, or a number of different antibodies screened, the Biorad Mini-Protean kit and Transblot cell were used. These kits, which allow SDS-PAGE to be run in 45 minutes and transfer to nitrocellulose in 1 hour, operate on the same principle as the larger kits, with the following modifications:

- (a) Gels were run at 200V, constant voltage, with no alteration in the running conditions between the stacking and separating gels or allowance made for the number of gels being run
- (b) Transfer was achieved at 100V for 1 hour in a Tris/glycine/methanol buffer (25mM Tris-HCl, 112mM glycine, 20% (v/v) methanol, pH8.3). To prevent excessive heat building up during transfer, a Bio-ice cooling unit was placed next to the gel cassettes to absorb the heat generated during transfer.

2.5.5 Antibodies

Cytochrome P450 antisera and purified rat P450 proteins which were run as standards on SDS-PAGE were provided by Dr. C.R. Wolf, and have been extensively characterised in this laboratory and others (Wolf *et al.*, 1983, 1984). Antibodies to purified rat cytochrome P450 proteins were isolated as described previously (Wolf & Oesch, 1983; Adams *et al.*, 1985; Wolf *et al.*, 1988). These antibodies have been extensively used in immunoblotting studies with both mouse and human microsomal samples (Meehan *et al.*, 1988a; Henderson *et al.*, 1990). In addition, the isozyme specificity of the antisera have been demonstrated by immunoblot analysis with expressed human recombinant P450 proteins (Forrester *et al.*, 1992). Cytochrome P450 CYP4A1 was isolated according to the method of Tamburini *et al.* (1984), and was of high purity, running as a single band on SDS-PAGE. Antibodies used in Western blot analysis were raised against rat CYP1A1, CYP2A1, CYP2B1, CYP2C6, CYP2E1, CYP3A1 and CYP4A1 proteins. CYP2E1 antisera was a generous gift from Dr. C.S. Yang, Department of Chemical Biology, College of Pharmacy, Piscataway, N.J., USA).

2.6 RNA analysis

2.6.1 Quantitation of RNA

(a) Spectrophotometric quantitation

RNA concentration was determined by measuring the UV absorbance at 260nm. The RNA sample of interest was diluted (usually 1:100) in depc-dH₂O to 1ml, mixed thoroughly and placed in a quartz cuvette. An identical reference cuvette contained depc-dH₂O only. The absorbance of the sample was read at 260nm and the RNA concentration calculated according to the following equation:

$$[\text{RNA}] = \frac{\text{OD}_{260} \times \text{D.F.}}{24}$$

where D.F. = dilution factor (usually 100); OD₂₆₀ is the absorbance at 260nm. The above equation is based on the assumption that OD₂₆₀ = 24.0 for a 1mg/ml solution of RNA.

To assess the purity of the sample, the absorbance at 280nm was also obtained and the ratio OD₂₆₀/OD₂₈₀ calculated. A pure RNA sample has an OD₂₆₀/280 ratio of 2.0. If the ratio of the sample was below 1.8, the sample was extracted with phenol/chloroform and sodium acetate/ethanol precipitated to eliminate any traces of contaminating DNA and protein.

(b) Ethidium bromide fluorescence quantitation

The RNA concentration measurements obtained spectrophotometrically were confirmed by running each sample on a horizontal 1% denaturing agarose gel (1% (w/v) agarose, 2 x GRB (0.1M 4-morpholinepropanesulphonic acid (MOPS), pH7.0, 40mM sodium acetate, 5mM EDTA, pH8.0), 18% (v/v) formaldehyde) before proceeding to Northern blot analysis. This method (Lehrach et al, 1977) involves the electrophoretic size fractionation of RNA samples on a denaturing formaldehyde/agarose gel which is then stained with the fluorescent interchelator ethidium bromide. RNA samples were prepared for electrophoresis as follows: samples were diluted in depc-dH₂O to 5µg, based on the concentration values obtained from the spectrophotometric analysis. 3 volumes of sample buffer (66% (v/v) formamide, 8% (v/v) formaldehyde, 1xMOPS) were then added to each sample, and the resulting mixture incubated at 55°C for 15 minutes. Loading dye (1/10 total volume) was added to each sample, which was mixed thoroughly before loading. Conditions for electrophoresis were dependent on the size of gel used: Mini-kits were run for 2-3 hours at 60V, midi-kits at 20V overnight. All gels were run in TBE buffer, which was freshly prepared from a 10 X stock (10 x TBE: 0.09M Tris base, 0.09M Boric Acid, 0.002M EDTA, pH 8.0) on each occasion to prevent contamination with formaldehyde. Following electrophoresis, the gel was removed from the apparatus, stained for 15 minutes in ethidium bromide (1.5mg/ml) and destained in dH₂O. UV-induced fluorescence of the RNA samples was visualised by placing the gel on a short wavelength transilluminator. The amount of fluorescence obtained was directly proportional to the [RNA] of the sample. Thus, an estimation of loading equality between a series of samples could be obtained when the gel was examined under UV light. Two sharp bands corresponding to the 18S (2366bp) and 28S (6333bp) ribosomal RNA species were present in all samples, indicating that the RNA was intact.

2.6.2 Northern blot analysis

Northern blot analysis was performed essentially as described by Meehan et al (1984). RNA was size-fractionated by denaturing gel electrophoresis as described in Section 2.6.1 (b). 10µg of each RNA sample was loaded onto a 1% formaldehyde-agarose midi-gel which was run at 20V overnight (~16 hours) in 1 x TBE buffer. A 3µl aliquot of RNA ladder was loaded in the final lane of the gel - this consisted of a mixture of DNA species (bacteriophage T7, yeast 2µ circle and bacteriophage lambda DNA) with molecular weights ranging from 0.24 - 9.5kb. Following

electrophoresis, the gel was removed from the apparatus and washed in an excess of 10 x SSC (1.5M NaCl, 0.15M tri-sodium citrate, pH 7-9) for 20 minutes to remove excess formaldehyde. Transfer to Hybond-N was achieved by capillary action in 10 x SSC (overnight, ~20 hours). The edges of the gel were sealed with Parafilm during transfer to ensure that all buffer flow was directed through the gel. The Hybond membrane was overlaid with several sheets of filter paper which had been soaked in 2 x SSC to create a buffer gradient and initiate transfer. Following transfer, the positions of the tracks were transferred from the gel to the Hybond filter, the transfer apparatus disassembled and the RNA permanently attached to the filter by "Stratalinking" i.e. exposure of the membrane to ultra-violet irradiation (254nm). To ensure that transfer was complete, the gel was stained with ethidium bromide as previously described and examined under UV-illumination. No RNA bands were visible, indicating that transfer to the Hybond membrane was complete. The portion of the membrane containing the RNA ladder was removed and stained (15 minutes) in methylene blue (0.04% (w/v) methylene blue, 0.5M sodium acetate). The RNA standards were clearly visible as a series of blue bands when the filter was destained in dH₂O. These were used to quantitate the size of the RNA transcript obtained on hybridising the filter with a cDNA probe. Hybridisation was performed at 65°C in a rotating glass tube in a Techne rotisserie oven. The filter was incubated in hybridisation mix (5 x SSC, 4 x Denhardt's solution (50 x Denhardt's: 1% Ficoll, 1% polyvinylpyrrolidone (PVP), 1% BSA), 10% (w/v) dextran sulphate, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate (NaPP_i)) for at least 3 hours before the addition of a ³²P-labelled cDNA probe. This "pre-hybridisation" allowed equilibration of the filter in the hybridisation solution and the blocking of non-specific binding sites. A ³²P-labelled cDNA probe, prepared and labelled as described in Sections 2.6.4 and 2.6.6, was denatured by heating to 100°C for 5 minutes and added directly to the tube containing the filter. Hybridisation with the probe was continued at 65°C for a further 16 -18 hours. Excess probe was then removed and the filter washed several times (2 x SSC, 0.1% (w/v) SDS, 0.1% (w/v) NaPP_i). All washes were carried out at 65°C to minimise any non-specific binding of the probe to the filter. Once the radioactive counts in the wash solution had returned to background, the filter was removed from the tube, air dried, wrapped in Saran-wrap and exposed to Kodak X-Omat AR-5 film at -70°C in a cassette with intensifying screens. If re-probing of the filter with a second cDNA

probe was required, the filter was stripped by incubation at 65⁰C in stripping solution (5mM Tris-HCl, pH 8.0, 2mM EDTA, 0.1 x Denhardt's solution) for 2 x 1 hour periods, according to the manufacturer's instructions.

2.6.3 cDNA probes

Details of the cDNA probes used are given in Table 2.6

The bacterial host for all clones was JM101, with the exception of pMP1, which was JM83; All clones were contained within pUC expression vectors - pUC9 for pMP1, 16, 26, 63 and 112; pUC19 for pMP17, 23, 81 and 90 and pUC12 for pMP193; All plasmids conferred Ampicillin resistance. The human cDNA probes have been shown to be specific for their respective gene families in mouse by mapping in recombinant inbred lines (Meehan *et al*, 1984; Miles *et al*, 1990). Equivalence of RNA loading was monitored by probing with a mouse β -actin cDNA probe, a generous gift from Dr. Joan McNab, Institute of Virology, Glasgow. The rat CYP2B1 probe was kindly provided by Dr. M. Adesnik, Department of Cell Biology, New York University School of Medicine, New York, USA and the rat CYP4A1 probe by Dr. G.G. Gibson, Department of Biochemistry, University of Surrey, Guildford, Surrey. The mouse metallothionein MT-IIa cDNA probe was kindly provided by Dr. Peter Searle, CRC Laboratories, Medical School, Birmingham and the mouse MUPS cDNA probe by Dr. Reya Al-Shawi, Department of Genetics, Edinburgh University. All other cDNA probes were produced and characterised in this laboratory.

Table 2.6 cDNA probes used in Northern blot analysis

Lab name	Clone	Species	Insert size	R.E.	Reference
pMP1	CYP1A1	human	1088bp	Eco RI	Spurr <i>et al.</i> , 1987
pMP16	CYP2E1	human	1.6kb	Eco RI	Song <i>et al.</i> , 1986
pMP17	CYP2B6	human	700bp	Eco RI	Miles <i>et al.</i> , 1989
pMP23	CYP2B1	rat	1.7kb	Eco RI	Friedberg <i>et al.</i> , 1986
pMP26	CYP3A4	human	727bp	Eco RI	Spurr <i>et al.</i> , 1989
pMP63	Cyp2c	mouse	1.4kb	Pst 1	Meehan <i>et al.</i> , 1988
pMP81	CYP2A6	human	1.75kb	Eco RI	Miles <i>et al.</i> , 1990
pMP90	Actin	mouse	1.2kb	Xba 1	Leader <i>et al.</i> , 1985
pMP112	CYP4A1	rat	2.1kb	Eco RI	Earnshaw <i>et al.</i> , 1988
pMP193	MTII	mouse	220bp	EcoRI/Hind III	Searle <i>et al.</i> , 1984
pMP240	MUPS	mouse	714bp	Sma I	Clark <i>et al.</i> , 1985

R.E. = Restriction enzyme required to release the cDNA from the plasmid

2.6.4 Preparation of cDNA probes

cDNA probes were prepared according to the small-scale alkaline lysis procedure of Birnboim & Doly (1979). An overnight culture of transformed bacteria was prepared as follows: 10mls of L-broth (10g/l bactotryptone, 5g/l yeast extract, 5g/l NaCl) was equilibrated to 37°C in a shaking incubator, before the addition of 10µl of selectable marker (ampicillin or tetracycline as appropriate). Transformed bacteria were added in one of two ways:

- (a) 10µl of the glycerol stock of the cDNA containing the plasmid of interest (see Section 2.6.5)
- (b) An aliquot (single colony) of the bacterial culture growing on an L-amp plate taken using a sterile hot loop (Section 2.6.5)

After overnight incubation, 1.5ml aliquots of the bacterial culture were transferred to eppendorf tubes and spun (13000rpm, 10 minutes) in a microfuge to pellet the bacteria. The supernatant was discarded and the pellet re-suspended in 0.15mls of Solution A (2mg/ml lysozyme, 50mM glucose, 25mM Tris-HCl pH8.0, 10mM EDTA) and left on ice for 30minutes to lyse the bacterial cell wall before the addition of 0.3mls Solution B (1% (w/v) SDS, 0.2M NaOH) to disrupt the bacterial cell membrane. After a further 5 minutes at 4°C, 0.25mls of Solution C (3M sodium

acetate, pH 4.8) was added and the resulting suspension left to precipitate for 1 hour at 4°C. Cell debris was removed by centrifugation (13000rpm, 5 minutes) and the nucleic acids precipitated from the plasmid-containing supernatant with 0.7 volumes of isopropanol for 30 minutes at -20°C. After further centrifugation (13000rpm, 10 minutes), the nucleic acids were re-suspended in 0.1ml TE buffer (10mM Tris-HCl, pH8.0, 0.1mM EDTA) and any contaminating proteins removed by phenol/chloroform extraction. The resulting extract was re-precipitated (10µl 2M sodium acetate, pH5.2, 0.25mls cold absolute alcohol) and the final pellet collected by centrifugation (13000rpm, 10 minutes), washed with cold ethanol, dried under vacuum and re-suspended in 25µl TE. The concentration of this final solution was usually between 25 and 50µg of plasmid DNA.

cDNAs were released from their carrier plasmids by digestion with an appropriate restriction enzyme. Plasmid DNA (20µl) was mixed in a sterile eppendorf tube with dH₂O (10µl), RNAaseA (3µl of a 10mg/ml stock), 10xRB (4µl) and 3µl of the appropriate restriction enzyme. RB is a commercially supplied reaction buffer specific for the particular restriction enzyme used. Digestion of the DNA was carried out at 37°C for 2 hours, before loading dye (1/10 total volume) was added to each tube and the contents loaded on a 0.8% low-melting point agarose midi-gel (0.8% LMA, 1 x TBE, 5µl EtBr). An aliquot (3µl) of 1kb DNA ladder was run to estimate the insert size(s) obtained and some uncut plasmid run as a control to assess the success of the digestion. The gel was run overnight (~18 hrs) at 20V in 1xTBE buffer and visualised on a transilluminator. The portions of gel which contained the insert cDNA of interest were excised and placed into a pre-weighed eppendorf tube. Sterile dH₂O was added (1.5mls per 1g of gel) and the DNA denatured by heating to 100°C for 5 minutes. The probe was then aliquoted and stored at -20°C prior to use.

2.6.5 Storage of plasmid DNA

(a) Permanent storage was achieved by making a glycerol stock of each plasmid as follows: transformed bacteria (JM101) were grown overnight in L-broth with appropriate antibiotic selection. Glycerol was added to a final concentration of 40% (v/v) and 1ml aliquots stored at -70°C.

(b) A working stock of commonly used plasmids was maintained on L-agar plates (L-broth plus 15g/l Difco agar) containing ampicillin (100µg/ml). L-agar plates were re-streaked with bacterial colonies every four weeks and were stored at

4°C.

2.6.6 Labelling of cDNA probes

cDNA probes were labelled according to the random priming method of Feinburg & Vogelstein (1983,1984). Purified double stranded DNA was mixed with an excess of nucleotide primers, denatured and synthesis of ^{32}P -labelled transcripts carried out by the Klenow fragment of DNA polymerase I. This fragment lacks 5'-3' exonuclease activity and the product is therefore synthesised exclusively by primer extension. The DNA was denatured (100°C, 5 minutes), added to dH_2O , and the following components added, in this order:

dH_2O	18 μl
denatured DNA	20 μl
OLB	5 μl
BSA	2 μl
^{32}P - αdCTP	3 μl
Klenow	2 μl

Notes:

1. The reaction is initiated by the addition of the Klenow fragment of E.Coli DNA polymerase I.
2. 3 μl of ^{32}P - αdCTP has a specific activity of 30 μCi .
3. OLB is a 2 : 5 : 1 mixture of the following solutions A, B, and C.
Solution A: 2M Tris-HCl, pH 8.0 (625 μl); dH_2O (82 μl); β -mercaptoethanol (18 μl); 1M MgCl_2 (125 μl); 10mM dATP, dGTP, cTTP (50 μl of each)
Solution B: 2M Hepes titrated to pH 6.6 with NaOH
Solution C: Hexadeoxyribonucleotides suspended in 3mM Tris-HCl, 0.2mM EDTA, pH 7.0, at a concentration of 90 O.D. units/ml.

The extent of incorporation of ^{32}P into the DNA was determined chromatographically. A 1 μl aliquot of each probe to be tested was spotted at the origin of a piece of DE81 chromatography paper and allowed to air-dry. The filter was then placed into a chromatography tank containing a small volume of 0.4M ammonium formate, pH8.0. Chromatographic separation was carried out until the solvent front was 1cm from the top of the filter paper (~15minutes), which was then removed from the tank, air dried, wrapped in Saran-wrap and exposed to X-ray film.

Incorporated radioactivity appeared as a spot at the origin of the chromatogram, while any unincorporated nucleotides eluted up the paper with the solvent front. Incorporation was routinely found to be >80%.

2.7 Cell culture

2.7.1 Cell line information

Five cell lines were used during this project. These lines were chosen as they have previously been shown to have constitutive monooxygenase activity (HepG2, C3H10T1/2, Hepa-1), or because they are derived from human breast (MCF7) or colon (HT29) tissues and thus provide an *in vitro* model for comparison with the *in vivo* studies. All cell lines were grown as monolayers and were maintained in a humidified atmosphere of 5% CO₂ / 37% air at 37°C. A brief description of each cell line is given below :

(a) C3H10T1/2 (Reznikoff *et al.*, 1973a,b) Culture conditions : BME (Basal Eagle's Medium) with 15% foetal calf serum (FCS). L-glutamine (0.2mM), penicillin (15U/ml) and streptomycin (5mg/ml) (P/S) were added to all media to prevent bacterial contamination : Doubling time 15.5 hours : C3H10T1/2 fibroblasts were established from C3H mouse embryos in order to study malignant transformation with polycyclic hydrocarbons. Recent reports (Jefcoate *et al.*, 1990, 1991) suggest that this cell line has constitutive mono-oxygenase activity and we and other workers have attributed this to an as yet uncharacterised cytochrome P450 protein from the Cyp2a family.

(b) Hepa-1 (Bernhard *et al.*, 1973) Culture conditions : RPMI + HEPES, 10% FCS, L-glutamine & P/S : Doubling time 28 hours : This cell line, isolated from the mouse hepatoma BW7756, retains many liver specific functions including albumin production, a function which is dramatically reduced in many immortalised cell lines. Hepa-1 cells have constitutive cytochrome P-450 dependent aryl hydrocarbon hydroxylase activity, illustrated by their ability to hydroxylate benzo(a)pyrene.

(c) HepG2 (Knowles *et al.*, 1980) Culture conditions : DMEM, 10%FCS, L-glutamine & P/S : Doubling time 60 hours: The Hep G2 cell line was derived from a childhood hepatoblastoma and has been shown to retain many of the differentiated features of human liver including detectable levels of cytochrome P450-dependent mono-oxygenase activity.

(d) MCF7 (Soule *et al.*, 1973) Culture conditions : RPMI, 10% FCS, L-glutamine & P/S : Doubling time 24 hours : This cell line, which is oestrogen-receptor positive,

was derived from a pleural effusion from a 69 year old female Caucasian patient with metastatic mammary carcinoma.

(e) HT29 (Fogh & Trempe, 1975) Culture conditions : RPMI, 10% FCS, L-glutamine & P/S: HT29 is derived from the primary colonic adenocarcinoma of a 44 year old female Caucasian patient.

2.7.2 Routine cell culture techniques

Routine cell culture was performed as described by Freshney (1987,1992).

(a) Cryopreservation of cells

Cell lines were prepared for storage in liquid nitrogen as follows: Confluent cell monolayers were washed 3 times in phosphate buffered saline (PBS) and harvested with trypsin-versene (0.1% trypsin, 0.01% EDTA). Once the cell monolayer had detached, fresh medium was added to inhibit the trypsin and the cells were collected by centrifugation (2500rpm, 5 minutes). The cell pellet was washed with PBS, spun down again and the resulting cell pellet re-suspended (10^7 cells/ml) in freezing-mix (90% newborn calf serum, 10% DMSO). 1ml aliquots of this cell suspension were frozen at -70°C for 24 hours before transfer to long term storage in liquid nitrogen (-196°C). When required, a vial of cells was removed from storage and thawed rapidly at 37°C , washed several times with pre-warmed media to eliminate any traces of DMSO, transferred to a 25cm^2 flask and allowed to adhere for 24 hours before re-feeding.

(b) Refeeding

Cells were re-fed by pouring off the spent medium and replacing it with fresh medium which had been pre-warmed to 37°C . If the cell density was low and there were viable cells present in the supernatant which had not yet adhered to the flask, these were recovered by centrifugation, washed in fresh medium and added back to the original flask. Refeeding was carried out as often as was required to maintain stable pH and optimal cell growth - frequency of refeeding was determined by the growth rate of the cells, but in most cases was required only 2-3 times per week. All media contained a pH-dependent indicator, thus any significant pH change was immediately visible.

(c) Subculture

Cells were subcultured when confluent by washing 3 times in PBS and treating with trypsin-versene to detach the cell monolayer. Once the cells had detached, fresh medium was applied to inhibit the trypsin and the cells were dispersed using a Pasteur pipette until a single cell suspension was achieved. If an accurate cell count was required, the cells were spun down, resuspended in a known volume of medium and counted using a Neubauer haemocytometer. The cells were then seeded at an appropriate density into new flasks.

2.7.3 Sterility testing of cell lines

Sterility was maintained as far as was practicable in the cell culture suite and all cell culture techniques were carried out aseptically in a Class 2 Biological Safety Cabinet which was maintained under UV light when not in use. Routine tests for mycoplasma contamination were regularly performed on all cell lines by Mr. W. Christie, MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Crewe Road, Edinburgh. The tests used involved staining cells with Hoescht 33258 fluorescent dye (Chen,1977) and growth of the cell supernatant on selective broth (Taylor-Robinson,1978). All media and other cell culture reagents were autoclaved or filter sterilised (0.22 μ m filter) as appropriate on preparation and tested for bacterial contamination by incubation of an aliquot of each solution in L-broth (2% bactotryptone, 1% bacto yeast, 2% NaCl) at 37 $^{\circ}$ C for at least one week prior to use. If any turbidity was observed, the solution was assumed to be contaminated and discarded.

2.7.4 Cell line induction experiments

TCPOBOP (dissolved in DMSO) was administered to all cell lines at a concentration of 10^{-5} M. The compound, at this dose, was not toxic to any of the cell lines tested. Higher doses resulted in decreased solubility in the culture media and crystals of TCPOBOP forming on the cell monolayer.

2.7.5 Preparation of protein from cultured cells

Cells were harvested when confluent by washing 3 times in PBS and treating with trypsin-versene to detach the cell monolayer. Fresh medium was added to inhibit the trypsin and the cells collected by centrifugation (2000rpm, 5 minutes). The resulting cell pellet was resuspended (0.4mls per 75cm² flask) in Buffer H

(10mM Na_2HPO_4 , 2mM MgCl_2 , 2mM DTT, 1mM EDTA, pH7.4) and homogenised with a glass-teflon homogeniser (30-40 strokes). The extent of cell disruption was assessed microscopically. When complete, the disrupted cells were transferred to an eppendorf tube and crude pellet/cytosol fractionation achieved by spinning (6500 rpm) in a microcentrifuge. This was carried out at 4°C to minimise protein degradation. The resulting supernatant (cytosolic fraction) was removed and stored at -40°C for future analysis. The pellet was washed several times with PBS and finally resuspended in a minimum volume of Buffer H and stored at -40°C .

2.7.6 Preparation of RNA from cultured cells

RNA was prepared from cultured cells according to the single-step acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski & Sacchi (1987). A 75cm^2 flask of cells was grown to confluency, washed several times with ice-cold phenol-red free PBS and 1.72mls of denaturing solution (DNS) (4M guanidinium isothiocyanate, 25mM sodium citrate, pH7.0, 0.5% (v/v) sarcosyl, 0.1M β -mercaptoethanol) added to detach the cell monolayer. The resulting cell suspension was immediately transferred to a depc-treated 15ml polypropylene tube and the following reagents added (in this order) : 0.401mls 2M sodium acetate, pH 4.0; 4.01mls dH_2O -saturated phenol; 0.802mls chloroform/isoamyl alcohol (49 : 1); with thorough mixing after the addition of each component. The final suspension was shaken vigorously for 10 seconds and cooled on ice for 15 minutes before centrifugation (15000 rpm, 20 minutes, 4°C). The lower (organic) layer and the interface contained DNA and protein and were discarded, while the upper (aqueous) phase contained RNA and was carefully removed and transferred to a fresh tube. Isopropanol (1.72mls) was added to precipitate the RNA (-20°C , 1 hour), which was collected by centrifugation (15000rpm, 20 minutes, 4°C). The resulting pellet was dissolved in 0.5mls DNS, transferred to a depc-treated eppendorf and re-precipitated with 1 volume of isopropanol (-20°C , 1 hour). After centrifugation in an Eppendorf centrifuge at 4°C , the RNA pellet was washed in 75% ethanol, vacuum dried and dissolved in depc- dH_2O . The purity and concentration of the product were determined as described in section 2.6.1.

2.7.7 The MTT cytotoxicity assay

This assay (Mossman, 1983) is based on the principle that only live metabolically active cells have the functional mitochondrial dehydrogenases required to reduce the water-soluble yellow dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to an insoluble blue formazan product which can be quantitated spectrophotometrically. The amount of formazan product generated (measured at 540nm) is proportional to cell number, and is therefore used as an estimate of cell survival following exposure to a cytotoxic drug. Although it is not possible to distinguish between cytotoxic and cytostatic effects, the assay provides a useful screening method to determine the maximum tolerated doses of a range of test compounds (Carmichael *et al.*, 1987). Cells were plated in 96-well microtitre plates so that several samples and a wide range of drug dilutions could be assayed simultaneously and were plated at a density such that the control (untreated) cells were in exponential growth at the time of harvest. Cell growth was found to be affected by evaporation of media from the outermost wells of the plate and these were therefore excluded from the assay. To minimise further evaporation, these wells were filled with PBS and the plates incubated in a sandwich box lined with damp tissues to maintain high humidity. The first column (6 wells) was filled with media only and was used to zero the spectrophotometer. Cells were plated at an appropriate density (e.g. 5×10^3 cells/well for the C3H10T1/2 cell line) in a volume of 180 μ l/well and allowed to adhere overnight before the addition of serial dilutions of a 10x concentrated stock of the drug of interest (added in 20 μ l aliquots in serum-free media). When it was necessary to prepare the stock drug in an organic solvent (e.g. DMSO), an additional control was included, where cells were treated with solvent only. Incubation with drug was carried out for 3 days - this was long enough for drug-induced cell death to occur but sufficiently short that re-feeding of the cells did not become necessary. The extent of cell kill was assessed by the addition of 50 μ l of MTT (2mg/ml in dH₂O) to each well. The plates were then incubated for a further 4 hours, the media removed by aspiration with a Pasteur pipette and 50 μ l of DMSO added to each well to dissolve the formazan crystals. The OD₅₄₀ of the wells was measured in a Biorad Model 2550 EIA plate reader and the % survival of the drug treated cells calculated relative to the controls. The results presented were the average of the OD₅₄₀ results obtained for 6 independent wells for each drug concentration. Each assay was carried out in triplicate and the results combined.

2.7.8 Transfections

Transfection of ZR-75 cells and the non-chromatographic assessment of CAT activity (Section 2.7.9 (b)) was carried out by Roger White, under the direction of Dr.M.G. Parker. ZR-75 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing phenol red with 10% FCS and 10^{-8} M estradiol. For transfection, cells were plated at 5×10^5 per 6cm dish in phenol-red free DMEM containing 5% FCS from which endogenous steroids had been removed by treatment with dextran coated charcoal. Transient transfections were performed using the calcium phosphate precipitation technique (Wigler *et al*, 1979), essentially as described by Ham *et al* (1988). The cells were then maintained for 48 hours in the appropriate hormones. Dexamethasone and R5020 were added to 10^{-7} M and phenobarbital and TCPOBOP in a range of concentrations from 10^{-6} to 10^{-9} M. CAT activity was assayed non-chromatographically (Sleigh, 1986) and normalised for transfection efficiency with luciferase activity as described in Section 2.7.9 (b). The construction of the reporter plasmid pLTR-CAT has been described previously (Parker *et al*, 1987). The control vector pJ3 Luciferase consists of the luciferase gene (De Wet *et al*, 1987) cloned into the eucaryotic expression vector pJ3W (Morgenstern & Land, 1990). C3H10T1/2 cells were transfected as described above with one of two constructs: (a) The GRE cloned into pLTR-CAT; (b) Two GRE sequences linked in series and cloned into pLTR-CAT. These cells were maintained in 10^{-5} M dexamethasone and TCPOBOP for 48 hours following transfection. CAT activity was determined chromatographically as described in section 2.7.9 (a).

2.7.9 CAT assays

The CAT assay provides a simple and sensitive measure of the effects of a range of compounds on a specific regulatory sequence. Compounds which interact with the sequence of interest cause activation of the CAT reporter gene, which is under the control of the SV40 early promoter. Chloramphenicol acetyltransferase (CAT) activity can be measured in a number of ways :

(a) Chromatographic determination of CAT activity

This was performed as described by Gorman *et al* (1982). Cells (25cm^2 flask) were harvested by washing 5 times in Ca^{2+} and Mg^{2+} free PBS before the

addition of 1ml/flask of TEN solution (40mM Tris-HCl, pH 7.5, 10mM EDTA, 150mM NaCl). When the monolayer had detached (~5 minutes), cells were removed from the flask by scraping, pelleted in an Eppendorf table-top centrifuge and resuspended in 150µl Tris-HCl (250mM, pH 8.0). The cells were lysed by 3 successive freeze-thaw cycles and incubated at 60°C for 10 minutes to inactivate endogenous acetylases. Denatured cell debris was removed by centrifugation and the resulting cell extract (supernatant) transferred to a fresh tube and stored at -20°C if analysis was to be carried out at a later date. CAT activity was determined by combining the following reagents in a sterile eppendorf tube - 50µl cell extract, 20µl acetyl CoA (3.5mg/ml, freshly prepared), 3µl [¹⁴C]- chloramphenicol & 250mM Tris-HCl, pH8.0 to a total volume of 180µl. The acetyl CoA was added last to initiate the reaction, which was incubated at 37°C for 60 minutes. The [¹⁴C]-labelled reaction products were extracted with two successive aliquots of ethyl acetate and evaporated to dryness under vacuum (the lower aqueous phase was discarded in each case), redissolved in 30µl ethyl acetate and applied (3x10µl aliquots) to the origin of a 25cm silica TLC plate. Chromatography was carried out in chloroform-methanol (95 : 5) in a closed tank, until the solvent front was ~1 inch from the top. The plate was dried, sprayed with enhancing reagent (En³Hance spray, Du Pont), and exposed to autoradiography film at -70°C. Unacetylated reaction products remained at the origin, while acetylated forms migrated with the solvent front. In cases of very high CAT activity, spots with a greater R_f value corresponding to doubly acetylated products could be detected. Although it was not necessary in this case, CAT activity can be quantitated by removing the radioactive spots from the TLC plate and determining their relative activities by liquid scintillation counting.

(b) A modified, non-chromatographic method of determining CAT activity was reported by Sleight (1986). Cell transfections and extract preparation were as previously described. CAT activity was determined by combining the following reagents in a sterile eppendorf tube: 20µl chloramphenicol (8mM), 30µl cell extract, 20µl [¹⁴C]-acetyl CoA (1mCi/ml), 0.25M Tris-HCl, pH7.8 to a total volume of 1ml. The reactants were incubated at 37°C for 1 hour, extracted with ethyl acetate and the product mixed with 1ml of Packard Instagel. The amount of incorporated radioactivity in each sample was determined by liquid scintillation counting. Determination of luciferase activity in each sample (De Wet et al, 1987)

acted as a control for transfection efficiency.

2.7.10 Hepatocyte isolation and culture

This experiment was carried out by Dr. R.G. Bars, ICI Central Toxicology Laboratories, Alderley Park, Macclesfield. Hepatocytes were isolated from adult male Alderley Park Swiss mice (20-25g) by *in situ* perfusion of the liver with collagenase, essentially as described by Mitchell *et al* (1984), with the following modifications: the superior vena cava was cut and the perfusate was not recirculated; flow rate was 12ml/min and collagenase digestion was 50mg collagenase/250ml perfusate for 10 mins. The viability of isolated hepatocytes was determined by trypan blue dye exclusion and was greater than 80%. Hepatocytes isolated from the two animals were pooled and primary monolayer cultures were prepared by seeding 2×10^6 viable cells in 4ml CL15 medium in 25cm² tissue culture flasks. Four hours after seeding, the spent medium was aspirated and fresh medium applied. Phenobarbital (PB) dissolved in 0.9% NaCl and TCPOBOP dissolved in dimethylformamide (DMF) were added to the medium when it was changed each day (at 24, 48 and 72h after seeding). Hepatocytes were therefore exposed for 3 days to 2mM PB; 2, 10, or 50µM TCPOBOP or vehicle alone (0.9% NaCl or 0.25% DMF). Observation under phase contrast microscope showed the presence of few or numerous crystal needles in the culture medium containing 10 and 50µM TCPOBOP respectively, indicating the limit of solubility of this compound in CL15 medium.. At 96h after seeding, the cells from individual flasks were harvested in 1ml of SET buffer (0.25M sucrose/5mM EDTA/20mM Tris-HCl (pH7.4)) and disrupted by sonication.

2.8 Cytochrome P450 activity measurements

2.8.1 Spectrophotometric determination of P450 content

This was performed according to Omura & Sato (1964). Microsomal samples were diluted to an approximate concentration of 2mg/ml. Each sample was divided equally between two identical glass cuvettes, and the base line absorbance between 550 and 400nm measured on a Shimadzu spectrophotometer. Carbon monoxide was then bubbled through one cuvette only (~2 bubbles / sec) for 1 minute, before the contents of both cuvettes were reduced by the addition of 3mgs of sodium dithionite and the absorbances re-measured over the same absorbance range as before. The concentration of P450 in the samples was then calculated according to the following equations:

$$S = 91\text{cm}^{-1}\text{mM}^{-1}$$

$\text{nmol/ml} = x / 0.091$, where $x = A450 - A490 / \text{full scale deflection}$

$\text{nmol/mg protein} = x / 0.091 \times \text{dilution factor} / [\text{sample}](\text{mg/ml})$

2.8.2 Cytochrome P450 reductase assay

P450 reductase activity was measured spectrophotometrically in microsomal samples as described by Yasukochi & Masters (1976). The assay was carried out in 0.3M KHPO_4 , pH 7.4, 0.1mM EDTA at 37°C. Two identical glass cuvettes were set up as follows: 970 μl of cytochrome C standard (1.5mgs/ml in phosphate buffer) were mixed with 20 μl of microsomes (diluted to 1mg/ml) and the spectrophotometer zeroed to generate a baseline. 10 μl of NADPH (16mg/ml in phosphate buffer) was added to each cuvette to initiate the reaction and the absorbance at 550nm recorded for several minutes. P450 reductase activity was calculated according to the following equation :

$$a/b \times [E \times 50 / 0.0187 \times t]$$

where a = deflection (mm),

b = total height of scale (mm), t = time (minutes), E = (upper-lower) scan limits

2.8.3 Resorufin assay

The O-dealkylation of a series of substituted phenoxazones (Resorufins) has been shown to be a sensitive and specific method to quantify cytochrome P450 activity in microsomal samples (Burke & Mayer, 1974). The nature of the alkoxy group determines the substrate specificity e.g. 7-ethoxyresorufin is a substrate for the CYP1A family and is therefore suitable for measuring induction by compounds which act through the Ah locus such as 3-MC (Burke & Mayer, 1974). Similarly, 7-pentoxyresorufin is a substrate for CYP2B proteins and its metabolism is increased on phenobarbital pretreatment and inhibited by CYP2B antisera (Lubet et al, 1985). Another substrate which is relatively specific for CYP2B proteins is 7-benzyloxyresorufin, although this substrate is also metabolised by other P450 isozymes (Burke et al, 1985). P450 activity was assayed fluorimetrically - the assay was carried out directly in a Perkin-Elmer LS3 Fluorescence Spectrometer with a built-in temperature regulator set to 37°C. The reaction mixture consisted of substrate (1ml of 1mM R-Resorufin in 100mM Tris-HCl, pH7.4), which was equilibrated to 37°C before use and sample (e.g. 10 μg of mouse liver microsomal protein). These were thoroughly mixed in a 1ml glass fluorimeter cuvette and the

background fluorescence (excitation 530nm, emission 585nm) of the mixture obtained. When the baseline was linear (~30seconds), the reaction was initiated by the addition of 10µl NADPH (10mM in Tris-HCl, pH 7.4) and the resulting fluorescence monitored continuously on a Perkin-Elmer 561 Chart Recorder. The reaction was allowed to proceed until a measurable gradient had been produced (~3 minutes). It was important to make all measurements during the period when the reaction was linear i.e. before the substrate concentration became rate limiting. A known amount of resorufin (10µl of a 10mM stock in ethanol) was then added as a standard to calibrate the reaction. Each assay was performed in triplicate and each result is the mean of at least 3 separate experiments.

2.9 Immunohistochemical analysis

All the experiments within this section were carried out under the direction of Dr. D.J. Harrison. Immunohistochemical staining was performed by Mrs. Helga Wolf and in situ hybridisation by Mrs. Frances Rae.

2.9.1 Tissue fixation

Tissue for immunohistochemical analysis was treated in one of two ways :

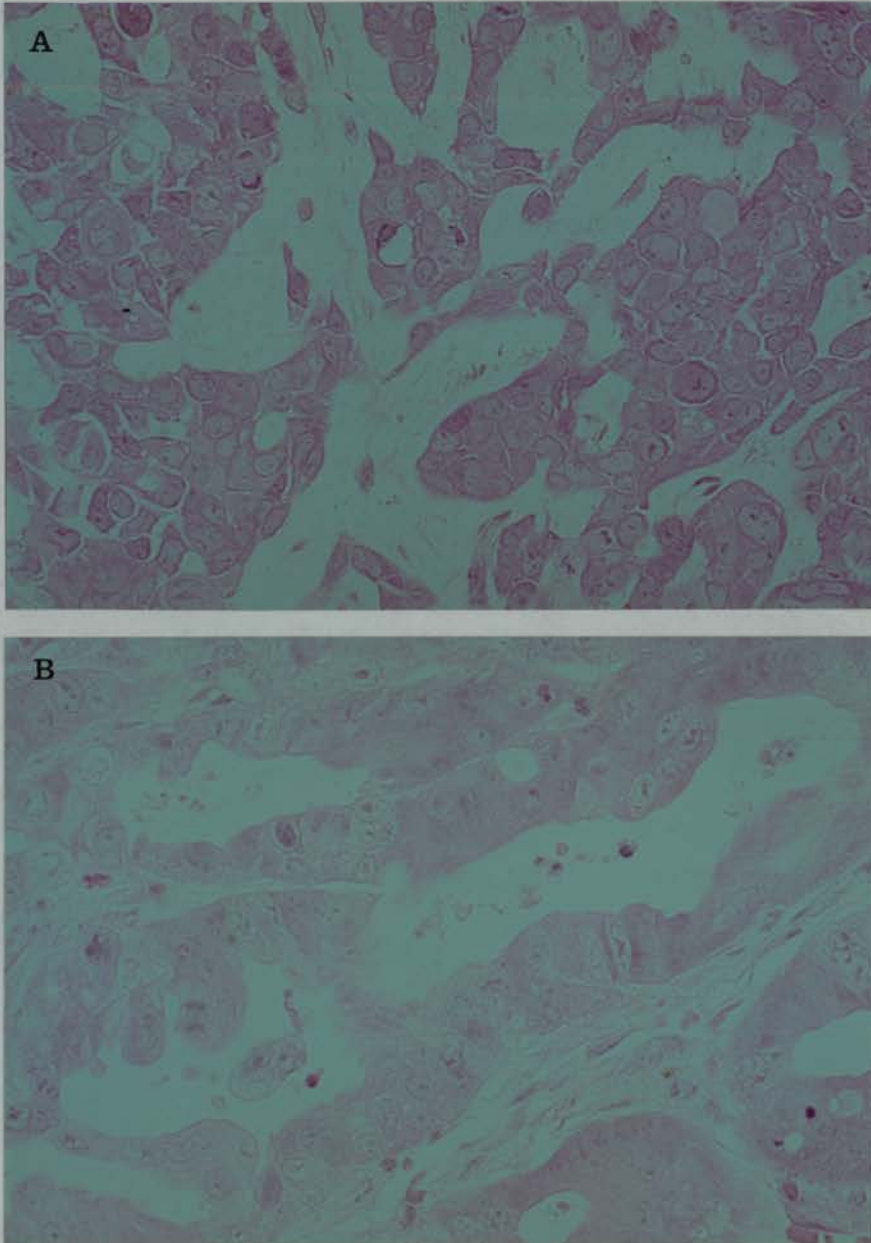
- (a) cut into small fragments and placed directly into cold buffered formalin on sacrifice
- (b) coated in OCT (Tissue-Tek OCT compound, Miles, USA) and snap-frozen. OCT is a sugar-based embedding medium which prevents the tissue from drying out.

All the analysis which is described in this section was performed on formalin-fixed tissue.

2.9.2 Haematoxylin and Eosin staining of tissue sections

This was performed as described by Lillie (1965). Nuclei were stained blue with haematoxylin, while cytoplasm and other structures stained pink with eosin. H&E staining of the breast and colon tumours used in these experiments is shown in Figure 2.9.2.

Figure 2.9.2 H & E staining of xenograft colon and breast tumours



The breast xenograft tumour NCH (A) was a ductal carcinoma (no special type). Cords and clumps of malignant cells are present. Nuclei show considerable variation in size (pleomorphism) and nucleoli are prominent. Necrosis in the centre of the tumour was marked.

The colon xenograft tumour GFH (B) was was a moderately differentiated adenocarcinoma. Cytologically, the tumour cells are obviously malignant. There was moderate focal mucin production.

2.9.3 Immunocytochemical detection of proteins

Sections for immunohistochemistry were cut at 3µm, dewaxed in xylene and rehydrated through graded alcohols. Slides were washed several times in buffer (100mM Tris, 0.1% (v/v) Tween 20, 5% (v/v) normal goat serum) before incubation overnight at 4°C with rabbit polyclonal P450 antisera, diluted 1:50 in buffer. Slides were then washed (3x10mins) and incubated (30 min) with biotinylated goat anti-rabbit IgG (1:500 in buffer), exposed to streptavidin-peroxidase conjugate (Dako, Ltd., UK) for 30 minutes and developed with 3,3' diaminobenzidine before light counterstaining with haematoxylin.

2.9.4 Antibodies

Antibodies to P450 proteins were as previously described (Section 2.5.5).

2.10 In situ hybridisation for mRNA

2.10.1 Experimental procedure

In situ hybridisation was performed essentially according to Herrington *et al* (1991), with the following modifications:

Formalin fixed sections were dewaxed and then permeabilised in 0.2M HCl followed by 0.3% Triton-X100 in phosphate-buffered saline. Proteinase K was used at 10-20µg/ml and reactions were postfixed in 0.4% paraformaldehyde. After 1h at 37°C in prehybridisation buffer (0.6M NaCl, 10% dextran sulphate, 30% deionised formamide, 0.1% sodium pyrophosphate, 0.2% polyvinylpyrrolidone, 0.2% Ficoll), 20-40ng of biotinylated probe was incubated overnight at 37°C. Detection of oligonucleotide binding was achieved using a mouse anti-biotin antibody, followed by alkaline phosphatase labelling using nitroblue tetrazolium (NBT)/ 5-bromo-4-chloroindolyl phosphate (BCIP), overnight at 4°C for visualisation.

2.10.2 Oligonucleotide probes

The oligonucleotides used were an 18mer from Exon 2 (bp 112-130) of the cDNA sequence of the human CYP2B6 gene, (5' CCCATATTTCTCTCGGAA 3' antisense); (5' TTCCGAGAGAAATATGGG 3' sense). These oligonucleotides had 6 mismatches relative to the mouse Cyp2b9 sequence (Figure 2.10). Three biotin molecules were added at the 5' end of each oligonucleotide using a monomer developed by Link Technologies, Cumbernauld, Scotland.

Figure 2.10 Comparison of human CYP2B and mouse Cyp2b nucleotide sequences

CYP2B6: 5' TTC CGA GAG AAA TAT GGG 3'

Cyp2b-9: 5' CTT CAA GAA AAA CAT GGC 5'

Cyp2b-10: 5' CTT CGA GAA AAA TAT GGC 3'

| = mismatch

Chapter 3 : Xenobiotic regulation of murine hepatic P450 expression

3.1 Introduction

In this chapter, the regulation of murine hepatic P450 expression by three compounds is discussed. Phenobarbital is a potent inducer of CYP2B1 expression in the rat where it has been shown to act by transcriptional gene activation. It appears to be equally potent, but more pleiotropic, in inducing hepatic P450 expression in the mouse. Dexamethasone, a synthetic glucocorticoid, also has marked effects on hepatic P450 expression, mediated in part at least, by binding to a glucocorticoid responsive promoter element in the upstream regions of certain P450 genes. The most profound effects on murine P450 expression were, however, observed with TCPOBOP. This compound, the effects of which are long-lasting and both tissue and species specific, shows similarity to both dexamethasone and phenobarbital in its patterns of induction. Regulation of cytochrome P450 expression by TCPOBOP has been characterised in mouse liver and attempts made to elucidate its mechanism of action. In Chapter 5, the regulation of murine hepatic P450 expression by these compounds will be compared to the effects observed in human tumour tissues.

3.2 Cytochrome P450 expression in mouse liver

Much of the work to date on murine hepatic P450 expression has centred on the regulation of Cyp1a by polycyclic aromatic hydrocarbons such as TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and 3-methylcholanthrene (3-MC). Two genes, Cyp1a1 and Cyp1a2, have been isolated from mouse liver. These are located on mouse chromosome 9, and are tightly linked to the Mpi-1 locus (Hildebrand *et al*, 1985). Both are inducible on treatment with polycyclic aromatic hydrocarbons, a process which is mediated by the Ah receptor (Nebert & Jensen, 1979). The Ah receptor (Aromatic hydrocarbon responsive) is a cytosolic protein which mediates Cyp1a expression by a mechanism similar to that of hormonal induction i.e. the inducer binds to the cytosolic receptor and the resulting complex is translocated to the nucleus where it binds chromatin (Tukey *et al*, 1982) resulting in transcriptional gene activation (Nebert & Eisen, 1984).

Inheritance of the Ah receptor has been studied in a number of inbred mouse strains, and has been shown to follow simple Mendelian genetics and to segregate as a single autosomal dominant trait (Thomas *et al*, 1972). Two thirds of the mouse strains studied have a high affinity Ah receptor ($K_d=1\text{nM}$ for TCDD binding) and

show a corresponding induction response when challenged with a suitable ligand. The remaining one third have a low affinity receptor and therefore require a much greater dose of TCDD for induction. Less potent inducers such as 3-MC do not cause induction in non-responsive strains. TCDD administration results in induction of Cyp1a expression in both strains, however, demonstrating that genetically non-responsive mice possess the structural and regulatory genes necessary for inducible P450 expression (Poland *et al.*, 1974). A later study (Poland *et al.*, 1976) demonstrated that there was no receptor mutation in non-responsive strains and the difference in response to polycyclic aromatic hydrocarbons was due to altered ligand binding affinity. The C57Bl/6 mouse is the prototype for the Ah-inducible phenotype, and the DBA/2 the prototype for the decreased response (Nebert *et al.*, 1982). Inducible Cyp1a1 mRNA expression is correlated with aromatic hydrocarbon hydroxylase activity, while inducible Cyp1a2 mRNA correlates with acetanilide 4-hydroxylase activity (Tuteja *et al.*, 1985). Constitutive expression of Cyp1a2 is higher in mouse liver and requires lower concentrations of inducer than Cyp1a1 to obtain the induction response, indicating that the inducer-receptor complex binds with different affinities to the regulatory regions of each gene.

Full-length cDNA clones of Cyp1a1 and Cyp1a2 have been isolated from hepatic C57Bl/6 mRNA (Kimura *et al.*, 1984a,b). Cyp1a1 is 2620 nucleotides in length, encoding a 521 amino acid protein with a molecular weight of 58,914, while Cyp1a2 is slightly smaller, with a molecular weight of 58,183. The isozymes are 68 and 73% homologous at nucleotide and protein level respectively. The Cyp1a1 upstream sequence contains a TCDD-inducible enhancer as well as additional regulatory element which controls constitutive expression (Neuhold *et al.*, 1986). Comparison of the upstream sequences of these genes reveals a highly conserved 11/12 base pair sequence, which has been proposed as a possible binding site for the Ah receptor-ligand complex (Gonzalez *et al.*, 1985). Alternatively, or in addition, this sequence may be involved in the binding of another DNA regulatory element. The mouse hepatoma cell line, Hepa-1, is Ah receptor-responsive and has proved to be a useful model for genetic and molecular analysis of the Ah receptor and Cyp1a gene expression (Hankinson *et al.*, 1979, 1991).

In the mouse, there is very good correlation between inducible Cyp1a expression and polycyclic aromatic hydrocarbon induced carcinogenesis (Kouri *et al.*, 1983). An analogous correlation has been proposed between CYP1A inducibility in humans and lung cancer incidence in cigarette smokers (Kouri *et al.*, 1982). The metabolic activation of environmental toxins to reactive oxygenated electrophiles by

Cyp1a genes therefore may lead to an increased cancer risk in man.

Constitutive expression of Cyp1a1 is observed during differentiation and is not dependent on stimulation by a foreign chemical inducer. Following partial hepatectomy, Cyp1a1 mRNA is increased in C57 (responsive) but not in DBA (non-responsive) mice, suggesting that the induction process is Ah receptor-dependant and may involve the binding of an endogenous ligand (Kimura et al., 1987). No endogenous ligand for the Ah receptor has, however, been isolated (Eisen et al., 1983).

Cycloheximide treatment lead to enhanced transcriptional activation of several genes involved in cell proliferation, suggesting the presence of such a labile protein repressor or, alternatively, an enhancer of RNA degradation. The presence of a labile protein repressor has been proposed for the rat CYP1A1 gene, where "superinduction" of gene transcription is observed when hepatoma cells are exposed to dioxin in the presence of cycloheximide (Israel et al., 1985). This superinduction is only observed in the presence of a functional Ah receptor.

Cyp2a expression in mouse liver is also represented by two genes, Cyp2a4 (55kD) and the 49kD Cyp2a5 (Squires & Negishi, 1988), which are located on chromosome 7 (Miles et al., 1990). Both genes have a glucocorticoid regulatory element (GRE) upstream of the transcription initiation site (Lindberg et al., 1989). The genes exhibit 98% sequence homology at nucleotide level and have only 11 out of 494 amino acid differences. Cyp2a5 is responsible for coumarin hydroxylase activity, catalysing the conversion of coumarin to its 7-hydroxy derivative (umbelliferone) (Lang et al., 1985; Lindberg & Negishi, 1989). This activity is highly and specifically inducible by pyrazole (Juvonen et al., 1985) and is also induced on treatment with the pleiotropic inducer phenobarbital. Cyp2a4, however, shows no Coh activity, but hydroxylates testosterone at the 15 α position (Negishi et al., 1989). Lindberg & Negishi (1989) compared the catalytic activities of these genes as a function of their structure by site directed mutagenesis, expressing the constructs in an appropriate expression vector in Cos-7 cells. Substitution of a single amino acid at position 209 in Cyp2a4 (Phe->Leu) resulted in an altered substrate specificity from coumarin to steroid hydroxylation. The nature of the amino acids at positions 117 and 365 were also shown to be important for catalytic activity.

Iwasaki et al. (1991) expressed a series of mutated Cyp2a P450s in *Saccharomyces cerevisiae* in which residue 209 was substituted by a variety of alternative amino acids. Hydrophobic residues (Phe, Leu, Val) produced P450 in the

high spin configuration, while substitution of a charged amino acid such as Lys or Asp produced the low spin form. Asn or Gly in position 209 produced a mixture of high and low spin forms of the protein. These results demonstrate that the nature of residue 209 is critical in determining the hydroxylase specificity of the P450 and therefore its activity towards a wide range of substrates. It has been proposed (Iwasaki et al, 1991) that residue 209 lies close to the sixth axial ligand on the distal surface of the heme. This is involved in binding dioxygen and thus plays a critical role in specifying catalytic activity.

Four P450 proteins were purified from phenobarbital-treated DBA mouse liver, one of which was the 49kD Cyp2a4 (Honkakoski & Lang, 1989). From NH₂-terminal sequence analysis, however, only one of these proteins, with an apparent molecular weight of 56.5kD, belonged to the Cyp2b subfamily. Noshiro et al (1988) isolated two distinct Cyp2b genes from mouse liver, Cyp2b9 (Mr 56,740) and Cyp2b10 (Mr 56,856). Both proteins have constitutive testosterone 16 α -hydroxylase activity. Meehan et al (1988a) reported that the synthetic glucocorticoid dexamethasone, like phenobarbital, was a potent inducer of Cyp2b gene expression, and demonstrated that the induction was regulated at the level of transcription. Dexamethasone also induced P450 levels in the Cyp2c and Cyp3a gene families. Hypophysectomy resulted in induced levels of these isozymes, implying that some sort of negative regulator or repressor is secreted by the pituitary, affecting either the transcription rate of certain P450 genes or the stability of their mRNAs. In contrast, administration of dexamethasone to the rat did not lead to induced levels of Cyp2b and Cyp2c proteins.

Cyp2c is present constitutively in mouse liver and is only marginally inducible by phenobarbital (Waxman et al, 1983; Wolf et al, 1984). The family is represented by a single gene, located on chromosome 9 (Meehan et al, 1988) which is tightly linked to the Ah locus. Indeed, Cyp2c proteins have constitutive aromatic hydrocarbon hydroxylase activity in rats, rabbits and humans and have been shown to be involved in both the activation and deactivation of polycyclic aromatic hydrocarbons.

Three Cyp2d genes have been isolated from mouse liver (Wong et al, 1987, 1989; Ichikawa et al, 1989) and are located on chromosome 15 (Gonzalez et al, 1987; Wong et al, 1989). Our experiments demonstrate that Cyp2d protein is constitutively expressed in mouse liver, and that it does not appear to be regulated to an appreciable extent by any of the xenobiotics tested. A male specific form, Cyp2d9 (Mr 56,948), responsible for the 16 α -hydroxylation of testosterone, was purified

from mouse liver by Harada et al (1984). Screening a cDNA library with a cDNA corresponding to this 16 α clone revealed the presence of at least 5 highly homologous genes. Cyp2d10 had the same number of amino acids as Cyp2d9 (504), but only 92% nucleotide and 85% deduced amino acid homology. Cyp2d11 had an extra amino acid, but showed 94 and 88% homology respectively. Two further Cyp2d genes (Cyp2d12 and Cyp2d13) have apparently been characterised but not yet reported (Wong et al, 1989).

The cDNA encoding the Cyp2e1 protein has recently been isolated from male Balb/c mouse liver (Freeman et al, 1992). The deduced amino acid sequence codes for a protein with a molecular weight of 56,781. Cyp2e mRNA and protein were dramatically decreased on treatment with diethylnitrosamine while pyrazole, a potent inducer of and substrate for CYP2E1 expression in rats and rabbits, was ineffective in the mouse, although its administration did lead to increased Cyp2a expression (Honkakoski et al, 1988). Treatment with acetone (1% (v/v)) lead to an increase in Cyp2e1 protein expression in both males and females. No corresponding increase in mRNA levels was observed, suggesting that the induced protein levels were as a result of increased translation of the mRNA, protein stabilisation or some contribution from each mechanism. It had previously been demonstrated (Song et al, 1989) demonstrated that acetone induction of CYP2E1 in the rat was the result of protein stabilisation. Johansson et al (1988) demonstrated that spontaneous and induced diabetes and starvation induce CYP2E1 in the rat. It has been proposed (Miller & Yang, 1984) that increased acetone production in these conditions are responsible, at least in part, for mediating this induction.

Cyp3a expression has not been well characterised, to date, in mouse liver. Recently, however, a full length Cyp3a cDNA was isolated from dexamethasone treated ddY mouse liver (Yanagimoto et al, 1992). The deduced amino acid sequence encodes a 504 amino acid protein with a molecular weight of 57 853. Meehan et al (1988) reported the regulation of Cyp3a expression by dexamethasone and hypophysectomy and demonstrated that induction was regulated at the level of transcription. This is in agreement with the findings of a recent report where dexamethasone and the antigluocorticoid pregnenolone 16 α -carbonitrile (PCN) were administered to a primary monolayer culture of rat hepatocytes which had been transfected with rat liver CYP3A1 (Burger et al, 1992). Induction in this system was mediated at the level of transcription.

Cyp4a was mapped to mouse chromosome 4 by Kimura et al (1989), and the cDNA has recently been isolated from clofibrate-treated C57Bl/6 mouse liver

(Henderson *et al*, manuscript in preparation). CYP4A genes in the rat are induced by hypolipidaemic compounds such as clofibrate and nafenopin (Reddy & Lalwani, 1983). Administration of these compounds also leads to the induction of the 4 ω -hydroxylation of lauric acid and enzymes within the peroxisomal fatty acid β -oxidation pathway. These compounds are classified as "peroxisome proliferators", from the marked proliferation of endoplasmic reticulum and resulting rapid induction of peroxisome proliferation associated with their administration. Xenobiotic induction of peroxisome proliferation has received considerable attention since a relationship was proposed between the induction of hydrogen peroxide producing peroxisomal enzymes and the development of hepatocellular carcinomas in rats and mice (Reddy & Lalwani, 1983). Peroxisome proliferators are classified as "non-genotoxic carcinogens" as they fail to cause DNA damage directly (Warren *et al*, 1980). It has been proposed that the increased hydrogen peroxide production resulting from the administration of these compounds leads to oxidative stress which leads in turn to DNA damage and possible tumour initiation (Kasai *et al*, 1989). Alternatively, or in addition, and similarly to PB and TCPOBOP, peroxisome proliferators can act as liver tumour promoters (Marsman *et al*, 1988).

Western blot analysis with rat CYP4A antisera demonstrated the presence of three Cyp4a isozymes in mouse liver and four in mouse kidney, with molecular weights of 51, 52, 53 and 55kD. These proteins are developmentally and hormonally regulated, with considerable variation in regulation observed between individual isozymes.

3.3 Introduction to TCPOBOP

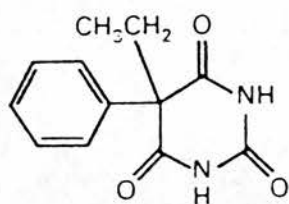
TCPOBOP, 1,4-bis(2-(3,5 dichloropyridyloxy)benzene) (Figure 3.3.1) was first isolated as a contaminant in a commercial herbicide, and was reported to be a potent "phenobarbital-like" inducer of hepatic mono-oxygenase activity in the mouse, as measured by aminopyrine N-demethylase activity (Poland *et al*, 1980). In addition, administration of TCPOBOP lead to an increase in liver weight, proliferation of the endoplasmic reticulum and induced levels of microsomal epoxide hydrolase and cytosolic glutathione S-transferase activities. The inductive effect was long lasting, maintained for up to 20 weeks following a single dose of 3mg/kg of the compound. Radiolabelling studies with ³H-TCPOBOP demonstrated that the compound was not metabolised to an appreciable extent within the body, but was stored as the parent compound, primarily in adipose tissue with a small amount of activity also detectable in the liver. The presence of such a potent agonist implies the presence of

a receptor-mediated mechanism of induction.

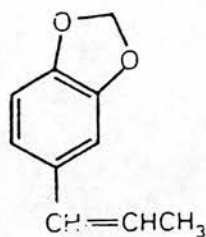
Previous workers had attempted, without success, to identify a phenobarbital receptor. Previous "phenobarbital-like" agonists had, however, been structurally dissimilar (Figure 3.3.1) and lacked potency. Administration of TCPOBOP and phenobarbital at a range of doses produced parallel dose-response curves, in agreement with both compounds acting through a common receptor, but having different binding affinities.

A companion study (Poland *et al*, 1981) reported that the inductive effects of TCPOBOP were highly species specific. Although active in the mouse and Syrian golden hamster, the compound was completely without effect in the rat and guinea pig, even at substantially higher doses. The effect was not strain dependent, as several different strains were examined in each species. Surprisingly, the compound was not metabolised in the non-responsive species, but was stored in adipose tissue as the parent compound. These workers proposed that the putative "TCPOBOP-receptor" was either absent from or mutated in the non-responsive species, but as no receptor had been purified this was purely speculative.

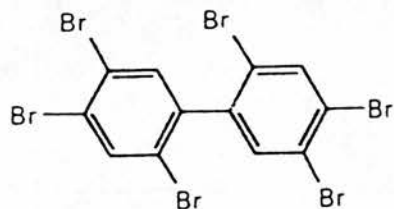
Figure 3.3.1 Structural diversity of PB-like inducers



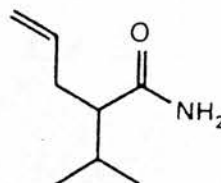
Phenobarbital



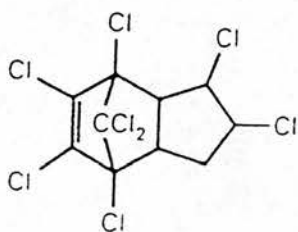
Isosafrole



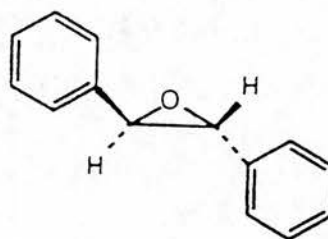
2,4,5,2',4',5'-Hexabromobiphenyl



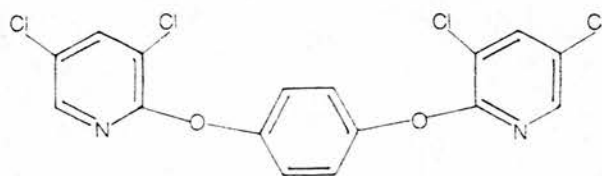
Allylisopropylacetamide



Chlordane



trans-Stilbene oxide



TCPOBOP

Adapted from Waxman & Azaroff, 1992

A number of studies have examined the structure-activity relationship of TCPOBOP and a series of structurally related analogues. Kende et al (1985) synthesised a series of 31 congeners and deduced the following minimum requirements for activity:

- (a) a central 1,4-dioxygenated benzene ring
- (b) lateral pyridine rings linked to the central benzene through ether bonds, although certain other heteroatomic rings had reduced activity
- (c) 5,5' substituents of Cl, Br or NO₂ on the lateral rings

X-ray crystallography was performed on TCPOBOP and two structural analogues, one which showed reduced activity and another which was completely inactive. These studies demonstrated that the pyridine rings must lie anti to the plane of the central benzene, with a dihedral angle of 60°. The ether oxygen is sp² bonded in conjugation with the heterodipolar bond C(2)-N(1), resulting in restricted rotation about the ether bonds.

The presence of a defined structure-activity relationship supports a receptor based induction mechanism, possibly with two similar and complementary binding sites. As phenobarbital is not structurally related to TCPOBOP, it was proposed that two molecules of phenobarbital might bind for every molecule of bound TCPOBOP. In a similar series of experiments, Kelley et al (1985) synthesised a series of 10 analogues and demonstrated that the central benzene could not be replaced with another ring structure and that para-substitution on the central benzene was necessary for maximum activity. A similar structure-activity relationship was observed for a group of 2,4-dichloro substituted polychlorinated biphenyl (PCB) compounds (Demomme et al, 1983). Interestingly, TCPOBOP administration (30mg/kg) to pregnant mice lead to transplacental induction of mono-oxygenase activity (Poland et al, 1980) in a similar manner to that observed on PCB administration (Demomme et al, 1983). Aniline hydroxylase activity, a marker substrate for Cyp2e1 was induced only slightly on TCPOBOP treatment, while coumarin hydroxylase activity was markedly increased (Raunio et al, 1988). Kelley et al (1990) reported that TCPOBOP did not induce aromatic hydrocarbon hydroxylase activity in Ah receptor positive strains of mice and, in agreement with our results, concluded that TCPOBOP effects are not mediated through the Ah receptor. An alternative mechanism was proposed by Fonne & Meyer (1987), who suggested that the P450 molecule itself was the TCPOBOP receptor and that TCPOBOP must bind to the P450 for the induction response to be observed. This was supported by the finding that rat CYP2B1 did not bind TCPOBOP.

In addition, Romano *et al* (1986) reported that TCPOBOP administration resulted in increased DNA synthesis in rodents and increased microsomal membrane fluidity in mice. Both TCPOBOP and phenobarbital enhanced the development of hepatic neoplasia when administered after an initiating carcinogen such as diethylnitrosamine (DEN). The doses of TCPOBOP required were, however, much greater than those required for induction of mono-oxygenase activity. Dragani *et al* (1985) reported that TCPOBOP stimulated murine hepatocyte proliferation without causing necrosis or cytotoxicity.

3.4 The effect of TCPOBOP on rodent hepatic cytochrome P450 levels

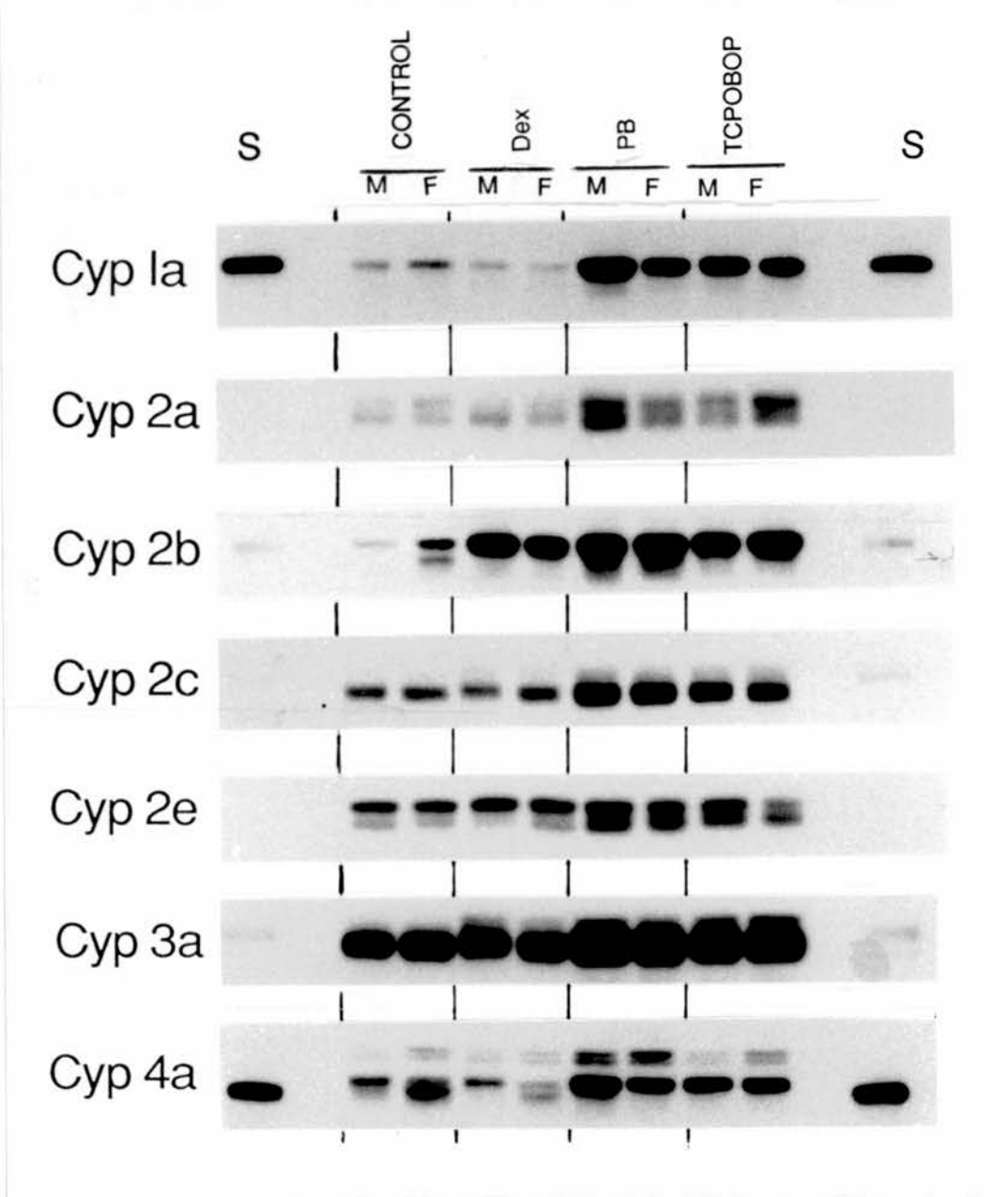
3.4.1 Xenobiotic induction of hepatic P450 proteins

Initial experiments were carried out to compare the effects of TCPOBOP, phenobarbital and the synthetic glucocorticoid dexamethasone on murine hepatic P450 expression. With the exception of Cyp2e1, the levels of protein from all the cytochrome P450 families and subfamilies examined were significantly increased, as determined by Western blot analysis, following a single injection of 75µg of TCPOBOP (Figure 3.4.1.1).

The inductive effect was particularly pronounced for Cyp1a, Cyp2a, Cyp2b and Cyp2c proteins. The induction of Cyp1a proteins by both phenobarbital and TCPOBOP is particularly interesting, as these isozymes are usually thought of as polycyclic aromatic hydrocarbon-inducible, mediated through the Ah receptor. Induction of Cyp1a by phenobarbital-like compounds has not previously been reported. Two distinct Cyp2a proteins were induced in mouse liver by TCPOBOP - the molecular weights of these proteins are consistent with their being Cyp2a4 (55kD, upper band) and Cyp2a5 (49kD, lower band). Interestingly, although Cyp2a genes contain a functional GRE in their upstream flanking region (Lindberg *et al*, 1989), no induction was seen with dexamethasone. Phenobarbital and TCPOBOP treatment did, however, lead to a marked increase in Cyp2a expression. P450s from the Cyp2c, Cyp3a and Cyp4a have not, to date, been well characterised in the mouse, but all were induced on treatment with TCPOBOP. Many similarities were observed with the induction patterns obtained on TCPOBOP and phenobarbital treatment. In agreement with a previous study (Meehan *et al*, 1988), dexamethasone, like TCPOBOP, was a potent inducer of Cyp2b expression. However, in contrast to the previous report, the levels of Cyp2c and Cyp3a proteins were not significantly increased by dexamethasone treatment in the experiments reported here. This could be due to the lower dose of dexamethasone administered (100 vs. 200mg/kg) or the

mouse strain used (C57Bl/6 vs. DBA/2). P450 isozyme expression was also studied in kidney, lung and testes following treatment with TCPOBOP, but no induction was observed.

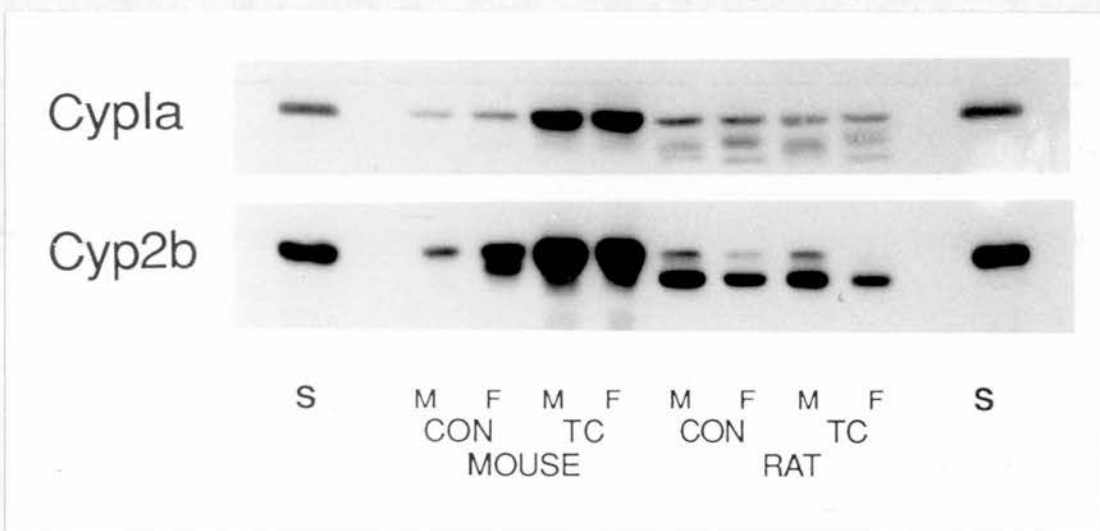
Figure 3.4.1.1 The effect of TCPOBOP on murine hepatic P450 protein expression



Male (M) and Female (F) DBA/2 mice were treated with dexamethasone (Dex), phenobarbital (PB) or a single injection of 3mg/kg TCPOBOP. Liver microsomal samples (15 μ g), prepared from pooled tissue from these animals, or suitable controls, were separated on 9% SDS-PAGE and P450 isozyme content determined by Western blot analysis using polyclonal antisera to various rat liver P450 proteins. S = purified rat liver P450 standard

Previous work (Poland *et al*, 1981) had demonstrated that TCPOBOP treatment did not influence hepatic P450 protein levels in the rat. Our experiments confirmed this finding (Figure 3.4.1.2), and we went on to investigate whether TCPOBOP treatment lead to increased mRNA expression in the rat, which would suggest that the lack of elevated protein expression was due to an inter-species difference in post-transcriptional control (Figure 3.4.3.2).

Figure 3.4.1.2 The effect of TCPOBOP on rat hepatic P450 protein levels



Male (M) or Female (F) Wistar rats were treated as controls (CON) or with 3mg/kg TCPOBOP (TC). Liver microsomal samples (15µg) were prepared from pooled tissue from these animals, separated on 9% SDS-PAGE and CYP1A and CYP2B isozyme content determined by Western blot analysis. S = purified rat liver P450 standard

3.4.2 The effect of TCPOBOP on P450 substrate metabolism

The induction patterns seen on Western blot analysis were confirmed by determining the rate of metabolism of a series of analogous alkoxy-substituted resorufin ethers (phenoxazones), which have previously been shown to be marker substrates for various of the P450 subfamilies (Burke *et al*, 1985). Figure 3.4.2.1 shows the metabolism of benzyloxyresorufin, a substrate for Cyp2b, by a series of microsomal samples prepared from mice treated as controls, or with dexamethasone, phenobarbital or TCPOBOP.

The Cyp2b protein induced is probably that described by Noshiro *et al* (1989) as the major phenobarbital-inducible enzyme, Cyp2b-9. The 40-fold induction of benzyloxyresorufin activity in females and the corresponding 8-fold increase in males is consistent with increased levels of this protein. This enzyme is sexually dimorphic in mice, and appears to be induced to a greater extent in females than in males. This effect was also observed, although less pronounced, on Western blot analysis (Figure 3.4.1.1). Similarly, the hepatic Cyp1a protein induced by TCPOBOP is likely to be Cyp1a2 as the activity towards 7-ethoxyresorufin, a substrate for Cyp1a1, was only slightly increased on TCPOBOP treatment (Figure 3.4.2.2).

Figure 3.4.2.1 Benzyloxyresorufin metabolism

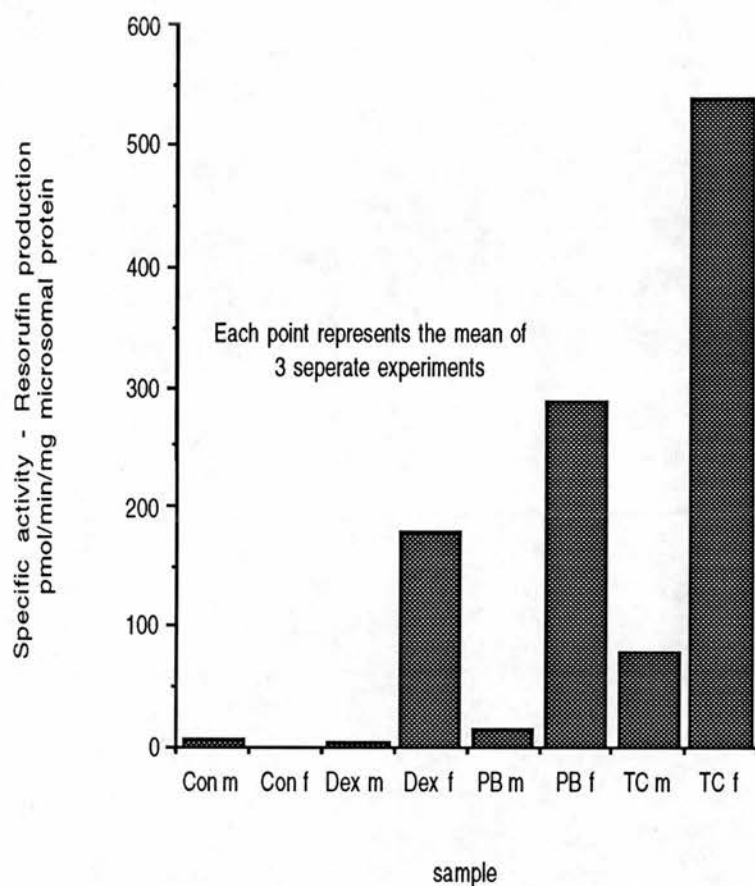
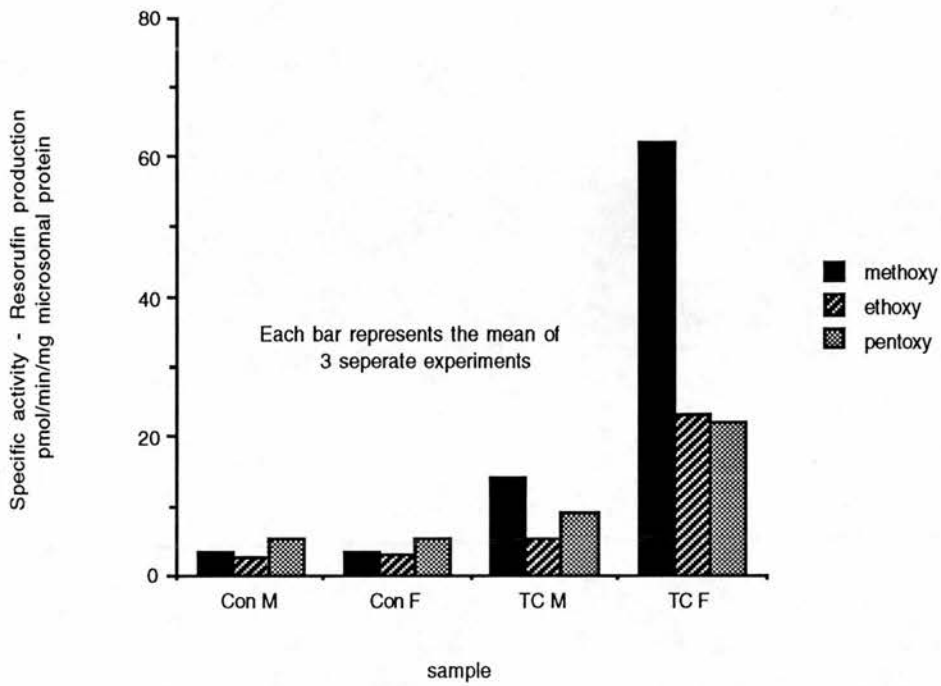


Figure 3.4.2.2 Alkoxyresorufin metabolism



TCPOBOP, although it has a profound inductive effect on murine Cyp4a gene expression, does not cause peroxisome proliferation (Anna-Karin Sohlenius, unpublished observations). No increase above control levels in catalase activity or peroxisomal β -oxidation was detectable in cytosolic or mitochondrial fractions from TCPOBOP-treated animals. TCPOBOP induction of Cyp4a protein expression is therefore unlikely to be mediated by the peroxisome proliferator activated receptor (PPAR)(Issemann & Green, 1990)

3.4.3 TCPOBOP effects are mediated at the level of transcription

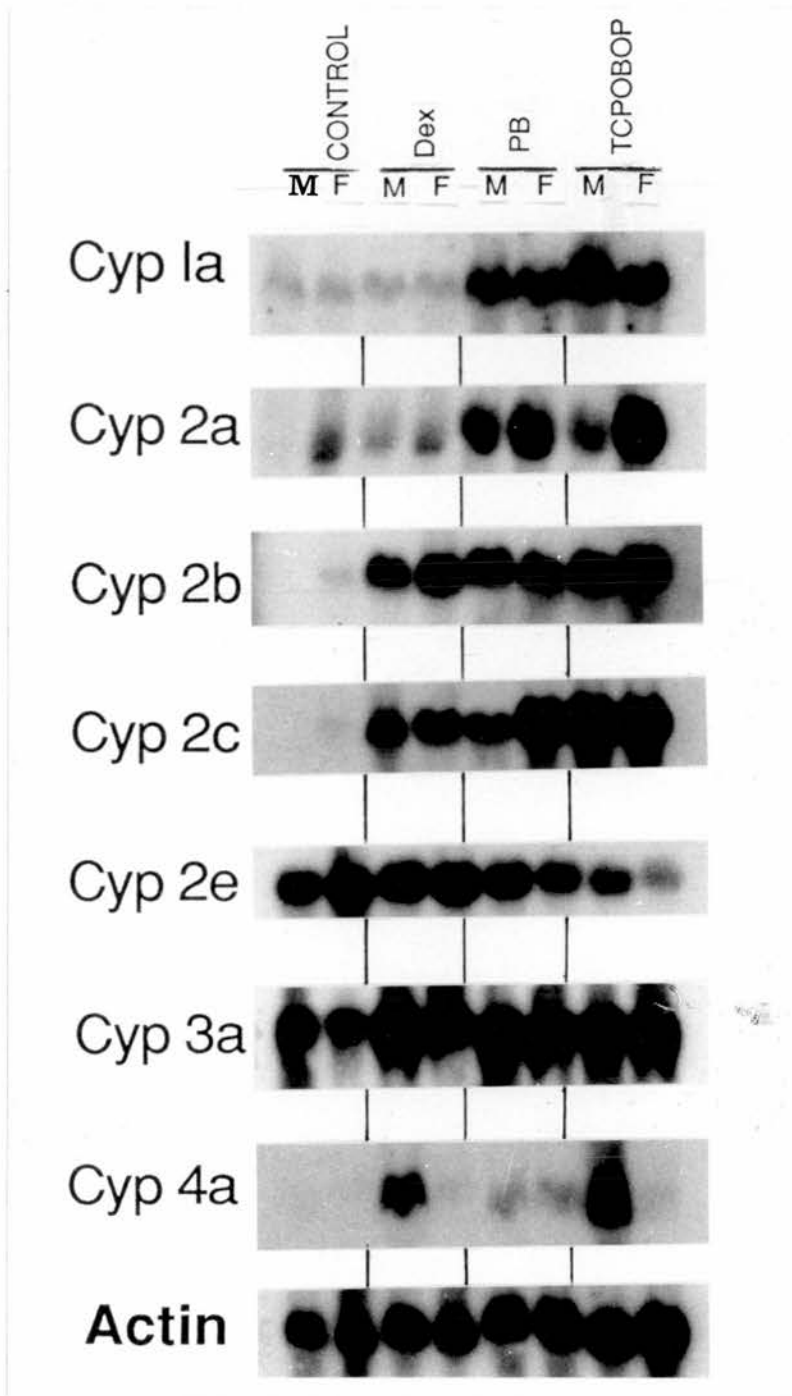
The effect of TCPOBOP, dexamethasone and phenobarbital on cytochrome P450 mRNA levels, in most cases, paralleled the changes in protein expression extremely closely (Figure 3.4.3.1).

Interestingly, and in contrast to the protein analysis, dexamethasone treatment lead to a marked increase in the levels of Cyp2c and Cyp3a mRNAs. Also, there appeared to be no correlation between the levels of Cyp4a mRNA and expressed protein. This may be attributed, in part at least, to the complexity of the gene families in the mouse and the possibility that the cDNA probe used in the Northern blot analysis is hybridising specifically to only one mRNA species. There is an interesting similarity between the ability of TCPOBOP and dexamethasone, but not phenobarbital to induce a male predominant Cyp4a mRNA.

The conclusion from the Northern blot analysis, therefore, was that the administration of both TCPOBOP and phenobarbital to mice lead to an increase in the mRNA levels encoded by genes from almost all the individual P450 gene families. This indicated that the observed changes in protein expression were due to an increased rate of transcription - this is agreement with the findings of Hardwick *et al* (1983) who demonstrated that phenobarbital induction of CYP2B1 expression in the rat occurred by transcriptional activation of the gene.

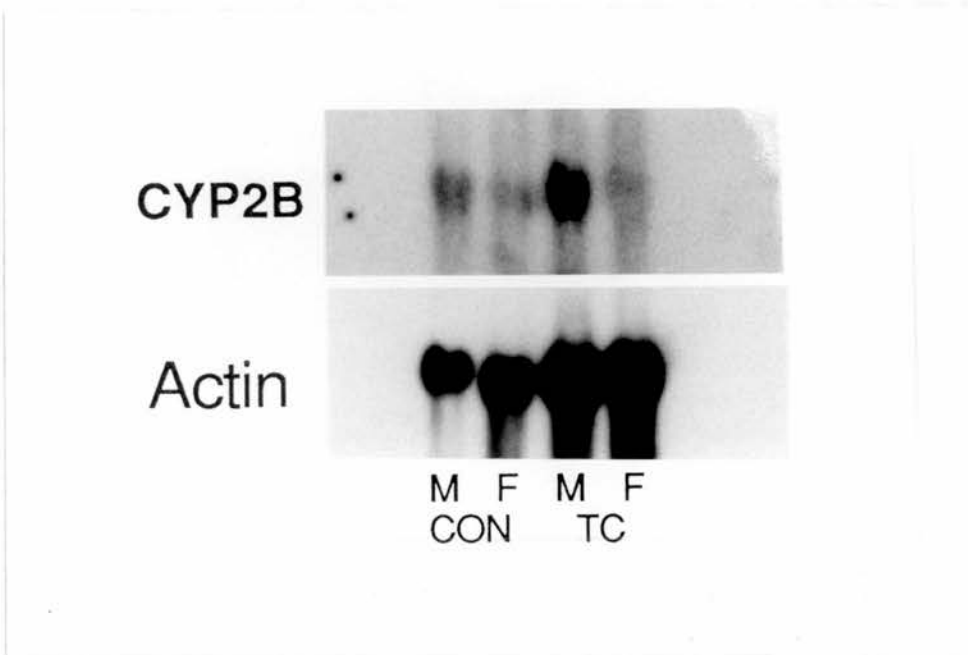
CY2B1 mRNA levels, the rat equivalent of the major murine TCPOBOP-inducible form, were relatively unaffected by TCPOBOP treatment, however (Figure 3.4.3.2), demonstrating that the compound has no inductive effect on P450 gene expression in the rat. This supports the theory that interaction with a species-specific receptor is necessary for the compound to exert its inductive effect.

Figure 3.4.3.1 Effect of TCPOBOP on murine hepatic cytochrome P450 mRNA levels



Liver RNA (10 μ g) from Male (M) and Female (F) DBA/2 mice from pooled tissue from control animals (Con) or animals treated with dexamethasone (Dex), phenobarbital (PB) or TCPOBOP, was separated on 1% denaturing formaldehyde-agarose gels, transferred overnight to Hybond-N, and hybridised with cDNA probes representing each of the individual P450 families. Equality of loading was determined by re-probing each filter with a mouse β -actin cDNA probe.

Figure 3.4.3.2 Effect of TCPOBOP on rat hepatic cytochrome P450 mRNA levels



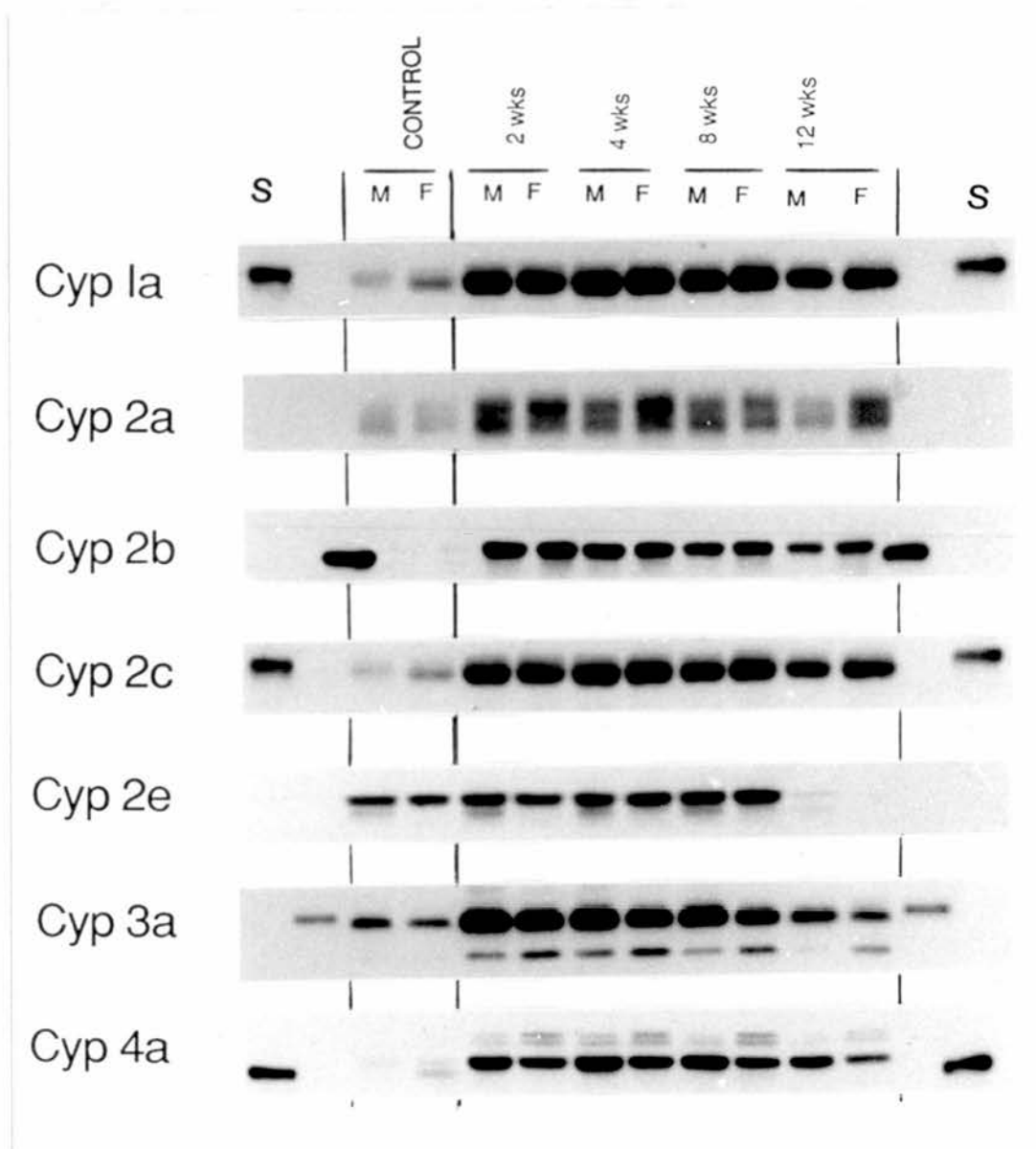
Liver RNA (10 μ g) from Male (M) and Female (F) Wistar rats from pooled tissue from control animals (CON) or those treated with 3mg/kg TCPOBOP (TC), was separated on 1% denaturing formaldehyde-agarose gels, transferred overnight to Hybond-N, and hybridised with a CYP2B1 cDNA probe. Equality of loading was determined by re-probing the filter with a mouse β -actin cDNA probe.

3.4.4 Long term effects of TCPOBOP on P450 expression

In order to establish the potency of TCPOBOP as a P450 inducer, liver samples were taken for a period of up to 3 months following a single injection of 3mg/kg of the compound. These experiments (Figure 3.4.4.1) clearly confirmed the ability of TCPOBOP to induce the expression of P450s from almost all the gene families studied and demonstrated that the induction was long-lasting. Although, in some cases, the level of induction at twelve weeks was reduced relative to the level of protein expression at two weeks, in others only a slight reduction was seen.

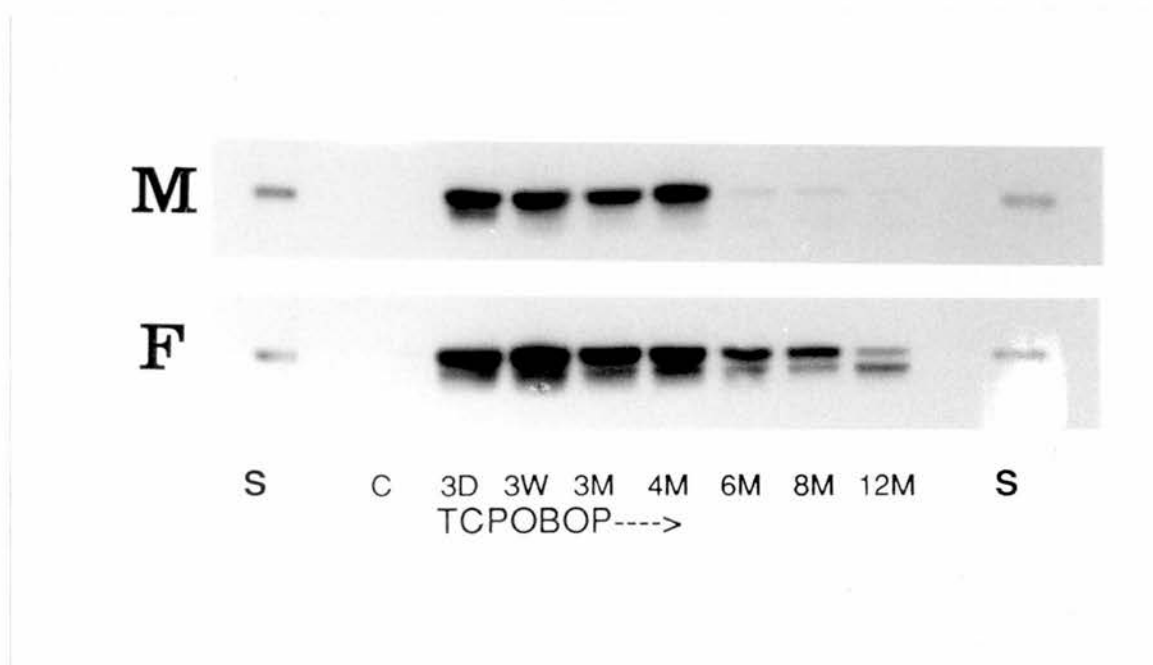
In a recent experiment, Cyp2b expression was monitored for 1 year following a single injection of 3mg/kg (75µg) of TCPOBOP. Protein expression remained above control level throughout the period of study (Figure 3.4.4.2), although Cyp2b levels in the male fell more quickly than in the female.

Figure 3.4.4.1 Long term induction of cytochrome P450 proteins by TCPOBOP



Liver microsomes (15 μ g) from pooled tissue from Male (M) and female (F) DBA/2 mice, treated with a single injection of 3mg/kg TCPOBOP or as Controls (Con), were separated on 9% SDS-PAGE, transferred to nitrocellulose and P450 isozyme content determined by Western blot analysis using polyclonal antisera to various rat liver P450 proteins. Samples were prepared 2, 4, 8 and 12 weeks following the administration of TCPOBOP. S = purified rat liver P450 standard

Figure 3.4.4.2 Hepatic Cyp2b protein expression 1 year after TCPOBOP administration

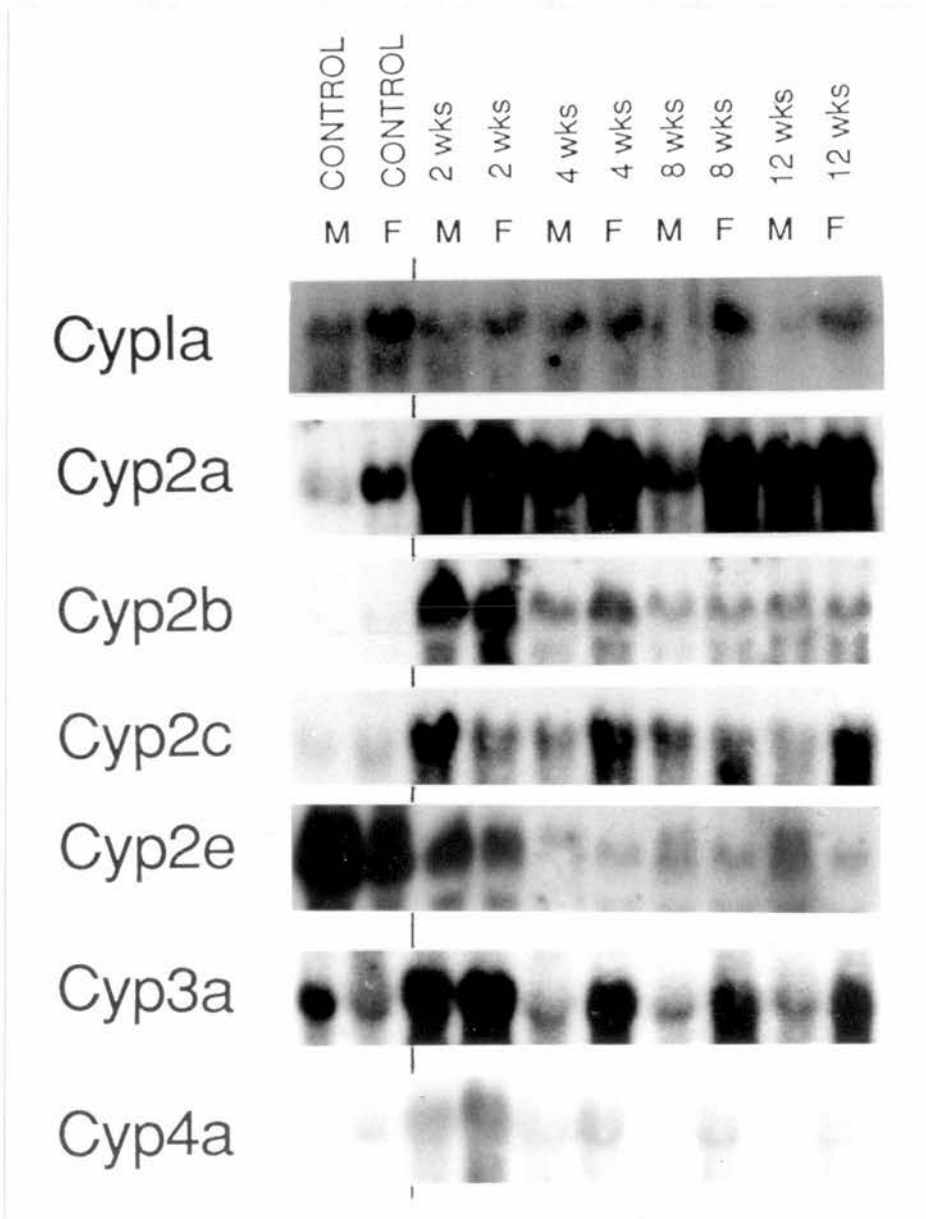


Liver microsomes (15 μ g) from pooled tissue from Male (M) and female (F) DBA/2 mice, treated with a single injection of 3mg/kg TCPOBOP or as Controls (Con), were separated on 9% SDS-PAGE, transferred to nitrocellulose and P450 isozyme content determined by Western blot analysis using polyclonal antisera to various rat liver P450 proteins. Samples were prepared at 3 days, 3 weeks, 3, 4, 6, 8 and 12 months following the administration of TCPOBOP. S = purified rat liver P450 standard

Analysis of P450 mRNA levels demonstrated that mRNAs encoding the various P450 subfamilies were also still elevated 3 months following a single 75µg injection of TCPOBOP (Figure 3.4.4.3). mRNA levels were observed to decline much more quickly than the corresponding protein levels, however, (Figure 3.4.4.1), indicating that the elevated protein expression was due, in part, to some form of post-translational stabilisation.

The long term effects of TCPOBOP were not only reflected in cytochrome P450 levels but also in liver size. An approximately two-fold increase in liver weight was observed 4 days after TCPOBOP treatment. This increase was still observed 3 months after administration of a single dose of the drug.

Figure 3.4.4.3 Long term induction of hepatic cytochrome P450 mRNA by TCPOBOP



Liver RNA (10 μ g) from pooled tissue from Male (M) and Female (F) DBA/2 mice from control animals (Con) or those treated with 3mg/kg TCPOBOP, was separated on 1% denaturing formaldehyde-agarose gels, transferred overnight to Hybond-N, and hybridised with cDNA probe representative of the individual P450 families. Mice were sacrificed at 2 weeks, 4 weeks, 8 weeks and 12 weeks following TCPOBOP administration. Equality of loading was determined by re-probing each filter with a mouse β -actin cDNA probe.

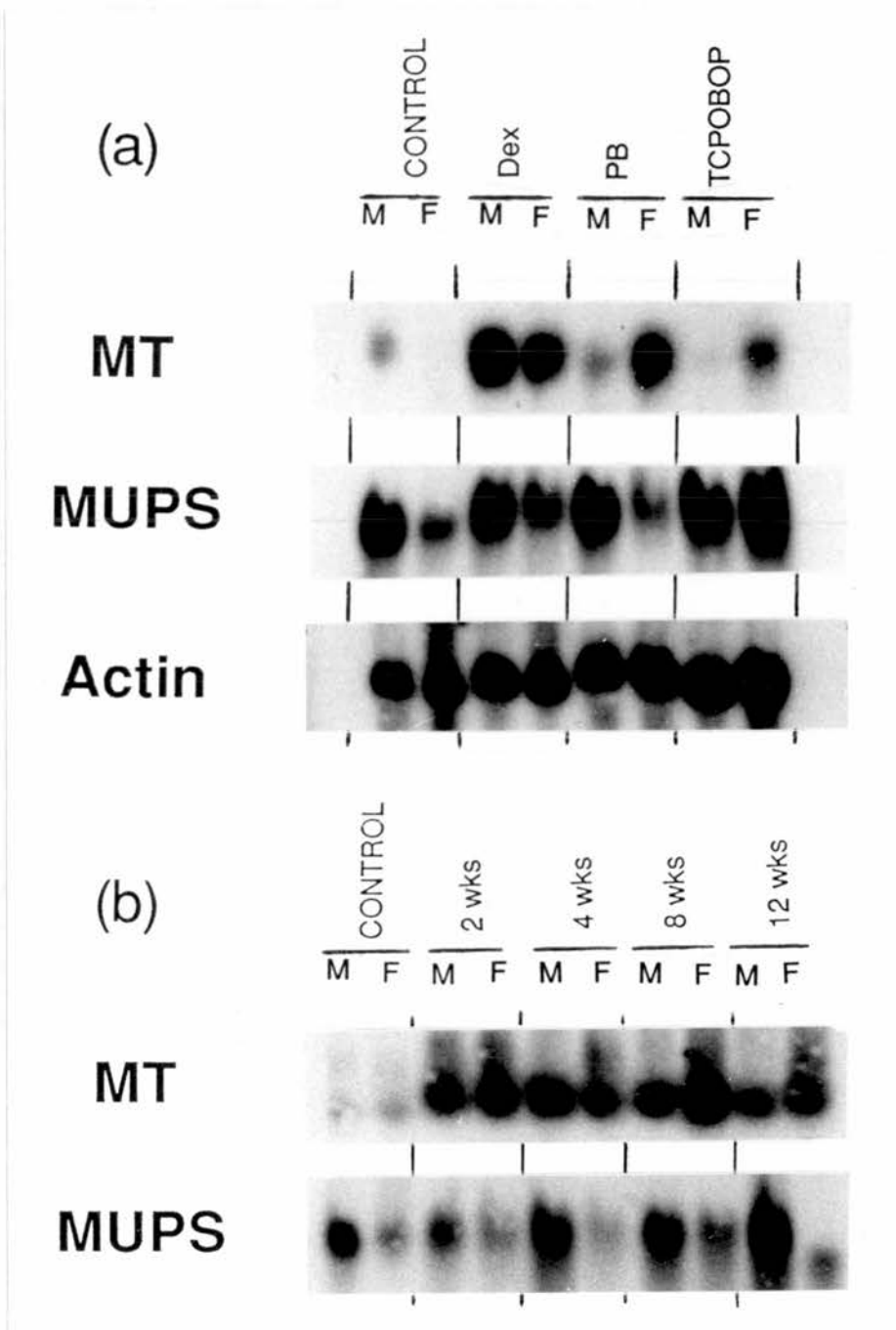
3.5 TCPOBOP - a synthetic glucocorticoid?

3.5.1 The effect of TCPOBOP on metallothionein and major urinary protein mRNA levels

Many similarities were observed between the abilities of TCPOBOP and dexamethasone to induce murine hepatic P450 expression, including a pronounced species specificity in their inductive effects. It seemed reasonable, therefore, that TCPOBOP may exert its inductive effects through the glucocorticoid receptor or another homologous steroid hormone-like receptor. To test this hypothesis, the effect of TCPOBOP was determined on the expression of genes which were known to be dexamethasone-inducible and to contain the glucocorticoid responsive promoter element (GRE). Two such genes are those encoding the mouse metallothionein IIa (Hu & Davidson, 1990) and the murine major urinary proteins (MUPS) (Knopf et al., 1983). The effect of TCPOBOP on the expression of these genes is shown in Figures 3.5.1.1(a) Short term analysis and 3.5.1.1(b) Long term analysis.

In the short term experiments, TCPOBOP induced metallothionein and MUPS mRNA levels in females and MUPS gene expression in both males and females. Interestingly, phenobarbital and dexamethasone also induced metallothionein mRNA to a greater extent in female tissue. In the long term experiment, although the sex differences in induction were less marked, the levels of both MUPS and metallothionein mRNA remained induced 3 months after TCPOBOP administration.

Figure 3.5.1.1 Analysis of metallothionein and major urinary protein mRNA levels following TCPOBOP treatment



(a) Short term experiment (b) Long term experiment

Liver RNA (10 μ g) from pooled tissue from Male (M) and Female (F) DBA/2 mice from control animals (Con) or those treated with dexamethasone (Dex), phenobarbital (PB) or 3mg/kg TCPOBOP, was separated on 1% denaturing formaldehyde-agarose gels, transferred overnight to Hybond-N, and hybridised with cDNA probes for metallothionein IIa (MT) or mouse major urinary proteins (MUPS). Equality of loading was determined by re-probing each filter with a mouse β -actin cDNA probe.

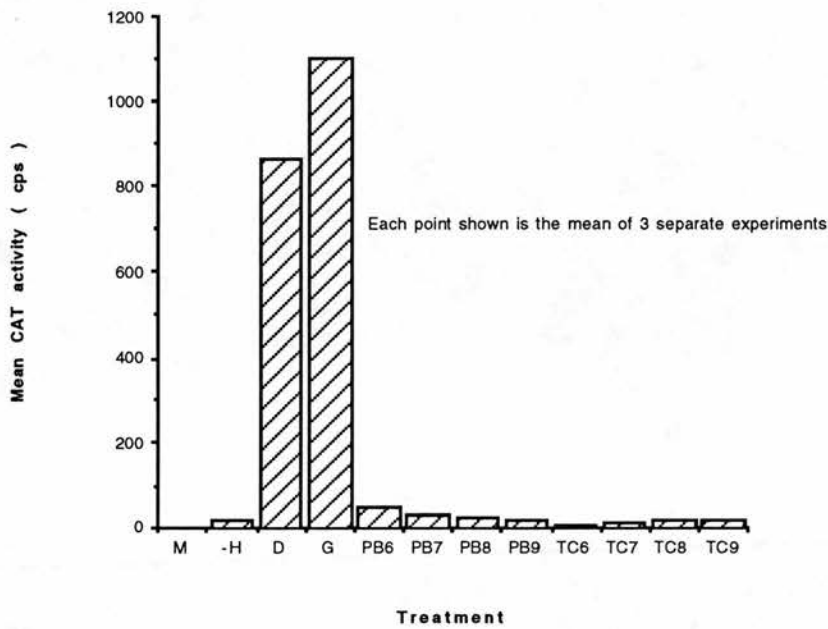
3.5.2 The effect of TCPOBOP and phenobarbital on the activation of the MMTV LTR

The above data confirmed that there were similarities in the ability of TCPOBOP and dexamethasone to influence hepatic gene expression and suggested that the GRE may mediate induction by TCPOBOP. In order to obtain more direct evidence of GRE involvement, the MMTV long terminal repeat (LTR) which contains a GRE was linked to the CAT reporter gene and transfected into the oestrogen-receptor positive breast cancer cell line ZR-75, with the efficiency of transfection monitored by co-transfection with the firefly luciferase gene. CAT activity was determined following exposure to dexamethasone, phenobarbital and TCPOBOP (Figure 3.5.2.1).

CAT activity was highly inducible by dexamethasone, but both phenobarbital and TCPOBOP were without effect at any of the concentrations tested. In addition, TCPOBOP did not antagonise the dexamethasone-mediated inducibility of CAT activity (not shown), indicating that the effects of the two compounds were not regulated by the same mechanism.

In a further experiment, however, the C3H10T1/2 cell line, which has constitutive P450 expression (Pottenger & Jefcoate, 1990, 1991) and is responsive to TCPOBOP (Section 4.6.2), was transfected with a construct comprising the GRE linked to CAT. In this system, very low levels of TCPOBOP-inducible CAT activity were detected (Figure 3.5.2.2). These data imply that although the effects of TCPOBOP do not appear to be mediated directly by the glucocorticoid receptor, a homologous "steroid hormone-like" receptor may be involved in regulating the pleiotropic effects of this compound.

Figure 3.5.2.1 Effect of TCPOBOP and phenobarbital on the activation of the MMTV LTR

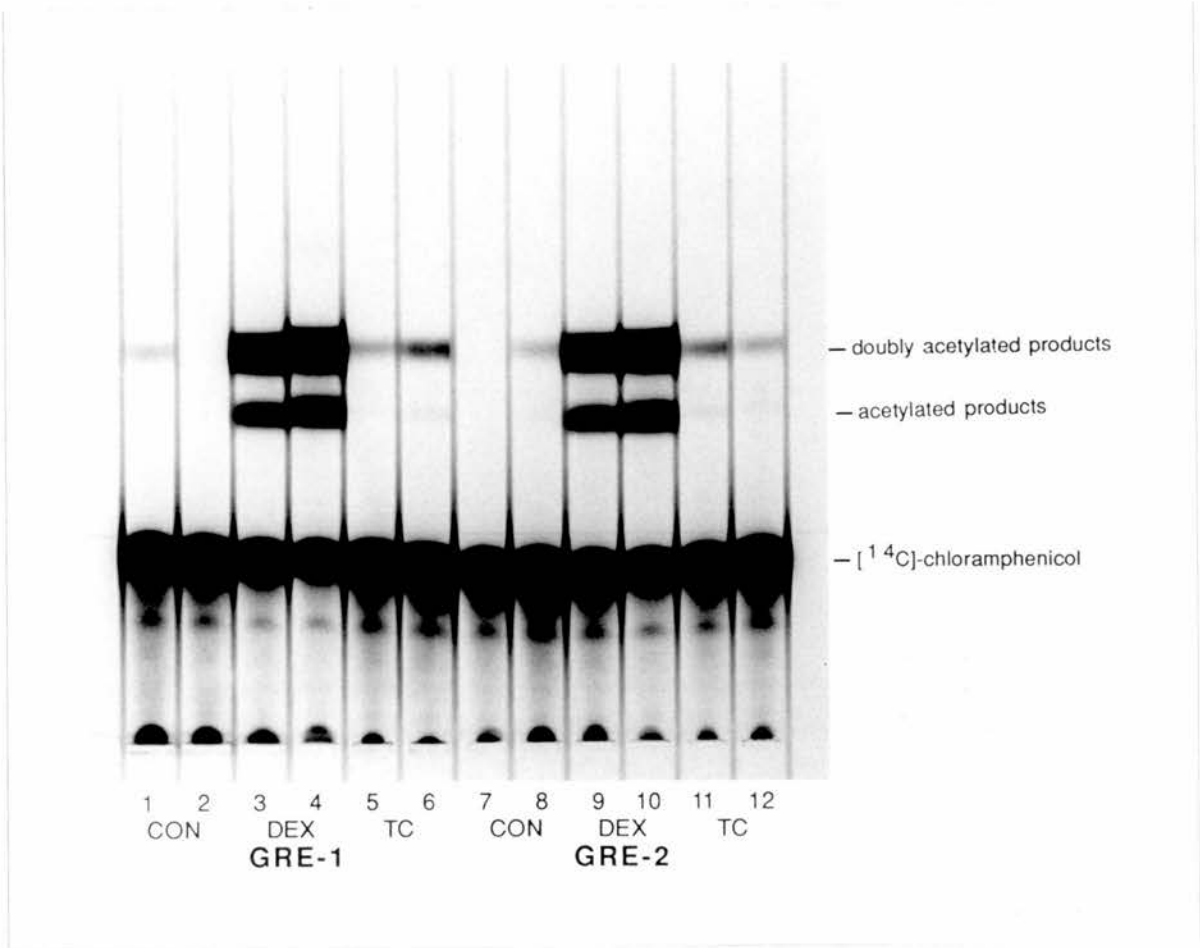


CAT activity was measured following transfection of the MMTV-LTR fused to CAT into ZR-75 cells and subsequent treatment with a range of inducing agents.

M = Mock transfection, -H = transfection without the addition of an inducing agent, G = R5020, a potent synthetic glucocorticoid, D = dexamethasone, P = phenobarbital, T = TCPOBOP.

Phenobarbital and TCPOBOP were administered at several doses, ranging from 10⁻⁶M to 10⁻⁹M (6,7,8 and 9 respectively).

Figure 3.5.2.2 TCPOBOP mediated induction of CAT activity in C3H10T1/2 cells

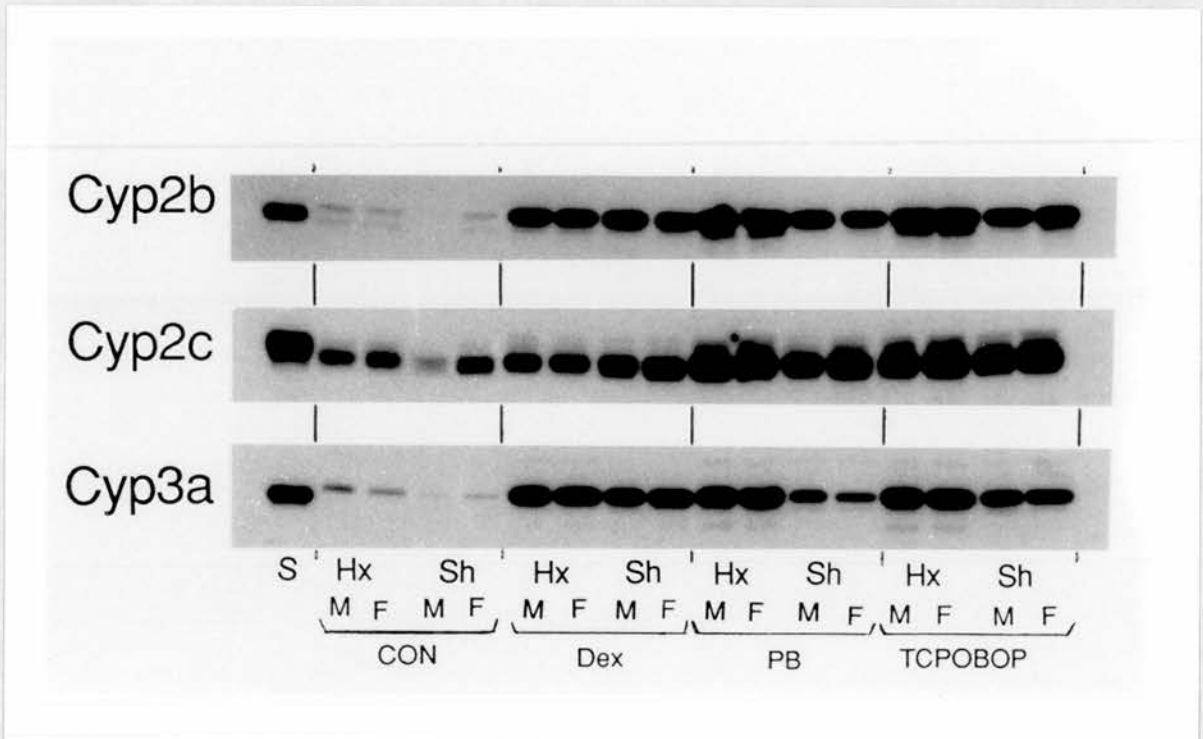


C3H10T1/2 cells were transfected with a construct comprising either (a) the GRE fused to the CAT reporter gene (GRE-1) or (b) 2 GRE elements linked in series fused to CAT (GRE-2). The transfected cells were then treated with vehicle only (CON) or with dexamethasone (Dex) or TCPOBOP (TC) at 10^{-5} M. CAT activity was determined chromatographically as described in Section 2.7.9 (a). Each sample was analysed in duplicate.

3.6 TCPOBOP induction is maintained in hypophysectomised animals

The pituitary has been shown to play a major role in the regulation of certain cytochrome P450 genes (Skett, 1987; Zaphiropoulos *et al.*, 1989). To determine whether induction of P450 expression by TCPOBOP was due to direct interaction with a “receptor-like” protein in hepatocytes or was mediated indirectly by the pituitary, TCPOBOP, dexamethasone and phenobarbital were administered to hypophysectomised animals (Figure 3.6.1).

Figure 3.6.1 The effect of hypophysectomy on TCPOBOP induction of hepatic cytochrome P450 proteins



Male (M) or Female (F) C57Bl/6 mice were hypophysectomised (Hx) or sham-operated (Sh) before being treated with as Controls (Con) or with dexamethasone (Dex), phenobarbital (PB) or TCPOBOP. Liver microsomal samples were prepared from pooled tissues from these animals and were separated (15µg) on SDS-PAGE using 9% gels. P450 isozyme content was determined by Western blot analysis using polyclonal rat antisera to the P450 isozymes shown. S = purified rat P450 protein standard

Neither hypophysectomy nor the control "sham" operations had any effect on the ability of any of these compounds to induce P450 levels. Interestingly, dexamethasone was found to be a potent inducer of both Cyp2c and Cyp3a proteins in these experiments. This finding is in agreement with Meehan *et al* (1988), but in contrast to the effect of administration of this compound to DBA/2 mice described above. It would appear, therefore that there is a strain difference in the murine response to dexamethasone administration. It is also interesting to note, in agreement with Meehan *et al*, that induction of P450 proteins by phenobarbital and TCPOBOP was significantly higher in hypophysectomised animals, implying that some form of negative regulator or repressor is secreted by the pituitary.

Further evidence that TCPOBOP acts predominantly by exerting a direct effect on hepatocytes was obtained by demonstrating that TCPOBOP could induce Cyp2b expression in a primary culture of mouse hepatocytes (section 4.7).

3.7 Discussion

These experiments demonstrate that TCPOBOP is an extremely potent and pleiotropic modulator of hepatic gene expression in the mouse. The inductive effects are both tissue and species specific, suggesting a receptor-based mechanism of induction. The compound shows a marked similarity to phenobarbital in the regulation of hepatic cytochrome P450, NADPH cytochrome P450 reductase, epoxide hydrolase and cytosolic glutathione S-transferase expression and, like phenobarbital, is an promoter of DEN-induced carcinogenesis. It has previously been proposed that phenobarbital exerts its inductive effects by interaction with a specific receptor, but no such receptor has, as yet, been isolated. This has been attributed to the considerable structural variation between "phenobarbital-like" ligands and their relative lack of potency. TCPOBOP, however, is 650 times more potent than phenobarbital in the induction of murine mono-oxygenase activity, and is therefore an ideal substrate to study the regulation of murine P450 genes.

The induction of Cyp1a gene expression by polycyclic aromatic hydrocarbons is regulated by the Ah locus (Nebert & Jensen, 1987). Inbred mice strains are defined as "responsive" or "non-responsive" depending on the ligand binding affinity of their Ah receptor protein. TCPOBOP, however, is equally effective in influencing Cyp1a expression in both C57Bl/6 (responsive) and DBA/2 (non-responsive) mice, suggesting that its action is not mediated by the Ah receptor.

TCPOBOP influences the expression of many more P450 genes than any inducer studied previously. Indeed, the levels of all the hepatic isozymes studied,

with the exception of Cyp2e1, were dramatically increased on treatment with a single injection of 3mg/kg TCPOBOP. The lack of inducibility of Cyp2e1 can be explained by the report of Freeman et al (1992), which demonstrated that induction of Cyp2e1 is regulated by post-translational protein stabilisation, with no concomitant increase in the levels of mRNA. In contrast, TCPOBOP, like phenobarbital, exerts its inductive effects at the level of transcription. There may however be an additional mechanism of P450 induction by both PB and TCPOBOP, which involves post-translational modification. Aida & Negishi (1991) report a 3-fold increase in mouse Cyp2a5 (P450Coh) protein which is not associated with changes in Cyp2a-5 mRNA levels or gene transcription rate. Long term studies of TCPOBOP induction of P450 expression demonstrate that mRNA levels decrease much more rapidly than the corresponding protein levels following TCPOBOP administration, implying that some form of protein stabilisation mechanism exists.

The mechanism of transcriptional regulation of cytochrome P450 genes by phenobarbital remains to be established, although recent progress has been made in the identification of a phenobarbital-responsive promoter element which is conserved between a *B.megeterium* gene and the rat P450 CYP2B1 gene (He & Fulco, 1991). The presence of this regulatory element has not been demonstrated in the mouse, however, and if the actions of TCPOBOP and phenobarbital were indeed regulated by the same mechanism, TCPOBOP would be expected to activate the phenobarbital-responsive promoter in the rat. The compound is ineffective in inducing monooxygenase activity in the rat, however, although the presence in vivo of further repressive regulatory elements which could mask or block TCPOBOP-promoter binding cannot be ruled out. The presence of this phenobarbital-specific element in CYP2B genes would also not explain the pleiotropic effects of TCPOBOP on hepatic P450 expression.

The effects of TCPOBOP on hepatic gene expression could be due to direct action on hepatocytes, or be mediated indirectly by, for example, the pituitary. Pituitary effects on the regulation of P450 expression have been previously extensively reported (Skett, 1987; Zaphiropoulos et al, 1989). Hypophysectomy, however, did not prevent TCPOBOP, phenobarbital or dexamethasone inducing hepatic cytochrome P450s, indicating that the observed effects of these compounds were exerted by a direct action on hepatocytes. This was confirmed by the finding that TCPOBOP and phenobarbital induced Cyp2b expression in mouse hepatocytes in primary culture.

The similarity between the effects of TCPOBOP and dexamethasone

administration on murine mono-oxygenase activities suggested that TCPOBOP may also possess glucocorticoid activity, and that its inductive effects may be mediated by the glucocorticoid receptor. Indeed, TCPOBOP treatment increased the expression of several genes which are known to be glucocorticoid responsive and to contain the glucocorticoid responsive promoter element (GRE). In a series of transfection experiments, however, TCPOBOP did not activate the GRE to a significant extent or compete with dexamethasone for receptor binding. Some slight activity was observed in the C3H10T1/2 cell line, however, suggesting that although TCPOBOP did not bind the GRE, its effects may be mediated by an analogous member of the steroid hormone superfamily which has a lower affinity for dexamethasone binding. Chasserot-Golaz *et al* (1990) also reported a similarity between the effects of phenobarbital and dexamethasone in mediating P450 induction, suggesting glucocorticoid receptor involvement. These workers, however, proposed that phenobarbital treatment increased the affinity of the GRE for its ligand and also increased the total number of glucocorticoid receptors available for binding. Whether this effect was as a consequence of phenobarbital itself being complexed with a specific receptor and thus no longer competing for GRE binding has not been established.

GRE-like elements have previously been reported in many P450 genes. A putative "GRE" lies 1.3kb upstream of the transcription initiation site in the rat CYP2B2 gene (Rangarajan *et al*, 1987; Jaiswal *et al*, 1990). This element (TGTCCT), which shows reasonable homology with the GRE (GGTACANNNTGTTCT) has been shown to be functional and to confer dexamethasone inducibility on the CAT reporter gene and is also present in the mouse Cyp2e1, Cyp2b9 and the hamster CYP2A genes. Hapgood *et al* (1989) demonstrated that the Ah receptor is a sequence-specific DNA binding protein which is both biochemically and structurally similar to the steroid hormone receptor superfamily. However, the Ah and the glucocorticoid receptors do not appear to share any common ligand binding specificity (Poellinger *et al*, 1985).

Fonne & Meyer (1989) proposed that the P450 molecule itself could be the TCPOBOP receptor. This seems inherently unlikely, however, as TCPOBOP administration leads to such profound effects on many distinct P450 genes. The drug would therefore have to bind to a very conserved region of P450 sequence. These conserved regions are found, in general, in regions which are important for catalytic activity, such as the heme binding site. It seems inconceivable that a drug-receptor complex could form without an accompanying disruption of heme or dioxygen binding. Substrate interaction would also be subject to considerable steric hindrance.

The potency and longevity of the inductive effects observed on TCPOBOP treatment also support the proposal that these effects are mediated by an intracellular receptor. The experiments described in this chapter demonstrate that the administration of a single dose of only 75µg of TCPOBOP to a mouse lead to increased Cyp2b expression one year after treatment. It is unlikely that metabolic effects could lead to such profound effects on hepatic gene expression. In addition, previous studies using radioligands demonstrated that TCPOBOP was stored within the body, primarily in adipose tissue, as the parent compound. No radiolabelled metabolites were detectable. Similarly, phenobarbital and not its major hydroxylated metabolite, p-hydroxy phenobarbital, appears to be the active inducing agent (Cresteil *et al.*, 1980).

Accumulation of TCPOBOP in adipose tissue may explain the profound differences in the activities of certain P450 isozymes between male and female mice. As females in general have a higher body fat content than males, it seems reasonable that the effective concentration of TCPOBOP is higher in females. In addition, accumulation of drug in adipose tissue implies that the circulating concentration is considerably lower than the administered dose and the reported potency is an underestimate.

Evans (1988) proposed that there exists a large superfamily of genes whose products are ligand-responsive transcription factors. Steroid hormone receptors and thyroid hormone receptors form part of this "receptor superfamily", the activity of which is mediated by a common mechanism. Gene activation is thought to occur by binding of receptor and ligand and translocation of the resulting receptor-ligand complex to the nucleus, where it binds chromatin with high affinity. Miyata & Yahara (1991) demonstrated that the glucocorticoid receptor exists in the cytoplasm of hormone-free cells as a complex with the 90kD heat shock protein (HSP90). Glucocorticoids induce dissociation of the HSP90/receptor complex and translocation of the receptor to the nucleus. The chromatin structure of each cell type is uniquely organised, and thus different sets of genes may be accessible to the hormone-receptor complex. Selectivity is further increased by the differential expression of the different receptors in specific cells and tissues.

Evans also proposed that a single ligand can simultaneously activate distinct genes, the DNA binding domains of which are similar but not identical. This mechanism seems plausible for the activation of P450 genes by TCPOBOP, where a series of genes with distinct but overlapping substrate specificities are simultaneously induced by the same ligand. Koracek *et al.* (1990) studied the co-

ordinate induction of rat CYP2A and CYP3A genes by phenobarbital in a Matrigel-supported hepatocyte culture, and demonstrated that phenobarbital was not equally effective in influencing the expression of individual P450 genes. These differential effects could, however, be explained if the putative ligand-receptor complex had a different affinity for the regulatory region of each gene. If this is the case, it should prove possible to identify the "TCPOBOP-responsive element" by deletion analysis of the 5' regions of genes which are highly inducible on TCPOBOP expression.

An alternative approach would be that adopted by Issemann & Green in cloning the mouse peroxisome proliferator-activated receptor. Having established that peroxisome proliferators could also act in a manner similar to that of steroid hormones (Green & Chambon, 1988; Evans, 1988), these workers compared the amino acid sequences of several nuclear receptors and identified a highly conserved region within the DNA binding domain. Oligos were constructed based on this consensus sequence and used to screen a mouse liver cDNA library. Several novel clones were isolated, one of which encoded a 468 amino acid protein (Mr 52400), which was shown to be the PPAR. The tissue-dependent expression of this protein was in agreement with the tissue specific effects of peroxisome proliferators. Similarly to the Ah receptor, the physiological role of and the endogenous ligand for the PPAR are, at present, unknown. The effects of peroxisome proliferators on the peroxisomal β -oxidation pathway, however, suggests a possible endogenous role for the receptor in triglyceride and cholesterol homeostasis.

It seems feasible that a similar approach could lead to the isolation of a "TCPOBOP-responsive " receptor. Several further questions must than be addressed:

1. It is not clear whether the putative receptor complex interacts directly with DNA or becomes associated with other transcriptional regulatory proteins
2. Is it necessary for the receptor-ligand complex to remain bound to DNA for the associated gene(s) to remain active or can a transiently bound receptor initiate long-term transcriptional activation ? The longevity of the inductive effects could be explained by a permanent "trickle" of TCPOBOP from adipose tissue to liver. It is not yet clear whether induction by TCPOBOP can be maintained in the absence of ligand. Becker et al (1986) demonstrated that hormone binding is necessary for the transcriptional activation of genes by glucocorticoid hormones. In contrast to these findings, however, Willman & Beato (1986) reported binding of the hormone responsive receptor to the MMTV GRE in the absence of a steroid ligand. Further studies are therefore necessary to determine which mechanism, if any, best

represents the action of TCPOBOP. The cell line C3H10T1/2 has constitutive expression of a "Cyp2a-like" protein (Jefcoate et al, 1990, 1991) which is inducible by TCPOBOP (Section 4.6.2). This may prove to be a suitable model in which to determine whether induction of P450 gene expression by TCPOBOP requires the presence of bound ligand.

Chapter 4: Cytochrome P450 expression and regulation in mammalian cell culture

4.1 Introduction and aims

Cell culture has become an invaluable *in vitro* technique for studying the expression and regulation of many genes. Cell lines have been established from a variety of species and tissues and have been extensively characterised with respect to their individual patterns of gene expression. The study of cytochromes P450 in this model is difficult, however, as the expression of these genes is usually either lost completely or, at best, significantly altered in established cell lines, often resulting in the loss of the characteristic P450 induction patterns observed *in vivo*. The study of cells in primary culture can circumvent this problem to some extent. As the liver is the primary metabolic site within the body, analysis of hepatic cells in culture has been widely used to study the expression and regulation of the enzyme systems involved in drug metabolism.

The aims of this section were twofold: 1) to identify a cell line(s) in which P450 expression could be regulated by xenobiotics; 2) to compare the effects of xenobiotic regulation of P450 expression in continuous culture with a primary culture model and the effects observed *in vivo*.

4.2 Primary hepatocyte culture

Hepatocyte culture is thought to be more physiologically relevant than isolated microsomes to study P450 regulation *in vitro*, as transport across the cell membrane and competition between cellular metabolic reactions can still occur (Begue *et al.*, 1983). Hepatocytes do not proliferate in primary culture however, and thus all experiments are restricted to the limited life-span of the cells. P450 expression and induction can be maintained in primary hepatocyte cultures under strictly defined conditions, albeit at a much lower level than observed *in vivo*. It has been demonstrated (Newman & Guzelian, 1982) that hepatocytes require a period of adaptation to the tissue culture environment before P450 expression is maximised and, in general, P450 expression declines with increased time in culture. Bissell & Guzelian (1979) demonstrated that as much as 80% of P450 activity was lost within 48 hours of establishing hepatocytes as a primary culture.

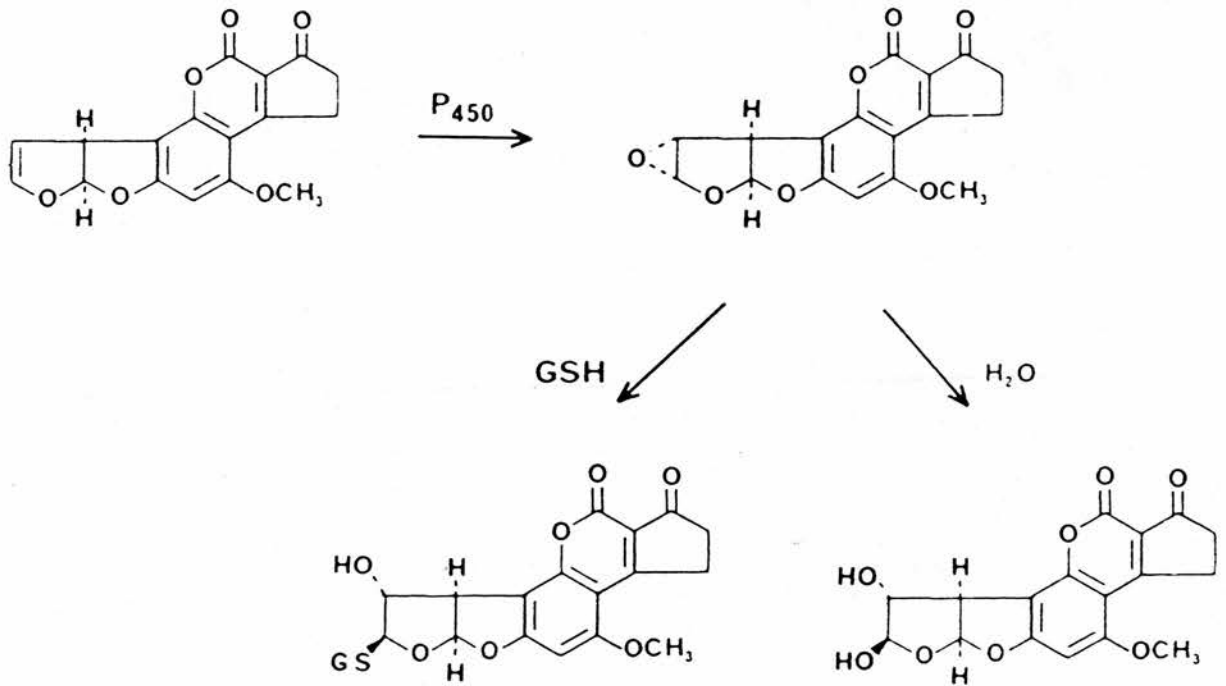
As human liver samples are difficult to obtain for obvious ethical reasons, almost all the primary culture models studied to date have used rodent (usually rat) hepatocytes. Great care must be taken in extrapolating these results to the human

response, as previous studies have demonstrated that there are significant differences between P450 activities in adult human and rat liver microsomes (Kremers *et al*, 1981). In certain cases, however, human liver samples have been obtained from patients undergoing kidney transplantation (Begue *et al*, 1983) or cholecistectomy (Donato *et al*, 1990) and hepatocytes isolated by collagenase perfusion. Human foetal hepatocytes have also been studied (Rollins *et al*, 1979; Rane & Tomson, 1980; Guillouzo *et al*, 1982). Using this model, direct effects of xenobiotics on hepatic gene expression can be determined, although the physiological relevance of studying a single cell type in isolation is often limited, and care must be taken in extrapolating the results of such experiments to the behaviour of a particular compound in the intact animal, where the regulation of hepatic gene expression is controlled by many hormonal and metabolic factors. There can be considerable variation in gene expression in hepatocytes isolated from different rats and it is therefore important to standardise culture conditions as far as is practicable between experiments.

For P450 expression to be maintained in cell culture, several factors must be present: (a) sufficient intracellular heme to be incorporated during protein synthesis; (b) a suitable membrane lipid environment to allow enzymatic and catalytic activities to occur; (c) NADPH-cytochrome P450 oxidoreductase and, in certain cases, cytochrome b_5 .

Other enzymes involved in drug metabolism, e.g. the glutathione S-transferases, do not have such strict requirements for activity and their expression is therefore maintained at close to *in vivo* levels in cell culture models. As drug metabolism is often dependent on the interaction of a number of different enzymes, it is important to determine that all the components of a particular metabolic pathway are both present and active in the hepatocyte population before metabolism studies are undertaken. A fine balance between detoxification and activation to a more toxic form exists in the metabolism of many chemicals, with the actual metabolic route dependent on the enzyme profile present on xenobiotic challenge. Aflatoxin B1 (Figure 4.2.1) is a good example of such a compound, the metabolism of which is dependent on both P450 and GST enzymes.

Figure 4.2.1 The metabolism of Aflatoxin B₁



Redrawn from Coles & Ketterer, 1990

Bars *et al* (1989) demonstrated that P450 expression in the rat is not distributed evenly throughout the hepatocyte population. This heterogeneous expression is also seen in untreated rodent liver where P450 expression is greater in centrilobular than in periportal hepatocytes. Induction by phenobarbital, for example, occurs predominantly in the centrilobular region. Several workers (Bars *et al*, 1989; Frey *et al*, 1984) however, have reported that the isozyme profile induced by a particular xenobiotic differs in this *in vitro* system from the intact liver. For example, the expression of the male-specific CYP2CII isozyme, which is pituitary-regulated, is lost in cell culture. This has been attributed (Waxman *et al*, 1990) to lack of the appropriate hormonal factors necessary for expression although expression was not restored on growth hormone treatment (Guzelian *et al*, 1988). This was attributed to the necessary episodic patterns of secretion being absent *in vitro*. In addition, Nemoto *et al* (1990) reported that in a series of experiments on mouse hepatocytes, aromatic hydrocarbon hydroxylase activity, although genetically determined in each strain, could be induced in the hepatocytes of previously non-responsive strains on treatment with aromatic hydrocarbons when the cells were transferred to primary culture.

In conclusion, therefore, xenobiotic regulation of P450 expression may be studied *in vitro* using this model, but significant differences from the *in vivo* metabolism of a particular compound may exist.

4.3 Optimisation of P450 expression in primary culture

Many attempts have been made to improve the levels of P450 expression in cell culture models and to extend the lifetime of the P450 expressing hepatocytes. Decad *et al* (1977) reported P450 expression close to *in vivo* levels, measured by the extent of Aflatoxin B1 metabolism, by the addition of a complex hormone mixture comprising testosterone, thyroxine, hydrocortisone, estradiol, glucagon, insulin, linoleic acid and oleic acid to serum-free medium. Each component was found to be necessary for maximum P450 expression and was added at the lowest effective concentration. Such complex culture conditions make reproducibility of experiments extremely difficult, however, as no two hepatocyte cultures are identical and each has a different hormonal requirement and therefore a different level of P450 expression. Paine (1990) demonstrated that the addition of 0.5mM metyrapone to the culture medium helped to minimise P450 loss.

The most significant advance in the maintenance of P450 activities in culture, however, came with the addition of components of the extra-cellular matrix

to the culture media. Lindblad et al (1991) demonstrated that the *in vivo* hepatocellular phenotype, both morphological and functional, was influenced by the nature of the collagenous substratum on which the cells were grown, with Matrigel being the most successful at potentiating P450 expression. Matrigel, a reconstituted basement membrane extracted from the Engelbreth-Holm-Swarm sarcoma (Kleinman et al, 1982) contains multiple attachment factors including laminin, type IV collagen and proteoglycan. Cells cultured on this matrix attach but do not spread and thus maintain their differentiated phenotype. Collagen-type matrices e.g. Vitrogen, do not maintain P450 expression as well as Matrigel, and other differentiated functions such as albumin production are also lost (Schuetz et al, 1988). It had previously been proposed (Wiebel et al, 1980) that there is a correlation between the ability of cultured hepatocytes to synthesise P450 and their degree of differentiation. Schuetz et al (1988) drew the conclusion from these data that cells grown on Matrigel are maintained in a more differentiated state.

Co-culturing adult rat hepatocytes with another rat liver epithelial cell type also potentiated P450 expression and inducibility (Guguen-Guillouzo et al, 1983; Begue et al, 1984). Cells were maintained in a differentiated state, as assessed by their capacity to secrete albumin, for up to several weeks, without the addition of hormonal supplements or the use of collagenous substrata. Aflatoxin B₁ metabolism by primary hepatocytes, usually undetectable after 48 hours in culture, was still easily measurable on Day 7 in a co-culture of the hepatocytes with another cell type. Donato et al (1991) studied a number of epithelial cell lines to determine which was the most suitable to form functional co-cultures with rat hepatocytes. The candidate cell line had to have no endogenous drug metabolising activity, be compatible with the growth requirements of the hepatocytes and be capable of forming a stable monolayer in the absence of serum. Monkey kidney MS cells proved to be the most successful at maintaining mono-oxygenase activities in co-culture, achieving 50% of the initial value. These results suggest that both specific cell-cell interactions and an extra-cellular matrix are needed to prevent rapid phenotypic changes in cultured hepatocytes. Interestingly, the levels of Phase II enzymes (UDP-glucuronosyl transferases and glutathione S-transferases) remained constant throughout the period of study in both pure and mixed cultures.

The mechanisms by which non-parenchymal cells support the expression of differentiated functions of hepatocytes in co-culture are not fully understood, although several mechanisms have been proposed. Goulet et al (1988) proposed that the non-parenchymal cells release soluble factors into the media thus conditioning it

for hepatocytes, while it has been demonstrated (Clement *et al*, 1984; Morin & Normand, 1986; Goulet *et al*, 1988) that these additional cells synthesise components of the extra-cellular matrix which are then deposited around the hepatocytes. This is in agreement with the findings of Guguen-Guillouzo *et al* (1983), who report that this production of insoluble extracellular material is the key function of co-cultures. Wright & Paine (1992) attempted to mimic cell density, cell-cell interactions and interactions with the extra-cellular matrix by culturing precision cut liver slices (250µm, 10 cells thick), but these also lost P450 expression after the first 24 hours in culture. DMSO (2% (v/v)) has been shown to protect against de-differentiation and loss of cytochrome P450 activity (Villa *et al*, 1991) although hepatocyte toxicity can become a problem at this concentration of solvent. DMSO increases the levels of δ -aminolaevulinic acid dehydratase, an enzyme necessary for heme biosynthesis. P450 loss has been associated with shortage of heme due to reduced levels of this enzyme in culture. It has also been proposed (Bissell & Guzelian, 1975) that loss of functional P450 expression is due to a concomitant rise in the activity of haem oxygenase, although this does not seem to be a general phenomenon. Paine & Legg (1978) have shown that high levels of P450 activity can be maintained along with high haem oxygenase activity in a primary culture of rat hepatocytes.

Immortalised differentiated cell lines have been created from primary cultures established from tissues from transgenic mice bearing the SV40 temperature-sensitive large T-antigen gene (Yanai *et al*, 1991). Hepatocytes from these animals had constitutive CYP1A1 expression which was 3-methylcholanthrene inducible and stable after 6 months in culture. Similarly, Bayad *et al* (1991) created the cell line SVHepB₄ from the SV40 transformation of adult rat hepatocytes. These cells also had a greatly extended lifespan (> 50 subcultures). γ -glutamyltransferase, a marker enzyme in dedifferentiated hepatocytes, was only present at the limit of detection in these cells, and the hepatospecific tyrosine aminotransferase was expressed at *in vivo* levels.

4.4 Continuous culture

The creation of immortalised cell lines has a number of advantages over primary cultures. Cells from a much wider range of species and tissues can be analysed, and a very wide range of human cell lines is available. Reproducibility of results is much easier to achieve as, in most cases, the profile of gene expression of a particular cell line remains constant within a defined passage range. It has proved

possible to establish cell lines from patients with many different stages of disease and often also from the same patient before and after drug treatment. This is of obvious importance in the study of enzymes involved in both drug metabolism and drug resistance. P450 expression in cell lines is low, however, and this has been attributed, in part at least, to the ability of cell lines to methylate genes which are not necessary for their survival, thus blocking transcription (Antequara et al, 1990). In many cases, when cell lines retained endogenous P450 expression, it did not prove possible to modulate the levels of these genes by exposing the cells to "classical" inducing agents such as phenobarbital and 3-methylcholanthrene. Although many cell lines have been established from extra-hepatic tissues, P450 expression in vivo in these tissues is low in comparison to liver, and thus becomes very difficult to detect in the cell culture environment. Again, P450 inducibility appeared to be related to the degree of differentiation of the cells. Corcos & Weiss (1988) studied a series of rat cell lines derived from the Reuber H35 hepatoma. These cells expressed P450s from the CYP1A family in both differentiated and undifferentiated cells, while phenobarbital-inducible forms were only detectable in differentiated cells.

P450 expression has been studied in five cell lines during this project. HepG2 (Knowles et al, 1980) and Hepa-1 (Bernhardt et al, 1973) are human and mouse hepatoma lines respectively. HepG2 is a highly differentiated human hepatoma cell line which has retained many of the specialised functions normally lost by hepatocytes in culture. Active inducible P450 has been reported in this cell line by a number of groups, but only at 10% of the activity of freshly isolated human hepatocytes (Dawson et al, 1985; Grant et al, 1987, 1988). The overall metabolic profile of these cells is, however, thought to be significantly different from human liver (Grant et al, 1987,1988), although the levels of NADPH cytochrome c reductase, UDP- glucuronosyl transferase and glutathione S-transferase proteins were very similar to in vivo levels. DMSO and δ -aminolaevulinic acid were again found to support P450 expression in these cells, but their influence was very dependent on the media used. Foetal calf serum had a very profound inhibitory influence on P450 expression (Doostdar et al, 1991).

The mouse hepatoma line, Hepa-1, has constitutive aromatic hydrocarbon hydroxylase activity which is polycyclic aromatic hydrocarbon inducible, mediated through the Ah receptor. Israel et al (1985) demonstrated functional "superinduction" of CYP1A mRNA in this cell line in the presence of cycloheximide,

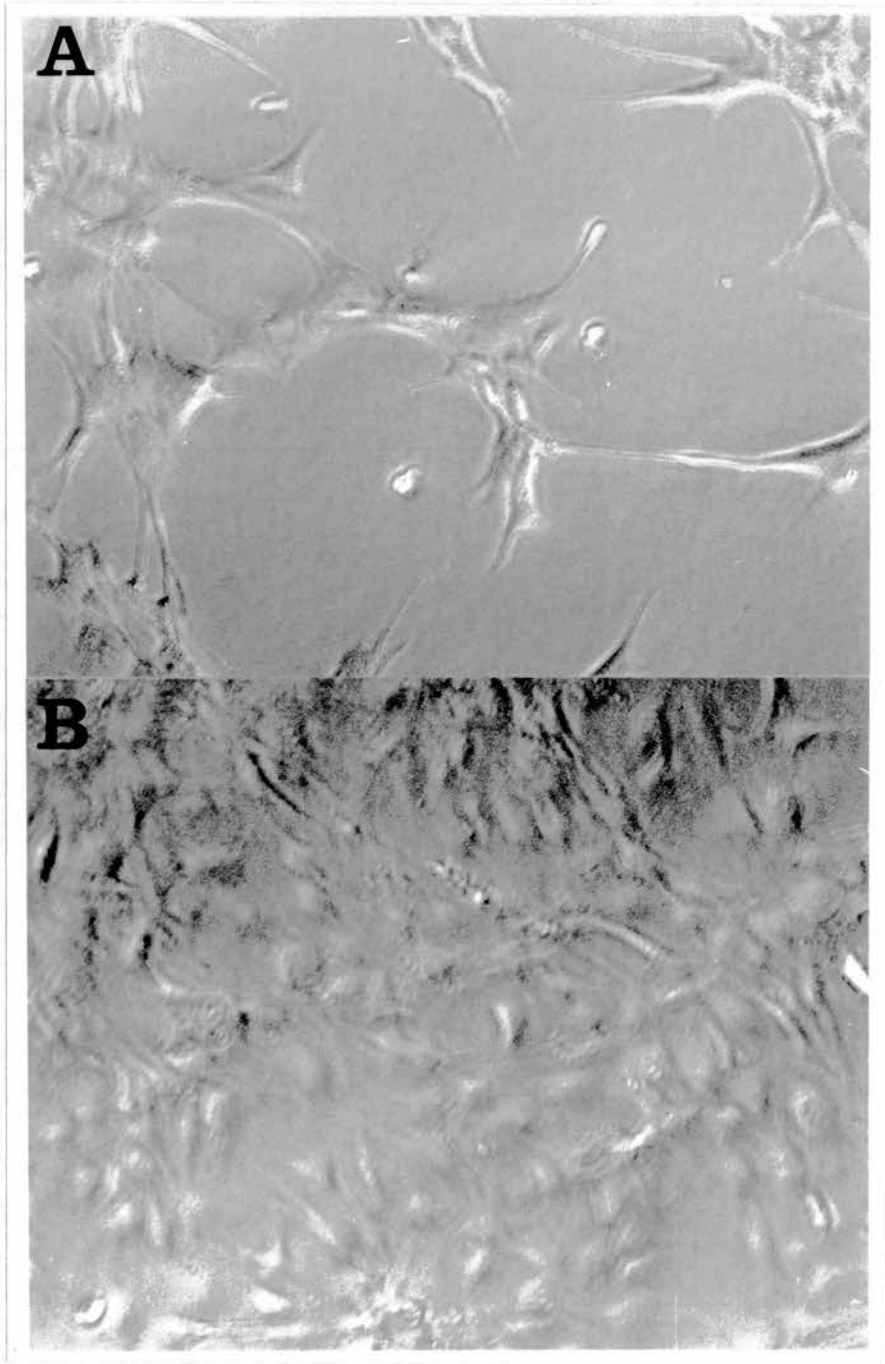
indicating that induction is regulated at the level of transcription. Interestingly, in agreement with our studies in DBA mice, Kärenlampi et al (1989) reported that this activity is very weakly inducible by phenobarbital, indicating that phenobarbital can act as a weak ligand for the Ah receptor. This effect was not observed in the rat.

MCF7 (Soule et al, 1973) and HT29 (Fogh & Trempe, 1975) were chosen as representative human breast (MCF7) and colon (HT29) tumour cell lines to provide an in vitro comparison with the xenograft tumours described in Chapter 5. P450 expression has previously been reported in both these cell lines. Pasenen et al (1988) described CYP1A1 expression in human MCF7 cells. Aromatic hydrocarbon hydroxylase (AHH) activity, characteristic of CYP1A expression, was inducible in this cell line on treatment with TCDD, although the presence of a functional Ah receptor could not be detected. MCF7 cells are oestrogen receptor positive. Interestingly, the subline AL-1, created from MCF7 cells but lacking oestrogen receptor expression lacked TCDD inducibility. These workers also demonstrated that there was considerable variation in constitutive P450 expression in a panel of breast tumour cell lines. This is in agreement with an in vivo study (Forrester et al, 1990) which described P450 expression in normal and tumour breast tissue. P450 expression in colon cell lines has also been previously described. White et al (1991) identified CYP1A1, CYP2C9 and CYP2E1 expression in LS174T cells, although CYP2E1 was only detectable by PCR amplification of mRNA from these cells. In a previous report, Hammond & Strobel (1990) had measured P450 reductase activity in the same cell line.

C3H10T1/2 cells, fibroblasts established from C3H mice embryos (Reznikoff et al, 1973 a,b) have constitutive P450 expression. This line proved to be the best in vitro model to study P450 regulation by xenobiotics. The cell line was created for the study of chemically induced malignant transformation by polycyclic hydrocarbons i.e. the ability of cells transformed in culture to induce tumours on inoculation into suitable recipient animals. These cells are unusually stable after prolonged culture and do not transform spontaneously. On treatment with 5-azacytidine however, the fibroblasts transform to yield a mixed population of contractile striated muscle cells, biochemically transformed adipocytes and chondrocytes, with the proportion of each dependent on the concentration of the inducing agent (Taylor & Jones, 1979). The wild-type cells exhibit post-confluence inhibition of cell division, which is abolished on transformation, providing an easily detectable morphological marker for differentiation. Wild-type cells (Figure 4.4.2)

grow with a fibroblast-like morphology with long cytoplasmic processes in sparse cultures. When confluent, they form flat even monolayers and appear epitheloid.

Figure 4.4.2 C3H10T1/2 cell morphology



(A) sub-confluent C3H10T1/2 cells, illustrating characteristic fibroblast morphology with long cytoplasmic processes
(B) confluent cells

As polycyclic aromatic hydrocarbons e.g. benzanthracene, benz(a)pyrene (B(a)P), are metabolised by the P450 system, it seemed reasonable to expect P450 to be present and active in the C3H10T1/2 cell line. Indeed, Gehly *et al* (1979) reported the presence of cytochrome P450, responsible for the metabolism of B(a)P, in this cell line. Exposure to this compound also resulted in the induction of AHH activity in these cells. A later report, however, suggested that CYP1A1, the major PAH-inducible isozyme was not present in the cell line (Pottenger & Jefcoate, 1990). The mouse hepatoma line, Hepa-1, has been shown to express CYP1A1 and to be regulated by polycyclic aromatic hydrocarbons, and was therefore used for comparison. It was discovered that a different metabolite profile was produced in each cell line on exposure to benzanthracene. As both BA and TCDD induce AHH activity in Hepa-1 cells, but 3-MC, the "classical" inducer does not, Okey *et al* (1983) suggested that there may be some inhibitory factor present in this cell line which prevents 3-MC binding to the Ah receptor. Alternatively, the Ah receptor itself may be mutated in some way such that binding is no longer possible. Further attempts to characterise the P450 (McGregor *et al*, 1991; Pottenger *et al*, 1990, 1991) strongly suggest that the isozyme expressed in the C3H10T1/2 cells is in fact a member of the Cyp2a subfamily, with a molecular weight of 55kD. As this protein is inducible by both BA and TCDD, it may well represent a novel protein. We have demonstrated that the level of expression of this protein is also inducible on treatment with TCPOBOP.

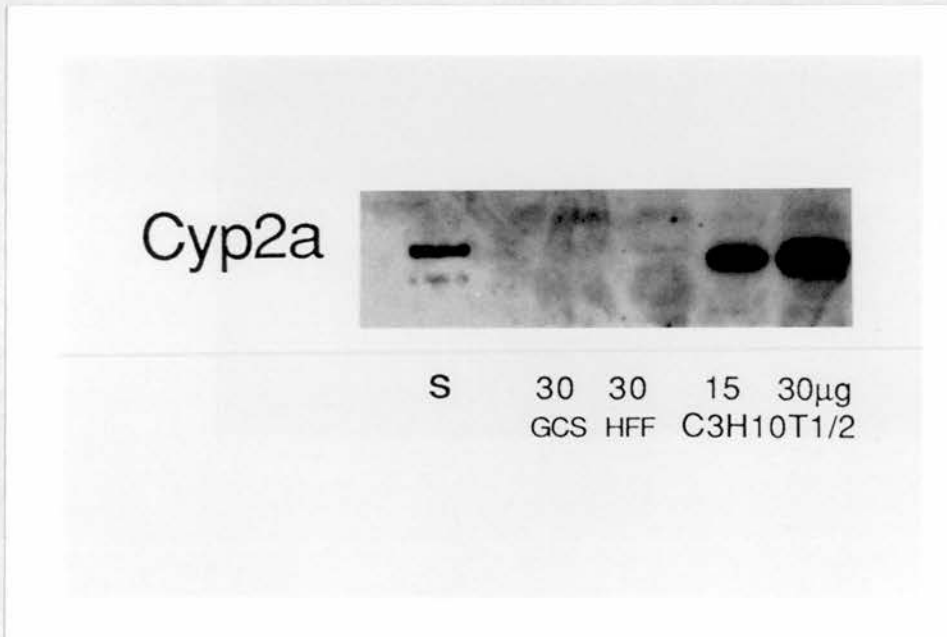
4.5 Characterisation of P450 expression in C3H10T1/2 cells

4.5.1 Western blot analysis

Western blot analysis of total cellular protein from C3H10T1/2 cells was carried out using a panel of antibodies to purified P450 proteins representative of each gene family (Figure 4.5.1). In agreement with Pottenger *et al*, the results demonstrated that Cyp2a protein was present constitutively in this cell line, as measured by a cross-reacting band at 55kD. This finding is in contrast to the report of McGregor *et al*, who reported that the constitutively expressed protein had a mobility of 48kD. In agreement with McGregor *et al*, however, very weak cross-reactivity was also observed using an antibody to CYP1A2, when the sensitive ECL detection system was used. The mobility of this protein, however, was much greater than the rat and mouse CYP1A proteins and there was therefore some doubt as to whether it did in fact represent P450 protein. Two further fibroblast lines (HF and GCS2) were also analysed for P450 expression (Figure 4.5.1, tracks 1 and 2) to

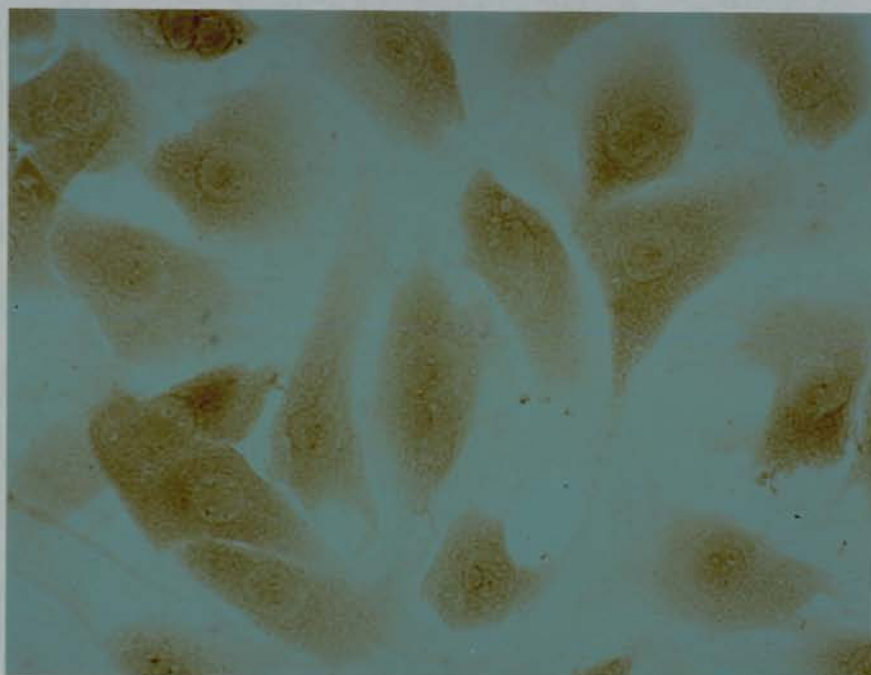
determine whether the ability to maintain P450 expression was related to the cell type studied or extent of differentiation of the cell line, but no expression was detected with any of the antibodies used.

Figure 4.5.1 Cyp2a expression in fibroblast cell lines



Tracks 1 and 2 contain protein from GCS and HFF cells respectively (30 μ g), both of which are human fibroblast cell lines. Tracks 3 and 4 contain protein from C3H10T1/2 cells, loaded at 15 and 30 μ g per track, respectively. S = purified rat CYP2A protein (0.8 pmol).

4.5.2 Immunohistochemical analysis of Cyp2a expression in wild-type C3H10T1/2 cells

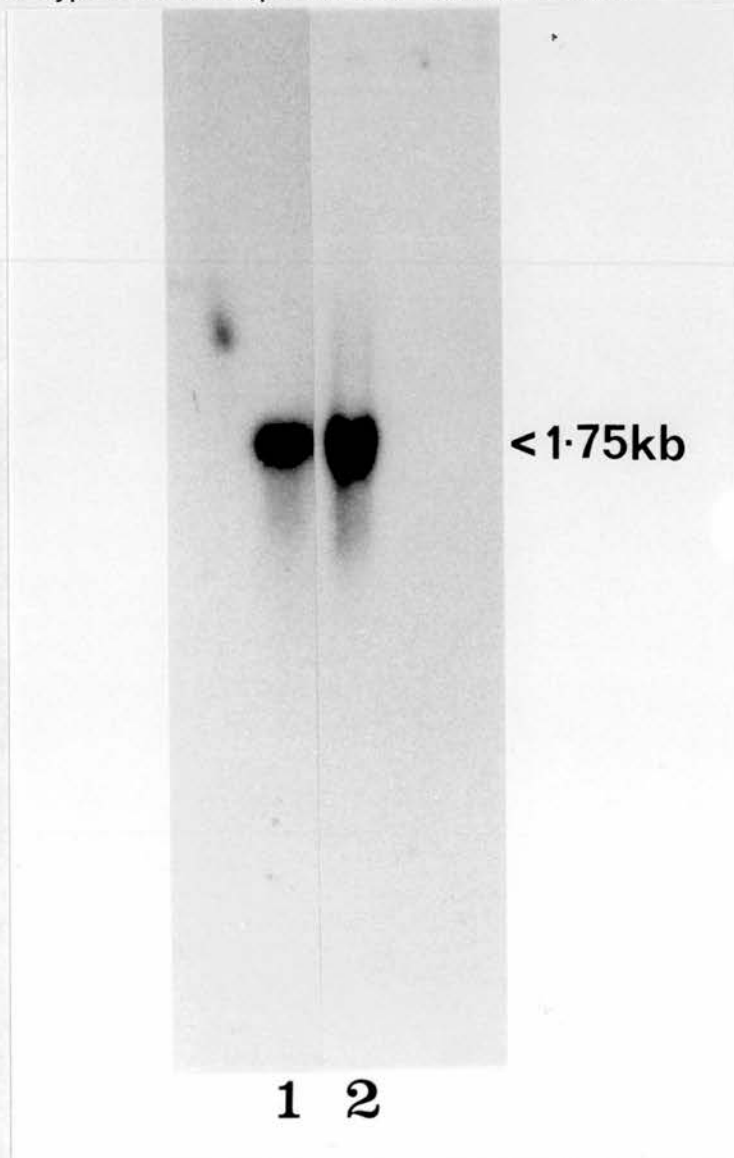


Constitutive expression of Cyp2a protein in C3H10T1/2 cells. There is granular cytoplasmic immunostaining visible in cells grown as a monolayer and fixed in methanol/acetone (50 : 50)

4.5.3 Northern blot analysis

Northern blot analysis was carried out on mRNA isolated from C3H10T1/2 cells, using a full-length cDNA probe specific for the CYP2A subfamily (Figure 4.5.3). A single mRNA transcript (1.75kb) was found to cross-hybridise with this probe. This mRNA species had the same mobility as mouse liver Cyp2a mRNA. Probes for the other P450 families were not found to cross-hybridise.

Figure 4.5.3 Cyp2a mRNA expression in C3H10T1/2 cells



Track 1 contains mouse liver RNA (10µg), and track 2 RNA isolated from C3H10T1/2 cells (20µg). The size of the cross-hybridising transcript (1.75kb) was determined from a marker track (not shown) containing RNA species with a range of molecular weights.

These data strongly suggest that the protein expressed in the C3H10T1/2 cell line is a member of the Cyp2a subfamily. This protein however, appears to be distinct from Cyp2a-5, the isozyme responsible for coumarin hydroxylase activity in the mouse, which has a molecular weight of 48kD and is inducible by pyrazole. A polyclonal antibody specific for Cyp2a-5 (Lang *et al.*, 1989) did not cross-react with the P450 expressed in this cell line, and the level of protein expression was not affected by pyrazole treatment. The precise identity of this expressed protein has yet to be determined, but it is hoped that reverse transcriptase PCR on the isolated RNA will lead to sufficient sequence information to determine whether the protein is indeed a member of the Cyp2a subfamily.

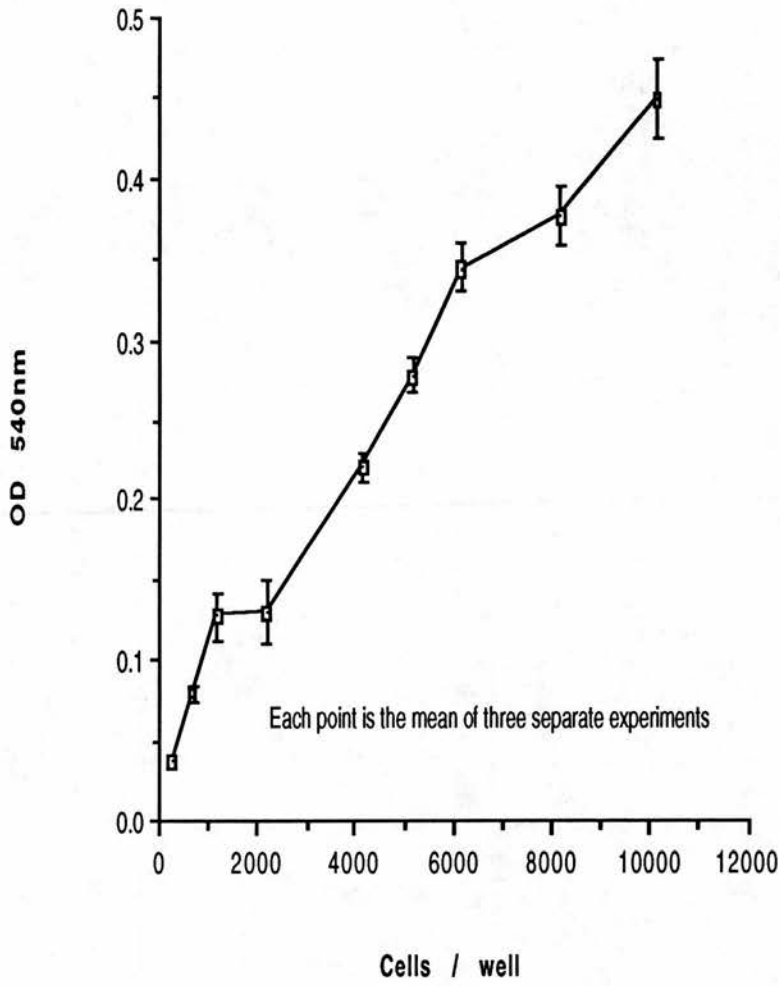
4.6 Xenobiotic regulation of P450 expression in mammalian cell lines

4.6.1 Assessment of cytotoxicity

The first step in the assessment of P450 induction by TCPOBOP, or any of the other compounds tested, was to determine the inherent cytotoxicity of the test compound on the candidate cell line, in order that the maximum non-toxic dose of inducing agent could be administered. This was assessed using the MTT assay. Experimental conditions were optimised by constructing a growth curve for each cell line used before drug treatment was begun. It was important to ensure that the cells were in logarithmic phase at the time of exposure to drug i.e. not so sparse that they could not tolerate drug treatment, yet not so dense that they would reach confluency before the end of the assay, thus inhibiting cellular metabolic reactions.

Cells were plated in a microtitre plate at a range of concentrations and incubated for 4 days, without re-feeding, to mimic the conditions of the MTT assay. 20µl of serum-free media was added to each well on Day 2 to represent the addition of drug. On Day 5, 50µl of MTT was added to each well and the absorbances corresponding to the formation of blue formazan crystals measured at 540nm and plotted as a function of cell density. A representative growth curve for the C3H10T1/2 cell line is shown in Figure 4.6.1.

Figure 4.6.1 C3H10T1/2 cells growth curve



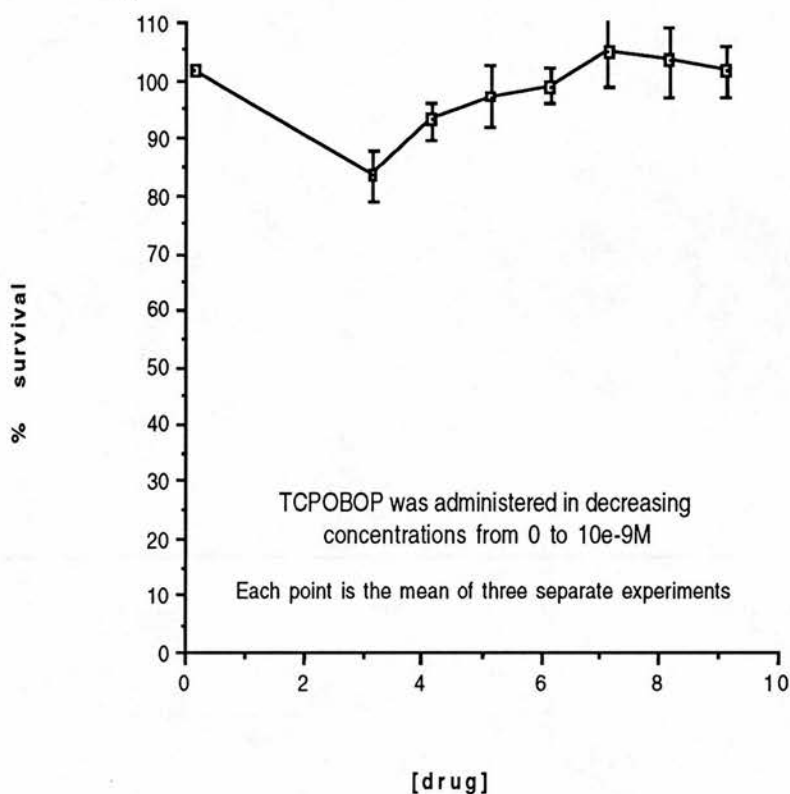
The relationship between cell density and OD₅₄₀ approximated to linearity over the range of cell densities studied, indicating that the cells were metabolically active at each concentration. On visual inspection of the plates, however, the cells plated at higher densities had reached confluency before the end of the assay and were therefore excluded from further studies. The cell density chosen for each MTT assay was a function of the growth rate of each individual cell line. Plating densities for each cell line tested are summarised in Table 4.1.

Table 4.1: Cell plating densities for the MTT assay

<u>Cell line</u>	<u>Plating density</u>
Hepa-1	2×10^3 cells/well
Hep G2	10^3 cells/well
MCF7	10^3 cells/well
HT29	10^4 cells/well
C3H10T1/2	10^3 cells/well

To assess the toxicity of TCPOBOP, cells were treated with the drug at a range of concentrations. Stock drug was prepared in DMSO at 10^{-2} M. This was the maximum solubility which could be achieved in any of the organic solvents or solvent combinations tested. When the drug was added to aqueous media, however, re-crystallisation resulted in the deposition of a fine layer of TCPOBOP crystals on the cell monolayer. The maximum possible solubility in aqueous media was found to be 10^{-5} M, and this concentration was therefore used in all subsequent experiments. At this concentration, no toxicity was observed to any of the cell lines tested. As an example, the effect of TCPOBOP on C3H10T1/2 cells is shown in Figure 4.6.2

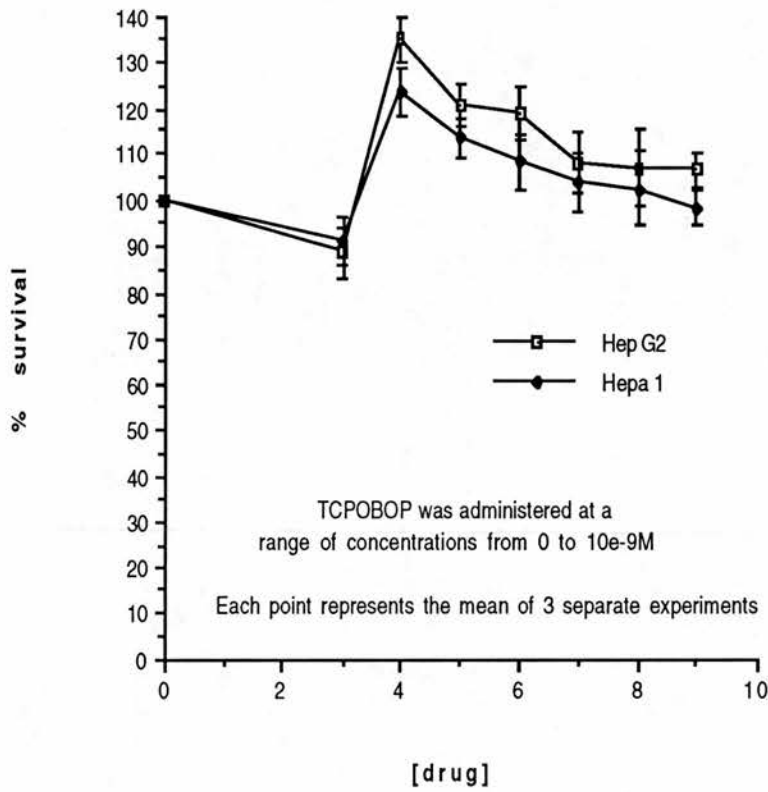
Figure 4.6.2 TCPOBOP administration to C3H10T1/2 cells



At the highest administered concentrations, only slight toxicity (~90% survival) was observed, but this slight decrease in cell survival was also noted on the addition of solvent alone. As the drug began to re-crystallise almost immediately at this concentration, however, it was not possible to accurately determine the actual concentration of drug to which the cells were exposed.

When TCPOBOP was administered to cultured liver cells, marked cellular proliferation was noted, even at low doses of the drug (Figure 4.6.3).

Figure 4.6.3 TCPOBOP causes cell proliferation in hepatoma cell lines

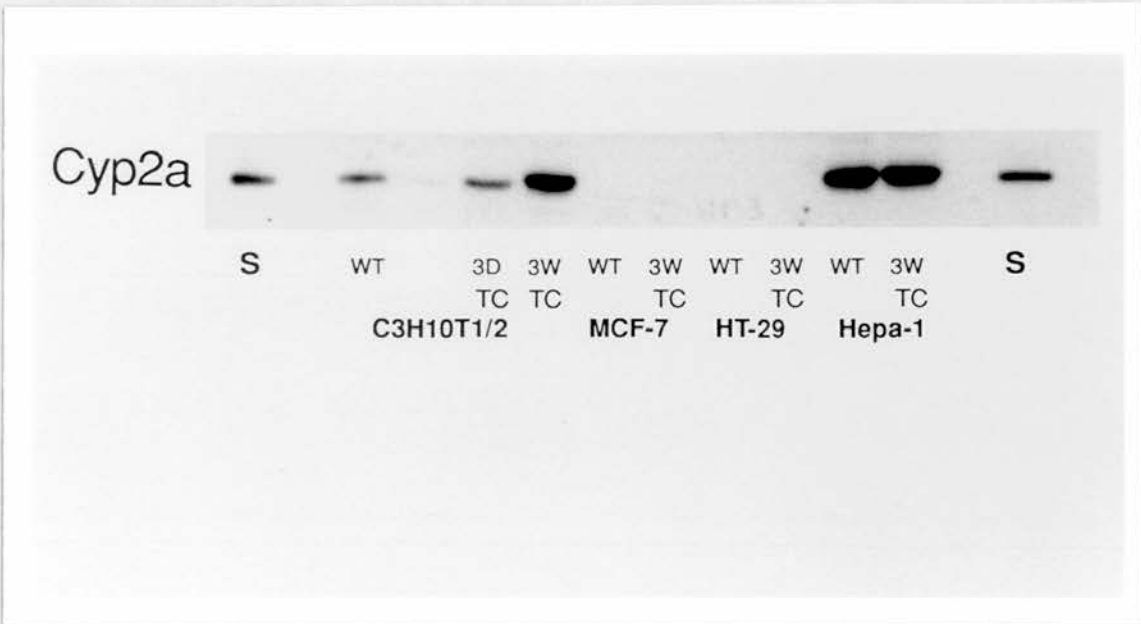


The effect was particularly pronounced in the Hep G2 cell line, and was not observed in non-liver derived lines. These findings are in agreement with the proliferative effects of TCPOBOP discussed in Chapters 3 and 6.

4.6.2 Induction of P450 expression by TCPOBOP

TCPOBOP administration ($10^{-5}M$) to C3H10T1/2 cells resulted in induction of the constitutively expressed "Cyp2a" protein. Hepa-1 cells also expressed a protein with the same molecular weight and immunological cross-reactivity but this protein was not inducible by TCPOBOP (Figure 4.6.4). Dexamethasone and phenobarbital had no effect on Cyp2a expression in these cell lines. Hep G2, MCF7 and HT29 did not have detectable constitutive or inducible Cyp2a expression. Constitutive Cyp2b expression was not detectable in any of the cell lines, and was not inducible on treatment with dexamethasone, phenobarbital or TCPOBOP. In contrast to a previous report (Kärenlampi *et al*, 1991), phenobarbital induction of Cyp1a1 expression in Hepa-1 cells was not observed.

Figure 4.6.4 Cyp2a expression in cell lines - effects of TCPOBOP

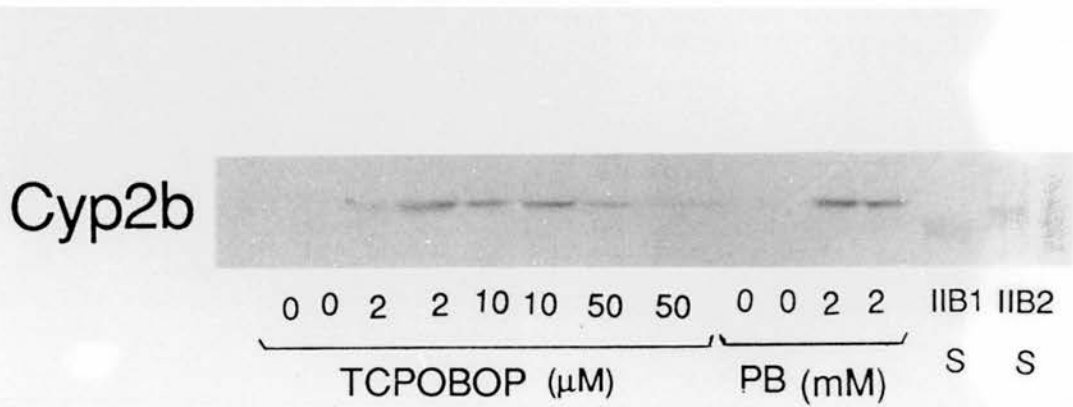


Constitutive (WT) Cyp2a expression was determined in four cell lines - C3H10T1/2, MCF-7, HT-29 and Hepa-1 and following TCPOBOP treatment ($10^{-5}M$) for 3 days (3D) and 3 weeks (3W)

4.7 TCPOBOP effects in primary culture

Hepatocytes were isolated from adult male Alderley Park Swiss mice (20-25g) by *in situ* perfusion of the liver with collagenase as described by Mitchell *et al* (1989). Phenobarbital (PB) dissolved in 0.9% NaCl and TCPOBOP dissolved in dimethylformamide (DMF) were added to the medium and hepatocytes exposed for 3 days to 2mM PB; 2, 10, or 50mM TCPOBOP or vehicle alone (0.9% NaCl or 0.25% DMF). Observation under phase contrast microscope showed the presence of few or numerous crystal needles in the culture medium containing 10 and 50mM TCPOBOP respectively, indicating the limit of solubility of this compound in CL15 medium. Phenobarbital was soluble at all the concentrations tested. Western blot analysis with Cyp2b antisera demonstrated that both PB and TCPOBOP treatment lead to an increase in the expression of Cyp2b protein. A single band was detected which had the same mobility as mouse liver Cyp2b protein (Figure 4.7).

Figure 4.7 The effect of PB and TCPOBOP on Cyp2b expression in mouse hepatocytes in primary culture



A primary monolayer culture of hepatocytes was established by collagenase perfusion of the livers of Alderley Park Swiss mice. The cells were treated with either 2mM phenobarbital (PB), TCPOBOP (2, 10 or 50mM) or vehicle alone (0.9% NaCl, 0.25% DMF). Cell sonicates were run in duplicate on SDS-PAGE using a 9% separating gel and Cyp2b isozyme content determined by Western blot analysis. IIB1 and IIB2 are rat CYP2B1 and CYP2B2 standards.

4.8 Conclusions

The experiments described in this section illustrate the limitations of cell culture as a model for studying P450 expression and regulation. Although primary culture models can often support inducible P450 expression, there are often many significant differences in the expression and regulation of P450 genes in culture and *in vivo*. TCPOBOP, which has been shown to be both a potent and pleiotropic inducer of murine hepatic P450 expression proved to be relatively ineffective in influencing P450 expression in cell lines. An exception to this, however, was the cell line C3H10T1/2, which has constitutive P450 expression and which was inducible on exposure of the cells to TCPOBOP. This, together with the other experiments described in this chapter, demonstrated that it was possible to induce only constitutively expressed P450 proteins in cultured cell lines, suggesting that those cell lines which did not express P450 constitutively had lost the inherent capacity to do so. The precise identity of the P450 expressed in C3H10T1/2 cells has yet to be determined, but our results strongly suggest that it is a member of the Cyp2a subfamily and may represent a novel protein. Experiments are currently underway to clone and sequence this gene from reverse transcriptase PCR amplification of RNA from cells treated with TCPOBOP. GST expression, which has been shown to be relatively unaffected by the culture environment, was also influenced by TCPOBOP in this cell line (not shown).

There have been many reports that cells in culture lose their differentiated phenotype very quickly (Grant *et al*, 1987), with a concomitant loss of tissue-specific functions. This is particularly true when a particular cell type is immortalised as an established cell line. The demonstration by Antequara *et al*, (1990) of methylation of CpG islands in genes which are not necessary for cell survival fits well with the loss of functional P450 expression.

Some valuable information can, however, be gained from these models. Transfection experiments have been widely used to study in isolation the regulation of and the range of substrates metabolised by a particular gene. Battula *et al* (1987) were the first to report the vaccinia virus controlled expression of mouse P₁-P450 and P₃-P450 (Cyp1a-1 and Cyp1a-2). These workers demonstrated that the transfected clones were translocated to the microsomal membranes, complexed with heme and adopted a configuration suitable for interaction with NADPH and reductase. As a result, functional protein was produced. Similar experiments have lead to the stable expression of a number of P450 genes. Reproducibility between experiments is good, particularly when a large stock of DNA coding for the gene of interest is

prepared and used in each successive experiment. Culture conditions (number of cells plated, media composition, etc.) can be precisely defined and transfection efficiency monitored by co-transfection with a reporter gene such as luciferase. Hansen et al (1989) transfected the rat CYP2B1 gene, the major phenobarbital inducible form, into C3H10T1/2 cells which have no constitutive Cyp2b expression. The activity of 2-acetyl amino fluorine (2-AAF) which requires metabolic activation by CYP2B1 was markedly increased in the transfected cells compared to the wild-type controls. Additionally, Northern blot analysis using the rat CYP2B1 cDNA as a probe, showed cross-reactivity with the transfected cells only.

These studies are of particular importance in the study of human P450 genes. Aoyama et al (1989 a,b) transfected human CYP1A2 into human hepatoma cells (Hep G2) which had no constitutive expression. This resulted in sufficiently high levels of expression that the P450 could be quantitated spectrophotometrically. Mouse Cyp1a2 was similarly expressed and thus comparisons could be made between the activities of these genes towards a range of substrates. The results obtained illustrated significant differences in the catalytic activities of orthologous P450s from different species. Again, the results of these experiments were highly reproducible - large viral stocks were prepared in each case and stored between experiments. In similar experiments, other workers (Yamano et al, 1989) have studied the regulation of NADPH-P450 oxidoreductase.

Although these and similar experiments have proved to be a useful model with which to determine the in vitro function of a particular P450 gene, it is important to realise that the in vivo role may be significantly different, and may be masked altogether by the activities of many other genes which are constitutively expressed at higher levels. Gene expression in vivo is controlled by a complex network of hormones and other metabolic factors which it is not possible to mimic in a cell culture model. In addition, isolated transfection experiments involve the expression of a single clone isolated from one individual or mouse and thus make no allowance for any inter-individual variability in the levels of expression or activity of the transfected gene. Genetic polymorphisms, a relatively common event in the genes coding for the drug metabolising enzymes, are also unlikely to be detected using this approach. These experiments have, however, proved invaluable in determining the role of a particular human P450 gene, without competitive binding or metabolic modification of the substrate by another P450 isozyme, which is often a complication in similar studies in animal models.

P450 expression can be maintained much more successfully in primary

cultures. This is of limited value, however, in the study of human gene expression, as human liver samples are not easily obtained. When it does become possible to obtain human tissue, the enzyme profiles within the tissue are often distorted by the disease state of the patient and it is virtually impossible to obtain true "control" samples. Primary cultures of rodent hepatocytes have, however, been widely used to study P450 expression. Long-term induction experiments are, of course, not possible in this system, as the life span of the cells defines the maximum length of any experiment. Indeed, both phenobarbital and TCPOBOP induced Cyp2b expression in mouse hepatocytes demonstrating that, although the action of these compounds may be influenced in vivo by factors such as pituitary hormones, both can exert a direct effect on hepatic gene expression. Although the considerable variability in gene expression in primary cultures created from different animals makes standardisation of experimental conditions difficult, it is a better representation of the in vivo situation than that achieved using established cell lines.

In conclusion, therefore, the regulation of P450 genes may be studied to a limited extent in cultured cells, although significant differences from the in vivo patterns of gene expression are often observed. It is therefore important to validate the results obtained from a cell culture model, wherever possible, by in vivo studies in the intact animal.

Chapter 5: Regulation of cytochrome P450 expression in human tumour xenografts

5.1 Introduction

Due in part to the limited availability of tissue for study, it is extremely difficult to identify the factors which regulate the expression of human cytochrome P450 genes, particularly in extra-hepatic tissues. As many anti-cancer drugs are dependent on P450-mediated activation to exert their anti-tumour effects or, alternatively, are metabolised to a less active form by cytochrome P450, it is clearly important to identify the factors which control the *in vivo* expression and regulation of this ubiquitous class of enzymes in man. The P450 system is thought to have evolved as an adaptive response to environmental insult and it therefore seems inherently unlikely that the defence systems, and thus P450 expression, of any two different organisms e.g. rat and man would be identical. Significant differences in the expression and regulation of P450 genes between species are indeed observed. It is therefore difficult to extrapolate the results obtained in one animal model to another. As discussed in the previous chapter, the use of cells in culture as a model to study P450 regulation is also of limited value, and it is therefore important to establish an *in vivo* model where the expression and regulation of human P450 genes can be assessed.

In this chapter, the development of a model system to address this problem is described, involving the use of human tumours grown as xenografts in immune deficient mice. This model has a number of applications:

- (a) Constitutive tumour P450 levels can be measured directly - the intra-tumour level of P450s involved in drug activation is more relevant than the hepatic concentration in terms of determining local concentrations of active drug
- (b) A wide range of tumour types can be analysed
- (c) A large number of tumours from the same tissue can be analysed simultaneously, thus providing information on any inter-individual variation in enzyme expression

5.2 The xenograft model

The xenograft model, where human tumours are implanted sub-cutaneously in the flanks of immune deficient mice and continue to grow *in situ*, has been widely used to determine the response of human tumours to therapy (Berger *et al*, 1991). A number of distinct tumour types and a wide variety of chemotherapy protocols have been assessed using this model. Inter-individual variations in response can be

evaluated by establishing a panel of xenograft tumours from the same tissue type, while the onset of drug resistance can be monitored by assessing the tumours before and after drug treatment.

Previous studies to evaluate the anti-tumour effects of new drugs or drug combinations involved the use of transplantable animal tumours such as the Walker 256 rat carcinosarcoma or murine L1210 leukaemia. These tumours, however, bore only a very vague histological and kinetic resemblance to the behaviour of human tumours (Mihian *et al*, 1974; Cobb & Mitchely, 1974; Steel, 1978).

Early studies using human tumour xenografts investigated whether these tumours retained human characteristics and behaviour after serial passage in mice. The results were very encouraging and related to a large variety of tumour types. Berenbaum *et al* (1974) transplanted 116 tumours comprising melanoma, ovarian, bladder, gastrointestinal, breast and uterine carcinoma into thymectomised, irradiated and anti-lymphocyte serum-treated mice and obtained established xenograft tumours from each tumour type. The histological appearance and characteristics of each tumour were retained in every case examined, as were differentiated functions such as mucin and keratin production (Shimosata *et al*, 1976). In addition, the response of these tumours to chemotherapy showed the same heterogeneity in response as was observed in the donor patients. Selby *et al* (1980) carried out a comprehensive study of a panel of xenograft tumours by histology, histochemistry, electron microscopy, chromosome analysis, immune fluorescence and measurement of growth rate and mitotic counts, and reported that, in all cases, the characteristics of the tumour of origin were retained. In addition, the chemotherapeutic response of a series of patients with bronchial carcinoma was compared to the response of the same tumours established as xenografts in immune suppressed mice (Shorthouse *et al*, 1980). Bronchial carcinoma was chosen as it demonstrates a comparatively wide spectrum of clinical response to chemotherapy. Twenty one distinct responses in sixteen individual tumours were very similar. These workers, however, emphasised that, due to the time required first to establish the tumour as a xenograft and to carry out subsequent drug testing, the xenograft model is of limited applicability in predicting appropriate chemotherapy for individual patients, although it is of great benefit in screening potential new therapeutic agents. This model has also been used to determine the response of human tumours to radiotherapy. For example, Rofstad (1992) demonstrated that the heterogeneous radiation sensitivity of a series of xenograft melanoma tumours were representative of the original tumour response.

More recent studies have concentrated on establishing xenograft tumours representative of a particular disease state, such as malignant mesothelioma (Chahinian *et al*, 1991) or cervical carcinoma (Han *et al*, 1991) and to assess the response of these tumours to a particular treatment regimen. Nio *et al* (1991) described the effects of co-administration of several independently effective anticancer drugs such as cisplatin and 5-fluorouracil to a number of digestive organ xenografts. These solid tumours are notoriously difficult to treat as they are often inherently resistant to chemotherapy. The co-administration of anticancer drugs in most cases, however, did not lead to an enhanced anti-tumour effect and often lead to increased side effects. Satta *et al* (1991) described the establishment of multidrug resistant gastric and colon xenografts by intra-tumoral injections of adriamycin. Systemic (intra-peritoneal) injections did not, however, lead to establishment of the MDR phenotype. In addition, an elegant study (Pearson *et al*, 1991) demonstrated the reversal of drug resistance in a MDR-expressing human colon cancer xenograft by administration of the anti P-glycoprotein monoclonal antibody MRK-16. In a similar study (Rittmann-Grauer *et al*, 1992), human melanoma xenografts were treated with a series of monoclonal antibodies to P-glycoprotein, resulting in the reversal of resistance to the Vinca alkaloids actinomycin D, vincristine and vinblastine. This approach, using monoclonal antibodies to chemosensitise previously drug resistant tumours is particularly attractive. The antibodies used are highly specific for tumour cells and thus produce fewer side effects than other small molecule modulators of MDR expression. In addition, this specificity leads to a longer residence time at the tumour site and a longer serum half-life. Resistance to adriamycin, however, was not reversed by antibody administration, suggesting that monoclonal antibody/P-glycoprotein interaction may result in a minor conformational change which affects the transport of certain drugs but not others.

Adriamycin sensitivity was determined in a series of xenograft tumours of mixed origin and the response to drug administration correlated with the expression of MDR, GST II and topoisomerase II, the expression of which has been implicated in drug resistance (Kim *et al*, 1992). These workers demonstrated that there was a significant relationship between the level of Topo II mRNA expression and tumour sensitivity to adriamycin. In contrast, no relationship was apparent between the levels of MDR and GST II expression and adriamycin sensitivity. There has been considerable controversy in recent literature over the level of expression of these genes within human tumours and their clinical response to chemotherapy (Ball *et al*, 1990; Moscow *et al*, 1989; Samuels *et al*, 1991).

For a number of reasons, tumours can undergo phenotypic change and hence have altered drug sensitivities when removed from source. Many tumours are composed of heterogeneous cell populations, the biological properties of which can vary widely. When challenged by a new environment e.g. when grown as a xenograft, selective pressures may lead to the dominance of one of the subpopulations (Heppner, 1974). Tumour cells are often inherently genetically unstable, and thus extensive genotypic and phenotypic changes can occur during growth in a new environment. In addition, drug sensitivity is influenced by cell cycle and the kinetics and distribution observed *in situ* may not necessarily be reproducible in a xenograft model. To address this problem, McQueen *et al* (1991) examined the stability of several genetic lesions in a series of human colorectal carcinoma xenografts. Cell lines established from the same source commonly acquired genetic rearrangements during culture (Brattain *et al*, 1983; Park *et al*, 1987). Haematoxylin and Eosin (H & E) stained paraffin sections of both primary tumour and xenografts, however, showed that the original histological pattern for each primary tumour was conserved through serial passage. In addition, the xenograft tumours had individual but stable expression of several of the oncosuppressor genes thought to be important in the initiation of colorectal cancer.

In summary, therefore, the xenograft model has been validated in the study of the response of human tumours to drug treatment. There are no reports, however, of the mechanisms of drug action in these xenograft tumours. The intra-tumour levels of expression of drug metabolising enzymes is central to the efficacy of many administered drugs which require *in situ* bioactivation to exert their anti-tumour effects. In this chapter, the intra-tumour expression of one of the most important of these enzyme systems, cytochrome P450, is investigated and the modulation of the levels of expression of these enzymes by the administration of a range of xenobiotic inducing agents described.

5.3 Characterisation of human P450 expression

Characterisation of human P450 gene expression, particularly in extra-hepatic tissues, has been greatly hampered by the lack of availability of tissue for analysis. Many of the hepatic genes which have been isolated have been identified by screening human hepatic libraries with cDNA or antibody probes to the analogous gene families in mouse and rat. Although this approach is very useful in determining the presence (or absence) of a homologous human gene in a particular tissue, it provides no information about the *in vivo* regulation of gene expression. Traditional

purification procedures are subject to the same limitations. As discussed in Chapter 4, cell culture models have limited applicability to the study of P450 gene regulation, although recent reports describe primary human hepatocytes cultured on the basement membrane "Matrigel", in which P450 expression can be modulated by foreign compounds (Burger *et al.*, 1992). Whether these experiments are physiologically relevant has yet to be determined. Vaccinia virus mediated expression of human P450 cDNAs in mammalian cells has, however, proved to be a convenient and reproducible way to study the function of these genes (Battula *et al.*, 1987; Estabrook *et al.*, 1991). This approach is particularly applicable to the isolation of low abundance proteins.

There are many similarities, but also some significant differences in the regulation and substrate inducibility of P450 genes between rodents and man (Gonzalez, 1990). For example, many of the sex differences observed in the expression of several P450 genes in the rat are not seen in the corresponding human genes. In addition, a number of the human P450 genes exhibit a genetic polymorphism in their expression, which is not seen in the corresponding animal models. These will be described more fully in Section 5.3.1.

The human P450 genes which have been characterised to date are summarised in Table 5.1. Current knowledge on the regulation of the individual P450 isozymes is summarised below:

CYP1A: To date, two members of the CYP1A family have been reported, CYP1A1 and CYP1A2. Members of the CYP1A subfamily are characterised by their inducibility following exposure to polycyclic aromatic hydrocarbons (Gonzalez, 1990), mediated by the inducing agent binding to a cytosolic protein, the "Ah receptor". CYP1A1 has not, as yet, been purified from human tissues. Screening human tissue libraries with a cDNA probe to the rodent CYP1A gene, however, yielded a 512 amino acid protein which, although detectable in lung (McLemore *et al.*, 1990; Omiecinski *et al.*, 1990), lymphocytes (Jaiswal *et al.*, 1985; Song *et al.*, 1985) and placenta (Song *et al.*, 1985) was undetectable in human liver. The expression of CYP1A1 mRNA in the lungs of cigarette smokers has been correlated with lung cancer incidence (McLemore *et al.*, 1990; Antilla *et al.*, 1991). Regulation of human CYP1A1 expression seems to be very closely related to the mechanism of regulation in rodents, where the presence of two cis-acting DNA elements is required (Fujisawa-Sehara *et al.*, 1986, 1987, 1988; Yanagida *et al.*, 1990). One of these, the inducible

enhancer XRE (xenobiotic response element) is located upstream of the gene, while the other, the BTE (basic transcription element) is found close to the TATA box and acts as a promoter element.

In contrast, CYP1A2 is highly expressed in human liver. The gene is 68% homologous (amino acid sequence) to CYP1A1 and has a higher molecular weight. This is in contrast to the situation in the rodent where CYP1A1 is the larger gene. Large inter-individual variations have been reported in the expression of CYP1A2 (up to 10-fold), but no genetic polymorphisms have yet been identified in the expression of this gene which could link the level of expression to cancer susceptibility. Regulation of CYP1A2 expression has been shown to occur both by transcriptional activation of the gene and mRNA stabilisation (Pasco *et al*, 1988).

CYP2A: The CYP2A gene cluster in man is tightly linked to CYP2B on chromosome 19 (Miles *et al*, 1988). Southern blot analysis of CYP2A revealed the presence of 2/3 genes (Miles *et al*, 1989). The most fully characterised of the genes, CYP2A6, has been identified as the human coumarin hydroxylase (Miles *et al*, 1990, Yamano *et al*, 1990), the human orthologue of the murine Cyp2a-5 gene (Lindberg & Negishi, 1989). Crespi *et al* (1990) reported that CYP2A6 was active in the metabolism of Aflatoxin B1, benzo(a)pyrene, N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) to mutagenic and cytotoxic products. CYP2A7, which has 94% amino acid homology with CYP2A6 (Davies *et al*, 1989) has been less well characterised. Recently, however, a full-length CYP2A7 clone was isolated from a human genomic cosmid library with a probe comprising a 0.7kB fragment of human CYP2A6 cDNA (Ding *et al*, unpublished observations). A core glucocorticoid-response sequence was identified in the 5'-flanking region of this gene.

CYP2B: Miles *et al* (1988) isolated 2 distinct CYP2B clones from a human liver cDNA library, using a cDNA probe to the rat CYP2B1 gene. On further characterisation, however, these clones were found to be alternatively spliced variants of the same gene, designated CYP2B6. These workers reported that there was considerable inter-individual variation in the level of expression of both the functional gene and its alternatively spliced variants. Yamano (1989) isolated a further CYP2B cDNA clone, CYP2B7, which had only 3 nucleotide and 1 amino acid differences from CYP2B6.

CYP2C: This gene family is thought to consist of at least 3 genes, CYP2C8, CYP2C9

and CYP2C10, all of which have been partially sequenced. The genes are thought to have individual but overlapping substrate specificities. The expression of CYP2C9 is genetically polymorphic, resulting in certain individuals having reduced ability to metabolise mephenytoin (see Section 5.3.1). This polymorphism is particularly prevalent in the Japanese population.

CYP2D: Distlerath et al (1985) isolated a P450 from human liver with an apparent molecular weight of 51,000 and a high catalytic activity towards debrisoquine and other drugs involved in the debrisoquine/sparteine polymorphism (see Section 5.3.1). This was presumably the same protein described by Gonzalez et al (1988) as CYP2D6, the human debrisoquine 4-hydroxylase. This enzyme is involved in the metabolism of many clinically important drugs, such as propranolol and dextromethorphan (Eichelbaum & Gross, 1990; Meyer et al, 1990). Kimura et al (1989) identified two further genes, CYP2D7 and CYP2D8, but both had mutations resulting in their inactivation.

CYP2E: The CYP2E family in humans is represented by a single gene (Song et al, 1986) which encodes a 493 amino acid protein. CYP2E metabolises and is induced by ethanol administration, catalysing the oxidation of ethanol to acetaldehyde. It is also inducible by a range of other substrates such as N-nitrosodimethylamine (NDMA), aniline and acetone. NDMA, which occurs as an environmental pollutant as well as in food, drink and cigarette smoke, is a potent rodent carcinogen which has also been implicated in human carcinogenesis. Again, variable levels of CYP2E1 mRNA and protein have been reported in a panel of human livers.

CYP2F: A cDNA encoding CYP2F1 and an alternatively spliced variant were isolated from a human lung library by screening with a cDNA probe to human CYP2C9 (Nhamburo et al, 1990). The gene has been localised to human chromosome 19. Little is known at present about the regulation of this gene family.

CYP3A: At least three CYP3A proteins have been identified in adult human liver, CYP3A3, CYP3A4 and CYP3A5. CYP3A4 is usually expressed at the highest level and there is considerable inter-individual variation in the expression of CYP3A3. A further CYP3A protein, CYP3A7 has recently been isolated from human foetal liver (Kitamura et al, 1992). The CYP3A7 cDNA was shown to be highly homologous to the cDNA for CYP3A4, isolated from an adult human liver cDNA library. Rifampicin,

barbiturates and glucocorticoids are all potent inducers of CYP3A gene expression.

CYP4A: Human CYP4A gene expression has been relatively poorly characterised to date. A recent report, however, described the isolation of a partial CYP4A clone from screening a human genomic cDNA library with a full-length rat cDNA probe (Hood *et al.*, manuscript in preparation). Partial sequencing of this clone demonstrated that it had 85% nucleotide homology with the rat form, but that homology was confined to the heme binding region.

Table 5.1 Classification of human P450 genes

Gene family	No. of genes	Chromosomal location	Regulated by
CYP1A	2	15q 22-qter	polycyclic aromatic hydrocarbons dioxins, isosafrole
CYP2A	2-3	19q 13.1-13.3	pyrazole, ethanol, hormones
CYP2B	2-3	19q 13.1-13.3	phenobarbital, dexamethasone, polychlorinated biphenyls
CYP2C	3-10	10q 24.1-24.3	hormones, phenobarbital
CYP2D	3-4	22q 11.2-qter	unknown
CYP2E	1	10	ethanol, diabetes, starvation, pyrazole, solvents
CYP2F	1-2	19	unknown
CYP3A	3-5	7q 21.3-q22	hormones
CYP4A	(2-4)	1	phenobarbital, clofibric acid
CYP4B	?	-	

Adapted from Wolf, 1990

The examples of regulating agents are illustrative only, and are by no means comprehensive. Individual members of a gene family or subfamily can be regulated differently and not all members within a gene family respond to the same inducing agents.

5.3.1 Genetic polymorphisms in human P450 expression

Genetic differences in the expression of carcinogen metabolising enzymes has long been thought to account, in part at least, for individual susceptibility to cancers attributable to environmental carcinogens. Several human P450 genes exhibit genetic polymorphisms in their expression which severely compromises the ability of certain individuals to metabolise a wide range of drugs.

Spurr et al (1987) demonstrated a polymorphism in the 3' flanking region of the CYP1A1 gene with the restriction endonuclease Msp1. Attempts have been made to link this polymorphism to susceptibility to lung cancer (Kawajira et al, 1991), but the results are equivocal and must be considered in terms of individual tumour types. Similarly, there is a polymorphism in the 5' flanking region of the CYP2E1 gene with Rsa1 (Watanabe et al, 1990). DNase 1 footprint analysis and gel retardation assays demonstrated that the polymorphic region interacts with a transcription factor, probably HNF-1, which leads to differential transcriptional regulation of the gene.

The best characterised P450 polymorphism, which affects between 5 and 10% of the Caucasian population, is that found in the human CYP2D6 debrisoquine hydroxylase gene. Affected individuals (poor metabolisers, PMs) have a compromised ability to metabolise a range of therapeutic drugs including debrisoquine, propranolol and dextromethorphan, leading to unpleasant side effects and, in extreme cases, death (Mahgoub et al, 1977). Three gene inactivating mutations have been identified in the CYP2D6 gene (Gonzalez et al, 1988; Gough et al, 1990): (a) a G-A transition at the intron 3/exon 4 junction; (b) deletion of the entire CYP2D6 gene and (c) a base pair deletion in Exon 5. A DNA-based assay has recently been developed (Gough et al, 1990) to unequivocally identify these defects and thus identify individuals with the poor metaboliser phenotype.

The polymorphic expression of CYP2D6 has been linked to the incidence of a number of cancers including lung and bladder cancer, leukaemia and melanoma, although the small number of samples analysed and limitations in the analytical methods used have made the results of many of these studies difficult to interpret. Wolf et al (1992) have recently studied a very large population of cancer patients

and reported that although there appeared to be no link between the polymorphic expression of CYP2D6 and lung cancer, a statistically significant increase in the proportion of poor metabolisers was seen in leukaemia, bladder cancer and melanoma patients. These results imply either a direct involvement of CYP2D6 in carcinogen detoxification or linkage of this gene to another "cancer-causing" gene on human chromosome 22.

The pathogenesis of Parkinson's disease may also be influenced by the polymorphic expression of CYP2D6. A recent study (Smith *et al*, 1992) demonstrated that individuals with the poor metaboliser phenotype were 2.54 times more likely to develop the disease compared to a control population. *In vitro* studies (Fonne-Pfister *et al*, 1987) have shown that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) can be metabolised by CYP2D6. This compound, a contaminant of synthetic meperidine narcotics, can induce Parkinsonism when oxidatively metabolised by the mitochondrial monoamine oxidase to its active form MPP⁺, the 1-methyl-4-phenyl pyridinium ion (Calne *et al*, 1983). This ion is selectively toxic to the substantia nigra. MPP⁺ can also bind to and be metabolised by CYP2D6, the expression of which has been identified in this region of the brain (Nisnik *et al*, 1990).

Other genetic polymorphisms which have been less well characterised exist in the CYP3A4 gene (Nakamura *et al*, 1985) which is responsible for the metabolism of nifedipine and in CYP2C9, which leads to a compromised ability to metabolise mephenytoin (Kupfer & Preseig, 1984). This polymorphism is particularly pronounced in the Japanese population. The expression of CYP2C10, which metabolises tolbutamide, is also genetically polymorphic. The genetic basis for these polymorphisms has not yet been characterised.

5.4 P450 expression and carcinogenesis in human breast and colon tissue

Colorectal cancer remains one of the most common malignancies in Western countries, and is the second commonest cause of cancer deaths in the world. The disease primarily affects the elderly, and incidence has been correlated with diet, smoking and alcohol consumption (Kikendall *et al*, 1989; Whittemore *et al*, 1990). Cytochrome P450-mediated metabolism of the carcinogen dimethylhydrazine has also been shown to increase with age (Newaz *et al*, 1983) and activation of this compound has been implicated in the aetiology of colonic cancer. Although certain dietary components are thought to reduce the risk of developing colon cancer, other

dietary factors as well as cigarette smoke and alcohol can be metabolised to toxic products by the P450 enzymes expressed in the colon.

Colonic carcinoma is extremely difficult to treat with chemotherapy, as many tumours are inherently drug resistant on presentation. A number of possible mechanisms have been proposed for this resistance, including:

(a) High levels of expression of P-glycoprotein in colon tumours. This protein, the product of the MDR1 gene, functions as an energy dependent efflux pump which excludes a wide range of structurally dissimilar drugs from the cell. Recent studies have demonstrated that in mouse liver, TCPOBOP administration can suppress MDR expression (Russell *et al*, manuscript in preparation). Whether this leads to increased drug accumulation and whether the same effect can be observed in human tumour tissue is currently being investigated.

(d) Down regulation of P450 expression. A number of drugs e.g. cyclophosphamide and the novel morpholino-doxorubicin anthracyclines require P450-mediated activation before exerting their anti-tumour effects, and are therefore of limited effectiveness in treating many poorly vascularised tumours where the expression of P450 is low or absent. Inducing the intra-tumour levels of these enzymes by compounds such as TCPOBOP may therefore lead to greater drug efficacy.

There have been several reports in recent literature of cytochrome P450 expression in human colon, but there are many inconsistencies between the findings of different research groups. This can, however, be partly attributed to inter-individual variation in the expression of these enzymes. Stralka & Strobel (1989) studied P450 activity and distribution in normal (i.e non-tumour) human colon mucosa. Activity was assessed by the rate of metabolism of the potent colon-specific carcinogen 1,2-dimethylhydrazine (DMH), the toxicity of which is mediated by the methyl diazonium ion. The involvement of P450 enzymes, particularly CYP2E1, in the metabolism of this chemical was demonstrated by antibody inhibition studies. The ability to metabolise DMH increases with age and carcinogen activation is therefore greater in old people. This is in agreement with the incidence of colon cancer which also increases with age. Peters *et al* (1989) reported that, in the rat, P450 content decreased from duodenum to ileum i.e. from proximal to distal small intestine. In the human, however, more than 50% of large bowel cancers are diagnosed in the distal section, suggesting either that the distribution of P450 enzymes is significantly different in human tissues or, alternatively, that P450 mediated carcinogen activation is not an important risk factor in colon cancer.

A number of studies have used animal models, usually rat, to study P450 expression in the colon. As constitutive expression was found to be very low, however, many animals were pre-treated with an inducing agent before their P450 content was determined, making it difficult to relate the results of these studies to true constitutive expression. Rosenberg (1991a) investigated P450 expression in rats treated with phenobarbital and β -naphthoflavone. Constitutive CYP1A1 expression was detectable in the colon and was inducible by β -NF, while CYP2B1 was detectable in the small intestine but not the colon. Stralka & Strobel (1991) detected CYP2B1, CYP2A1 and P450 reductase protein in rat colon, while de Waziers *et al* (1991) reported that CYP3A4 was the only detectable form in both normal colonic mucosa and colorectal carcinoma, although the level of expression in the tumour was greatly reduced compared to normal tissue. GST Π was, however, significantly increased in tumour tissue, resulting in an altered enzyme balance and therefore response to drug treatment. As constitutive expression in these tissues has been shown to be dependent on diet and is inducible by many dietary components, however, it is of limited relevance to make direct comparisons between the level of P450 expression in human colon and levels of expression in rodent models. Rosenberg (1991b) reported that P450 expression could be affected by components of the animal bedding, while Vang *et al* (1991) reported induction of CYP1A1, 1A2, 2B1, 2B2 and 2E1 by broccoli in rat liver and colon. Although these effects being the indirect result of a pesticide contaminant has not been ruled out, the active inducing agents are thought to be indoles and flavones e.g. 1-methoxy-indole-3-carbaldehyde. In addition, benzanthracene was reported to induce the expression of CYP1A1 and CYP1A2 in the colon carcinoma cell line LS174T (White *et al*, 1991). In the same study, trace amounts of CYP2C9 and CYP2E1 were also detectable, but only by reverse transcriptase polymerase chain reaction amplification of cellular mRNA.

The experiments described in this chapter were designed to investigate the level of expression of individual cytochrome P450 isozymes in a panel of human colon tumours, and describe the regulation of P450 expression by a number of xenobiotic modulators in a human xenograft colonic tumour.

In a detailed study of P450 and GST expression in a panel of normal and tumour breast tissues, Forrester *et al* (1990) demonstrated that P450 expression was very low, with a single P450 isozyme from the CYP2C family the only detectable form on Western blot analysis. This protein had a different mobility from the human hepatic CYP2C isozyme, but was apparently identical to the form expressed in an

ovarian tumour. Immunohistochemical analysis demonstrated that the expression of this protein was very heterogeneous, although strong uniform staining was observed for CYP3A. This isozyme had not been detectable by Western blotting, although these experiments were performed before the advent of the more sensitive ECL detection system. Recent studies have demonstrated that an enzyme within the CYP3A family is responsible for the metabolism of the anti-oestrogen Tamoxifen in human liver microsomes (Jalacot *et al*, 1991). Whether the same isozyme is active within breast tumour tissue remains to be established. Murray *et al* (1991) reported the heterogeneous expression of CYP1A protein in a panel of human breast tumours. Again, however, this was not confirmed by Western blot analysis. The expression of CYP1A protein in the breast carcinoma cell line MCF7, although low, has been correlated with reduced oestrogen receptor status, decreased drug accumulation and overexpression of P-glycoprotein (Vickers, 1989).

Variation in the levels of these proteins, whether by an environmental pollutant or therapeutic drug, alters the cellular response to a toxic challenge. Using a human breast tumour grown as a xenograft as a model, the expression of human tumour P450 proteins has been investigated, before and after xenobiotic administration. Modulation of the intra-tumour levels of certain of these isozymes may be of benefit in tumour targeting anti-cancer drugs which require P450 mediated activation.

5.5 P450 expression in human colorectal mucosa

The expression of individual cytochrome P450 isozymes and P450 reductase was investigated by immunohistochemical analysis in normal and neoplastic colon tissue from a panel of 20 patients. Tumour P450 levels were confirmed in 5 cases by Western blotting.

5.5.1 Immunohistochemical analysis

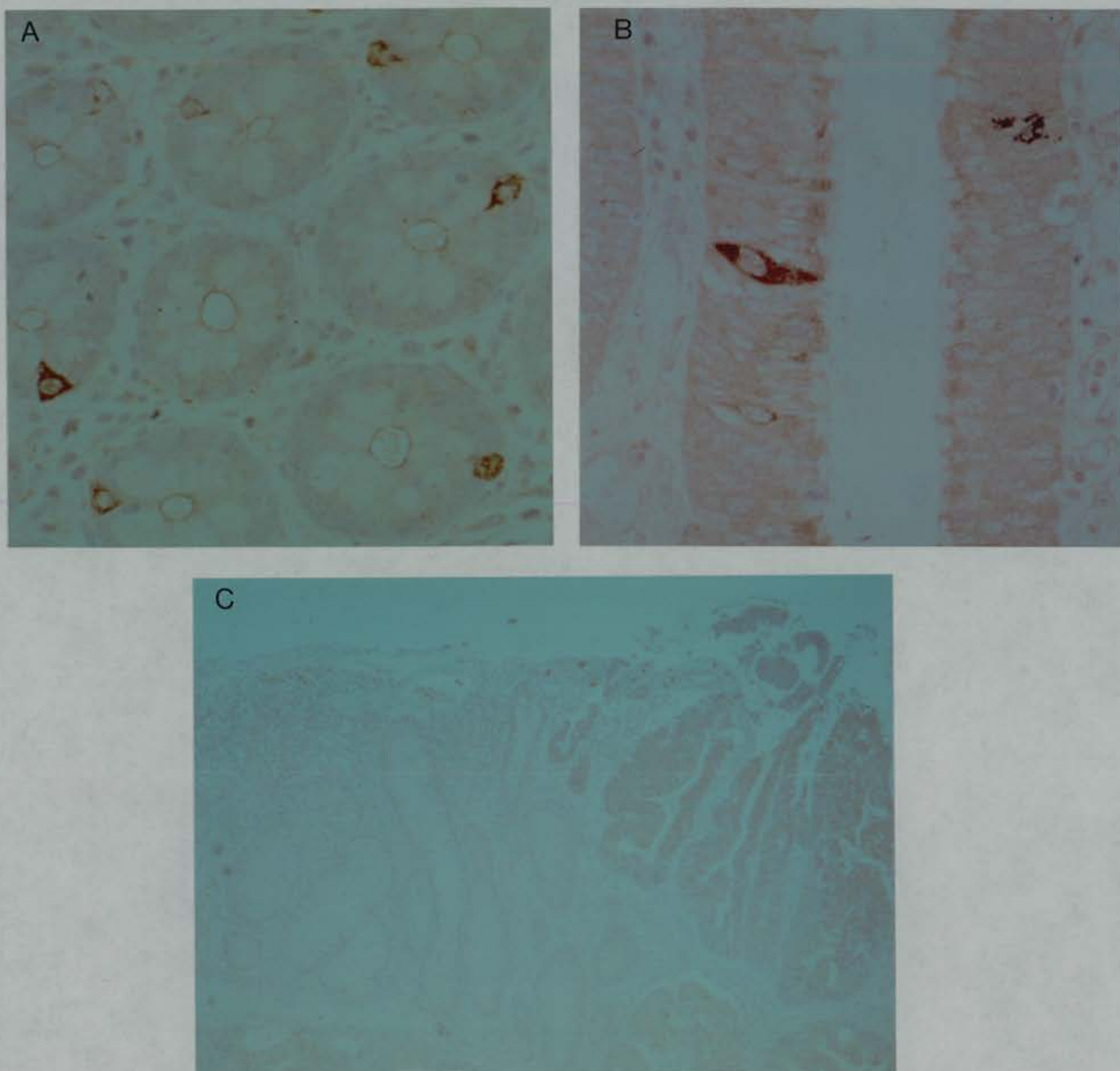
Both normal and tumour tissues had low, but detectable, levels of P450 expression. There was considerable variation in staining intensity between individual cases, although no consistent differences were observed between normal and neoplastic tissue. In addition, there appeared to be no correlation between the site of origin of the tumour and the intensity of P450 staining. Figure 5.5.1 shows the distribution of CYP4A, which was strongly expressed in enterochromaffin cells in normal mucosa and also focally within neoplastic tissue. Distal colon and rectal mucosa, which have the highest incidence of neoplasia, contain more

enterochromaffin cells and therefore a greater local concentration of CYP4A expression. CYP4A has an important endogenous role in eicosanoid metabolism and prostaglandin production (Capdevila *et al*, 1981), suggesting a possible autocrine function for these cells. The presence of CYP4A positive cells in some tumours is consistent with the reported appearance of cells with neuroendocrine differentiation in certain colorectal cancers. Expression of CYP3A, which has also been implicated in carcinogenesis, was found in vascular smooth muscle, but also weakly at the surface in colonic mucosal epithelium. Cytochrome P450 reductase was detected in both normal and neoplastic tissue.

5.5.2 Western blot analysis

Western blot analysis confirmed the results obtained by immunohistochemical staining and highlighted the considerable inter-individual variation between cases. P450 expression was, in general, very low and was only detectable using the sensitive ECL detection system. The expression of P450 isozymes from all of the individual gene families with the exception of CYP2C was, however, detectable (Figure 5.5.2). P450 reductase was present in each of the tumour samples analysed.

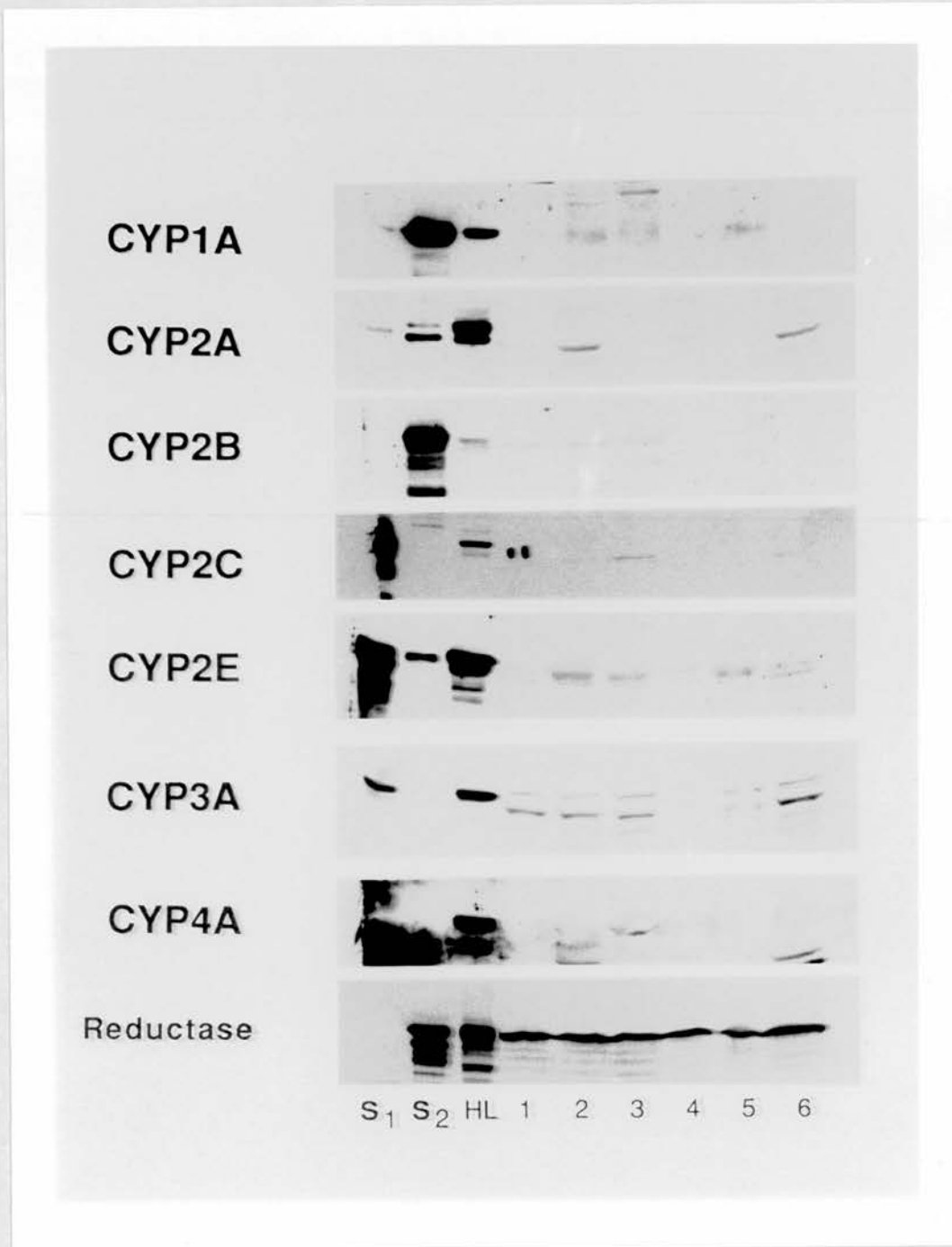
Figure 5.5.1 Immunohistochemical analysis of P450 expression in human colon tissue



Immunohistochemical analysis (A) demonstrated that CYP4A protein expression was restricted to the neuroendocrine cells of colonic crypts. CYP4A positive cells were more numerous in the distal colon (i.e. left side) in keeping with the known distribution of neuroendocrine cells in the gastrointestinal tract. Colonic carcinomas also contained occasional CYP4A positive cells (B).

Normal colon (left) showed very weak expression of CYP3A (C). In some cases, as shown here (right and lower), malignant epithelium in the adenocarcinoma demonstrated increased, but still weak, expression of the gene product.

Figure 5.5.2 P450 isozyme expression in a series of human colon tumours



The level of expression of individual P450 isozymes and of NADPH-cytochrome P450 reductase was determined by Western blotting in a panel of six human colon tumours. 40µg of microsomal protein was loaded per track. HL = human liver microsomes; S₁ and S₂ are purified P450 proteins

5.6 Xenobiotic regulation of P450 expression

5.6.1 Western blot analysis

A variety of compounds, known to induce P450s from a range of gene families or subfamilies in animal models, was administered to mice bearing either human breast or colon tumours as xenografts. The effectiveness of the induction protocol was confirmed by demonstrating that the predicted changes in murine hepatic cytochrome P450 gene expression had occurred.

Control untreated xenograft samples had extremely low P450 content. However, a protein which reacted with the antibody to CYP2A1 was identified by Western blot analysis (Figure 5.6.1). This protein had a different mobility to recombinant human CYP2A6, which has been associated with hepatic coumarin hydroxylase activity (Miles *et al*, 1990; Yamano *et al*, 1990). Very low constitutive levels of proteins reacting with antibodies to CYP2B1 and CYP2C6 were detected in the colon tumour using the highly sensitive ECL detection system (Figure 5.6.2). The relative mobility of the protein detected with the CYP2B1 antibody (54.5kD) was different from human hepatic CYP2B6 (51.0kD) while the mobility of the protein detected with anti CYP2C6 was the same as human hepatic CYP2C9 (54.5kD).

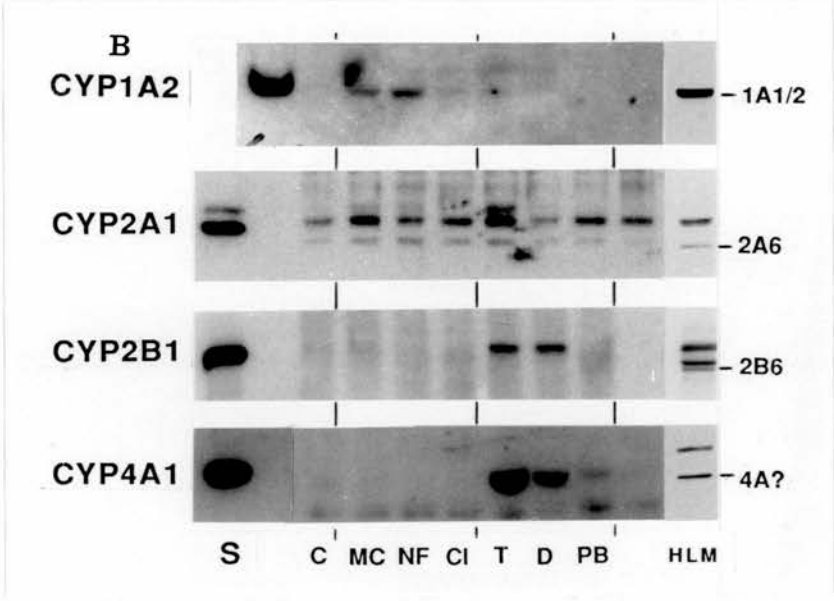
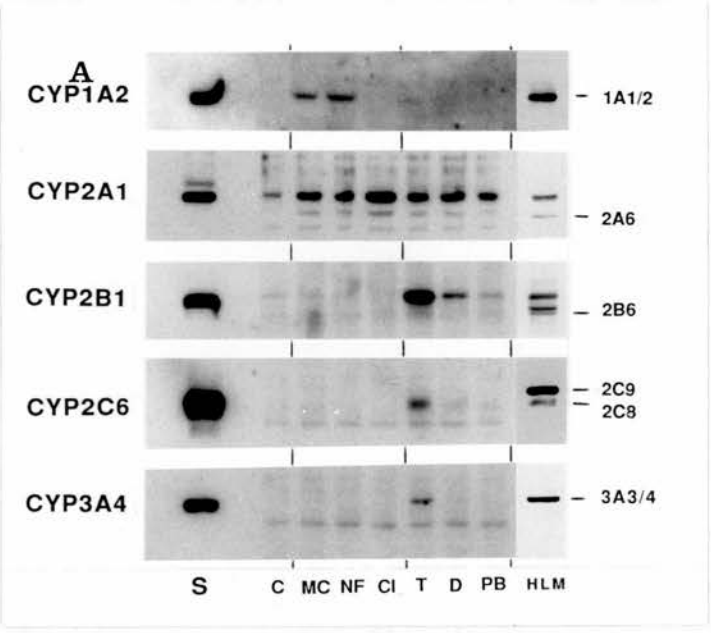
Tumour cytochrome P450 content was significantly altered by the administration of several of the P450-inducing agents tested (Figure 5.6.1). 3-Methylcholanthrene (3-MC) and β -naphthoflavone (β -NF) are both well characterised inducers of P450 proteins in the CYP1A and CYP2A gene families (Guengerich, 1987). Both of these compounds could induce the level of a CYP1A protein, probably CYP1A1, within both the colon and breast tumour tissues. The level of a protein reacting with the CYP2A1 antibody (Figure 5.6.1, lower band) was also increased 2-3 fold and approximately 5-fold in the breast and colon samples respectively, by both 3-MC and β -NF. A further protein with a higher apparent molecular weight (55.5kD, upper band) was also very slightly induced by these compounds in the breast, but not the colon tissue. Interestingly, in further experiments using ECL as the detection system, 3-MC also caused slight induction (2-3 fold) in the level of the protein reacting with the antibody to CYP2B1 (Figure 5.6.2).

TCPOBOP and dexamethasone are potent "phenobarbital-like" inducing agents in the mouse, but have virtually no effect on the expression of the major phenobarbital-inducible isozymes in the rat (Poland *et al*, 1980; Meehan *et al*,

1988, Chapter 3). The ability of these two compounds to induce human tumour P450 levels was therefore compared to the effects of phenobarbital. TCPOBOP, at a dose of 3mg/kg was a potent modulator of human P450 expression, inducing proteins in the CYP1A, CYP2A, CYP2B, CYP2C, CYP3A and CYP4A gene families. In breast tissue, the constitutively expressed CYP2A form (lower band) and that slightly induced by 3-MC (upper band) were both significantly induced. In colon tissue, only the 51.5kD protein (lower band) was induced. Proteins reacting with antisera to CYP2B1 were markedly increased on TCPOBOP treatment in both the breast and colon tissues, with the induction of CYP2B in the colon being particularly pronounced. This induced protein had the same mobility as the constitutively expressed form and appears to be distinct from CYP2B6. Whether this protein is CYP2B7 or a novel cytochrome P450 isozyme is currently being investigated. Proteins reacting with the CYP2C6 and CYP3A1 antibodies were also induced in the xenograft tissue by TCPOBOP. The mobilities of these induced proteins were identical to CYP2C9 and CYP3A5 respectively. TCPOBOP administration also lead to a profound increase in the expression of a protein reacting with the antibody to CYP4A1. This induction was particularly pronounced in the breast tumour, but was also observed in the colon samples.

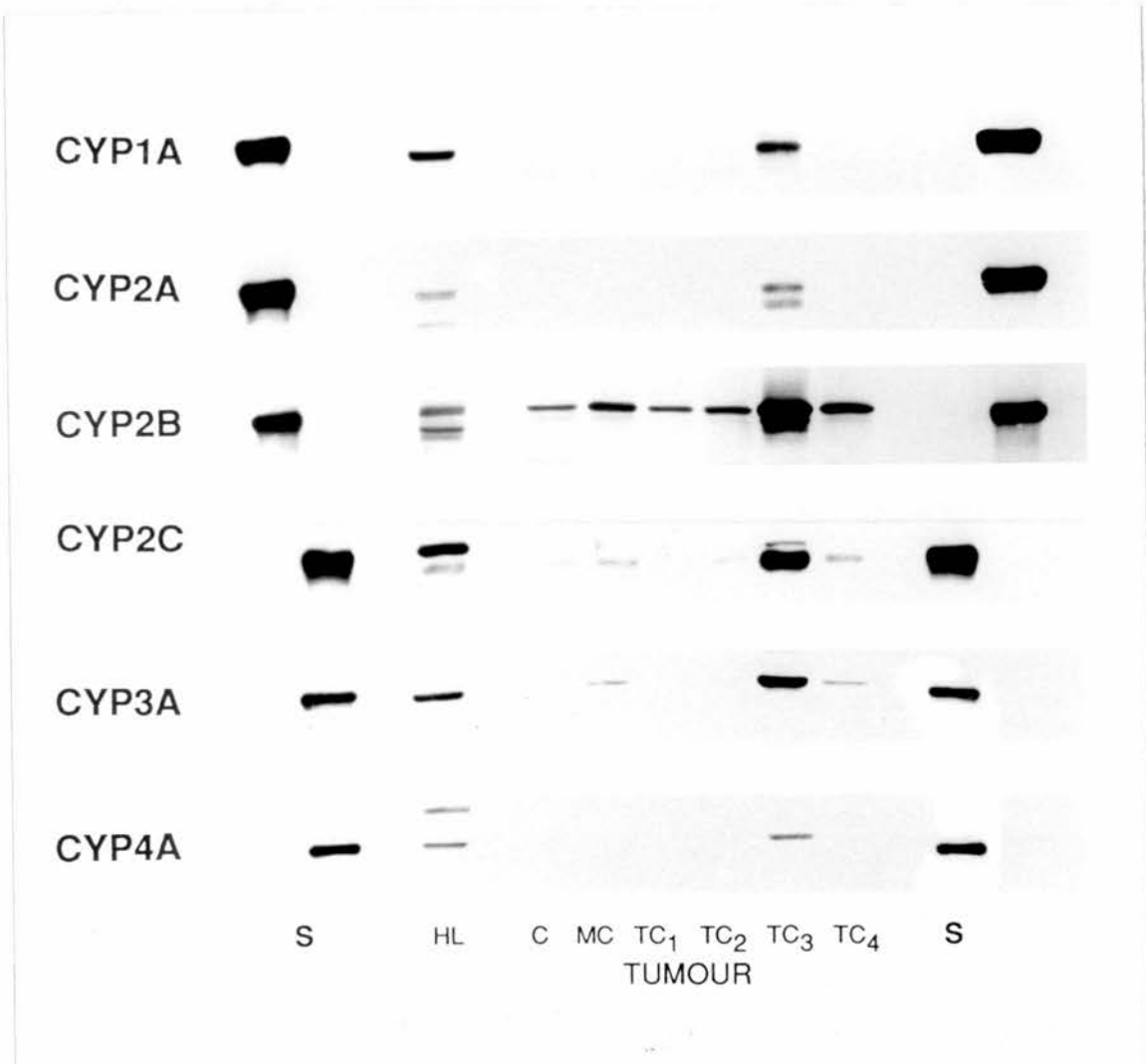
In a similar manner to TCPOBOP, dexamethasone also induced proteins in the CYP2B gene family in both the breast and colon tumours (Figure 5.6.1). Interestingly, this compound also caused a profound induction of the protein reacting with the CYP4A antibody in colon but not in breast tissue. Relative to TCPOBOP and dexamethasone, phenobarbital was much less effective as an inducing agent in the xenograft tumours, but did cause some increase in the levels of CYP2A and CYP2B proteins in both tumours and CYP4A in the breast xenograft. Clofibrilic acid had very little effect on tumour P450 expression but did appear to induce the protein reacting with the CYP2A antibody (lower band). Surprisingly, this compound did not induce proteins reacting with the antibody to CYP4A1.

Figure 5.6.1.1 P450 expression in human breast and colon tumours grown as xenografts



Human colon (A) or breast (B) tumours were grown as xenografts in immune deficient mice to a diameter of 1cm. Animals were then treated intra-peritoneally with a variety of compounds, as described in Materials and Methods. Microsomal fractions were prepared from the tumour samples and analysed for cytochrome P450 content by Western blotting using the antibodies shown. 15µg of microsomal protein was loaded per track. C = Control, MC = 3-methylcholanthrene, NF = β-naphthoflavone, CI = clofibric acid, T = TCPOBOP, D = dexamethasone, PB = phenobarbital, S = purified rat P450 standard. The relative mobility of recombinant human P450s are shown in the right hand track.

Figure 5.6.2 ECL detection of P450 expression in a human colonic xenograft tumour



P450 content was determined by Western blotting in a human colon tumour grown as a xenograft. Animals were treated intra-peritoneally with vehicle only (Con), 3MC (100mg/kg, 3 injections) or a single injection of TCPOBOP (1mg/kg (TC₁), 3mg/kg (TC₂), 10mg/kg (TC₃) or 15mg/kg (TC₄)). HL = human liver microsomes, S = purified

5.6.2 Localisation of the induced proteins

A potential problem in the use of tumour xenografts to study human gene regulation is the possibility that the observed changes in gene expression are due to infiltration of the tumour with cells of the host and that the enzymes studied are, in fact, murine. Immunohistochemical studies with P450 polyclonal antisera unequivocally demonstrated that the induced proteins were localised within malignant human epithelial cells rather than mouse derived tissues (Figure 5.6.3). Staining of P450 proteins was often found to be heterogeneous, with some areas of tumour staining strongly, while others were weak or even negative. Good agreement was obtained between the levels of protein detected on Western blot analysis and immunohistochemical staining.

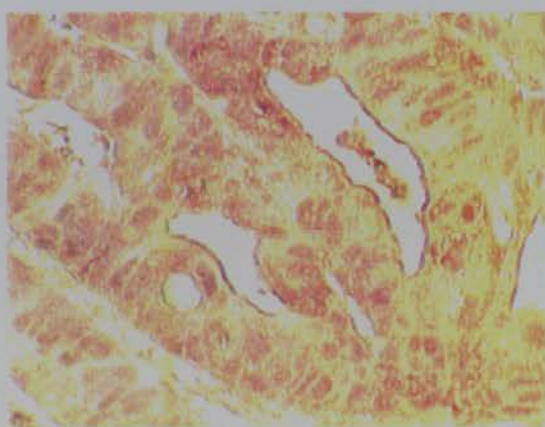
Figure 5.6.3 Immunohistochemical localisation of the induced proteins

P450 expression in xenograft colon (A) and breast (B) tumours was determined using antibodies against rat (i) CYP1A2 (reactive with both CYP1A1 and CYP1A2) (ii) CYP2B1 and (iii) CYP4A1 proteins. Control tumours showed low or negative expression except for breast which expressed CYP2B at low levels and colon which demonstrated focal reactivity for all three antibodies. Induction of CYP4A was less marked in colon and the expression of CYP1A in treated colon tumour remained very heterogeneous

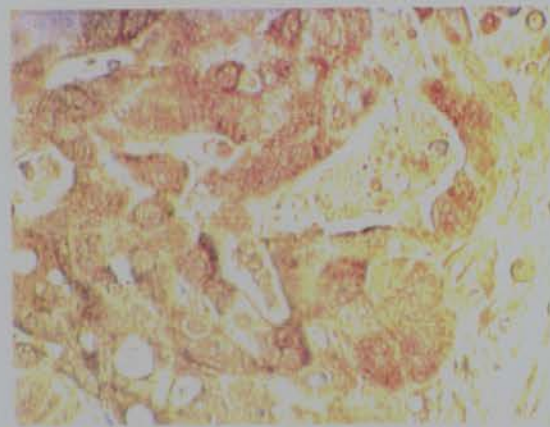
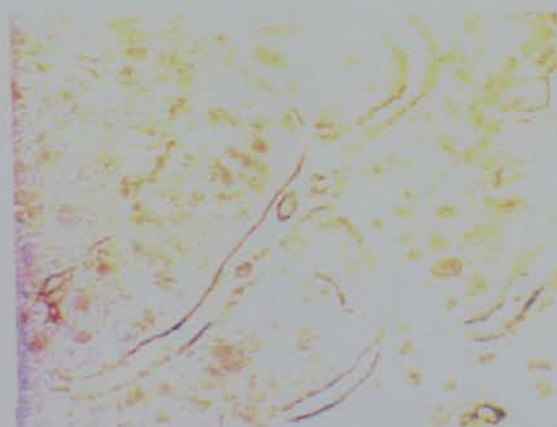
(A) Colon tumour

Control

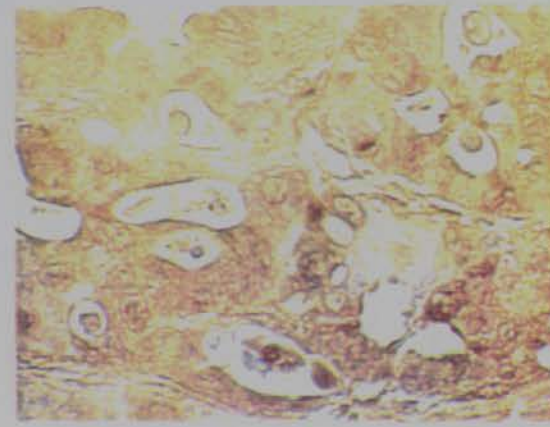
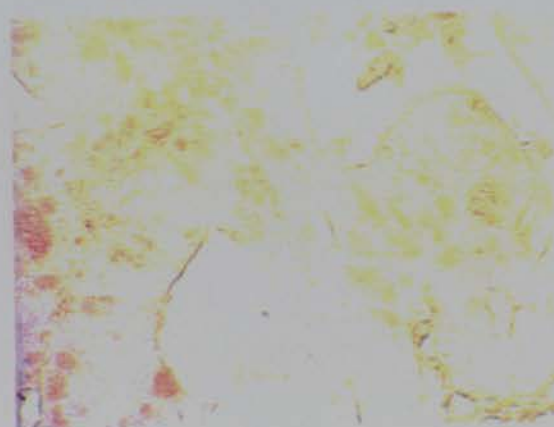
After TCPOBOP treatment



(i) CYP1A



(ii) CYP2B

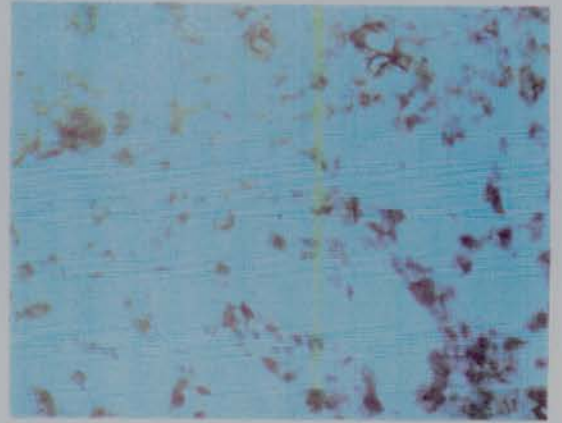


(iii) CYP4A

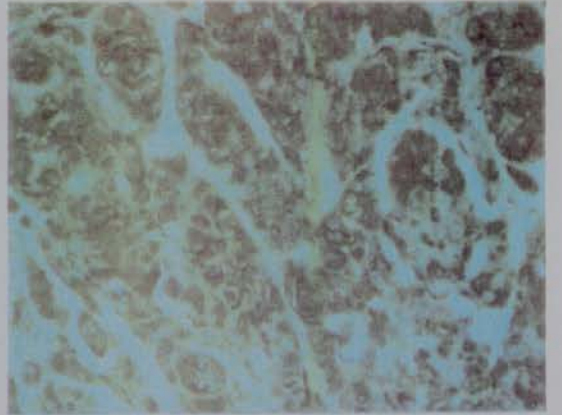
(B) Breast tumour

Control

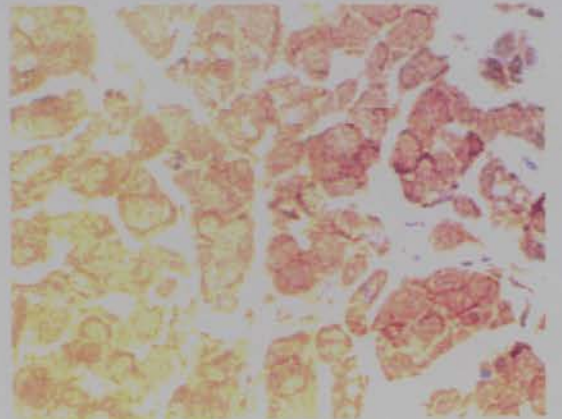
After TCPOBOP treatment



(i) CYP1A



(ii) CYP2B



(iii) CYP4A

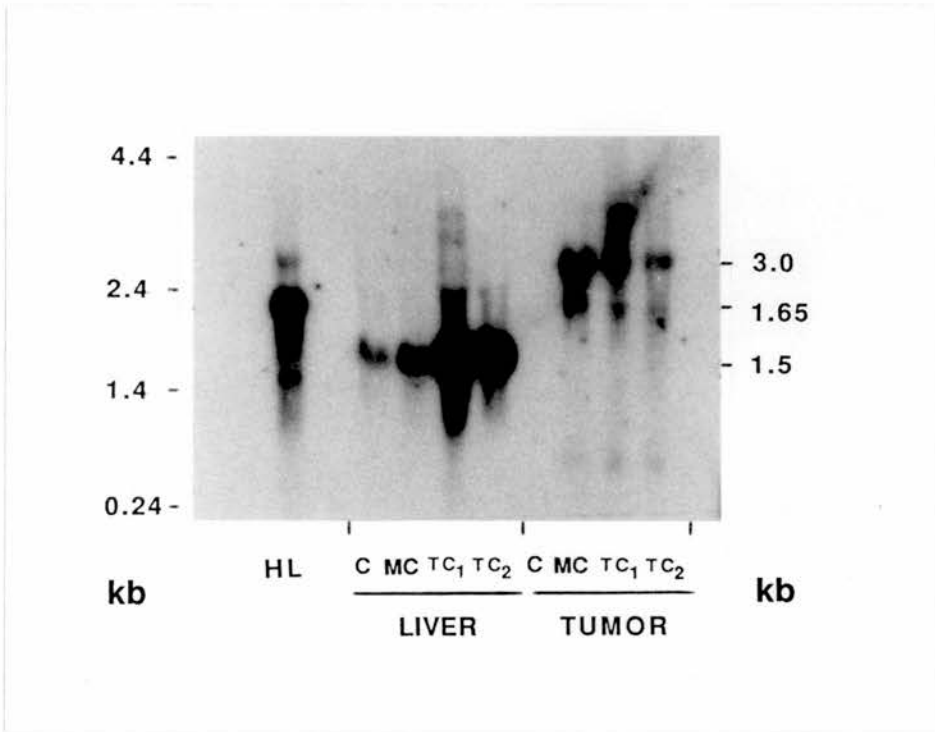
5.6.3 Evidence that TCPOBOP induces CYP2B protein expression by transcriptional activation of the gene

1. Northern blot analysis

CYP2B mRNA levels were determined in the human colon xenograft by Northern blot analysis using a cDNA probe for human CYP2B6 (Figure 5.6.3.1). Two major bands of 3.0kb and 1.65kb respectively were detected in both the human liver mRNA and the xenograft samples. Cyp2b mRNA isolated from the liver of the host mouse had a much smaller transcript size (1.5kb), and was clearly distinguishable from the mRNA species identified in the tumour tissue. It is interesting to note that the predominant mRNA species induced in the tumour tissue is the 3.0kb transcript, while that in the liver runs at 1.65kb. This supports the possibility that the major inducible P450 isozyme within the tumour is distinct from CYP2B6, but is from the same gene family. Alternatively, the differences in the relative abundance of the mRNA transcripts may be due to alternative splicing of the mRNA as reported by Miles *et al.*, (1988).

CYP2B mRNA from both the liver of the host mouse and the associated xenograft tumour was highly induced on xenobiotic treatment. In the liver, induction by TCPOBOP was far greater than that observed on 3-methylcholanthrene treatment. Within the tumour tissue, however, mRNA levels for both the 3-methylcholanthrene and TCPOBOP treated groups were equally induced.

Figure 5.6.3.1 Northern blot analysis of CYP2B mRNA levels in a human colonic tumour xenograft

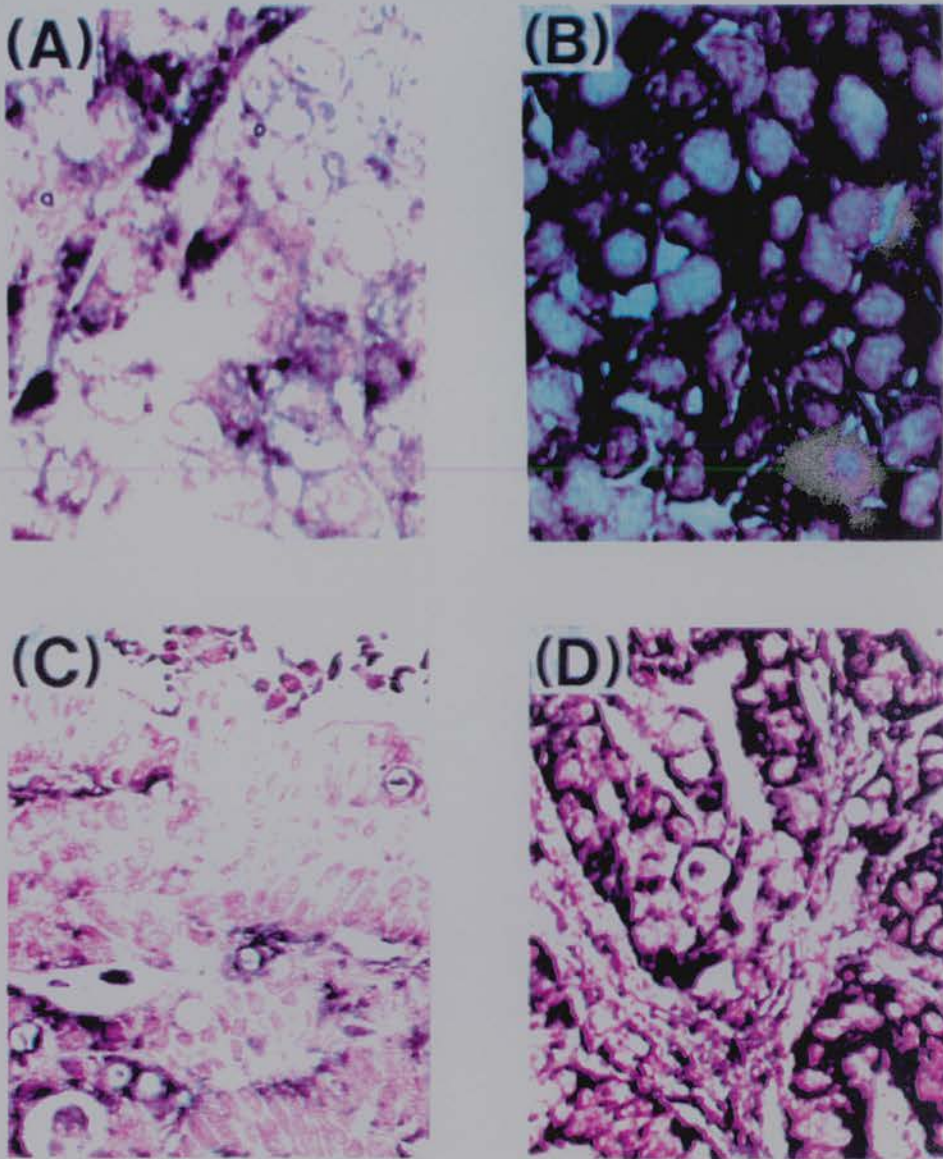


Northern blot analysis of CYP2B mRNA levels in the xenograft colon tumour was performed as described in Materials and Methods. Animals were treated intraperitoneally with either vehicle alone, or with 3-MC or TCPOBOP. HL = human liver RNA, C = control xenograft, or tumours from MC = 3-methylcholanthrene (100mg/kg x 3), TC₁ = TCPOBOP (3mg/kg) or TC₂ = TCPOBOP (15mg/kg) treated animals

2. In situ hybridisation for mRNA

The induction of a CYP2B protein within the xenograft tumours was confirmed by in situ hybridisation for mRNA using an oligonucleotide probe derived from the human CYP2B6 cDNA sequence (Fig 5.6.3.2). This oligonucleotide has 6 mismatches with mouse Cyp2b9 (see Section 2.10), and would therefore not be expected to hybridise to Cyp2b mRNA. These studies confirmed the localisation of P450 mRNA to the human breast and colon tumour cells and, in agreement with the Northern blot analysis, indicated that the observed induction of CYP2B protein had occurred at the level of transcription.

Figure 5.6.3.2 *In situ* hybridisation for mRNA in colon and breast tumours using a probe to CYP2B6



In situ hybridisation was performed using oligonucleotide probes derived from Exon 2 of the human CYP2B6 gene. (A) Breast xenograft from control animals using the antisense oligonucleotide probe showing little significant labelling; (B) TCPOBOP-treated breast xenograft using antisense probe showing widespread, but heterogeneous cytoplasmic reactivity; (C) TCPOBOP-treated colon xenograft using a sense probe, showing no specific binding; (D) TCPOBOP treated colon xenograft using antisense probe showing strong, fairly homogeneous cytoplasmic reactivity

5.6.6 Does TCPOBOP administration have a synergistic effect on the anti-tumour effect of cyclophosphamide?

Cyclophosphamide is metabolised to its active form by CYP2B proteins (see Section 1.5.3). As the intra-tumour levels of these proteins can be modulated by TCPOBOP administration, it seemed reasonable to investigate whether inducing the intra-tumour CYP2B levels would lead to greater metabolism of cyclophosphamide within the tumour and thus greater efficacy. TCPOBOP and cyclophosphamide were therefore administered singly and in combination to mice bearing breast and colon tumours as xenografts and the tumour surface areas monitored on a weekly basis. Cyclophosphamide administration lead to marked tumour regression in the breast model, but was without any significant effect in the colon tumour. In addition, TCPOBOP appeared to have potent anti-tumour effects in its own right (Figure 5.6.6). Some enhanced anti-tumour activity was indeed observed when the compounds were administered in combination. Experiments to investigate the possible anti-tumour effects of TCPOBOP and to determine whether the effect, if observed, is dose-dependant, are currently being carried out.

Although these experiments are preliminary, it seems that co-administration of TCPOBOP and cyclophosphamide did indeed lead to enhanced tumour kill. The synergistic effect was particularly pronounced for the colon tumour which did not respond to cyclophosphamide administered alone. This is in agreement with the low and heterogeneous expression of CYP2B enzymes, responsible for the metabolic activation of cyclophosphamide, observed in a panel of human colon tumours (Section 5.5.2). Whether this is simply a function of the inherent anti-tumour effects of TCPOBOP has yet to be determined. If further experiments validate these results, however, this would represent an extremely powerful strategy to increase the intra-tumour levels of active drug, leading in turn to a selective increase in cell kill.

Figure 5.6.6 (A) Breast tumour

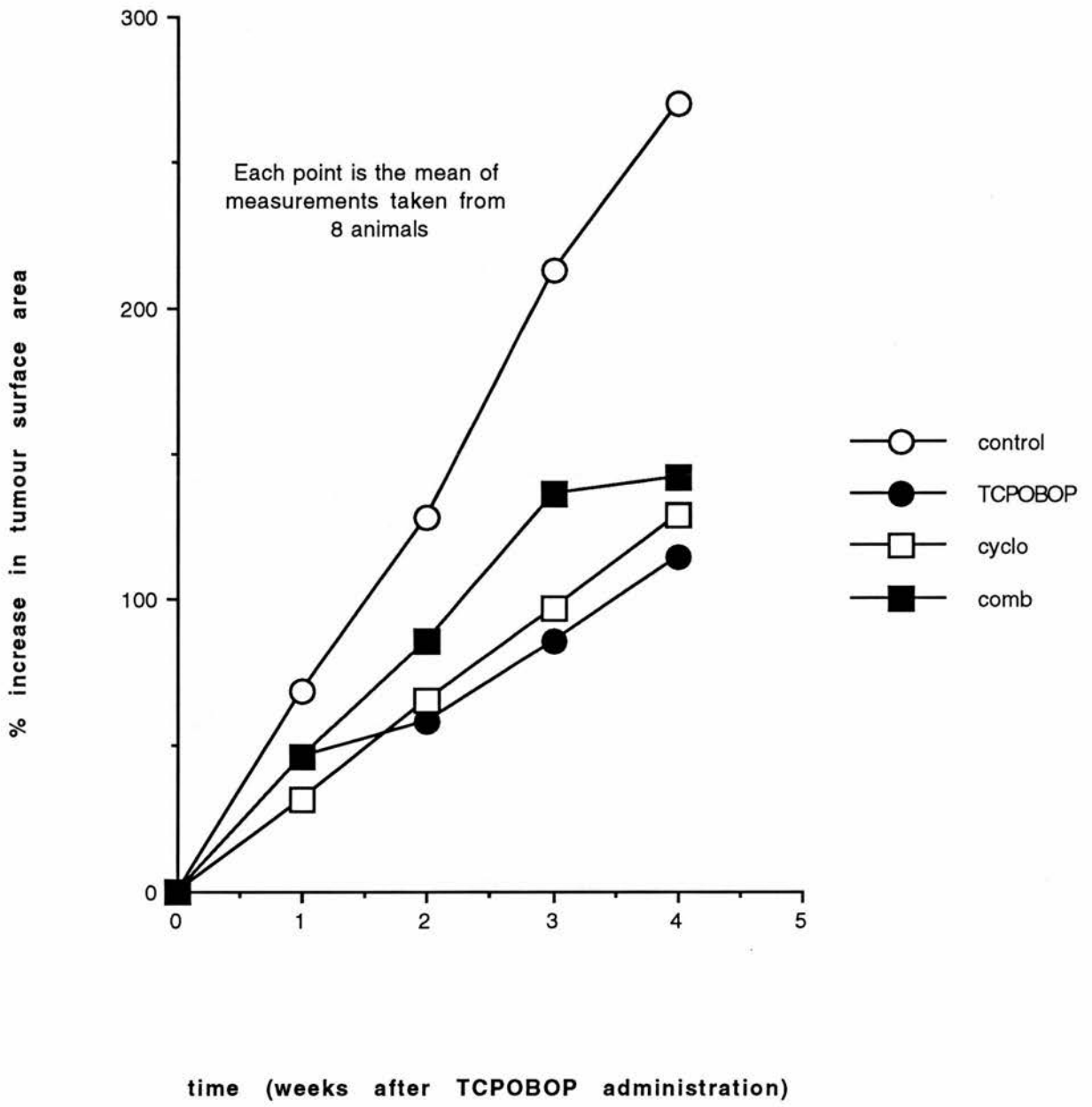
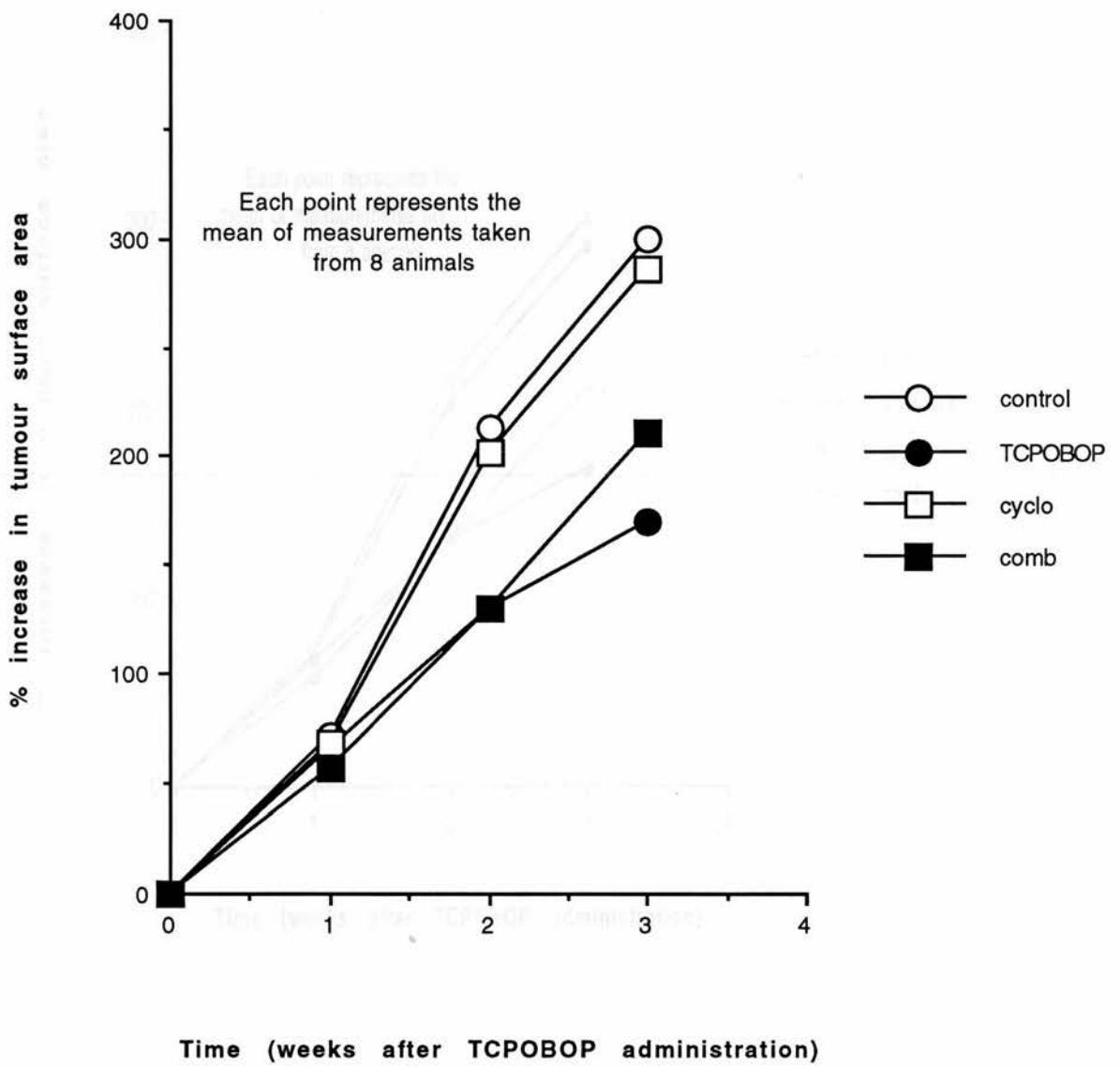


Figure 5.6.6 (B) Colon tumour



5.6.8 Xenobiotic regulation of P450 expression in a xenograft tumour derived from the cell line Hepa 1.

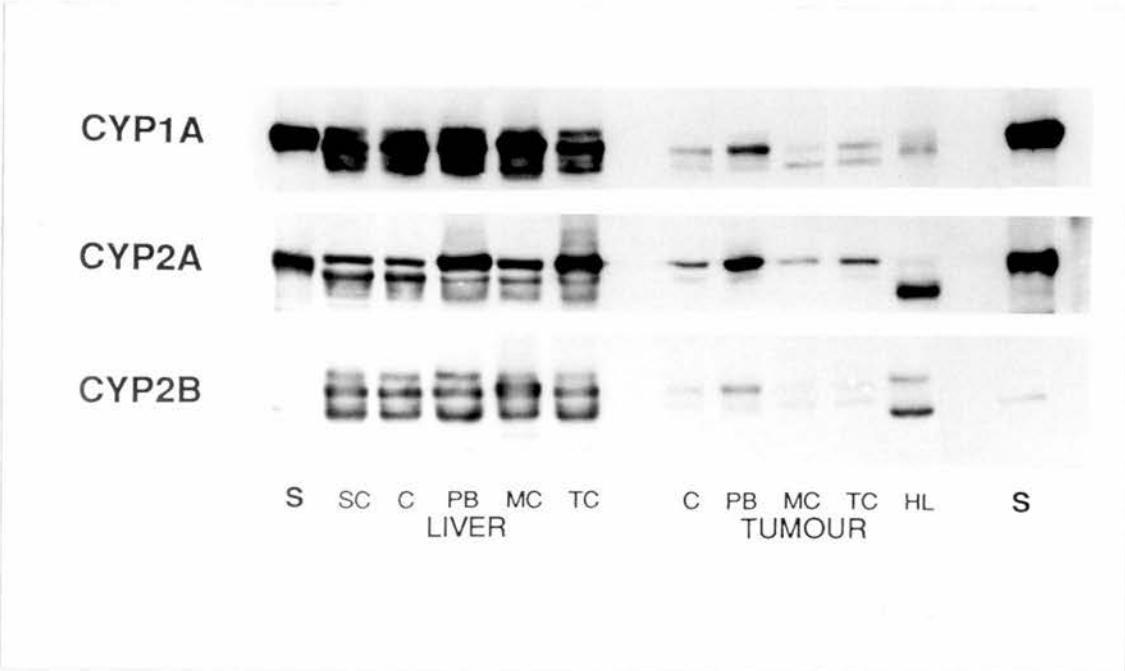
Hepa 1 cells have constitutive P450 expression, but this was not inducible by any of the xenobiotics tested when the cells were grown in culture (Section 4.6.2). To investigate whether this lack of inducibility was a function of the limited ability of cells in culture to maintain regulable P450 expression, Hepa 1 cells were established as a solid tumour which was grown as a xenograft in nude mice. These mice were treated intra-peritoneally with phenobarbital, 3-MC and TCPOBOP. As illustrated in Figure 5.6.8, the level of intra-tumour P450 expression was influenced by xenobiotic administration. Although 3-MC and phenobarbital were effective inducing agents^{*} in both liver and tumour in this model, TCPOBOP administration lead to induced levels of P450 protein only in the liver of the host mouse.

If the effects of TCPOBOP are indeed receptor mediated, it seems plausible that the expression of this receptor was lost when the Hepa-1 cells were grown in continuous culture and thus the tumour established from this cell line remained non-responsive to TCPOBOP administration in vivo. Neither of the other xenograft tumours had previously been grown as cell lines.

This experiment, although preliminary, demonstrated that cell lines grown in culture can be established as solid tumour xenografts and shows that the intra-tumour levels of drug metabolising enzymes can be modulated by xenobiotic administration. This is potentially of great benefit for future experiments in which stably transfected cell lines expressing a particular gene of interest will be created and established as transgenic tumours and thus their in vivo metabolic profiles determined.

^{*}of Cyp1a and Cyp2b expression respectively,

Figure 5.6.8 Xenobiotic modulation of P450 levels in a xenograft tumour created from the cell line Hepa-1



Mice bearing solid tumour xenografts established from the cell line Hepa-1 were treated as controls (C) or with phenobarbital (PB), 3-methylcholanthrene (3-MC) or TCPOBOP (TC). HL = human liver microsomes, S = purified rat P450 proteins, SC = stock control i.e. liver from a non-tumour bearing mouse

5.7 Discussion

In this chapter, a model is described which can be applied to study how environmental and hormonal factors regulate the expression of human cytochrome P450 genes. The strength of this model is that it circumvents the previously intractable problem that the expression and induction of cytochrome P450 expression in cell lines is lost, possibly due to methylation of the cytochrome P450 genes (Antequara *et al*, 1990). It also allows the effects of humoral or metabolic factors such as hormones and lymphokines which can not be easily studied in *in vitro* systems to be evaluated.

Although the influence of the mouse host on P450 expression cannot be ruled out, immunohistochemical analysis clearly demonstrated that the observed changes in gene expression occurred within human tumour tissues and therefore reflected the responsiveness of human cells to the administered agents. Both the tumour tissues studied maintained their human phenotype throughout these experiments and remained characteristic of the tumour of origin.

The data presented in this chapter demonstrated that human breast and colon tumours constitutively express low levels of certain cytochrome P450 isozymes. This is agreement with previous findings which demonstrate that P450 expression is detectable in tumour biopsy samples from these tissues (Senler *et al*, 1985; Forrester *et al*, 1990; Murray *et al*, 1991). The constitutive expression of a protein in the CYP2A gene family in both the breast and colon tumours is interesting in view of the recent report of the isolation of a CYP2A protein from mouse liver tumours (Maurice *et al*, 1991). In addition, there was considerable inter-individual variation in the level of expression of many of the P450 isozymes detected within a panel of human colon tumour samples, implying that the constitutive levels of these proteins may be inducible.

Indeed, these experiments demonstrated that cytochrome P450 levels in human colon and breast tumour tissues can be profoundly influenced by environmental agents. The patterns of gene regulation observed were often consistent with what is known about hepatic P450 regulation in rats and mice. In certain case, however, e.g. 3-MC induction of CYP2B expression, significant differences from the rodent model were observed. These data imply that human breast and colon tissues have the capacity to express P450s from many of the gene families involved in foreign compound metabolism. Many of the proteins identified in the xenograft tissues had different mobilities on SDS-PAGE from the human hepatic P450s and

may therefore represent novel enzymes. This is currently being investigated.

Of particular interest in these studies was the capacity of dexamethasone and TCPOBOP to induce human tumour P450 expression. The marked species specificity in the capacity of these compounds to induce P450 expression raised the question of whether they would also be active in man. Both compounds however, especially TCPOBOP, had marked effects on the expression of many human cytochrome P450 isozymes, particularly in the induction of CYP2B and CYP4A proteins. Immunohistochemistry and *in situ* hybridisation studies demonstrated that the induction of CYP2B occurred within human tissue and indicated that it was the result of transcriptional activation of the gene. These studies also illustrated the marked heterogeneity in both the constitutive expression and inductive response of many of the P450 isozymes observed to several of the inducing agents tested.

It is conceivable that the species-specific effects of TCPOBOP are due to effects on another tissue such as the pituitary and that this compound may still be inactive in man. This is unlikely to be the case however, as we have previously shown that TCPOBOP-mediated P450 induction in the liver is due to direct action on hepatocytes (Section 4.7). Species differences in the metabolism of TCPOBOP to the active inducer could also explain the specificity of this compound. However, this also seems unlikely as TCPOBOP is an inert compound, and there is no evidence that metabolism is required before it exerts its inductive effects (Poland *et al.*, 1980). The immunohistochemical data unequivocally confirmed that TCPOBOP has the capacity to alter human gene expression.

TCPOBOP and dexamethasone administration also had a profound effect on the expression of CYP4A proteins in the human breast tumour. The metabolic consequences of CYP4A induction in this tissue have not yet been determined, but CYP4A proteins have been shown to have an important endogenous role in fatty acid metabolism and prostaglandin and leukotriene biosynthesis (Capdevila *et al.*, 1981). These enzymes are also important mediators of peroxisome proliferation (Reddy & Lalwani, 1983). There are currently no reports of CYP4A expression in either colon or breast tissue.

Mechanisms of regulation of human P450s in extra-hepatic tissues, by agents other than polycyclic aromatic hydrocarbons (McLemore *et al.*, 1990; Vang *et al.*, 1991; Stralka & Strobel, 1991), are essentially unknown. Although animal models have previously shown that colon tissue will respond to compounds such as phenobarbital (Degawa *et al.*, 1991; Hammond & Strobel, 1990), the particular P450 isozymes affected are poorly characterised. A similar situation exists for

rodent mammary tissues. It also remains to be established how closely P450 regulation within tumour tissues reflects that of the normal tissue of origin. It has been demonstrated, however, that tumour cell lines, as well as rat hepatic preneoplastic lesions, retain many of the transcription factors required for P450 induction (Degawa *et al*, 1991). In this regard, the cell line Hepa 1, which did not respond to a range of xenobiotics in culture, became responsive when the cells were grown as a solid tumour xenograft. The only exception to this was TCPOBOP which, in contrast to the previous results, had no effect on P450 expression in this tumour. These studies imply that the expression of a specific receptor or transcription factor is necessary for TCPOBOP-mediated P450 induction, and that expression is lost or down regulated when cells are grown in continuous culture. The xenograft tumours which were used in all the other experiments described in this chapter had been established directly from the primary tumour.

The technique of establishing cell lines as solid tumour xenografts is potentially very powerful. Cell lines can be readily transfected with DNA coding for a particular P450 gene and used to determine the substrate specificity of a particular isozyme. Establishing this transgenic cell line as a solid tumour xenograft provides an *in vivo* model to study the regulation of a particular gene by a range of xenobiotics. This system can be extended to include genes with precisely engineered mutations, such as those which lead to polymorphisms in the expression of the P450 protein.

These studies have a variety of implications. Individuality in the expression of tumour P450 levels may relate to the responsiveness of patients to chemotherapeutic agents which require P450-mediated activation to exert their anti-tumour effects. If the P450 expression within the tumour is very heterogeneous, some cells will be able to activate the anti-tumour drug more effectively than others. Cells with low P450 expression and thus reduced activating capacity will proliferate to a greater extent than those which can activate the pro-drug and are, in turn, killed. In addition, heterogeneous expression of the enzymes involved in the onset of drug resistance can lead to the clonal expansion of a proliferating cell population and tumour progression. Establishing a number of xenograft lines from different tumours of the same tissue type should provide information on any inter-individual variation in constitutive P450 expression or heterogeneity in response to induction.

The ability to modulate tumour P450 levels raises the possibility that the efficacy of chemotherapeutic agents which require P450-mediated activation in

order to exert their anti-tumour effects could be increased by prior or concomitant administration of compounds such as TCPOBOP. Of particular interest in this regard are cyclophosphamide, Tamoxifen and the novel morpholinodoxorubicin derivatives. These compounds require *in situ* bioactivation by specific cytochrome P450 isozymes which are inducible within the human tumours by TCPOBOP (Powis & Prough, 1987; Jalacot *et al*, 1991; Mani & Kupfer, 1991; Lewis *et al*, 1992). The anti-oestrogenic compound Tamoxifen is widely used clinically in the treatment of breast cancer. This drug is metabolised *in vivo* by enzymes from the CYP3A family, the expression of which has been shown to be regulable within the breast tumour by TCPOBOP. These enzymes have also been shown to bioactivate the morpholinodoxorubicins, a new class of anti-cancer drugs which are derived from adriamycin, but do not have the same cardiotoxic side effects (Acton *et al*, 1984). Cyclophosphamide is activated by CYP2B which is maximally increased within the tumour cells by TCPOBOP. Although optimisation of the experimental protocols is clearly necessary to maximise the effect, the experiments described in Section 5.6.6 suggest that the efficacy of cyclophosphamide administration was indeed increased by concomitant administration of compounds such as TCPOBOP.

Chapter 6 : Summary of results and future prospects

In this thesis, the regulation of cytochrome P450 gene expression by a number of foreign compounds has been described. In particular, the effects of TCPOBOP (1,4 bis 2(3,5-dichloropyridyloxy)benzene) on P450 gene expression has been evaluated in a number of model systems. The major findings from this work are summarised below.

6.1 Xenobiotic regulation of murine hepatic P450 expression

Murine hepatic P450 expression was markedly increased by the intra-peritoneal administration of several xenobiotic inducing agents - the synthetic glucocorticoid dexamethasone, phenobarbital and the "phenobarbital-like" inducer, TCPOBOP. There were many similarities in the regulation of individual P450 isozymes by each of the administered compounds. The most profound and pleiotropic inductive effects were, however, observed with TCPOBOP.

TCPOBOP, first isolated as a pesticide contaminant, exhibited a marked species specificity in its inductive effects. Although a potent inducer of monooxygenase activity in mice and Syrian golden hamsters, the compound was without effect in rats and guinea pigs, thus implying that induction by TCPOBOP may be receptor mediated.

The administration of TCPOBOP, at a dose much less than that required for induction by any of the other inducing agents tested, lead to increased expression of proteins within each of the P450 subfamilies, with the exception of Cyp2e. The highest levels of induction were observed for Cyp2b, the major "phenobarbital-inducible" form. Induction appeared to be regulated at the level of transcription, as the corresponding P450 mRNA levels were also significantly increased. The lack of Cyp2e induction is in agreement with the report of Freeman *et al* (1991), which demonstrated that induction of murine Cyp2e protein expression by compounds such as ethanol and acetone was not accompanied by an increase in the corresponding mRNA, indicating that the increased protein expression was due to post-translational modification.

The inductive effects of TCPOBOP were confined to the liver - analysis of P450 protein expression in lung, kidney and testes showed no induction following TCPOBOP administration. This further supports the proposal that the effects of TCPOBOP are mediated by a tissue and species specific receptor. It has been proposed that the effects of phenobarbital are exerted through a receptor-mediated

mechanism, although, to date, such a receptor has not been isolated. In addition, the effects of dexamethasone have been shown to be mediated, in part at least, through a cytosolic glucocorticoid-responsive receptor.

Analysis of the long term effects of TCPOBOP administration demonstrated that the induction of P450 gene expression was long lasting. Twelve weeks following a single injection of the compound, P450 protein expression was still significantly elevated above control levels. The corresponding mRNA levels, although elevated, were observed to fall much more quickly, however, indicating that although induction was regulated at the level of transcription, some form of post-translational protein stabilisation was also involved in the expression of these genes. The mRNA levels in male mice fell much more quickly than in the corresponding females. This effect was also observed, although less markedly, in the expression of P450 proteins following acute treatment with TCPOBOP. In agreement with this, a longer term study of hepatic P450 protein expression demonstrated that Cyp2b, the major phenobarbital and TCPOBOP-inducible isozyme, remained above control levels for up to one year following treatment in female mice, while the level of expression in males declined more rapidly. A possible explanation for this is the relatively higher proportion of adipose tissue in female mice compared to males. As radiolabelling studies with TCPOBOP have shown that the compound is stored primarily in adipose tissue, it may be that the stored concentration of active drug is higher in females. Alternatively, if TCPOBOP action is indeed receptor mediated, there may be a different affinity of the drug for the receptor in male and female mice. Alternatively, the decrease in protein expression in male mice may represent selective loss of the TCPOBOP-responsive receptor.

There are several similarities between the inductive effects of TCPOBOP and the synthetic glucocorticoid dexamethasone with respect to their effects on P450 expression. Experiments were therefore performed to investigate whether the effects of TCPOBOP were mediated through a steroid hormone-like receptor, analogous to the glucocorticoid receptor. The GRE containing MMTV LTR linked to the CAT reporter gene was transfected into the hormone-responsive cell line ZR-75, but no increase in CAT activity was detectable when the cells were exposed to either phenobarbital or TCPOBOP. A similar experiment, carried out using the cell line C3H10T1/2 which had been shown to be TCPOBOP-responsive, however, resulted in the generation of acetylated reaction products on TCPOBOP treatment. Although the effect of glucocorticoids such as dexamethasone were much more pronounced than TCPOBOP, the possibility that TCPOBOP acts through a response element similar to

the GRE is worthy of further investigation.

Pituitary hormones do not play a significant role in the regulation of gene expression by TCPOBOP. Induction of P450 proteins by dexamethasone, phenobarbital and TCPOBOP was maintained in hypophysectomised animals. Indeed, a slight increase in hepatic P450 expression was observed following hypophysectomy, implying that some form of repressor may be secreted by the pituitary.

In addition to the profound effects on hepatic gene expression observed on TCPOBOP administration, a marked increase in liver size was also observed. Preliminary immunohistochemical studies indicate that the increase is due to hyperplasia, illustrated by increased expression of proliferating cell nuclear antigen and increased bromodeoxyuridine uptake in TCPOBOP-treated liver. In agreement with the increase in microsomal monooxygenase activity, hyperplasia was confined to the liver.

6.2 Cytochrome P450 expression and regulation in mammalian cell culture

The experiments described in Chapter 4 illustrate the limitations of attempting to study P450 regulation in cultured cell lines. The three human cell lines studied - the hepatoma HepG2, breast carcinoma MCF7 and colon carcinoma HT29, all lacked constitutive P450 expression and did not respond to TCPOBOP administration. Although this raised the possibility that human cells were not responsive to this compound, possibly due to a lack of a functional "TCPOBOP-receptor", later experiments in human tumours grown as xenografts demonstrated that this was not the case. Indeed, TCPOBOP administration had a profound effect on the expression of P450 proteins in both breast and colon tumour tissues. Antequara *et al* (1990), however, demonstrated that many cells in continuous culture down-regulate the expression of genes which are not necessary for their survival. This mechanism is thought likely to account for the loss of functional P450 expression in these cell lines.

Interestingly, although P450 expression was not inducible in the human cell lines on TCPOBOP treatment, in agreement with similar studies in mouse liver, administration of TCPOBOP to the human hepatoma cell line HepG2 lead to marked cellular proliferation. This phenomenon was also observed in the murine hepatoma cell line Hepa-1. In agreement with the *in vivo* studies, therefore, the proliferative effects of TCPOBOP appear to be liver specific. Another important observation was the low apparent cytotoxicity of TCPOBOP to any of the cell lines tested. This again

was in agreement with *in vivo* studies in the mouse, which demonstrated profound induction of hepatic P450 gene expression without any detectable associated systemic toxicity.

In contrast to the human cell lines, the expression of one P450 isozyme was maintained in both the mouse cell lines studied. Hepa-1 cells, a mouse hepatoma cell line, had constitutive expression of a protein which cross-reacted with Cyp2a antisera, as did the mouse embryo fibroblast cell line C3H10T1/2. While Hepa-1 cells did not respond to TCPOBOP treatment, the levels of Cyp2a protein in C3H10T1/2 cells were markedly increased on exposure to this compound. Although the expression of further P450 isozymes was not detectable in wild-type or TCPOBOP-treated cells, the increased level of Cyp2a expression demonstrated that the C3H10T1/2 cell line represents a possible model for studying the regulation of P450 gene expression by TCPOBOP.

The "Cyp2a" protein in this cell line has not yet been identified, although it has been demonstrated that it is distinct from Cyp2a-5, the murine coumarin hydroxylase. Previous reports suggested that the expressed P450 was active in the metabolism of some, but not all, polycyclic aromatic hydrocarbons. Whether this activity is due to very low levels of a protein which cross-reacted with the Cyp1a antibody has yet to be determined. In addition, mRNA prepared from C3H10T1/2 cells hybridised with a cDNA probe for Cyp2a, and had the same mobility as mRNA isolated from mouse liver. Pcr amplification of the cDNA generated from reverse transcription of mRNA isolated from C3H10T1/2 cells should yield the cDNA for the expressed "Cyp2a" protein. Sequence analysis will then confirm whether the protein is indeed a member of the Cyp2a subfamily, and will show whether it is, in fact, a novel gene. If a Cyp1a protein is indeed present at very low concentrations, the Cyp1a cDNA should also be isolable using this procedure. As the regulation of the expressed protein appears to be significantly different from Cyp1a1, this may also represent a novel protein.

C3H10T1/2 cells have previously been used in transfection experiments, where the cDNA encoding a P450 gene which is not constitutively expressed is inserted into the genome in order that, for example, its substrate specificity may be determined. As the cell line has also been shown to be TCPOBOP-responsive, transfection of deletion mutants of highly TCPOBOP-inducible genes such as Cyp2b9 or the human CYP2B6 linked to a reporter gene such as CAT, may lead to the identification of a TCPOBOP-responsive promoter region which is common to or similar among all responsive P450 genes. Such experiments involving transfection

of the MMTV-LTR into suitable recipient cell lines have already been performed in order to determine whether the effects of TCPOBOP were mediated by the GRE.

Mouse hepatocytes in primary culture proved to be the most sensitive *in vitro* model in which to study the regulation of P450 genes. In agreement with the effects observed *in vivo*, treatment of a primary hepatocyte culture with either phenobarbital or TCPOBOP lead to a significant increase in the expression of Cyp2b protein. This data unequivocally demonstrated that TCPOBOP can act directly on the liver to regulate P450 gene expression and, while the effects of metabolic factors in mediating this induction cannot be ruled out, further regulatory mechanisms are clearly not essential for regulable P450 expression.

6.3 Regulation of cytochrome P450 expression in human tumour xenografts

Establishing human tumours as xenografts in immune deficient mice has proved to be an extremely useful model in which to study the *in vivo* regulation of human P450 genes. Intra-peritoneal treatment of tumour bearing mice with a range of xenobiotic agents, known from animal studies to be powerful modulators of P450 gene expression, lead to a marked induction of the intra-tumour levels of several distinct P450 isozymes. The patterns of induction observed in the liver of the host mouse were as expected from previous animal studies, but there were several significant differences in the regulation of gene expression in the human tumour tissues. Most notable of these was the marked induction of CYP2B expression, both mRNA and protein, by the polycyclic aromatic hydrocarbon 3-methylcholanthrene, which had previously been considered as an Ah receptor mediated specific agonist for the CYP1A subfamily. In addition, clofibric acid, a potent modulator of hepatic CYP4A gene expression in rodent models, did not induce intra-tumour levels of CYP4A protein, although it did lead to increased expression of CYP2B protein.

TCPOBOP exhibited a marked species specificity in its inductive effects - it was a potent inducer of monooxygenase activity in the mouse, but was completely ineffective in the rat. Administration of TCPOBOP to mice bearing either human breast or colon tumours as xenografts lead to marked induction of the intra-tumour levels of expression of CYP1A, CYP2A, CYP2B, CYP2C, CYP3A and CYP4A proteins. In agreement with the effects of this compound in mouse liver, CYP2B was the major TCPOBOP-inducible isozyme. That the induction occurred specifically in human tumour tissue rather than in the surrounding mouse stromal cells was demonstrated by immunohistochemical analysis. In all cases, the staining intensities observed in

control and TCPOBOP-treated tissues mirrored the patterns of induction obtained on Western blot analysis.

For several P450 isozymes, the mobility of the proteins detected within the human tumours differed from the mobility of the proteins expressed in human liver microsomes. It seems likely, therefore, that these tumour P450s may represent novel proteins.

Northern blot analysis of mRNA prepared from the xenograft tumours demonstrated that induction was regulated at the level of transcription. Using a cDNA probe specific for CYP2B6, induction of intra-tumour mRNA was observed on administration of both TCPOBOP and 3-methylcholanthrene. This was in agreement with the induction of CYP2B proteins by 3-methylcholanthrene observed on Western blot analysis and implies that either 3-MC can induce P450 expression by a mechanism which does not involve the Ah receptor or, alternatively, that the Ah receptor-ligand complex can also bind to the upstream region of the CYP2B6 gene. It was also interesting to note that the mRNA transcript sizes recognised in mouse liver and in the human tumours by the CYP2B6 cDNA were significantly different and that, in addition, the tumour mRNA had the same mobility as mRNA isolated from human liver.

To further confirm that the observed induction was indeed occurring at the level of transcription and was specific to the human tumour tissues, in situ hybridisation for mRNA was performed on both the breast and colon tumours using oligonucleotide probes to a region of the human CYP2B6 gene which would not be expected to cross-hybridise with the murine Cyp2b genes. Marked induction of human CYP2B6 mRNA was again observed in the TCPOBOP-treated tumours.

The anti-tumour agent cyclophosphamide is metabolised in vivo to its active form by CYP2B enzyme(s). It seemed reasonable to propose, therefore, that increasing the expression of CYP2B protein within a tumour would lead to greater metabolic activation of cyclophosphamide and hence greater drug efficacy. A preliminary experiment where cyclophosphamide was administered following TCPOBOP treatment indicated that this may indeed be the case, and demonstrated that TCPOBOP itself may have an anti-tumour effect. Later experiments, however, administering TCPOBOP alone, did not lead to such marked tumour regression.

A solid tumour xenograft was also established from the cell line Hepa-1. These cells, although responsive to 3-MC, did not respond to phenobarbital or TCPOBOP in culture, but were responsive to both 3-MC and phenobarbital when these agents were administered in vivo. TCPOBOP was, however, without effect in

influencing P450 expression in the Hepa-1 tumour. This lends further support to the theory that the effects of TCPOBOP are indeed receptor dependent, and suggests that the expression of such a receptor is lost when cells are established in continuous culture as cell lines. Neither the breast or colon tumours used in the previous induction experiments had been passaged in tissue culture before establishment as solid tumour xenografts.

6.4 Future prospects

The experiments described in this thesis illustrate the potency of TCPOBOP as a species-specific inducer of monooxygenase activity.

Using the mouse as a model system, TCPOBOP was shown to be a potent and pleiotropic inducer of hepatic monooxygenase activity. Administration of TCPOBOP to a primary culture of mouse hepatocytes demonstrated that the inductive effects of this compound could be reproduced without the need for any external metabolic factors such as pituitary hormones. Experiments where P450 induction was also maintained when TCPOBOP was administered to hypophysectomised animals confirmed this finding.

There were many similarities in the ability of TCPOBOP, phenobarbital and the synthetic glucocorticoid dexamethasone to influence hepatic P450 expression. It had previously been proposed that both phenobarbital and dexamethasone exerted their inductive effects through a receptor-mediated mechanism, in a similar manner to the Ah receptor-mediated induction of CYP1A gene expression by polycyclic aromatic hydrocarbons. Much of the data presented in this thesis supports the possibility the regulation of P450 gene expression by TCPOBOP is also receptor mediated. Experiments were therefore performed to determine whether TCPOBOP could, like dexamethasone, activate the GRE, the glucocorticoid responsive promoter element which is present in the upstream region of many P450 genes. Although TCPOBOP was much less active than dexamethasone in activating the GRE, activity which was significantly higher than control levels was achieved. Therefore, although TCPOBOP does not appear to bind the GRE directly, it seems feasible that an analogous steroid hormone-like receptor may mediate the effects of this compound. To test this hypothesis, it will be necessary to create a series of constructs comprising deletion mutants of a highly TCPOBOP-inducible gene such as CYP2B6 linked to the CAT reporter gene. These constructs will then be transfected into a cell line which responds to TCPOBOP administration such as C3H10T1/2. Administration of TCPOBOP to the cells containing the full length gene should lead to the activation of

the CAT reporter gene. Analysis of the various deletion mutants should reveal regions of the gene which are necessary for TCPOBOP activity. In order to determine the *in vivo* effects of xenobiotic modulators on P450 gene expression, it will be necessary to establish the cell lines containing the transfected genes of interest as solid tumour xenografts, as described for the Hepa-1 cell line in Chapter 5. These "transgenic tumours" can then be challenged with a range of xenobiotic modulators and their effects on gene expression *in vivo* determined.

Establishing human tumours as xenografts has proved to be an extremely powerful model for studying the regulation of human P450 genes. TCPOBOP was shown to be a potent and pleiotropic inducer of human P450 gene expression in human tumour tissues. To date, a breast tumour and a colon tumour have been studied, and TCPOBOP was equally active in influencing P450 expression in each model. Further experiments are planned, extending the studies to include a greater number of tumours and different tumour types. Xenobiotic regulation of the expression of Phase II drug metabolising enzymes such as the glutathione S-transferases and UDP-glucuronosyl transferases has also been studied in this model - TCPOBOP was equally effective at influencing the intra-tumour levels of expression of these proteins.

The precise identity of the P450 proteins induced within the tumours by TCPOBOP, or any of the other xenobiotic inducing agents studied has yet to be determined. In particular, it will be of great interest to determine which, if any, of these isozymes are the same proteins as those expressed in human liver, and which represent novel enzymes. As several of the proteins expressed within the xenograft tumours have different mobilities on SDS-PAGE from those expressed in human liver, it seems likely that they may indeed represent novel isozymes.

In order to unequivocally identify these expressed proteins, it will be necessary to isolate and sequence the corresponding cDNAs. A relatively straightforward approach to this is to reverse transcribe mRNA isolated from the treated tumours and amplify the resulting cDNA by the polymerase chain reaction. Each of the tumours studied appeared to have very low, but detectable, constitutive expression of certain of the P450 isozymes. The cDNAs encoding these proteins should also be isolable using this approach.

Once any novel genes have been isolated, it will be necessary to determine their substrate specificity. An initial *in vitro* approach involves transfecting the cDNA of the gene of interest into a suitable recipient cell line which can then be challenged with a number of candidate drugs. Compounds identified as substrates for

the particular gene of interest may then be administered to tumour-bearing mice in order to determine whether the effects on P450 gene expression observed in vitro are similar in vivo. C3H10T1/2 cells have been shown to support functional P450 expression and are responsive to TCPOBOP. As such, this is an ideal cell line in which to perform the transfection experiments.

The administration of TCPOBOP to experimental animals has been shown to cause hyperplasia in the liver. The xenograft model can also be used in the study of cell proliferation, both in the liver of the host mouse and also in the associated tumour tissues. Several of the inducing agents which induced intra-tumour P450 expression also had profound effects on the expression of genes such as p53 and pcna which are involved in cell cycle control. This represents an important area for further study.

Many anti-tumour drugs which are commonly used in cancer treatment, including cyclophosphamide and tamoxifen, are inactive as administered and are metabolised in vivo to their active forms by one or more P450 isozymes. Modulation of the intra-tumour levels of these enzymes is therefore potentially of clinical significance. If the tumour does not express P450, or the levels of expression are very low, the pro-drug will be metabolised in the liver and the anti-tumour effects will be dependent on transport of active drug to the tumour site. In this situation, hepatic metabolism often leads to the conjugation and/or excretion of the majority of the administered drug before it can be transported to the site of the tumour. If, however, the levels of the P450 isozyme which is active in the metabolism of the pro-drug can be significantly increased by prior or concomitant administration of an inducing agent such as TCPOBOP, then the pro-drug may undergo activation in situ leading to greater efficacy resulting in enhanced tumour kill. In addition, administration TCPOBOP should also lead to highly induced levels of human hepatic P450s. Thus, the concentration of circulating active drug will be higher and more will be transported to the tumour site. As many anti-tumour drugs are highly cytotoxic as administered, it may also be possible to reduce the amount of drug administered if the conversion to active metabolites is increased. It may also be possible to specifically target the tumour cells by, for example, conjugating the anti-tumour drug to a monoclonal antibody raised against a human tumour-specific antigen. This has the added benefit of increased binding specificity of the drug to the tumour cells, leading to a longer residence time of the drug at the tumour and, thus, greater efficacy.

Many tumours are either inherently drug resistant on presentation or

acquire resistance during chemotherapy. Several mechanisms have been proposed to account for this phenomenon. One of these, the overexpression of P-glycoprotein, an energy dependent efflux pump which is the product of the MDR gene leads to the exclusion of active drug from the cell. Previous work in this laboratory has demonstrated that TCPOBOP administration leads to a marked reduction in MDR expression in mouse liver. This fits well with the report of Romano et al (1986) that TCPOBOP administration leads to increased membrane fluidity. The xenograft model is ideally suited to determining whether the same mechanism of regulation is present in human tumour tissues. If so, administration of xenobiotic modulators such as TCPOBOP may result in the reversal of multidrug resistance and the resensitisation of tumours to therapy. Another mechanism which has been proposed to account for cellular resistance is the low expression or complete lack of expression within tumours of drug metabolising enzymes such as cytochrome P450 which are responsible for drug activation. This is particularly true for many tumours which are poorly vascularised. It may be possible, therefore, to sensitise or re-sensitise these tumours to chemotherapy by raising intra-tumour P450 levels by the administration of exogenous inducing agents. The administration of TCPOBOP as such an inducing agent is particularly attractive - this compound is a highly potent modulator of P450 gene expression in human tumour tissues at an administered dose which is significantly lower than the doses required for induction for any of the other inducing agents tested. In addition, studies in experimental animals and in a cell culture model indicated that the compound has very low associated cytotoxicity and the inductive effects are persistent.

A pilot experiment where cyclophosphamide and TCPOBOP were administered in combination to mice bearing human breast and colon tumours as xenografts indicated that enhanced cyclophosphamide activation may indeed have occurred. A much more sophisticated series of experiments is obviously required in order to fully investigate this strategy. For example, it will be necessary to measure the concentration of active drug with and without prior TCPOBOP administration, and to investigate the optimum doses and dosing schedules for each compound. It will also be necessary to develop a more rigorous strategy for assessing tumour kill. Histopathological examination of many of the xenograft tumours revealed a high degree of necrosis. Hence, although there may be no observed decrease in tumour volume on drug treatment, the number of viable cells within the tumour may be significantly reduced.

In addition, it will be necessary to determine whether the levels of P450

protein within the xenograft tumours, as assessed by Western blotting and immunohistochemistry, represents catalytically active enzyme. Although it did not prove possible to quantitate P450 substrate metabolism in frozen xenograft tumour samples, functional P450 was identified spectrophotometrically. In addition, NADPH-cytochrome P450 reductase was readily measurable in the tumour samples, demonstrating that reductase concentration was not rate limiting. Analysis of fresh (i.e. not frozen) tissue samples should allow measurement of substrate metabolism by the P450 isozymes expressed within the xenograft tumours which will, in turn, yield valuable information about the nature of the expressed proteins.

In summary, therefore, the xenograft model provides an opportunity to study the *in vivo* regulation of gene expression in human tumours, not only by xenobiotic modulators, but by endogenous factors such as hormones and lymphokines. Preliminary experiments have been performed which suggest that lymphokine administration can alter constitutive P450 expression in the xenograft tumours and, in addition, can alter the response of these tissues to administered xenobiotics. Although these effects have not yet been fully characterised, it is already apparent that infection and inflammation can have major metabolic consequences, not only in altering the response of patients to drugs which are metabolised by cytochrome P450, but in the regulation of the enzyme systems which protect against toxicological challenge.

References :

- Acton, E.M., Tong, G.L., Mosher, C.W. & Wolgemuth, R.L., (1984) *J. Med. Chem.*, **27**, 638-645 Intensely potent morpholinyl anthracyclines
- Adams, D.J., Seilman, S., Amelizad, Z., Oesch, F. & Wolf, C.R., (1985) *Biochem. J.*, **232**, 869-876 Identification of human cytochromes P-450 analogous to forms induced by phenobarbital and 3-methylcholanthrene in the rat
- Aida, K. & Negishi, M., (1991) *Biochemistry*, **30**, 8041-8045 Posttranscriptional regulation of coumarin 7-hydroxylase induction by xenobiotics in mouse liver: mRNA stabilisation by pyrazole
- Antequera, F., Boyes, J. & Bird, A., (1990) *Cell*, **62**, 503-514 High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines
- Antilla, S., Hietman, E., Vainio, H., Canns, A.M., Gelboin, H.V., Park, S.S., Heikkila, L., Karalainen, A.M. & Bartsch, H., (1991) *Int. J. Cancer*, **47**, 681-685 Smoking and peripheral types of cancer are related to high levels of pulmonary cytochrome P450IA in lung cancer patients
- Aoyama, T., Korekwa, K., Nagata, K., Adesnik, M., Reiss, A., Lapenson, D.P., Gillette, J., Gelboin, H.V., Waxman, D.J. & Gonzalez, F.J., (1989) *J. Biol. Chem.*, **264**, 21327-21333 Sequence requirements for cytochrome P-450IIB1 catalytic activity. Alteration of the stereospecificity and regioselectivity of steroid hydroxylation by a simultaneous change of two hydrophobic amino acid residues to phenylalanine
- Aoyama, T., Gonzalez, F.J. & Gelboin, H.V., (1989) *Molecular Carcinogenesis*, **2**, 192-198 Human cDNA-expressed cytochrome P450 IA2: Mutagen activation and substrate specificity
- Aoyama, T., Yamano, S., Waxman, D.J., Lapenson, D.P., Meyer, U.A., Fischer, V., Tyndale, R., Inaba, T., Kalow, W., Gelboin, H.V. & Gonzalez, F.J., (1989) *J. Biol. Chem.*, **264**, 10388-10395 Cytochrome P-450 hPCN3, a novel cytochrome P-450 IIIA gene product that is differentially expressed in adult human liver
- Arnold, H. & Bourseaux, F., (1958) *Angewandte Chemie*, **70**, 539-544 Synthese und abbau cytostatisch wirksamer cyclischer N-Phosphamidester des bis-(β -chloräthyl)-amins
- Bars, R.G., Mitchell, A.M., Wolf, C.R. & Elcombe, C.R., (1989) *Biochem. J.*, **262**, 151-158 Induction of cytochrome P450 in cultured rat hepatocytes: The heterogeneous localisation of specific isoenzymes using immunocytochemistry
- Bartlomowicz, B., Waxman, D.J., Utesch, D., Oesch, F. & Friedberg, T., (1989) *Carcinogenesis*, **10**, 225-228 Phosphorylation of carcinogen metabolising enzymes: regulation of the phosphorylation status of the major phenobarbital-inducible cytochromes P-450 in hepatocytes

Bartoszek, A. & Wolf, C.R., (1992) *Biochem. Pharmacol.*, **42**, 1449-1457
Enhancement of doxorubicin cytotoxicity following activation by NADPH cytochrome P450 reductase

Basra, J., Wolf, C.R., Brown, J.R. & Patterson, L.H., (1985) *Anti-cancer Drug design*, **1**, 45-52
Evidence for human liver mediated free-radical formation by doxorubicin and mitozantrone

Batist, G., Tulpule, A., Sinha, B.K., Kataki, A.G., Myerces, C.E. & Cowan, K.H., (1986) *J. Biol. Chem.*, **261**, 15544-15549
Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells

Battula, N., Sagara, J. & Gelboin, H.V., (1987) *Proc. Natl. Acad. Sci. (USA)*, **84**, 4073-4077
Expression of P₁-450 and P₃-450 DNA coding sequences as enzymatically active cytochromes P-450 in mammalian cells

Bayad, J., Bagrel, D., Sabolovic, N., Magdalon, J. & Siest, G., (1991) *Biochem. Pharmacol.*, **42**, 1345-1351
Expression and regulation of drug metabolising enzymes in an immortalised rat hepatocyte cell line

Beatson, G.T., (1896) *Lancet*, **2**, 104-107
On the treatment of inoperable cases of carcinoma of the mamma. Suggestions for a new method of treatment with illustrative cases

Beaune, P.H., Dansette, P.M., Mansuy, D., Kiffel, L., Finck, M., Amar, C., Leroux, J.P. & Homberg, J.C., (1987) *Proc. Natl. Acad. Sci., (USA)*, **84**, 551-555
Human anti-endoplasmic reticulum autoantibodies appearing in a drug-induced hepatitis are directed against a human liver cytochrome P450 that hydroxylates the drug

Becker, P.B., Gloss, B., Schmid, W., Strahle, U. & Schutz, G., (1986) *Nature*, **324**, 686-688
In vivo protein-DNA interactions in a glucocorticoid response element require the presence of the hormone

Begue, J.M., LeBigot, J.F., Gugen-Guillouzo, C., Kiechel, J.R., Guillouzo, A., (1983) *Biochem. Pharmacol.*, **32**, 1643-1646
Cultured human adult hepatocytes: a new model for drug metabolism studies

Begue, J.M., Gugen-Guillouzo, C., Padeloup, N. & Guillouzo, A., (1984) *Hepatology*, **4**, 839-842
Prolonged maintenance of active cytochrome P-450 in adult rat hepatocytes co-cultured with another liver cell type

Berenbaum, M.C., Sheard, C.E., Reittie, J.R. & Bundick, R.V., (1974) *Br. J. Cancer*, **30**, 13-32
The growth of human tumours in immunosuppressed mice and their response to chemotherapy

Berger, D.P., Winterhalter, B.R. & Fiebig, H.H., (1991), pp 165-184, In: "The Nude Mouse in Oncology Research", Boren, B. & Winograd, B., (eds), CRC Press

- Bernhard, H.P., Darlington, G.J. & Ruddle, F.H., (1973) *Devel. Biol.*, **35**, 83-96
Expression of liver phenotypes in cultured mouse hepatoma cells: synthesis and secretion of serum albumin
- Bernhardt, R., Makower, A., Janig, G.R. & Ruckpaul, K., (1984) *Biochem. Biophys. Acta.*, **785**, 186-190 Selective chemical modification of a functionally linked lysine in cytochrome P-450 LM2
- Bernhardt, R., Kraft, R., Otto, A. & Ruckpaul, K., (1988) *Biomed. Biochim. Acta.*, **47**, 581-592 Electrostatic interactions between cytochrome P-450 LM2 and NADPH-cytochrome P-450 reductase
- Birnboim, H.C. & Doly, J.A., (1979) *Nucleic Acids Res.*, **7**, 1513-1523 A rapid alkaline extraction procedure for screening recombinant plasmid DNA
- Bissell, D.M. & Guzelian, P.S., (1975), pp 119-136, In: "Gene expression and carcinogenesis in cultured liver", Gerschenson, L.E. & Thompson, E.B., (eds), Academic Press
- Bissell, D.M. & Hammaker, L.E., (1976) *Arch. Biochem. Biophys.*, **176**, 91-102
Cytochrome P-450 heme and the regulation of hepatic heme oxygenase activity
- Bissell, D.M. & Guzelian, P.S., (1980) *Ann. N.Y. Acad. Sci.*, **349**, 85-98
Phenotypic stability of adult rat hepatocytes in primary monolayer culture
- Bissell, D.M. & Guzelian, P.S., (1980) *J. Clin. Invest.*, **176**, 1135-1140
Degradation of endogenous hepatic heme by pathways not yielding carbon monoxide
- Black, S.D., (1992) *Faseb J.*, **6**, 680-685 Membrane topology of the mammalian P450 cytochromes
- Black, S.M., Ellard, S., Meehan, R.R., Parry, J.M., Adesnik, M., Beggs, J.D. & Wolf, C.R., (1989) *Carcinogenesis*, **10**, 2139-2143 The expression of cytochrome P450IIB1 in *Saccharomyces cerevisiae* results in an increased mutation frequency when exposed to cyclophosphamide
- Bradfield, C.A., Glover, E. & Poland, A., (1991) *Mol. Pharmacol.*, **39**, 13-19
Purification and N-terminal amino acid sequence of the Ah receptor from the C57Bl/6 mouse
- Brattain, M.G., Marks, M.E., McCombs, J., Finely, W. & Brattain, D.E., (1983) *Br. J. Cancer*, **47**, 373-381 Characterisation of human colon carcinoma cell lines isolated from a single primary tumour
- Brock, N. & Hohorst, H.J., (1963) *Arzneimittel-Forschung, Drug Research* **13**, 1021-1031 Über die Aktivierung von Cyclophosphamid in vivo und in vitro
- Brock, N. & Hohorst, H.J., (1967) *Cancer*, **20**, 900-904 Metabolism of cyclophosphamide

Brock, N., (1967) *Cancer Chemotherapy Reports*, **5** 1, 315-325 Pharmacologic characterisation of cyclophosphamide (NSC-26271) and cyclophosphamide metabolites

Brock, N., Pohl, J. & Stejar, J., (1981) *Eur. J. Cancer*, **1** 7, 595-607 Studies on the urotoxicity of oxazophosphorine cytostatics and its prevention. I. Experimental studies on the urotoxicity of alkylating compounds

Buchmann, A., Schwartz, M., Schmidt, R., Kuhlmann, W.D., Wolf, C.R. & Oesch, F., (1987) *Arch.Toxicol.*, **6** 0, 198-203 Expression and inducibility of drug metabolising enzymes in preneoplastic and neoplastic lesions of rat liver during nitrosamine-induced hepatocarcinogenesis

Burger, H.J., Scheutz, J.D., Scheutz, E.G. & Guzelian, P.S., (1992) *Proc. Natl. Acad. Sci., (USA)*, **8** 9, 2145-2149 Paradoxical transcriptional activation of rat-liver cytochrome-P-450 3A1 by dexamethasone and the antigluocorticoid pregnenolone 16-alpha-carbonitrile - analysis by transient transfection into primary monolayer cultures of adult-rat hepatocytes

Burke, M.D. & Mayer, R.T., (1974) *Drug Metab. Disp.*, **2**, 583-588 Ethoxyresorufin: Direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene

Burke, M.D. & Mayer, R.T., (1974) *Drug Metab. Dispos.*, **3**, 245-253 Inherent specificities of purified cytochromes P-450 and P-448 toward biphenyl hydroxylation and ethoxyresorufin deethylation

Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J. Haaparanta, T. & Mayer, R.T., (1985) *Biochem. Pharmacol.*, **3** 4, 3337-3345 Ethoxy-, pentoxy- and benzyloxyphenoxazones: A series of substrates to distinguish between different induced cytochromes P-450

Cairney, A.E., Sladek, N.E. & Woods, W.G., (1984) *Proc. Am. Assoc. for Cancer Res.*, **2** 5, 339 DNA crosslinking by 4-hydroperoxycyclophosphamide in sensitive and resistant mouse leukaemia cells

Calabresi, P. & Parks, R.E., (1985) *Chemotherapy of neoplastic diseases* pp 1240-1258 In: "The pharmacological basis of therapeutics", 7th edition, Gillman, A.G., Goodman, L.S., Rall, T.W. & Mured, F., (eds) Macmillan Publishing Co., New York

Calne, D.B & Lanston, J.W., (1983) *Lancet*, **2**, 1457-1459 Etiology of Parkinson's disease

Capdevila, J., Chacos, N., Werringloer, J., Prough, R.A. & Estabrook, R.W., (1981) *Proc. Natl. Acad. Sci., (USA)*, **7** 8, 5362-5366 Liver microsomal cytochrome P-450 and the oxidative metabolism of arachadonic acid

Capdevila, J.H., Karara, A., Waxman, D.J., Martin, M.V., Falck, J.R. & Guengerich, F.P., (1990) *J. Biol. Chem.*, **2** 6 5, 10865-10871 Cytochrome P-450 enzyme-specific control of the regio- and enantiofacial selectivity of the microsomal arachadonic acid epoxygenase

- Carmichael, J., De Graft, W.G., Gazder, A.F., Minna, J.D. & Mitchell, J.B., (1987) *Cancer Research*, **47**, 936-942 Evaluation of a tetrazolium based semi-automated colorimetric assay: assessment of chemosensitivity testing
- Celier, C. & Cresteil, T., (1991) *Arch. Biochem. Biophys.*, **290**, 407-410 Control of cytochromes P450 expression in Gunn rat liver: implication of the intracellular heme pool
- Chahinian, A.P., Kirschner, P.A., Gordon, R.E., Szrajer, L. & Holland, J.F., (1991) *Cancer*, **68**, 558-560 Usefulness of the nude mouse model in mesothelioma based on a direct patient-xenograft comparison
- Chasserot-Golaz, S., Beck, G., Venetianer, A. & Corcos, L., (1990) *Biochem. Pharmacol.*, **40**, 1815-1819 Effect of phenobarbital on the glucocorticoid receptor in rat hepatoma cells
- Chen, T.R., (1977) *Exp. Cell Res.*, **104**, 255-262 In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoescht 33258 stain
- Chomczynski, P. & Sacchi, N., (1987) *Anal. Biochem.*, **162**, 156-159 Single-step method of RNA isolation by acid guanidinium thiocyanate - phenol - chloroform extraction
- Clark, A.J., Chare-Cox, A., Ma, X. & Bishop, J.O., (1985) *EMBO J.*, **4**, 3167-3171 Analysis of mouse major urinary protein genes: variation between the exonic sequences of Group 1 genes and a comparison with an active gene outwith group 1 both suggest that gene conversion has occurred between MUP genes
- Clement, B., Guguen-Guillouzo, C., Campion, J-P., Glaise, D., Bourel, M. & Guillouzo, A., (1984) *Hepatology*, **4**, 373-380 Long-term co-cultures of adult human hepatocytes with rat liver epithelial cells: Modulation of albumin secretion and accumulation of extracellular material
- Cobb, L.M. & Mitchley, B.C.V., (1974) *Cancer Chemother. Rep.*, **58**, 645-651 Development of a method for assessing the antitumour activity of chemotherapeutic agents using human tumour xenografts
- Colby, H.D., (1980) *Adv. Sex Hormone Res.*, **4**, 27-71 Regulation of hepatic drug and steroid metabolism by androgens and estrogens
- Coles, B. & Ketterer, B., (1990) *CRC Crit. Rev. Biochem. Mol. Biol.*, **25**, 47-70 The role of glutathione and glutathione transferases in chemical carcinogenesis
- Conney, A.H., (1982) *Cancer Research.*, **42**, 4875-4917 Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G.H.A. Clowes memorial lecture
- Corcos, L. & Weiss, M.C., (1988) *FEBS Lett.*, **233**, 37-40 Phenobarbital, dexamethasone and benzanthracene induce several cytochrome P450 mRNAs in rat hepatoma cells

- Correia, M.A., (1991) *Methods Enzymol.*, **206**, 315-325 Cytochrome P450 turnover
- Cox, R. A. (1986) *Methods Enzymol.*, **12**, 120-129 The use of guanidinium chloride in the isolation of nucleic acids
- Crespi, C.L., Penman, B.W., Leakey, J.A., Arlotto, M.P., Stark, A., Parkinson, A., Turner, T., Steimel, D.T., Rudo, K. & Davies, R.L., (1990) *Carcinogenesis*, **11**, 1293-1300 Human cytochrome P450IIA3: cDNA sequence, role of the enzyme in the metabolic activation of promutagens, comparison to nitrosamine activation by human cytochrome P450IIE1
- Cresteil, T., Matin, J.L., Dansette, P.M. & Leroux, J.P., (1980) *Biochem. Pharmacol.*, **29**, 1127-1133 *In vivo* administration of hydroxylated phenobarbital metabolites: effect on rat hepatic cytochromes P-450, epoxide hydrolase and UDP-glucuronosyltransferase
- Daughaday, W.H., (1989) *Perspect. Biol. Med.*, **52**, 194-211 Personal history of the origin of the somatomedin hypothesis and recent challenges to its validity
- Davies, R.L., Crespi, C.L., Kudo, K., Turner, T.R. & Langenbach, R., (1989) *Carcinogenesis*, **10**, 885-891 Development of a human cell line by selection and drug-metabolizing gene transfection with increased capacity to activate promutgens
- Dawson, J.R., Adams, D.J. & Wolf, C.R., (1985) *FEBS Lett.*, **183**, 219-222 Induction of drug metabolising enzymes in human liver cell line Hep G2
- Dearfield, K.L., Jackson-Kram, D., Juber, B.E. & Williams, J.R., (1986) *Biochem. Pharmacol.*, **35**, 2199-2205 Indication of sister chromatid exchanges in human and rat hepatoma cell lines by cyclophosphamide and phosphoramidate mustard and the effects of cytochrome P-450 inhibitors
- Decad, G.M., Hsien, D.P.H. & Byard, J.L., (1977) *Biochem. Biophys. Res. Comm.*, **78**, 279-287 Maintenance of cytochrome P-450 and metabolism of Aflatoxin B₁ in primary hepatocyte cultures
- DeLuca, H.F., (1982) *Biochem. Soc. Trans.*, **10**, 147-158 Metabolism and molecular mechanism of action of Vitamin D
- De Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R. & Subrami, S., (1987) *Mol. Cell. Biol.*, **7**, 725-737 Firefly luciferase gene: Structure and expression in mammalian cells
- Degawa, M., Miura, S. & Hashimoto, Y., (1991) *Carcinogenesis*, **12**, 2151-2156 Expression and induction of cytochrome P450 isozymes in hyperplastic nodules of rat liver

Distlerath, L.M., Reilly, P.E.B., Martin, M.V., Davis, G.G., Wilkinson, G.R. & Guengerich, F.P., (1985) *J. Biol. Chem.*, **260**, 9057-9067 Purification and characterisation of the human liver cytochromes P-450 involved in debrisoquine 4-hydroxylation and phenacetin O-deethylation, two prototypes for genetic polymorphism in oxidative drug metabolism

Doll, R. & Peto, R., (1981) *Causes of Cancer*, Oxford University Press, Oxford

Donato, M.T., Gomez-Lochon, M.J. & Castell, J.V., (1990) *Biochem. Pharmacol.*, **39**, 1321-1326 Effect of xenobiotics on monooxygenase activities in cultured human hepatocytes

Donato, M.T., Castell, J.V. & Gomez-Lochon, M.J., (1991) *Cell Biology and Toxicology*, **7**, 1-14 Co-cultures of hepatocytes with epithelial-like cell lines: expression of drug-biotransformation activities by hepatocytes

Doostder, H., Burke, M.D., Melvin, W.T. & Grant, M.H., (1991) *Biochem. Pharmacol.*, **42**, 1307-1313 The effects of dimethylsulphoxide and 5-aminolaevulinic acid on the activities of cytochrome P450-dependent mixed function oxidase and UDP-glucuronosyltransferase activities in human HepG2 hepatoma cells

Dragani, T.A., Manenti, G., Galliani, G. & Della Porta, G., (1985) *Carcinogenesis*, **6**, 225-228 Promoting effects of 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene in mouse hepatocarcinogenesis

Earnshaw, D., Dale, J.W., Goldfarb, P.S. & Gibson, G.G., (1988) *FEBS Lett.*, **236**, 357-361 Differential splicing in the 3' non-coding region of rat cytochrome P452 (P450 IVA1) mRNA

Eden, S., (1979) *Endocrinology*, **105**, 555-560 Age- and sex-related differences in episodic growth hormone secretion in the rat

Eichelbaum, M. & Gross, A.S., (1990) *Pharmacol. Ther.*, **46**, 377-394 The genetic polymorphism of debrisoquin/sparteine metabolism - clinical aspects

Eisen, H.J., Hannah, R.R., Legraverend, C., Okey, A.B., & Nebert, D.W., (1983) The Ah receptor: Controlling factor in the induction of drug-metabolising enzymes by certain chemical carcinogens and other environmental pollutants pp227-252 In: "Biochemical action of hormones", Litwack, G., (ed), Academic Press, New York

Eliasson, E., Johansson, I. & Ingelman-Sundberg, M., (1990) *Proc. Natl. Acad. Sci., (USA)*, **87**, 3225-3229 Substrate-, hormone-, and cAMP-regulated cytochrome P450 degradation

Erickson, L.C., Ramonas, L.M., Zaharko, D.S. & Kohn, K.W., (1980) *Cancer Research*, **40**, 4216-4220 Cytotoxicity and DNA cross-linking activity of 4-sulfidocyclophosphamides in mouse leukaemia cells in vitro

Estabrook, R.W., Franklin, M.R., Cohen, B., Shigamatzu, A. & Hildebrandt, A.G., (1971) *Metabolism*, **20**, 187-199 Influence of hepatic microsomal mixed function oxidation reactions on cellular metabolic control

Estabrook, R.W., Mason, J.I., Simpson, E.R., Peterson, J.A. & Waterman, M.R., (1991) *Adv. Enzyme Regul.*, **31**, 365-383 The heterologous expression of the cytochromes P450: a new approach for the study of enzyme activities and regulation

Evans, R.M., (1988) *Science*, **240**, 889-895 The steroid and thyroid hormone receptor superfamily

Eyer, C.S. & Backes, W.L., (1992) *Arch. Biochem. Biophys.*, **293**, 231-240 Relationship between the rate of reductase-cytochrome P450 complex formation and the rate of first electron transfer

Feinberg, A.P. & Vogelstein, B., (1983) *Anal. Biochem.*, **136**, 6-13; (1984) *Anal. Biochem.* (Addendum), **137**, 266-267 A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity

Fischer, M.T. & Sligar, S.G., (1983) *J. Am. Chem. Soc.*, **107**, 5018-5019 Control of heme redox potential and reduction rate: a linear free energy relation between potential and ferric spin state equilibrium

Fogh, J. & Trempe, G., (1975) New human tumour cell lines, pp115-159, In: "Human tumour cell lines *in vitro*" Fogh, J., (ed), Plenum Press, London

Fonne, R. & Meyer, U.A., (1987) *Pharmac. Ther.*, **33**, 19-22, Mechanisms of phenobarbital-type induction of cytochrome P-450 isozymes

Fonne-Pfister, R., Bargetzi, M.J.A. & Meyer, U.A., (1987) *Biochem. Biophys. Res. Comm.*, **148**, 1144-1150 MPTP the neurotoxin inducing Parkinson's disease, is a potent inhibitor of human and rat P450 enzymes (P450_{buf1}, P450_{db1}) catalysing debrisoquine 4-hydroxylation

Fornander, T.L., Cedermark, B., Mattsson, A., Skoog, L., Theve, T., Askergren, J., Rutqvist, L.E., Glas, U., Silfersward, C., Somell, A., Wilking, N. & Hjalmar, M-L., (1989) *Lancet*, **1**, 117-119 Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers

Forrester, L.M., Hayes, J.D., Millis, R., Barnes, D., Harris, A.L., Schlager, J.J., Powis, G. & Wolf, C.R., (1990) *Carcinogenesis*, **11**, 2163-2170 Expression of glutathione S-transferase and cytochrome P450 in normal and tumour breast tissue

Forrester, L.M., Neal, G.E., Judah, D.J., Glancey, M.G. & Wolf, C.R., (1990) *Proc. Natl. Acad. Sci., (USA)* **87**, 8306-8310 Evidence for involvement of multiple forms of cytochrome P-450 in aflatoxin B₁ metabolism in human liver

Forrester, L.M., Henderson, C.J., Glancey, M.G., Back, D.J., Park, B.K., Ball, S.E., Kitteringham, N.R., McLaren, A.W., Miles, J.S., Skett, P. & Wolf, C.R., (1992) *Biochem. J.*, **281**, 359-368 Relative expression of cytochrome P450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics

Freeman, J.E., Stirling, D., Russell, A.L. & Wolf, C.R., (1992) *Biochem. J.*, **281**, 689-695 cDNA sequence, deduced amino acid sequence, predicted gene structure and chemical regulation of mouse Cyp2e1

Freshney, R.I., (1987, 1992) *Culture of animal cells: A manual of basic technique*, Liss, A.R., Inc

Frey, A.B., Rosenfeld, M.G., Dolan, W.J., Adesnik, M. & Kreibich, G., (1984) *J. Cellular Physiol.*, **120**, 169-180 Induction of cytochrome P-450 isozymes in rat hepatoma-derived cell cultures

Friedberg, T., Waxman, D.J., Atchison, M., Kumar, A., Haaparanta, T., Raphael, C. & Adesnik, M., (1986) *Biochemistry* **25**, 7975-7983 Isolation and characterisation of cDNA clones for cytochromes P-450 immunochemically related to rat hepatic P-450 form PB-1

Fujisawa-Sehara, A., Sogawa, K., Nishi, C. & Fujii-Kuriyama, Y., (1986) *Nucleic Acids Res.*, **14**, 1465-1477 Regulating DNA elements localised remotely upstream from the drug-metabolising cytochrome P-450c gene

Fujisawa-Sehara, A., Sogawa, K., Yamano, M. & Fujii-Kuriyama, Y., (1987) *Nucleic Acids Res.*, **15**, 4179-4191 Characterisation of xenobiotic responsive elements upstream from the drug-metabolising cytochrome P-450c gene: a similarity to glucocorticoid regulatory elements

Fujisawa-Sehara, A., Yamano, M. & Fujii-Kuriyama, Y., (1988) *Proc. Natl. Acad. Sci., (USA)*, **85**, 5859-5863 A DNA-binding factor specific for xenobiotic responsive elements of P-450c exists as a cryptic form in cytoplasm: its possible translocation to nucleus

Gandolfi, A.J., White, R.D., Sipes, I.G. & Poul, L.R., (1980) *J. Pharmacol. Exp. Ther.*, **414**, 721 Bioactivation and covalent binding of halothane in vitro: studies with [³H]- and [¹⁴C]-halothane

Gehly, E.B., Fahl, W.E., Jefcoate, C.R., Heidelberger, C., (1979) *J. Biol. Chem.*, **254**, 5041-5048 The metabolism of benzo(a)pyrene by cytochrome P-450 in transformable and non-transformable C3H mouse fibroblasts

Ghezzi, P., Donelli, M.G., Panticrotto, C., Facchinetti, T. & Grattini, S., (1981) *Biochem. Pharmacol.*, **30**, 175-177 Evidence for covalent binding of adriamycin to rat liver microsomal proteins

Gonzalez, F.J., Mackenzie, P.I., Kimura, S., Nebert, D.W., (1984) *Gene*, **29**, 281-292 Isolation and characterisation of full-length mouse cDNA and genomic clones of 3-methylcholanthrene-inducible cytochrome P₁-450 and P₃-450

Gonzalez, F.J., Kimura, S. & Nebert, D.W., (1985) *J. Biol. Chem.*, **260**, 5040-5049 Comparison of the flanking regions and introns of the mouse 2,3,7,8-tetrachlorodibenzo-p-dioxin-inducible cytochrome P₁-450 and P₃-450 genes

Gonzalez, F.J., Song, B.J. & Hardwick, J.P., (1986) *Mol. Cell Biol.*, **6**, 2969-2976
Pregnenolone 16 α carbonitrile-inducible P-450 gene family: gene conversion and differential regulation

Gonzalez, F.J., Matsunaga, T., Nagata, K., Meyer, U.A., Nebert, D.W., Patsekwa, J., Kozak, C.A., Gillette, J., Gelboin, H.V. & Hardwick, J.P., (1987) *DNA*, **6**, 149-161
Debrisoquine 4-hydroxylase: characterisation of a new P450 subfamily, regulation, chromosomal mapping, and molecular analysis of the DA rat polymorphism

Gonzalez, F.J., Skoda, R.C., Kimura, S., Umeno, M., Zanger, U.M., Nebert, D.W., Gelboin, H.V., Hardwick, J.P. & Meyer, U.A., (1988) *Nature*, **331**, 442-446
Characterisation of the common genetic defect in humans deficient in debrisoquine metabolism

Gonzalez, F.J., Vibois, F., Hardwick, J.P., McBride, O.W., Nebert, D.W., Gelboin, H.V. & Meyer, U.A., (1988) *Genomics*, **2**, 174-179
Human debrisoquine 4-hydroxylase (P450IID1): cDNA and deduced amino acid sequence and assignment of the CYP2D locus to chromosome 22

Gonzalez, F.J. & Nebert, D.W., (1990) *Trends in Genetics*, **6**, 182-186
Evolution of the P450 gene superfamily: animal-plant "warfare", molecular drive and human genetic differences in drug oxidation

Gonzalez, F.J., (1990) *Pharmacol. Ther.*, **45**, 1-38
Molecular genetics of the P-450 superfamily

Gorman, C.M., Moffat, L.F. & Howard, B.H., (1982) *Mol. Cell Biol.*, **2**, 1044-1051
Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells

Gotoh, O., (1992) *J. Biol. Chem.*, **267**, 83-90
Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analysis of amino acid and coding nucleotide sequences

Göttlicher, M., Widmark, E., Li, Q. & Gustafsson, J-A., (1992) *Proc. Natl. Acad. Sci. (USA)*, **89**, 4653-4657
Fatty acids activate a chimera of the clofibrilic acid-activated receptor and the glucocorticoid receptor

Gough, A.C., Miles, J.S., Spurr, N.K., Moss, J.E., Gaedigk, A., Eichelbaum, M. & Wolf, C.R., (1990) *Nature*, **347**, 773-776
Identification of the primary gene defect at the cytochrome P450 CYP2D6 locus

Goulet, F., Normand, C. & Morin, O., (1988) *Hepatology*, **8**, 1010-1018
Cellular interactions promote tissue-specific function, biomatrix deposition and junctional communication of primary cultured hepatocytes

Grant, M.H., Burke, M.D., Hawksworth, G.M., Duthie, S.J., Engeset, J. & Petrie, J.C., (1987) *Biochem. Pharmacol.*, **34**, 2311-2316
Human adult hepatocytes in primary monolayer culture: Maintenance of mixed function oxidase and conjugation pathways of drug metabolism

Grant, M.H., Duthie, S.J., Gray, A.G. & Burke, M.D., (1988) *Biochem. Pharmacol.*, **37**, 4111-4116 Mixed function oxidase and UDP-glucuronosyl transferase activities in the human HepG2 hepatoma cell line

Green, S. & Chambon, P., (1988) *Trends Genetics*, **4**, 309-314 Nuclear receptors enhance our understanding of transcription regulation

Guengerich, F.P., Mueller-Enoch, D. & Blair, I.A., (1986) *Mol. Pharmacol.*, **30**, 287-295 Oxidation of quinidine by human liver cytochrome P-450

Guengerich, F.P., (ed) (1987) *Mammalian Cytochrome P450*, Vols.I & II, CRC Press, Boca Raton

Guengerich, F.P., (1989) *Ann. Rev. Pharmacol. Toxicol.*, **29**, 241-264 Characterisation of human microsomal cytochrome P-450 enzymes

Guguen-Guillouzo, C., Clement, B., Baffet, G., Beaumont, C., Morel-Chany, E., Glaise, D. & Guillouzo, A., (1983) *Exp. Cell Res.*, **143**, 47-54 Maintenance and reversibility of active albumin secretion by adult rat hepatocytes co-cultured with another liver epithelial cell type

Guillouzo, A., LeBigot, J.F., Guguen-Guillouzo, C. & Kiechel, J.R., (1982) *Biochem. Pharmacol.*, **31**, 2427-2430 Presence of Phase I and Phase II drug metabolising enzymes in cultured human foetal hepatocytes

Gustafsson, J-A., (1978) *Methods in Enzymology*, **52**, 377-388 Steroid hydroxylations catalysed by cytochrome P450

Gustafsson, J-A., Mode, A., Norstedt, G., Hokfelt, T., Sonnenschein, C., Eneroth, P. & Skett, P., (1980) The hypothalamo-pituitary-liver axis, a new hormonal system in control of hepatic steroid metabolism pp47-89 In: "Biochemical actions of hormones", Volume 7, Litwack, G., (ed), Academic Press, New York

Guzelian, P.S., Li, D., Scheutz, E.G., Thomas, P., Levin, W., Mode, A. & Gustafsson, J-A., (1988) *Proc. Natl. Acad. Sci., (USA)*, **85**, 9783-9787 Sex change in cytochrome P-450 phenotype by growth hormone treatment of adult rat hepatocytes maintained in a culture system on matrigel

Ham, J., Thompson, A., Needham, M., Webb, P. and Parker, M.G., (1988) *Nucleic Acids Res.*, **16**, 5263-5276 Characterisation of response elements for androgens, glucocorticoids and progestins in mouse mammary tumour virus

Hammond, D.K. & Strobel, H.W., (1990) *Mol. Cell. Biochem.*, **93**, 95-105 Human colon tumor cell line LS174T drug metabolizing system

Han, X., Lyle, R., Eustace, D.L.S., Jewers, R.J., Parrington, J.M., Das, A., Chana, T., Dagg, B., Money, S., Bates, T.D., Kenney, A. & Heyderman, E., (1991) *Br. J. Cancer*, **64**, 645-654 XH1 - a new cervical carcinoma cell line and xenograft model of tumour invasion, "metastasis" and regression

- Hankinson, O., (1979) Proc. Natl. Acad. Sci., (USA), **7 6**, 373-376 Single-step selection of clones of a mouse hepatoma line deficient in aryl hydroxylase
- Hankinson, O., Brooks, B.A., Weir-Brown, K.I., Hoffman, E.C., Johnson, B.S., Nanthur, J., Reyes, H. & Watson, A.J., (1991) Biochimie, **7 3**, 61-66 Genetic and molecular analysis of the Ah receptor and of Cyp1a1 gene expression
- Hansen, S.K., Ross, J.A., Siegfried, J.M., Leavitt, S., Kudo, K., Langenbach, R. & Neshow, S., (1989) Molecular Carcinogenesis, **2**, 261-267 Transfection of a rat cytochrome P450b cDNA into C3H10T1/2 Cl8 mouse embryo fibroblasts
- Hapgood, J., Cutlill, S., Denis, M., Poellinger, L. & Gustafsson, J.A., (1989) Proc. Natl. Acad. Sci., (USA), **8 6**, 60-64 Specific protein-DNA interactions at a xenobiotic-responsive element: Co-purification of dioxin receptor and DNA-binding activity
- Harada, N. & Negishi, M., (1984) J. Biol. Chem., **2 5 9**, 12285-12287 Mouse liver testosterone 16 α -hydroxylase (Cytochrome P-450_{16 α}): Purification, regioselectivity, stereospecificity and immunological characterisation
- Hapgood, J., Cuthill, S., Denis, M., Poellinger, L. & Gustafsson, J.A., (1989) Proc. Natl. Acad. Sci., (USA), **8 6**, 60-64 Specific protein DNA-interactions at a xenobiotic-response element: Co-purification of dioxin receptor and DNA-binding affinity
- Hardwick, J.P., Gonzalez, F.J. & Kasper, C.B., (1983) J. Biol. Chem., **2 5 8**, 8081-8085 Transcriptional regulation of rat liver epoxide hydratase, NADPH-cytochrome P-450 oxidoreductase, and cytochrome P-450b genes by phenobarbital
- Hayes, J.D. & Mantle, T., (1986) Biochem. J., **2 3 3**, 779-788 Use of immunoblot techniques to discriminate between the glutathione S-transferases Yf, Yk, Ya, Yn/Yb and Yc subunits and to study their distribution in extrahepatic tissues
- He, J. & Fulco, A.J., (1991) J. Biol. Chem., **2 6 6**, 7864-7869 A barbiturate-regulated protein binding to a common sequence in the cytochrome P450 genes of rodents and bacteria
- Henderson, C.J., Scott, A.R. & Wolf, C.R., (1990) Biochem. J., **2 6 6**, 675-681 Testosterone-mediated regulation of mouse renal cytochrome P-450 isoenzymes
- Henderson, C.J. & Wolf, C.R., (1991) Biochem. J., **2 7 8**, 499-503 Evidence that the androgen receptor mediates sexual differentiation of mouse renal cytochrome P450 expression
- Henry, H.L. & Norman, A.W., (1984) Ann. Rev. Nutr., **4**, 493-520 Vitamin D: metabolism and biological effects
- Heppner, G.H., (1984) Cancer Res., **4 4**, 2259-2265 Tumor heterogeneity

Herrington, C.S., Flannery, D.M.J., McGee, J.O'D. (1990) In: "In Situ Hybridisation", Polak, J.M. & McGee, J.O'D., (eds), Oxford, University Press, Oxford

Hildebrand, C.E., Gonzalez, F.J., Kozak, C.A., Nebert, D.W., (1985) Biochem. Biophys. Res. Comm., **130**, 396-406 Regional linkage analysis of the dioxin-inducible P-450 gene family on mouse chromosome 9

Hinson, J.A., Pohl, L.R., Monks, T.J. & Gillette, J.R., (1981) Life Sci., **29**, 107-116 Acetaminophen-induced hepatotoxicity

Hoffman, E.C., Reyes, H., Chu, F.F., Sander, F., Conley, L.H., Brooks, B.A. & Hankinson, O., (1991) Science, **252**, 954-958 Cloning of a factor required for activity of the Ah (dioxin) receptor

Homberg, J-C., Andre, C. & Abauf, N., (1984) Clin. Exp. Immunol., **55**, 561-570 A new anti-liver-kidney microsomal antibody (anti-LKM-2) in tienilic acid-induced hepatitis

Honkakoski, P., Autio, S., Juvonen, R., Raunio, H., Gelboin, H.V., Park, S.S., Pelkonen, O. & Lang, M.A., (1988) Arch. Biochem. Biophys., **267**, 589-598 Pyrazole is different from acetone and ethanol as an inducer of the polysubstrate monooxygenase system in mice: evidence that pyrazole-inducible P450_{Coh} is distinct from acetone-inducible P450_{ac}

Honkakoski, P. & Lang, M.A., (1989) Arch. Biochem. Biophys., **273**, 42-57 Mouse liver phenobarbital-inducible P450 system: purification, characterisation and differential inducibility of four cytochrome P450 isozymes from the D2 mouse

Hu, M.C.T. & Davidson, N., (1990) Mol. Cell Biol., **10**, 6141-6151 A combination of derepression of the *lac* operator-repressor system with positive induction by glucocorticoid and metal ions provides a high-level-inducible gene expression system based on the human metallothionein-II_A promoter

Ichikawa, T., Itakura, T. & Negishi, M., (1989) Biochemistry, **28**, 4779-4784 Functional characterisation of two cytochrome P450s within the mouse male-specific steroid 16 α -hydroxylase gene family: Expression in mammalian cells and chimeric proteins

Iino, Y., Wolf, D.M., Langan-Fahey, S.M., Johnson, D.A., Ricchio, M., Thompson, M.E. & Jordan, V.C., (1991) Br. J. Cancer **64**, 1019-1024 Reversible control of oestradiol-stimulated growth of MCF-7 tumours by tamoxifen in the athymic mouse

Israel, D.I., Estolano, M.G., Galeazzi, D.R. & Whitlock, J.P., (1985) J. Biol. Chem., **260**, 5648-5653 Superinduction of cytochrome P₁-450 gene transcription by inhibition of protein synthesis in wild-type and variant mouse hepatoma cells

Issemann, I. & Green, S., (1990) Nature, **347**, 645-650 Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators

Iwasaki, M., Juvonen, R., Lindberg, R. & Negishi, M., (1990) *J. Biol. Chem.*, **266**, 3380-3382 Alteration of high and low spin equilibrium by a single mutation of amino acid 209 in mouse cytochromes P450

Jaiswal, A.K., Gonzalez, F.J. & Nebert, D.W., (1985) *Nucleic Acids Res.*, **13**, 4503-4520 Human P₁-450 gene sequence and correlation of mRNA with genetic differences in benzo[a]pyrene metabolism

Jaiswal, A.K., Haaparanta, T., Luc, P.V., Schembri, J. & Adesnik, M., (1990) *Nucleic Acids Res.*, **18**, 4327-4342 Glucocorticoid regulation of a phenobarbital-inducible cytochrome P-450 gene: the presence of a functional glucocorticoid response element in the 5'-flanking region of the CYP2B2 gene

Jalacot, F., Simon, I., Dreano, Y., Beaune, P., Rioche, C. & Berthon, F., (1991) *Biochem. Pharmacol.*, **41**, 1911-1919 Identification of the cytochrome P450III_A family of the enzymes involved in the N-demethylation of tamoxifen in human liver microsomes

Jesson, M.I., Johnston, J.B., Anhalt, C.D. & Begleiter, A., (1987) *Cancer Research*, **47**, 5935-5938 Effects of 3'-(3-cyano-4-morpholinyl)-3'-deamino adriamycin and structural analogues on DNA in HT-29 human colon cancer cells

Jeudes, M.J., Bulger, W.H. & Kupfer, D., (1987) *Drug Metab. Dispos.*, **15**, 786-793 Monooxygenase-mediated activation of chlorotrianisene (TACE) in covalent binding of rat hepatic microsomal proteins

Jeudes, M.J. & Kupfer, D., (1990) *Drug Metab. Dispos.*, **18**, 131-137 Role of P-450c in the formation of a reactive intermediate of chlorotrianisene (TACE) by hepatic microsomes from methylcholanthrene treated rats

Johansson, I., Eskman, G., Scholte, B., Puzychi, D., Jornvall, H. & Ingelman-Sunberg, M., (1988) *Biochemistry*, **27**, 1925-1934 Ethanol-, fasting- and acetone-inducible cytochromes P-450 in rat liver: regulation and characteristics of enzymes belonging to the IIB and IIE gene subfamilies

Johansson, I., Eliasson, E., Johansson, A., Hagbjorg, A-L., Linders, K. & Ingelman-Sunberg, M., (1989) pp 592-595 In: "Cytochrome P450 Biochemistry and Biophysics", Schuster, I., (ed), Taylor & Francis, London

Johnson, E.F., Kronbach, T. & Hsu, M-H., (1992) *Faseb J.*, **6**, 700-705 Analysis of the catalytic specificity of cytochrome P450 enzymes through site-directed mutagenesis

Johnson, P.F., (1990) *Cell growth & differentiation*, **1**, 47-52 Transcriptional activators in hepatocytes

Jordan, V.C., (1982) *Breast Cancer Res. Treatment*, **3**, 123-138 Metabolites of tamoxifen in animals and man: identification, pharmacology and significance

- Jordan, V.C., (1983) *Breast Cancer Res. Treat.*, **3**, 73-86 Laboratory studies to develop general principles for the adjuvant treatment of breast cancer with antioestrogens: problems and potential for future clinical applications
- Jordan, V.C., (1990) *J. Natl. Cancer Inst.*, **82**, 1662-1663 Estrogen-receptor-mediated direct and indirect antitumour effects of tamoxifen
- Jordan, V.C. & Robinson, S.P., (1987) *Fed. Proc.*, **46**, 1870-1874 Species-specific pharmacology of anti-oestrogens: role of metabolism
- Juvonen, K.O., Kaipainen, P.K. & Lang, M.A., (1985) *Eur. J. Biochem.*, **152**, 3-8 Selective induction of coumarin 7-hydroxylase by pyrazole in D₂ mice
- Karara, A., Dishman, E., Blair, I., Falck, J.R. & Capdevila, J.H., (1989) *J. Biol. Chem.*, **264**, 19822-19827 Endogenous epoxyeicosatrienoic acids. Cytochrome P450 controlled stereoselectivity of the hepatic arachadonic acid epoxygenase
- Kärenlampi, S.O., Tuomi, K., Korkalainen, M. & Raunio, H., (1989) *Biochem. Pharmacol.*, **38**, 1517-1525 Induction of cytochrome P4501A1 in mouse hepatoma cells by several chemicals: Phenobarbital and TCDD induce the same form of cytochrome P450
- Kasai, H., Okadi, Y., Nishimura, S., Rao, M.S. & Reddy, J.K., (1989) *Cancer Research*, **49**, 2603-2605 Formation of 8-hydroxydeoxyguanosine in liver DNA of rats following long-term exposure to a peroxisome proliferator
- Kawajiri, K., Youkawa, H., Harada, N., Noshiro, M., Omura, T. & Tagershira, Y., (1980) *Cancer Res.*, **40**, 1652-1657 Immunochemical study on the role of different types of microsomal P-450 in mutagenesis by chemical carcinogens
- Kawajiri, K., Nakachi, K., Imai, K., Hayashi, S-I. & Watanabe, J., (1991) Individual differences in lung cancer susceptibility in relation to polymorphisms of P4501A1 gene and cigarette dose pp55-61 In: "Xenobiotics and cancers", Ernster, L., Esumi, H., Furi, Y., Gelboin, H.V., Kato, R. & Sugimura, T., (eds) Japan Scientific Societies Press, Tokyo
- Kelley, M., Lambert, I., Merrill, J. & Safe, S., (1985) *Biochem. Pharmacol.*, **19**, 3489-3494 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) and related compounds as inducers of hepatic mono-oxygenases: Structure-activity effects
- Kelley, M., Womack, J. & Safe, S., (1990) *Biochem. Pharmacol.*, **39**, 1991-1998 Effects of cytochrome P-450 monooxygenase inducers on mouse hepatic microsomal metabolism of testosterone and alkoxyresorufins
- Kelly, K., Rospendowski, B.N., Smith, W.E. & Wolf, C.R., (1987) *FEBS Lett.*, **222**, 120-124 Surface enhanced resonance Raman as a probe of the spin state of structurally related cytochromes P-450 from rat liver

Kemp, J.V., Adam, H.K., Wakeling, A.E. & Slater, R., (1983) *Biochem. Pharmacol.*, **3 2**, 2045-2052 Identification and biological activity of tamoxifen metabolites in human serum

Kende, A.S., Ebetino, F.H., Drendel, W.B., Sundralingham, M., Glover, E. & Poland, A., (1985) *Mol. Pharmacol.*, **2 8**, 445-453 Structure-activity relationship of bispyridyloxybenzenes for induction of mouse hepatic aminopyrine N-demethylase activity: Chemical, biological and X-Ray crystallographic studies

Kikendall, J.W., Bowen, P.C., Burgess, M.B., Magnetti, C., Woodward, J. & Langenberg, C., (1989) *Gastroenterology*, **9 7**, 660-664 Cigarettes and alcohol as independent risk factors for colonic adenomas

Kim, S.G. & Novak, R.F., (1990) *Biochim. Biophys. Res. Comm.*, **1 6 6**, 1072-1079 Induction of rat hepatic P450IIE1 (CYP2E1) by pyridine: evidence for a role of protein synthesis in the absence of transcriptional activation

Kim, R., Hirabayashi, N., Nishiyama, M., Jinushi, K., Toge, T. & Okada, K., (1992) *Anticancer Research*, **1 2**, 241-246 Factors contributing to adriamycin sensitivity in human xenograft tumours: the relationship between expression of the MDR1, GST- Π and topoisomerase II genes and tumour sensitivity to adriamycin

Kimura, S., Gonzalez, F.J. & Nebert, D.W., (1984) *Nucleic Acids Research*, **1 2**, 2917-2928 Mouse cytochrome P₃-450: complete cDNA and amino acid sequence

Kimura, S., Gonzalez, F.J. & Nebert, D.W., (1984) *J. Biol. Chem.*, **2 5 9**, 10705-10713 The murine Ah locus: Comparison of the complete cytochrome P₁-450 and P₃-450 cDNA nucleotide and amino acid sequences

Kimura, S., Gonzalez, F.J., & Nebert, D.W., (1986) *Mol. Cell Biol.*, **6**, 1471-1477 Tissue-specific expression of the mouse dioxin-inducible P₁450 and P₃450 genes: differential transcriptional activation and mRNA stability in liver and extrahepatic tissues

Kimura, S., Donovan, J.C. & Nebert, D.W., (1987) *J. Exp. Pathology*, **3**, 61-74 Expression of the mouse P₁450 gene during differentiation without foreign chemical stimulation

Kimura, S., Umeno, M., Skoda, R.C., Meyer, U.A. & Gonzalez, F.J., (1989) *Am. J. Hum. Genet.*, **4 5**, 889-904 The human debrisoquine 4-hydroxylase (CYP2D) locus: sequence and identification of the polymorphic CYP2D6 gene, a related gene, and a pseudogene

Kitamura, R., Sato, K., Sawada, M., Hoh, S., Kitada, M., Komori, M. & Kamataki, T., (1992) *Arch. Biochem. Biophys.*, **2 9 2**, 136-140 Stable expression of cytochrome P450 IIIA7 cDNA in human breast cancer cell line MCF-7 and its application to cytotoxicity testing

Kleinman, H.K., McGarvey, M.L., Liotta, L.A., Robey, P.G., Tryggvason, K. & Martin, G.R., (1982) *Biochemistry*, **21**, 6188-6193 Isolation and characterisation of type IV procollagen, laminin and heparin sulfate proteoglycan from the EHS sarcoma

Klotz, U. & Reimann, I., (1980) *New Engl. J. Med.*, **302**, 1012-1014 Delayed clearance of diazepam due to cimetidine

Knodell, R.G., Holtzman, J.L., Crankshaw, D.L., Steele, N.M. & Stanley, L.N., (1982) *Gastroenterology*, **82**, 84-88 Drug metabolism by rat and human hepatic microsomes in response to interaction with H₂-receptor antagonists

Knodell, R.G., Browne, D.G., Gwozdz, G.P., Brian, W.R. & Guengerich, F.P., (1991) *Gastroenterology*, **101**, 1680-1691 Differential inhibition of individual human liver cytochromes P-450 by cimetidine

Knopf, J.L., Gallagher, J.F. & Held, W.A., (1983) *Mol. Cell Biol.*, **3**, 2232-2340 Differential, multihormonal regulation of the mouse major urinary protein gene family in the liver

Knowles, B.B., Howe, C.C & Aden, D.P., (1980) *Science*, **209**, 497-499 Human hepatocellular carcinoma cell lines secrete the major plasma proteins and Hepatitis B surface antigen

Koch, J.A. & Waxman, D.J., (1989) *Biochemistry*, **28**, 3145-3152 Posttranslational modification of hepatic cytochrome P-450. Phosphorylation of phenobarbital-inducible P-450 forms PB-4 (IIB1) and PB-5 (IIB2) in isolated rat hepatocytes and *in vivo*

Koracek, T.A., Scheutz, E.G. & Guzelian, P.S., (1990) *Mol. Pharmacol.*, **38**, 440-444 Differentiated induction of cytochrome P450b/e and P450p mRNAs by dose of phenobarbital in primary cultures of adult rat hepatocytes

Kouri, R.E., McKinney, C.E., Siomiany, D.J., Snodgrass, D.R., Wray, N.P. & McLemore, T., (1982) *Cancer Research*, **42**, 5030-5037 Positive correlation between high aromatic hydrocarbon hydroxylase activity and primary lung cancer as analysed in cryopreserved lymphocytes

Kouri, R.E., Lubet, R.A. & McKinney, C.E., (1983) Aryl hydrocarbon hydroxylase of mice and humans pp179-197 In: "Application of biological markers to carcinogen testing", Milman, H.A. & Sell, S., (eds) Plenum Press, New York

Kronbach, T., Kemper, B. & Johnson, E.F., (1991) *Biochemistry*, **30**, 6097-6102 A hypervariable region of P450IIC5 confers progesterone 21-hydroxylase activity to P450IIC1

Kremers, P., Goujon, F., De Graeve, F., Confort, J.P. & Gielen, J., (1981) *Eur. J. Biochem.*, **116**, 67-72 Multiplicity of cytochrome P-450 in primary fetal hepatocytes in culture

- Kupfer, A. & Preisig, R., (1984) *Eur. J. Clin. Pharmacol.*, **26**, 753
Pharmacogenetics of mephenytoin: A new drug hydroxylation polymorphism in man
- Lacroix, D., Desrochers, M., Lambert, M. & Naderson, A., (1990) *Gene*, **86**, 201-207
Alternative slicing of mRNA encoding rat liver cytochrome P450e (P450IIB2)
- Laemmli, U. K., (1970) *Nature (London)* **277**, 680-685
Cleavage of structural proteins during the assembly of the head of bacteriophage T4
- Lam, H. Y-P., (1984) *Biochem. Biophys. Res. Comm.*, **118**, 27-32
Tamoxifen is a calmodulin antagonist in the activation of cAMP phosphodiesterase
- Lambeth, J.D., Saybert, D.W. & Kamin, H., (1980) *J. Biol. Chem.*, **255**, 4667-4672
Adrenodoxin reductase . adrenodoxin complex. Rapid formation and breakdown of the complex and a slow conformational change in the flavoprotein
- Landaw, S.A., Callahan, E.W. & Schmid, R., (1970) *J. Clin. Invest.*, **49**, 914-925
Catabolism of heme *in vivo*: comparison of the simultaneous production of bilirubin and carbon monoxide
- Lang, M.A., Juvonen, R., Javvinen, P., Honkakoski, P. & Raunio, H., (1989) *Arch. Biochem. Biophys.*, **271**, 139-148
Mouse liver P450 Coh: Genetic regulation of the pyrazole-inducible enzyme and comparison with other P450 isoenzymes
- Lau, D.H.M., Lewis, A.D. & Sikic, B.I., (1989) *J. Natl. Cancer Inst.*, **81**, 1034-1038
Association of DNA cross-linking with potentiation of the morpholino derivative of doxorubicin by human liver microsomes
- Leader, D.P., Gall, I. & Lehrach, H., (1985) *Gene* **36**, 369-374
The structure of a cloned mouse γ -actin processed pseudogene
- LeBlanc, G.A. & Waxman, D.J., (1990) *Cancer Res.*, **50**, 5720-5726
Mechanisms of cyclophosphamide action on hepatic P-450 expression
- Lee, I.P. & Lucier, G.W., (1976) *J. Pharmacol. Exp. Ther.*, **196**, 586-593
The potentiation of barbiturate-induced necrosis by procarbazine
- Leeman, T., Dayer, P. & Meyer, U.A., (1986) *Eur. J. Clin. Pharmacol.*, **29**, 739-741
Single-dose quinidine treatment inhibits metoprolol oxidation in extensive metabolisers
- Lehrach, H., Diamond, D., Wozney, J.M. & Boedtker, H., (1977) *Biochemistry*, **16**, 4743-4751
RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical re-examination
- Leo, M.A., Iida, S. & Lieber, C.S., (1984) *Arch. Biochem. Biophys.*, **234**, 305-312
Retinoic acid metabolism by a system reconstituted with cytochrome P-450
- Leo, M.A. & Lieber, C.S., (1985) *J. Biol. Chem.*, **260**, 5228-5231
New pathway for retinol metabolism in liver microsomes

Lephart, E.D., Peterson, K.G., Noble, J.F., George, F.W. & McPhaul, M.J., (1990) *Mol. Cell Endocrinol.*, **70**, 31-40 The structure of cDNA clones encoding the aromatase P-450 isolated from a rat Leydig cell tumour line demonstrated differential processing of aromatase mRNA in rat ovary and a neoplastic cell line

Lewis, A.D., Hickson, I.D., Robson, C.N., Harris, A.L., Hayes, J.D., Griffiths, S.A., Manson, M.M., Hall, A.E., Moss, J.E. & Wolf, C.R. (1988) *Proc. Natl. Acad. Sci. (USA)*, **85**, 8511-8515 Amplification and increased expression of alpha class glutathione S-transferase encoding genes associated with resistance to nitrogen mustards

Lewis, A.D., Lau, D.H.M., Duran, G.E., Wolf, C.R. & Sikic, B.I., (1992) *Cancer Research*, in press, Role of cytochrome P450 from the human CYP3A gene family in the potentiation of morpholinodoxorubicin by human liver microsomes

Lillie, R.D., (1965), pp176-177, In: "Histopathologic Technique and Practical Histochemistry", McGraw-Hill, New York

Lindberg, R.L.P. & Negishi, M., (1989) *Nature*, **339**, 632-634 Alteration of mouse cytochrome P450 Coh substrate specificity by mutation of a single amino-acid residue

Lindberg, R., Burkhart, B., Ichikawa, T. & Negishi, M., (1989) *J. Biol. Chem.*, **264**, 6465-6471 The structure and characterisation of type I P-450 (15) alpha gene as major steroid 15 alpha-hydroxylase and its comparison with type II P-450 (15) alpha gene

Lindblad, W.J., Schuetz, E.G., Redford, K.S. & Guzelian, P.S., (1991) *Hepatology*, **13**, 282-288, Hepatocellular phenotype *in vitro* is influenced by biophysical features of the collagenous substratum

Lippmann, M.E. & Dickson, R.B., (1989) *Yale J. Biol. Med.*, **62**, 459-480 Studies on the urotoxicity of oxazophosphorine cytostatics and its prevention I. Experimental studies on the urotoxicity of alkylating compounds

Liu, G-T., (1991) *Drug Metab. Rev.*, **23**, 439-465 Effects of some compounds isolated from Chinese medicinal herbs on hepatic microsomal cytochrome P-450 and their potential biological consequences

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) *J. Biol. Chem.*, **193**, 265-275 Protein measurement with the Folin Phenol reagent

Lubet, R.A., Mayer, R.T., Cameron, J.W., Nims, R.W., Burke, M.D., Wolff, T. & Guengerich, F.P., (1985) *Arch. Biochem. Biophys.*, **238**, 43-48 Dealkylation of pentoxyresorufin: A rapid and sensitive assay for measuring induction of cytochrome(s) P-450 by phenobarbital and other xenobiotics in the rat

Lund, J., Zaphiropoulos, P.G., Mode, A., Warner, M. & Gustafsson, J-A., (1991) *Advances in Pharmacology*, **22**, 325-354 Hormonal regulation of cytochrome P-450 gene expression

- MacLeod, J.W. & Shapiro, B.H., (1989) *Biochem. Pharmacol.*, **38**, 1673-1677
Growth hormone regulation of hepatic drug-metabolising enzymes in the mouse
- Mahgoub, A., Idle, J.R., Dring, L.G., Lancaster, R. & Smith, R.L., (1977) *Lancet*, **2**, 584-588
Polymorphic hydroxylation of debrisoquine in man
- Maines, M.D. & Kappas, A., (1977) *Science*, **198**, 1215-1221
Metals as regulators of heme metabolism
- Malik, S.T.A., Griffin, D.B., Fiers, W. & Balkwill, F.R., (1989) *Int. J. Cancer*, **44**, 918-925
Paradoxical effects of tumour necrosis factor in experimental ovarian cancer
- Mani, C. & Kupfer, D., (1991) *Cancer Research*, **51**, 6052-6058
Cytochrome P-450-mediated activation and irreversible binding of the antioestrogen tamoxifen to proteins in rat and human liver: Possible involvement of flavin-containing monooxygenases in tamoxifen metabolism
- Manns, M., (1989) *J. Hepatol.*, **9**, 272-280
Autoantibodies and antigens in liver diseases
- Manns, M.P., (1991) *Immunol. Res.*, **10**, 503-507
Cytochrome P450 enzymes as human autoantigens
- Marsman, D.S., Cattley, R.C., Conway, J.G. & Popp, J.A., (1988) *Cancer Research*, **48**, 6739-6744
Relationship of hepatic peroxisome proliferation and replicative DNA synthesis to the hepatocarcinogenicity of the peroxisome proliferators Di (2-ethylhexyl)phthalate and [4-chloro-6-(2,3-xylidino)-2-pyrimidinyl thio] acetic acid (Wy 14,463) in rats
- Matsunaga, T., Nagata, K., Holsztynska, E.J., Lapenson, D.P., Smith, A., Kato, R., Gelboin, H.V., Waxman, D.J. & Gonzalez, F.J., (1988) *J. Biol. Chem.*, **263**, 17995-18002
Gene conversion and differential regulation in the rat P-450IIA gene subfamily
- Matsunaga, T., Nomoto, M., Kozak, C.A. & Gonzalez, F.J., (1990) *Biochemistry*, **29**, 1329-1341
Structure and *in vitro* transcription of the rat CYP2A1 and CYP2A2 genes and regional localisation of the CYP2A gene subfamily on mouse chromosome 7
- Maurice, M., Emiliani, S., Dalet, B.I., Derancourt, J. & Lange, R., (1991) *Eur. J. Biochem.*, **200**, 511-517
Isolation and characterisation of a cytochrome P-450 of the IIA subfamily from human liver microsomes
- May, H.E., Boose, R. & Reed, D.J., (1974) *Biochemistry*, **13**, 4723-4730
Microsomal monooxygenation of the carcinostatic 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea. Synthesis and identification of *cis* and *trans* monohydroxylated products
- Mays, D.C., Camisa, C., Cheney, P., Pacula, C.M., Nawoot, S. & Gerber, N., (1987) *Clin. Pharmacol. Ther.*, **42**, 621-626
Methoxsalen is a potent inhibitor of the metabolism of caffeine in humans

McGregor, D.B., Edwards, I., Wolf, C.R., Forrester, L.M. & Caspary, W.J., (1991) *Mutation Res.*, **261**, 29-39 Endogenous xenobiotic enzyme levels in mammalian cells

McLellan, L.I. & Hayes, J.D., (1989) *Biochem. J.*, **263**, 393-402 Differential induction of class α glutathione S-transferase by the anti-carcinogenic antioxidant butylated hydroxyanisole - purification and characterisation of glutathione S-transferase Ya₁ Ya₁

McLemore, T.L., Adelberg, S., Liu, M.C., McMahon, N.A., Yu, S.J., Hubbard, W.C., Czerwinski, M., Wood, T.G., Storeng, R., Lubet, R.A., Eggleston, J.C., Boyd, M.R. & Hines, R.N., (1990) *J. Natl. Cancer Inst.*, **82**, 1333-1339 Expression of CYP1A1 gene in patients with lung cancer: evidence for cigarette smoke-induced gene expression in normal lung tissue and for pulmonary carcinomas

McQueen, H.A., Wyllie, A.H., Piris, J., Foster, E. & Bird, C.C., (1991) *Br. J. Cancer*, **63**, 94-96 Stability of critical genetic lesions in human colorectal carcinoma xenografts

Meehan, R.R., Barlow, D.P., Hill, R.E., Hogan, B.L.M. & Hastie, N.D., (1984) *EMBO J.*, **3**, 1881-1885 Pattern of serum protein gene expression in mouse visceral yolk sac and foetal liver

Meehan, R.R., Forrester, L.M., Stevenson, K., Hastie, N.D., Buchmann, A., Kunz, H.W. & Wolf, C.R., (1988a) *Biochem. J.*, **254**, 789-797 Regulation of phenobarbital-inducible cytochrome P450s in rat and mouse liver following dexamethasone administration and hypophysectomy

Meehan, R.R., Speed, J.M., Gosden, J.R., Rout, D., Hutton, J.J., Taylor, B.A., Hilkens, J., Kroezen, V., Hilgers, J., Adesnik, M., Friedberg, T., Hastie, N.D. & Wolf, C.R., (1988b) *Proc. Natl. Acad. Sci. (USA)*, **85**, 2662-2666 Chromosomal organisation of the cytochrome P450 2C gene family in the mouse: a locus associated with constitutive aryl hydrocarbon hydroxylase activity

Meyer, U.A., Skoda, R.C. & Zanger, U.M., (1990) *Pharmacol. Ther.*, **46**, 297-308 The genetic polymorphism of debrisoquine/sparteine metabolism -molecular mechanisms

Miles, J.S., Spurr, N.K., Gough, A.C., Jowett, T., McLaren, A.W., Brook, J.D. & Wolf, C.R., (1988) *Nucleic Acids, Res.*, **16**, 5783-5795 A novel human cytochrome P450 gene (P450IIB): chromosomal localisation and evidence for alternative splicing

Miles, J.S., Bickmore, W.A., Brook, J.D., McLaren, A.W., Meehan, R.R. & Wolf, C.R., (1989) *Nucleic Acids Res.*, **17**, 2907-2919 Close linkage of the human cytochrome P450 IIA and P450IIB gene subfamilies: implications for the assignment of substrate specificity

Miles, J.S., Moss, J.E., Taylor, B.A., Burchell, B. & Wolf, C.R., (1991) *Genomics*, **1 1**, 309-316 Mapping genes encoding drug-metabolising enzymes in recombinant inbred mice

Miles, J.S., McLaren, A.W., Forrester, L.M., Glancey, M.J., Lang, M.A. & Wolf, C.R., (1990) *Biochem.J.*, **2 6 7**, 365-371 Identification of the human liver cytochrome P450 responsible for coumarin 7-hydroxylase activity

Miller, K.W. & Yang, C.S., (1984) *Arch. Biochem. Biophys.*, **2 2 9**, 483-491 Studies on the mechanisms of induction of N-nitrosodimethylamine demethylase by fasting, acetone and ethanol

Mitchell, A.M., Bridges, D.W. & Elcombe, C.R., (1984) *Arch.Tox.*, **5 5**, 239-246 Factors influencing peroxisome proliferation in cultured rat hepatocytes

Miwa, G.T., West, S.B., Huang, M.T. & Lu, A.Y.H., (1979) *J. Biol. Chem.*, **2 5 4**, 5695-5700 Studies on the association of cytochrome P-450 and NADPH-cytochrome c reductase during catalysis in a reconstituted hydroxylating system

Miwa, G.T. & Lu, A.Y.H., (1984) *Arch. Biochem. Biophys.*, **2 3 4**, 161-166 The association of cytochrome P-450 and NADPH-cytochrome P-450 reductase in phospholipid membranes

Miyata, Y. & Yahara, I., (1991) *J. Biol. Chem.*, **2 6 6**, 8779-8783 Cytoplasmic 8S glucocorticoid receptor binds to actin filaments through the 90kDa heat shock protein moiety

Monfardidi, S., Brunner, K., Crowther, D., Eckhardt, S., Olive, D., Tanneberger, S., Veronesi, A., Whitelouse, J.M.A. & Witte, R., (1987) *Manual of adult and paediatric medical oncology* International Union against Cancer, Springer-Verlag

Morin, O. & Normand, C., (1986) *J. Cell Physiol.*, **1 2 9**, 103-110 Long-term maintenance of hepatocyte functional activity in co-culture: requirements for sinusoidal endothelial cells and dexamethasone

Morin, O., Goulet, F. & Normand, C., (1988) *Revis. Biol. Cellular*, **1 5**, 1-85 Liver sinusoidal endothelial cells: isolation, purification, characterisation and interaction with hepatocytes

Morgan, E.T., Koop, D.R. & Coon, M.J., (1983) *Biochem. Biophys. Res. Comm.*, **1 1 2**, 8-13 Comparison of six rabbit liver cytochrome P-450 isozymes in formation of a reactive intermediate of acetaminophen

Morgan, E.T., MacGeoch, C. & Gustafsson, J-A., (1985) *J. Biol. Chem.*, **2 6 0**, 11895-11898 Hormonal and developmental regulation of expression of the hepatic microsomal steroid 16 α -hydroxylase cytochrome P-450 apoprotein in the rat

Morgenstern, J.P. & Land, H., (1990) *Nucleic Acids Res.*, **1 8**, 1068 A series of mammalian expression vectors and characterisation of their expression of a reporter gene in stably and transiently transfected cells

Moscow, J.A., Fairchild, C.R., Madden, M.J., Ransom, D.T., Wieand, H.S., O'Brien, E.E., Poplack, D.G., Cossman, J., Myers, C.E. & Cowe, K.H., (1989) *Cancer Research*, **49**, 1422-1428 Expression of anionic glutathione-S-transferase and P-glycoprotein genes in human tissues and tumours

Mossman, T., (1983) *J. Immunol. Methods.*, **65**, 55-63 Rapid colorimetric assay for cellular growth and survival: applications to proliferation and cytotoxicity

Murray, G.I., Foster, C.O., Barnes, T.S., Weaver, R.J., Ewen, S.W., Melvin, W.T. & Burke, M.D., (1991) *Br. J. Cancer*, **63**, 10021-1023 Expression of cytochrome P450IA in breast cancer

Murray, G.I., Foster, C.O., Barnes, T.S., Weaver, R.J., Snyder, C.P., Ewen, S.W.B., Melvin, W.T. & Burke, M.D., (1992) *Carcinogenesis*, **13**, 165-169 Cytochrome P450IA expression in adult and fetal human livers

Nebert, D.W., (1979) *Mol. Cell Biochem.*, **27**, 27-46 Multiple forms of inducible drug-metabolising enzymes: a reasonable mechanism by which any organism can cope with adversity

Nebert, D.W. & Jensen, N.M., (1979) *CRC Crit. Rev. Biochem.*, **6**, 401-437 The Ah locus: Genetic regulation of the metabolism of carcinogens, drugs and other environmental chemicals by cytochrome P-450 mediated monooxygenases

Nebert, D.W., Negishi, M., Lang, M.A., Hjelmeland, L.M. & Eisen, H.J., (1982) *Adv. Genet.*, **21**, 1-52 The Ah locus, a multigene family necessary for survival in a chemically adverse environment: Comparison with the immune system

Nebert, D.W. & Eisen, H.J., (1984) *Biochem. Pharmacol.*, **33**, 917-924 The Ah receptor: Binding specificity only for foreign chemicals?

Nebert, D.W., Adesnik, M., Coon, M.J., Estabrook, R.W., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Kemper, B., Levin, W., Philips, I.R., Sato, R. & Waterman, M.R., (1987) *DNA*, **6**, 1-11 The P450 gene superfamily: recommended nomenclature

Nebert, D.W., Nelson, D.R., Adesnik, M., Coon, M.J., Estabrook, R.W., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Kemper, B., Levin, W., Philips, I.R., Sato, R. & Waterman, M.R., (1989) *DNA*, **8**, 1-14 The P450 gene superfamily: update on listing of all genes and recommended nomenclature of chromosomal loci

Nebert, D.W., Nelson, D.R., Coon, M.J., Estabrook, R.W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Loper, J.C., Sato, R., Waterman, M.R. & Waxman, D.J., (1991) *DNA and Cell Biology*, **10**, 1-14 The P450 superfamily: Update on new sequences, gene mapping and recommended nomenclature

Nebert, D.W. & Gonzalez, F.J., (1987) *Annual Reviews in Biochemistry*, **56**, 945-993 P450 genes: Structure, evolution and regulation

- Negishi, M., Lindberg, R., Burkhart, B., Ichikawa, T., Honkakoski, P. & Lang, M.A., (1989) *Biochemistry*, **28**, 4169-4172 Mouse steroid 15 α -hydroxylase gene family: Identification of type II P-450_{15 α} as coumarin 7-hydroxylase
- Nelson, D.C., Avant, G.R., Speeg, K.V., Hoyumpa, A.M., Schenker, S., (1985) *Hepatology*, **5**, 305-309 The effect of cimetidine on hepatic drug elimination in cirrhosis
- Nelson, D.R. & Strobel, H.W., (1989) *Biochemistry*, **28**, 656-660 Secondary structure prediction of 52 membrane-bound cytochromes P-450 shows a strong structural similarity to P-450_{cam}
- Nemoto, N., Sakurai, J., Tazawa, A. & Ishikawa, T., (1990) *Cancer Research*, **50**, 3226-3230 Acquisition of aryl hydrocarbon hydroxylase inducibility by aromatic hydrocarbons in monolayer-cultured hepatocytes from non-responsive mouse strains
- Neuhold, L.A., Gonzalez, F.J., Jaiswal, A.K. & Nebert, D.W., (1986), *DNA*, **5**, 403-411 Dioxin-inducible enhancer region upstream from the mouse P(1)450 gene and interaction with a heterologous SV40 promoter
- Newaz, S.N., Fang, W.F. & Strobel, H.W., (1983) *Cancer*, **52**, 794-798 Metabolism of the carcinogen 1,2-dimethylhydrazine by isolated human colon microsomes and human colon tumor cells in culture
- Newman, S. & Guzelian, P.S., (1982) *Proc. Natl. Acad. Sci., (USA)*, **79**, 2922-2926 Stimulation of *de novo* synthesis of cytochrome P-450 by phenobarbital in primary nonproliferating cultures of adult rat hepatocytes
- Nhamburo, P.T., Kimura, S., McBride, O.W., Kozak, C.A., Gelboin, H.V. & Gonzalez, F.J., (1990) *Biochemistry*, **29**, 5491-5499 The human CYP2F gene subfamily: identification of a cDNA encoding a new cytochrome P450, cDNA-directed expression and chromosome mapping
- Nio, Y., Tseng, C., Tsubono, M., Kawabata, K., Masau, Y., Shiraishi, T., Fukomoto, M. & Tobe, T., (1991) *J. Surgical Oncology*, **48**, 252-259 Combining independantly effective anticancer agents may not augment the independent antitumor effects: A study using human digestive organ cancer xenograft lines in nude mice
- Niznik, H.B., Tyndale, R.F., Sallee, F.R., Gonzalez, F.J., Hardwick, J.P., Inaba, T. & Kalow, W., (1990) *Arch. Biochem. Biophys.*, **276**, 424-432 The dopamine transporter and cytochrome P450IID1 (debrisoquine 4-hydroxylase) in brain: resolution and identification of two distinct [³H] GBR-12935 binding proteins
- Noshiro, M., Lasko, M., Kawajiri, K. & Negishi, M., (1988) *Biochemistry*, **27**, 6434-6443 Rip locus: Regulation of female-specific isozyme (I-P-450_{16 α}) of testosterone 16 α -hydroxylase in mouse liver, chromosome localisation and cloning of P-450 cDNA

O'Brian, C.A., Liskamp, R., Solomon, D.H. & Weinstein, I.B. (1985) *Cancer Research*, **45**, 2462-2465 Inhibition of protein kinase C by tamoxifen

O'Brian, C.A., Liskamp, R.M., Solomon, D.H. & Weinstein, I.B., (1986) *J. Natl. Cancer Inst.*, **76**, 1243-1246 Triphenylethylenes: a new class of protein kinase C inhibitors

O'Brian, C.A., Housey, G.M. & Weinstein, I.B., (1988) *Cancer Research*, **48**, 3626-3629 Specific and direct binding of protein kinase C to an immobilised tamoxifen analogue

Okey, A.B., Mason, M.E., Gehly, E.B., Heidelberger, C., Muncan, J. & Dufresne, M.J., (1983) *Eur. J. Biochem.*, **132**, 219-227 Defective binding of 3-methylcholanthrene to the Ah receptor within C3H10T1/2 Clone 8 mouse fibroblasts in culture

Okey, A.B., (1990) *Pharmacol. Ther.*, **45**, 241-298 Enzyme induction in the cytochrome P450 system

Omiecinski, C.J., Redlich, C.A. & Costa, P., (1990) *Cancer Research*, **50**, 4315-4321 Induction and developmental expression of cytochrome P450IA1 messenger RNA in rat and human tissues: detection by the polymerase chain reaction

Omura, T. & Sato, R., (1964) *J. Biol. Chem.*, **239**, 2370-2378 The carbon monoxide binding pigment of liver microsomes: 1. Evidence for its hemoprotein nature

Osborne, C.K., Boldt, D.H., Clark, G.M. & Trent, J.M., (1983) *Cancer Research*, **43**, 3583-3585 Effects of tamoxifen on human breast cancer cell cycle kinetics: accumulation of cells in early G₁ phase

Osborne, C.K., Wiebe, V.J., McGuire, W.L., Ciocca, D.R. & DeGregario, M.W., (1992) *J. Clin. Oncol.*, **10**, 304-310 Tamoxifen and the isomers of 4-hydroxytamoxifen in tamoxifen-resistant tumours from breast

Pace-Asciak, C.R. & Edwards, N.S., (1981) *J. Biol. Chem.*, **255**, 6106-6110 Distinct age-related changes in the urinary catabolic profile of prostaglandin F₂ alpha in the rat

Paine, A.J., (1990) *Chem. Biol. Interact.*, **74**, 1-31 The maintainance of cytochrome P-450 in rat hepatocyte cultures: some applications of liver cell cultures to the study of drug metabolism, toxicity and the induction of the P-450 system

Park, J., Oie, H.K., Sugarbaker, P.H., Henslee, J.G., Chen, T.R., Johnson, B.E. & Gazder, A., (1987) *Cancer Res.*, **47**, 6710-6718 Characteristics of cell lines established from human colorectal carcinoma

Paine, A.J., Hockin, L.J. & Allen, C.M., (1982) *Biochem. Pharmacol.*, **3 1**, 1175-1178 Long term maintenance and induction of cytochrome P-450 in rat liver cell culture

Paine, A.J. & Legg, R.F., (1978) *Biochem. Biophys. Res. Comm.*, **8 1**, 672-679 Apparent lack of correlation between the loss of cytochrome P-450 in hepatic parenchymal cell culture and the stimulation of haem oxygenase activity

Parker, M.G., Webb, P., Needham, M., White, R. & Ham, J., (1987) *J. Cell Biochem.*, **3 5**, 285-292 Identification of androgen response elements in mouse mammary tumour virus and the rat prostate C3 gene

Parkinson, A., Thomas, P.E., Ryan, D. & Levin, W., (1983) *Arch. Biochem. Biophys.*, **2 2 5**, 216-236 The *in vivo* turnover of rat liver microsomal epoxide hydrolase and both the apoprotein and heme moieties of specific cytochrome P-450 isozymes

Pasco, D.S., Boyum, K.W., Merchant, S.N., Chalberg, S.C. & Faggan, J.B., (1988) *J. Biol. Chem.*, **2 6 3**, 8671-8676 Transcriptional and post-transcriptional regulation of the genes encoding cytochromes P450c and P450d *in vivo* and in primary hepatocyte cultures

Pasenen, M., Stacey, S., Lykkesfeldt, A., Briand, P., Hines, R. & Autrup, H., (1988) *Chem. Biol. Interact.*, **6 6**, 223-232 Induction of cytochrome P4501A1 gene expression in human breast tumour cell lines

Pearson, J.W., Fogler, W.E., Volker, K., Usui, N., Goldenberg, S.K., Gruys, E., Riggs, C.W., Komschlies, K., Wiltrott, R.H., Tsuruo, T., Pastan, I., Gottesman, M.M. & Longo, D.M., (1991) *J. Natl. Cancer Inst.*, **8 3**, 1386-1391 Reversal of drug resistance in a human colon cancer xenograft expressing MDR1 complementary DNA by *in vivo* administration of MRK-16 monoclonal antibody

Peters, W.H.M., Nagengast, F.M. & van Tongeren, J.H.M., (1989) *Gastroenterology*, **9 6**, 783-789 Glutathione S-transferase, cytochrome P450 and uridine 5'-diphosphate glucuronosyltransferase in human small intestine and liver

Peterson, J.A., Ebel, R.E., O'Keefe, D.H., Matsubara, T. & Estabrook, R.W., (1976) *J. Biol. Chem.*, **2 5 1**, 4010-4016 Temperature dependence of cytochrome P-450 reduction. A model for NADPH-cytochrome P450 reductase : cytochrome P-450 interaction

Poellinger, L., Lund, J., Gillner, M. & Gustafsson, J.A., (1985) In: "Molecular mechanism of steroid hormone action", pp755-790, Mondgil, V.K., (ed), de Gruyter Press, New York

Poland, A. & Glover, E., (1973) *Science*, **1 7 9**, 476-477 2,3,7,8-Tetrachlorodibenzo-p-dioxin: a potent inducer of 5-aminolevulinic acid synthetase

Poland, A., Glover, E., Robinson, J.R. & Nebert, D.W., (1974) *J. Biol. Chem.*, **249**, 5599-5606 Genetic expression of aromatic hydrocarbon hydroxylase activities and cytochrome P₁-450 formation by 2,3,7,8-tetrachlorodibenzo-p-dioxin in mice genetically "nonresponsive" to other aromatic hydrocarbons

Poland, A., Glover, E. & Kende, A.S., (1976) *J. Biol. Chem.*, **251**, 4936-4946 Stereospecific high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol: Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase

Poland, A., Mak, I., Glover, E., Boatman, R.J., Ebetino, F.H. & Kende, A.S., (1980) *Mol. Pharmacol.*, **18**, 571-580 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, a potent phenobarbital-like inducer of microsomal monooxygenase activity

Poland, A., Mak, I. & Glover, E., (1981) *Mol. Pharmacol.*, **20**, 442-450 Species differences in responsiveness to 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, a potent phenobarbital-like inducer of microsomal monooxygenase activity

Poland, A., Glover, E. & Bradfield, C.A., *Mol. Pharmacol.*, **39**, 20-26 Characterisation of polyclonal antibodies to the Ah receptor prepared by immunisation with a synthetic peptide hapten

Porter, T.D., Khani, S.C. & Coon, M.J., (1989) *Mol. Pharmacol.*, **36**, 61-65 Induction and tissue-specific expression of rabbit cytochrome P450IIE1 and IIE2 genes

Pottenger, L.H. & Jefcoate, C.R., (1990) *Carcinogenesis*, **11**, 321-327 Characterisation of a novel cytochrome P450 from the transformable line, C3H10T1/2

Pottenger, L.H., Christou, M. & Jefcoate, C.R., (1991) *Arch. Biochem. Biophys.*, **286**, 488-497 Purification and immunological characterisation of a novel cytochrome P450 from C3H10T1/2 cells

Poul, L.R. & Gillette, J.R., (1982) *Anaesth. Analg.*, **61**, 809 A perspective on halothane-induced hepatotoxicity

Poulos, T.L., Finzel, B.C., Gunsalus, I.C., Wagner, G.C. & Kraut, J., (1985) *J. Biol. Chem.*, **260**, 16122-16130 The crystal structure of cytochrome P450_{cam}

Poulos, T.L., Finzel, B.C., Howard, A.J., (1987) *J. Molec. Biol.*, **192**, 687-700 The high resolution crystal structure of cytochrome P450_{cam}

Poulos, T.L., (1989) *Pharm. Res.*, **5**, 67-75 Cytochrome P450: molecular architecture, mechanism and prospects for rational inhibitor design

Powis, G. & Prough, R.A., (eds) (1987) In: "Metabolism and action of anticancer drugs", Taylor & Francis, London

Powles, T.J., Tillyer, C.R., Jones, A.L., Ashley, S.E., Treleaven, J., Davey, J.B. & McKinna, J.A., (1990) *Eur. J. Cancer*, **26**, 680-684 Prevention of breast cancer with tamoxifen - an update on the Royal Marsden Hospital pilot programme

Prescott, L.F., Wright, N., Roscoe, P. & Brown, S.S., (1971) *Lancet*, **1**, 519-522 Plasma-paracetamol half-life and hepatic necrosis in patients with paracetamol overdose

Ptachcinski, R.J., Carpenter, B.J., Burckert, G.J., Venkatatamanan, R., (1985) *N. Engl. J. Med.*, **313**, 1416-1417 Effect of erythromycin on cyclosporin levels

Pyerin, W., Taniguchi, H., Horn, F., Oesch, F., Amelizad, Z. & Friedberg, T., (1987) *Biochem. Biophys. Res. Comm.*, **142**, 885-892 Isoenzyme-specific phosphorylation of cytochromes P-450 and other drug metabolising enzymes

Ram, P.A. & Waxman, D.J., (1992) *J. Biol. Chem.*, **267**, 3294-3301 Thyroid hormone stimulation of NADPH P450 reductase expression in liver and extrahepatic tissues

Rane, A. & Tomson, G., (1980) *Eur. J. Clin. Pharmacol.*, **18**, 9-15 Prenatal and neonatal drug metabolism in man

Rangarajan, P.N., Ravishankar, H. & Padmanaban, G., (1987) *Biochem. Biophys. Res. Comm.*, **144**, 258-263 Isolation of a cytochrome P-450 gene variant and characterisation of its 5' flanking sequences

Raucy, J.L., Lasker, J.M., Lieber, C.S. & Black, M., (1989) *Arch. Biochem. Biophys.*, 270-283 Acetaminophen activation by human liver cytochromes P450IIE1 and P450IA2

Raunio, H., Kojo, A., Juvonen, R., Honkakoski, P., Jarvinen, P., Lang, M.A., Vahakangas, K., Gelboin, H.V., Park, S. & Pelkonen, O., (1988) *Biochem. Pharmacol.*, **37**, 4141-4147 Mouse hepatic cytochrome P-450 isozyme induction by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, pyrazole and phenobarbital

Reddy, J.K. & Lalwani, N.D., (1983) *CRC Crit. Rev. Toxicol.*, **12**, 1-58 Carcinogenesis by hepatic peroxisome proliferators: Evaluation of the risk of hypolipidemic drugs and industrial plasticisers to humans

Reitberg, D., Bernhard, H. & Schentag, J., (1981) *Ann. Intern. Med.*, **95**, 582-585 Alteration of theophylline clearance and half-life by cimetidine in normal volunteers

Relling, M.V., Evans, R., Dass, C., Desiderio, D.M. & Nemecek, J., (1992) *J. Pharmacol. Exp. Ther.*, **261**, 491-498 Human cytochrome P450 metabolism of teniposide and etoposide

Reyes, H., Reisz-Porszasz, S. & Hankinson, O., (1992) *Science*, **256**, 1193-1195 Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor

Reznikoff, C.A., Brankow, D.W. & Heidelberger, D.W., (1973a) *Cancer Research*, **33**, 3231-3238 Establishment and characterisation of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division

Reznikoff, C.A., Bertram, J.S., Brankow, D.W. & Heidelberger, D.W., (1973b) *Cancer Research*, **33**, 3239-3249 Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division

Rittman-Grauer, L.S., Yong, M.A., Sanders, V. & Mackensen, D.G., (1992) *Cancer Research*, **52**, 1810-1816 Reversal of Vinca alkaloid resistance by anti-P-glycoprotein monoclonal antibody HYB-241 in a human tumor xenograft

Robertson, I.G.C., Philpot, R.M., Zeiger, E. & Wolf, C.R., (1981) *Mol. Pharmacol.*, **20**, 662-668 Specificity of rabbit pulmonary cytochrome P-450 isozymes in the activation of several aromatic amines and aflatoxin B1

Rofstad, E.K., (1992) *Cancer Research*, **52**, 1764-1769 Retention of cellular radiation sensitivity in cell and xenograft lines established from human melanoma surgical samples

Rollins, D.E., Von Bahr, C., Glaumann, H., Moldeus, P. & Rane, A., (1979) *Science*, **205**, 1414-1416 Acetaminophen: potentially toxic metabolite formed by human fetal and adult liver microsomes and isolated fetal liver cells

Romano, M., Esteve, A., Coccia, P., Masturzo, P., Galliani, G., Ghezzi, P. & Salmona, M., (1986) *Tox. Appl. Pharmacol.*, **33**, 379-385 Biochemical characterisation of the hepatic effects in mice and rats of 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, a hepatic neoplasm promoter

Rosenberg, D.W., (1991) *Arch. Biochem. Biophys.*, **284**, 223-226 Tissue-specific induction of the carcinogen inducible cytochrome P450 isoform, P450IA1, in colonic epithelium

Rosenberg, D.W., (1991) *Pharmacology*, **43**, 36-46 Dietary modulation of cytochrome P450 in the small intestinal epithelium

Rospendowski, B.N., Kelly, K., Wolf, C.R. & Smith, W.E., (1991) *J. Am. Chem. Soc.*, **113**, 1217-1225 Surface-enhanced resonance Raman scattering from cytochromes P-450 adsorbed on citrate-reduced silver sols

Sadano, H. & Omura, T., (1983) *Biochem. Biophys. Res. Comm.*, **116**, 1013-1019 Reversible transfer of heme between molecular species of microsome-bound cytochrome P-450 in rat liver

Samuels, B.L., Murray, J.L., Cohen, M.B., Safa, A.R., Sinha, B.K., Townsend, A.J., Beckett, M.A. & Weichselbaum, R.R., (1991) *Cancer Res.*, **51**, 521-527 Increased glutathione peroxidase activity in a human sarcoma cell line with inherent doxorubicin resistance

Satoh, H., Takemura, T., Ferransm V.J., Davies, H.W., Gillette, J.R. & Pohl, I.R., (1985) The binding of a reactive metabolite of halothane to cytochrome P-450 on the cell surface of hepatocytes In: "Abstracts of the 3rd Int. Symposium Biol. Reactive Intermediates"

Satta, T., Isobe, K., Yamauchi, I., Akiyama, S., Itou, K., Watanabe, T. & Takagi, H., (1991) *Jpn. J. Cancer Res.*, **8 2**, 593-598 Establishment of drug resistance in human gastric and colon carcinoma xenograft lines

Scheutz, E.G., Li, D., Omiecinski, C.J., Muller-Eberhard, U., Kleinman, H.L., Elswick, B. & Guzelian, P.S., (1988) *J. Cell Physiol.*, **1 3 4**, 309-323 Regulation of gene expression in adult rat hepatocytes cultured on a basement membrane matrix

Scudder, S.A., Brown, J.M. & Sikic, B.I., (1988) *J. Natl. Cancer Inst.*, **8 0**, 1294-1298 DNA cross-linking and cytotoxicity of the alkylating cyanomorpholino derivative of doxorubicin in multidrug resistant cells

Searle, P.F., Davison, B.L., Stuart, G.W., Wilkie, T.M., Norstedt, G. & Palmiter, R.D., (1984) *Mol. Cell Biol.*, **4**, 1221-1230 Regulation, linkage and sequence of mouse metallothionein I and II genes

Selby, P.J., Thomas, J.M., Monaghan, P., Sloane, J. & Peckam, M.J., (1980) *Br. J. Cancer*, **4 1**, 52-61 Human tumour xenografts established and serially transplanted in mice immunologically deprived by thymectomy, cytosine arabinoside and whole-body irradiation

Senler, T.I., Dean, W.L., Murray, L.F. & Wittliff, J.L., (1985) *Biochem. J.*, **2 2 7**, 379-387 Quantification of cytochrome P-450-dependent cyclohexane hydroxylase activity in normal and neoplastic reproductive tissues

Shen, S. & Strobel, H.W., (1992) *Arch. Biochem. Biophys.*, **2 9 4**, 83-90 The role of cytochrome P450 lysine residues in the interaction between cytochrome P450IA1 and NADPH-cytochrome P450 reductase

Shimizu, T., Tateishi, T., Hatano, M. & Fujii-Kuriyama., (1991) *J. Biol. Chem.*, **2 6 6**, 3372-3375 Probing the role of lysines and arginines in the catalytic function of cytochrome P450d by site-directed mutagenesis

Shimosato, Y., Kameya, T., Nagai, K., Hirohashi, S., Koide, T., Hayashi, H. & Nomura, T., (1976) *J. Natl. Cancer Inst*, **5 6**, 1251-1260 Transplantation of human tumours in nude mice

Shiraka, H. & Guengerich, F.P., (1984) *Arch. Biochem. Biophys.*, **2 3 5**, 86-96 Turnover of membrane proteins: kinetics of induction and degradation of seven forms of rat liver microsomal cytochrome P-450, NADPH-cytochrome P-450 reductase, and epoxide hydrolase

Shorthouse, A.J., Smyth, J.F., Steel, G.G., Ellison, M., Mills, J. & Peckham, M.J., (1980) *Br. J. Surg.*, **6 7**, 715-722 The human tumour xenograft - a valid model in experimental chemotherapy?

- Simmons, D.L. & Kasper, C.B., (1983) *J. Biol. Chem.*, **258**, 9585-9588 Genetic polymorphisms for a phenobarbital-inducible cytochrome P-450 map to the *Cdh* locus in mice
- Simmons, D.L., McQuiddy, P. & Kasper, C.B., (1987) *J. Biol. Chem.*, **262**, 326-332 Induction of the hepatic mixed function oxidase system by synthetic glucocorticoids: transcriptional and post-transcriptional regulation
- Sinha, B.K., (1980) *Chem. Biol. Interact.*, **30**, 67-77 Binding specificity of chemically and enzymatically activated anthracycline anticancer agents to nucleic acids
- Sinha, B.K., (1989) *Chem. Biol. Interact.*, **69**, 293-317 Free radicals in anticancer drug pharmacology
- Skett, P., (1987) *Drug Metab.*, **10**, 85-140 Hormonal regulation and sex differences of xenobiotic metabolism
- Sladek, N.E., Smith, P.C., Brati, P.M., Low, J.E., Powers, J.F., Borch, R.F. & Coveney, J.R., (1982) *Cancer Treatment Reports*, **66**, 1889-1900 Influence of diuretics on urinary general base catalytic activity and cyclophosphamide induced bladder toxicity
- Sleigh, M., (1986) *Anal. Biochem.*, **156**, 251-256 A non-chromatographic assay for expression of the chloramphenicol acetyltransferase gene in eucaryotic cells
- Sladek, N.E., (1972) *Cancer Research*, **32**, 535-542 Therapeutic efficacy of cyclophosphamide as a function of its metabolism
- Smith, C.A.D., Gough, A.C., Leigh, P.N., Summers, B.A., Harding, A.E., Maranganore, D.M., Sturman, S.G., Schapira, A.H.V., Williams, A.C., Spurr, N.K. & Wolf, C.R., (1992) *Lancet*, **339**, 1375-1377 Debrisoquine hydroxylase gene polymorphism and susceptibility to Parkinson's disease
- Song, B.J., Friedman, F.K., Park, S.S., Tsokos, G.C. & Gelboin, H.V., (1985) *Science*, **228**, 490-492 Monoclonal antibody-directed radioimmunoassay detects cytochrome P-450 in human placenta and lymphocytes
- Song, B.J., Gelboin, H.V., Park, S.S., Yang, C.S., Gonzalez, F.J., (1986) *J. Biol. Chem.*, **261**, 16689-16697 Complimentary cDNA and protein sequences of ethanol inducible rat and human P450s: transcriptional and post-transcriptional regulation of the rat enzyme
- Song, B.J., Veech, R.L., Gelboin, H.V. & Gonzalez, F.J., (1989) *J. Biol. Chem.*, **264**, 3568-3572 Induction of rat hepatic N-nitrosodimethylamine demethylase by acetone is due to protein stabilisation
- Soule, H.D., Vasquez, J., Long, A., Albert, S & Brennan, M., (1973) *J. Natl. Cancer Inst.*, **51**, 1409-1416 A human cell line from a pleural effusion derived from a breast carcinoma

Spurr, N.K., Gough, A.C., Stevenson, K. & Wolf, C.R., (1987) *Nucleic Acids Res.*, **15**, 5901, Msp-1 polymorphism detected with a cDNA probe for the P450 I family on chromosome 15

Spurr, N.K., Gough, A.C., Stevenson, K. & Wolf, C.R., (1989) *Hum. Genetics*, **81**, 171-174, The human cytochrome P450 CYP3 locus: assignment to chromosome 7q 22-qter

Squires, E.J. & Negishi, M., (1988) *J. Biol. Chem.*, **263**, 4166-4171 Reciprocal regulation of sex-dependant expression of testosterone 16 α -hydroxylase (P-450_{16 α}) in liver and kidney of male mice by androgen: Evidence for a single gene

Steel, G.G., (1978) *Bull. Cancer*, **65**, 465-472 The growth and therapeutic response of human tumours in immune deficient mice

Stralka, D. & Strobel, H.W., (1989) *Cancer*, **64**, 2111-2116 Cytochrome P450 activity and distribution in the human colon mucosa

Stralka, D. & Strobel, H.W., (1991) *Cancer*, **68**, 2363-2369 Characterisation of cytochrome P450-dependent dimethylhydrazine metabolism in human colon microsomes

Streeter, D.G., Taylor, D.L., Acton, E.M. & Peters, J.H., (1985) *Cancer Chemother. Pharmacol.*, **14**, 160-164 Comparative cytotoxicities of various morpholinyl derivatives

Sutherland, R.L., Green, M.D., Hall, R.E., Reddel, R.R. & Taylor, I.W., (1983) *J. Cancer Clin. Oncol.*, **19**, 615-621 Tamoxifen induces accumulation of MCF₇ human mammary carcinoma cells in the G₀/G₁ phase of the cell cycle

Swenson, D.H., Miller, J.A. & Miller, E.C., (1975) *Cancer Research*, **35**, 3811-3823 The reactivity and carcinogenicity of Aflatoxin B₁-2,3-dichloride, a model for the putative 2,3-oxide metabolite of Aflatoxin B₁

Swenson, D.H., Liu, J.K., Miller, E.C. & Miller, J.A., (1977) *Cancer Research*, **37**, 172-181 Aflatoxin B₁-2,3-oxide as a probable intermediate in the covalent binding of Aflatoxins B₁ and B₂ to rat liver DNA and ribosomal RNA *in vivo*

Tagaki, Y., Otake, T., Kataoke, M., Murata, Y., Aburada, S., Akasaka, S., Hashimoto, K., Uda, H. & Kitaura, T., (1976) *Tox. Appl. Pharmacol.*, **38**, 549-558 Studies on the transfer and distribution of [¹⁴C]polychlorinated biphenyls from maternal to fetal and suckling rats

Tamburini, P.P., Mason, H.A., Bains, S.K., Makowski, R.J., Morris, B. & Gibson, G.G., (1984) *Eur. J. Biochem.*, **139**, 235-246 Multiple forms of cytochrome P450: purification, characterisation and comparison of a novel-clofibrate induced isoenzyme with other forms of cytochrome P450

- Tannenbaum, G.S., (1981) *Endocrinology*, **108**, 176-2182 Growth hormone secretory dynamics in streptozotocin diabetes: evidence of a role for endogenous circulating somatostatin
- Taylor, S.M. & Jones, P.J., (1979) *Cell*, **17**, 771-779 Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine
- Taylor-Robinson, D., (1978) Cultural and serologic procedures for mycoplasmas in tissue culture, pp 47-56 In: "Mycoplasma infection of cell cultures" McGarrity, G., Murphy, D.G. & Nichols, W.W., (eds) Plenum Press, London
- Tenhunen, R., Marver, H.S. & Schmid, R., (1969) *J. Biol. Chem.*, **244**, 6388-6394 Microsomal heme oxygenase
- Thomas, P.E., Kowi, R.E. & Hutton, J.J., (1972) *Biochemical Genetics*, **6**, 157-168 The genetics of aromatic hydrocarbon hydroxylase induction in mice: a single gene difference between C57Bl/6J and DBA/2J
- Tinel, M., Belghiti, J., Descatoire, V., Amouyal, G., Letteron, P., Geneve, J., Larvey, D. & Pessayre, D., (1987) *Biochem. Pharmacol.*, **36**, 951-955 Inactivation of human liver cytochrome P-450 by the drug methoxsalen and other psoralen derivatives
- Towbin, H., Staehlin, T. & Gordon, J., (1979) *Proc. Natl. Acad. Sci. (USA)*, **76**, 4350-4354 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications
- Tukey, R.H., Hannah, R.R., Negishi, M., Nebert, D.W. & Eisen, H.J., (1982) *Cell*, **31**, 275-284 The Ah locus: Correlation of intranuclear appearance of inducer-receptor complex with induction of cytochrome P₁-450 mRNA
- Turesky, R.J., Lang, N.P., Butler, M.A., Teitel, C.H. & Kadlubar, F.F., (1991) *Carcinogenesis*, **12**, 1839-1845 Metabolic activation of carcinogenic heterocyclic aromatic amines by human liver and colon
- Turner, M.J., Fields, C.E. & Everman, D.B., (1991) *Biochem. Pharmacol.*, **41**, 1701-1705 Evidence for superoxide formation during hepatic metabolism of tamoxifen
- Tuteja, N., Gonzalez, F.J. & Nebert, D.W., (1985) *Developmental Biology*, **112**, 177-184 Developmental and tissue-specific differential regulation of the mouse dioxin-inducible P₁-450 and P₃-450 genes
- Umeno, M., Morris, M., Matsunaga, E., Gelboin, H.V. & Gonzalez, F.J., (1988) *Nucleic Acids Research*, **16**, 6249 Sequence of the 5' end of the developmentally regulated rat P450 PB1 (P450IIC6) gene
- Vang, O., Jensen, H. & Autrup, H., (1991) *Chem. Biol. Interact.*, **78**, 85-96 Induction of cytochrome P-450 IA1, IA2, IIB1, IIB2 and IIE1 by broccoli in rat liver and colon

Vickers, P.J., Dufresne, M.J. & Cowan, K.H., (1989) *Molecular Endocrinology*, **3**, 157-164 Relation between cytochrome P450IA1 expression and estrogen receptor content of human breast cancer cells

Villa, P., Arioli, P & Guitani, A., (1991) *Exp. Cell Research*, **194**, 157-160 Mechanism of maintenance of liver-specific function by DMSO in cultured rat hepatocytes

Vodicnik, M.J. & Lech, J.J., (1980) *Tox. App. Pharmacol.*, **54**, 293-300 The transfer of 2,4,5,2',4',5'-hexachlorobiphenyl to fetuses and nursing offspring I. Disposition in pregnant and lactating mice and accumulation in young

Wagner, S.L., Dean, W.L. & Gray, R.D., (1984) *J. Biol. Chem.*, **259**, 2390-2395 Effect of a zwitterionic detergent on the state of aggregation and catalytic activity of cytochrome P-450_{LM2} and NADPH-cytochrome P-450 reductase

Warren, J.R., Simmon, V.F. & Reddy, J.K., (1980) *Cancer Research*, **40**, 36-41 Properties of hypolipidemic peroxisome proliferators in the lymphocyte [³H] thymidine and *Salmonella* mutagenesis assays

Watanabe, J., Hayashi, S-I., Nakachi, K., Imai, K., Suda, Y., Sekine, T. & Kawajiri, K., (1990) *Nucleic Acids Res.*, **18**, 7194 Pst I and Rsa I RFLPs in complete linkage disequilibrium at the CYP2E gene

Watkins, P.B., Wrighton, S.A., Scheutz, E.G., Maurel, P. & Guzelian, P.S., (1986) *J. Biol. Chem.*, **261**, 6264-6271 Macrolide antibiotics inhibit the degradation of the glucocorticoid-responsive cytochrome P-450p in rat hepatocytes *in vivo* and in primary monolayer culture

Watkins, P.B., (1990) *Seminars in liver disease*, **10**, 235-250 Role of cytochromes P450 in drug metabolism and hepatotoxicity

Waxman, D.J., Ko, A. & Walsh, C., (1983) *J. Biol. Chem.*, **258**, 11937-11944 Regioselectivity and stereoselectivity of androgen hydroxylations catalysed by cytochrome P-450 isozymes purified from phenobarbital-induced rat liver

Waxman, D.J., (1988) *Biochem. Pharmacol.*, **37**, 71-81 Interactions of hepatic cytochromes P-450 with steroid hormones: regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression

Waxman, D.J., Morrissey, J.J., Naik, S. & Jauregui, H.O., (1990) *Biochem. J.*, **271**, 113-119 Phenobarbital induction of cytochromes P-450: High-level long-term responsiveness of primary rat hepatocyte cultures to drug induction and glucocorticoid dependence of the phenobarbital response

Waxman, D.J., Ram, P.A., Notani, G., LeBlanc, G.A., Alberta, J.A., Morrissey, J.J. & Sundseth, S.S., (1990) *Mol. Endocrinology*, **4**, 447-454 Pituitary regulation of the male specific steroid 6 β -hydroxylase P-450 2a (gene product IIIA2 in adult rat liver). Suppressive influence of growth hormone and thyroxine acting at a pretranslational level

- de Waziers, I., Cugnenc, P.H., Berger, A., Leroux, J.P. & Beaune, P.H., (1991) *Carcinogenesis*, **12**, 905-909 Drug-metabolising enzyme expression in human normal, peritumoral and tumoral colorectal tissue samples
- de Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R. & Subramani, S., (1987) *Mol. Cell Biol.*, **7**, 725-737 Firefly luciferase gene: Structure and expression in mammalian cells
- White, T.B., Hammond, D.K., Vasquez, H. & Strobel, H.W., (1991) *Mol. Cell. Biochem.*, **102**, 61-69 Expression of two cytochromes P450 involved in carcinogen activation in a human colon cell line
- Whitlock, J.P., (1990) *Ann. Rev. Pharmacol. Toxicol.*, **30**, 251-277 Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-p-dioxin
- Wickner, W.T. & Lodish, H.A., (1985) *Science*, **230**, 400-407 Multiple mechanisms of protein insertion into and across membranes
- Wiebel, F.J., Wolffe, T. & Lambiotte, M., (1980) *Biochem. Biophys. Res. Comm.*, **94**, 466-472 Presence of cytochrome P-450 and P-448-dependent monooxygenase functions in hepatoma cell lines
- Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G. and Chasin, L., (1979) *Proc. Natl. Acad. Sci., (U.S.A.)*, **156**, 251-256 DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells
- Willman, T. & Beato, M., (1986) *Nature*, **324**, 688-691 Steroid-free glucocorticoid receptor binds specifically to mouse mammary tumour virus DNA
- Wittemore, A.S., Williams, A.H., Lee, M., Shu, Z., Gallagher, R.P., Deng-Ao, J., Lu, Z., Xianghui, W., Hui, C., Jung, D., Ten, C., Chengde, L., Yao, X., Paffenberger, R.S. & Henderson, B.E., (1990) *J. Natl. Cancer Inst.*, **82**, 915-926 A population-based case-control study evaluating lifestyle characteristics in Chinese men and women with and without colorectal cancer in the two countries
- Wolf, C.R. & Oesch, F., (1983) *Biochem. Biophys. Res. Comm.*, **111**, 504-511 Isolation of a high spin form of cytochrome P-450 induced in rat liver by 3-methylcholanthrene
- Wolf, C.R., Moll, E., Friedberg, T., Oesch, F., Buchmann, A., Kuhlmann, W. D. & Kunz, H.W., (1984) *Carcinogenesis*, **5**, 993-1001 Characterisation, localisation and regulation of a novel PB-inducible form of cytochrome P450 compared with 3 further P450 isoenzymes, NADPH-P450 reductase, glutathione S-transferase and microsomal epoxide hydrolase
- Wolf, C.R., Seilman, S., Oesch, F., Mayer, R.T. & Burke, M.D., (1986) *Biochem. J.*, **240**, 27-33 Multiple forms of cytochrome P-450 related to forms induced marginally by phenobarbital: Differences in structure and in the metabolism of alkoxyresorufins

Wolf, C.R., Miles, J.S., Seilman, S., Burke, M.D., Rospendowski, B.N., Kelly, K. & Smith, W.E., (1988) *Biochemistry*, **27**, 1597-1603 Evidence that the catalytic differences of two structurally homologous forms of cytochrome P-450 relate to their heme environment

Wolf, C.R., (1991) *Cancer Surveys*, **9**, 437-474 Metabolic factors in cancer susceptibility

Wolf, C.R., Smith, C.A.D., Gough, A.C., Moss, J.E., Vallis, K.A., Howard, G., Carey, F.J., Mills, K., McNee, W., Carmichael, J. & Spurr, N.K., (1992) *Carcinogenesis*, **13**, 1035-1038 Relationship between the polymorphisms in debrisoquine hydroxylase and cancer susceptibility

Wong, G., Kawajiri, K. & Negishi, M., (1987) *Biochemistry*, **26**, 8683-8690 Gene family of male-specific testosterone 16 α -hydroxylase (C-P-450_{16 α}) in mouse liver: cDNA sequences, neonatal imprinting and reversible regulation by androgen

Wong, G., Hakura, T., Kawajiri, K., Skow, L. & Negishi, M., (1989) *J. Biol. Chem.*, **264**, 2920-2927 Gene family of male-specific testosterone 16 α -hydroxylase (C-P-450_{16 α}) in mice

Wood, A.W., Ryan, D.E., Thomas, P.E. & Lewin, W., (1983) *J. Biol. Chem.*, **258**, 8839-8847 Regio- and stereoselective metabolism of two C₁₉ steroids by five highly purified and reconstituted rat hepatic cytochrome P-450 isozymes

Wright, M.C. & Paine, A.J., (1992) *Biochem. Pharmacol.*, **43**, 237-243 Evidence that the loss of rat liver cytochrome P450 *in vitro* is not solely associated with the use of collagenase, the loss of cell-cell contacts and/or the absence of an extracellular matrix

Yamano, S., Aoyama, T., McBride, O.W., Hardwick, J.B., Gelboin, H.V. & Gonzalez, F.J., (1989) *Mol. Pharmacol.*, **35**, 83-88 Expression of P450 cDNAs and NADPH-P450 oxidoreductase

Yamano, S., Tatsuno, J. & Gonzalez, F.J., (1990) *Biochemistry*, **29**, 1322-1329 The CYP2A3 gene product catalyzes coumarin 7-hydroxylation in human liver microsomes

Yamazoe, Y., Murayama, N., Shimada, M., Yamanchi, K. & Kato, R., (1989) *Arch. Biochem. Biophys.*, **268**, 567-575 Cytochrome P450s in livers of diabetic rats: Regulation by growth hormone and insulin

Yanagida, A., Sogawa, K., Yasumaoto, K. & Fujii-Kuriyama, Y., (1990) *Mol. Cell Biol.*, **10**, 1470-1475 A novel cis-acting DNA element required for a high level of inducible expression of the rat P-450c gene

Yanagimoto, T., Hoh, S., Muller-Enoch, D. & Kamataki, T., (1992) *Biochim. Biophys. Acta.*, **1130**, 329-332 Mouse liver cytochrome P-450 (P-450III_{A1}): its cDNA cloning and inducibility by dexamethasone

Yanai, N., Suzuki, M. & Obinata, M., (1991) *Exp. Cell Research.*, **197**, 50-56 Hepatocyte cell lines established from transgenic mice harboring temperature-sensitive Simian virus 40 large T-antigen gene

Yano, M., Falvey, E. & Gonzalez, F.J., (1992) *Mol. Cell Biol.*, **12**, 2847-2854 Role of the liver-enriched transcription factor DBP in expression of the cytochrome P450 CYP2D6 gene

Yasukochi, Y. & Masters, B.S.S., (1976) *J. Biol. Chem.*, **251**, 537-5344 Some properties of a detergent solubilised NADPH-cytochrome C (Cytochrome P450) Reductase purified by biospecific affinity chromatography

Zanger, U.M., Hauri, H.P., Loeper, J., Homberg, J-C., Meyer, U.A., (1988) *Proc. Natl. Acad. Sci. (USA)*, **85**, 8256-8260 Antibodies against human cytochrome P450db1 in autoimmune hepatitis Type II

Zaphiropoulos, P.G., Mode, A., Storm, A., Moller, C., Fernandez, C. & Gustafsson, J.A., (1988) *Proc. Natl. Acad. Sci. (USA)*, **85**, 4214-4217 cDNA cloning, sequence and regulation of a major female-specific and growth-hormone inducible rat liver cytochrome P-450 active in 15 β -hydroxylation of steroid sulfates

Zaphiropoulos, P.G., Westin, S., Strom, A., Mode, A. & Gustafsson, J-A., (1990) *DNA Cell Biol.*, **9**, 49-56 Structural and regulatory analysis of a cytochrome P450 gene (CYP2C12) expressed predominantly in female rat liver

Zaphiropoulos, P.G., Strom, A., Robertson, J.A. & Gustafsson, J.A., (1990) *Mol. Endocrinol.*, **4**, 53-58 Structural and regulatory analysis of the male-specific rat liver cytochrome P-450g: repression by continuous growth hormone administration

Zaphiropoulos, P.G., Mode, A., Norstedt, G. & Gustafsson, J.A., (1989) *Trends Pharmacol. Sci.*, **10**, 149-157 Regulation of sexual differentiation in drug and steroid metabolism

Appendix 1: Sources of materials

Aldrich Fine Chemicals Ltd., The Old Brickyard, New Road,
Gillingham, Dorset, SP8 4JL

hydroquinone

Amersham International plc, UK Sales Office, Lincoln Place, Green
End, Aylesbury, Buckinghamshire

^{125}I -Protein A, ^{32}P - α dCTP, [^{14}C]-chloramphenicol, ECL Western blotting
detection reagents, Hybond N,

BDH, Macfarlane Robson Ltd., Burnfield Avenue, Thornliebank,
Glasgow, G46 7TP

sodium citrate, sarcosyl, phenol, chloroform, isoamyl alcohol, isopropanol, ethyl
acetate, copper sulphate, potassium sodium (+)-tartrate, sodium carbonate.10H₂O,
sodium orthophosphate.12H₂O, dimethylsulphoxide, sodium hydroxide, carbon
tetrachloride, sodium chloride, dipotassium hydrogen phosphate, potassium
dihydrogen phosphate, di-Na ethylenediamminetetraacetic acid, potassium chloride,
glacial acetic acid, methanol, ammonium acetate, sodium acetate, Tris-HCl, glycerol,
bromophenol blue, acrylamide, bis-acrylamide, ammonium persulphate, glycine,
chloroform, formaldehyde, tris base, boric acid, tri-sodium citrate, glucose,
propan-2-ol, butan-2-ol, sodium pyrophosphate, magnesium chloride, Hepes,
ammonium formate

Boehringer Mannheim, Boehringer Mannheim House, Bell Lane,
Lewes, East Sussex, BN7 1LG

Klenow fragment, dATP, dGTP, dTTP, RNAase A, restriction enzymes

DAKO Ltd, 16 Manor Courtyard, Highenden Avenue, High Wycombe,
Bucks., HP13 5RE

AB complex

Difco Ltd., PO Box 14B, Central Avenue, West Molesey, Surrey
Agar, bactotryptone, yeast extract, trypsin

Du Pont (UK) Ltd., Wedgewood Way, Stevenage, Hertfordshire, SG1
4YH

En³Hance spray

Fluka, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4JL
2,3,5-trichloropyridine

Gibco-BRL Ltd., PO Box 35, Trident House, Renfrew Road, Paisley
BME, RPMI, RPMI + HEPES, DMEM, foetal calf serum, penicillin-streptomycin, new
born calf serum, phenol, RNA ladder, low melting point agarose, 1kb ladder,
guanidinium hydrochloride, guanidinium isothiocyanate

Kodak Ltd., Box 33, Swallowdale Lane, Hemel Hempstead,
Hertfordshire, HP2 7EU
X-Omat AR5 X-ray film

Mackay and Lynn Ltd., 2, West Bryson Road, Edinburgh, EH11 1EH
Whatmann 3 MM paper, DE81 paper

Oxoid, Wade Road, Basingstoke, Hampshire
phosphate buffered saline

Pharmacia, Pharmacia House, Midsummer Boulevard, Milton
Keynes, MK9 3HP
Ficoll, dextran sulphate, hexadeoxyribonucleotides

Scottish Antibody Production Unit, Glasgow and West Scotland
Blood Transfusion Service, Law Hospital, Carluke, Lanarkshire,
ML8 5ES

anti-mouse HRP, anti-rabbit HRP

Sigma Chemical Co., Ltd., Fancy Road, Poole, Dorset

ethidium bromide, agarose, lysozyme, MOPS, methylene blue, L-glutamine,
dithiothreitol, diethylpyrocarbonate, bovine serum albumin, β -mercaptoethanol,
Coomassie brilliant blue R, TEMED, 4-chloro-1-naphthol, sucrose,
cyclophosphamide, 3-methylcholanthrene, β -naphthoflavone, dexamethasone, Folin
& Coicalteau's phenol reagent, Tween 20, BCIP, NBT, formamide